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Morphology and physiology of mammalian (hamster) eggs before and after fertilization

Yang, Cheng-Hsiung, Ph.D.

University of Hawaii, 1989

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MORPHOLOGY AND PHYSIOLOGY OF MAMMALIAN (HAMSTER) EGGS BEFORE AND AFTER FERTILIZATION

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN BIOMEDICAL SCIENCES

(Anatomy and Reproductive Biology)

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BY

Cheng-Hsiung Yang

Dissertation Committee:

Ryuzo Yanagimachi, Chairman
Thomas T. F. Huang, Jr.
Ruth G. Kleinfeld
Vincent J. DeFeo
Samuel R. Haley

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ABSTRACT

Preovulatory mature ovarian hamster eggs were compared with recently ovulated eggs. Although these two groups of eggs were at metaphase of the second meiotic division, there were quantitative differences between the two. The most striking difference was found in the zona pellucida. The zona of the oviductal egg was "heterogeneous" in its optical density and had a stronger acrosome reaction-inducing ability than that of the ovarian egg. When cultured in artificial media, the ooplasm of ovarian eggs became like that of oviductal eggs. However, their zonae remained unchanged. Zonae of ovarian eggs became like those of oviductal eggs only when they were exposed to oviductal fluid.

To investigate egg morphology after centrifugation, and how egg fragments respond to spermatozoa, zona-free hamster eggs were centrifuged in a Percoll gradient. Each egg was separated into a light and a heavy half. Chromosomes remained in their original cortical position in the egg during centrifugation or after separation into either light or heavy half. When the eggs were treated with cytochalasin D (CD) and then centrifuged, the chromosomes were extruded quickly before each egg was separated into halves or fragment. Fertilizability was different between light and heavy halves. Most of the heavy halves supported development of sperm nuclei into pronuclei, whereas only a

few light halves could do so. When the light and heavy halves were centrifuged further, each separated into two quarters. The lightest quarter was fragile and few of them could fuse with spermatozoa. In this quarter, the sperm nucleus could decondense but could not develop into a pronucleus, whereas in the other quarters the sperm nuclei could develop into well-formed pronuclei.

The rigidity of eggs at various stages were examined by determining the minimum time needed for complete separation of all eggs in a group into halves. The rigidity of the egg diminished sharply with the progression of meiotic maturation, reaching a minimum at metaphase of second meiosis, then increasing progressively after fertilization. Cytochalasin D reduced the rigidity of any stages of eggs, indicating that the actin-based cytoskeleton in the cortical region is largely responsible for the rigidity of the egg.

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CHAPTER 1

Some of the material contained in this dissertation was previously published or is currently in press. All relevant text and figures have been reproduced with the express permission of the publishers (see appendix 2). Chapter 2 was previously published under the title "Differences between mature ovarian and oviductal oocytes: a study using the golden hamster" (Yang, CH and Yanagimachi, R) in Human Reproduction, volume 4, pages 63-71 (appendix 1). Chapter 3 is currently in press under the title "Morphology and fertilizability of zona-free hamster eggs separated into halves and quarters by centrifugation" (Yang, CH, Yanagimachi, R and Yanagimachi, H) in Biology of Reproduction. Chapter 4 is currently in press under the title "Changes in the rigidity of the hamster egg during meiotic maturation and after fertilization" (Yang, CH and Yanagimachi, R) in Development Growth and Differentiation. With respect to the multiple authorship of these publications, I, as senior author of the publications, was responsible for experimental design, conduct of the experimentation, interpretation of the results and the writing of the manuscript. The contributions of the co-authors include technical assistance (H. Yanagimachi), assistance in interpretation of the results and preparation of the manuscript (R. Yanagimachi).

INTRODUCTION

Fertilization is the link between one generation and the next. The fertilized egg divides repeatedly to give rise to the numerous cell lines which constitute the body (soma) and also the germ cells that maintain the continuity of the species. Wilson (1925) stated that "The ovum has to supply most of the material for the body of the embryo, and often also to provide for its protection and maintenance during development". In mammals, the egg plays a leading role in fertilization and development. For example, the mature egg and its investments induce the sperm acrosome reaction (Bleil and Wassarman, 1980, 1983, 1986; Cherr, et al., 1986; Uto, et al., 1988). The cytoplasm of the egg induces decondensation of sperm nucleus leading to the formation of the male pronucleus (Usui and Yanagimachi, 1976; Thibault, 1977). Fertilization of eggs with immature cytoplasm can result in certain abnormalities such as polyspermy (Jaffe and Gould, 1985), failure of sperm nucleus decondensation (Usui and Yanagimachi, 1976), failure of male pronucleus development (Yanagimachi and Noda, 1970; Thibault, 1977) and inability of the embryo to continue to develop (First, et al., 1988; Leibfried-Rutledge, et al., 1989).

There is overwhelming experimental evidence that a zygote receives from the egg not only genetic material, but also an extensive reserve of macromolecules and organelles

essential for supporting the nutritional, synthetic, energetic and regulatory processes involved in early embryonic development (Austin and Short, 1982). It is obvious that the egg is a highly complex cell and is an essential component of mammalian reproduction. Studies relating to differentiation are thus of crucial importance to understanding the process of fertilization and successful mammalian reproduction.

The purpose of this dissertation is to contribute to a better understanding of morphological and physiological aspects of mammalian egg maturation in relation to the process of fertilization. I have chosen the golden (Syrian) hamster as an animal model (Orsini, 1961). A considerable amount of basic information about the gametes and fertilization processes for this species has been established and will form the foundation for the present study.

This dissertation consists of three parts. In the first part, I raise a question relevant to the procedures applied in human in vitro fertilization (IVF). In human IVF, eggs are collected from ovaries shortly before ovulation and then inseminated in vitro. Since these eggs can be fertilized in vitro and develop into viable offspring (Edwards, 1981; Edwards and Purdy, 1982; Trounson and Wood, 1984; Wolf and Quigleg, 1984), it is generally assumed that preovulatory eggs which have reached metaphase of the second meiotic division are fully mature; i.e., they are morphologically and physiologically identical with ovulated eggs. In order to

determine whether this is the case, I have carefully compared mature ovarian eggs to ovulated eggs collected from the oviduct. The parameters examined include: (1) the morphology of the cumulus oophorus, zona pellucida and vitellus (Talbot and DiCarlantonio, 1984), (2) the acrosome reaction-inducing ability of the zona and (3) the response of the vitellus to spermatozoa (Cummins and Yanagimachi, 1986), to calcium ionophore (Steinhardt, et al., 1974) and to centrifugal force. Any differences observed between mature ovarian eggs and oviductal eggs may prove to be significant in the application of human IVF technology.

After learning that the vitellus of the oviductal egg is readily divided into "halves" by centrifugation (Yang and Yanagimachi, 1989; Yang, et al., 1989), I performed a series of experiments to determine (1) how the cell organelles of the egg are distributed before and after centrifugation (Karasiewicz and Solytnska, 1985; Longo and Chen, 1985; Maro, 1986a, b; Cran, 1987; Longo, 1987;) and (2) how the separated halves and quarters respond to spermatozoa (Austin, 1951; Tarkowski, 1980; Yanagimachi, 1981, 1982, 1988; Huang and Yanagimachi, 1985; Clarke and Masui, 1987). This constitutes the second part of my dissertation.

The third part of my dissertation is concerned with changes in the physical properties, i.e., the "rigidity" of the egg cortex during egg maturation, fertilization and early embryonic development. Changes in the "rigidity" of the egg cortex during egg maturation is expected based upon previous

studies of amphibian (Selman and Waddington, 1955; Sawai and Yoneda, 1974;) and echinoderm (Hiramoto, 1963, 1976; Mitchison and Swann, 1965; Nemoto and Yoneda, 1980) eggs. However, no such study has been reported in mammals. I examined changes in rigidity of the egg cortex (Hiramoto, 1962; Uehara and Yanagimachi, 1976, 1977; Barak, et al., 1980; Alberts, et al., 1983; Battaglia and Gaddum-Rosse, 1986; 1987) not only during egg maturation, but also during fertilization and early preimplantation development.

REFERENCES

Alberts, B, Bray, D, Lewis, J, Raff, M, Roberts, K and Watson, JD (1983) The Cytoskeleton. In: Molecular Biology of The Cell. pp. 549-607.

Austin, CR (1951) Observation on the penetration of sperm into the mammalian egg. Aust J Sci Res 4:581-596.

Austin, CR and Short, R, (eds) (1982) Germ Cells and Fertilization. In: Reproduction in Mammals, vol. 1, Cambridge University Press, Cambridge. pp. 46-62.

Barak, LS, Yocum, RR, Nothnagel, EA and Web, WW (1980) Fluorescence staining of the actin cytoskeleton in living cells with NBD-ph. Proc Natl Acad Sci USA 77:980-984.

Battaglia, DE and Gaddum-Rosse, P (1986) The distribution of polymerized actin in the rat egg and its sensitivity to cytochalasin B during fertilization. J Exp Zool 237:97-105.

Battaglia, DE and Gaddum-Rosse, P (1987) Influence of Ca ionophore on rat egg behavior and cortical F actin. Gamete Res 18:141-152.

Bleil, JD and Wassarman, PM (1980) Mammalian sperm-egg interaction: Identification of a glycoprotein in mouse egg zona pellucida possessing receptor activity for sperm. Cell 20:873-882.

Bleil, JD and Wassarman, PM (1983) Sperm egg interaction in the mouse: Sequence of events and induction of the acrosome reaction by a zonae pellucida glycoprotein. Dev Biol 95:317-324.

Bleil, JD and Wassarman, PM (1986) Autoradiographic visualization of the mouse egg's sperm receptor bound to sperm. J Cell Biol 102:1363-1371.

Cherr, GN, Lambert, H, Meizel, S and Katz, DF (1986) In vitro studies of the golden hamster sperm acrosome reaction. Dev Biol 114:119-131.

Clarke, HJ and Masui, Y (1987) Dose-dependent relationship between oocyte cytoplasmic volume and transformation of sperm nuclei to metaphase chromosome. J Cell Biol 104:831-840.

Cran, DG (1987) The distribution of organelles in mammalian oocytes following centrifugation prior to injection of foreign DNA. Gamete Res 18:67-76.

Cummins, JM and Yanagimachi, R (1986) Development of ability to penetrate the cumulus oophorus by hamster spermatozoa capacitated in vitro, in relation to the timing of the acrosome reaction. Gamete Res 15:187-212.

Edwards, RG (1981) Test tube babies. Nature 293:253-256.

Edwards, RG and Purdy, JM (eds) (1982) Human Conception In Vitro. Academic Press, London.

First, NL, Leibfried-Ruthledge, ML and Sirard, MA (1988) Cytoplasmic control of oocyte maturation and species differences in development of competence to mature. In: Meiotic Inhibition, Molecular Control of Meiosis: Progress in Clinical and Biological Research, Haseltine, J and First, NL (eds) Alan R Liss, New York. pp. 1-46.

Hiramoto, Y (1962) Microinjection of the live spermatozoa into sea urchin eggs. Exp Cell Res 27:461-466.

Hiramoto, Y (1963) Mechanical properties of sea urchin eggs. Exp Cell Res 32:76-88.

Hiramoto, Y (1976) Mechanical properties of starfish oocytes. Dev Growth Diff 18:205-209.

Huang, TTF and Yanagimachi, R (1985) pH and proteinase control of acrosomal content stasis and release during the guinea pig acrosome reaction. Biol Reprod 32:451-462.

Jaffe, LA and Gould, M (1985) Polyspermy Preventing Mechanisms. In: Biology of Fertilization., vol. 3, Metz, CB and Monroy, A (eds), Academic Press, New York. pp. 223-250.

Karasiewicz, J and Solytnska MS (1985) Ultrstructural evidence for the presence of actin filaments in mouse eggs in fertilization. Roux's Arch Dev Biol 194:369-372.

Leibfried-Ruthledge, ML, Florman, HM and First, NL (1989) The Molecular Biology of Mammalian Oocyte Maturation. In: The Molecular Biology of Fertilization. Schatten, H and Schatten, G (eds) Academic Press, New York. pp. 259-301.

Longo, FJ and Chen, DY (1985) Development of cortical polarity in mouse eggs: Involvement of the meiotic apparatus. Dev Biol 107:382-394.

Longo, FJ (1987) Actin - plasma menbrane association in mouse eggs and oocytes. J Exp Zool 243:299-309.

Maro, B (1986a) Cytoskeletal dynamics in the mouse egg. J Cell Sci (suppl.) 5:343-359.

Maro, B (1986b) Fertilization and the cytoskeleton in the mouse. Bioessays 3:18-21.

Mitchison, JM and Swann, MM (1965) The mechanical properties of the cell surface. J Exp Biol 32:734-750.

Nemoto, S, Yoneda, M, and Uemura, I (1980) Marked decrease in the rigidity of starfish oocytes induced by 1-methyladenine. Dev Growth Diff 22:315-325.

Orsini, MW (1961) The external vaginal phenomena characterizing the stages of the estrous cycle, pregnacy, pseudopregnacy, lactation and the anestrous hamster. Proc. animal care panel 11:193-206.

Sawai, T and Yoneda, M (1974) Wave of stiffness propagating along the surface of the newt egg during cleavage. J Cell Biol 60:1-7.

Selman, GG and Waddington, CH (1955) The mechanism of cell division in the cleavage of the newt's egg. J Exp Biol 32:700-733.

Steinhardt, RA, Epel, D, Carroll, EJ and Yanagimachi, R (1974) Is calcium ionophore a universal activator for unfertilized egg? Nature 252:41-43.

Talbot, P and Dicarlantonio, G (1984) Architecture of the hamster oocyte cumulus complex. Gamete Res 9:261-272.

Tarkowaski, AK (1980) Fertilization of nucleate and anucleate egg fragments in the mouse. Exp Cell Res 128:73-77.

Thibault, C (1977) Are follicular maturation and oocyte maturation independent processes? J Reprod Fertil 51:1-15

Trounson, A and Wood, C (eds) (1984) In Vitro Fertilization and Embryo Transfer. Churchill Livingston, Edinburgh.

Uehara, T and Yanagimachi, R (1976) Microsurgical injection of spermatozoa into hamster eggs with subsequent transformation of sperm nuclei into male pronucleus. Biol Reprod 16:467-470.

Uehara, T and Yanagimachi, R (1977) Activation of hamster egg by pricking. J Exp Zool 199:269-274.

Usui, N and Yanagimachi, R (1976) Behavior of hamster sperm nuclei incorporated into eggs at various stages of maturation, fertilization and early development. J Ultrastruc Res 57:276-287.

Uto, N, Yoshimatsu, N, Lopata, A and Yanagimachi, R. (1988) The zona-induced acrosome reaction of hamster spermatozoa. J Exp Zool 248:113-120

Wolf, DP and Quigleg, MM (eds) (1984) Human In Vitro Fertilization and Embryo Transfer. Plenum Press, New York.

Yanagimachi, R (1981) Mechanisms of Fertilization in Mammals. In: Fertilization and Embryo Development In Vitro, Mastroianni, L and Biggers, JD (eds), Plenum Press, New York. pp. 61-182

Yanagimachi, R (1982) In vitro sperm capacitation and fertilization of golden hamster eggs in a chemically defined media. In: In Vitro Fertilization and Embryo Transfer, Hafez, ESE and Semm, K (eds), MTP Press, Lancaster. pp. 65-76.

Yanagimachi, R (1988) Mammalian Fertilization. In: The Physiology of Reproduction, vol. 1, Knobil, E and Neill, JP (eds), Raven Press, New York. pp. 135-185.

Yanagimachi, R and Noda, YD (1970) Electron microscope studies of sperm incorporation into the golden hamster egg. Am J Anat 12:429-462.

Yang, CH and Yanagimachi, R (1989) Differences between mature ovarian and oviductal oocytes: a study using the golden hamster. Human Reprod 4:63-71.

Yang, CH, Yanagimachi, H and Yanagimachi, R (1989) Morphology and fertilizability of zona free hamster egg separated into halves and quarters by centrifugation. Biol Reprod (in press).

CHAPTER II

DIFFERENCES BETWEEN MATURE OVARIAN AND OVIDUCTAL EGGS: A STUDY USING THE GOLDEN HAMSTER

In mammals, with the possible exception of the fox, dog and certain primitive insectivores, ovulation occurs when the egg reaches the metaphase of the second meiotic division (Austin and Walton, 1960; Baker, 1982). Since human ovarian eggs, collected from ovaries shortly before ovulation, can be fertilized in vitro and develop into normal viable offspring (Edwards, 1981; Edwards and Purdy, 1982; Trounson and Wood, 1984; Wolf and Quigley, 1984), investigators have gained the impression that ovarian eggs which have reached the metaphase of the second meiotic division are fully mature and physiologically identical to ovulated eggs. The present study was designed to determine whether this is the case. Here we chose the golden hamster as an animal model, because we have much basic information on the gametes and fertilization processes of this species, which are needed for the present study.

MATERIALS AND METHODS

Media

The media used in this study were M2 medium containing 0.4% bovine serum albumin (BSA) (Quinn et al., 1982), m-TALP-4 medium containing 1.5% BSA (Cummins and

Yanagimachi, 1986) and m-TALP-7 medium containing 1.2% BSA (Uto, et al., 1988). Albumin was added to these media immediately before use. These media had a pH value of approximately 7.4 when they were kept under mineral oil (gas phase, air) during experiments.

Animals

Golden hamsters (Mesocricetus auratus) were raised and maintained in an air-conditioned room with controlled light conditions (light, 05:00-19:00 hr; dark, 19:00-05:00 hr). Under these conditions, mature females came into behavioral estrus around 18:00 hr of every fourth day (day 4) and ovulated early next morning (01:00-02:00 hr of day 1). An average of 11 ± 1.7 (Mean ± SD) eggs are ovulated in each cycle. The age of the females used this study was 2-4 months, and that of the males was 4-6 months.

Collection and examination of ovarian and oviductal eggs

The ovarian eggs shortly before natural ovualtion were collected by sacrificing females between 23:30-24:00 hr of day 4, placing isolated ovaries in M2 medium and breaking the wall of distended follicles using a sharp needle. The eggs shortly after natural ovulation were collected by flushing oviducts with M2 medium beteween 05:30 and 06:00 hr of day 1. Thus, these ovarian and oviductal eggs were considered to be approximately 1.5-3.0 hr before and

2.5-5.0 hr after ovulation, respectively. Superovulation was induced by intramuscular injection of pregnant mare serum gonadotropin (30 IU/female; Calbiochem, La Jolla, CA) in the morning of day 1 followed by injection of human chorionic gonadotropin (HCG) (30 IU/female; Organon, West Orange, NJ) in the afternoon or evening of day 3. Ovulation took place between 13 and 14 hr after HCG injection. An average of 50 eggs were ovulated in each female. We collected ovarian eggs at 13 hr after HCG injection when 98% of enlarged follicles were still "unruptured." Oviductal eggs were flushed out of oviducts 17 hr after HCG injection. Thus, these ovarian and oviductal eggs were considered to be within 1 hr before and 3-4 hr after superovulation, respectively. Karyologically, all the ovarian and oviductal eggs used in this study were at the metaphase of the second meiotic division.

To examine the arrangement of cumulus cells around the egg, the cumuli containing eggs were mounted between a slide and coverslip supported by four pillars of Vasoline-paraffin mixture, compressed strongly under the coverslip, and examined with a phase-contrast microscope. To examine the details of the zona pellucida and the egg itself (the vitellus), eggs were freed from cumulus cells by treating them for 5 min (at 25-30^{o}C) with 0.1% bovine testicular hyaluronidase (300 USP U/mg; ICN Pharmaceut., Cleveland, OH) in M2 medium. The eggs were rinsed with enzyme-free M2 medium and mounted between the slide and coverslip. Care

was taken not to compress the eggs under the coverslip. Measurements and photography of the zona pellucida, perivitelline space and vitellus were made within 15 min after isolation of eggs from the ovary or oviduct. Since the diameter and volume of the vitellus was altered by the osmolality of medium, every precaution was taken to avoid evaporation of M2 medium.

Examination of the susceptibility of cumulus oophorus to hyaluronidase and capacitating spermatozoa

Each ovarian egg was surrounded by a cumulus oophorus, whereas oviductal eggs were in a large mass of cumulus. The "sticky" cumulus matrix of the ovulatated eggs must have united into a large mass while the eggs were moved around by a peristaltic movement of the oviduct. To compare the susceptibility of the cumulus matrix of ovarian and oviductal eggs to hyaluronidase, it was necessary to reduce the thickness of the cumulus of oviductal eggs close to that of the cumulus surrounding an ovarian egg. This was done by flushing a cumulus mass through the utero-tubal junction. A blunt-ended fine needle (28-30 gauge) was inserted into the infundibulum and a cumulus mass in the ampulla was flushed out through the utero-tubal junction. A string-like cumulus, containing eggs, was collected. The "string" was cut into several pieces before exposure to 0.1% bovine hyaluronidase in M2 medium. The enzyme treatment was performed at room temperature (23-25^{o}C) with constant

agitation of the medium. The time needed for the complete "denudation" of all eggs was recorded.

The susceptibility of the cumulus to capacitating spermatozoa was tested as follows. The cumuli containing either ovarian or oviductal eggs (see above) were transferred into 300 ul of m-TALP-4 medium which had been previously placed under mineral oil (Squibb and Sons, Princeton, NJ) in a plastic petri dish. A drop (40 ul) of sperm suspension which had been incubated for 1 hr in m-TALP-4 medium (see: Insemination and examination of eggs) was added to the drop containing cumuli and mixed thoroughly so that the concentration of spermatozoa was 1-2 $x10^5$ cells/ml. The preparations, incubated at 37-38^{o}C, were examined from time to time until cumulus cells had been dispersed completely.

"Staining" of eggs with Janus green

Janus green is known as a vital stain for mitochondria. While trying to stain mitochondria and cortical granules of hamster eggs (unpublished experiments), it was found that this dye "stains" the inner half of the zonae pellucidae of oviductal eggs. Ovarian and oviductal eggs, which had been freed from cumulus cells by hyaluronidase treatment, were immersed, for 10 min, in 0.9% NaCl solution containing 0.03% Janus green B (Allied Chemical, Morristown, NJ) and 20 mM Tris (pH 7.4). They were rinsed thoroughly with M2 medium, mounted between a slide and coverslip, and examined

immediately with ordinary optics. Immediate examination was important because the staining was not permanent.

Examination of the responsiveness of eggs to Ca^{2+} ionophore

Cumulus-free ovarian and oviductal eggs were induced to activate by Ca^{2+} ionophore, A-23187 (Sigma Chemical, St. Louios, MO), using a procedure similar to that used by Steinhardt, et al. (1974). Briefly, eggs were treated with 10 uM ionophore in Ca^{2+}/Mg^{2+}-free M2 medium for 3 min, rinsed thoroughly and kept in the same medium but without ionophore for 30 min, then transferred into regular M2 medium. An egg was recorded activated when it had resumed meiosis. The density of cortical granules (mean number in 25 um^2) in the cortical layer of the egg was determined before and after activation of eggs.

Peparation of zona-free eggs

Some ovarian and oviductal eggs were freed from zonae pellucidae by treating them for 1-2 min with M2 medium containing 0.1% bovine pancreatic trypsin (120 TAME U/mg; ICN Pharmmaceutical, Cleveland, OH). They were thoroughly rinsed with M2 medium before use.

Examination of "viscosity" of the vitellus

The "viscosity" of the vitellus of ovarian and oviductal eggs was examined using centrifugal force. One hundred microliters of 45% Percoll (Sigma) in M2 medium was placed

in the bottom of a 400 ul plastic micro-centrifuge tube (5 X 45 mm, Heparin-lithum coated; Beckman Instruments, Palo Alto, CA), which was overlaid by 100 ul of 30% Percoll followed by 100 ul of 7.5% Percoll. M2 medium (about 50 ul) containing zona-free eggs was placed gently on the 7.5% Percoll layer and the tube was centrifuged at 9500 g using a high speed microcentrifuge (Model Z-230M, Vangard International, Neptune, NJ). During centrifugation, eggs migrated to the 30% Percoll layer, the vitellus elongated and, in time, divided into heavy and light halves. The minimum time needed for complete separation of all vitelli into heavy and light halves was determined. All procedures were carried out at room temperature (23-25^{o}C).

In vitro culture of ovarian eggs

Ovarian eggs were cultured in various media for 4 hr to see if the properties of the zona and ooplasm were altered by these media. The media tested include: (a) M2 medium supplemented with 10% heat-intactivated (60^{o}C, 30 min) hamster serum, (b) Ham's F10 medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% heat-inactivated hamster serum, (c) M2 medium with oviduct extract, and (d) ampullary fluid and (e) isthmic fluid of the hamster oviduct.

Oviduct extract was prepared as follows. Oviducts were collected from females 17 hr after HCG injection. After eggs were removed from the ampullae, the entire oviducts

(the ampulla and isthmus) were frozen at -20°C. For one experiment, 20 frozen oviducts were thawed and homogenized in 1.5 ml of M2 medium. The homogenate was centrifuged at 400 g for 10 min to sediment the tissue debris. The supernatant was collected and its pH was adjusted to 7.3-7.4. Ampullary and isthmic fluids were collected as follows. Between 13 and 14 hr before expected time of ovulation, females were anesthetized with trifluoroethane vapor (Sigma) and the oviducts were exposed through dorsolateral incisions. To collect ampullary fluid, the oviduct was ligated at the infundibulum and the ampullary-isthmic junction. A thin nylon surgical suture (6-0, American Cyanamid Co., Danbury, CT) was used for the ligation.

To collect the isthmic fluid, the ampullary-isthmic junction and the utero-tubal junction were ligated. Oviductal fluid (a mixture of the ampullary and isthmic fluids) was collected by ligating the infundibulum and the utero-tubal junction. Every precaution was taken to avoid blocking of blood supply to the oviduct. Sixteen to 17 hr after the surgery (i.e., 3-4 hr after ovulation), the ampulla and/or the isthmus was/were distended, depending on the ligation sites. From a distended ampulla, 20-30 ul of fluid (ampullary fluid) could be collected. From a distended isthmus, 10-15 ul of fluid (isthmic fluid) could be collected.

Ovarian eggs (surrounded by the cumulus oophorus) in 10 ul of M2 medium were transferred into (a) 300 ul of M2 medium with 10% serum, (b) 300 ul of Ham F-10 medium with 10% serum, (c) 300 ul of M2 medium with oviduct extract, (d) 60 ul of ampullary fluid, and (e) 40 ul of isthmic fluid, all of which were previously placed under mineral oil in plastic petri dish. Each test medium received 10-12 oocytes. Preparations were incubated under either pure air (a,c) or 5% CO_2 in air (b,d,e). At the end of a 4 hr incubation at 37°C, the morphology of the eggs as well as the responsiveness of eggs to Ca^{2+} ionophore and centrifugal force were examined.

Examination of eggs collected from ligated oviducts

Between 13 and 14 hr before the expected time of ovulation, females were anesthetized and oviducts were ligated as described above, but at the first, second or last loop of the ampulla (cf. Plate VI, figure 6). The females were killed 17 hr later (i.e., 3-4 hr after ovulation) to examine the morphology of eggs collected from ligated ampullae.

Insemination and examination of eggs

Spermatozoa, collected from the cauda epididymis, were incubated in m-TALP-4 medium (Cummins and Yanagimachi, 1986). The concentration of spermatozoa during incubation was 1-2 $x10^6$ cells/ml. Under these conditions,

spermatozoa began to undergo spontaneous acrosome reactions about 2 hr after the start of incubation, and the majority completed the reaction during next 2 hr (Cummins and Yanagimachi, 1986). All the eggs to be inseminated were freed from cumulus cells by hyaluronidase treatment. Some of them were further treated with trypsin to remove their zonae pellucidae. The eggs were thoroughly rinsed with M2 medium, and transferred to an aliquot (300 ul) of m-TALP-4 medium which had been previously placed under mineral oil in a plastic petri dish. Insemination was performed by adding 20-40 ul of the sperm suspension to the medium containing eggs. Zona-intact eggs were inseminated with 1 hr pre-incubated spermatozoa, incubated at 37^{o}C and examined for zona penetration at 1 hr intervals. Zona-free eggs were inseminated with 4 hr preincubated spermatozoa and examined at hourly intervals for cortical granule exocytosis, the resumption of meiosis, and development of sperm and egg pronuclei.

Assessment of the acrosome reaction-inducing ability of the zona pellucida

The ability of the zona pellucida to induce the acrosome reaction of spermatozoa was determined using the procedure described by Uto, et al. (1988). Briefly, cumulus-free eggs with intact zonae were inseminated with spermatozoa which had been incubated for 2 hr in m-TALP-7 medium. Five minutes after insemination, when 30-50 spermatozoa had

attached to each egg (zona), eggs were rinsed and transferred into fresh m-TALP-7 medium. Incubation was carried out at 37-38^{o}C. The incidence of the acrosome reaction in lively motile spermatozoa on the zona was determined at 30 min intervals.

Statistical analysis

Where appropriate, statistical analysis was made using Student's t-test for paired samples.

RESULTS

Since my preliminary study revealed that naturally ovulated and superovulated eggs were identical in morphology and several physiological characteristics (e.g., response to spermatozoa and Ca^{2+} ionophore), data obtained from these two groups of eggs were combined. However, most information reported here was obtained from superovulated (superovulating) eggs.

Difference in the cumulus oophorus

The cumulus cells surrounding an ovarian egg were tightly and radially arranged (Plate I, figure 1A), whereas those around an oviductal egg were loosely and randomly arranged (Plate I, figure 1B). When exposed to 0.1% hyaluronidase, most of the cumulus cells surrounding the ovarian egg were dispersed rapidly except those on the zona

pellucida. It took 3.6 ± 0.5 min (Mean ± SD, n=24) before all cumulus cells were dispersed. The cumulus cells around the oviductal egg were dispersed by hyaluronidase much more readily than those surrounding the ovarian egg. All the oviductal eggs became free of cumulus cells in 2.2 ± 0.3 min (n=25). This difference was significant ($p<0.01$). When exposed to capacitating spermatozoa (1-2 $x10^5$ cells/ml), the cumulus cells surrounding oviductal eggs dispersed faster (37.5 ± 7.5 min, n=30) than those surrounding ovarian eggs (67.5 ± 7.5 min, n=30). This difference was also significant ($p<0.01$).

Differences in the general structure of the eggs

Examination of 24 ovarian eggs and 25 oviductal eggs revealed that the diameter of the vitellus of the ovarian egg was significantly larger (81 ± 1.3 um) than that of the oviductal egg (78 ± 1.0 um) ($p<0.01$). The thickness of the zona was about the same in the ovarian egg (11 ± 0.5 um) and oviductal egg (12 ± 0.7 um) ($p>0.05$). Partly because of an expansion of the zona and partly because of a shrinkage of the vitellus, the oviductal egg had a considerably larger perivitelline space (150 ± 6.2 $x10^3$ um^3) than the ovarian egg (64 ± 10.5 $x10^3$ um^3) ($p<0.001$) (Plate I, figure 1C) (Table 2).

Differences in the zona pellucida

There was a distinct difference between ovarian and oviductal eggs with respect to the appearance of the zona pellucida. When viewed with the phase-contrast microscope equipped with positive-contrast optics, the outer half of the zona of the oviductal egg appeared very bright, whereas its inner half appeared very dark (Plate I, figure 1D, top). Such an optical heterogeneity was not detected in the zona of ovarian egg (Plate I, figure 1D, bottom). The heterogeneity of the zona of the oviductal egg could also be appreciated when the zona was examined through a microscope with regular optics after the staining of the egg (zona) with Janus green B. When oviductal eggs were immersed in 0.03% solution of Janus green for 10 min, and then washed thoroughly, the inner half of the zona appeared dark blue (Plate I, figure 1E, top). The zona of the ovarian egg reduced its thickness and remained unstained except for its inner surface (Plate I, figure 1E, bottom).

The zonae of the ovarian and oviductal eggs were equally sensitive to trypsin. There was no significant difference between zonae of ovarian eggs and those of oviductal eggs with respect to the miminum time needed for dissolution by trypsin. A dilute solution of trypsin (0.01%) in M2 medium completely dissolved the zonae of all of 30 ovarian and 30 oviductal eggs in 356 $\pm$ 23 sec at 23-25^{o}C.

When eggs were inseminated with 1 hr preincubated spermatozoa, sperm penetration through the zona took place

faster in oviductal eggs than in ovarian eggs (Plate II, figure 2). The zonae of oviductal eggs appeared to have a stronger acrosome reaction-inducing ability than those of ovarian eggs (Plate III, figure 3).

Differences in the vitellus

Hamster eggs are activated by Ca^{2+} ionophore (Steinhardt, et al., 1974). When 78 oviductal eggs were treated with 10 uM ionophore and examined 1 hr later, 93% of them were activated as evidenced by the exocytosis of cortical granules and the presence of the female pronucleus in the ooplasm. Only 37% of 70 ovarian eggs were activated when they were treated and examined under the same conditions, indicating that the ovarian eggs were less responsive to the ionophore than the oviductal eggs (Table 3).

When 32 zona-free ovarian and 44 zona-free oviductal eggs were inseminated with acrosome-reacted spermatozoa and examined 1 hr later, all of of them contained decondensing sperm nuclei and/or early sperm pronuclei. The ratio of the decondensing sperm nuclei and the early sperm pronuclei was 1:9 in both ovarian and oviductal eggs, indicating that sperm nuclei transform into pronuclei at about the same rate in these 2 groups. The situation was different for egg nuclei. At 1 hr after insemination, 90% of the 32 ovarian eggs were at the telophase of the second meiosis, whereas 91% of the 44 oviductal eggs were in the early pronuclear stage. Apparently, after activation by spermatozoa, meiosis

was completed more quickly in oviductal than in ovarian eggs (Table 4).

When zona-free oviductal eggs (Plate IV, figure 4A) were centrifiged at 9500 g, they elongated (Plate IV, figure 4 B,C) and then divided into light and heavy halves (Plate IV, figure 4D). It took 594 $\pm$ 13 sec before all of the oviductal eggs were divided into two halves (6 experiments using 120 eggs). Ovarian eggs took a significantly ($p<0.01$) longer time (637 $\pm$ 10 sec) to be divided under the same conditions (6 experiments using 100 oocytes). Apparently, the ooplasm of ovarian oocytes is more viscous than that of ovidcutal eggs.

Effects of in vitro culture of ovarian eggs on the characteristics of the zona pellucida and vitellus

As stated above, ovarian eggs were different from oviductal eggs in several morphological and physiological characteristics. What are the causes of these differences? Chronologically, the oviductal eggs we used were 3-4 hr older than ovarian eggs. If time alone is solely or primarily responsible for the differences, ovarian eggs should become like oviductal eggs when they are cultured in vitro for 3-4 hr. Table 1 summarizes the results of experiments in which ovarian eggs (with surrounding cumulus cells) were cultured for 4 hr in various media. When cultured in M2 and Ham F10 media supplemented with hamster sera (Experiments A and B), the cumulus oophorus expanded,

but the egg itself remained morphologically unchanged. Its perivitelline space remained small and the zona pellucida retained its optical homogenity. The vitellus, however, did become more responsive to the Ca^{2+} ionophore. The vitellus also became less viscous after 4 hr of culture. In other words, the vitelli of ovarian eggs became somewhat like those of oviductal eggs, whereas their zonae and the perivitelline space remained unaltered. It can be seen from Table 1 that both the zonae and vitelli of ovarian eggs became like those of the oviductal eggs when they were cultured in the medium containing an oviduct extract (Experiment C) or in the ampullary and isthmic fluids (Experiments D and E). Plate V, figure 5 summarizes the results of experiments in which ovarian eggs were cultured for 4 hr in vitro in the presence or absence of oviductal fluid. When cultured in M2 medium supplemented with hamster serum, the zonae of ovarian eggs increased their acrosome reaction-inducing ability slightly, but not significantly. The ability increased significantly when eggs were cultured in the oviductal fluid.

<u>When and where are the characteristics of the zonae of in vivo ovulated eggs altered</u> ?

The eggs released from the ovary stay on the ovarian surface and in the ovarian bursa for some time before they are picked up by the infundibulum (fimbria). All the eggs we collected from the bursa appeared exactly like ovarian

eggs with a small perivitelline space and an optically homogeneous zona pellucida. The eggs which were believed to have been in the ampulla of the oviduct for about 1 hr had a large perivitelline space and an optically heterogeneous zona, just like those which had been in the ampulla for 3-4 hr or more. When the ampulla was ligated at its first or second loop 13-14 hr before ovulation and the eggs were collected and examined 3-4 hr after ovulation (Plate VI, figure 6A,B), 62-73% of the eggs had the typical appearance of oviductal eggs with a large perivitelline space and an optically heterogenous zona. The remaining eggs looked like the typical ovarian eggs (9-22%) or were in intermediate stages (16-18%). When the third loop of the ampulla was ligated (Plate VI, Figure 6C), all the eggs had the typical appearance of the oviductal eggs, just like those collected from unligated oviducts (Plate VI, figure 6D).

DISCUSSION

Maturation of the cumulus oophorus

In some species, eggs are penetrated by spermatozoa almost immediately after entering the oviduct, while in other species eggs stay in the oviduct for some time before they are penetrated by spermatozoa (cf. Braden, 1962). In the mouse, the time interval between ovulation and sperm penetration is 3-5 hr in naturally ovulating females, and it is 1-3 hr in females ovulating following treatments with

exogenous gonadotropins (Braden, 1962). Braden (1962) postulated that mouse cumuli undergo a "maturation" in the oviduct before they become penetrable by spermatozoa. In naturally ovulating hamsters, eggs stay in the oviduct (ampulla) for at least 1.5-2 hr before they are penetrated by spermatozoa (Austin, 1956; Strauss, 1956; Yanagimachi, 1966). The hamster cumulus may not require maturation in the oviduct, because, according to Corselli and Talbot (1986), the cumuli of ovarian eggs collected shortly before ovulation are readily penetrable by spermatozoa in vitro. In the hamster, the time interval of 3 hr between ovulation and the onset of sperm penetration must represent the minimum time needed for the spermatozoa to migrate from the lower segment of the oviduct to the ampulla where eggs and spermatozoa meet (Smith et al., 1987).

It is well established that an expansion of the cumulus or the synthesis of hyaluronic acid by cumulus cells is triggered by the preovulatory gonadotropin surge (Eppig, 1979, 1980). The expansion begins from the periphery of the cumulus and proceeds inward (Eppig, 1982). In the hamster, the cumulus expands during and even after ovulation (Talbot and DiCarlantonio, 1984). The fact that the cumulus matrix surrounding the oviductal hamster egg is more susceptible to hyaluronidase than that of the ovarian egg seems to support the notion that the expansion of the cumulus or the synthesis of hyaluronic acid continues even after ovulation.

Maturation of the vitellus

We have confirmed the previous report that the vitellus of hamster egg shrinks slightly after ovulation, whereas the perivitelline space expands considerably after ovulation (Yanagimachi and Chang, 1961). Partial exocytosis of cortical granules (Okada et al., 1986) and accumulation of oviductal glycoproteins in the perivitelline space (Kapur and Johnson, 1986) must be resposible, at least in part, for such postovulatory changes of the egg.

It is interesting that sperm nuclei incorporated into the vitelli of ovarian and oviductal eggs decondensed and transformed into pronuclei at about the same rate, whereas egg nuclei completed their meiosis at different rates. The facts that ovarian eggs (a) complete meiosis slower than oviductal eggs and (b) respond to Ca^{2+} ionophore less efficiently than oviductal eggs seem to suggest that the ooplasm of the ovarian egg is not as mature as that of the oviductal egg. The relatively high viscosity of the vitellus of the ovarian egg may be another indication of the immaturity of the ovarian egg.

The vitellus of the ovarian egg appears to be able to mature without contribution from the oviduct. The ovarian eggs cultured in relatively simple media without any oviductal factors became like oviductal eggs as far as responsiveness to Ca^{2+} ionophore and the viscosity of the vitellus are concerned (cf. Table 1).

Changes in the zona pellucida

According to Fowler and Grainge (1985), the glycosaminoglycans content of the mouse zona pellucida reduces drastically during the several hours proceeding ovulation. There is convincing evidence that glycoproteins of oviductal origin are integrated into the zonae of hamster and porcine eggs after the eggs have been transferred from the ovary to the oviduct (Fox and Shivers, 1975; Brown and Cheng, 1986; Oikawa, et al., 1986, 1988; St-Jacques, et al., 1986; Araki, et al., 1987; Hedrick, et al., 1987; Leveille, et al., 1987). As reported here, the zona pellucida of the hamster egg changes its optical properties and affinity for Janus green rather dramatically after exposure to the oviduct or culture in vitro in the presence of oviductal fluid. This is probably due to an integration of oviductal glycoproteins into the intrinsic zona glycoproteins. According to Leveille, et al. (1987), the epithelium of the hamster isthmus secretes more oviductal immunogens (glycoproteins) than the ampulla and infundibulum. The results of these experiments seem to support this view. However, the ampulla alone seems to able to secrete enough oviductal factors (glycoproteins) to alter the zona (cf. Table 1). It is known that the hamster oviduct displays a very active adovarian peristaltic movment during the periovulatory period (Battalia and Yanagimachi, 1979). Therefore, it is possible that the isthmus fluid is

transported upward and mixed with the ampullary fluid to alter zonae synergistically.

When I examined eggs collected from the ovarian surface or from the ovarian bursa, their zonae were optically homogeneous. In other words, they were just like the zonae of ovarian eggs. By examining oviductal eggs at various times after ovulation, it is estimated that the zona changes from the optically homogeneous "ovarian type" to the optically heterogenous "oviductal type" within 1 hr after entering the oviduct. Since hamster eggs, under normal in vivo conditions, reside in the oviduct for at least 1.5 hr before meeting spermatozoa (Austin, 1956; Strauss, 1956; Yanagimachi, 1966), all eggs must have the zonae of the "ovidutal type" when they meet spermatozoa.

What is the biological function of the oviductal glycoproteins added to the intrinsic zona? Apparently, these glycoprteins are not essential for fertilization. At least in the human, mature ovarian eggs which have never been exposed to the oviductal glycoproteins can be fertilized "normally" and develop into viable offspring (Edwards, 1981; Edwards and Purdy, 1982; Trounson and Wood, 1984; Wolf and Quigley, 1984). The function of the oviductal glycoproteins could be to enhance the functions of the intrinsic zona glycoproteins. As reported previously (Uto et al., 1988) and confirmed in the present study, the zonae of oviductal eggs are more efficient than those of ovarian eggs in inducing the acrosome reaction of

spermatozoa. We have demonstrated that the zonae of ovarian eggs increase their acrosome reaction-inducing ability when they are exposed to the oviduct fluid (glycoproteins) either in vivo or in vitro. The oviductal glycoproteins integrated into the zona must work synergistically with the native zona glycoproteins in inducing the acrosome reaction. By the same token, these two groups of glycoproteins may work synergistically to achieve other fuctions of the zona, including (a) the prevention of polyspermy via the zona reaction and (b) the protection of developing preimplantation embryos.

REFERENCES

Araki, Y, Kurota, S, Oikawa, T, Yamashita, T, Hiroi, M, Nakai, M and Sendo, F (1987) A monoclonal antibody reacting with the zona pellucida of the oviductal egg but not with that of the ovarian egg of the golden hamster. J Reprod Immunol 11:193-208.

Austin, CR (1956) Ovulation, fertilization, and early cleavage in the hamster. J Roy Micro Soc 75:141-154.

Austin, CR and Walton, A (1960) Fertilisation. In: Marshall's Physiology of Reproduction, vol. 1, pt. 2. Parkes, AS (ed), Longmans, London. pp. 310-416.

Baker, TG (1982) Oogenesis and Ovulation. In: Reproduction in Mammals, vol. 1. Austin, CR and Short, RV (eds), Cambridge University Press, London. pp. 17-45.

Battalia, DB and Yanagimachi, R (1979) Enhancement and co-ordinated movement of the hamster oviduct during the periovulatory period. J Reprod Fertil 56:515-520.

Braden, AWH (1962) Spermatozoon penetration and fertilization in the mouse. Symp Genet et Biol Italica, Pavia, Italy, Sept. 1-8.

Brown, CR and Cheng, WK (1986) Changes in composition of the porcine zona pellucida during development of the oocyte to the 2- to 4-cell embryo. J Embryol Exp Morph 92:183-191.

Corselli, J and Talbot, P (1986) An in vitro technique to study penetration of hamster cumulus complexes by using physiological numbers of sperm. Gamete Res 13:293-308.

Cummins, JM and Yanagimachi, R (1986) Development of ability to penetrate the cumulus oophorus by hamster spermatozoa capacitated in vitro, in relation to the timing of the acrosome reaction. Gamete Res 15:187-212.

Edwards, RG (1981) Test-tube babies. Nature 293:253-256.

Edwards, RG and Purdy, JM (eds) (1982) Human Conception In Vitro. Academic Press, London.

Eppig, JJ (1979) FSH stimulates hyaluronic acid synthesis by oocyte-cumulus cell complxes from mouse preovulatory follicles. Nature 281:483-494.

Eppig, JJ (1980) Regulation of cumulus oophorus expansion by gonadotropins in vivo and in vitro. Gamete Res 23:545-552.

Eppig, JJ (1982) The relationship between cumulus cell-oocyte coupling, oocyte meiotic maturation, and cumulus expansion. Dev Biol 89:268-272.

Fowler, RE and Grainge, C (1985) A histochemical study of the changes in the protein-carbonhdrate composition of the cumulus-oocyte complex and zona pellucida in immature mice in response to gonadotropin stimulation. Histochem J 17:1235-1249.

Fox, LL and Shivers, CA (1975) Immunological evidence for addition of oviductal compoments to the hamster zona pellucida. Fertil Steril 26:599-608.

Hedrick, JL, Wardlip, NJ and Berger, T (1987) Differences in the macromolecular composition of the zona pellucida isolated from pig oocytes, eggs and zygotes. J Exp Zool 241:257-262.

Kapur, RP and Johnson, LV (1986) Selective sequestration of an oviductal fluid glycoprotein in the perivitelline space of mosue oocytes and embryos. J Exp Zool 238:249-260.

Leveille, MC, Roberts, KD, Chevalier, S, Chapdelaine, A and Bleau, B (1987) Uptake of an oviductal antigen by the hamster zona pellucida. Biol Reprod 36:227-238.

Oikawa, T, Kurata S and Sendai, Y (1986) Discovery of a novel zona glycoprotein (ZP-0) having a sperm binding activity. J Reprod Immunol (suppl) pp. 81.

Oikawa, T, Sendai, Y, Kurata, S and Yanagimachi, R (1988) A glycoprotein of oviductal origin alters biochemical properties of the zona pellucida of hamster egg. Gamete Res 19:113-122.

Okada, A, Yanagimachi, H and Yanagimachi, R (1986) Development of a cortical granule-free area of cortex and the perivitelline space in the hamster oocyte during maturation and following ovulation. J Submicro Cytol 18:233-247.

Quinn, P, Barros, C and Whittingham, DG (1982) Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. J Repro Fertil 66:161-168.

Smith, TT, Koyanagi, F and Yanagimachi, R (1987) Distribution and number of spermatozoa in the oviduct of the golden hamster after natural mating and artificial insemination. Biol Reprod 37:225-234.

Steinhardt, RA, Epel, D, Carroll, EJ and Yanagimachi, R (1974) Is calcium ionophore a universal activator for unfertilized eggs? Nature 252:41-43.

Strauss, F (1956) The time and place of fertilization of the golden hamster egg. J Embryol Exp Morph 4:42-56.

St-Jacques, S and Forget, A, Roberts, KD, Chevalier, S, Chapdelaine, A and Bleau, G (1986) Monoclonal antibodies specific for an ovidcutal component associated with the zona pellucia. J Reprod Immunol (suppl) p. 52.

Talbot, P and DiCarlantonio, G (1984) Architecture of the hamster oocyte-cumulus complex. Gamete Res 9:261-272.

Trounson, A and Wood, C (eds) (1984) In Vitro Fertilization and Embryo Transfer. Churchill Livingston, Edinburgh.

Uto, N, Yoshimatsu, N, Lopata, A and Yanagimachi, R (1988) The zona-induced acrosome reaction of hamster spermatozoa. J Exp Zool 248:113-120.

Wolf, DP, and Quigley, MM (eds) (1984) Human In Vitro Fertilization and Embryo Transfer. Plenum Press, New York.

Yanagimachi, R (1966) Time and process of sperm penetration into hamster ova in vivo and in vitro. J Reprod Fertil 11:359-370.

Yanagimachi, R and Chang, MC (1961) Fertilizable life of golden hamster ova and their morphological changes at the time of losing fertilizability. J Exp Zool 148:185-294.

Table 1

Changes in ovarian eggs cultured in vitro for 4 hr

Eggs (Exp.)	Vol. of perivit. space (um^3)	Optical heterogeneity of zona	% Egg activated by Calciume ionophore	Time (sec) needed for division of vitellus by centrifugation
Ovarian eggs not cultured (control)	64 ± 11	-	37	637 ± 10
Ovarian eggs cultured in:				
(A) M2 with 10% serum	70 ± 2	-	86	622 ± 14 ($p < 0.05$)
(B) Ham F-10 with 10% serum	63 ± 2	-	77	N.D.
(C) M2 with oviduct extract	128 ± 3	+	92	N.D.
(D) Ampullary fluid	134 ± 4	+	87	614 ± 12[e] ($p<0.01$)
(E) Isthmic fluid	140 ± 3	+	86	N.D.
Oviductal eggs, not cultured	150 ± 6	+	93	594 ± 13 ($p < 0.001$)

Legend to Table 1

N.D., no data

[a] Each value is based on measurement of 24 eggs collected from 6 different females (4 eggs/female).

[b] The appearance of the zona under the phase-contrast microscope with positive contrast optics. In oviductal eggs, the outer half of the zona appears very bright in contrast to the relatively dark inner half, indicating optical heterogenity (+) of the zona (cf. Plate I, figure 1D, top). The zona of ovarian eggs freshly collected from the ovary appears uniform thoughout its depth (cf. Plate I, fig. 1D, bottom).

[c] Examined 1 hr after ionophore treatment (cf. Materials and Methods). Each value is based on 3-7 experiments using a total of 22-70 eggs.

[d] Time needed for complete separation of vitelli of all eggs into light and heavy halves (cf. Materials and Methods). Each value is based on 6-7 experiments using a total of 100-120 eggs.

[e] The fluid used in this centrifugation experiment was a mixture of the ampullary and isthmic fluids, which was collected after ligating the infundibulum and utero-tubal junction of oviduct (cf. Materials and Methods).

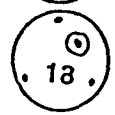
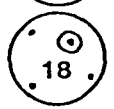
Table 2

Dimensional differences in ovarian and oviductal eggs (numbers are mean ± S.D.)

	Diameter of zona pellucida (u)	Diameter of vitellus (u)	Diameter of zona cavity (u)	Thickness of zona pellucida (u)	Volume of perivitelline space (10^3u^3)
Ovarian eggs	109±1.8	81±1.3	87±1.5	11±0.5	64±10.5
Oviductal eggs	115±1.5	78±1.0	92±1.3	12±0.7	150±6.2
	p<0.01	p<0.01	p<0.01	N.S.	p<0.001

Table 3

NUCLEAR AND CYTOPLASMIC ACTIVATION OF OVARIAN AND OVIDUCTAL OOCYTES BY CA IONOPHORE

	Hrs post-treatment	Nucleus unactivated		Nucleus activated	
		% of egg	% of C.G. remained	% of egg	% of C.G. remained
Ovarian Oocytes	0	100	100	0	
	1	63	35	37	19
	3	58	30	42	 18
Oviductal Oocytes	0	100	100	0	
	1	7	30	93	17
	3	6	29	94	 18

C.G. : Cortical granule

Table 4

CHANGES IN SPERM AND EGG NUCLEI AFTER THE PENETRATION OF SPERM INTO OVARIAN AND OVIDUCTAL OOCYTES

	Hrs post-Inseml.	% of sperm nuclei					% of egg nuclei				
Ovarian Oocytes	1	–	10	90	–	–	6	90	4	–	–
	2	–	9	71	21	–	–	2	67	17	14
	3	–	3	62	35	–	–	3	6	77	14
Ovlductal Oocytes	1	–	9	89	2	–	–	11	82	7	–
	2	–	–	72	28	–	–	–	13	54	33
	3	–	–	52	46	2	–	–	–	15	85

PLATE I

COMPARISON OF OVARIAN AND OVIDUCTAL EGGS

Figure 1.

A. Ovarian eggs and cumulus cells, phase-contrast: note that cumulus cells are tight and radially arranged.

B. Oviductal eggs and cumulus cells: cumulus cells are loose and randomly arranged.

C. Eggs seen through regular optics: oviductal egg has a larger perivitelline space than ovarian egg; zonae appear the same.

D. Oviductal and ovarian eggs seen through phase-contrast optics: note an optical heterogeneity of the zona of oviductal egg.

E. Oviductal and ovarian eggs seen through regular optics after staining with Janus green: note that the inner half of the zona of oviductal egg is stained dark; the zona of ovarian egg has reduced its thickness and is unstained except its inner surface (an arrow in the inset indicates that the inner surface of the zona is darkly stained).

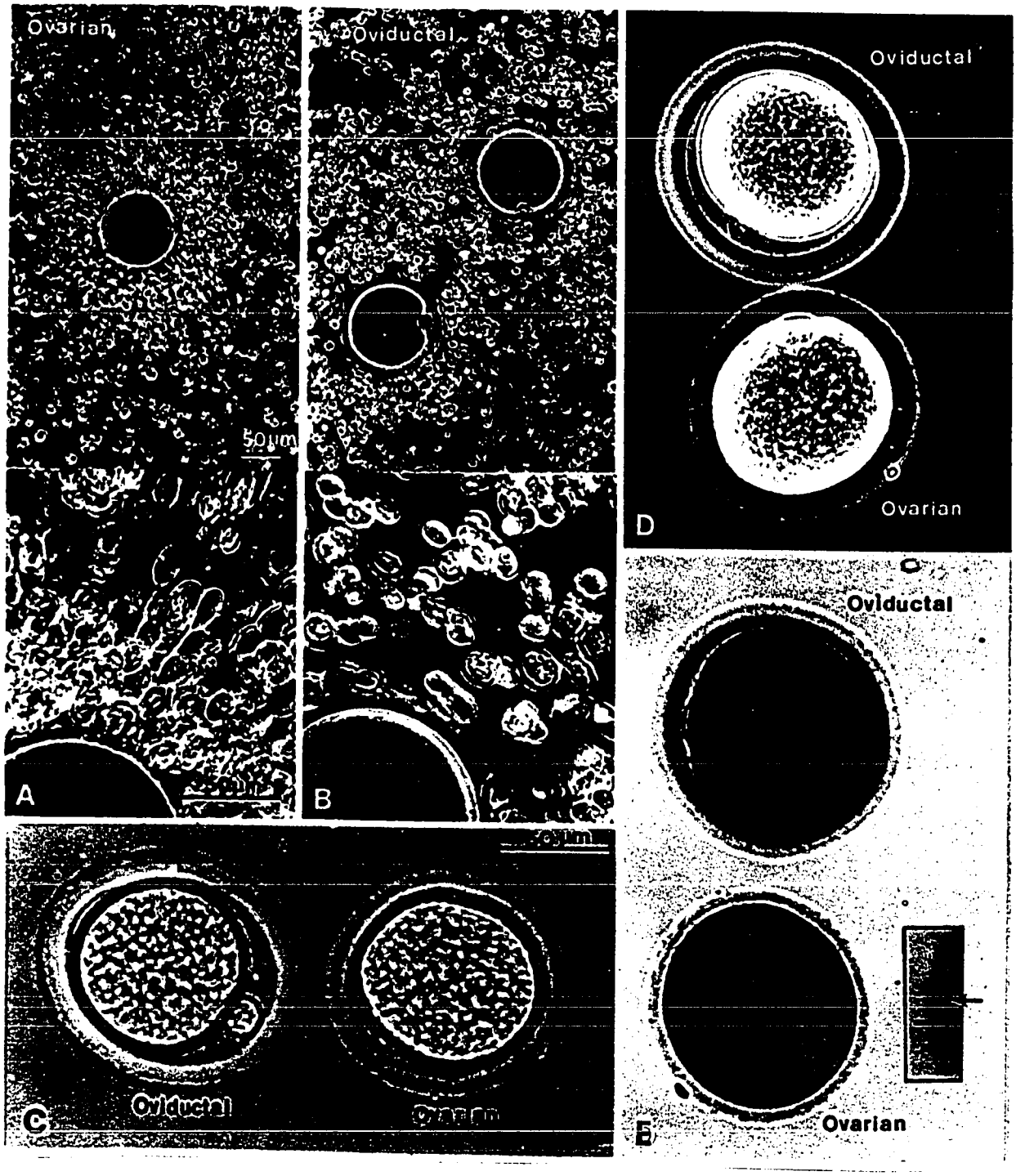

Ovarian
50 µm
A
Oviductal
B
Oviductal
Ovarian
D
Ovarian
Oviductal
C
Ovarian
E

PLATE II

PROPORTION OF PENETRATED EGGS AT VARIOUS TIMES AFTER IN VITRO INSEMINATION

Figure 2.

Oviductal and ovarian eggs were inseminated in vitro with 1 hr preincubated spermatozoa and examined for zona penetration. Those with sperm heads (nuclei) either in the perivitelline space or the vitellus were recorded "penetrated". Each value is based on examination of about 40 eggs (4 experiments).

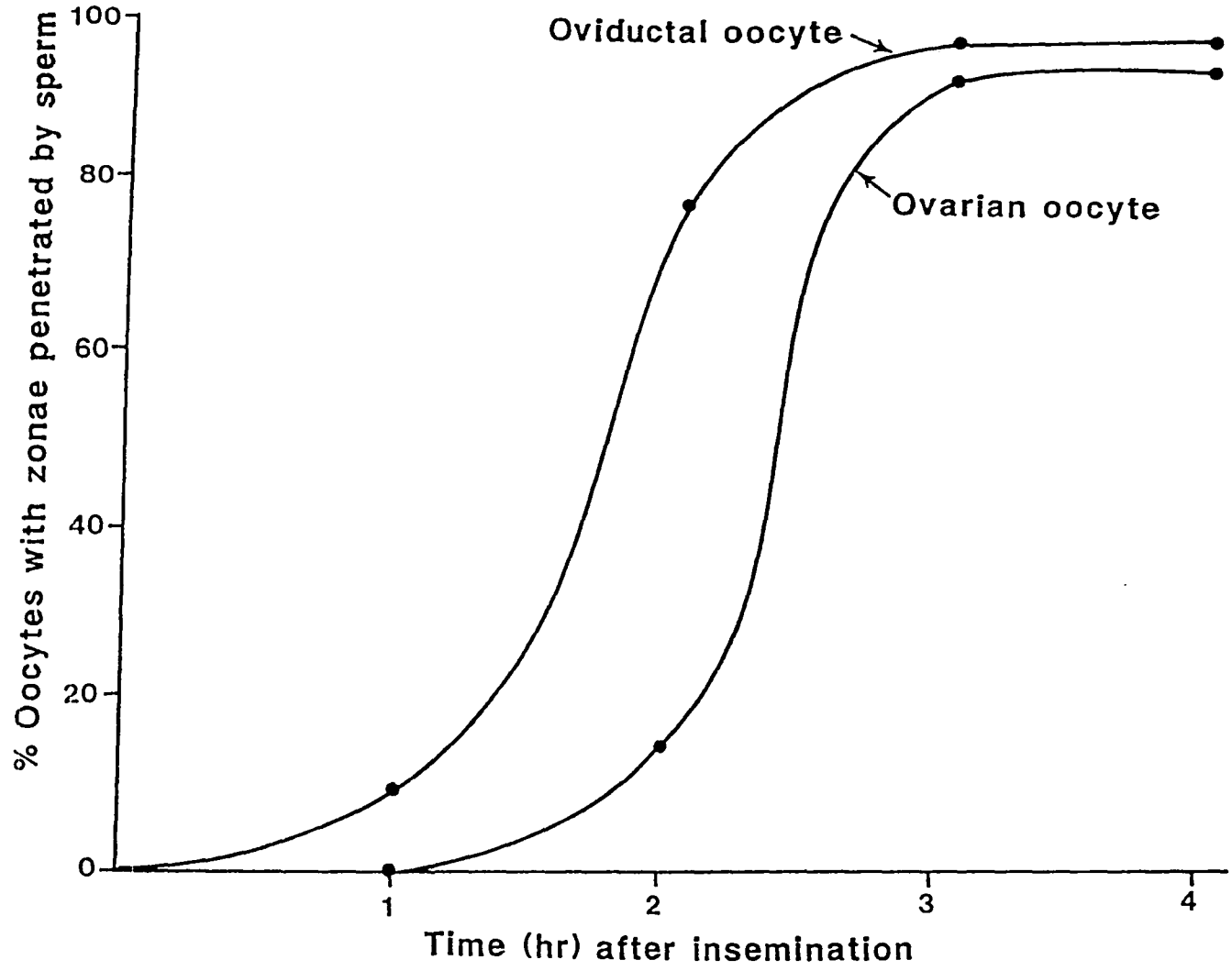

% Oocytes with zonae penetrated by sperm
100
80
60
40
20
0
1
2
3
4
Time (hr) after insemination
Oviductal oocyte
Ovarian oocyte

PLATE III

PROPORTION OF ACROSOME-REACTED SPERMATOZOA ON THE SURFACE OF THE ZONA PELLUCIDA

Figure 3.

Ovarian (A) and oviductal eggs (B) were inseminated in vitro with 2 hr preincubated spermatozoa and the incidence of acrosome-reacted spermatozoa on the zona was determined. Each value (Mean ± S.D.) is based on examination of 700-1200 spermatozoa on 25-40 zonae (5-6 experiments). (C) Incidence of spontaneous acrosome reaction in m-TALP-7 medium.

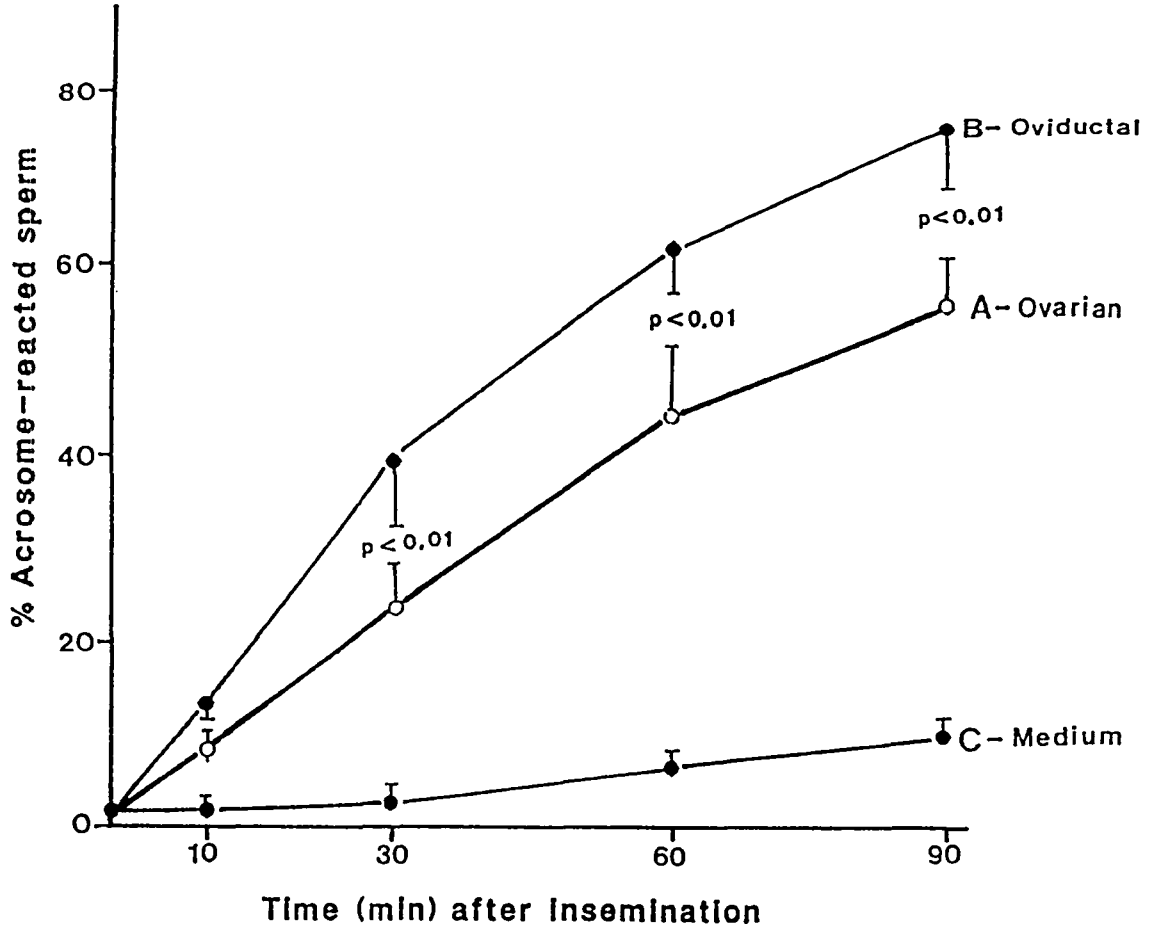

% Acrosome-reacted sperm
80
60
40
20
0
10
30
60
90
Time (min) after Insemination
B- Oviductal
A- Ovarian
C- Medium
p<0.01
p<0.01
p<0.01

PLATE IV

SEPARATION OF ZONA-FREE EGG INTO LIGHT AND HEAVY HALVES BY CENTRIFUGATION

Figure 4.

A. Zona-free hamster egg before centrifugation.

B-C. Elongated zona-free hamster egg after centrifugation

D. Zona-free hamster egg separated into light and heavy halves after centrifugation.

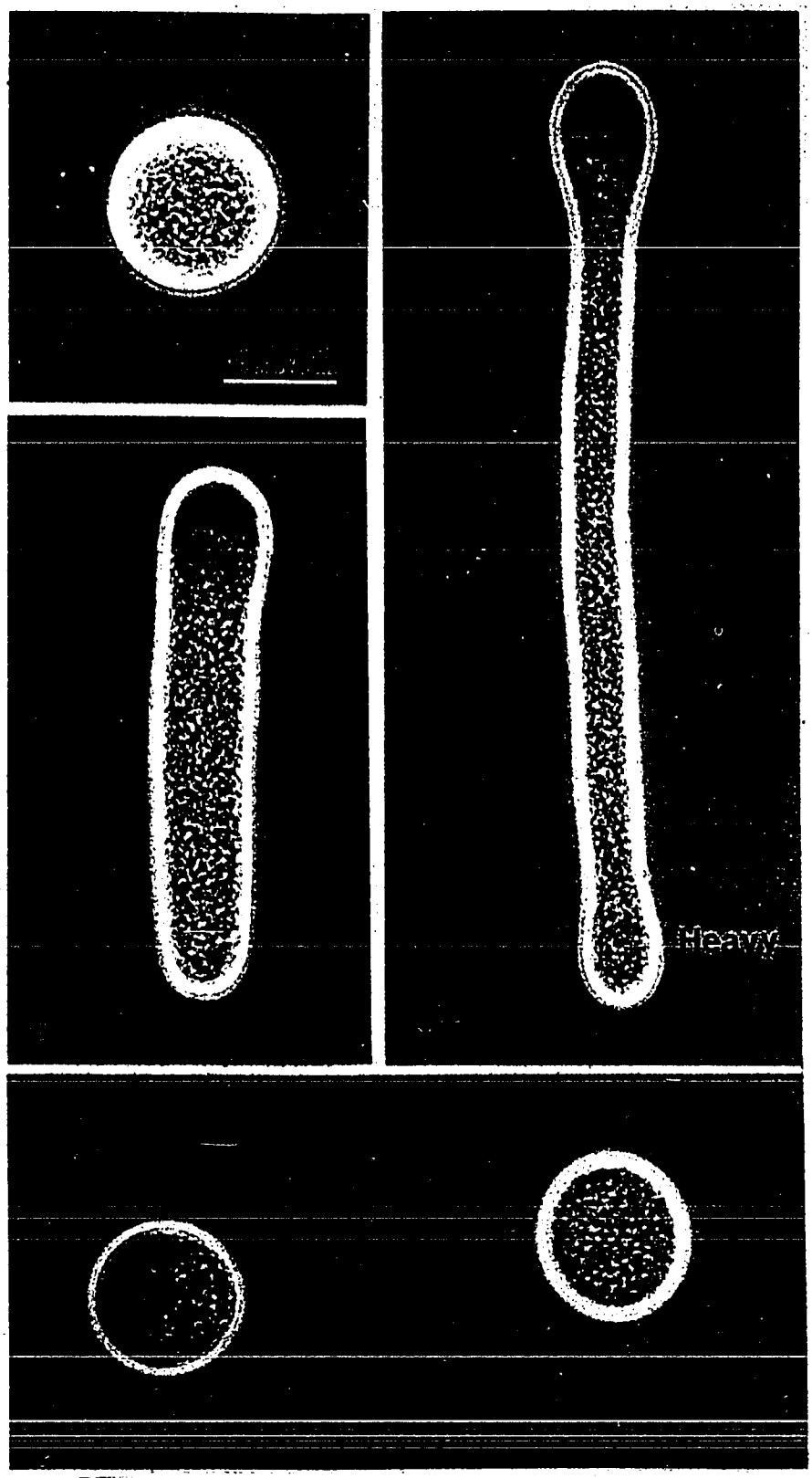

Heavy

PLATE V

INCIDENCE OF THE ACROSOME REACTION OF SPERMATOZOA ON THE ZONA SURFACE OF OVARIAN EGGS

Figure 5.

A. Zonae of eggs not cultured.

B. Zonae of eggs cultured for 4 hr in M2 medium with 10% hamster serum.

C. Zonae of eggs cultured 4 hr in oviductal fluid (a mixture of ampullary and isthmic fluid) (cf. Materials and Methods).

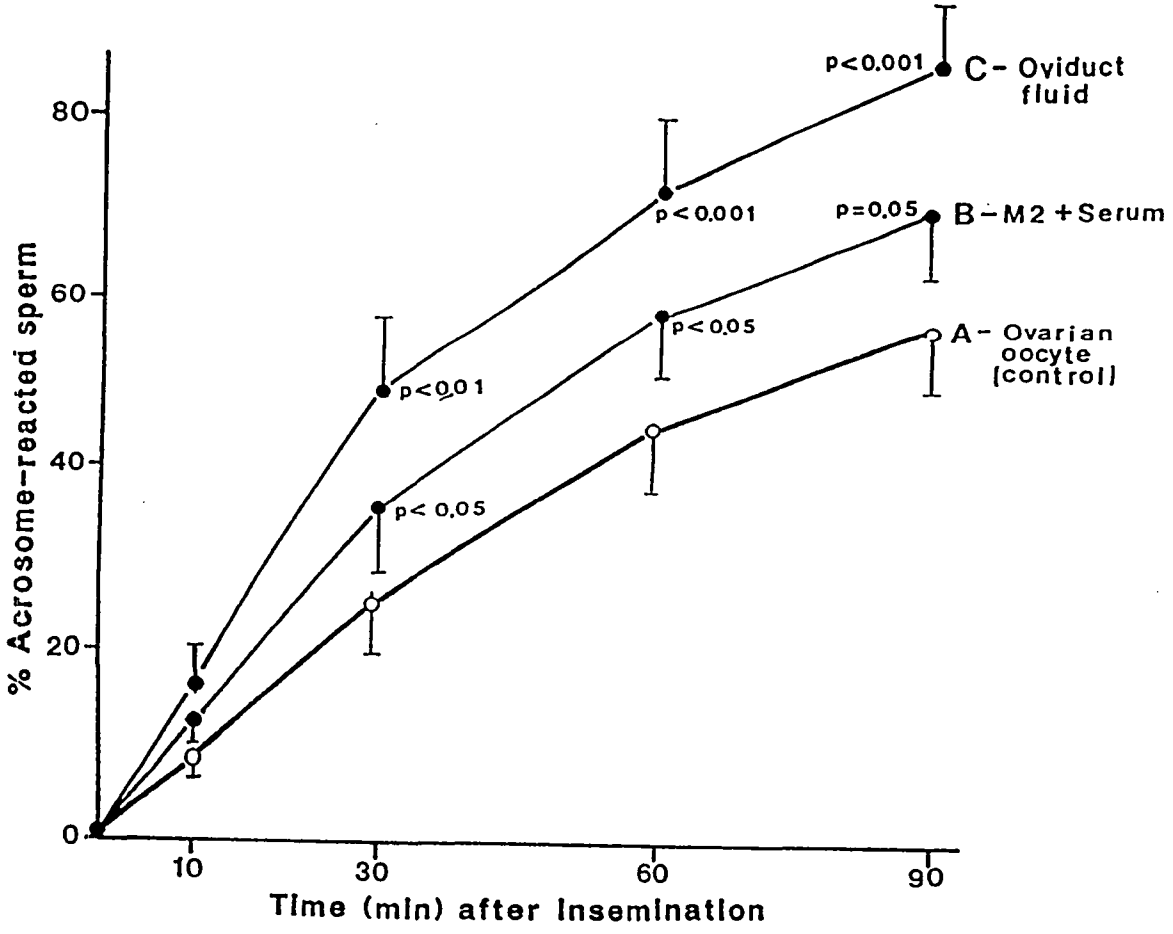

% Acrosome-reacted sperm
0
20
40
60
80
10
30
60
90
Time (min) after Insemination
p<0.001
C- Oviduct fluid
p<0.001
p=0.05
B-M2 +Serum
p<0.05
A- Ovarian oocyte (control)
p<0.01
p<0.05

PLATE VI

RELATIONSHIPS BETWEEN THE SITE OF AMPULLA LIGATION (13-14 HR PRIOR TO OVULATION) AND THE APPEARANCE OF EGGS COLLECTED 3-4 HR AFTER OVULATION

Figure 6.

A. Ligation of first loop of ampulla.

B. Ligation of second loop of ampulla.

C. Ligation of third loop of ampulla.

D. No ligation (control).

Sector diagrams on the right illustrate the percentage of eggs with typical and intermediate appearances of ovarian and oviductal eggs. Each datum is based on examination of 160-200 eggs collected from four superovulated females.

OOCYTES
Ovarian type
Intermediate
Oviductal type
100%
100%
C
D
62%
22
15
73%
9
18
A
B

CHAPTER III

MORPHOLOGY AND FERTILIZABILITY OF ZONA-FREE HAMSTER EGGS SEPARATED INTO HALVES AND QUARTERS BY CENTRIFUGATION

Sea urchin and starfish eggs are surrounded by a very thin vitelline envelope. When they are centrifuged in suspension, each egg elongates and then separates into a heavy and a light half (e.g., Harvey, 1936; Maruyama, et al., 1986). Mammalian eggs with a thick vitelline envelope (zona pellucida) do not separate into halves under the same conditions, although cellular inclusions become stratified according to their specific gravities (e.g., Wall, et al., 1985; Cran, 1987; Tellez, et al., 1988). This study reports that mammalian (hamster) eggs free from zonae pellucidae are readily separated into light and heavy halves by centrifugation. Each half can be separtaed into two quarters by additional centrifugation. It was of prime interest to find out how these halves and quarters respond to spermatozoa.

MATERIALS AND METHODS

Medium

The media used in this study were M2 containing 0.4% bovine serum albumin (BSA) (Quinn, et al., 1982), and m-TALP-4 containing 1.5% BSA (Cummins and Yanagimachi, 1986). BSA was added to these media immediately before use.

The pH of media was adjust to 7.4 by adding a small quantity of 1 M NaOH. The M2 medium was used for handling oocytes. The m-TALP-4 medium was used for inducing the acrosome reaction of spermatozoa and inseminating oocytes. Incubation atmospheres were pure air for M2 and 1.5% CO_2 in air for m-TALP-4. Throughout the experiments, pH of the media was about 7.4.

Collection and centrifugation of eggs

Collection and centrifugation of hamster eggs were performed as described previously (Yang and Yanagimachi, 1989). Briefly, eggs were collected from superovulated females and freed from both the cumulus oophorus and the zonae pellucidae by consecutive treatments with hyaluronidase and trypsin in M2. Zona-free eggs thus obtained were thoroughly rinsed in M2, loaded on the top layer of Percoll gradient (7.5%, 30% and 45%) and centrifuged at 9500 g. During centrifugation, the eggs came to rest in the 30% Percoll layer, the vitellus became elongated and, in time, separated into heavy and light halves. In some experiments, eggs were treated for 30 min with 10 uM cytochalasin D (Sigma Chemical, St. Louis, MO) or 25 uM colcemid (Grand Island Biol., Grand Island, NY) in M2 before they were centrifuged. All the procedures mentioned were carried out at room temperature (about 25^{o}C) unless otherwise stated.

Examination of centrifuged eggs

At various times after the start of centrifugation, eggs were recovered from the centrifuge tube by withdrawing the layered solutions. The eggs were rinsed with M2 and examined immediately either in a watchglass, or after being mounted between a slide and coverslip (Yang and Yanagimachi, 1989). Some eggs were fixed with 2.5% glutaraldehyde at the end of centrifugation to maintain their shape for subsequent examination. The position of the nucleus (metaphase II chromosomes) was determined by staining live eggs with 20 ug/ml of bisBenzimide (Hoechst 33342; Sigma) in M2 for 30 min and examining them with a fluorescence microscope. The distribution of F-actin was examined after staining paraformaldehyde-fixed eggs with NBD-phallacidine (Molecular Probe Inc., Eugene, OR) according the procedure described by Battaglia and Gaddum-Rosse (1986). Some of the eggs were fixed for 1-2 hr with 2.5% glutaraldehyde in 0.15 M cacodylate buffer and processed for electron microscopy (Okada et al., 1986).

Insemination and examination of centrifuged eggs

The light and heavy halves of centrifuged eggs were inseminated with acrosome-reacted spermatozoa to determine whether they were capable of fertilization. Hamster spermatozoa from the cauda epididymis were allowed to undergo capacitation and the acrosome reaction by incubating them in 300 ul of m-TALP-4 for 4-5 hr at 38^{o}C (Cummins and

Yanagimachi, 1986). Eggs to be inseminated were placed in another drop (300 ul) of m-TALP-4 which had been previously placed under mineral oil (38°C) in a plastic petri dish (10 X 30 mm). Insemination was performed by introducing 10-20 ul of the preincubated sperm suspension to the egg-containing droplet so that the final concentration of spermatozoa was 2-3 $x10^4$ cells/ml. After incubation for 3 hr at 38°C (in a few instances, up to 20 hr), the eggs were examined for evidence of fertilization. A egg containing at least one decondensed sperm nucleus (or sperm pronucleus) with an associated tail was recorded as fertilized (Yanagimachi, 1978). Some fertilized eggs were fixed and processed for electron microscopy as previously described.

RESULTS

Separation of an egg into light and heavy halves

When zona-free eggs were centrifuged at 9500 g, each egg became elongated (Plate VII, figures 1A-C) and then separated into light (centripetal) and heavy (centrifugal) halves (Plate VII, figure 1D). It took an average of 590 sec (about 10 min) before all the eggs had separated into halves (Yang and Yanagimachi, 1989). The separated halves regained their spherical shape within 3-5 min after centrifugation (Plate VII, figures 1E,1E'). The light half (Plate VII, figure 1E) of the egg was much more transparent

than the heavy half (Plate VII, figure 1E'), and could be distinguished easily from the latter even with a dissecting microscope. The light halves were buoyant and tended to float in the medium, whereas the heavy halves sank to the bottom of the dish quickly. The relative sizes of the heavy and light halves were not constant, but in general the heavy half was larger than the light half. Occasionally, we found small fragments which presumably came from the mid-segment of elongated eggs. The relative size differences between light and heavy halves and the appearance of small fragments were probably due to a variation in the site of separation of the eggs during centrifugation (Plate VIII, figure 2).

Strictly speaking, "half" or "halves" may not be appropriate terms to use because the light and heavy halves were usually not exactly half of an intact egg. Nevertheless, this term (as well as the term "quarters") is used because they are accepted and convenient terms in the literature (Harvey, 1936; Beams and Kessel, 1987).

The time needed for complete separation of an egg into light and heavy halves was greatly reduced when eggs were treated with 10 uM cytochalasin D. It took only 3-4 min before all the eggs were separated. Treatment of eggs with 25 uM colcemid did not change the time required for separation.

Examination of the light and heavy halves by electron microscopy revealed that the former contained much fewer organelles (Plate IX, figures 3 A,A') than the latter

(Plate IX, figures 3B,B'). The size and number of microvilli appeared smaller in the light half compared to the heavy half. The density of F-actin in the cortex, as revealed by NBD-phallacidine fluorescence staining, was not significantly different between the two (Plate X, figure 4).

When light and heavy halves were centrifuged at 9500 g for an additional 11-14 min, each half separated into two quarters (Plate VII, figures 1F,G). The lightest quarter was transparent, buoyant and prone to rupture. Many of them broke when they were compressed between a slide and coverslip. The other quarters were stable and could tolerate compression under a coverslip. There was no distinct difference between the lightest quarter and the other quarters with respect to the density of F-actin in the cortex as judged by the intensity of NBD-phallacidine fluorescence (micrograph not shown).

Location of metaphase II chromosomes in centrifuged eggs"

Before centrifugation, the metaphase II chromosomes were located in the cortex of a microvilli-free area of the egg (Okada, et al., 1986). This area of the cortex was also free of cortical granules. After 10 min of centrifugation at 9500 g, the chromosomes were found in either the light or heavy half (Table 5). This indicates that the chromosomes do not move from their original position in the egg during centrifugation. The result was different when the eggs were treated with cytochalasin D prior to centrifugation.

Following 10 min of centrifugation, neither half retained the chromosomes (Table 5). To determine where the chromosomes went during centrifugation, cytochalasin D-treated eggs were examined after brief centrifugation. Following 30 sec of centrifugation at 9500 g, the chromosomes were located in the centripetal pole of the elongated oocytes (Plate XI, figure 5B). After 60-90 sec of centrifugation, none of the 12 eggs examined had retained their chromosomes (Plate XI, figures 5C,D). Perhaps, the chromosomes (and a small amount of cytoplasm surrounding chromosomes) were extruded from the centripetal end of the elongated eggs before each egg separated into halves or fragments.

Location of cortical granules in centrifuged eggs

In the cortex of a mature unfertilized egg, the cortical granules (CGs) are evenly distributed except for a microvilli-free area of the cortex where the metaphase II chromosomes are located (Okada, et al., 1986). The density of CGs in the equatorial region of the egg before centrifugation was about 25 per 25 um^2 (Table 6). When eggs were centrifuged and separated into halves, the density of CGs in the cortex remained unchanged except for the centripetal region of the light half where the density of CGs was reduced by 50% (Plate XII, figure 6 and Table 6). When eggs were treated with cytochalasin D prior to centrifugation, the density of CGs in the cortex was reduced

significantly in both light and heavy halves, particularly in the centripetal region of the light half (Table 6).

Fertilizability of halves and quarters

When a total of 66 light and 73 heavy halves were inseminated and examined 3 hr later, all of them were fertilized as evidenced by the presence of decondensed sperm nuclei or sperm pronuclei. There was, however, a notable difference between light and heavy halves. About 85% of the heavy halves contained well developed sperm pronuclei (Plate XIII, figures $7A_1$,A_2), whereas only about 15% of the light halves contained sperm pronuclei and these pronuclei were considerably smaller (Plate XIII, figure $7B_1$) than those found in the heavy halves. The remaining 85% of light halves had only decondensed sperm nuclei or small pronuclei without nucleoli (Plate XIII, figures $7B_{2-4}$). This was true even when the period of post-insemination incubation was extended to 20 hr. Metaphase II chromosomes could transfrom into well developed egg pronuclei in both light and heavy halves (Plate XIV, figure 8).

As previously stated, the light and heavy halves were each separated into two quarters after additional centrifugation. When these quarters were inseminated and examined 10 min later, several spermatozoa were seen attached to each of all but the lightest quarter. Very few to no spermatozoa were seen attached to the surface of the lightest quarter (Plate XIV, left of figure 9A). When

examined 3 hr after insemination, only about 20% of the 34 lightest quarters were fertilized. No pronuclei were found in these quarters; only slightly decondensed sperm nuclei were seen in the ooplasm (Plate XIV, figure 9B). On the other hand, 80 to 90% of 65 heavier quarters contained well-developed sperm pronuclei (Plate XIV, Figure 9C). Well developed egg pronuclei were found in some of heavier quarters, but not in any of the lightest quarters (Tables 7,8).

DISCUSSION

Centrifugation of mammalian eggs is not a new technique. For example, Beams and King (1938) centrifuged isolated guinea pig ovaries for 30 min at 150,000 g and recorded stratification of the cytoplasmic components of the egg. Others have made similar reports for the mouse (e.g., Gresson, 1938, 1940) and human (e.g., Aykroyd, 1941). The purpose of these studies was to determine the relative specific gravities of various cytoplasmic components. During centrifugation, the eggs in the ovary may have become elongated, but would not divide because of the presence of a rigid zona pellucida and the ovarian stroma surrounding each egg. In the present study, hamster eggs freed from their zonae pellucidae separated into halves under a relatively low centrifugal force (9500 g). It took about 10 min for all eggs to become separated into light and heavy halves.

This time was greatly shortened when eggs were treated with cytochalasin D, suggesting that the stiffness of the egg is largely dependent on the actin-based cytoskeleton.

When the light and heavy halves were further centrifuged, each half separated into two quarters. The lightest quarter was very transparent and buoyant. It was so prone to rupture during handling that it could not be processed for electron microscopy. The other three quarters were very stable. The reason for the fragility of the lightest quarter is not clear. Although significant differences in F-actin concentration between the lightest quarter and other quarters were not detected, the cytoskeletal network might have become disorganized or non-functional in this quarter.

According to Cran (1987), the metaphase chromosomes of pig, sheep and cattle eggs migrated to the mid-centripetal region when oocytes were centrifuged at 65000 g for 1 hr. We did not observe such a migration of chromosomes when hamster eggs were centrifuged at 9500 g for 10 min; the chromosomes remained in their original position even after the egg had separated into halves. This discrepancy is probably due to differences in the intensity and duration of the centrifugal force employed, ours being much weaker than that used by Cran. According to Karasiewicz and Soltynska (1985) and Longo (1987), there is a dense network of actin filaments lying between metaphase chromosomes and the egg plasma membrane. It is conceivable that this filamentous

network is disrupted by very strong centrifugal force (65000 g), but not by a moderate force (9500 g). When hamster eggs were treated with cytochalasin D and then centrifuged, the metaphase chromosomes were moved very rapidly. By 30 sec of centrifugation at 9500 g, the chromosomes were near the centripetal pole. When centrifugation continued for another 30 sec, all the chromosomes were extruded from the main body of elongated egg. At the moment, it is not known how the chromosomes were extruded from the eggs. Perhaps the chromosomes and a small amount of cytoplasm surrounding the chromosomes are "pinched off" from the main body of an elongated egg before the egg is separated into two or more fragments. Extrusion of nuclei from cytochalasin-treated somatic cells has been reported (Goldman, et al., 1973; Shay, et al., 1974). The combination of cytochalasin-treatment and centrifugation would be an ideal method to eliminate nuclei from a large number of mammalian eggs simultaneously. In the past, enucleated eggs were prepared by laborious manual dissection (e.g., Tarkowski, 1980).

When hamster eggs were centrifuged, the density of cortical granules in the centripetal region was reduced. This was particularly evident when the eggs were pretreated with cytochalasin D (Table 2). Cytochalasin-treatment alone did not change the density and distribution pattern of cortical granules. It was during subsequent centrifugation when the density of the granules was reduced. According to

Cran (1987), cortical granules in intact eggs of the pig and sheep remained in the original position after centrifugation at 65000 g for 1 hr. When the eggs were treated with cytochalasin D (1 ug/ml or 1.8 uM) and then centrifuged at 10000 g, virtually all the granules underwent exocytosis within a few minutes. Why did the granules undergo exocytosis instead of forming a stratified layer or band? Cran (1987) imagined that the cytochalasin D treatment altered the Ca^{2+} status within the cytoplasm and/or the fusibility of the granules with the plasma membrane and that these changes together with a "stress" produced by centrifugation initiated events leading to fusion between the membranes of the cortical granules and the plasma membrane. In the present study, a sharp reduction in the density of cortical granules in hamster eggs was noted when the eggs were centrifuged after cytochalasin-treatment (Table 6). This could have been due to exocytosis of the granules as reported by Cran (1987), but the possibility of extrusion of the granules from the centrifugal region of the egg cannot be ruled out.

Both light and heavy halves of hamster eggs were fertilizable. There was no significant difference between the two with respect to the rate of sperm penetration. The major observable difference was that the light half was unable to support the development of a sperm (male) pronucleus consistently (Plate XIII, figure 7B), whereas the heavy half could do so (Plate XIII, figure 7A). Apparently,

most of the light halves did not contain, in high enough concentration, the materials or factors necessary for development of a sperm pronucleus. According to Hirao and Yanagimachi (1979) and Clarke and Masui (1987), the capacity of an oocyte to support the transformation of a sperm nucleus into a male pronucleus (and to metaphase chromosomes) is limited. When more than four to five spermatozoa enter a mature egg, the sperm nuclei compete for the materials necessary for the development of male pronuclei. Under the conditions, either none or only a few sperm nuclei develop into pronuclei. When an egg is dissected into half, no more than two sperm nuclei can be transformed into metaphase chromosomes (Clarke and Masui, 1987). Thus, the ability of an egg to support the development of a sperm pronucleus (and chromosomes) is dependent on the volume of egg. As shown in Plate VII, figure 1, the heavy half of the centrifuged hamster egg is usually larger than the light half, but this is not always the case (Plate VII, figure 2). Regardless of their relative size, light halves were consistently inferior in supporting the development of sperm pronucleus. Apparently, the volume of the cytoplasm per se is not the determining factor for the development of sperm pronucleus. A lack or deficiency in organelles (cf. Plate IX, figure 3A) may be responsible for the apparent poor ability of the light halves to support the development of sperm pronuclei. This notion seems to be supported by the fact that the two

quarters produced by centrifugation of a light half were very different in their abilities to support the development of a sperm pronucleus. The lightest quarter, which was almost free of organelles, could not support the development of a sperm pronucleus (Plate XV, figure 9B), whereas the next to the lightest quarter which was rich in organelles could support pronuclear development (Plate XV, figure 9C). Thus, the density of organelles, rather than their absolute number in the eggs, seems to be of critical importance for the successful development of a sperm nucleus into a sperm (male) pronucleus. Which organelles play a key role in the development of the sperm pronucleus remains to be determined.

Finally, it is important to note some of the interesting characteristics of the lightest quarter. As mentioned previously, this quarter was almost devoid of organelles. It was very buoyant and prone to rupture by ordinary mechanical handling or chemical fixation. The reason for the fragility is unknown, but it may be due to a disruption of the cytoskeletal network and/or the deficiency in membrane precursors. Spermatozoa could fuse with this quarter, but the incidence of the fusion was low. None of the sperm nuclei incorporated in this quarter were able to develop into a male pronucleus. On the other hand, all other quarters fused readily with spermatozoa and could support the development of sperm pronuclei. Thus, a detailed biological and biochemical comparison of the

lightest quarter with the other quarters may contribute to better understanding of the mechanisms controlling sperm-egg fusion and pronuclear development.

REFERENCES

Aykroyd, OE (1941) The effects of ultracentrifuging human oocytes. Proc Roy Irish Acad Dublin, Sec B, 46:101-111.

Battaglia, DE and Guddum-Rosse, P (1986) The distribution of polymerized actin in the rat egg and its sensitivity to cytochalasin B during fertilization. J Exp Zool 237:97-105.

Beams, HW and Kessel, RG (1987) Development of centrifuges and their use in the study of living cells Int J Cytol 100:15-48.

Beams, HW and King, RL (1938) A study of the cytoplasmic components and inclusions of the developing guinea pig egg. Cytologia 8:353-367.

Clarke, HJ and Masui, Y (1987) Dose-dependent relationship between oocyte cytoplasmic volume and transformation of sperm nuclei to metaphase chromosomes. J Cell Biol 104:831-840.

Cran, DG (1987) The distribution of organelles in mammalian oocytes following centrifugation prior to injection of foreign DNA. Gamete Res 18:67-76.

Cummins, JM and Yanagimachi, R (1986) Development of ability to penetrate the cumulus oophorous by hamster spermatozoa capacitated in vitro, in relation to the timing of the acrosome reaction. Gamete Res 15:187-212.

Goldman, RD, Pollack, R and Hopkins, NH (1973) Preservation of normal behavior by enucleated cells in culture. Proc Natl Acad Sci USA 70:750-754.

Gresson, RAR (1938) Effect of ultra-centrifuging the oocyte of the mouse. Nature 142:957-958.

Gresson, RAR (1940) A cytological study of the centrifuged oocyte of the mouse. Quart J Micr Sci 81:569-584.

Harvey, EB (1936) Parthenogenetic merogony or cleavage without nuclei in Arbacia Punctulata. Biol Reprod 71:101-121.

Hirao, Y and Yanagimachi, R (1979) Development of pronuclei in polyspermic eggs of the golden hamster. Zool Mag Tokyo 88:24-33.

Karasiewicz, J and Solytnska, MS (1985) Ultrastructural evidence for the presence of actin filaments in mouse eggs in fertilization. Roux's Arch Dev Biol 194:369-372.

Longo, FJ (1987) Actin-plasma membrane association in mouse eggs and oocytes. J Exp Zool 243:299-309.

Maruyama, YK, Yamamoto, K, Mita-Miyazaki, I, Kominami, T and Nemoto, S (1986) Manipulative methods for analyzing embryogenesis. In: Methods in Cell Biology, Schroeder, TE (ed), Academic Press, New York. pp. 325-344.

Okada, A, Yanagimachi, R and Yanagimachi, H (1986) Development of a cortical granule-free area of cortex and the perivitelline space in the hamster oocyte during maturation and following ovulation. J Submicr Cytol 18:233-247.

Quinn, P, Barros, C and Whittingham, DG (1982) Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. J Reprod Fert 66:161-168.

Shay, JW, Porter, KR and Prescott DM (1974) The surface morphology and fine structure of CHO (Chinese hamster ovary) cells following enucleation. Proc Natl Acad Sci USA 71:3059-3063.

Tarkowski, AK (1980) Fertilization of nucleate and anucleate egg fragments in the mouse. Exp Cell Res 128:73-77.

Tellez, V, Ahumada, A, Muro, J, Sepulveda, S, and Izquierdo, L (1988) Centrifugation of 2-cell mouse ova: Cytoplasmic stratification and recovery. Roux's Arch Dev Biol 197:360-365.

Wall, RJ, Pursel, VG, Hammer, RE and Brinster, RL (1985) Development of porcine ova that were centrifuged to permit visualization of pronuclei and nuclei. Biol Reprod 32:645-651.

Yanagimachi, R (1978) Calcium requirement for sperm-egg fusion in mammals. Biol Reprod 19:949-958.

Yanagimachi, R (1981) Mechanisms of fertilization in mammals. In: Fertilization and Embryonic Development In Vitro, Mastroianni, L and Biggers, BJ (eds), Plenum Press, New York. pp. 81-182.

Yang, CH and Yanagimachi, R (1989) Differences between mature ovarian and oviductal oocytes: A study using the golden hamster. Human Reprod 4:63-71.

Table 5

Distribution of metaphase II chromosomes (spindles) in light and heavy halves of centrifuged hamster eggs.

Eggs	Total no. of eggs examined	% Eggs with chromosomes
Not treated with cytochalasin D		
Not centrifuged	20	100
Centrifuged, light half	63	49
Centrifuged, heavy half	66	50
Pretreated with cytochalasin D		
Not centrifuged	25	100
Centrifuged, light half	59	0
Centrifuged, heavy half	79	0

Table 6

Density of cortical granules (CGs) in light and heavy halves of centrifuged hamster eggs

Eggs	Total no. of eggs examined	Region in egg examined	No. of CGs in 25 um^2 (Mean ± S.D.)
Not treated with cytochalasin D			
Not centrifuged	20	Equatorial	25 ± 1.2
Centrifuged, light half	31	Centripetal	12 ± 2.1
		Centrifugal	24 ± 2.0
Centrifuged, heavy half	26	Centripetal	25 ± 1.9
		Centrifugal	25 ± 1.9
Pretreated with cytochalasin D			
Not centrifuged	20	Equatorial	25 ± 1.4
Centrifuged, light half	24	Centripetal	10 ± 2.1
		Centrifugal	18 + 2.4
Centrifuged, heavy half	27	Centripetal	18 ± 3.0
		Centrifugal	18 ± 3.8

Table 7

Fertilizablity of heavy and light halves of centrifuged eggs

	No. of exp.	Total No. of egg/halves inseminated	% egg/halves fertilized by 3 hr insemination
Egg not centrifuged	6	36	100
Centrifuged heavy half	6	73	95 ± 5.5
light half	6	66	94 ± 4.5

Table 8

Fertilizability of quarters obtained by double centrifugation

	No. of exp.	Total no. of quarters inseminated	% egg quarters fertilized by 3 hr after insemination
Lightest quarter	4	34	26.4 (9/34)
Next to lightest quarter	4	32	90.6 (29/32)
Heavy quarters	4	33	93.9 (31/33)

PLATE VII

SEPARATION OF A ZONA-FREE HAMSTER EGG INTO HALVES AND QUARTERS BY CENTRIFUGATION

Figure 1.

Separation of a zona-free hamster egg into halves and quarters by centrifugation at 9500 g. Magnification, x440.

A. Zona-free hamster egg before centrifugation.

B-C. Elongated eggs, 6 and 9 min after the start of centrifugation, respectively.

D. An egg separated into a light (top) and heavy (bottom) halves, 10 min after the start of centrifugation.

E-E. Light (E) and heavy (E′) halves which have regained their spherical shape.

F. Two egg quarters produced by additional centrifugation of the light half: top, the lightest quarter; bottom, next to the lightest quarter.

G. Two egg quarters produced by additional centrifugation of the heavy half.

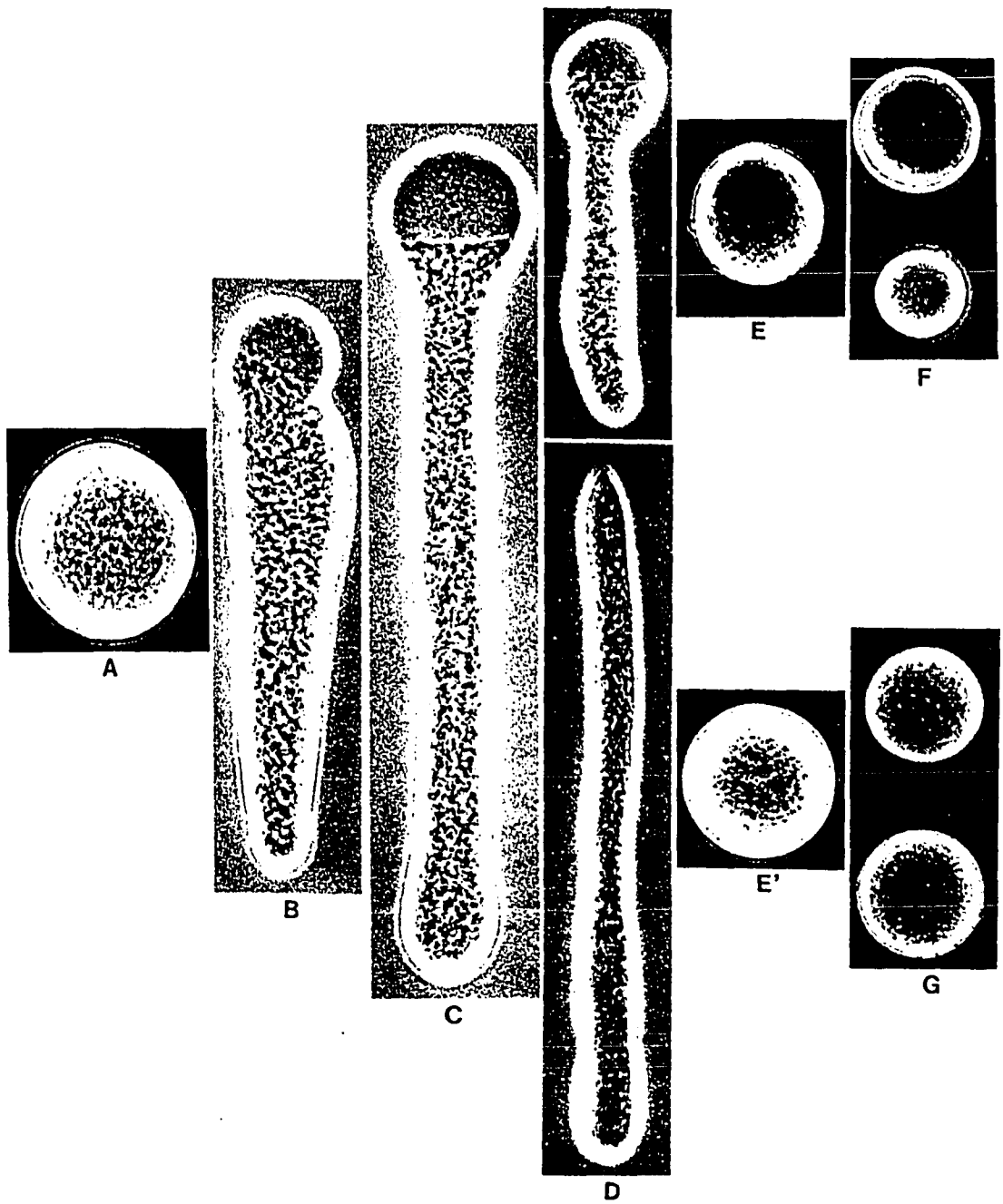

A
B
C
D
E
E'
F
G

PLATE VIII

PROBABLE RELATIONSHIP BETWEEN THE SITE OF SEPARATION AND VARIATION IN THE SIZE OF LIGHT AND HEAVY HALVES OF EGGS

Figure 2.

Probable relationship between the site of separation and variation in the size of light and heavy halves. Arrows indicate the site of separation during centrifugation. The incidence of A appeared to be highest followed by B, C and D. H, heavy half; L, light half; M, midfragment.

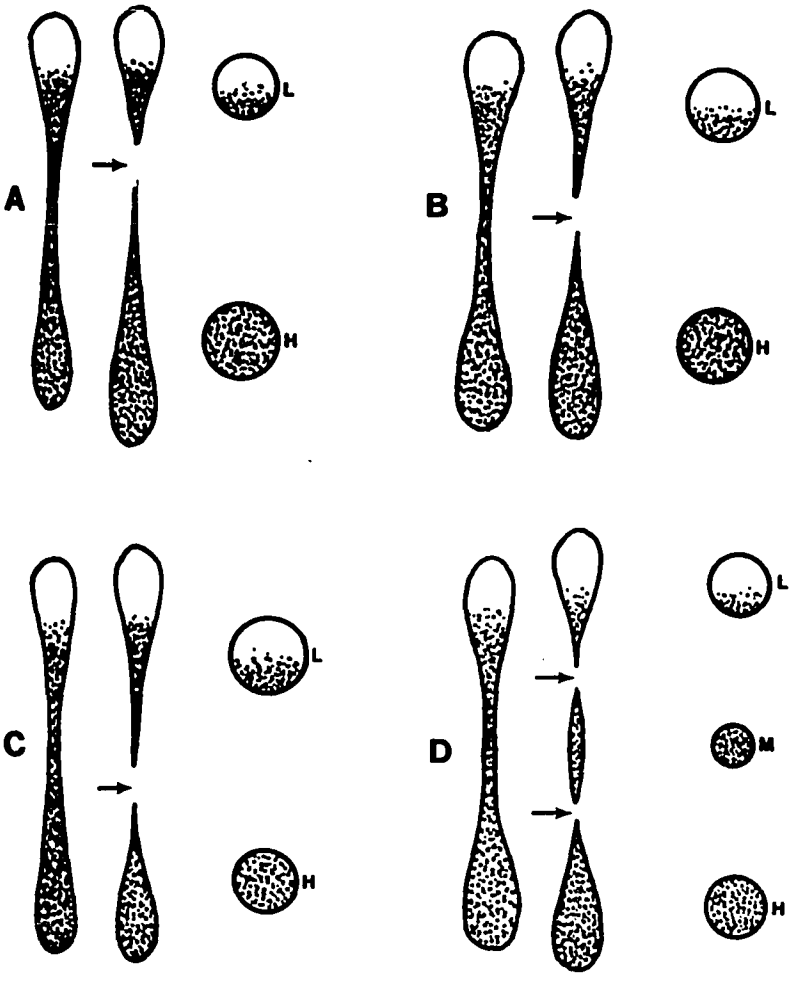

A
L
H
B
L
H
C
L
H
D
L
M
H

PLATE IX

ELECTRON MICROSCOPIC SECTIONS THROUGH LIGHT AND HEAVY HALVES OF EGGS

Figure 3.

Sections through light (A) and heavy (B) halves. The arrow in 3A indicates the direction of centrifugal force. Magnification, A and B, x3,100; A'and B' x10,800. CGs, cortical granules.

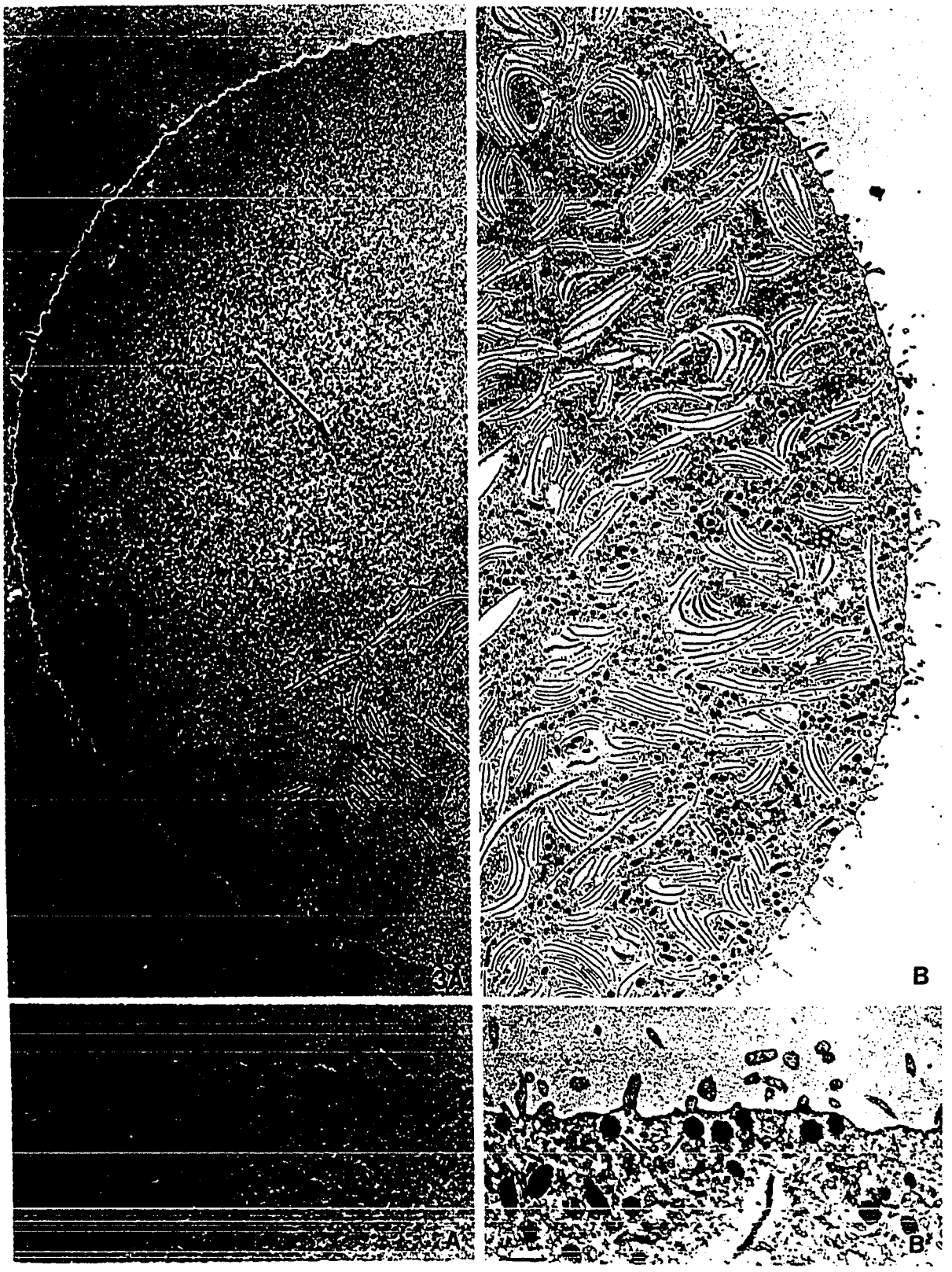

3A
B
A
B

PLATE X

NBD-PHALLACIDINE FLUORESCENCE STAINING OF

LIGHT AND HEAVY HALVES OF EGGS

Figure 4.

NBD-phallacidine fluorescence of light (L) and heavy (H) halves of eggs. Note the strong fluorescence at the top of the heavy half, where metaphase chromosome was located. Magnification, x570.

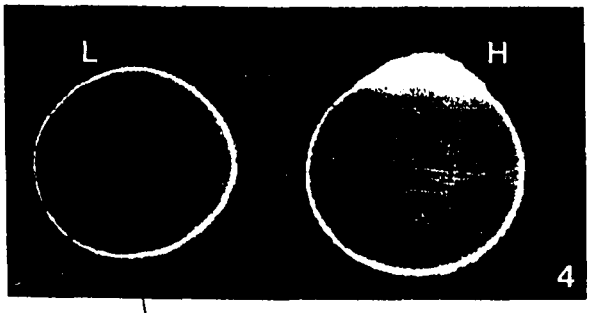

L
H
4

PLATE XI

DISAPPEARANCE OF CHROMOSOMES FROM

CYTOCHALASIN-TREATED EGGS

Figure 5.

Disappearance of chromosomes from cytochalasin-treated eggs. Eggs were stained with Hoechst 33342 before or after centrifugation and examined with a fluorescence microscope.

A. Before centrifugation.

B. 30 sec after the start of centrifugation; chromosomes were at the centripetal pole.

C-D. 60-90 sec after the start of centrifugation; no chromosomes were seen in the eggs. Arrow indicate the direction of centrifugal force. Magnification, x440.

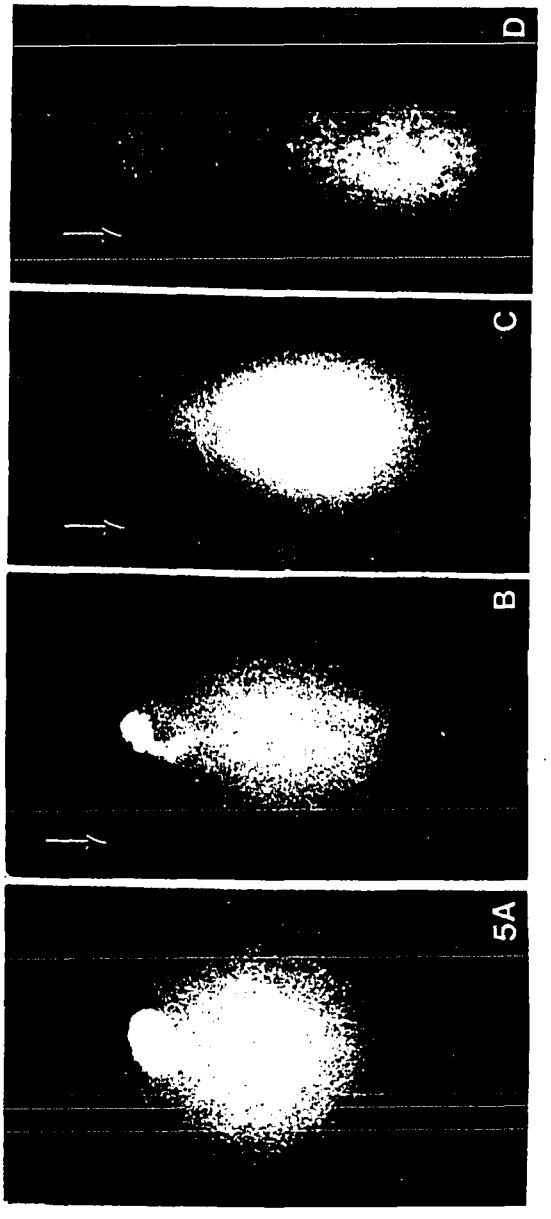

5A
B
C
D

PLATE XII

CORTICAL GRANULES IN LIGHT AND HEAVY EGG HALVES REVEALED BY PHASE CONTRAST AND ELECTRON MICROGRAPHS

Figure 6.

Cortical granules (cg) in light (A) and heavy (B) egg halves. Note that the density of cortical granules is low in the centripetal region (top) of the light half (6A). A and B, phase-contrast micrographs, magnification, x1,400; A'and B', electron micrographs, magnification x27,700.

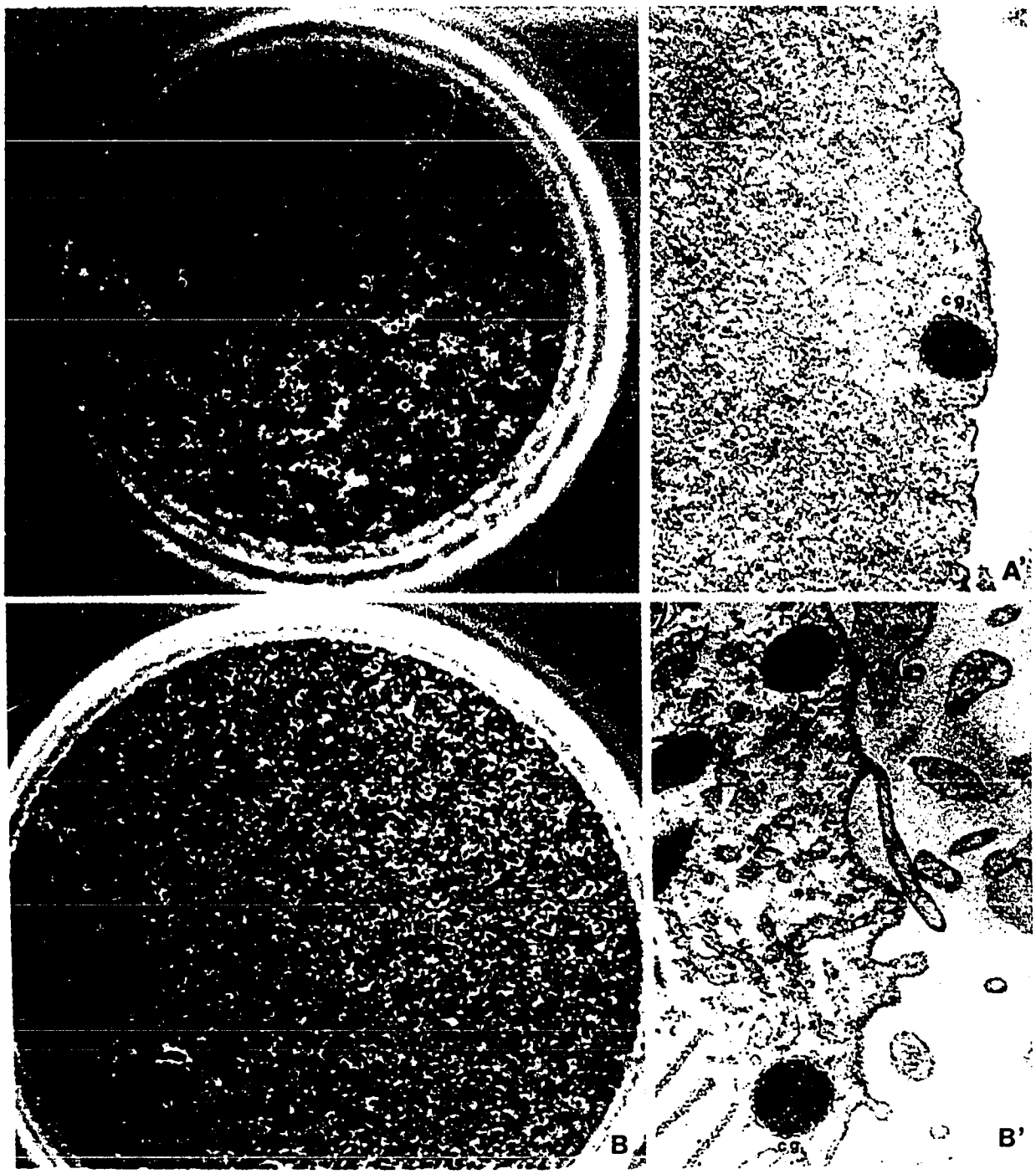

A'
B
B'

PLATE XIII

SPERM NUCLEI DECONDENSATION IN LIGHT AND HEAVY HALVES OF EGGS

Figure 7.

Sperm nuclei in light (A) and heavy (B) egg halves, at 3 hr after insemination, showing that large sperm pronuclei were found in heavy halves, but not in light halves. Magnification, A1 and B1-3, x1,000; A2 and B4, x7,600.

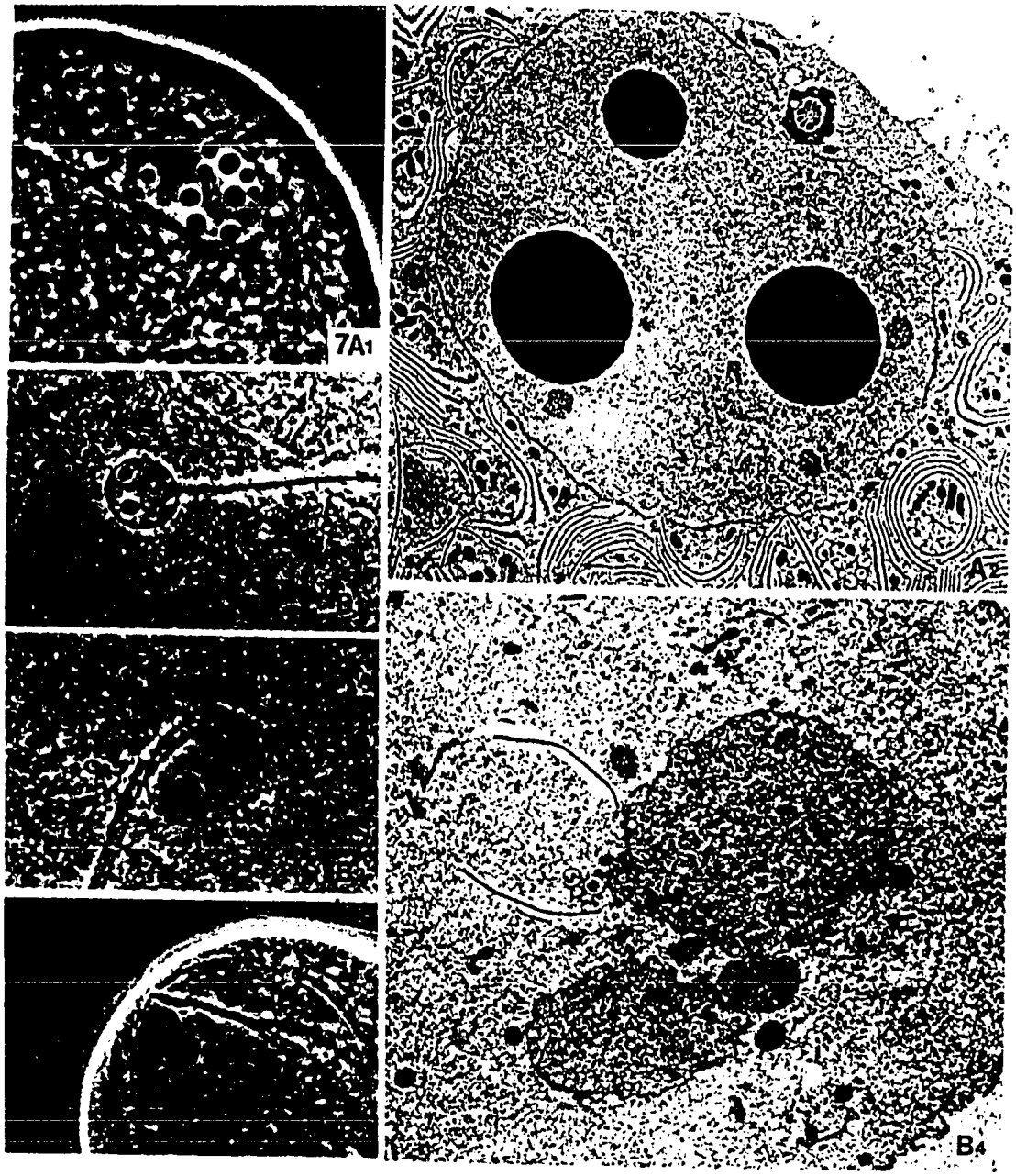

7A1
A2
B4

PLATE XIV

SPERM NUCLEI DECONDENSATION AND FEMALE PRONUCLEUS FORMATION IN LIGHT EGG HALVES

Figure 8.

A light egg half showing two decondensed sperm nuclei (s) and one egg pronucleus (e), at 3 hr after insemination.

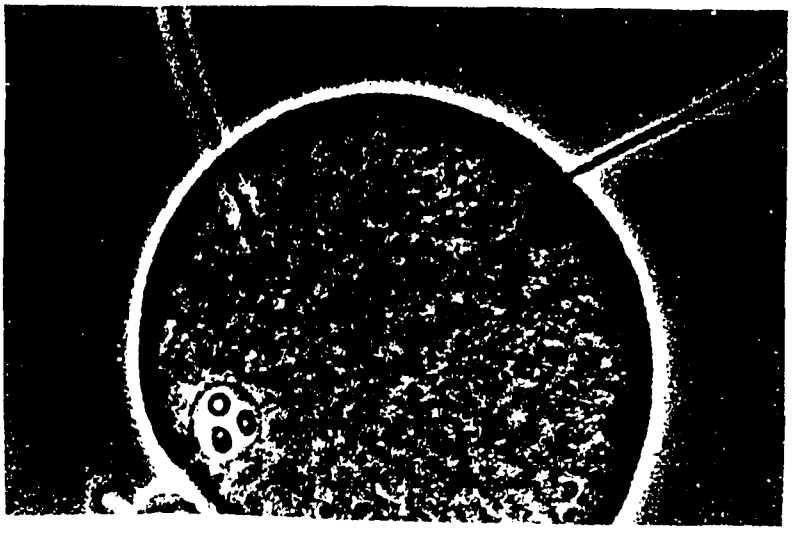


PLATE XV

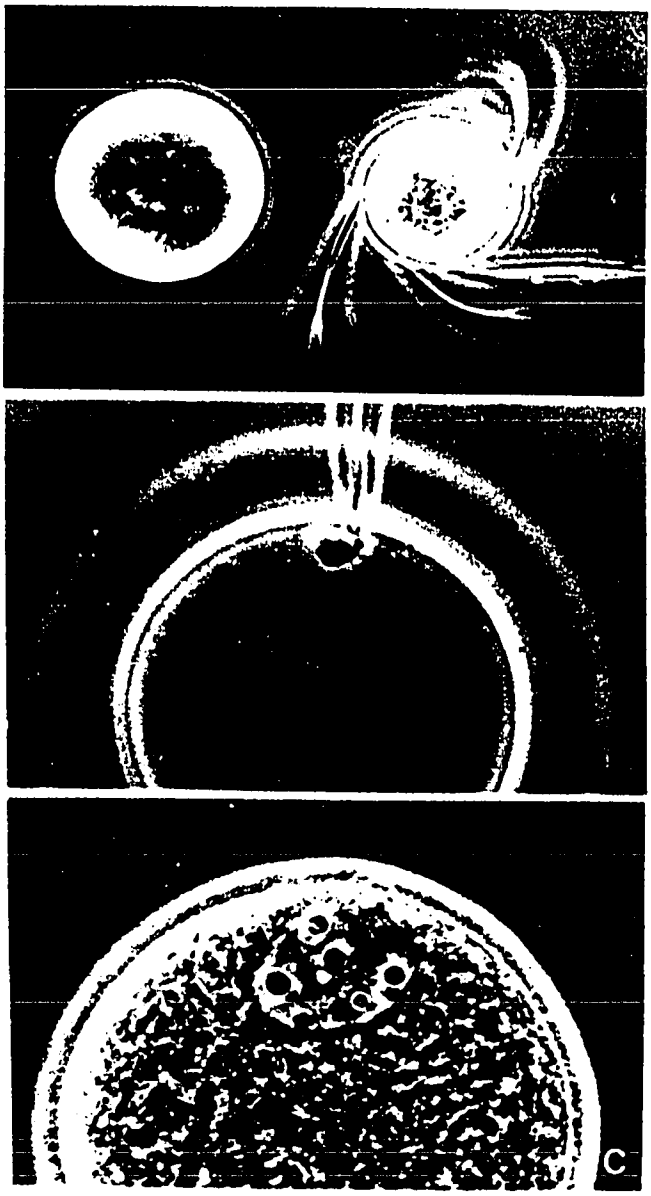
DIFFERENCES IN THE BEHAVIOR OF SPERMATOZOA TO THE TWO LIGHT QUARTERS OF CENTRIFUGED EGGS

Figure 9.

A. Very few to no spermatozoa were attached to the lightest quarter (left) at 10 min after insemination, whereas several spermatozoa were seen attached to the surface of another (heavier) quarter.

B. Some of the lightest quarters, as shown here, contained a decondensed sperm nucleus at 3 hr after insemination.

C. The next to the lightest quarters contained well-developed sperm pronuclei at 3 hr after insemination. Magnification, A, x650; B-C, x1,000.


C

CHAPTER IV

CHANGES IN THE RIGIDITY OF THE HAMSTER EGG DURING MEIOTIC MATURATION AND AFTER FERTILIZATION

When mature hamster eggs are freed from their zonae pellucidae and centrifuged in a Percoll gradient, each egg elongates and then separates into a light and heavy half (Yang and Yanagimachi, 1989; Yang, et al., 1989). Under these conditions, eggs collected from the oviduct shortly after ovulation separate faster than those isolated from the ovary immediately before ovulation (Yang and Yanagimachi, 1989), suggesting that the former is less "rigid". Thus, a reduction in egg rigidity could be a feature of meiotic maturation. This prompted an investigation into the "rigidity" of the egg changes during meiotic maturation, fertilization and preimplantation development.

MATERIALS AND METHODS

Medium and Reagents

M2 medium (Quinn, et al., 1982) containing 0.4% bovine serum albumin fraction v (BSA) and 50 ug/ml gentamicin sulfate was used throughout this study. All inorganic salts were purchased from either Mallinckrodt Chemical Co. (St. Louis, Mo) or Sigma Chemical Co. (St. Louis, MO). The sources of the other compounds were as follows: D-glucose, Na-lactate, Na-pyruvate, HEPES, gentamicin sulfate,

cytochalasin D (CD), dimethyl sulfoxide (DMSO), Percoll, and human chorionic gonadotropin (HCG) (Sigma Chemical Co., St. Louis, MO); BSA and pregnant mare serum gonadotropin (PMSG) and colcemid (Calbiochem, La Jolla, CA); bovine pancreatic trypsin and bovine testicular hyaluronidase (ICN Biochemicals, Cleveland, OH); lysolecithin (Avanti Polar Lipids, Birmingham, AL); N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) phallacidin or NBD-phallacidin (Molecular Probes Inc., Eugene, OR). Stock solutions of cytochalasin D (1 mM), colcemid (0.27 mM) and NBD-Ph (100 units/ml) were prepared with 100% DMSO, 0.9% NaCl and 100% methanol, respectively.

Collection of Eggs at Various Stages of Maturation and Preimplantation Development

Golden hamsters were raised and maintained in an air-conditioned room with controlled light conditions (light, 05:00-19:00 hr; dark, 19:00-05:00 hr). Under these conditions, mature females came into behavioral estrus around 18:00 hr of every fourth day (day 4) and ovulated early the next morning (01:00-02:00 hr of day 1). The age of females used in this study was 2-4 months and that of males was 4-6 months.

Females were each injected intramuscularly with 30 IU PMSG in the morning of day 1 followed by injection of 30 IU HCG in the afternoon or evening of day 3. Ovulation took place between 13 and 14 hr after HCG injection. The number

of eggs ovulated was about 50 per female. Ovarian eggs with intact germinal vesicles were collected 1 hr after HCG injection by puncturing enlarged follicles. Ovarian eggs in the process of germinal vesicle breakdown and those at metaphase I, anaphase to telophase I and metaphase II of meiotic division were collected in the same manner at 3.5, 7, 10 and 13 hr after HCG injection, respectively. Ovulated eggs were collected 17 hr after HCG injection by flushing them from the oviduct. To obtain fertilized eggs at various stages of development, estrous females which did not receive any hormones were allowed to mate in the evening of day 4. Fertilized eggs, at pronuclear stage of development, were flushed from the oviduct around 10:00 and 16:00 hr of the next day (day 1 of pregnancy). Eggs undergoing the first cleavage were obtained in the evening (18:00 hr) of day 1 of pregnancy. Eggs at 2-,4- and 8-cell stages were collected 10:00 hr of day 2, 23:00 hr of day 2 and 09:00 hr of day 3, respectively.

Prior to centrifugation, all eggs were freed from both cumulus oophorus and zona pellucida. All ovarian eggs, except those at metaphase II, were first treated for 5 min with 0.2% hyaluronidase in M2 to loosen their cumuli and then drawn in and out of a small bore pipette to disperse the remaining cumulus cells. No pipetting was necessary for mature ovarian and oviducal eggs which had reached metaphase II. A 5 min treatment with 0.1% hyaluronidase in M2 dispersed the cumulus cells from these eggs. Most

pronuclear eggs and all developing eggs were already free of cumulus cells. All of cumulus-free eggs thus obtained were treated for 1-2 min with 0.1% trypsin in M2 to dissolve their zonae pellcudae. Zona-free eggs were rinsed thoroughly and kept in M2 for no longer than 20 min before they were centrifuged.

Examination of egg rigidity by centrifugation

The rigidity of an egg was assessed by determining the minimum time needed for complete separation of all eggs or blasotomeres into heavy and light halves. Centrifugation was carried out as described by Yang and Yanagimachi (1989). Briefly, zona-free eggs (usually, 10 to 15) were loaded onto the top layer of a Percoll gradient (7.5%, 30% and 45% Percoll in M2) and centrifuged at 9500 g (in a few instances, at 16000 g). During centrifugation, the eggs came to rest in the 30% Percoll layer. Each egg became elongated and, in time, separated into a heavy and a light half. The time needed for complete separation of all the eggs in a particular group into halves was determined by varying the duration of centrifugation. Experiments were repeated at least 6 times for each determination. In some experiments, eggs were treated for 30 min at 25^{o}C with 10-40 uM cytochalasin D, 1-4% DMSO, or 25 uM colcemid (all in M2) prior to centrifugation.

Egg rigidity was also examined by drawing a small region of the cortex into a capillary tube attached to a micromanipulator.

Distribution of F-actin in eggs

The distribution of F-actin was examined using a F-actin-specific fluorescent probe, NBD-phallacidin (abbr. NBD-ph), according to the procedure described by Battaglia and Gaddum-Rosse (1986). Briefly, eggs were fixed 10-15 min with 2% paraformaldehyde in Dulbecco's phosphate buffered saline (PBS), rinsed with PBS, and stained for 15 min with NBD-ph (5 units/ml) in PBS containing 0.1 mg/ml lysolecithin. After rinsing in PBS, eggs were mounted between a slide and coverslip and examined with a fluorescence microscope.

RESULTS

Rigidity of eggs at various stages of maturation and development

When unfertilized oviducal eggs were centrifuged at 9500 g, each egg separated into halves by 10 min after the start of centrifugation (Plate XVI, figures 1A-C) (Yang and Yanagimachi, 1989; Yang, et al., 1989). Ovarian eggs at the germinal vesicle (GV) stage did not separate after 40 min of centrifugation at 9500 g (Plate XVI, figures 2A,B). An even stronger centrifugation (16000 g for 40 min) could not

separate these eggs, indicating that ovarian eggs at GV stage were very rigid. Soon after GV breakdown, eggs were separated by 32 min of centrifugation at 9500 g. Apparently, the rigidity of the egg diminishes during or after GV breakdown. Changes in the rigidity of the egg during maturation, fertilization and development, as determined by the minimum time needed for separation of all eggs or blastomeres into halves, are shown in Plate XVII, figure 3. It can be seen that egg rigidity diminishes during or after GV breakdown and increases markedly after the first cleavage. It should be noted that the mature, unfertilized eggs collected from the oviduct are the least rigid. Plate XVIII, figure 4 and Plate XIX, figure 5 show fertilized eggs at the pronuclear and 2-cell stage which were elongated and separated by centrifugation at 9500 g.

The rigidity of eggs or blastomeres was not affected by 30 min pretreatment with 25-40 uM colcemid or 1-4% DMSO. On the other hand, cytochalasin D (CD) diminished egg and blastomere rigidity significantly. Regardless of the stage of meiotic maturation and development, all the eggs or blastomeres treated with 10-40 uM CD for 30 min separated into halves within 3-12 min of centrifugation at 9500 g (Plate XVII, figure 3). It should be noted that reduction in the rigidity by CD is more marked in GV eggs than in developing eggs. Plate XX, figure 6 shows GV eggs separated by centrifugation after CD-treatment.

Results of our micromanipulation experiments indicated that it was the cortex, not the endoplasm, that was largely responsible for the rigidity of the egg. When a GV egg was held and drawn into a micropipette by applying a negative pressure, the egg resisted the pressure until the tip of evagination broke abruptly (Plate XXI, figures 7A-D). The contents of the egg (endoplasm) did not appear to be very viscous, mingling with the medium rapidly (Plate XXI, figure 7E). In contrast, the mature oviducal egg did not resist the negative pressure. Using the same degree of pressure, it was easily drawn into the capillary tube (Plate XXI, figures 8A-D). The entire egg could be drawn into the tube without being ruptured. When the portion of an egg which had been drawn into the capillary tube (Plate XXI, figure 8E) was then pushed out, it resembled a soft sausage (Plate XXI, figure 8F-I). GV eggs treated with CD behaved like mature oviducal eggs.

Distribution of F-actin in eggs at various stages of meiotic maturation and development

When unfertilized oviductal eggs were stained with NBD-ph and then examined with a fluorescence microscope, the entire cortex exhibited strong fluorescence, indicating the presence of F-actin in the egg cortex. The fluorescence was particularly strong in the area of cortex above the metaphase II chromosomes (Plate XXII, figure 9A), supporting previous reports that this area of cortex is rich

in F-actin (Maro, et al., 1984, 1986; Longo, 1987). There were differences in the fluorescence pattern between CD-treated and untreated (control) eggs. The fluorescence of CD-treated eggs was strong during the first 5-10 sec of exposure to UV light (Plate XXII, figure 9B), but it faded quickly thereafter. The fluoresence of control eggs faded with time, but not as fast as that of CD-treated eggs. The cortical fluorescence of control eggs was evenly distributed, whereas that of treated egg was patchy (Plate XXI, figure 9C).

When a GV egg and a mature unfertilized egg were stained with NBD-ph simultaneously in the same dish and examined side-by-side, the former always displayed stronger fluorescence (Plate XXII, figure 9D). A 2-cell egg and a mature egg exhibited similar cortical fluorescence (Plate XXII, figure 9E). Perinuclear fluorescence was evident in the 2-cell egg.

DISCUSSION

In this study the rigidity of hamster eggs was evaluated by determining the minimum time needed for complete separation of all eggs in a group into halves. The results of these experiments indicate that hamster eggs at the GV stage are much more rigid than those undergoing meiotic maturation. In this respect, hamster eggs are similar to starfish eggs. Nemoto, et al. (1980) measured the rigidity

of the starfish egg by compressing it under a coverslip and found that its rigidity diminishes progressively during and after GV breakdown. In both the starfish (Nemoto et el., 1980) and the hamster (this study), the rigidity of eggs is reduced significantly by cytochalsin, suggesting that the rigidity of both starfish and hamster eggs is largely dependent on the actin-based cytoskeleton.

According to Kaplan, et al. (1982), cytoskeletal proteins, including actin, are synthesized and accumulated during the growth of the egg. Actin synthesis declines during meiosis and is insignificant in mature unfertilized eggs as well as recently fertilized eggs (Bachvarova, et al., 1985). Our study, using NBD-ph as an F-actin probe, has revealed that the density of F-actin in the cortex and endoplasm of hamster egg changes, but not very significantly, during meiotic maturation and after fertilization. It may be the manner of interaction of F-actin with other cytoskeletal proteins (e.g., filamin and alpha-actinin), not the concentration of actin per se, that changes significantly during meiotic maturation and after fertilization.

In the sea urchin, Temmnopleurus toreumaticus, the rigidity of the egg cortex increases and reaches a maximum about 1.5 min after fertilization. It then decreases to a minimum about 4 min after fertilization, followed by a gradual increase until the onset of first cleavage (Hiramoto, 1974). Such periodic changes in the rigidity of

the hamster egg during fertilization and first cleavage were not detected; only a slow, gradual increase in rigidity was noted (Plate XVII, figure 3). It is possible that short-term changes in rigidity were not detected because eggs were not examined continuously during the 14 hr between fertilization and first cleavage.

In the mouse, actin synthesis is low in both unfertilized and 2-cell eggs. It becomes greater after the egg reaches the 4-to 8-cell stage (Van Blerkom and Brockway, 1975; Howe and Solter, 1979; Abreu and Brinster, 1978; Giebelhaus, et al., 1983). In this respect, it is somewhat surprising that the 2-cell hamster egg is considerably more rigid than the unfertilized egg (Plate XVII, figure 3). The density of cortical actin in the 2-cell egg appeared to be equal to or only slightly greater than that in the unfertilized egg (Plate XXII, figure 9E and Table 9). The striking difference between unfertilized and 2-cell eggs with respect to the rigidity may not be explained in terms of the density and distribution of actin alone. As stated previously, the rigidity of an egg (and blastomere) may be influenced greatly by other cytoskeletal proteins. The manner of interaction of F-actin with other cytoskeleton proteins could be a prime factor controlling the rigidity of an egg (or blastomere).

REFERENCES

Abreu, SL and Brinster, RL (1978) Synthesis of tubulin and actin during the preimplantation development of the mouse. Exp Cell Res 114:135-141.

Bachvaroova, R, De Leon, V, Johnson, A, Kaplan, G and Paynton, BV (1985) Changes in total RNA, polyadenylate RNA, and actin mRNA during meiotic maturation of mouse oocytes. Dev Biol 108:325-331.

Battaglia, DE and Gaddum-Rosse, P (1986) The distribution of polymerized actin in the rat egg and its sensitivity to cytochalasin B during fertilization. J Exp Zool 237:97-105.

Giebelhaus, DH, Hekkila, JJ, Schuetz, GS (1983) Changes in the quantity of histone and actin messenger RNA during the development of preimplantation embryos. Dev Biol 98:148-154.

Hiramoto, Y (1974) Mechanical properties of the surface of the sea urchin egg at fertilization and during cleavage. Exp Cell Res 89:320-326.

Howe, CC and Solter, D (1979) Cytoplasmic and nuclear protein synthesis in preimplantation mouse embryo. J Embryol Exp Morph 52:209-225.

Kaplan, G, Abreu, SL and Bachvarova, R (1982) rRNA accumulation and protein synthetic pattern in growing mouse oocytes. J Exp Zool 220:361-370.

Longo, FJ (1987) Actin-plasma membrane association in mouse eggs and oocytes. J Exp Zool 243:299-309.

Maro, B, Johnson, MH, Pickering, SJ and Flach, G (1984) Changes in actin distribution during fertilization of the mouse egg. J Embryo Exp Morph 81:211-237.

Maro, B, Howlett, SK and Houlinston, E (1986) Cytoskeletal dynamics in the mouse egg. J Cell Sci (suppl) 5:343-359.

Nemoto, S, Yoneda, M and Uemura, I (1980) Marked decrease in the rigidity of starfish oocytes induced by 1-methyladenine. Dev Growth Diff 22:315-325.

Quinn, P, Barros, C, Whittingham, DG (1982) Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. J Fert Steril 66:161-168.

Van Blerkom, J and Brockway, G (1975) Qualitative patterns of protein synthesis in the preimplantation mouse embryo. I. Normal pregnancy. Dev Biol 44:148-157.

Yang, CH and Yanagimachi, R (1989) Differences between mature ovarian and oviductal oocytes: A study using the golden hamster. Human Reprod 4:63-71.

Yang, CH, Yanagimachi, R and Yanagimachi, H (1989) Morphology and fertilizability of zona-free hamster oocytes separated into halves and quarters by centrifugation. Biol Reprod (in press)

Table 9

The distribution and intensity of NBD-ph staining among germinal vesicle (GV) and metaphase II (M-II) eggs and two cell embryos

Eggs	Eggs treated	Intensity of fluorescence	Thickness of cortical staining	Distribution of F actin staining
GV	CCD(+)	++++ - +++	1.5-2.5 um	uneven with patches,cortical
	CCD(-)	+++	2.5 um	evenly, cortical
M-II	CCD(+)	+++ - ++	1.5-2 um	uneven with patches,cortical
	CCD(-)	++	1.5-2 um	evenly,cortical * thick at M-II site
2-cell	CCD(+)	+++ - ++	1-1.5 um	diffuse in cytoplasm cortex with patches
	CCD(-)	+++ - ++	1.5-2 um	Cortical and nuclear ** thick at cell contact region

* F actin staining is heavy subcortically, between cortex and metaphse II nucleus. (about 3-4 um).

** F actin staining is cortical and perinuclear in the 2-cell embryo, with heavy staining in cell contact regions.

PLATE XVI

SEPARATION OF AN UNFERTILIZED OVIDUCTAL EGGS

INTO HALVES BY CENTRIFUGATION

IMMATURE EGGS AT THE GERMINAL VESICLE STAGE

CENTRIFUGED FOR 40 MIN AT 9500 G

Figure 1.

Separation of an unfertilized oviductal egg into halves by centrifugation (9500 g).

A. Before centrifugation.

B-B'. Light and heavy halves, after 10 min of centrifugation (an arrow in figure 1B indicates the direction of centrifugation).

C. Light (l) and heavy (h) halves which have "round-up" after removal of centrifugal force. Magnification, x270.

Figure 2.

A-B: An immature egg at germinal vesicle stage, centrifuged for 40 min at 9500 g; the egg elongated, but did not separate into halves. Before (A) and after (B) compression between a slide and coverslip. The germinal vesicle (gv) is clearly visible at the border between clear and granular regions of the compressed, B. An arrow in 2 A indicates the direction of centrifugation. Magnification, x380.

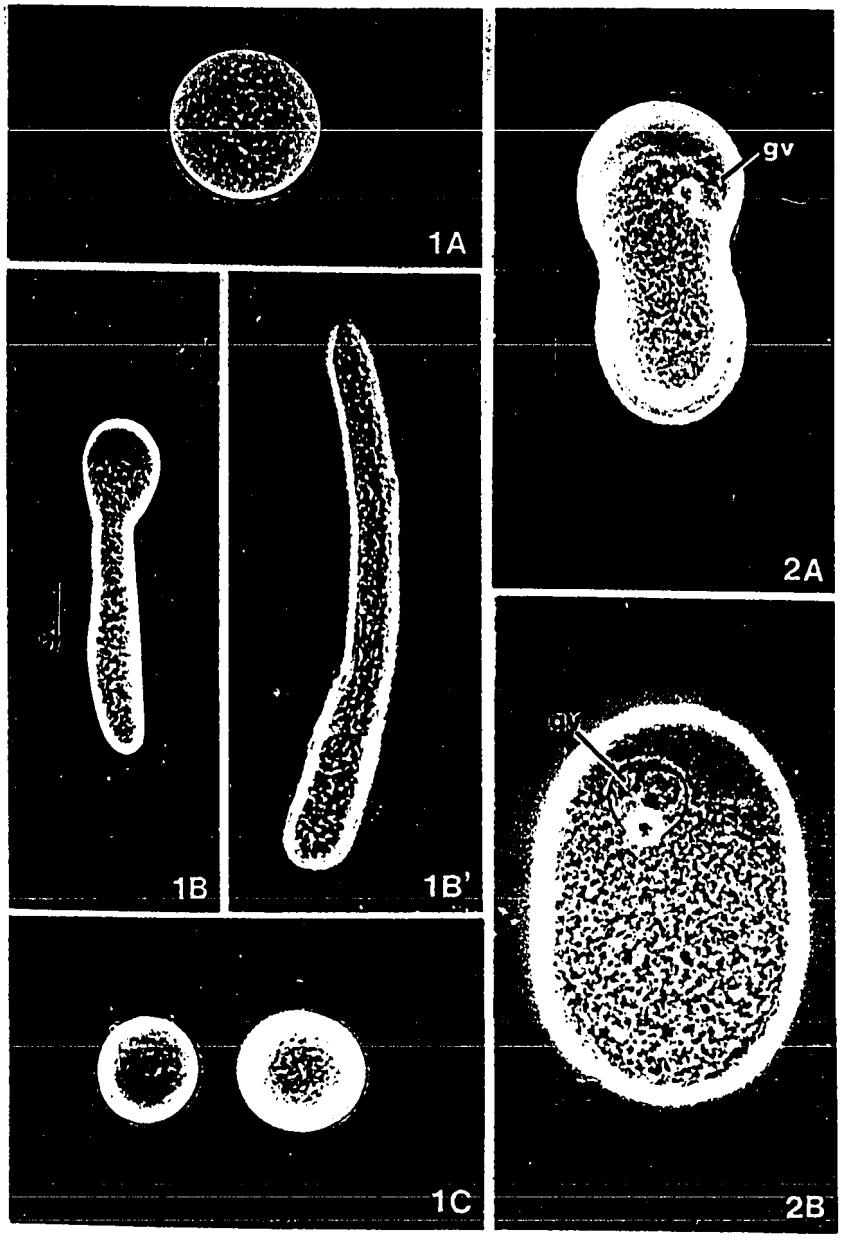

1A
gv
2A
1B
1B'
gv
1C
2B

PLATE XVII

CHANGES IN THE EGG RIGIDITY DURING MEIOTIC MATURARTION AND AFTER FERTILIZATION AS REVEALED BY CENTRIFUGATION

Figure 3.

Abscissa: stages of egg maturation and development.

Ordinate: minimum time (min) needed for separating all eggs (or blastomeres) in a group into halves by centrifugation at 9,500 g, mean ± S.D. It can be seen that the rigidity of intact egg (-o-) diminishes with the progression of meiotic maturation, reaches a minimum with the completion of maturation, and then increases after fertilization. Ten to 40 uM CD (-o-) reduces egg rigidity regardless of the stages of egg maturation and development, whereas 10-40 uM colcemid (*) and 1-4% DMSO ( ) do not.

GV, germinal vesicle stage; GVBD, during and immediately after germinal vesicle breakdown; M1, T1, M2, metaphase and telophase of the 1st and 2nd meiosis; ovarian and oviductal, mature ovarian and oviductal eggs at metaphase of the 2nd meiosis; pron., pronuclear stage. 1st cleav., during the 1st cleavage.

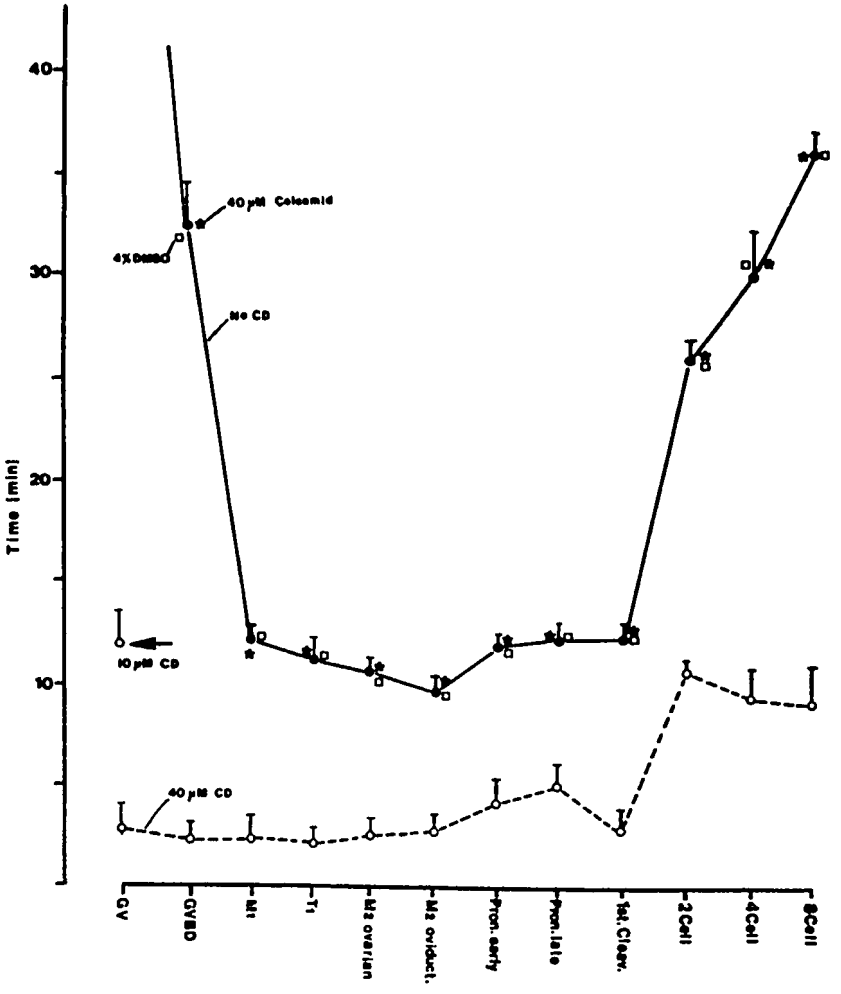

40
30
20
10
Time (min)
40 μM Colcemid
4X DMSO
No CD
10 μM CD
40 μM CD
GV
GVBD
MI
TI
MII ovarian
MII oviduct.
Pron. early
Pron. late
1st. Cleav.
2Cell
4Cell
8Cell

PLATE XVIII

SUCCESSIVE STAGES OF SEPARATION OF A

PRONUCLEAR EGG INTO HALVES

Figure 4.

Successive stage of separation of a pronuclear egg into halves.

A. Before centrifugation.

B-C. Seven and 11 min after start of centrifugation at 9500 g (an arrow in Figure 4B indicates the direction of centrifugation).

D-E. Light and heavy halves; in some instances, an egg can separate into three or more fragments.

Magnification, x330 except for the main figure of C which is x65.

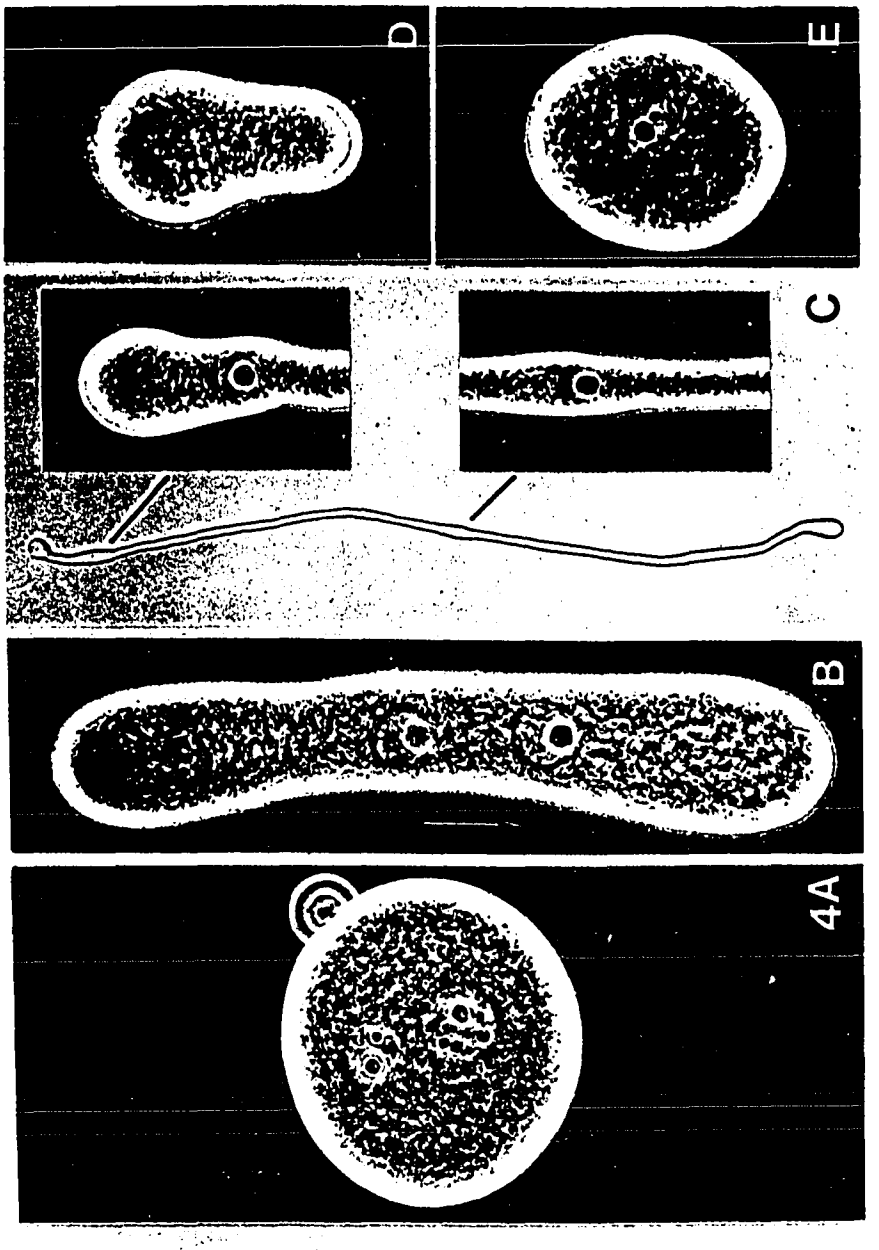

D
E
C
B
4A

PLATE XIX

SUCCESSIVE STAGES OF SEPARATION OF THE BLASTOMERES OF A TWO CELL EGG INTO LIGHT AND HEAVY HALVES

Figure 5.

Successive stages of separation of the blastomeres of a two cell egg into light and heavy halves.

A. Before centrifugation.

B. The blastomeres of a two cell egg 5-7 min after the start of centrifugation at 9500 g.

C. The blastomeres of a two cell egg 15 min after the start of centrifugation at 9500 g; only one of the blastomeres is shown in the figure

D. The blastomeres of a two cell egg 20-22 min after the start of centrifugation at 9500 g; only one of the blastomeres is shown in the figure

E-F. Light and heavy halves before "rounding-up". Magnification, x200.

E
F
D
C
5A
B

PLATE XX

SEPARATION OF A CYTOCHALASIN-TREATED GERMINAL VESICLE EGG INTO HALVES

Figure 6.

Separation of a cytochalasin-treated germinal vesicle egg into halves.

A. Before separation into two, an arrow indicates the direction of centrifugation.

B-C. Separated into light (B) and heavy (C) halves. The cord-like portion of the light half may or may not be integrated into the main body of the light half during "rounding-up" process. GV, germinal vesicle. Magnification, x430.

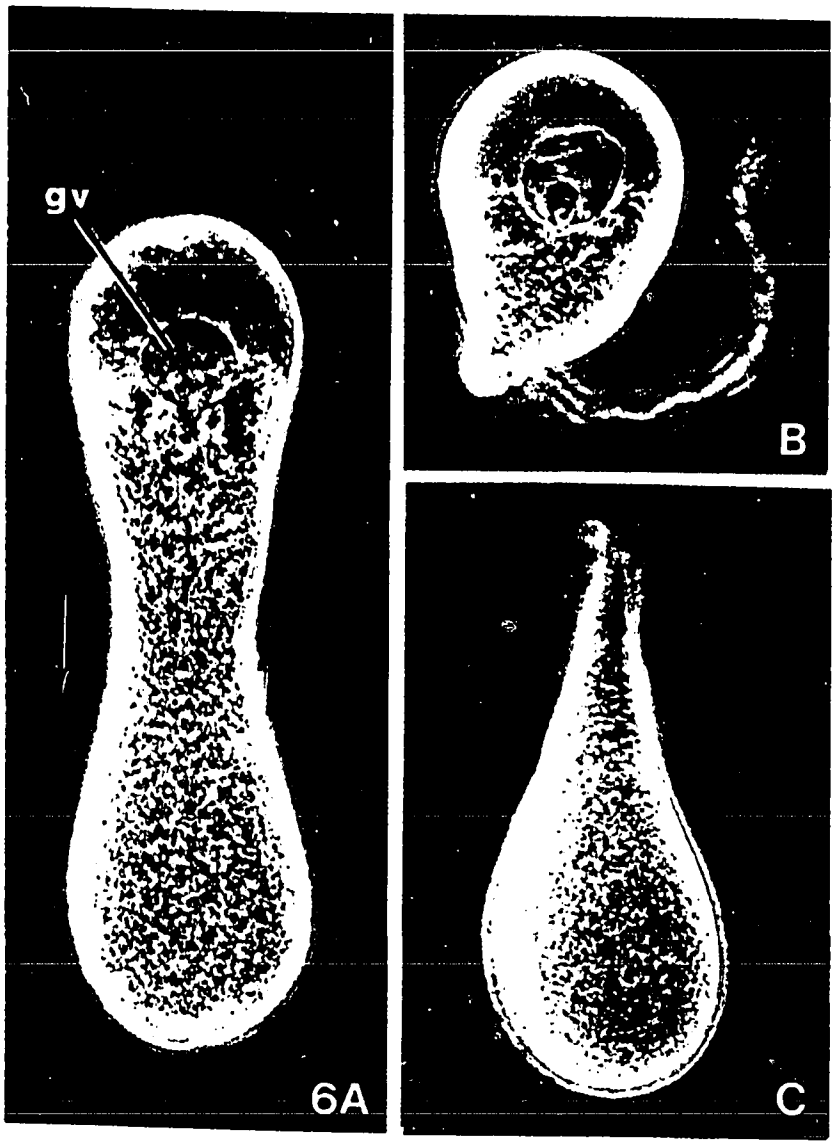

gv
6A
B
C

PLATE XXI

REACTION OF A MATURE OVIDUCTAL EGGS AND GERMINAL VESICLE EGGS TO SUCTION OF MICROMANIPULATOR

Figure 7.

Reaction of a germinal vesicle egg to suction. Arrows in the capillary tube indicate directions of fluid flow. The egg resists to suction force (A-C) until the tip of evagination breaks abruptly and the broken egg is drawn into the tube (D); the contents of broken egg are pushed out of the tube as granular and amorphous materials (E).

Figure 8.

Reaction of mature oviductal eggs to suction. Arrows in capillary tube indicate directions of fluid flow.

A-D An egg is easily drawn into the tube (the entire egg can be drawn into the tube without being ruptured).

E-I When the portion of an egg, which has been drawn into the tube, is push out, it resembles a soft sausage. Two eggs are shown here, one of which (b) being drawn into the tube deeper than the other (a).

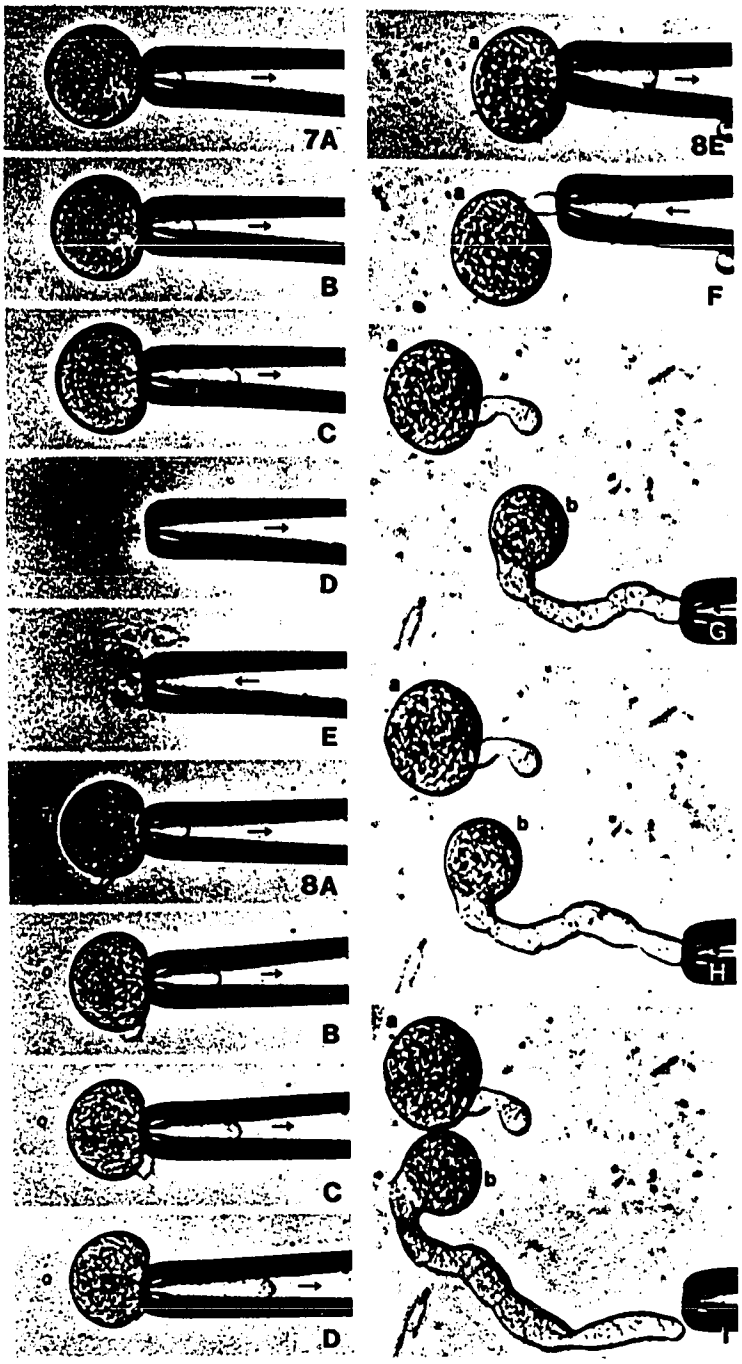

7A
B
C
D
E
8A
B
C
D
8E
F
G
H

PLATE XXII

NBD-PHALLACIDINE FLUORESCENCE STAINING OF EGGS

Figure 9.

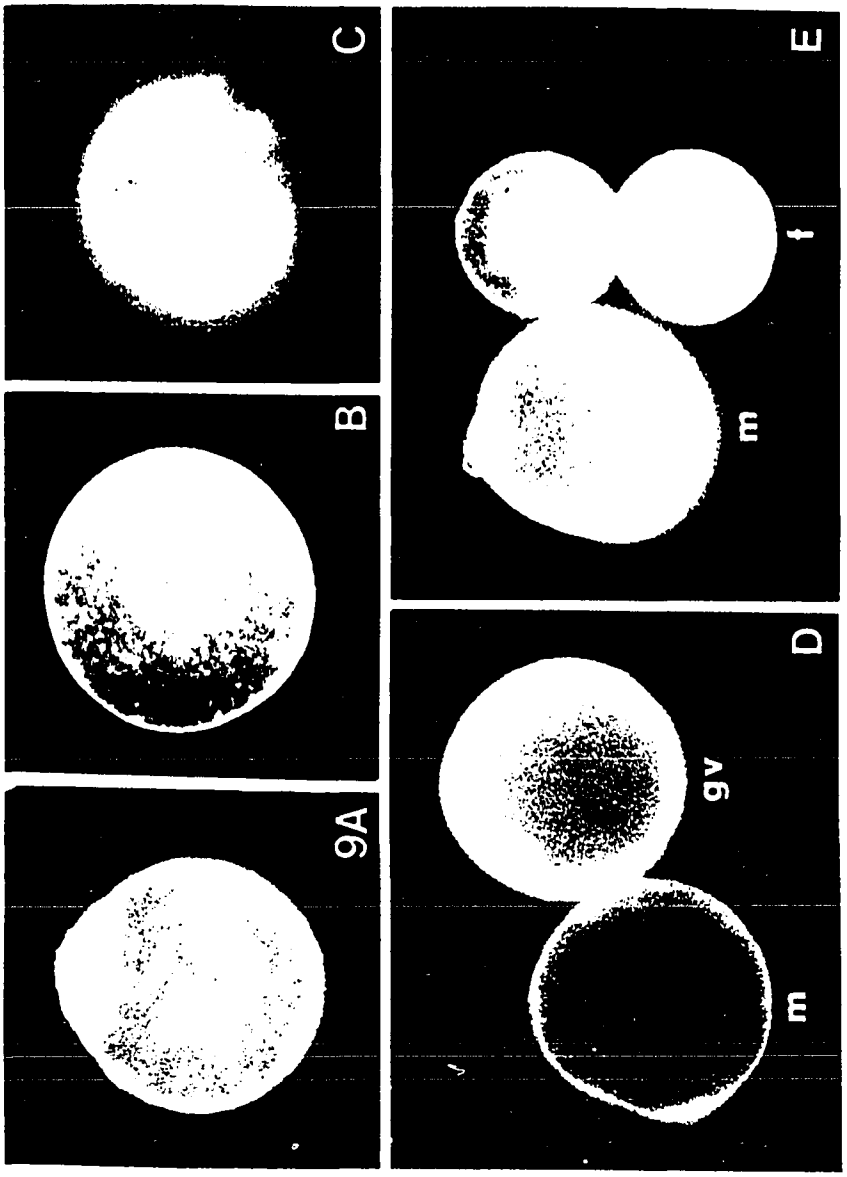
NBD-phallacidine fluorescence of eggs.

A. A mature oviductal egg, showing strong fluorescence of the cortex, particularly the area where metaphase chromosome are located, the top of the figure.

B-C. Side and surface view of a mature oviductal egg treated with cytochalasin D; note the endoplasmic fluorescence (B) and spotty fluorescence of the egg surface (C).

D. Mature oviductal egg (m) and germinal vesicle egg (gv) side by side; note that cortical fluorescence is stronger in the germinal vesicle egg.

E. Mature oviductal egg (m) and 2-cell egg (f) side by side; in this micrograph, intensity of cortical fluorescence appears same in (m) and (t), but in most case, (t) showed stronger fluorescence than (m) within 5-10 sec of exposure to UV light. Magnification, x270.


9A
B
C
D
gv
m
E
m
f

APPENDIX I

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Differences between mature ovarian and oviductal oocytes: a study using the golden hamster

Cheng-Hsiung Yang and R.Yanagimachi[1]

Department of Anatomy and Reproductive Biology, University of Hawaii School of Medicine, Honolulu, Hawaii 96822, USA

[1]To whom correspondence should be addressed

To investigate whether mature ovarian oocytes are physiologically identical with recently ovulated oocytes, hamster oocytes collected from ovaries ~1 h before ovulation were compared with the oocytes which had been in the oviduct for 3–4 h. Although these two groups of oocytes were at the metaphase of the second meiosis, there were quantitative differences between the two with respect to (i) sensitivity of cumulus matrix to hyaluronidase and spermatozoa (oviductal > ovarian), (ii) size of the perivitelline space (oviductal > ovarian), (iii) viscosity of the ooplasm (ovarian > oviductal), (iv) responsiveness to Ca^{2+}-ionophore (oviductal > ovarian), and (v) time needed in completing meiosis (ovarian > oviductal). The most prominent difference was found in the zona pellucida. The zona of the oviductal oocyte was 'heterogeneous' in its optical density and had a stronger acrosome-reaction-inducing ability than that of the ovarian oocyte. When cultured in artificial media (e.g. F-10 medium with serum) for 4 h, the ooplasm of ovarian oocytes became like that of oviductal oocytes. However, their zonae remained unchanged. Zonae of ovarian oocytes became like those of oviductal oocytes only when they were exposed to ampullary and/or isthmic fluids. The zona-altering factors in the oviductal fluid (oviductal glycoproteins), which are apparently integrated into the native zona, may act to enhance the various functions of the zona.

Key words: oocytes/maturation/cumulus oophorus/zona pellucida

Introduction

In mammals, with the possible exception of the fox, dog and certain primitive insectivores, ovulation occurs when an oocyte reaches the metaphase of the second meiotic division (Austin and Walton, 1960; Baker, 1982). Since human ovarian oocytes, collected from ovaries shortly before ovulation, can be fertilized *in vitro* and develop into normal viable offspring (Edwards, 1981; Edwards and Purdy, 1982; Trounson and Wood, 1984; Wolf and Quigley, 1984), investigators have gained the impression that ovarian oocytes which have reached the metaphase of the second meiosis are fully mature and physiologically identical to ovulated oocytes. The present study was initiated to determine whether this is the case. The golden hamster was chosen as an animal model, because much basic information is available on the gametes and fertilization processes of this species, which was needed for the present study.

Materials and methods

Media

The media used in this study were M2 medium containing 0.4% bovine serum albumin (BSA) (Quinn *et al.*, 1982), m-TALP-4 medium containing 1.5% BSA (Cummins and Yanagimachi, 1986) and m-TALP-7 medium containing 1.2% BSA (Uto *et al.*, 1988). Albumin was added to these media immediately before use. These media had a pH value of ~7.4 when they were kept under mineral oil (gas phase, air) during experiments.

Animals

Golden hamsters (*Mesocricetus auratus*) were raised and maintained in an air-conditioned room with controlled light conditions (light, 05.00–19.00 hours; dark, 19.00–05.00 hours). Under these conditions mature females came into behavioral oestrus around 18.00 hours of every fourth day (day 4) and ovulated early next morning (01.00–02.00 hours of day 1). An average of 11 ± 1.7 (mean ± SD) oocytes were ovulated in each cycle. The age of the females used in this study was 2–4 months and that of the males was 4–6 months.

Collection and examination of ovarian and oviductal oocytes

The ovarian oocytes were collected shortly before natural ovulation by killing females between 23.30 and 24.00 hours of day 4, placing isolated ovaries in M2 medium and breaking the wall of distended follicles using a sharp needle. The oocytes were collected shortly after natural ovulation by flushing oviducts with M2 medium between 05.30 and 06.00 hours of day 1. Thus, these ovarian and oviductal oocytes were considered to be ~1.5–3.0 h before and 2.5–5.0 h after ovulation respectively. Superovulation was induced by i.m. injection of pregnant mare serum gonadotrophin (30 IU/female; Calbiochem, La Jolla, CA) in the morning of day 1 followed by injection of human chorionic gonadotrophin (HCG) (30 IU/female; Organon, West Orange, NJ) in the afternoon or evening of day 3. Ovulation took place between 13 and 14 h after HCG injection. An average of 50 oocytes was ovulated in each female. Ovarian oocytes were collected at 13 h after HCG injection when 98% of enlarged follicles were still 'unruptured'. Oviductal oocytes were flushed out of oviducts 17 h after HCG injection. Thus, these ovarian and oviductal oocytes were considered to be within 1 h before

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and 3–4 h after superovulation, respectively. Karyologically, all the ovarian and oviductal oocytes used in this study were at the metaphase of the second meiosis.

To examine the arrangement of cumulus cells around the oocyte, the cumuli containing oocytes were mounted between a slide and coverslip supported by four pillars of Vaseline–paraffin mixture, compressed strongly under the coverslip and examined with a phase-contrast microscope. To examine the details of the zona pellucida and the oocyte itself (the vitellus), oocytes were freed from cumulus cells by treating them for 5 min (at 25–30°C) with 0.1% bovine testicular hyaluronidase (300 USP U/mg; ICN Pharmaceut., Cleveland, OH) in M2 medium. The oocytes were rinsed with enzyme-free M2 medium and mounted between the slide and coverslip with care being taken not to compress the oocytes under the coverslip. Measurements and photography of the zona pellucida, perivitelline space and vitellus were made within 15 min after the isolation of oocytes from the ovary or oviduct. Since the diameter and volume of vitellus was altered by the osmolality of the medium, every precaution was taken to avoid evaporation of M2 medium.

Examination of the susceptibility of cumulus oophorus to hyaluronidase and capacitating spermatozoa

The ovarian oocyte was individually surrounded by a cumulus oophorus, whereas oviductal oocytes were in a large mass of the cumulus. The 'sticky' cumulus matrix of the ovulated oocytes must have united into a large mass while the oocytes were moved around by a peristaltic movement of the oviduct. To compare the susceptibility of the cumulus matrix of ovarian and oviductal oocytes to hyaluronidase, it was necessary to reduce the thickness of the cumulus of oviductal oocytes close to that of the cumulus surrounding an ovarian oocyte. This was done by flushing a cumulus mass through the utero-tubal junction. A blunt-ended fine needle (no. 28–30 gauge) was inserted into the infundibulum and a cumulus mass in the ampulla was flushed out through the utero-tubal junction. A string-like cumulus containing oocytes was collected. The 'string' was cut into several pieces before they were exposed to 0.1% bovine hyaluronidase in M2 medium. The enzyme treatment was performed at room temperature (23–25°C) with constant agitation of the medium. The time needed for the complete 'denudation' of all oocytes was recorded.

The susceptibility of the cumulus to capacitating spermatozoa was tested as follows. Cumuli containing either ovarian or oviductal oocytes (see above) were transferred into 300 μl of m-TALP-4 medium which had been previously placed under mineral oil (Squibb and Sons, Princeton, NJ) in a plastic Petri dish. A drop (40 μl) of sperm suspension which had been incubated for 1 h in m-TALP-4 medium (see section Insemination and Examination of oocytes) was added to the drop containing cumuli and mixed thoroughly so that the concentration of spermatozoa was $1-2 \times 10^5$/ml. The preparations, incubated at 37–38°C, were examined from time to time until cumulus cells had been dispersed completely.

'Staining' of oocytes with Janus green

Janus green is known as a vital stain for mitochondria. While trying to stain mitochondria and cortical granules of hamster oocytes (unpublished experiments), it was found that this dye 'stains' the inner half of the zona pellucida of oviductal oocytes. Ovarian and oviductal oocytes, which had been freed from cumulus cells by hyaluronidase treatment, were immersed for 10 min in 0.9% NaCl solution containing 0.03% Janus green B (Allied Chemical, Morristown, NJ) and 20 mM Tris (pH 7.4). They were rinsed thoroughly with M2 medium, mounted between a slide and coverslip and examined immediately with ordinary optics. Immediate examination was important because the staining was not permanent.

Examination of the responsiveness of oocytes to Ca^{2+} ionophore

Cumulus-free ovarian and oviductal oocytes were induced to activate by the Ca^{2+} ionophore, A-23187 (Sigma Chemical, St Louis, MO), using a procedure similar to that used by Steinhardt *et al.* (1974). Briefly, oocytes were treated with 10 μM ionophore in Ca^{2+}/Mg^{2+}-free M2 medium for 3 min, rinsed thoroughly and kept in the same medium but without ionophore for 30 min, then transferred into regular M2 medium. An egg was recorded activated when it had resumed meiosis. The density of cortical granules (mean number in 25 μm^2) in the cortical layer of the oocyte was determined before and after activation of oocytes.

Preparation of zona-free oocytes

Some ovarian and oviductal oocytes were freed from zonae pellucidae by treating them for 1–2 min with M2 medium containing 0.1% bovine pancreatic trypsin (120 TAME U/mg; ICN Pharmaceutical, Cleveland, OH). They were thoroughly rinsed with M2 medium before use.

Examination of the 'viscosity' of the vitellus

The 'viscosity' of the vitellus of ovarian and oviductal oocytes was examined using centrifugal force. One-hundred μl of 45% Percoll (Sigma) in M2 medium was placed in the bottom of a 400-μl plastic micro-centrifuge tube (5 × 45 mm, heparin-lithium coated; Beckman Instruments, Palo Alto, CA), which was overlaid by 100 μl of 30% Percoll followed by 100 μl of 7.5% Percoll. M2 medium (~50 μl) containing zona-free oocytes was placed gently on the 7.5% Percoll layer and the tube was centrifuged at 9500 *g* using a high-speed microcentrifuge (Model Z-230M, Vangard International, Neptune, NJ). During centrifugation, oocytes migrated to the 30% Percoll layer, the vitellus elongated then, in time, divided into heavy and light halves. The minimum time needed for complete separation of all vitelli into heavy and light halves was determined. All procedures were carried out at room temperature (23–25°C).

In-vitro culture of ovarian oocytes

Ovarian oocytes were cultured in various media for 4 h to see if the properties of the zona and ooplasm were altered by these media. The media tested included: (i) M2 medium supplemented with 10% heat-inactivated (60°C, 30 min) hamster serum; (ii) Ham's F10 medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% heat-inactivated hamster serum; (iii) M2 medium with oviduct extract; and (iv) ampullary fluid and (v) isthmic fluid of the hamster oviduct.

Oviduct extract was prepared as follows. Oviducts were collected from females 17 h after HCG injection. After oocytes

were removed from the ampullae, the entire oviducts (the ampulla and isthmus) were frozen at −20°C. For one experiment, 20 frozen oviducts were thawed and homogenized in 1.5 ml of M2 medium. The homogenate was centrifuged at 400 g for 10 min to sediment the tissue debris. The supernatant was collected and its pH was adjusted to 7.3−7.4. Ampullary and isthmic fluids were collected as follows. Between 13 and 14 h before expected time of ovulation, females were anaesthetized with trifluoroethane vapour (Sigma) and the oviducts were exposed through dorsolateral incisions. To collect ampullary fluid, the oviduct was ligated at the infundibulum and the ampullary−isthmic junction. A thin nylon surgical suture (6-0, American Cyanamid Co., Danbury, CT) was used for the ligation.

To collect the isthmic fluid, the ampullary−isthmic junction and the utero-tubal junction were ligated. Oviductal fluid (a mixture of the ampullary and isthmic fluids) was collected by ligating the infundibulum and the utero-tubal junction. Every precaution was taken to avoid blocking the blood supply to the oviduct. The ampulla and/or the isthmus was/were distended, depending on the ligation sites, 16−17 h after surgery (i.e. 3−4 h after ovulation). From a distended ampulla, 20−30 μl of fluid (ampullary fluid) could be collected. From a distended isthmus, 10−15 μl of fluid (isthmic fluid) could be collected.

Ovarian oocytes (surrounded by the cumulus oophorus) in 10 μl of M2 medium were transferred into (i) 300 μl of M2 medium with 10% serum, (ii) 300 μl of Ham F-10 medium with 10% serum, (iii) 300 μl of M2 medium was oviduct extract, (iv) 60 μl of ampullary fluid, and (v) 40 μl of isthmic fluid, all of which were previously placed under mineral oil in a plastic Petri dish. Each test medium received 10−12 oocytes. Preparations were incubated under either pure air (i,iii) or 5% CO_2 in air (ii,iv,v). At the end of a 4-h incubation at 37°C, the morphology of the oocytes as well as the responsiveness of oocytes to Ca^{2+} ionophore and centrifugal force were examined.

Examination of oocytes collected from ligated oviducts

Between 13 and 14 h before the expected time of ovulation, females were anaesthetized and oviducts were ligated as described already, but at the first, second or last loop of the ampulla (cf. Figure 6). The females were killed 17 h later (i.e. 3−4 h after ovulation) to examine the morphology of oocytes collected from ligated ampullae.

Insemination and examination of oocytes

Spermatozoa, collected from the cauda epididymis, were incubated in m-TALP-4 medium (Cummins and Yanagimachi, 1986). The concentration of spermatozoa during incubation was $1-2 \times 10^6$ cells/ml. Under these conditions, spermatozoa began to undergo spontaneous acrosome reactions ~2 h after the start of incubation and the majority completed the reaction during the next 2 h (Cummins and Yanagimachi, 1986). All the oocytes to be inseminated were freed from cumulus cells by the hyaluronidase treatment. Some of them were further treated with trypsin to remove their zonae pellucidae. The oocytes were thoroughly rinsed with M2 medium, and transferred to an aliquot (300 μl) of m-TALP-4 medium which had been placed previously under mineral oil in a plastic Petri dish. Insemination was performed by adding 20−40 μl of the sperm suspension to the medium containing oocytes. Zona-intact oocytes were inseminated with 1-h pre-incubated spermatozoa, incubated at 37°C and examined for zona penetration at 1-h intervals. Zona-free oocytes were inseminated with 4-h preincubated spermatozoa and examined at hourly intervals for cortical granule exocytosis, the resumption of meiosis and development of sperm and egg pronuclei.

Assessment of the acrosome-reaction-inducing ability of the zona pellucida

The ability of the zona pellucida to induce the acrosome reaction of spermatozoa was determined using the procedure described by Uto *et al.* (1988). Briefly, cumulus-free oocytes with intact zonae were inseminated with spermatozoa which had been incubated for 2 h in m-TALP-7 medium. Five minutes after insemination, when 30−50 spermatozoa attached to each oocyte (zona), oocytes were rinsed and transferred into fresh m-TALP-7 medium. Incubation was carried out at 37−38°C. The incidence of the acrosome reaction in lively motile spermatozoa on the zona was determined at 30-min intervals.

Statistical analysis

Where appropriate, statistical analysis was performed using Student's *t*-test for paired samples.

Results

Since preliminary studies revealed that naturally ovulated and superovulated oocytes were identical with respect to their morphology and several physiological characteristics (e.g. response to spermatozoa and Ca^{2+} ionophore), data obtained from these two groups of oocytes were combined. However, most information reported in this paper was obtained from superovulated (ovulating) oocytes.

Difference in the cumulus oophorus

The cumulus cells surrounding an ovarian oocyte were tightly and radially arranged (Figure 1A), whereas those around an oviductal oocyte were loosely and randomly arranged (Figure 1B). When exposed to 0.1% hyaluronidase, most of the cumulus cells surrounding the ovarian oocyte were dispersed rapidly except those on the zona pellucida. It took 3.6 ± 0.5 min (mean ± SD, n = 24) before all the cumulus cells were dispersed. The cumulus cells around the oviductal oocyte were dispersed by hyaluronidase much more readily than those surrounding the ovarian oocyte. All the oviductal oocytes became free of cumulus cells in 2.2 ± 0.3 min (n = 25). This difference was significant ($P < 0.01$). When exposed to capacitating spermatozoa ($1-2 \times 10^5$ cells/ml), the cumulus cells surrounding oviductal oocytes dispersed faster (37.5 ± 7.5 min; n = 30) than those surrounding ovarian oocytes (67.5 ± 7.5 min, n = 30). The difference was also significant ($P < 0.01$).

Differences in the general structure of the oocytes

Examination of 24 ovarian oocytes and 25 oviductal oocytes revealed that the diameter of the vitellus of the ovarian oocyte was significantly larger (81 ± 1.3 μm) than that of the oviductal oocyte (78 ± 1.0 μm) ($P < 0.01$). The thickness of the zona

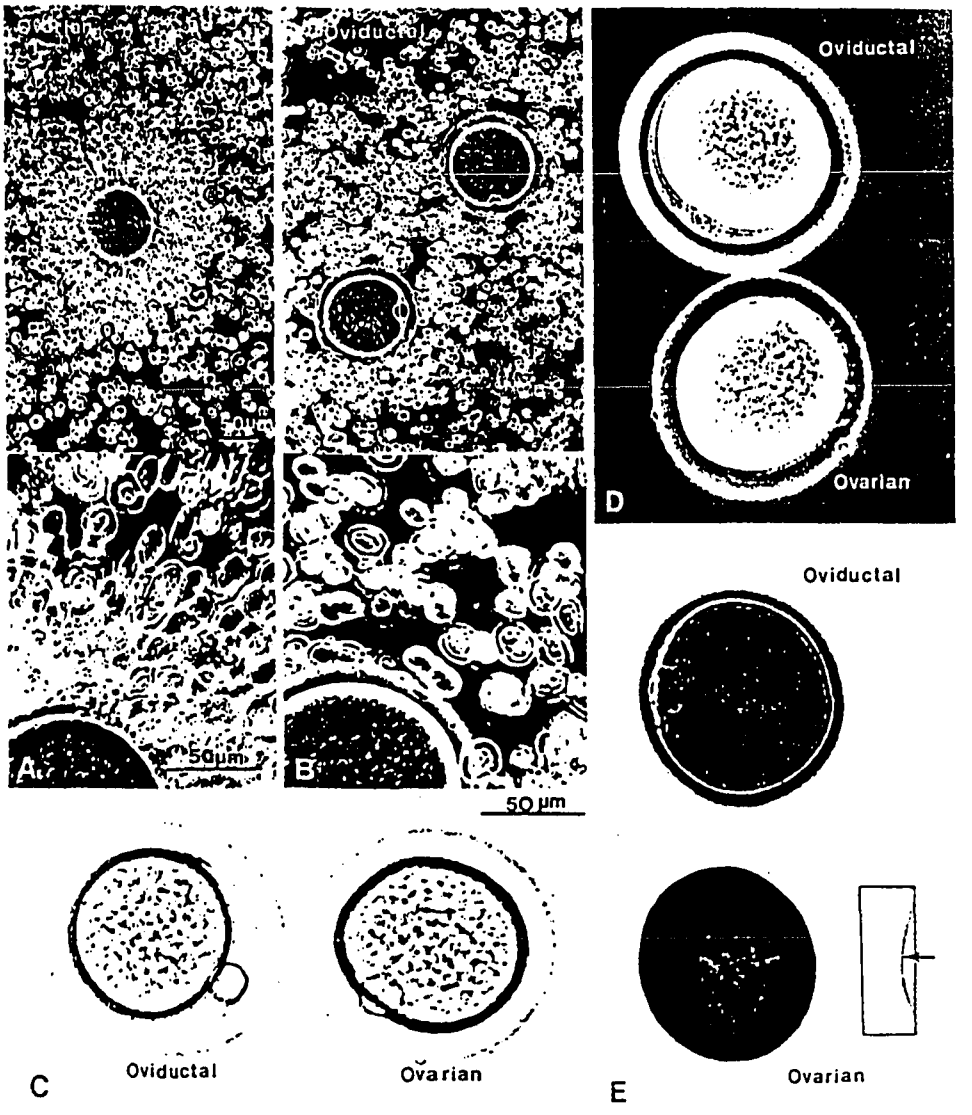

Fig. 1. Comparison of ovarian and oviductal oocytes. (A) Ovarian oocytes and cumulus cells, phase-contrast: note that cumulus cells are tightly and radially arranged. (B) Oviductal oocytes and cumulus cells: cumulus cells are loosely and randomly arranged. (C) Oocytes seen through regular optics: oviductal oocyte has a larger perivitelline space than ovarian oocyte: zonae appear the same. (D) Oviductal and ovarian oocytes seen through phase-contrast optics: note an optical heterogeneity of the zona of oviductal oocyte. (E) Oviductal and ovarian oocytes seen through regular optics after staining with Janus green: note that the inner half of the zona of oviductal oocyte is stained dark; the zona of the ovarian oocyte has reduced its thickness and is unstained except its inner surface (an arrow in the insert indicates that the inner surface of the zona is darkly stained).

was about the same in the ovarian oocyte (11 ± 0.5 μm) and oviductal oocyte (12 ± 0.7 μm) ($P > 0.05$). Partly because of an expansion of the zona and partly because of a shrinkage of the vitellus, the oviductal oocyte had a considerably larger perivitelline space (150 ± 6.2 × 10^3 μm^3) than the ovarian oocyte (64 ± 10.5 × 10^3 μm^3) ($P < 0.001$) (Figure 1C).

Differences in the zona pellucida

There was a distinct difference between ovarian and oviductal oocytes with respect to the appearance of the zona pellucida. When viewed with the phase-contrast microscope equipped with positive-contrast optics, the outer half or the zona of the oviductal oocyte appeared very 'bright', whereas its inner half appeared very 'dark' (Figure 1D, top). Such an optical heterogeneity was not detected in the zona of ovarian oocyte (Figure 1D, bottom). The heterogeneity of the zona of the oviductal oocyte could also be appreciated when the zona was examined through a microscope with regular optics after 'staining' of the oocyte (zona) with Janus green B. When oviductal oocytes were immersed in 0.03% solution of Janus green for 10 min, and then washed thoroughly, the inner half of the zona appeared dark blue (Figure 1E, top). The zona of the ovarian oocyte reduced its thickness and remained unstained except for its inner surface (Figure 1E, bottom).

The zonae of the ovarian and oviductal oocytes were equally sensitive to trypsin. There was no significant difference between the zonae of ovarian oocytes and those of oviductal oocytes with respect to the minimum time needed for dissolution by trypsin. A dilute solution of trypsin (0.01%) in M2 medium completely dissolved zonae of all of 30 ovarian and 30 oviductal oocytes in 356 ± 23 s at 23–25°C.

When oocytes were inseminated with 1-h pre-incubated spermatozoa, sperm penetration through the zona took place faster in the oviductal oocytes than in the ovarian oocytes (Figure 2). The zonae of oviductal oocytes appeared to have a stronger acrosome-reaction-inducing ability than those of ovarian oocytes (Figure 3).

Differences in the vitellus

Hamster oocytes are activated by Ca^{2+} ionophore (Steinhardt *et al.*, 1974). When 78 oviductal oocytes were treated with 10 μM ionophore and examined 1 h later, 93% of them were activated as shown by the exocytosis of cortical granules and the presence of the female pronucleus in the ooplasm. Only 37% of 70 ovarian oocytes were activated when they were treated and examined under the same conditions, indicating that the ovarian oocytes were less responsive to the ionophore than the oviductal oocytes.

When 32 zona-free ovarian and 44 zona-free oviductal oocytes were inseminated with acrosome-reacted spermatozoa and examined 1 h later, all of them contained decondensing sperm nuclei and/or early sperm pronuclei. The ratio of the decondensing sperm nuclei and the early sperm pronuclei was 1:9 in both ovarian and oviductal oocytes, indicating that sperm nuclei transform into pronuclei at about the same rate in these two groups of oocytes. The situation was different for oocyte nuclei. At 1 h after insemination, 90% of the 32 ovarian oocytes were at the telophase of the second meiosis, whereas 91% of the 44

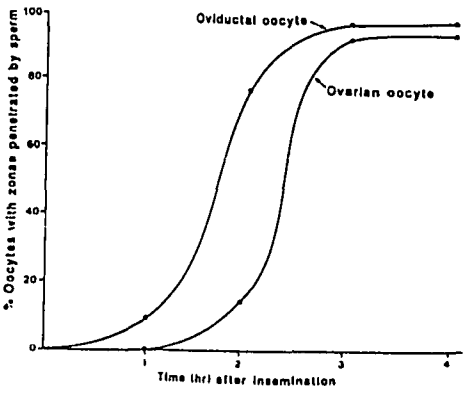

Fig. 2. Proportion of penetrated oocytes at various times after in-vitro insemination. Oviductal and ovarian oocytes were inseminated *in vitro* with 1-h preincubated spermatozoa and examined for zona penetration. Those with sperm heads (nuclei) either in the perivitelline space or the vitellus were recorded 'penetrated'. Each value is based on examination of ~40 oocytes (four experiments).

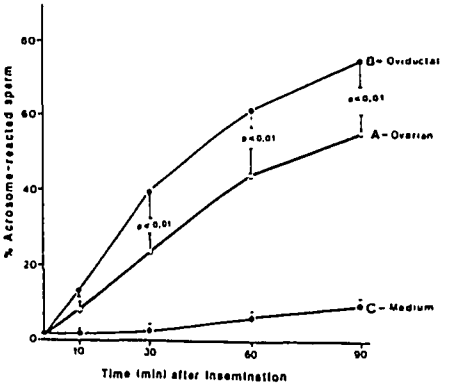

Fig. 3. Proportion of acrosome-reacted spermatozoa on the surface of zona pellucida. Ovarian (A) and oviductal oocytes (B) were inseminated *in vitro* with 2-h preincubated spermatozoa and the incidence of acrosome-reacted spermatozoa on the zona was determined. Each value (mean ± SD) is based on examination of 700–1200 spermatozoa on 25–40 zonae (five or six experiments). (C) Incidence of spontaneous acrosome reaction in m-TALP-7 medium.

oviductal oocytes were in the early pronuclear stage. Apparently, after activation by spermatozoa, meiosis was completed more quickly in oviductal oocytes than in ovarian oocytes.

When zona-free oviductal oocytes (Figure 4A) were centrifuged at 9500 *g*, they elongated (Figure 4B–C) and then divided into

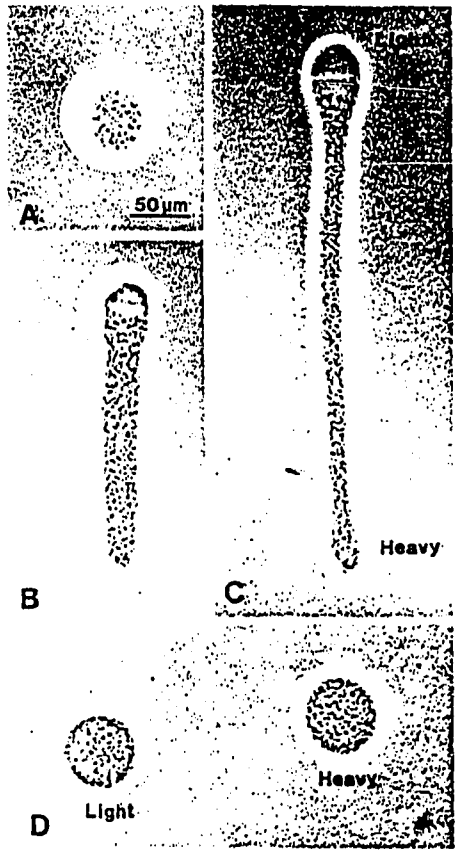

Fig. 4. Separation of zona-free eggs into light and heavy halves by centrifugation: (A) before centrifugation; (B and C) elongated; (D) separated into light and heavy halves.

light and heavy halves (Figure 4D). It took 594 ± 13 s before all of the oviductal oocytes were divided into two halves (six experiments using 120 oocytes). Ovarian oocytes took a significantly ($P < 0.01$) longer time (637 ± 10 s) to be divided under the same conditions (six experiments using 100 oocytes). Apparently, the ooplasm of ovarian oocytes is more 'viscous' than that of oviductal oocytes.

Effects of in-vitro culture of ovarian oocytes on the characteristics of the zona pellucida and vitellus

As stated above, ovarian oocytes were different from oviductal oocytes in several morphological and physiological characteristics. What are the causes of these differences? Chronologically, the oviductal oocytes used were 3–4 h older than ovarian oocytes. If the time alone is solely or primarily responsible for the differences, ovarian oocytes should become like oviductal oocytes when cultured *in vitro* for 3–4 h. Table I summarizes the results of experiments in which ovarian oocytes (with surrounding cumulus cells) were cultured for 4 h in various media. When cultured in M2 and Ham's F10 media supplemented with hamster sera (experiments A and B), the cumulus oophorus expanded, but the oocyte itself remained morphologically unchanged. Its perivitelline space remained small and the zona pellucida retained its optical homogeneity. The vitellus, however, did become more responsive to the Ca^{2+} ionophore. The vitellus also became 'less viscous' after 4 h of culture. In other words, the vitelli of ovarian oocytes became somewhat like those of oviductal oocytes, whereas their zonae and the perivitelline space remained unaltered. It will be seen from Table I that both the zonae and vitelli of ovarian oocytes became like those of the oviductal oocytes when they were cultured in the medium containing an oviduct extract (experiment C), or in the ampullary and isthmic fluids (experiments D and E). Figure 5 summarizes the results of experiments in which ovarian oocytes were cultured for 4 h *in vitro* in the presence or absence of oviductal fluid. When cultured in M2 medium supplemented with hamster serum, the zona of ovarian oocyte increased its acrosome-reaction-inducing ability slightly, but not significantly. The ability increased significantly when oocytes were cultured in the oviductal fluid.

When and where are the characteristics of the zonae of in-vivo ovulated oocytes altered?

The oocytes released from the ovary stay on the ovarian surface and the ovarian bursa for some time before they are picked up by the infundibulum (fimbria). All the oocytes collected from the bursa appeared exactly like ovarian oocytes, with a small perivitelline space and an optically homogeneous zona pellucida. The oocytes which were believed to have been in the ampulla of the oviduct for ~1 h had a large perivitelline space and an optically heterogeneous zona, just like those which had been in the ampulla for 3–4 h or more. When the ampulla was ligated at its first or second loop 13–14 h before ovulation and the oocytes were collected and examined 3–4 h after ovulation (Figure 6A,B), 62–73% of the oocytes had the typical appearance of oviductal oocytes with a large perivitelline space and an optically heterogeneous zona. The remaining oocytes looked like the typical ovarian oocytes (9–22%) or were in intermediate stages (16–18%). When the third loop of the ampulla was ligated (Figure 6C), all the oocytes had the typical appearance of the oviductal oocytes, just like those collected from unligated oviducts (Figure 6D).

Discussion

Maturation of cumulus oophorus

In some species, oocytes are penetrated by spermatozoa almost immediately after entering the oviduct, while in some other species oocytes stay in the oviduct for some time before they are penetrated by spermatozoa (Braden, 1962). In the mouse, the time interval between ovulation and sperm penetration is 3–5 h in naturally ovulating females, and it is 1–3 h in the females ovulating following treatments with exogenous

Table I. Changes in ovarian oocytes cultured *in vitro* for 4 h

Oocytes (Exp.)	Vol. of perivitelline space (μm^3)[a]	Optical heterogeneity of zona[b]	% Oocyte activated by Ca ionophore[c]	Time (s) needed for division of vitellus by centrifugation[d]
Ovarian oocytes not cultured (control)	64 ± 11	–	37	637 ± 10
Ovarian oocytes cultured in:				
(A) M2 with 10% serum	70 ± 2	–	86	622 ± 14 ($P < 0.05$)
(B) Ham F-10 with 10% serum	63 ± 2	–	77	N.D.
(C) M2 with oviduct extract	128 ± 3	+	92	N.D.
(D) Ampullary fluid[e]	134 ± 4	+	87	614 ± 12[e] ($P < 0.01$)
(E) Isthmic fluid	140 ± 3	+	86	N.D.
Oviductal oocytes, not cultured	150 ± 6	+	93	594 ± 13 ($P < 0.001$)

N.D., no data.
[a]Each value is based on the measurement of 24 oocytes collected from six different females (four oocytes/female).
[b]The appearance of the zona under the phase-contrast microscope with positive contrast optics. In oviductal oocytes, the outer half of the zona appears very 'bright' in contrast to the relatively dark inner half, indicating optical heterogeneity (+) of the zona (cf. Figure 1D, top). The zona of ovarian oocytes freshly collected from the ovary appears 'uniform' throughout its depth (cf. Figure 1D, bottom).
[c]Examined 1 h after ionophore treatment (cf. Materials and methods). Each value is based on three to seven experiments using a total of 22–70 oocytes.
[d]Time needed for complete separation of vitelli of all oocytes into light and heavy halves (cf. Materials and methods). Each value is based on six or seven experiments using a total of 100–120 oocytes.
[e]The fluid used in this centrifugation experiment was a mixture of the ampullary and isthmic fluids, which was collected after ligating the infundibulum and utero-tubal junction of the oviduct (cf. Materials and methods).

gonadotrophins. Braden (1962) postulated that mouse cumuli undergo a 'maturation' in the oviduct before they become penetrable by spermatozoa. In naturally ovulating hamsters, oocytes stay in the oviduct (ampulla) for at least 1.5–2 h before they are penetrated by spermatozoa (Austin, 1956; Strauss, 1956; Yanagimachi, 1966). The hamster cumulus may not require maturation in the oviduct, because, according to Corselli and Talbot (1986), the cumuli of ovarian oocytes collected shortly before ovulation are readily penetrable by spermatozoa *in vitro*. In the hamster, the time interval of 1.5–2 h between ovulation and the onset of sperm penetration must represent the minimum time needed for the spermatozoa to migrate from the lower segment of the oviduct to the ampulla where oocytes and spermatozoa meet (Smith *et al.*, 1987).

It is well established that an expansion of the cumulus or the synthesis of hyaluronic acid by cumulus cells is triggered by the pre-ovulatory gonadotrophin surge (Eppig, 1979, 1980). The expansion begins from the periphery of the cumulus and proceeds inward (Eppig, 1982). In the hamster, the cumulus expands during and even after ovulation (Talbot and DiCarlantonio, 1984). The fact that the cumulus matrix surrounding the oviductal hamster oocyte is more susceptible to hyaluronidase than that of the ovarian oocyte seems to support the notion that the expansion of the cumulus or the synthesis of hyaluronic acid continues even after ovulation.

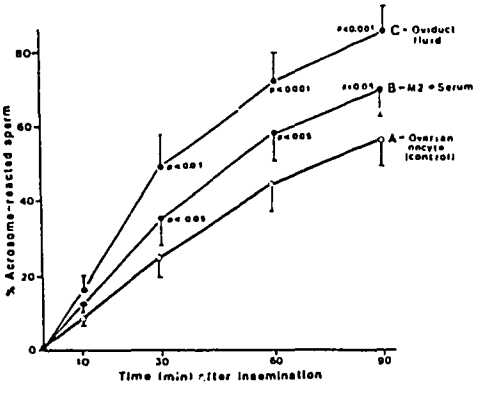

Fig. 5. Incidence of the acrosome reaction of spermatozoa on the zona surface of ovarian oocytes: (A) zonae of oocytes not cultured; (B) zonae of oocytes cultured for 4 h in M2 medium with 10% hamster serum; (C) zonae of oocytes cultured 4 h in oviductal fluid (a mixture of ampullary and isthmic fluids; cf. Materials and methods).

Maturation of the vitellus

This study confirmed the previous report that the vitellus of hamster oocyte 'shrinks' slightly after ovulation, whereas the perivitelline spoace 'expands' considerably after ovulation (Yanagimachi and Chang, 1961). Partial exocytosis of cortical granules (Okada *et al.*, 1986) and accumulation of oviductal glycoproteins in the perivitelline space (Kapur and Johnson, 1986) must be responsible, at least in part, for such postovulatory changes of the oocyte.

It is interesting that sperm nuceli incorporated into the vitelli of ovarian and oviductal oocytes decondensed and transformed into pronuclei at about the same rate, whereas oocyte nuclei completed their meiosis at different rates. The facts that ovarian oocytes (i) complete meiosis more slowly than oviductal oocytes

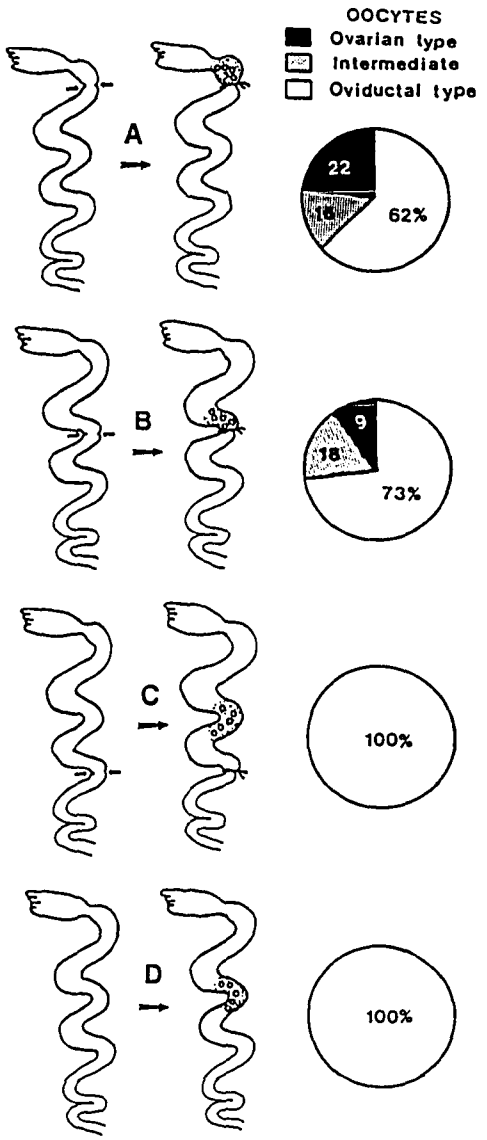

Fig. 6. Relationships between the site of ampulla ligation (13–14 h prior to ovulation) and the appearance of oocytes collected 3–4 h after ovulation: (A) the first loop of the ampulla was ligated; (B) the second loop of the ampulla was ligated. (C) the third loop of the ampulla was ligated; (D) no ligation, the control. Sector diagrams in the right row illustrate the percentage of oocytes with typical and intermediate appearances of ovarian and oviductal oocytes. Each datum is based on examination of 160–200 oocytes collected from four superovulated females.

and (ii) respond to Ca^{2+} ionophore less efficiently than oviductal oocytes seem to suggest that the ooplasm of the ovarian oocyte is not as mature as that of the oviductal oocyte. The relatively high 'viscosity' of the vitellus of the ovarian oocyte may be another indication of the immaturity of the ovarian oocyte.

The vitellus of the ovarian oocyte appears to be able to mature without contribution from the oviduct. The ovarian oocytes, cultured in relatively simple media without any oviductal factors, became like the oviductal oocytes as far as the responsiveness to Ca^{2+} ionophore and the viscosity of the vitellus were concerned (cf. Table I).

Changes in the zona pellucida

According to Fowler and Grainge (1985), the glycosaminoglycan content of the mouse zona pellucida reduces drastically during the several hours preceding ovulation. There is convincing evidence that glycoproteins of oviductal origin are integrated into the zonae of hamster and porcine oocytes after the oocytes have been transferred from the ovary to the oviduct (Fox and Shivers, 1975; Brown and Cheng, 1986; Oikawa *et al.*, 1986; 1988; St-Jacques *et al.*, 1986; Araki *et al.*, 1987; Hedrick *et al.*, 1987; Leveille *et al.*, 1987). As reported in this paper, the zona pellucida of the hamster oocyte changes its optical property and affinity to Janus green rather dramatically, after the oocyte has resided in the oviduct or has been cultured *in vitro* in the presence of oviductal fluid. This is probably due to an integration of oviductal glycoproteins into the intrinsic zona glycoproteins. According to Leveille *et al.* (1987), the epithelium of the hamster isthmus secretes more oviductal immunogens (glycoproteins) than the ampulla and infundibulum. The results of these experiments seem to support this view. However, the ampulla alone seems to be able to secrete enough oviductal factors (glycoproteins) to alter the zona (Table I). It is known that the hamster oviduct displays a very active adovarian peristaltic movement during the peri-ovulatory period (Battalia and Yanagimachi, 1979). Therefore, it is possible that the isthmic fluid is transported upward and mixed with the ampullary fluid to alter zonae synergistically.

When oocytes collected from the ovarian surface or from the ovarian bursa were examined, their zonae were optically homogeneous. In other words, they were just like the zonae of ovarian oocytes. By examining oviductal oocytes at various times after ovulation, it was estimated that the zona changes from the optically homogeneous 'ovarian type' to the optically heterogeneous 'oviductal type' within 1 h after entering the oviduct. Since hamster oocytes, under normal in-vivo conditions, reside in the oviduct for at least 1.5 h before meeting spermatozoa (Austin, 1956; Strauss, 1956; Yanagimachi, 1966), all oocytes must have zonae of the 'oviductal type' when they meet spermatozoa.

What is the biological function of the oviductal glycoproteins added to the intrinsic zona? Apparently, these glycoproteins are not essential for fertilization. At least in the human, mature ovarian oocytes, which have never been exposed to the oviductal glycoproteins, can be fertilized 'normally' and develop into viable offspring (Edwards, 1981; Edwards and Purdy, 1982; Trounson and Wood, 1984; Wolf and Quigley, 1984). The function of the oviductal glycoproteins could be to enhance the functions of the

intrinsic zona. As reported previously (Uto *et al.*, 1988) and confirmed in the present study, the zonae of oviductal oocytes are more efficient than those of ovarian oocytes in inducing the acrosome reaction of spermatozoa. It has been demonstrated that the zonae of ovarian oocytes increase their acrosome-reaction-inducing ability when they are exposed to the oviduct fluid (glycoproteins) either *in vivo* or *in vitro*. The oviductal glycoproteins integrated into the zona must work synergistically with the native zona glycoproteins in inducing the acrosome reaction. By the same token, these two groups of glycoproteins may work synergistically to achieve other functions of the zona, including (i) the prevention of polyspermy via the zona reaction and (ii) the protection of developing preimplantation embryos.

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References

Araki,Y., Kurata,S., Oikawa,T., Yamashita,T., Hiroi,M., Nakai,M. and Sendo,F. (1987) A monoclonal antibody reacting with the zona pellucida of the oviductal egg but not with that of the ovarian egg of the golden hamster. *J. Reprod. Immunol.*, **11**, 193–208.

Austin,C.R. (1956) Ovulation, fertilization, and early cleavage in the hamster. *J. R. Microsc. Soc.*, **75**, 141–154.

Austin,C.R. and Walton,A. (1960) Fertilisation. In Parkes,A.S. (ed.), *Marshall's Physiology of Reproduction*. Longmans, London, Vol. 1, pt 2, pp. 310–416.

Baker,T.G. (1982) Oogenesis and ovulation. In Austin,C.R. and Short,R.V. (eds), *Reproduction in Mammals*. Cambridge University Press, London, Book 1, pp. 17–45.

Battalia,D.B. and Yanagimachi,R. (1979) Enhancement and co-ordinated movement of the hamster oviduct during the periovulatory period. *J. Reprod. Fertil.*, **56**, 515–520.

Braden,A.W.H. (1962) Spermatozoon penetration and fertilization in the mouse. *Symp. Genet. Biol. Italica, Pavia, Italy*, **9**, 1–8.

Brown,C.R. and Cheng,W.K. (1986) Changes in composition of the porcine zona pellucida during development of the oocyte to the 2- to 4-cell embryo. *J. Embryol. Exp. Morph.*, **92**, 183–191.

Cornelli,J. and Talbot,P. (1986) An in vitro technique to study penetration of hamster cumulus complexes by using physiological numbers of sperm. *Gamete Res.*, **13**, 293–308.

Cummins,J.M. and Yanagimachi,R. (1986) Development of ability to penetrate the cumulus oophorus by hamster spermatozoa capacitated *in vitro*, in relation to the timing of the acrosome reaction. *Gamete Res.*, **15**, 187–212.

Edwards,R.G. (1981) Test-tube babies, 1981. *Nature*, **293**, 253–256.

Edwards,R.G. and Purdy,J.M. (eds) (1982) *Human Conceptum In Vitro*. Academic Press, London.

Eppig,J.J. (1979) FSH stimulates hyaluronic acid synthesis by oocyte–cumulus cell complexes from mouse preovulatory follicles. *Nature*, **281**, 483–494.

Eppig,J.J. (1980) Regulation of cumulus oophorus expansion by gonadotropins *in vivo* and *in vitro*. *Gamete Res.*, **23**, 545–552.

Eppig,J.J. (1982) The relationship between cumulus cell–oocyte coupling, oocyte meiotic maturation, and cumulus expansion. *Dev. Biol.*, **89**, 268–272.

Fowler,R.E. and Grainge,C. (1985) A histochemical study of the changes in the protein–carbohydrate composition of the cumulus–oocyte complex and zona pellucida in immature mice in response to gonadotropin stimulation. *Histochem. J.*, **17**, 1235–1249.

Fox,L.L. and Shivers,C.A. (1975) Immunological evidence for addition of oviductal components to the hamster zona pellucida. *Fertil. Steril.*, **26**, 599–608.

Hedrick,J.L., Wardlip,N.J. and Berger,T. (1987) Differences in the macromolecular composition of the zona pellucida isolated from pig oocytes, eggs and zygotes. *J. Exp. Zool.*, **241**, 257–262.

Kapur,R.P. and Johnson,L.V. (1986) Selective sequestration of an oviductal fluid glycoprotein in the perivitelline space of mouse oocytes and embryos. *J. Exp. Zool.*, **238**, 249–260.

Leveille,M.C., Roberts,K.D., Chevalier,S., Chapdelaine,A. and Bleau,B. (1987) Uptake of an oviductal antigen by the hamster zona pellucida. *Biol. Reprod.*, **36**, 227–238.

Oikawa,T., Kurata,S. and Sendai,Y. (1986) Discovery of a novel zona glycoprotein (ZP-O) having a sperm binding activity. *J. Reprod. Immunol.*, Suppl., pp. 81.

Oikawa,T., Sendai,Y., Kurata,S. and Yanagimachi,R. (1988) A glycoprotein of oviductal origin alters biochemical properties of the zona pellucida of hamster egg. *Gamete Res.*, **19**, 113–122.

Okada,A., Yanagimachi,R. and Yanagimachi,H. (1986) Development of a cortical granule-free area of cortex and the perivitelline space in the hamster oocyte during maturation and following ovulation. *J. Submicrosc. Cytol.*, **18**, 233–247.

Quinn,P., Barros,C. and Whittingham,D.G. (1982) Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. *J. Reprod. Fertil.*, **66**, 161–168.

Smith,T.T., Koyanagi,F. and Yanagimachi,R. (1987) Distribution and number of spermatozoa in the oviduct of the golden hamster after natural mating and artifical insemination. *Biol. Reprod.*, **37**, 225–234.

Steinhardt,R.A., Epel,D., Carroll,E.J. and Yanagimachi,R. (1974) Is calcium ionophore a universal activator for unfertilized eggs? *Nature*, **252**, 41–43.

Strauss,F. (1956) The time and place of fertilization of the golden hamster egg. *J. Embryol. Exp. Morphol.*, **4**, 42–56.

St-Jacques,S. and Forget,A., Roberts,K.D., Chevalier,S., Chapdelaine,A. and Bleau,G. (1986) Monoclonal antibodies specific for an oviductal component associated with the zona pellucida. *J. Reprod. Immunol.*, Suppl., pp. 52.

Talbot,P. and DiCarlantonio,G. (1984) Architecture of the hamster oocyte–cumulus complex. *Gamete Res.*, **9**, 261–272.

Trounson,A.O. and Wood,C. (eds) (1984) *In Vitro Fertilization and Embryo Transfer*. Churchill Livingston, Edinburgh.

Uto,N., Yoshimatsu,N., Lopata,A. and Yanagimachi,R. (1988) The zona-induced acrosome reaction of hamster spermatozoa. *J. Exp. Zool.*, **248**, 113–120.

Wolf,D.P. and Quigley,M.M. (eds) (1984) *Human In Vitro Fertilization and Embryo Transfer*. Plenum Press, New York.

Yanagimachi,R. (1966) Time and process of sperm penetration into hamster ova *in vivo* and *in vitro*. *J. Reprod. Fertil.*, **11**, 359–370.

Yanagimachi,R. and Chang,M.C. (1961) Fertilizable life of golden hamster ova and their morphological changes at the time of losing fertilizability. *J. Exp. Zool.*, **148**, 185–294.

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APPENDIX II

University of Hawaii at Manoa

John A. Burns School of Medicine
Department of Anatomy and Reproductive Biology
1960 East-West Road • Honolulu, Hawaii 96822

May 22, 1989

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University of Hawaii at Manoa

John A. Burns School of Medicine
Department of Anatomy and Reproductive Biology
1960 East-West Road • Honolulu, Hawaii 96822

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Journal: Zoological Science, Manuscript No. MS0-89-067

I am looking forward to hearing from you soon.

Sincerely yours,

Cheng-Hsiung Yang

Cheng-Hsiung Yang

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Directors
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Washington DC office
PO Box Q
McLean VA 22101-08
USA
Tel: (703) 437 3334
Telex: 880579
Fax: (703) 689 0660

LITERATURE CITED

Abreu, SL and Brinster, RL (1978) Synthesis of tubulin and actin during the preimplantation development of the mouse. Exp Cell Res 114:135-141.

Alberts, B, Bray, D, Lewis, J, Raff, M, Roberts, K and Watson, JD (1983) The Cytoskeleton. In: Molecular Biology of The Cell, Garland Publishing, New York. pp. 549-607.

Araki, Y, Kurota, S, Oikawa, T, Yamashita, T, Hiroi, M, Nakai, M and Sendo, F (1987) A monoclonal antibody reacting with the zona pellucida of the oviductal egg but not with that of the ovarian egg of the golden hamster. J Reprod Immunol 11:193-208.

Austin, CR (1951) Observation on the penetration of sperm into the mammalian egg. Aust J Sci Res 4:581-596.

Austin, CR (1956) Ovulation, fertilization, and early cleavage in the hamster. J Roy Micro Soc 75:141-154.

Austin, CR and Walton, A (1960) Fertilization. In: Marshall's Physiology of Reproduction, vol. 1, pt. 2, Parkes, AS (ed) Longmans, London. pp. 310-416.

Austin, CR and Short, R (eds) (1982) Germ Cells and Fertilization. In: Reproduction in Mammals, vol. 1, Cambridge University Press, Cambridge. pp. 46-62.

Aykroyd, OE (1941) The effects of ultracentrifuging human oocytes. Proc Roy Irish Acad Dublin, Sec B, 46:101-111.

Bachvaroova, R, De Leon V, Johnson A, Kaplan, G, Paynton, BV (1985) Changes in total RNA, polyadenylate RNA, and actin mRNA during meiotic maturation of mouse oocytes. Dev Biol 108:325-331.

Baker, TG (1982) Oogenesis and Ovulation. In: Reproduction in Mammals, vol. 1, Austin, CR and Short, RV (eds), Cambridge University Press, London, pp. 310-416.

Barak, LS, Yocum, RR, Nothnagel, EA and Web, WW (1980) Fluorescence staining of the actin cytoskeleton in living cells with NBD-ph. Proc Nat Acad Sci USA 77:980-984.

Battaglia, DE and Gaddum-Rosse, P (1986) The distribution of polymerized actin in the rat egg and its sensitivity to cytochalasin B during fertilization. J Exp Zool 237:97-105

Battaglia, DE and Gaddum-Rosse, P (1987) Influence of Ca ionophore on rat egg behavior and cortical F actin. Gamete Res 18:141-152.

Battalia, DE and Yanagimachi, R (1979) Enhancement and co-ordinated movement of the hamster oviduct during the periovulatory period. J Reprod Fertil 56:515-520.

Beams, HW and Kessl, RG (1987) Development of centrifuges and their use in the study of living cells. Int J Cytol 100:15-48

Beams, HW and King, RL (1938) A study of the cytoplasmic components and inclusions of the developing guinea pig egg. Cytologia 8:353-367.

Bleil, JD and Wassarman, PM (1980) Mammalian sperm-egg interaction: Identification of a glycoprotein in mouse egg zonae pellucida processing receptor activity for sperm. Cell. 20:873-882.

Bleil, JD and Wassarman, PM (1983) Sperm egg interaction in the mouse: Sequence of events and induction of acrosome reaction by a zonae pellucida glycoprotein. Dev Biol 95:317-324.

Bleil, JD and Wassarman, PM (1986) Autoradiographic visualization of the mouse egg's sperm receptor bound to sperm. J Cell Biol 102:1363-1371.

Braden, AWH (1962) Spermatozoa penetration and fertilization in the mouse. Symp Genet et Biol Italica, Pavia, Italy Sept. 1-8.

Brown, CR and Cheng, WK (1986) Changes in composition of the porcine zona pellucida during development of the oocyte to the 2- to 4- cell embryo. J Embryol Exp Morph 92:183-191.

Cherr, GN, Lambert, H, Meizel, S and Katz, DF (1986) In vitro studies of the golden hamster sperm acrosome reaction. Dev Biol 114:119-131.

Clarke, HJ and Masui, Y (1987) Dose-dependent relationship between oocyte cytoplasmic volume and transformation of sperm nuclei to metaphase chromosome. J Cell Biol 104:831-840.

Corselli, J and Talbot, P (1986) An in vitro technique to study penetration of hamster cumulus complexes by using physiological numbers of sperm. Gamete Res 13:293-308.

Cran, DG (1987) The distribution of organelles in mammalian oocytes following centrifugation prior to injection of foreign DNA. Gamete Res 18:67-76.

Cummins, JM and Yanagimachi, R (1986) Development of ability to penetrate the cumulus oophorus by hamster spermatozoa capacitated in vitro, in relation to the timing of the acrosome reaction. Gamete Res 15:187-212.

Edwards, RG (1981) Test tube babies. Nature 293:253-256.

Edwards, RG and Purdy, JM (eds) (1982) Human Conception In Vitro, Academic Press. London.

Eppig, JJ (1979) FSH stimulates hyaluronic acid synthesis by oocyte-cumulus cell complexes from mouse preovulatory follicles. Nature 281:483-494.

Eppig, JJ (1980) Regulation of cumulus oophorus expansion by gonadotropins in vivo and in vitro. Gamete Res 23:545-552.

Eppig, JJ (1982) The relationship between cumulus cell-oocyte coupling, oocyte meiotic maturation, and cumulus expansion. Dev Biol 89:268-272.

First, NL, Leibfried-Ruthledge, ML and Sirard, MA (1988) Cytoplasmic control of oocyte maturation and species differences in development of competence to mature. In: Meiotic Inhibition, Molecular Control of Meiosis: Progress in Clinical and Biological Research, Haseltine J and First, NL (eds), Alan R Liss, New York. pp. 1-46.

Fowler, RE and Grainge, C (1985) A histochemical study of the changes in the protein-carbonhydrate composition of the cumulus-oocyte complex and zona pellucida in immature mice in response to gonadotrophin stimulation. Histochem J 17:1235-1249.

Fox, LL and Shivers, CA (1975) Immunological evidence for addition of oviductal components to the hamster zona pellucida. Fertil Steril 26:599-608.

Giebelhaus, DH, Hekkila, JJ and Schuetz, GS (1983) Change in the quantity of histone and actin messenger RNA during the development of preimplantation embryos. Dev Biol 98:148-154.

Goldman, RD, Pollack, R and Hopkins, NH (1973) Preservation of normal behavior by enucleated cells in culture. Proc Nat Acad Sci USA 70:750-754.

Gresson, RAR (1938) Effect of ultra-centrifuging the oocytes of the mouse. Nature 142:957-958.

Gresson, RAR (1940) A cytological study of the centrifuged oocyte of the mouse. Quart J Micr Sci 81: 569-584.

Harvey, EB (1936) Parthenogenetic merogony or cleavage without nuclei in Arbacia Punctulata. Biol Reprod 71:101-102.

Hedrick, JL, Wardlip, NJ and Berger, T (1987) Differences in the Macromolecular composition of the zona pellucida isolated from pig oocytes, eggs and zygotes. J Exp Zool 241:257-262.

Hirao, Y and Yanagimachi, R (1979) Development of pronuclei in polyspermic eggs of the golden hamster. Zool Mag Tokyo 88:24-33.

Hiramoto, Y (1962) Microinjection of the live spermatozoa into sea urchin eggs. Exp Cell Res 27:461-466

Hiramoto, Y (1963) Mechanical properties of sea urchia eggs. Exp Cell Res 32:76-88.

Hiramoto, Y (1974) Mechanical properties of the surface of the sea urchin egg at fertilization and during cleavage. Exp Cell Res 9:320-326.

Hiramoto, Y (1976) Mechanical properties of starfish oocytes. Dev Growth Diff 18:205-209.

Howe, CC and Solter, D (1979) Cytoplasmic and nuclear protein synthesis in preimplantation mouse embryo. J Embryol Exp Morph 52:209-225.

Huang, TTF and Yanagimachi, R (1985) pH and proteinase control of acrosomal content stasis and release during the guinea pig acrosome reaction. Biol Reprod 32:451-462.

Jaffe, LA and Gould, M (1985) Polyspermy Preventing Mechanisms. In: Biology of Fertilization, vol. 3, Metz, CB and Monroy, A (eds), Academic Press, New York. pp.223-250.

Kaplan, G, Abreu, SL and Bachvarova, R (1982) rRNA accumulation and protein synthetic in growing mouse oocytes. J Exp Zool 220:361-370.

Kapur, RP and Johnson, LV (1986) Selective sequestration of an oviductal fluid glycoprotein in the perivitelline space of mouse oocytes and embryos. J Exp Zool 238:249-260.

Karasiewicz, J and Solytnska, MS (1985) Ultrastructural evidence for the presence of actin filaments in mouse eggs in fertilization. Roux's Arch Dev Biol 194:369-372.

Leibfried-Ruthledge, ML, Florman, HM and First, NL (1989) The Molecular Biology of Mammalian Oocyte Maturation. In: The Molecular Biology of Fertilization, Schatten, H and Schatten, G (eds) Academic Press, New York. pp. 259-301.

Leveille, MC, Roberts, KD, Chevalier, S, Chapdelaine, A and Bleau, B (1987) Uptake of an oviductal antigen by the hamster zona pellucida. Biol Reprod 36:227-238.

Longo, FJ and Chen, DY (1985) Development of cortical polarity in mouse eggs: Involvement of the meiotic apparatus. Dev Biol 107:382-394.

Longo, FJ (1987) Actin-plasm menbrane association in mouse eggs and oocytes. J Exp Zool 243:299-309.

Maro, B, Howlett, SK and Houlinston, E (1986a) Cytoskeletal dynamics in the mouse egg. J Cell Sci (suppl) 5:343-359.

Maro, B (1986b) Fertilization and the cytoskeleton in the mouse. Bioessay 3:18-21.

Maro, B, Johnson, MH, Pickering, SJ and Flach, G (1984) Changes in actin distribution during fertilization of the mouse egg. J Embryol Exp Morph 81:211-237.

Maruyama, YK, Yamamoto, K, Mita-Miyazaki, I, Kominami, T and Nemoto, S (1986) Manipulative methods for analyzing embryogenesis. In: Methods in Cell Biology, Schrieder, TE (ed), Academic Press, New York. pp. 325-244.

Mitchison, JM and Swann, MM (1965) The mechanical properties of the cell surface. J Exp Biol 32:734-750

Nemoto, S, Yoneda, M, and Uemura, I (1980) Marked decrease in the rigidity of starfish oocytes induced by 1-methyladenine. Dev Growth Diff 22:315-325.

Oikawa, T, Kurata, S and Sendai, Y (1986) Discovery of a novel zona glycoprotein (ZP-0) having a sperm binding activity. J Repro Immunol (suppl) p. 81.

Oikawa, T, Sendai, Y, Kurata, S and Yanagimachi, R (1988) A glycoprotein of oviductal origin alters biochemical properties of the zona pellucida of hamster egg. Gamete Res 19:113-122.

Okada, A, Yanagimachi, R and Yanagimachi, H (1986) Development of a cortical granule-free area of cortex and the perivitelline space in the hamster oocyte during maturation and following ovulation. J Submicr Cytol 18:233-247.

Orsini, MW (1961) The external vaginal phenomena characterizing the stages of the esterous cycle, pregancy, pseudopreganacy, lactation and the anestrous hamster. Proc animal care panel 11:193-206.

Quinn, P, Barros, C and Whittingham, DG (1982) Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. J Fertil Steril 66:161-168.

Sawai, T and Yoneda, M (1974) Wave of stiffness propagating along the surface of the newt egg during cleavage. J Cell Biol 60:1-7.

Selman, GG and Waddington, CH (1955) The mechanism of cell division in the cleavage of the newt's egg. J Exp Biol 32:700-733.

Shay, JW, Porter, KR and Prescott, DM (1974) The surface morphology and fine structure of chinese hamster ovary cells following enucleation. Proc Nat Acad Sci USA 71:3059-3063.

Smith, TT, Koyanagi, F and Yanagimachi, R (1987) Distribution and number of spermatozoa in the oviduct of the golden hamster after natural mating and artificial insemination. Biol Reprod 37:225-234.

Steinhardt, RA, Epel, D, Carrol, EJ and Yanagimachi, R (1974) Is calcium ionophore a universal activator for unfertilized eggs? Nature 252:41-43.

Strauss, F (1956) The time and place of fertilization of the golden hamster. J Embryol Exp Morph 4:42-56.

St-Jacques, S, Forget, A, Roberts, KD, Chevalier, S, Chapdelaine, A and Bleau, G (1986) Monoclonal antibodies specific for an oviductal component associated with the zona pellucida. J Reprod Immunol (suppl) p. 52.

Talbot, P and Dicarlantonio, G (1984) Architecture of the hamster oocyte cumulus complex. Gamete Res 9:261-172.

Tarkowaski, AK (1980) Fertilization of nucleate and anucleate egg fragments in the mouse. Exp Cell Res 128: 73-77.

Tellez, V, Ahumada, A, Muro, J, Sepulveda, S and Izquierdo, L (1988) Centrifugation of 2-cell mouse ova: Cytoplasmic stratification and recovery. Roux's Arch Dev Biol 197:360-365.

Thibault, C (1977) Are follicular maturation and oocyte maturation independent processes? J Reprod Fert 51:1-15

Trounson, A. and Wood, C. (eds) (1984) In Vitro Fertilization and Embryo Transfer. Churchill Livingston, Edinburgh.

Uehara, T and Yanagimachi, R (1976) Microsurgical injection of spermatozoa into hamster eggs with subsequent transformation of sperm nuclei into male pronucleus. Biol Reprod 16:467-470.

Uehara, T and Yanagimachi, R (1977) Activation of hamster egg by pricking. J Exp Zool 199:269-274.

Usui, N and Yanagimachi, R (1976) Behavior of hamster sperm nuclei incorporated into eggs at various stages of maturation, fertilization and early development. J ultrastruc Res 57:276-287.

Uto, N, Yoshimatsu, N, Lopata, A and Yanagimachi, R (1988) The zona-induced acrosome reaction of hamster spermatozoa. J Exp Zool 248:113-120.

Van Blerkom, J and Brockway, G (1975) Qualitative patterns of protein synthesis in the preimplantation mouse embryo. I. Normal pregnancy. Dev Biol 44:148-157.

Wall, RJ, Pursel, VG, Hammer, RE and Brinster, RL (1985) Development of porcine ova that were centrifugated to permit visualization of pronuclei and nuclei. Biol Reprod 32:645-651.

Wassarman, PM (1988) The Mammalian Ovum. In: The Physiology of Reproduction, vol.1., Knobil, E and Neill, JD (eds)., Raven Press, New York. pp. 69-102.

Wolf, DP and Quigleg, MM (eds) (1984) Human In Vitro Fertilization and Embryo Transfer, Plenum Press, New York.

Yanagimachi, R (1966) Time and process of sperm penetration into hamster ova in vivo and in vitro. J Reprod Fertil 11:359-379.

Yanagimachi, R (1978) Calcium requirement for sperm-egg fusion in mammals. Biol Reprod 19:949-958.

Yanagimachi, R (1981) Mechanism of Fertilization in Mammals. In: Fertilization and Embryonic Development In Vitro, Mastroianni, L Biggers, BJ (eds), Plenum Press, New York. pp.81-182.

Yanagimachi, R (1982) In vitro sperm capacitation and fertilization of golden hamster egss in a chemically defined medium. In: In Vitro Fertilization and Embryo Transfer, Hafez, ESE and Semm, K (eds), MTP Press, Lancaster. pp. 65-76.

Yanagimachi, R. (1988): Mammalian Fertilization. In: The Physiology of Reproduction, vol. 1, Knobil, E and Neill, JP (eds), Raven Press, New York, pp. 135-185.

Yanagimachi, R and Chang, MC (1961) Fertilizable life of golden hamster ova and their morphological changes at the time of losing fertilizability. J Exp Zool 148:285-294

Yanagimachi, R and Noda, YD (1970) Electron microscope studies of sperm incorporation into the golden hamster egg. Am J Anat 12:429-462.

Yang, CH and Yanagimachi, R (1989) Differences between mature ovarian and oviductal oocytes: a study using the golden hamster. Human Reprod 4:63-71.

Yang, CH, Yanagimachi, R and Yanagimachi, H (1989) Morphology and fertilizability of zona free hamster egg separated into halves and quarters by centrifugation. Biol Reprod (in press).