



1980

Aging Effects on Ova Maturation and RNA and Protein Synthesis In Vitro

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AGING EFFECTS ON OVA MATURATION AND
RNA AND PROTEIN SYNTHESIS IN VITRO

by

Reinhold J. Hutz

A Thesis Submitted to Graduate School of
Loyola University of Chicago in Partial Fulfillment of the
Requirements for the Degree of Master of Science

April

1980

ACKNOWLEDGMENTS

The author desires to express his gratitude to his advisor, Dr. John Peluso, in appreciation for his instruction in relating a clearer understanding of the world of science, and his perseverance, guidance and friendship. The author is appreciative of the services rendered by the members of his committee, Dr. Genaro Lopez and Dr. Albert Rotermund, in reviewing the manuscript and for making the author's graduate career a valuable experience.

The expert technical assistance of Ms. Marcia Xenakis is gratefully acknowledged.

Special thanks must go to the author's parents and a special friend, Ms. Irene O'Shaughnessy, for their love and understanding.

VITA

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CHAPTER I

REVIEW OF LITERATURE

AGING AND REPRODUCTIVE DECLINE

The general decline in reproductive capacity due to advancing maternal age is evident as measured by several parameters. There is a gradual decrease in the number of oocytes present within the aging ovary (Mandl and Shelton, 1959; Jones and Krohn, 1961). Yet, the wane in fertility occurs long before the population of ovarian oocytes is depleted (Talbert, 1968; Jones, 1970), and ovulation rate is not altered by increasing age (Jones, 1970; Fugo and Butcher, 1971; Harman and Talbert, 1970, 1974; Peluso et. al., 1979). Fertilization and implantation rates do, however, diminish with age (Talbert, 1968, 1971; Harman and Talbert, 1970; Fugo and Butcher, 1971; Maurer and Foote, 1972). Litter size also decreases with advancing age (Ingram et. al., 1958; Blaha, 1964b), and the incidence of chromosomal and developmental abnormalities increases (Carr, 1969; Fechheimer, 1972; Gosden, 1973; Yamamoto et. al., 1973; Tsuji and Nakano, 1978). Clearly, defects associated with aging must be due to intrinsic functional factors. Consequently, the decline in fertility characteristic of aged females has been attributed to alterations at all levels

of the hypothalamic-pituitary-ovarian-uterine axis (Talbert, 1968).

HYPOTHALAMIC-PITUITARY FUNCTION AND AGE

The reproductive pattern of female rats appears to be sequentially altered with age due mainly to a progressive desensitization of the hypothalamo-hypophyseal complex (Huang et. al., 1978). Consequently, the regular 4- or 5-day estrous cycle of the rat becomes irregular (irregularly-cycling, IRC) at 10-12 months, exhibits persistent cornification (constant estrus, CE) at 19 months, subsequently undergoes prolonged diestrus (pseudopregnant, PP) with intermittent estrous cycles and ultimately develops into a persistent diestrous or anestrous (AS) state at 25-27 months (Huang and Meites, 1975; Lu et. al., 1979). Although anestrous rats possess atrophic ovaries, when transplanted to young ovariectomized (OVX) rats these ovaries grow and develop large follicles and corpora lutea (CL) and therefore remain responsive to pituitary gonadotropin stimulation (Peng and Huang, 1972). This indicates aging results in a malfunction of this neuroendocrine axis.

Although basal serum LH and FSH levels in old CE and PP rats are not appreciably different from young cyclers (Huang et. al., 1976), LH and FSH secretion is

decreased in response to castration and/or to the positive feedback action of estrogen (Howland and Preiss, 1975; Shaar et. al., 1975; Huang et. al., 1976; Lu et. al., 1977; Peluso et. al., 1977). The impaired positive feedback effect of estrogen correlates with a decreased hypothalamic and pituitary uptake of ^3H -estradiol (Peng and Peng, 1973). However, old anestrous rats have extremely low LH and FSH levels and a decreased capacity to release the gonadotropins in response to synthetic gonadotropin-releasing hormone (GnRH) (Bruni et. al., 1977). Exogenous estrogen treatment of aged non-cycling rats restores the capacity to release gonadotropins in response to GnRH (Watkins et. al., 1975; Peluso et. al., 1977). However, aged cycling rats are still able to respond to exogenous GnRH alone (Steger and Peluso, 1979).

In very old male rats (21 months of age), the biogenic amine content of the hypothalamus is altered (Meites et. al., 1979). There is a decrease in the hypothalamic catecholamines (norepinephrine, NE, and dopamine, DA) and an increase in serotonin (5-hydroxytryptamine, 5-HT). NE increases gonadotropin release, DA inhibits prolactin (PRL) release (Meites et. al., 1977; Simpkins et. al., 1977) and 5-HT inhibits the gonadotropins and stimulates PRL (Meites et. al., 1979). Therefore, reciprocal changes in these amines would

reduce LH and FSH levels and enhance PRL levels to those characteristic of very aged rats (Meites et. al., 1979; Simpkins et. al., 1977; Lu et. al., 1979; Clemens and Meites, 1971; Shaar et. al., 1975; Huang et. al., 1976). The hypothalamus of old CE rats also exhibits lower GnRH and prolactin inhibiting factor (PIF) activity which may be due to a decrease in NE and an increase in 5-HT levels. This would also result in lowered LH and FSH (Clemens and Meites, 1971) and enhanced PRL levels (Riegler et. al., 1977; Shaar et. al., 1975).

CHANGES IN OVARIAN FUNCTION WITH AGE

There is also a significant reduction in ovarian function in aging rats due to the alteration of the hypothalamic-pituitary complex (Aschheim, 1979). The pattern of estrous cycles is changed considerably with advancing age as previously stated. In addition, serum levels of gonadotropins and gonadal steroids are mutually dependent and both are influenced by advancing age and the particular reproductive state (Huang et. al., 1978). Hence, CE rats have lowered LH and progesterone and elevated FSH and estradiol levels, thereby enhancing vaginal cornification and follicular cyst formation (Huang et. al., 1978; Steger et. al., 1976; Peluso et. al., 1979). Old PP rats possess high progesterone and moderate estradiol levels due to many corpora lutea

present. Finally, anestrous rats have very low levels of gonadotropins and gonadal steroids and hence atrophic ovaries (Huang et. al., 1978).

Aged rats show fewer compensatory ovulations (Peppler, 1971) and varying degrees of contralateral ovarian compensatory hypertrophy in response to unilateral OVX, ranging from normal hypertrophic compensation (Peppler, 1971), to moderate (Howland and Preiss, 1975) or very limited compensation (Labhsetwar, 1970; Lu et. al., 1977). Alterations in luteal cell morphology also appear in aged PP rats, although luteal LH binding in PP rats (Steger et. al., 1976) and granulosa LH binding in CE rats is maintained (Erickson et. al., 1979). The ovaries of aging IRC rats also have a decrease in the total number of both atretic and non-atretic follicles, although ovulation rate is maintained (Peluso et. al., 1979). Therefore, a compensatory "rescue" mechanism appears to exist that allows the normal number of preovulatory follicles to develop and ovulate (Peluso et. al., 1979, 1980).

Ovarian estradiol levels in aged cycling rats (Peluso et. al., 1979) and ovarian androgen levels in aged CE and PP rats (Chan and Leathem, 1977) are elevated. Deficiencies of ovarian enzymes regulating steroidogenesis have also been demonstrated: glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate

dehydrogenase (6PGD) (Leathem and Appel, 1977) and 5-3B-hydroxysteroid dehydrogenase (3B-HSD) (Leathem and Shapiro, 1975). However, granulosa aromatase activity is unaffected by age (Erickson et. al., 1979). Any alterations in the ovaries' ability to synthesize steroids would also be detrimental to the normal functioning of their target organ, the uterus.

AGING EFFECTS ON UTERINE FUNCTION

Aging exerts drastic effects on the capacity of the uterus to function normally. These alterations are evident in that the aged uterus has a reduced decidual cell reaction (DCR) in response to mechanical stimulation or intraluminal oil injection (Blaha, 1967; Biggers, 1969; Finn, 1970; Holinka et. al., 1977; Holinka and Finch, 1977; Gosden, 1979) and a decreased blastocyst implantation rate (Harman and Talbert, 1970; Talbert, 1971; Maurer and Foote, 1972; Butcher, 1975). Some investigators have shown a reduced sensitivity of the aged uterus to exogenous steroids (Blaha, 1967; Finn, 1970; Larson et. al., 1973; Peng and Peng, 1973). Aging impairs ^{14}C -estradiol and ^3H -progesterone uptake in vivo by uterine muscle tissue (Larson et. al., 1972). In addition, the estrogen receptor content of the aged uterus is reduced, although receptor affinity remains constant (Hsueh et. al., 1979). An alteration in estrogen receptor content may, in part,

account for the reduced capacity of the uterus to undergo normal implantation.

Embryonic transfer experiments have indicated uterine complicity in aging anomalies to a certain extent. Gosden (1974, 1979) demonstrated a significant reduction in survival of embryos collected from young donor mice and transferred to aged mice uteri. Talbert and Krohn (1966) demonstrated a 14% survival rate of morulae and blastocysts transferred from young mice donors to old recipients as compared to 48% in "young-to-young" transfers. However, Blaha (1964a) observed a significant increase in fetal viability only in "young-to-young" transfers in hamsters. Both young-to-old and old-to-young embryo transfers resulted in resorption and abnormal fetal development, indicating that defective oocytes may also be at fault. Thus, alterations in the intrauterine environment and defects within the oocyte may be responsible for decreased implantation rates and increased embryonic death associated with age (Butcher, 1975).

ABERRATIONS IN THE AGED OOCYTE AND RESULTING ANOMALIES

Alterations at any level of the hypothalamic-pituitary-ovarian-uterine axis could ultimately cause the deterioration of the oocyte, which would result in chromosomal and developmental errors. Several theories have been espoused regarding chronological aging effects

on the oocyte's chromosomal complement. Penrose (1966) has suggested that kinetochore weakening during the greatly-prolonged dictyene stages in the rat and human may be responsible for random distribution of disrupted bivalents at the first metaphase plate.

Evans (1967) has proposed that failure of nucleolar dissolution in the aged oocyte would result in non-dissolution of chromosomal pairs. Recent electron-micrographic analysis has demonstrated Evan's contention that the nucleolus is shared by bivalent pairs; thus, its retention would lead to a physical difficulty in chromosomal separation (Calarco et. al., 1972). This is particularly true in the case of human chromosomes 21 and 22, which maintain nucleolar remnants (Polani et. al., 1960).

Several investigators have demonstrated a precipitous decline in chiasma frequency, a change in their chromosomal location when present, and a concomitant sharp increase in the frequency of univalents with advancing age in the mouse oocyte (Henderson and Edwards, 1968; Luthardt et. al., 1973). Although Polani and Jagiello (1976) produced similar findings, no parallelism was found in old female mice between univalents present at metaphase I (MI) and chromosomal errors at the second metaphase (MII) plate. They therefore postulated that much of what had previously been designated MI univalents was actually tech-

nical artifact. MII mouse oocytes show increased hyperploidic frequency to intermediate age and then a reduction in old age (Martin et. al., 1976). The peculiar decrease in hyperploid oocytes in the aged group may be due to the decreased number of oocytes reaching MII, in vitro, with age (Martin et. al., 1976). However, the preponderance of hypoploid oocytes in all groups, particularly the middle-aged group, must be partly attributed to chromosome loss during oocyte fixation (Rohrborn, 1972; Uchida and Lee, 1974; Martin et. al., 1976). Any or all three of the above mechanisms may be responsible for the non-disjunction of chromosomal pairs within the chronologically-aged oocyte. As a consequence, there exists a higher incidence of aneuploidy in embryos of aged mice (Yamamoto et. al., 1973; Gosden, 1973), and embryos and abortuses of women reaching the climacteric (Carr, 1969; Fechheimer, 1972; Tsuji and Nakano, 1978).

Other types of oocyte "aging" also contribute to chromosomal aberrations. These may be associated with chronological age of the mother (Butcher, 1972). Spindle fiber degeneration and chromosomal and developmental anomalies due to delayed ovulation (follicular aging of the oocyte), either spontaneous or artificially-induced, have been demonstrated in Xenopus laevis (Mikamo, 1968), the rat (Fugo and Butcher, 1966; Butcher and Fugo, 1967; Butcher, 1969; Butcher et. al., 1969; Fugo and Butcher,

1971; Butcher, 1975; Butcher et. al., 1975), and man (Iffy, 1963; Hertig, 1967; Arrata and Iffy, 1971). Observations of 34 human ova showed that 1 of 13 ova ovulated on or before day 14 of the menstrual cycle was cytologically abnormal, while 12 of 21 ova ovulated after day 14 were abnormal (Hertig, 1967). Similarly, Iffy (1963) demonstrated that of 19 abortuses recovered from women, 14 were conceived after day 17 of the cycle, indicating that delayed ovulation contributed to alterations within the oocyte.

Aging alters the morphology and chromosomal complement of the oocyte such that embryonic viability is diminished. However, since even a brief exposure of the oocyte to the environment of the aged uterus could affect its viability, the viability of the aged oocyte prior to ovulation needs to be assessed. An indicator of the pre-ovulatory oocyte's viability is its ability to resume meiosis, both in vivo and in vitro.

RESUMPTION OF MEIOTIC MATURATION WITHIN THE OOCYTE

In Vivo Oocyte Maturation

Resumption of meiotic divisions within the oocyte (oocyte maturation) can be induced by a hormonal stimulus in vivo (Freeman et. al., 1970; Tsafiriri and Kraicer, 1972; Ayalon et. al., 1972). Oocyte maturation is also temporally associated with estrous behavior and gonadotropin release in the rat.

The female rat exhibits a regular 4- or 5-day estrous cycle (Long and Evans, 1922), with acceptance of the male on the afternoon of proestrus (4-10 P.M.). Ovulation occurs 9-10 h after the onset of "heat" or estrous behavior (Blandau et. al., 1941). The surge of LH found during a critical period on the afternoon of proestrus is responsible for the ensuing oocyte maturation-al changes (Everett and Sawyer, 1950; Ayalon et. al., 1972; Tsafiriri et. al., 1972). The oocyte nucleus or germinal vesicle (GV) remains intact up to 2 h after the LH surge in the rat. The GV persists throughout chromosomal condensation and up to spindle formation. At the end of chromatin condensation, chromosomes are circularly arranged at the first metaphase plate (circularly arranged chromosome, or CAC, stage), 2-4 h after the LH surge. Telophase follows at 4-7 h, with polar body abstriction and formation of the second metaphase spindle occurring at 7-10 h, and ovulation 2 h later (Odor, 1955; Calarco et. al., 1972; Tsafiriri and Kraicer, 1972; Butcher et. al., 1975).

Follicularly-enclosed oocytes explanted prior to the LH-surge undergo meiotic maturation only in suitable medium supplemented with LH, FSH or prostaglandin E₂ (PGE₂) (Tsafiriri et. al., 1972). Microinjection of dibutyryl 3',5'-cyclic-AMP (dbcAMP) into cultured follicles also stimulates oocytes to resume meiotic divisions. LH,

FSH and PGE_2 have been shown to increase cAMP activity as evidenced by ^3H -adenine uptake and actual measurement of cAMP. Cyclic-AMP, in turn, increases protein kinase activity (Tsafriri et. al., 1972; Tsafriri et. al., 1976a). Although LH and FSH increase cAMP levels, FSH's effects on ovum maturation, ovulation and steroidogenesis in the rat could be regarded as largely pharmacological (Schwartz et. al., 1973; Schwartz et. al., 1975). Therefore, LH is the dominant hormone responsible for these physiological effects, and its action appears to be mediated via cAMP and prostaglandins. Further work with follicularly-enclosed oocytes allowed Tsafriri and associates (1973) to propose the involvement of two different proteins in cAMP-mediated LH action on the follicle: one protein necessary for the resumption of meiosis, regulated at the translational level, and another, essential for steroidogenesis, which is under transcriptional control (Lindner et. al., 1974).

Since Chang (1955) first indicated the presence of a meiotic inhibitor in follicular fluid, it has been postulated that LH may remove this inhibitory influence of the granulosa cells on oocyte maturation (Foote and Thibault, 1969; Tsafriri and Channing, 1975a). This oocyte maturation inhibiting factor (OIF) has been derived from porcine follicular fluid (PFF) (Tsafriri and Channing, 1975b) and its effect can be overcome by exogenous LH

(Tsafriri et. al., 1976b). Working with highly-purified porcine OIF, several experimenters have demonstrated a molecular weight of approximately 2000 and heat stability to 60°C, indicating that OIF is a small polypeptide (Tsafriri et. al., 1976b; Stone et. al., 1978).

In Vitro Oocyte Maturation

Resumption of meiosis can also be induced by removing the oocyte from the follicle and placing it in suitable culture medium (Chang, 1955; Edwards, 1965; Cross and Brinster, 1970; Donahue, 1968). A timing sequence for in vitro oocyte maturation has been delineated in the mouse (Donahue, 1968), rat (Tsafriri and Kraicer, 1972; Zeilmaker and Verhamme, 1974; Zeilmaker et. al., 1974) and human (Edwards, 1965b; Jacobson et. al., 1970). Donahue found that 90-95% of mouse oocytes cultured in a Krebs-Ringer salt solution with pyruvate resumed meiosis, i.e., had undergone GVB and proceeded to metaphase I. GVB in vitro requires 2-6 h in the mouse, 2 h in the rat, and 30-40 h in man. Donahue also characterized three chromatin condensation stages occurring during the first 1.5 h in vitro: 1) filament shortening, 2) condensation about the nuclear and nucleolar periphery as the nucleolus itself disperses, and 3) discrete bivalents (tetrads), circularly arranged. As early as 8 minutes after follicular liberation, the oocyte's nuclear envelope appears undulated, an event occurring prior to GVB both in vivo

and in vitro (Calarco et. al., 1972; Szollosi et. al., 1972). Furthermore, no significant ultrastructural differences were detected between in vivo and in vitro oocytes regarding meiotic maturation.

In vitro activation of oocyte maturation requires specific metabolic substrates. Biggers and associates (1967) demonstrated that oocytes denuded of cumulus cells matured in pyruvate- or oxaloacetate (OAA)-supplemented medium, but required follicular cells when cultured with phosphoenolpyruvate (PEP), lactate or glucose as added energy sources. Donahue and Stern (1968) noted that the mouse oocyte undergoes GVB in medium containing glucose if the cumulus cells are present, indicating that the cumulus cells convert glucose to pyruvate which can then be used by the oocyte. Limited maturation of rat oocytes can occur with lactate alone or no energy substrate available in the medium, implying a possible endogenous energy substrate (Zeilmaker and Verhamme, 1974; Zeilmaker, 1978). Therefore, metabolic requirements for maturation of the rat oocyte are different from those which exist in the mouse. This may be responsible for the 4 h shorter maturation in vitro for the rat oocyte (Van Vliet and Zeilmaker, 1972).

Recent metabolic studies have indicated that several other factors are required for oocyte maturation in vitro. Cytochrome oxidase involvement has been implicated in GVB

of mouse oocytes as cyanide effectively blocks GVB (Zeilmaker et. al., 1974). The use of phosphorylation-uncoupling agents also prevents GVB, indicating ATP-dependence of mouse oocyte maturation. Dekel and co-workers (1976) showed that 90% of the oxygen uptake of the cumulus-oocyte complex was due to the cumulus. With the onset of maturation, there follows a decrease in cumulus oxygen consumption and a corresponding increase in oxygen uptake by the oocyte. Magnusson and associates (1977) demonstrated that the increased oocyte oxygen consumption associated with GVB and PBF is due to the meiotic process rather than hormonal stimulation. However, LH is postulated to have a direct effect on in vitro oocyte maturation via an accumulation of lysosome-like organelles about the GV and apparently involved in its dissolution (Ezzell and Szego, 1979).

Macromolecular Synthesis During Oocyte Maturation, In Vitro

RNA and protein synthesis is required for in vitro oocyte maturation. Intense incorporation of ^3H -uridine occurs within the GV of the preovulatory oocyte following a two-hour incubation period with ^3H -uridine (Bloom and Mukherjee, 1972; Wassarman and Letourneau, 1976a). Bloom and Mukherjee (1972), using actinomycin-D, demonstrated that RNA synthesis is required for GVB and chromo-

somal arrangement at the first metaphase plate. The pre-m-RNA synthesized prior to GVB is associated with the condensing chromosomes of porcine ovarian oocytes undergoing meiotic maturation in vitro. This RNA synthesized within the GV is subsequently transferred to the cytoplasm during maturation (Rodman and Bachvarova, 1976; Bloom and Mukherjee, 1972; Wassarman and Letourneau, 1976a; Motlik et. al., 1978) and appears to serve as a template to code for specific maturational proteins (Rodman and Bachvarova, 1976; McGaughey and Van Blerkom, 1977).

Proteins synthesized prior to GVB are also necessary for GVB and polar body extrusion (Wassarman and Letourneau, 1976b; Stern et. al., 1972; Ekholm and Magnusson, 1979). Rate of protein synthesis is greatest prior to GVB (McGaughey and Van Blerkom, 1977; Warnes et. al., 1977; Stern and Wassarman, 1974), and thereafter decreases with time (Schultz et. al., 1978a). However, the most marked changes in the pattern of protein synthesis appear after GVB (Schultz and Wassarman, 1977a,b; Schultz et. al., 1978b; Wassarman and Letourneau, 1979), and are associated with specific maturational events, including PBF (McGaughey and Van Blerkom, 1977; Van Blerkom and McGaughey, 1978).

Inhibitors of meiotic maturation can be used to elucidate the role of de novo protein synthesis in oocyte

maturation. Dibutyryl 3',5'-cyclic-AMP (dbcAMP) arrests mouse oocytes in the dictyate stage of the first meiotic prophase (Stern and Wassarman, 1974; Schultz and Wassarman, 1977a). However, protein synthesis was not affected, as dictyate oocytes accumulated exogenous valine to the same extent as those undergoing maturation (Stern and Wassarman, 1974). Therefore, it appears that cytoplasmic maturation of mammalian oocytes proceeds independently of nuclear progression in the first meiotic division, in vitro, (Stern and Wassarman, 1974; Schultz et. al., 1978b). DbcAMP, in conjunction with puromycin, blocks short-lived proteins which are necessary for GVB (Ekholm and Magnusson, 1979). However, puromycin alone inhibits incorporation of several amino acids, preventing oocyte maturation beyond the CAC stage (Stern et. al., 1972; Wassarman and Letourneau, 1976; Schultz and Wassarman, 1977a).

CHAPTER II

STATEMENT OF THE PROBLEM

The general decline in reproductive capacity associated with advancing maternal age has been almost entirely attributed to defects within the hypothalamic-pituitary-ovarian axis (Huang and Meites, 1975; Aschheim, 1979) or a steady deterioration of the uterine environment (Finn, 1970; Butcher, 1975). Several investigators have suggested that defects within the aged oocyte may be one cause of the reproductive wastage characteristic of aged females (Blaha, 1964a; Butcher, 1975; Peluso, 1976). However, little has been done concerning the effect of chronological age on the preovulatory oocyte. Therefore, the viability of the aged oocyte prior to ovulation must be assessed. An indicator of the preovulatory oocyte's viability is its ability to resume meiotic divisions (oocyte maturation), in vitro. A study was therefore designed to determine the effect of age on oocyte maturation, in vitro.

De novo RNA and protein synthesis is required for oocyte maturation (GVB and PBF) (Bloom and Mukherjee, 1972; Wassarman and Letourneau, 1976a,b; Ekholm and Magnusson, 1979). Consequently, experiments were designed to determine the effects of age on ³H-uridine and

^3H -leucine incorporation in preovulatory oocytes, in vitro.

It has been demonstrated that follicular (preovulatory) aging of the oocyte is a potent cause of alterations within the oocyte which leads to developmental anomalies (Butcher and Fugo, 1967; Butcher, 1969; Butcher et. al., 1969; Butcher, 1975). Since cycle length increases in the older female rat, the effect of follicular aging on the ability of the oocyte to mature, in vitro, was also examined in aged oocytes collected from irregularly-cycling rats.

CHAPTER III

MATERIALS AND METHODS

Female Sprague-Dawley rats were housed under controlled conditions of temperature (22°C), humidity (50%), and photoperiod. The animals were exposed to equal hours of light and dark with midnight corresponding to the midpoint of darkness. Estrous cycles were monitored by vaginal smears taken daily between 0800 and 1000 hours. Only those mature animals exhibiting three consecutive 4-day cycles and those aged rats with cycles between 4 and 9 days in length were used for the experiment. Also healthy-appearing rats without signs of respiratory distress, mammary tumors, or other gross pathologies were selected for these experiments.

EXPERIMENT I: RATE OF GERMINAL VESICLE BREAKDOWN (GVB) AND POLAR BODY FORMATION (PBF) IN AGED OOCYTES

In this study, mature rats (4-5 months old) on day 3 (proestrus) of the estrous cycle, and aged rats (10-11 months-old) on days 3, 4 and 5 of the estrous cycle were autopsied. In the aged rats, days 3-5 were considered to be proestrus if the vaginal smears were epithelial or epithelial/cornified and the uteri ballooned. The ovaries

were excised and oocytes collected from the largest pre-ovulatory follicles by puncturing with a 26-gauge needle. Thirty minutes elapsed between time of sacrifice and initiation of oocyte culture.

Oocytes were placed in a microdroplet (0.1 ml) of Brinster's Ova Culture Medium (BMOC-3) and incubated under paraffin oil for 20 h at 37°C in a humidified atmosphere of 5% CO₂ and air. After incubation, the cumulus cells were removed from oocytes by incubating them for 10 to 15 minutes in either 2.5% pancreatic trypsin or 800 I.U./ml hyaluronidase. Oocytes were then examined under phase-contrast optics for germinal vesicle breakdown (GVB) and first polar body formation (PBF). The percentage of ova undergoing GVB, PBF and degeneration or fragmentation were calculated. Maturation parameters were statistically evaluated using either Fisher exact or chi-square test.

EXPERIMENT II: RNA AND PROTEIN SYNTHESIS IN AGED OOCYTES

In this study, oocytes were collected from preovulatory follicles of mature day-3 (proestrus) rats and aged day-3 (proestrus) rats. Oocytes were then placed in a microdroplet (0.1 ml) of BMOC-3 supplemented with 50 uCi/ml ³H-uridine (specific activity = 5Ci/mmmole) for 1.5 and 3 hours. After incubation with radioactive media, oocytes were washed three times in non-radioactive media, fixed in Carnoy's solution for 15 minutes and prepared for radio-

autography (Weitlauf and Greenwald, 1971). Oocytes were embedded in paraffin and serially sectioned at 5 μ m. Alternate paraffin sections were mounted on two sets of slides and deparaffinized. One set was treated with ribonuclease A (specific activity = 3798 U/mg) in phosphate buffer (1 mg/l; pH = 7.4). The other set of slides received only buffer treatment. All slides were incubated at 37°C for one hour and then treated with 5% TCA for 10 minutes at 4°C. Finally, the slides were washed in tap water for 15 minutes, air-dried, and dipped in Kodak NTB-3 emulsion. Slides were exposed for 14 days, developed, and stained with hematoxylin and eosin (Peluso and Butcher, 1974). In addition to the autoradiographic analysis, these slides were also examined to determine the percentage of oocytes undergoing GVB and nucleolar dispersion.

In this second study, liberated oocytes were incubated in a microdroplet of BMOC-3 containing 10 μ Ci/ml 4,5-³H-leucine (specific activity = 52 Ci/mmol) for 1.5, 3 and 4.5 hours. After incubation, these oocytes were washed with non-radioactive media, fixed in Bouin's fluid for 24 hours and prepared for radioautography. The slides were exposed for 18 days.

Autoradiographic Analysis

The relative amount of ³H-uridine incorporation

into RNA at 1.5 and 3 hours was quantitated by counting the total number of silver grains over 78.54 um^2 of either nucleoplasm or cytoplasm of oocytes subjected to buffer and TCA treatment using a microdensitometer (Hughes et. al., 1977). RNase treatment reduced grain density to that within the emulsion adjacent to the oocyte section. Therefore, background readings were taken 200 um from the oocyte and the number of grains associated with background subtracted from non-RNase-treated sections. This grain density represented the relative amount of newly-synthesized RNA.

The amount of protein synthesis at 1.5 h was quantitated by counting grains over 78.54 um^2 of cytoplasm using the microdensitometer and subtracting background readings taken 200 um from the oocyte. Since previous studies have shown that fixation in Bouin's fluid removes incorporated amino acids (Weitlauf and Greenwald, 1971), this measurement was considered to represent the relative amount of protein synthesized. Oocytes too heavily labelled with ^3H -leucine to be quantitated were considered to have maximally incorporated this amino acid.

The area read by the densitometer was converted to 100 um^2 to facilitate calculation and graphical representation. Relative amounts of RNA and protein synthesis within the oocyte were statistically evaluated using either Student's "t" test or Mann-Whitney U test. Maturation

parameters were evaluated using either Fisher exact or chi-square test.

CHAPTER IV

RESULTS

EXPERIMENT I: RATE OF GERMINAL VESICLE BREAKDOWN (GVB) AND POLAR BODY FORMATION (PBF) IN AGED OOCYTES

After 20 h of incubation, 95% of control oocytes had undergone GVB. Aged oocytes showed no alteration in GVB rate (Table 1). Of aged ova undergoing GVB, three had retained a clearly visible nucleolus. Aged oocytes showed a reduced ability to form a polar body and an increased tendency to fragment or degenerate, with respect to mature controls ($p < 0.05$) (Table 1). No further effects of day of cycle (follicular aging) were observed as judged by the parameters tested.

EXPERIMENT II: RNA AND PROTEIN SYNTHESIS IN AGED OOCYTES

After 1.5 and 3 h of incubation, 43.8% and 61.5% of control oocytes, respectively, had undergone GVB. Rate of GVB in aged oocytes was not affected (Fig. 1). While over 80% of control oocytes showed nucleolar dissolution at both 1.5 and 3 h, the percentage of aged oocytes undergoing nucleolar dispersion was reduced by 50% at both times tested ($p < 0.05$) (Fig. 1; compare Fig. 2A and 2B).

Preliminary observations using the densitometer demonstrated that a linear relationship between amount of

Table 1. Effect of age and length of the cycle on the ability of the oocyte to undergo germinal vesicle breakdown (GVB) and polar body formation (PBF) in vitro.

Day of Cycle/Age	No. of Rats	No. of Ova Examined	% of GVB ^a	% of PBF	% Fragmented &/or Degenerated
Day 3 Mature	4	45	95	30	0
Day 3 Aged	6	52	98 ^b	9.6 ^a	15.4 ^a
Day 4 Aged	5	31	100	9.7 ^a	9.7 ^a
Day 5 Aged	4	27	93 ^c	7.4 ^a	22.2 ^a

- a. Significantly different from mature day 3 controls (p 0.05)
 b. Nucleolus retained in two ova after GVB
 c. Nucleolus retained in one ovum after GVB

Figure 1. In vitro maturation of liberated mature and aged oocytes. Parameters measured were percentage of oocytes undergoing GVB and nucleolar dispersion. Data from the mature oocytes are represented in the open bars, while the shaded bars represent the data from the aged oocytes. Fifteen to twenty oocytes were examined in both age groups at each time tested.

There was no significant difference in the percentage of oocytes with nucleolar dispersion between mature and aged oocytes at each time of incubation. However, when the data for both incubation periods were pooled, a significant decrease in nucleolar dispersion in the aged oocytes was apparent.

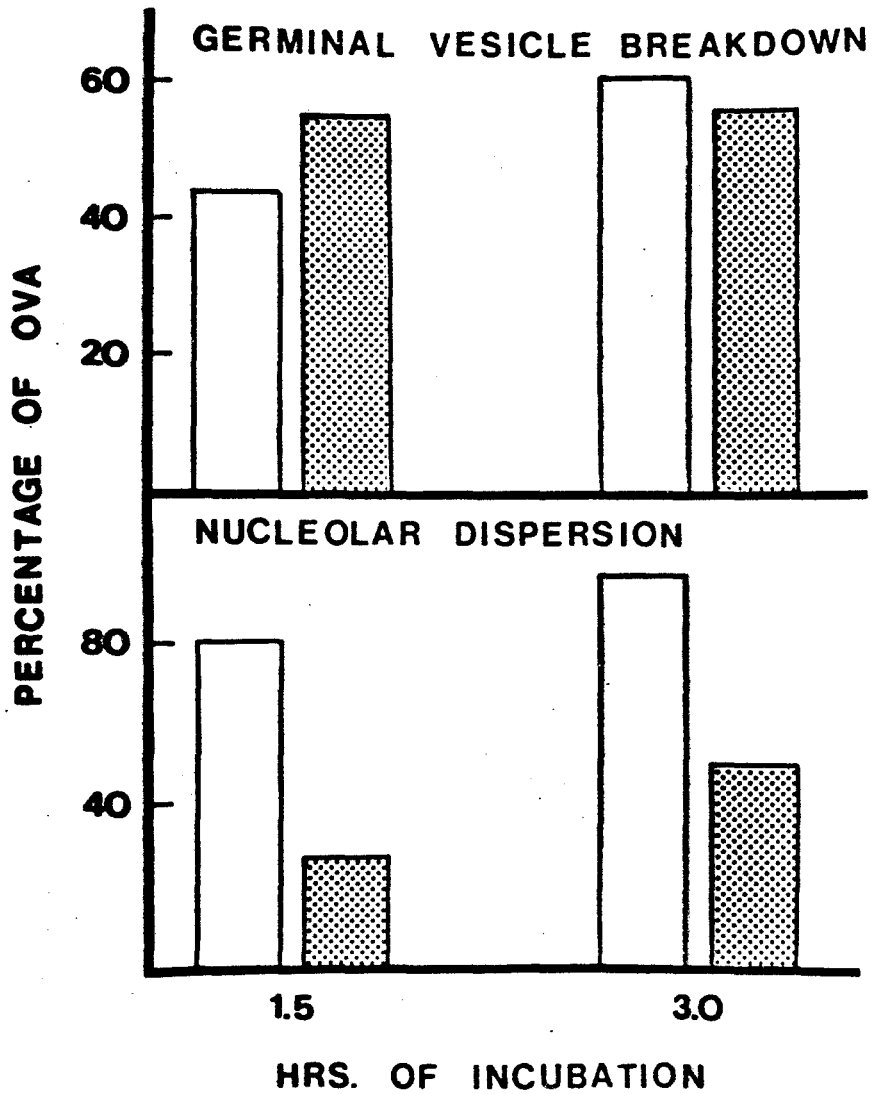
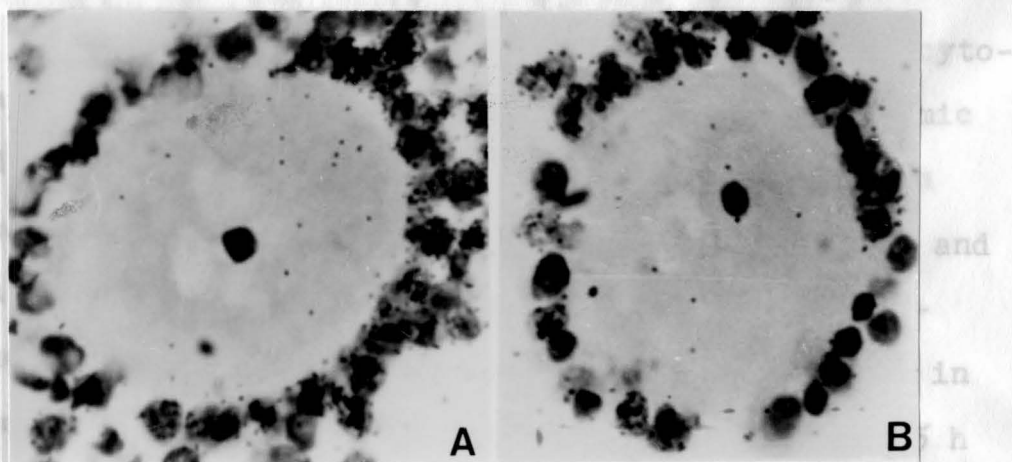


Figure 2A. An autoradiograph of an aged oocyte incubated for 3 h in ^3H -uridine-supplemented medium and treated with RNase. Note the distinct nucleolus still surrounded by remnants of the GV (x 1000).

Figure 2B. An autoradiograph of an aged oocyte incubated for 3 h in ^3H -uridine-supplemented medium and treated with RNase. Note presence of intact nucleolus after completion of GVB (x 1000).

light (as represented by Δ transmittance) and voltage exists (Fig. 3). A standard curve regressing densitometry on grain density as determined by visual counting and voltage showed a positive correlation (Fig. 4).



(Fig. 7). As with ^{125}I , the percentage of ova incorporating ^3H -leucine maximally was not altered by age (Fig. 8). Although cumulus cells were more closely associated with control oocytes, maximal ^3H -leucine uptake by oocytes was neither dependent on density nor proximity of the cumulus mass, regardless of age (compare Fig. 5C and 5D).

light (as represented by % transmission) and voltage exists (Fig. 3). A standard curve regressing densitometry on grain density as determined by visual counting and voltage showed a positive correlation (Fig. 4).

^3H -Uridine was incorporated into nuclei and cytoplasm of control oocytes (Fig. 5A). Both cytoplasmic and nuclear incorporation of ^3H -uridine into RNA in aged oocytes was significantly reduced at both 1.5 and 3 h ($p < 0.05$) (Fig. 6; compare Fig. 5A and 5B). ^3H -Leucine incorporation into protein was not altered in aged oocytes with respect to mature controls at 1.5 h (Fig. 7). In addition, the percentage of ova incorporating ^3H -leucine maximally was not altered by age (Fig. 8). Although cumulus cells were more closely associated with control oocytes, maximal ^3H -leucine uptake by oocytes was neither dependent on density nor proximity of the cumulus mass, regardless of age (compare Fig. 5C and 5D).

Figure 3. Transmission curve correlating percent light transmission and voltage.

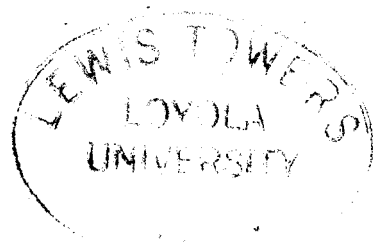
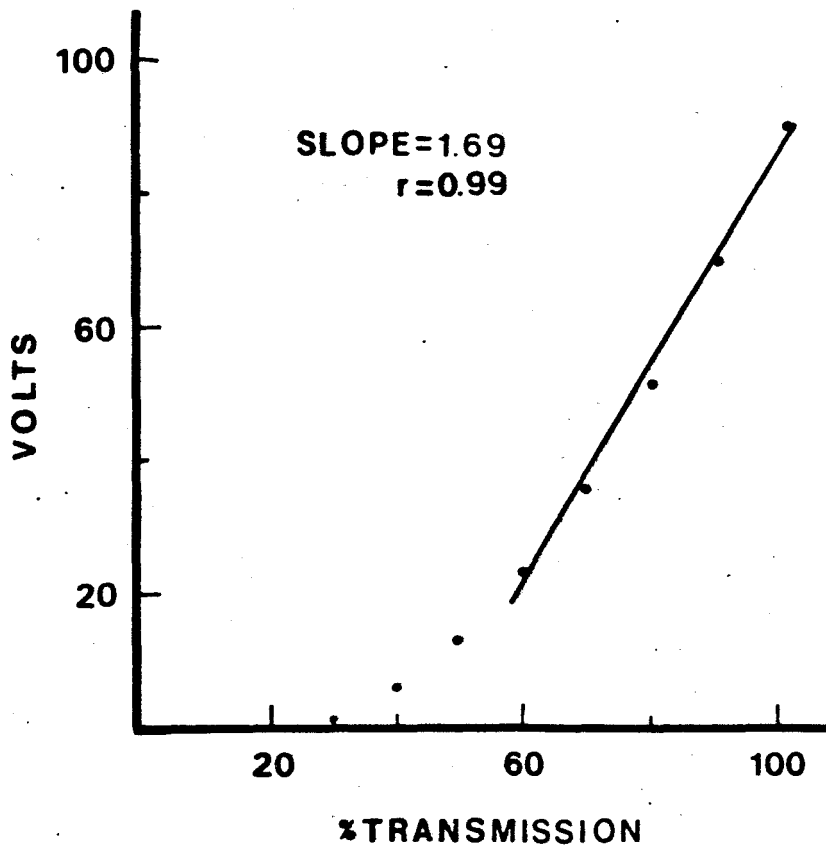
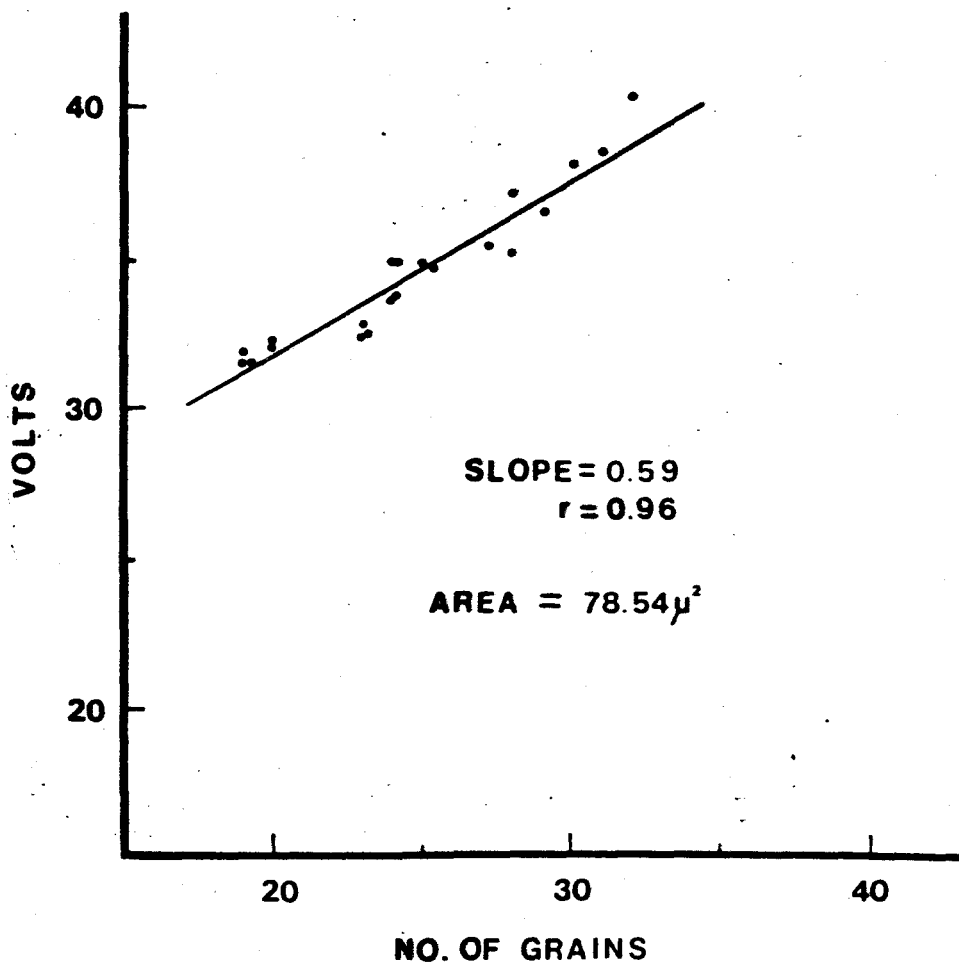


Figure 4. Standard curve correlating densitometry readouts (volts) and grain densities.



- Figure 5A. An autoradiograph of a mature oocyte, incubated for 1.5 h in ^3H -uridine-supplemented medium. Note the high grain density localized within the germinal vesicle (x 900).
- Figure 5B. An autoradiograph of an aged oocyte incubated for 1.5 h in ^3H -uridine-supplemented medium. The number of silver grains within the GV is reduced (x 900).
- Figure 5C. An autoradiograph of two mature oocytes incubated for 1.5 h in ^3H -leucine-supplemented medium. The oocyte on the left incorporated ^3H -leucine maximally. The grain density of the oocyte on the right was 52 grains/100 μm^2 of ooplasm (x 600).
- Figure 5D. An autoradiograph of an aged oocyte incubated for 1.5 h in ^3H -leucine-supplemented medium. Although the cumulus cells were not closely associated with the oocyte, the aged oocyte incorporated ^3H -leucine maximally (x 600).

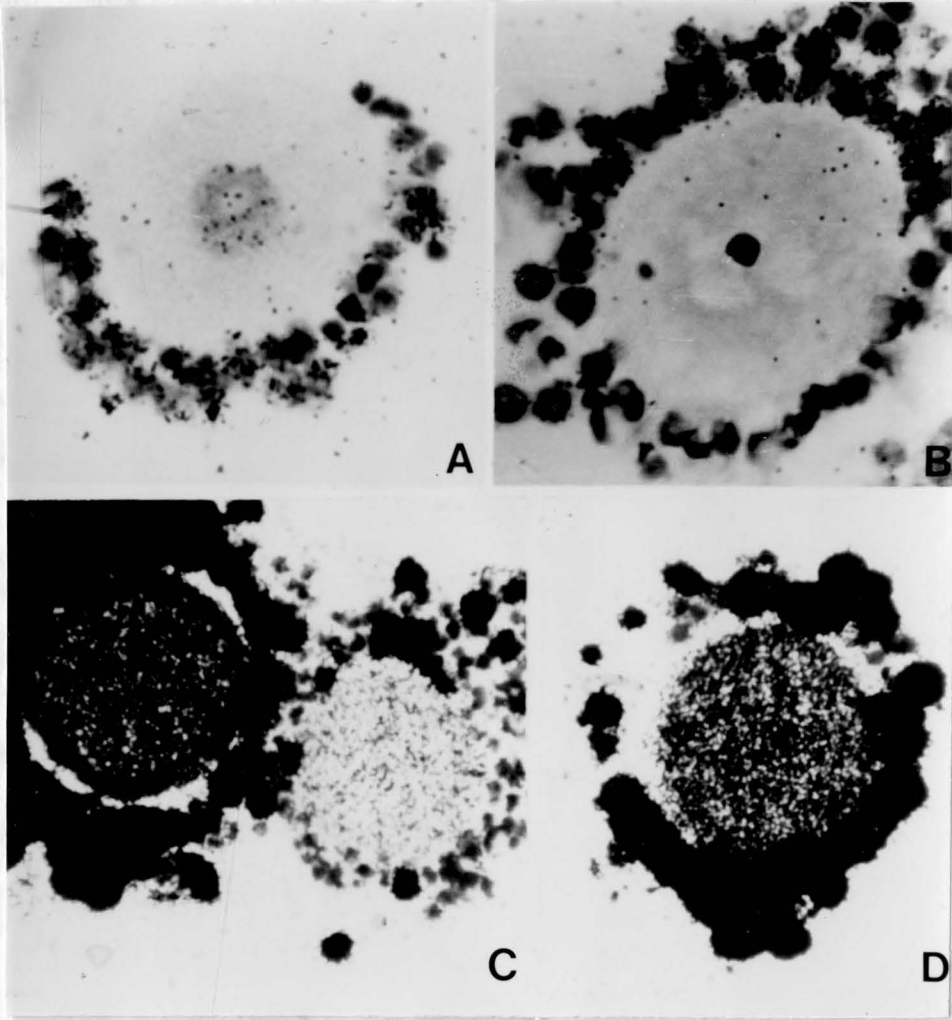


Figure 6. RNA synthesis in mature and aged oocytes. Fifteen to twenty oocytes were examined in each group. Values are expressed as mean \pm one standard error. Data from the mature oocytes are presented in the open bars, while the data from the aged oocytes are represented by the shaded bars.

*Significantly different from respective mature control group ($p < 0.05$).

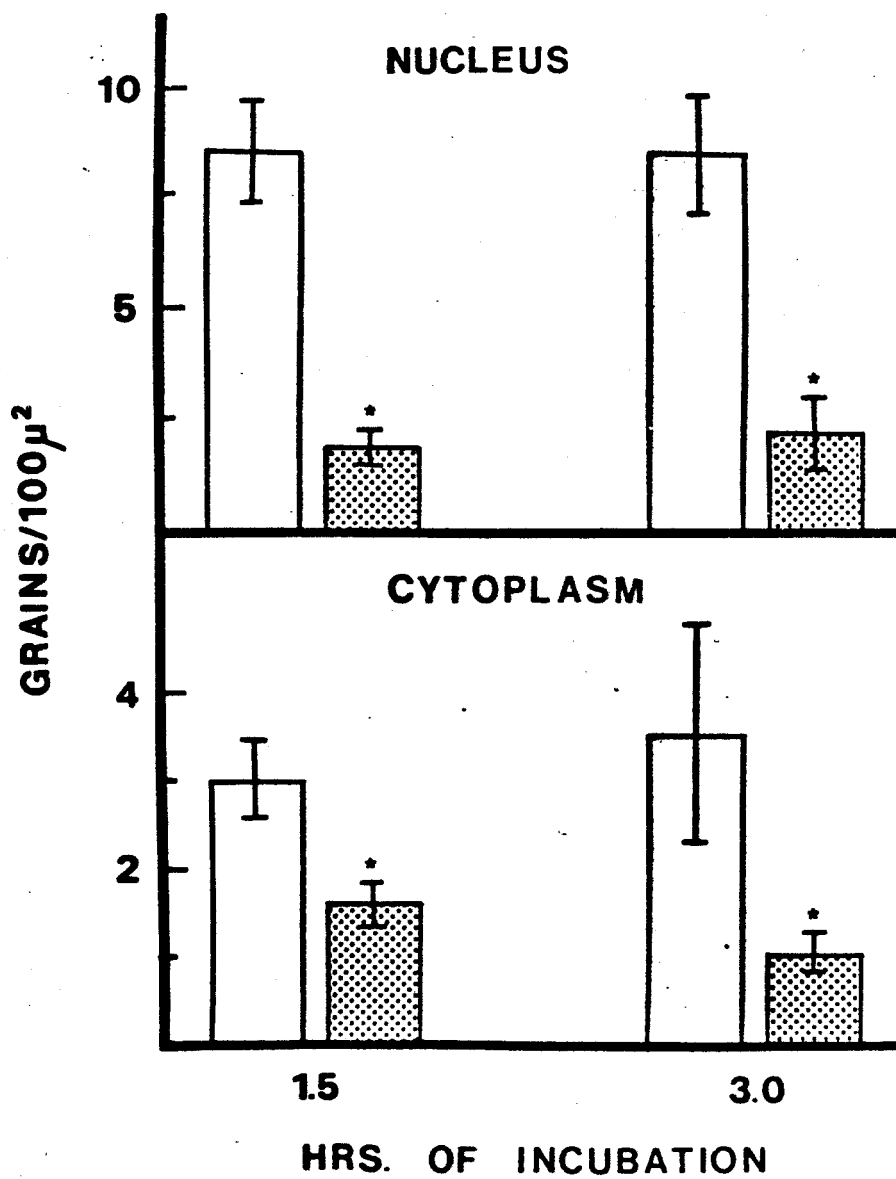


Figure 7. Protein synthesis in mature and aged oocytes incubated in ^3H -leucine-supplemented medium for 1.5 hours. Twenty-seven mature and twenty-one aged oocytes were examined.

N.S. Not significantly different from respective mature control group.

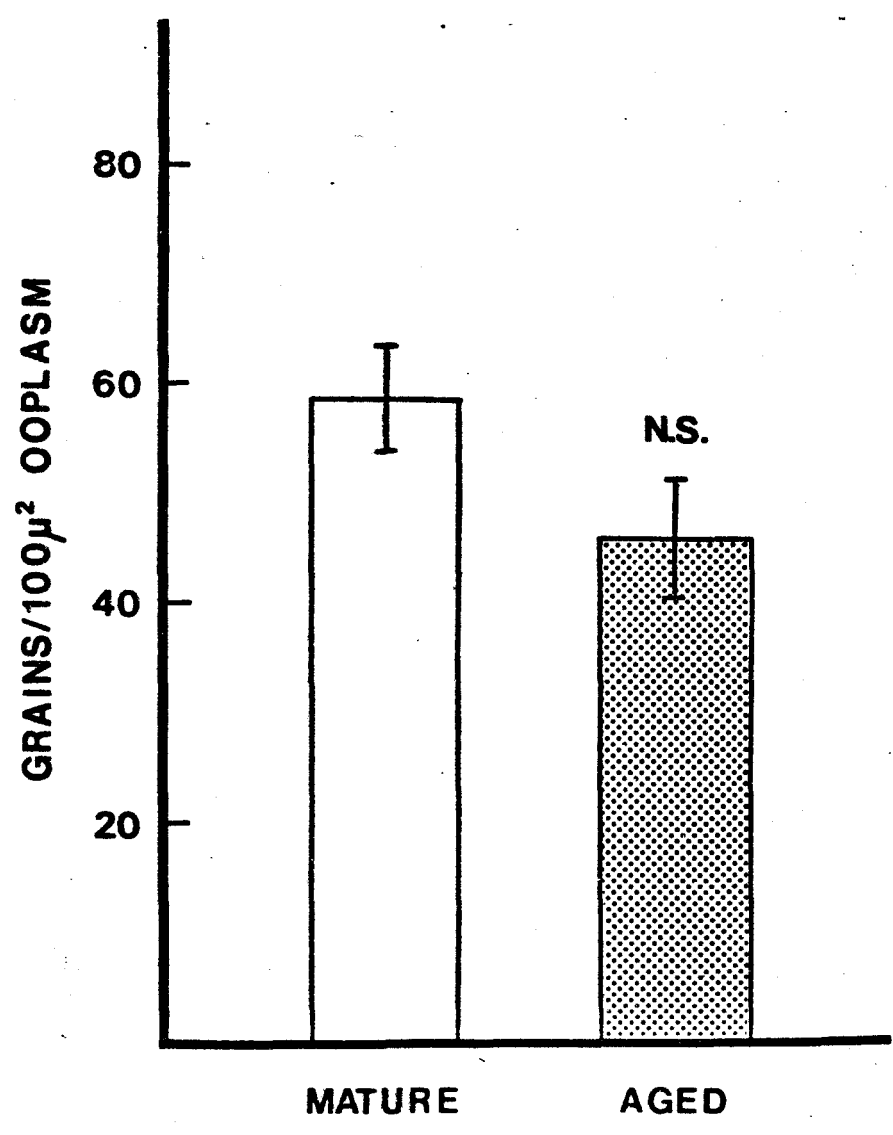
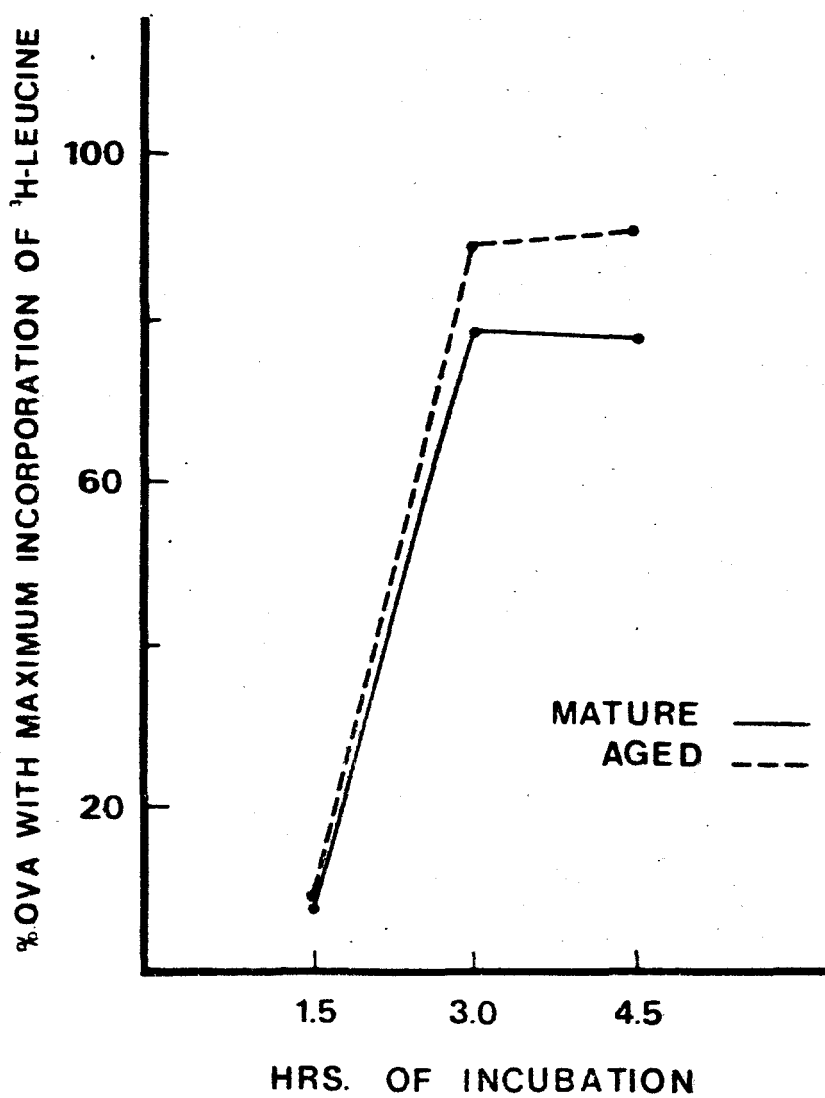


Figure 8. Time course for maximal incorporation of ^3H -leucine into protein by both control and aged oocytes. Twenty to thirty oocytes were examined at each time in each group studied.

There was no significant difference between mature and aged oocytes with regard to the percentage of oocytes maximally incorporating ^3H -leucine at all times examined.



CHAPTER V

DISCUSSION

The decline in fertility characteristic of aged animals may be due to a number of factors: defects in the hypothalamic-pituitary-ovarian axis (Huang and Meites, 1975), an inadequate intrauterine environment (Biggers, 1969; Finn, 1970; Butcher, 1975), or defects within the aged oocyte itself (Butcher, 1975; Peluso, 1976; present study).

The results from the present study indicate that chronological aging affects the oocyte such that these ova 1) have a reduced ability to extrude the first polar body, 2) tend to degenerate and/or fragment in culture, 3) are prone towards nucleolar retention and 4) are impaired in their ability to synthesize RNA, although protein synthesis appears unaltered by age. However, no further effects of follicular aging were observed with respect to the parameters examined in this study.

Therefore, it appears that aging alters both the cytoplasm and nucleus of many oocytes such that they are not allowed to complete meiotic maturation. Failure to extrude the first polar body would result in the retention of an extra chromosomal complement. Failure of nucleolar dissolution would result in non-disjunction of

chromosomal pairs since the nucleolus is shared by several bivalent pairs, and its presence would lead to a physical difficulty in chromosome separation (Calarco et. al., 1972; Polani et. al., 1960; Evans, 1967). Subsequent fertilization of digynic eggs or those retaining the nucleolus would produce triploid (Chang and Hunt, 1968) or aneuploid embryos (Yamamoto et. al., 1973; Gosden, 1973), respectively.

De novo RNA and protein synthesis is necessary for oocyte maturation. RNA synthesis is required for GVB and subsequent stages of oocyte meiotic maturation and persists for 2-6 h in vitro (Bloom and Mukherjee, 1972; Wassarman and Letourneau, 1976a; Rodman and Bachvarova, 1976). RNA synthesized within the GV is subsequently transferred to the ooplasm during maturation (Rodman and Bachvarova, 1976; Bloom and Mukherjee, 1972; Wassarman and Letourneau, 1976a; Motlik et. al., 1978). Proteins synthesized prior to GVB are also necessary for GVB and polar body extrusion (Wassarman and Letourneau, 1976b; Stern et. al., 1972; Ekholm and Magnusson, 1979). Rate of protein synthesis is greatest prior to GVB (McGaughey and Van Blerkom, 1977; Warnes et. al., 1977; Stern and Wassarman, 1974), and thereafter decreases with time (Schultz et. al., 1978a). However, the most marked changes in the protein synthetic pattern appear after GVB (Schultz and Wassarman, 1977a,b; Schultz et. al., 1978b; Wassarman

and Letourneau, 1979) and are associated with specific maturational events, including polar body formation (PBF) (McGaughey and Van Blerkom, 1977; Van Blerkom and McGaughey, 1978).

It is apparent from the autoradiographic analyses of this study that the capability of the aged oocyte to incorporate ^3H -leucine into protein, in vitro, was not significantly altered with respect to mature controls. However, the ability of the aged oocyte to synthesize RNA was impaired. Since RNA synthesized prior to GVB appears to serve as a template for specific maturational proteins (Rodman and Bachvarova, 1976; McGaughey and Van Blerkom, 1977), defects in RNA synthesis in the aged oocyte could alter these specific proteins. Such an alteration in protein synthesis may be responsible for the decrease in first polar body formation characteristic of aged rat oocytes observed in the present study.

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APPROVAL SHEET

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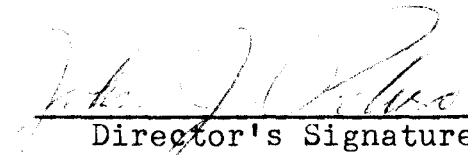
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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science.

April 14, 1980
Date


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