Zygote development after delayed fertilization

A cytological and genetical analysis of embryos of the mouse

De invloed van post-ovulatoire veroudering op de ontwikkeling van zygoten

Een cytologische en genetische analyse van embryo's van de muis

Promotor: dr. C. Heyting
hoogleraar in de generatieve en somatische celgenetica

Co-promotor: dr. ir. P. de Boer
universitair hoofddocent

M. L. Boerjan

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Opinions concerning reproduction have altered considerably since Antonie van Leeuwenhoek 'saw' a miniature human (homunculus) within the spermhead. He thought the women's role to be restricted to protection and nutrition of the developing embryo. Nowadays everyone accepts that contributions of maternal and as well as paternal gametes to the next generation are necessary. In a sexual life cycle, development of new offspring starts, by definition, with the fusion of maternally and paternally derived haploid germ cells, resulting in a diploid 1-cell embryo, the zygote. The fusion, called fertilization, triggers a sequence of morphological and molecular changes in the maternally derived germ cell, the oocyte.

The present thesis deals with a descriptive investigation concerning the order and timing of morphological, genetical and molecular changes during the first cell cycle in zygotes from Swiss mice after delayed fertilization.

STELLINGEN

1. De snelheid waarmee post-ovulatoir verouderde eicellen reageren op activerende prikkels, is aanleiding om het begrip "arrest van de tweede meiotische metafase" niet op te vatten als het stilstaan van celbiologische processen.

   Dit proefschrift

2. De ontkoppeling van morfologische en moleculaire ontwikkelingskenmerken na eicel veroudering is een aanwijzing dat meerdere parameters noodzakelijk zijn voor een kwaliteitsbeschrijving van het zoogdier preimplantatie embryo.

   Dit proefschrift

3. De bijdrage van het sperma aan de vroeg embryonale ontwikkeling moet ruimer opgevat worden dan louter mikroinjectie van erfelijk materiaal.

   Swann K. (1989): Winter Meeting Society for the Study of Fertility, Warwick, UK.

4. De locatie van het sperma intreepunt en de afsnoering van het tweede poollichaampje krijgen te weinig aandacht in discussies over het al dan niet aanwezig zijn van polariteit in de zoogdier zygote.

   Petersen R.A. (1986): In: Experimental approaches to mammalian embryonic development. Eds. J. Rossant & R.A. Pedersen, pp 16.

5. De plannen tot privatisering van de voorlichting over de insektenbestuiving en bijenhouderij getuigen van een onderschatting van het ecologisch en economisch belang van de imkerij.

6. De export van embryotransplantatie technieken vanuit de eerste naar de tweede en derde wereld vereist een zorgvuldige integratiestudie.
   Een integratiestudie omvat: economische, culturele, sociale en technologische dimensies.

7. Centralisatie van het beroepsonderwijs bevordert het vergrijzingsproces van plattelandskernen.

8. Het aantal geitenwollensokkendragers in het onderwijs is niet afhankelijk van vrijstelling van de dienstplicht voor toekomstige leraren, wel van het aantal geiten dat beschikbaar is voor de wolproduktie.

Behandeling onderwijsbegroting 15 februari 1990.

9. Kennis van de wordingsgeschiedenis van de geabstraheerde figuratieve kunst is in hoge mate bepalend voor de waardering van deze kunst.

10. Voorwaarden voor de ontwikkeling van empirische theorieën zijn én toepassing én evaluatie, én de eventueel daardoor vereiste correctie, herziening en uitbreiding. Dit geldt ook voor planeconomische theorieën.

Stellingen behorend bij het proefschrift "Zygote development after delayed fertilization, a cytological and genetical analysis of embryos of the mouse" van Marleen Boerjan

Wageningen, 27 april 1990

CONTENTS

SUMMARY

Chapter I gives (1) a brief review of morphological and molecular changes in mammalian oocytes initiated by fertilization, (2) a summary of published data on several aspects of post-ovulatory ageing in relation to embryonic development and (3) a description of methods to time ovulation and fertilization.

Precise timing of ovulation and fertilization was a prerequisite for the study of the consequences of delayed fertilization for embryonic development.

Chapter II describes the methods of ovulation induction and artificial insemination. We induced ovulation with injections of luteinizing hormone releasing hormone (LHRH) at pro-oestrus of the oestrus cycle. The number of oocytes that ovulated after LHRH administration was similar to that after spontaneous ovulation. Although LHRH was administered 8-12 hrs before the expected endogenous luteinizing hormone (LH) surge, we found no significant effect of LHRH induced ovulation on embryonic mortality. This method of ovulation induction, supplemented with artificial insemination to achieve *in vivo* fertilization, provided a tool to study aspects of early embryonic development after delayed fertilization.

Investigations were carried out to determine in detail the timing of the distinct morphological changes triggered by fertilization of unaged oocytes and oocytes aged post-ovulation for 12 hrs. Sperm penetration was shown to be accelerated by 1 hr 30 min after delayed insemination compared with sperm penetration in unaged oocytes. The rate of progression to the first cleavage division was also influenced by the post-ovulation age of mouse oocytes prior to fertilization: penetrated aged oocytes needed less time (1 hr 30 min) to reach the 2-cell stage than zygotes from unaged oocytes. This observation was confirmed by experiments carried out later: the percentages of 3- and 4-cell embryos from aged and unaged oocytes, collected 36 hrs after insemination, were 14% and 7% respectively (Chapter IV).

Fertilization activates, among other things, a cascade reaction of protein synthetic alterations.

Chapter III deals with protein synthetic activity in unaged and aged LHRH induced oocytes, in unaged superovulated oocytes and in zygotes

derived from these types of oocytes. Polypeptides with a relative molecular weight of 35 kDa were predominantly synthesized by LHRH induced and superovulated secondary oocytes and zygotes from these oocytes. This study of patterns of 35 kDa proteins synthesized by zygotes from aged and unaged LHRH induced oocytes revealed that fertilization dependent protein synthetic changes of the 35 kDa protein complex were advanced in zygotes from aged oocytes with reference to pronuclear development.

A fraction (16.7%) of morphologically normal zygotes from unaged superovulated oocytes did not synthesize the 35 kDa protein complex at all.

It was of interest to learn more about the *in vitro* developmental capacity of embryos from aged oocytes.

In **Chapter IV** the results of these investigations are arranged and discussed. One-cell and late 2-cell embryos from aged and unaged oocytes were cultured in the presence and absence of DNA damage or DNA-damaging agents for different periods of time. On the one hand we studied the progression to metaphase of the first cleavage division in zygotes from aged and unaged oocytes fertilized with X-irradiated sperm. On the other hand the *in vitro* developmental capacity of late 2-cell embryos was evaluated in presence and absence of the thymidine analogue 5-Bromodeoxyuridine (BrdU).

Post-ovulatory ageing had an effect on the morphology of male as well as female pronuclear chromosomes of the first cleavage metaphase. We also found a detrimental effect of fertilization with X-irradiated spermatozoa on the morphology of male and female pronuclear chromosomes. This effect was particularly observed in male pronuclear chromosomes of zygotes from aged oocytes. Furthermore, fertilization with X-irradiated spermatozoa led to an arrest at interphase in 27% and 7% of zygotes from aged and unaged oocytes respectively. This arrest was not shown after fertilization with sperm not irradiated with X-rays.

This experimental setup also enabled us to compare the amount of radiation induced chromosome damage in zygotes from aged and unaged oocytes. Zygotes from aged oocytes did not contain more chromosome damage than zygotes from unaged oocytes, when the visible chromosome mutations originating from the X-irradiated spermatozoa were analyzed at metaphase of the first cleavage division.

In **Chapter IV** we also have shown that zygotes from aged oocytes and

unaged oocytes develop *in vitro* at similar rates from the late 2-cell stage, collected at 36 hrs after insemination, to the 8-cell stage (24 hrs cultures). However, *in vitro* development of 2-cell embryos from aged oocytes collected 30 hrs after insemination and cultured for 66 hrs is impaired.

To determine the number of sister-chromatid exchanges, we cultured 2-cell embryos from aged and unaged oocytes, collected 36 hrs after insemination, in the presence of BrdU for 24 hrs. SCE levels were not significantly different between embryos from aged and unaged oocytes.

Cell proliferation of late 2-cell embryos from aged oocytes, collected 36 hrs post-insemination, from aged oocytes was clearly retarded and asynchronous during the 24 hrs culture period in the presence of 10^{-6} M BrdU.

In **Chapter V** a cytochemical method is described to determine in individual oocytes the distribution of the activity of SDH (succinate dehydrogenase), an enzyme which is located on the inner membrane of mitochondria. We showed that treatment of oocytes with the drug caffeine prior to cytochemical staining resulted in an intens staining of the cells by a formazan precipitate. We applied the cytochemical staining procedure to preovulatory oocytes of mice. In a maturation experiment *in vitro* we found that the location of formazan correlated well with the location of mitochondria in subsequent stages of maturation. Unfortunately, this cytochemical staining procedure could not be applied to ovulated and fertilized oocytes, since these cells acquired a poor morphology during the staining procedure and displayed high levels of non-dehydrogenase formazan production.

CHAPTER I

GENERAL INTRODUCTION

THE SECONDARY MOUSE OOCYTE

Mammalian oocytes acquire their full competence to complete embryonic development during growth and after resumption and completion of the first meiotic maturation division in the follicle. Maturation starts after the binding of luteinizing hormone to receptors on hormonally primed granulosa cells of the cumulus mass (Niswender and Nett, 1988), and is characterized by the resumption of meiosis which involves a sequence of reorganizations in the nucleus, the cytoplasm (Zamboni, 1970; Van Blerkom and Runner, 1984) and the plasma membrane (Nicosia et al., 1978; Maro et al., 1986a). At the molecular level, oocyte maturation is characterized by both the cessation of transcription prior to germinal vesicle breakdown (GVB, Wassarman and Letourneau, 1976) and stage related alterations in quantitative and qualitative patterns of protein synthesis and post-translational modifications (mouse: Schulz and Wassarman, 1977; Huarte et al., 1987; sheep: Moor and Crosby, 1986).

Maturation culminates in the ovulation of highly organized secondary oocytes, arrested in metaphase of the second meiotic division with the spindle orientated with its long axis parallel to the oocyte membrane (Fig. 1a, Szollosi et al., 1972). The cortical cytoplasm overlying the meiotic spindle appears to be devoid of cortical granula (Nicosia et al.,1977) and mitochondria (Zamboni, 1970), the plasmalemma is free of microvilli (Nicosia et al., 1978) and concanavalin A-binding sites (Maro et al., 1984). The differentiation of the cytocortical region associated with the meiotic spindle is mediated by condensed meiotic chromosomes (Maro et al., 1986b; Van Blerkom and Bell, 1986). It has been demonstrated that the presence of condensed meiotic chromosomes is correlated with:
(1) A local disappearance of concanavalin A-binding sites and microvilli at the cell surface. (2) An actin-rich filamentous area in the cell cortex between the chromosomes and the plasma membrane (Maro et al., 1986b; Van Blerkom and Bell, 1986). The equatorial region of the meiotic spindle is attached to the membrane and it is therefore suggested that the subcortical layer of actin plays an important role in spindle rotation during second polar body extrusion after fertilization (Maro et al., 1984; Webb et al., 1986). (3) The formation of microtubules, which appears to occur

because of a lowering of the critical concentration for the polymerization of tubulin in the presence of chromosomes (Maro et al., 1986a). In the freshly ovulated mouse oocyte the microtubules are located exclusively within the body of the spindle (Maro et al., 1985), although microtubule organizing centers (MTOCs) are present in the cytocortex (Calarco-Gillam et al., 1983; Maro et al., 1985).

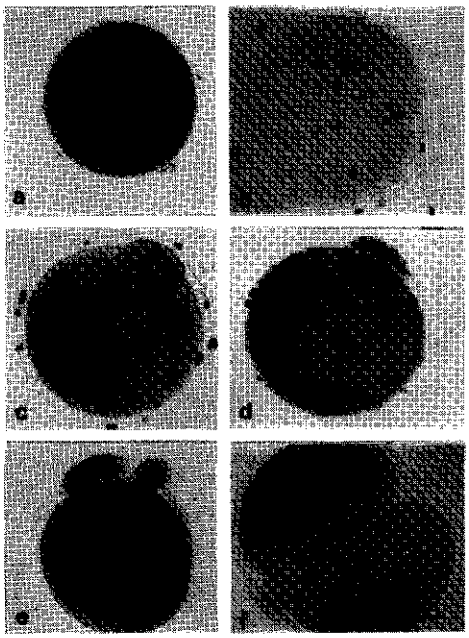

Figure 1. Light micrographs of mouse embryos at different stages of zygote development. (bar represents 30 μm).

a. an unaged unfertilized oocyte; the spindle (arrow) is tangentially orientated along the plasma membrane.

b. an unfertilized oocyte post-ovulatory aged for 16-18 hrs; the spindle (arrow) is located more to the center of the cell and has a radial orientation.

c. an unaged fertilized oocyte; the second meiotic cleavage division has been resumed and a second polar body (arrow) is formed.

d. an unaged fertilized oocyte; the spermatozoon (arrow) has penetrated the vitellus (micrographs c-d represent the same oocyte, but in different focal planes).

e. a zygote in pronuclear (arrow) interphase.

f. a 2-cell embryo.

FERTILIZATION

The sequence of morphological changes upon activation

Upon fusion of the sperm plasma membrane with the microvilli rich part of the oocyte, the second meiotic cleavage division is resumed. This becomes visible by the extrusion of the second polar body (Fig. 1c). Subsequently, the chromosomes of the activated egg decondense and form a haploid interphase nucleus; in the second polar body the chromosomes clump and a nuclear envelope is formed (Zamboni, 1972).

The spermhead penetrates the vitellus and also forms an interphase pronucleus (Fig. 1d). The sequence of events leading to a mature male pronucleus is different from that for female pronuclear development. In the male pronucleus the sperm-specific protamines are replaced by oocyte derived histones; this process involves the reduction of many SS-cross-links present in mature sperm (reviewed by Yanachimachi, 1988).

By definition, pronucleus formation is complete if the chromatin has decondensed and a pronuclear membrane is formed. The nuclear membrane is thought to be derived from endoplasmatic reticulum membranes (Zamboni, 1972). In this stage of development the female pronucleus is located close to the second polar body and the male pronucleus is often accompanied by a sperm tail. The maternally and paternally derived genomes can, therefore, be distinguished in the interphase of the first cell cycle. DNA-replication takes place in both the male and the female pronucleus during interphase (Ambramczuk and Sawicki, 1975; Luthardt and Donahue, 1973; Krishna and Generoso, 1977; Molls et al., 1983; Howlett and Bolton, 1985).

Following pronuclear formation the progression to the first cleavage division is, at the morphological level, characterized by: (1) migration of the pronuclei towards the center of the cell, (2) disappearance of the pronuclear membranes, (3) formation of two sets of condensed chromosomes which align in a common metaphase plate and (4) cytokinesis resulting in a 2-cell embryo (Fig. 1f).

The role of the cytoskeleton becomes clear in studies on intracellular reorganizations following fertilization. Firstly, the cleavage furrow of meiosis II and thus of second polar body formation can only be formed in the actin-rich domain overlying the meiotic spindle and an uniform layer of microfilaments is associated with the plasma membrane after fertilization. Secondly, the critical concentration for the polymerization of microtubules is lowered upon fertilization and therefore many asters of microtubules are formed around the cytoplasmic MTOCs (Maro, 1985; Schatten

et al., 1985). These asters enlarge to form a dense cytoplasmic network of microtubules by the pronuclear stage. It has been suggested that these cytoskeletal elements mediate the migration of the pronuclei. The network of microtubules disassembles at the end of interphase and MTOCs located close to the condensing chromosomes form the spindle of the first mitotic division.

Molecular changes observed in mammalian oocytes after fertilization

The sequence of morphological changes described above and characteristic for the first cell cycle, is accompanied and possibly regulated by a cascade of molecular changes. The intact oocyte is normally fertilized by a single spermatozoon; shortly after fertilization modifications of the zona pellucida and the plasma membrane occur. These inhibit the penetration of surplus spermatozoa and it has long been recognized that these modifications are the result of the cortical reaction or exocytosis of cortical granules (Austin, 1956; Gwatkin et al., 1973; Gulyas, 1980). Evidence is accumulating that the cortical reaction is a secondary response of the oocyte to the fusion of sperm and oocyte membranes. In the sea urchin (reviewed by Yanachimachi, 1988) and in the hamster (Miyazaki, 1988) the fusion of both membranes induces, probably via the inositide-triphosphate cycle, a wave of Ca^{2+}-release and an intracellular increase of pH. The Ca^{2+}-release initiates exocytosis of cortical granules as shown in hamster and sheep oocytes (Miyazaki et al., 1988; Cran et al., 1988). The increase of the intracellular pH could be a trigger that activates metabolic processes necessary for pronuclear formation and progression to the first cleavage division (reviewed by Yanachimachi, 1988). Among these are processes like modifications of proteins (Cullen et al., 1980; Van Blerkom, 1981; Howlett and Bolton, 1985) and the unmasking of maternally derived messenger RNAs (mmRNA) for the translation of fertilization dependent proteins (Howlett, 1985). It should be noted that in the early mouse embryo transcription does not occur before early the two-cell stage (Clegg and Piko, 1983; Bolton et al., 1984) and it has been suggested that this embryonic directed transcription is dependent on pronuclear DNA-synthesis (Bolton et al., 1984).

ASPECTS OF DELAYED FERTILIZATION

In mammals, except man, the time of mating is restricted to a period shortly before the moment of ovulation. Thus, delayed mating is not a

natural occurrence in most mammals. Delayed fertilization can, however, occur after artificial insemination.

It is known that oocytes aged post-ovulation loose their capacity for normal development before the end of their fertilizable life: in the mouse fertilizable life is at least 15 hrs, but 17% of zygotes from oocytes aged *in vivo* for 12 hrs showed abnormal *in vivo* development (Marston and Chang, 1964); rabbit eggs are fertilizable until at least 7 hrs post-ovulation, but pre-implantation development of oocytes fertilized after 7 hrs post-ovulatory ageing is impaired (Austin, 1967); hamster oocytes aged *in vivo* for 12 hrs could be fertilized *in vitro*, only 21% of these penetrated aged oocytes underwent first cleavage (Juetten and Bavister, 1983).

Delayed fertilization induces two distinct abnormalities of fertilization, namely (1) triploidy as a result of dispermic penetration or retention of the second polar body (reviewed by Szollozi, 1975) and (2) extrusion of an abnormal second polar body. In the mouse, di- or polyspermy is ascribed to the migration of cortical granules to the center of the vitellus during ageing (Szollozi, 1975). Abnormal extrusion of the second polar body could be a result of post-ovulatory changes in the organization of cytoskeleton elements (Webb et al., 1986; Eichenlaub-Ritter, 1986).

The organization of the cytoskeleton in aged oocytes shows features which are normally observed in the fertilized oocytes: the actin-rich layer overlying the meiotic spindle disappears during the post-ovulatory ageing period and this could be the cause of abnormal abstriction of second polar bodies (Webb et al., 1986). In 30% of oocytes aged for 12 hrs the cortical actin shows an uniform distribution along the plasma membrane (Webb et al., 1986). The disappearance of the actin rich region is followed by the migration of the meiotic spindle towards the center of the oocyte 14-18 hrs after ovulation (Fig 1b, Szollozi et al., 1972; Webb, 1986). Furthermore, cytoplasmic asters of microtubules can be visualized with immunofluorescence in oocytes aged post-ovulation for 12 hrs (Eichenlaub-Ritter, 1986). In fertilized unaged oocytes these asters of microtubules appear 6-12 hrs after sperm penetration (Maro, 1985; Schatten et al., 1985); apparently the formation of asters is independent of, but accelerated by, fertilization. Eichenlaub-Ritter (1986) postulated that these premature alterations of the cytoskeleton enables the aged oocytes to catch up with the normal developmental schedule. Evidence of an accelerated early embryonic development has been found in mice (Fraser, 1979; Smith and Lodge, 1987; the present study) and in the rat (Shalgi, 1985).

PROCEDURES FOR TIMING OF OVULATION AND FERTILIZATION

The timing of fertilization related events is, by definition, controlled by the fertilization programme and can be related to (1) the time of mating in the case of spontaneous ovulation (Abramczuk and Sawicki, 1975; Schrabonath, 1988), (2) the moment of human chorionic gonadotrophin (hCG) administration in a superovulation procedure (Luthardt and Donahue, 1973) or (3) the moment of mixing of gametes *in vitro* fertilization experiments (Howlett and Bolton, 1985). Each of these different experimental designs have provided time schedules for the sequence of fertilization events. However, the timing of fertilization events and the duration of the cell cycle phases appear to vary with the experimental designs (Table 1). The observed differences in timing can partly be explained by interstrain variations. The length of the zygotic DNA synthetic phase appears to be dependent on the paternal genotype (Schrabonath, 1988).

In many *in vivo* experiments the moment of ovulation is related to the midpoint or the end of the dark period. However, the interval between the midpoint of the dark period and ovulation is dependent on the length of the dark period and varies between mouse strains (Braden 1957; Bingel and Schwartz, 1969). Therefore, the moment of ovulation should be determined for each mouse strain used in investigations concerning the timing of fertilization events. There are also strain differences in the transport of spermatozoa and as a consequence differences in the moment of sperm penetration (Nicol and McLaren, 1974).

Superovulation (Fowler and Edwards, 1957) is another widely used method for the induction and timing of ovulation. Administration of pregnant mare serum gonadotrophin (PMSG) induces follicular growth in mature and immature female mice, and a subsequent injection of human chorionic gonadotrophin (hCG) triggers preovulatory maturation and ovulation (Fowler and Edwards, 1957; Marston and Chang, 1964). The release of oocytes is related to the moment of the hCG administration, so ovulation can be timed within a few hours. However, the accuracy in estimating the moment of ovulation is dependent upon age and strain of the females: ovulation occurs 10-12 hrs and 10-14 hrs after the hCG injection in mature and in immature Swiss females respectively (Marston and Chang, 1964). In CF_1 female mice ovulation occurs between 10 and 14 hrs after hCG adminstration (Donahue, 1972). Also, the number of oocytes released after superovulation differs between strains (Fowler and Edwards, 1957).

Despite the advantages of superovulation, the procedure is a target for

criticism. Abnormal embryonic development has been described after superovulation (Foote and Ellington, 1988): the incidence of pre-implantation embryonic death has been shown to be increased to 44% in the LACA stock after *in vivo* fertilization of superovulated oocytes (Beaumont and Smith, 1975); abnormal development *in vivo* and increased sister chromatid exchange in embryos from superovulated oocytes have been described (Swiss females: Elbling and Colot, 1985); the incidence of polyploidy, which can be ascribed to the failure of second polar body extrusion, has found to be increased in superovulated fertilized oocytes from A/HE females (Takagi and Sasaki, 1976). In sheep, follicle maturation by PMSG induces a pattern of protein synthesis in the oocytes that is normally seen in luteinizing hormone (LH) activated oocytes. This phenomenon is called premature activation and leads to aged and morphological abnormal oocytes (Moor et al., 1985). Moreover, administration of hCG at pro-oestrus of the oestrus cycle has been shown to increase pre-implantation embryonic death in the rat (Mattheij et al, 1986 and 1987) and in Swiss-random bred female mice (De Boer et al., submitted). Administration of luteinizing hormone releasing hormone (LHRH) at pro-oestrus has no deleterious effect on embryonic development, however (Mattheij et al., 1986 and de Boer et al., submitted). These observations made us decide to time ovulation by the administration of LHRH at pro-oestrus of the cycle. Pro-oestrus was simply determined by evaluation of vaginal smears taken daily (Thung et al., 1956).

The age of embryos derived after *in vitro* fertilization is related to the moment of mixing female and male gametes and therefore timing of fertilization is precise after *in vitro* fertilization. However, embryos grown *in vitro* can exhibit retarded development because of sub-optimal culture conditions (Bavister, 1987). Although the quality of culture media for *in vitro* pre-implantation development is improving, they will nevertheless never mimic the oviductal environment completely.

It will be clear that an exact timing of fertilization events is impossible and can only be approached in comparative studies using one stock or strain of mice and the same set of experimental conditions. In the present study we used LHRH to time ovulation and artificial insemination to time fertilization. The maternal germ cells derived from Swiss random-bred females and Swiss x LIII males were sperm donors.

TABLE 1: Length (hrs) of cell cycle phases of the first embryonic cell cycle in the mouse as estimated by different authors.

Authors plus genotype	G_1*	S	G_2+M	time of cleavage (50% point)
Natural mating				
Schabronath (1988)				
(AKR/NHAN)	4	6.4	8.5	19.1 p.c.
(CBA/JHAN)	6	11.1	5	21.1 p.c.
Molls et al. (1983)				
NMRI		5	5	
Krishna and Generoso (1977)				
(C3HxC57BL)F_1 females x				
(SEC x C57BL)F_1 males	8	7-8	1-2	18.0 p.c.
Superovulation				
Donahue (1972)				
CF1 males and females				32-34**
Luthardt and Donahue (1973)				
CF1 males and females		4		
Abramczuk and Sawicki (1975)				
Swiss	4	3.5-4.0		
<u>in vitro</u> fertilization after natural ovulation				
Kaufman (1973),				
CFLP x CFLP			2	31.5**
(C57BLxA^2G)F_1 x (C57BLxA^2G)F_1			1h 39m	29.8**
<u>in vitro</u> fertilization after superovulation				
Howlett and Bolton (1985)				
(C57BL.10 x CBA)F_1	11	6	4	18-22***

Footnote: *includes completion of meiosis II; **post hCG; ***post-insemination; p.c. post-conception.

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CHAPTER II.

THE FIRST CELL CYCLE OF ZYGOTES OF THE MOUSE DERIVED FROM OOCYTES AGED POST-OVULATION *IN VIVO* AND FERTILIZED *IN VIVO*

M.L. Boerjan and P. de Boer,
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SUMMARY

This paper describes an analysis of the first cell cycle of mouse oocytes aged post-ovulation and fertilized *in vivo*. For this purpose we developed a procedure for inducing ovulation *in vivo* that allows accurate timing of ovulation. The method is based on a luteinizing hormone releasing hormone (LHRH) administration at pro-oestrus. This ovulation procedure had no detectable effect on the rate of ovulation or post-implantation embryonic death.

We used this method of ovulation induction in an analysis of the separate stages of the first cell cycle of *in vivo* fertilized post-ovulation aged oocytes. All stages assessed were shorter in aged oocytes (12 hrs post-ovulation) than in zygotes from unaged oocytes (1 hr post-ovulation): (1) the time interval between insemination and penetration of the aged oocytes was 1 hr 30 min shorter than the time interval of the unaged oocytes; (2) pronuclear formation in the fertilized aged oocytes was somewhat quicker than pronuclear formation in fertilized unaged oocytes; (3) in zygotes from aged oocytes the time between formation of pronuclei and the pronuclear membrane breakdown was 1 hr shorter than in zygotes from unaged oocytes; (4) the first cleavage division was 3 hrs advanced in zygotes from aged oocytes compared with the moment of the first cleavage division in zygotes from unaged oocytes.

We also determined the glutathione (GSH) content of unaged and aged oocytes to investigate a possible relationship between the rate of pronuclear formation and GSH. The level of GSH was two times lower in oocytes aged post-ovulation for 12 hrs than in unaged oocytes. The level of GSH in fertilized unaged oocytes was half that in unfertilized unaged oocytes; this decline was not observed after fertilization of aged oocytes, however.

In summary, we developed a procedure that allows *in vivo* fertilization at defined points of time after LHRH-induced ovulation. Under the conditions used in this procedure we could detect several differences between zygotes derived from unaged and post-ovulation aged oocytes.

Key words:ageing, glutathione, luteinizing hormone releasing hormone.

INTRODUCTION

The first cell cycle of mammalian embryonic development can be divided in three distinct phases: (1) formation of the female and male pronuclei, (2) DNA synthesis, (3) onset of mitosis and first cleavage. The analysis of the first cell cycle after post-ovulatory ageing could provide a tool

to study the regulation of the first cell cycle, because in mice the duration of the first embryonic cell cycle is influenced by the post-ovulatory age of the oocyte (Fraser, 1979). Little is known about the regulation of first cell cycle phases, although the synthesis of cell cycle dependent proteins in the mouse zygote has been described (Howlett and Bolton, 1985).

Several authors have reported that oocytes aged *in vivo* for 4-7 hrs and fertilized *in vitro* have a shorter first cell cycle than zygotes derived from unaged oocytes (Shalgi et al.,1985; Smith and Lodge, 1987). A detailed study of the first cell cycle of oocytes aged and fertilized *in vivo* has not been published, however. The aim of this investigation was to analyze in detail the first cell cycle in zygotes derived from oocytes that were aged post-ovulation *in vivo* and fertilized *in vivo*. The main problem to be solved was the exact determination of the moment of ovulation. One way to circumvent this problem is activation of follicles by pregnant mare's serum gonadotrophin (PMSG), followed by induction of ovulation by human chorionic gonadotrophin (hCG) (Fowler and Edwards, 1957); the age of the oocytes can then be referred to the moment of hCG administration. However, this superovulation procedure may have detrimental effects on embryonic development (Foote and Ellington, 1988). We therefore tested an alternative procedure of ovulation induction, namely injection of luteinizing hormone releasing hormone (LHRH) shortly before the expected endogenous luteinizing hormone (LH) surge. In this study fertilization was precisely timed by means of artificial insemination. We did not find a detectable effect of these ovulation and fertilization conditions on the rate of ovulation or post-implantation embryonic death.

We subsequently used this system in an investigation of some aspects of the first cell cycle of oocytes that were aged and fertilized *in vivo*, namely (1) the rate of sperm penetration, (2) the rate of pronucleus formation, (3) the rate of pronuclear membrane breakdown, and (4) the moment of the first cleavage.

We also measured the glutathione (GSH) content of unaged and post-ovulation aged oocytes, before and after fertilization, because it has been suggested that pronuclear development is dependent on the level of GSH in the oocyte (Perreault et al., 1988; Calvin et al., 1986).

In summary, we developed a procedure that allows *in vivo* fertilization at defined intervals of time after LHRH induced ovulation. This procedure gave us the opportunity to study some aspects of the first cell cycle in

oocytes aged post-ovulation for 12 hrs and fertilized *in vivo*. We could detect several differences between zygotes from unaged and aged oocytes.

MATERIAL AND METHODS

Animals and housing conditions

Females were random bred Swiss (CpbSE(S)) mice, and males were F_1(CpbSE(S) x LIII), with LIII being a linkage testing stock from the MRC Radiobiology Unit, Chilton, U.K. Throughout all experiments the mice were kept in two air-conditioned rooms (20 °C, 60% relative humidity) with a dark/light schedule of 4 hrs dark and 20 hrs light. In one of the two animal rooms the dark period coincided with the natural night, in the other room the dark period was 11 hours later. In each cage we kept three females and one male; the male was separated from the females.

Determination of post-implantation embryonic death

The influence of the 4 hrs dark/20 hrs light schedule on ovulation rate and on post-implantation embryonic death was determined as follows: females that were adapted to the dark-light schedule for at least 3 weeks were mated to F_1 males. The first day of pregnancy was the day a vaginal plug was found; on day 13 of pregnancy the number of decidua and the number of moles were determined. The percentage of post-implantation embryonic death is expressed as: (no. of moles)/(no. of decidua)*100. We also analyzed the effect of LHRH induced ovulation on post-implantation embryonic death: LHRH (Sigma no: L7134, 200 ng/ female) was administered 14 hrs before the expected LH peak. Females were mated to F_1 males after the LHRH injection and post-implantation embryonic death was determined on day 13 of pregnancy.

Determination of natural and induced ovulation

Vaginal smears were taken daily. In the animal room with the dark period during the day the smears were taken between 8:00 - 9:00 hrs A.M. in the other room we took smears between 5:00 - 6:00 hrs P.M. The smears were stained and classified according to Thung et al. (1956).

Mice with at least two consecutive regular 4-day cycles were selected by inspection of the vaginal smears. Pro-oestrus females were killed at appropriate intervals after the midpoint of the dark period, and the numbers of oocytes in the ampullae were counted.

LHRH (200 ng/female) was injected intraperitoneally 8-12 hrs before the expected LH peak which occurs in mice 12 hrs before the moment of ovulation (Runner and Palm, 1953). Females were killed at appropriate time points after the injection and the number of secondary oocytes in the ampullae was counted.

Artificial insemination (AI)

Fertilization was performed by means of artificial insemination (West et al., 1977): the cauda epididymis of an F_1 male were cut into 3-4 pieces and sperm was allowed to disperse in 0.3 ml Dulbeccos medium (Dulbecco and Vogt, 1954) supplemented with 3 mg/ml bovine serum albumin (BSA, BDH, no. 44004), during 15 min at 35 °C. The sperm suspension was then carefully sucked off. Only those sperm suspensions with at least 40.10^6 cells/ml and more than 70% moving and normal looking spermatozoa were used for insemination, 50 μl of the sperm suspension was brought into the uterus via the cervix with a blunt 21 gauge injection needle. The females were not anaesthetized before the insemination. The sperm suspension from one male was sufficient for the insemination of 4 females of which two carried unaged oocytes and two had aged oocytes.

The females were inseminated at 13 hrs (1 hr post-ovulation) and 24 hrs (12 hrs post-ovulation) after the LHRH injection. In these experiments LHRH was injected 8-12 hrs before the expected LH peak.

Light microscopic analysis of zygotes

The cumulus-oocyte complexes were released from the ampullae at appropriate intervals after insemination. Cumulus cells were removed by incubation in 300 U/ml hyaluronidase (Sigma, type I-S bovine testis, no. H 3506)in Dulbeccos medium. Subsequently the oocytes were washed in Dulbeccos medium and fixed in Heidenhain fixative (2 parts $HgCl_2$ sat., 2 parts H_2O and one part formaldehyde 40%) for at least 15 min. After fixation we transferred the oocytes to a microscope slide and carefully covered them with a cover glass provided with vaseline walls without squashing the cells. After a post-fixation in Carnoy's fixative for 15 min the cells were stained with aceto-orcein (0.75% in 45% acetic acid).

We defined oocytes as fertilized when a sperm head had penetrated the vitellus. Pronucleus formation was considered to be complete when the nuclear membrane could be clearly observed in the aceto-orcein stained cells. Due to the fixation with $HgCl_2$ the nucleoli were obvious and their development could be evaluated.

For aged as well as unaged oocytes, the median interval between AI and pronuclear formation, pronuclear breakdown and first cleavage was determined.

Determination of GSH in secondary oocytes and zygotes

We determined the level of glutathione (GSH, L-glutamyl-L-cysteinylglycine) in groups of oocytes or zygotes by means of a fluorometric procedure (Hissin and Hilf, 1976) as modified by Rietjens et al. (1985).

We disrupted cumulus free oocytes or zygotes collected from one female by suspending them in 30 μl PE-buffer (0.1 M sodium phosphate buffer, 5 mM EDTA, pH 8.0), and freezing them in liquid N_2. Subsequently, we thawed the samples and quickly added 30 μl o-Phthaldialdehyde (Sigma, no. P-1378) solution (1 mg/ml in methanol) and 540 μl PE-buffer. Twenty minutes after mixing we measured the fluorescence at 412 nm (excitation at 335 nm). Stock solutions of GSH (Merck no. 4090) (5-50 ng/ml) were used as a reference.

RESULTS

Effect of the ovulation induction procedure on post-implantation embryonic death

The mice used in this investigation were maintained in a 4 hrs dark/20 hrs light schedule, because there appears to be less variation in the moment of ovulation among females maintained in a 4 hrs dark/20 hrs light cycle than among females maintained in a 10 hrs dark/14 hrs light cycle (Braden, 1957; Bingel and Schwartz, 1969). We examined whether this dark/light schedule had any harmful effects on the number of ovulated oocytes or on post-implantation embryonic death (Table 1). The number of ovulated oocytes was similar in all four experimental groups (Table 1, $P>0.05$, Student's t test among paired groups). There appears to be a slight effect of the 4 hrs dark/ 20 hrs light schedule on the mean and

variability of percentage post-implantation embryonic death, but this is not statistically significant (Table 1, P>0.05 Mann-Whitney U test; group 1 vs group 4; group 3 vs group 4). The embryonic death in females kept on a schedule with the dark period coinciding with the natural night did not differ significantly from that in females which were maintained in a schedule with the dark period coinciding with day time (Table 1; Mann-Whitney U test, P>0.05, group 2 vs. group 3).

TABLE 1. The effects of LHRH administration and of a short (D)ark (4hrs)/long (L)ight (20hrs) regime on post-implantation development.

treatment	total no. of females	total no. of decidua mean ± s.d.	post-implantation death (%) [1] mean ± s.d.
1. LHRH induced, 4 hrs D(day)/20 hrs L[3]	46	9.8 ± 2.3	19.6 ± 23.9
2. 4 hrs D(night)/ 20 hrs L [2]	74	10.9 ± 2.2	12.4 ± 16.9
3. 4 hrs D(day)/ 20 hrs L [3]	19	10.3 ± 3.1	15.0 ± 22.6
4. Control 10 hrs D (night)/14 hrs L	20	10.8 ± 2.3	9.0 ± 12.7

Footnote: 1) death (%)=no. of moles/decidua * 100.
2) 4 hrs D(night)/20 hrs light: midpoint dark period 1:00 AM
3) 4 hrs D(day)/20 hrs light: midpoint dark period 2:00 PM

We induced ovulation by an LHRH injection 14 hrs before the expected endogenous LH-peak; follicle disruption was therefore advanced and the resulted mating frequency was 65% instead of 100%. This advanced ovulation caused no significant increase in pre-implantation or post-implantation death (Table 1; P>0.05 Mann-Whitney U test;group 1 vs. group 3).

Ovulation in spontaneously ovulating females

The day of ovulation was determined by inspection of vaginal smears, taken before the midpoint of the dark period. Females with a pro-oestrus vaginal smear (see materials and methods) were killed at appropriate intervals after the midpoint of the dark period and the numbers of oocytes

in the ampullae were counted (Table 2). The mice that had not ovulated at the time of observation showed big swollen follicles. Two out of seven females had ovulated at 3 hrs 45 min after the midpoint of the dark period; at 5 hrs 15 min all females had ovulated. Thus ovulation essentially occurs between 3 hrs 30 min and 5 hrs after the midpoint of the dark period. Because in mice ovulation occurs about 12 hrs after the LH peak (Runner and Palm, 1953) we conclude that in our experimental system the endogenous LH surge occurs 7-8 hrs before the midpoint of the dark period. Within a female, follicles appear to ovulate almost simultaneously; we never found swollen follicles and cumulus-oocyte complexes on the ovaries of the same female (Table 2).

TABLE 2: Rate of natural and LHRH-induced ovulation.

	no. of			
	females	eggs in ampullae of ovulated females mean/female ± s.d.	mice with eggs in the ampullae	eggs on the ovaries mean/females ± s.d. (range)
a) natural ovulation				
time (hrs) after midpoint of the dark period				
3h 15 - 3h 45 min	4	0	0	0
3h 45 - 4h	7	11.5 ± 3.5	2	0
4h - 4h 15	6	12.8 ± 1.1	5	0
4h 15 - 4h 45	5	9.7 ± 3.0	4	0
4h 45 - 5h 15	5	9.8 ± 3.6	5	0
b) LHRH-induced ovulation				
time (hrs) after LHRH injection				
11	3	0	0	0.3 ± 0.6 (0-1)
12	8	8.3 ± 2.3	7	0.6 ± 0.7 (0-2)
13	13	8.8 ± 2.1	13	0
15-32	49	9.3 ± 2.3	49	0

Ovulation induced by LHRH

The experiment described in Table 2b was carried out to assess whether ovulation induced with LHRH had any influence on the rate of ovulation. In this experiment we administered LHRH 8-12 hrs before the expected endogenous LH peak, so that the time point of induced ovulation was slightly earlier than that of the expected natural ovulation. Eleven hrs after the LHRH injection we did not find any cumulus-oocyte complexes in the ampul-

lae, but in one female we did find a cumulus-oocyte complex on one of the ovaries (Table 2b). All other females ovulated between 11 and 13 hrs after the LHRH injection (Table 2). Thus, the time between the LHRH-induced LH peak and ovulation is the same as that between the endogenous LH peak and ovulation, namely about 12 hrs (Table 2b; Runner and Palm, 1953). The number of ovulated oocytes per female after LHRH induction does not differ significantly from the number of naturally ovulated oocytes per female (Table 2b, $P>0.05$ Student's t test). In this paper we refer the age of the oocyte to the moment of the LHRH injection: in the experiments described below the unaged oocytes were collected at 13 hrs after the LHRH injection and the aged oocytes were collected at 24 hrs after the injection. This corresponds to 1 hr and 12 hrs after ovulation, respectively.

TABLE 3: The effect of the mode of ovulation and insemination on the fertilization rate of unaged oocytes[1].

	females	mean no. of of eggs/female ± s.d.	mean no. of fertilized eggs/ female ± s.d.	fertilization rate (%) ± s.d.
natural ovulation				
natural mating	10	9.4 ± 2.2	8.6 ± 3.1	87.8 ± 19.7
artificial insemination	10	9.8 ± 2.3	9.5 ± 2.6	95.6 ± 8.2
induced ovulation				
natural mating	8	10.1 ± 1.6	9.7 ± 1.7	96.4 ± 7.3
artificial insemination	10	9.5 ± 2.6	8.4 ± 3.6	85.4 ± 29.1

Footnote: 1) The rate of fertilization was determined 15 hrs after artificial insemination

Natural mating vs. AI

The rate of fertilization of naturally ovulated or LHRH induced unaged oocytes was not affected by AI (Table 3; $P>0.05$, Mann-Whitney U test).

Secondary oocytes could be fertilized until 28 hrs after the LHRH injection, although the morphology of the resulting zygotes deteriorated and the number of dispermic zygotes increased if AI was delayed until 25 hrs after the LHRH injection (Table 4, Fig. 1c). In most of the abnormal one-cell embryos there were cytoplasmic fragments adjacent to an enlarged second polar body, whereas the pronuclear membranes were less visible in light microscopic preparations (Fig. 1c,d). In addition, the fraction of unfertilized oocytes with an abnormal meiotic spindle increased with

TABLE 4: The efficiency of fertilization of freshly ovulated and aged oocytes[1].

	no. of			percent zygotes with an abnormal abstriction second polar body	percent dispermic zygotes
	females	eggs/female (± s.d.)	fertilization (%)		
13 (freshly ovulated	7	7.6 ± 2.5	77	1.9	0.0
24 (aged)	4	10.7 ± 1.5	100	2.3	2.3
25 (aged)	4	9.5 ± 1.3	87	69.7	3.3
26 (aged)	4	9.0 ± 1.4	42	66.6	6.7
27 (aged)	4	10.0 ± 2.8	53	52.4	9.5
28 (aged)	2	9.5 ± 0.7	84	62.5	0.0

Footnote: 1) The rate of fertilization was determined 6 hrs after artificial insemination.

TABLE 5: Duration of the subsequent stages of the first cell cycle of zygotes, derived from aged and unaged oocytes.

	UNAGED			AGED		
event	time of observation hrs after AI	n	50% time point	time of observation hrs after AI	n	50% time point
sperm penetration (t_1) pronucleus formation	2-4	148	3 hrs 15 min	1.5-4	176	1 hrs 45 min
female and male (t_2)	3-7	173	5 hrs 15 min	3-6	167	3 hrs 15 min
pronucleus breakdown (t_3)	15-22	165	17 hrs 30 min	12-19	193	14 hrs 30 min
first cleavage (t_4)	15-22	165	20 hrs 30 min	12-19	193	17 hrs 30 min

longer post-ovulatory ageing periods (not shown).

Effect of <u>in vivo</u> ageing on sperm penetration, pronucleus formation and first cleavage

After artificial insemination, unaged oocytes (Table 5 and Fig. 2, t=13) were penetrated later than oocytes that were aged post-ovulatory for 12 hrs (Table 5 and Fig. 2, t=24). At 3 hrs 15 min after AI 50% of the unaged oocytes were fertilized (Table 5 and Fig. 2, t_1). For the aged oocytes this 50% time point was 1 hr 45 min after AI (Table 5 and Fig. 2, t_1). The difference in the rate of penetration between the two experimental groups was statistically significant as tested with a Chi-square test on independence ($P< 0.001$).

Pronuclei were found in 50% of the fertilized unaged oocytes at 5 hrs 15 min after AI: for the post-ovulatory aged oocytes this 50% time point was at 3 hrs 15 min after insemination (Table 5 and Fig. 2, t_2). The difference in 50% time points between zygotes from unaged and aged oocytes is statistically significant ($P<0.001$, Chi-square test). The time interval between penetration and pronucleus formation (t_2-t_1) was 30 min shorter in fertilized aged oocytes than in fertilized unaged oocytes. No difference was observed in the rate of female or male pronucleus formation in both types of zygotes.

In female as well as male pronuclei the number of nucleolus-like particles per nucleus decreases with time (Table 6 and Fig. 3). Most of the younger zygotes had pronuclei with several small nucleolus-like particles (Table 6 and Fig. 3) whereas the number of nucleolus-like particles was lower in more developed pronuclei. This phenomenon occurred in fertilized aged and unaged oocytes. Taking into account the time difference of fertilization between unaged and aged oocytes, we observed a rate of "nucleoli fusion" which was comparable for both groups of zygotes. However, in zygotes from aged oocytes the fusion of the nucleolus-like particles was 1 hrs later than in zygotes from unaged oocytes with respect to pronucleus membrane formation (Table 5, t_2; Table 6).

TABLE 6: Development of pronuclear nucleoli in zygotes derived from unaged (a) and aged (b) oocytes.

Hours after AI	no. of zygotes	no. of nucleolus-like particles in the female pronucleus (n) 1<n<10 (%)	n≥10 (%)	particles in the male pronucleus (n) 1<n<10 (%)	n≥10 (%)
a) zygotes from unaged oocytes					
5	13	38	62	22	78
6	27	78	22	74	26
7	39	82	18	62	38
b) zygotes from aged oocytes					
3	11	27	73	27	73
4	24	33	67	28	72
5	33	76	24	72	28

The time between formation of pronuclei (t_2) and the pronuclear membrane breakdown in 1-cell embryos (t_3) from aged oocytes was 11 hrs 15 min (Table 5, Fig. 2, t_3), whereas in the 1-cell embryos from unaged oocytes the pronuclear stage was 12 hrs 15 min ($P<0.001$, Chi-square test).

Seventeen hours and 30 min after AI 50% of the fertilized aged oocytes were in the 2-cell stage (Table 5 and Fig. 2, t_4), for the fertilized unaged oocytes this 50% time point was 20 hrs 30 min (Table 5 and Fig.2, t_4).

The glutathione content of aged and unaged oocytes

We determined the level of glutathione (GSH) in unfertilized unaged oocytes and oocytes that were aged post-ovulation *in vivo* for 12 hrs. In the unfertilized unaged oocytes the concentration of GSH was 21 mM, which is two times the highest concentration found in somatic cells (Meister and Anderson, 1983). In the fertilized unaged oocytes the GSH concentration was only 9 mM (Table 7, $P< 0.05$ Student's t test). We found no difference in GSH content between fertilized unaged oocytes at the start of the first cell cycle, i.e. 7 hrs after AI, and fertilized unaged oocytes which were collected shortly before pronuclear membrane breakdown (16 hrs after AI).

TABLE 7: GSH concentration in unfertilized and fertilized unaged oocytes in and oocytes that were aged post-ovulatory for 12 hrs[1].

	unaged			aged		
	no. of females	ng/oocyte ± s.d.	mM[4]	no. of females	ng/oocyte ± s.d.	mM[4]
Unfertilized	6	2.3 ± 1.0[3]	21	6	1.1 ± 0.5	9
Fertilized, hours after artificial insemination						
5[2]				7	0.9 ± 0.2	8
7[2]	7	1.0 ± 0.2	9			
13[2]				7	0.9 ± 0.3	7
16[2]	11	0.9 ± 0.4	8			

Footnotes: 1) The oocytes were pooled per female.
2) The zygotes were collected at phases of the first cell cycle that were comparable for the "unaged" and "aged" zygotes (Fig. 3).
3) Compared with the other experimental groups $P<0.05$.
4) For the calculation of the molarity we used a volume of 0.36 nl/oocyte, based on a mean radius of 44 μm/oocyte (Fig. 1)

In the unfertilized aged oocytes the GSH concentration was half of that in the unfertilized unaged oocytes (Table 7, $P<0.05$ Student's t-test). The aged oocytes demonstrated no further decline in GSH content after fertilization (Table 7, $P>0.05$ Student's t-test).

In summary, the concentration of GSH in unfertilized unaged oocytes was higher than that of unfertilized *in vivo* aged oocytes. In fertilized aged oocytes the level of GSH was similar to that in fertilized unaged oocytes.

DISCUSSION

Delayed mating or insemination results in an increased embryonic mortality in mice (Sakai and Endo, 1988; Marston and Chang, 1964), rats (Blandau and Jordan, 1941), rabbits (Adams and Chang, 1962), pigs (Hunter, 1967), hamsters (Juetten and Bavister, 1983) and man (Bomsel-Helmreich, 1976). This embryonic loss might be a result of chromosomal anomalies that could arise after delayed fertilization (Vickers, 1969; Shaver and Carr, 1967). Another factor involved in this increased embryonic mortality might be an asynchrony, in zygotes from aged oocytes, between cytoplasmic functions on the one hand and the rate of the second meiotic division or first cleavage on the other hand. Several authors reported that, *in vitro*, the first cell cycle is shorter in mouse zygotes derived from aged oocytes than in those derived from unaged oocytes (Fraser, 1979; Smith and Lodge, 1987). Many *in vivo* aged and *in vitro* fertilized hamster oocytes fail to extrude the second polar body (Juetten and Bavister, 1983). The extrapolation of these observations to the effects of *in vivo* fertilization of *in vivo* aged oocytes is not unambiguous; mouse oocytes aged post-ovulation *in vivo* for 12 hrs are activated instead of being penetrated in *in vitro* fertilization experiments (Kaufman, 1973), whereas *in vivo* these oocytes are readily penetrated (this study and Marston and Chang, 1964). Moreover, the possible interactions between oviduct and the oocyte are not implicated in the above mentioned *in vitro* studies. Futhermore, the oocytes that were used for the above cited studies were obtained by superovulation, induced with PMSG and hCG. Thus obtained oocytes might differ from naturally ovulated oocytes (Foote and Ellington, 1988).

In this study we have tried to complement the *in vitro* investigations with an *in vivo* analysis of the effects of post-ovulatory ageing of oocytes on the first embryonic cell cycle. For this purpose, we developed

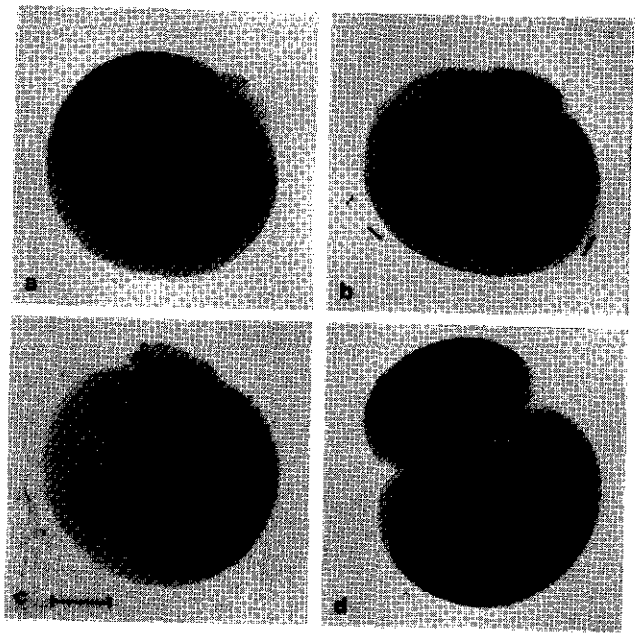

Figure 1. Light micrographs of unaged and postovulation aged ooyctes fertilized in vivo. Zygotes were stained with aceto-orcein after fixation in Häidenheins fixative as described in materials and methods. Bar represents 22 μm.

(a) Fertilized unaged oocyte collected 20 hrs after artificial insemination (AI). Pronuclear membranes and nucleoli are clearly visible in this zygote.

(b) Fertilized aged oocyte; females were inseminated 24 hrs after the LHRH injection. The zygote was collected 15 hrs after AI. Pronuclear membranes are visible in this zygote derived from an oocyte aged postovulation for 12 hrs.

(c,d) Fertilized aged oocytes; females were inseminated 28 hrs after the LHRH injection. The zygote was collected 6 hrs after AI. The pronuclear membranes are very vague in this zygote from oocytes aged postovulation for 16 hrs.

(d) An example of the abstriction of an abnormal large second polar body in a zygote derived from an oocyte aged postovulation for 16 hrs.

a procedure that allows a precise timing of ovulation as well as *in vivo* fertilization, and that has minimal effects on embryonic development. We chose to induce ovulation by injection of LHRH at proestrus; thus the endogeneous LH-surge was advanced by about 8-12 hrs. Fertilization could be precisely timed by means of AI. These procedures mimic more closely the natural processes of ovulation and fertilization than commonly used methods of superovulation by PMSG and hCG and *in vitro* fertilization. Our procedure has no detectable effect on the rate of ovulation or post-implantation embryonic death (Table 1). In contrast, superovulation is correlated with a higher embryonic mortality in the rat (Sherman et al., 1982) and mouse (Beaumont and Smith, 1975). Furthermore, ovulation induced by hCG is correlated with embryonic death in mice (de Boer et al., in

preparation) as well as rats (Mattheij et al., 1986). The observed increase in variability in percentage of post-implantation embryonic death among females, kept in a short dark/long light regime, could probably be ascribed to a genetical variation in the outbred Swiss strain used throughout this study.

With this *in vivo* system we analyzed the effects of ageing of the oocyte on the following parameters of the first embryonic cell cycle: (1) the rate of sperm penetation, (2) the rate of pronucleus formation, (3) the rate of pronucleus membrane breakdown and (4) the moment of first cleavage (Fig. 2). All these stages were advanced in zygotes derived from aged oocytes; this is particularly striking with respect to the rate of sperm penetration (Fig. 2, t_1). Two possible explanations can be considered with respect to this effect. First, post-ovulation changes in the composition of the oviductal fluid may influence sperm transport and capacitation of spermatozoa (Nieder and Corder, 1983; Hunter, 1987; Fraser and Ahuja, 1988). Or second, post-ovulation changes of the cumulus, zona pellucida or

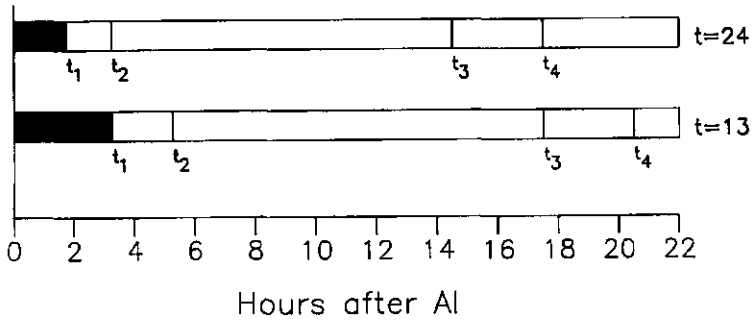

Figure 2. Time intervals between artificial insemination (AI) and the 50% time points of the seperate stages of the first cell cycle in zygotes derived from unaged oocytes (t=13), and from oocytes aged postovulatory for 12 hr (t=24).
t_1: Interval between AI and penetration of 50% of the secondary oocytes.
t_2: Interval between AI and pronuclear formation of 50% of the zygotes.
t_3: Interval between AI and pronuclear breakdown of 50% of the zygotes.
t_4: Interval between AI and first cleavage of 50% of the zygotes.

plasmalemma of the oocytes could result in an altered rate of sperm penetration. The observation (Fraser, 1979) of more rapid penetration of sperm into aged oocytes *in vitro*, where sperm transport and capacitation would not be a factor, is consistent with this. We think it unlikely that changes in sperm transport are responsible for the difference in the rate of sperm penetration, because sperm are found in the oviduct within 15 min after mating (Blandau, 1973). It also seems unlikely that changes in the cumulus cause a more rapid penetration of sperm, because the cumulus of unaged oocytes appears to stimulate sperm penetration in mouse (Wolf et al., 1977) and hamster (Bavister, 1982). On the other hand, the cumulus of aged oocytes appears thinner than that of unaged oocytes, and one might argue that sperm meet less resistance while passing an aged cumulus. It is also possible that post-ovulation changes in the zona pellucida, plasma membrane and cytocortex caused an altered rate of sperm penetration: several authors have reported that such changes do occur. For instance, Gaunt (1985) decribes the post-ovulation accumulation in the oocyte membrane of an oviductal protein and Longo (1981) observed a more uniform distribution of lectin-binding sites on the plasma membrane. Also, a post-ovulatory reorganization of the cytocortex has been described (Webb et al., 1986). However, none of these alterations have been associated with an altered rate of sperm penetration.

An other effect of *in vivo* post-ovulatory ageing of oocytes is the more rapid formation of pronuclei after fertilization (Fig. 2; t_2). This has also been observed after *in vitro* fertilization of oocytes aged *in vivo* for 4-7 hrs (Fraser, 1979; Smith and Lodge, 1987). In the young maternal and paternal pronuclei several small nucleolus-like bodies could be observed, more developed pronuclei contained one or a few larger nucleoli. The replacement of several smaller nucleolus-like particles by a single large nucleolus has also been described by Fraser (1979). The nature of these nucleolus-like particles is not clear, however.

There is some evidence that GSH is involved in the reduction and thereby decondensation of sperm protamine (Perreault et al., 1984, 1988; Calvin et al. 1986). The observation that the GSH concentration decreases after fertilization is in agreement with this hypothesis (Perreault et al. 1988, and Table 7). The more rapid formation of pronuclei in zygotes from aged oocytes could then be due to a higher concentration of GSH in these cells. However, we measured a lower concentration of GSH in unfertilized aged oocytes than in unfertilized unaged oocytes (Table 7). Moreover, there is

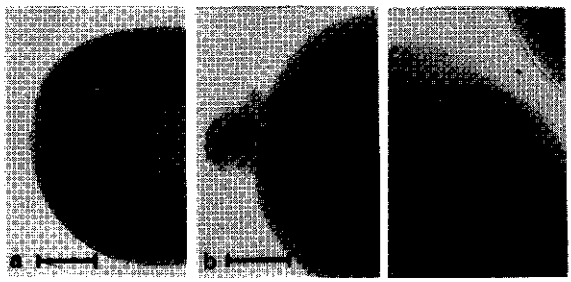

Figure 3. Fusion of nucleolus-like particles in zygotes from aged oocytes.
(a,b) Male (a) and female (b) pronucleus in the same zygote derived from an aged oocytes. The zygote was collected at 3 hrs after artificial insemination (AI), many nucleolus-like particles are visible in the early zygote.
(c) Female pronucleus in a zygote collected at 4 hrs after AI. In this more advanced zygote the nucleolus-like particles are replaced by one distinct nucleolus. (Bar represents 22 μm).

hardly a detectable decrease of GSH after fertilization of aged oocytes (Table 7). Thus it is doubtful that GSH plays a major role in sperm chromatin decondensation in at least aged oocytes. In aged oocytes, either relatively small amounts of GSH are involved or additional factors, for instance synthesis of GSH, play a role.

In summary, we have developed a procedure that allows *in vivo* ovulation induction and fertilization at defined points of time. This procedure provides us with a tool to compare the earliest stages of development *in vivo* of zygotes derived from aged and unaged oocytes. By such a comparison we hope to obtain more insight in the cellular functions that are important for early embryonic development.

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CHAPTER III

ASYNCHRONY BETWEEN PRONUCLEAR DEVELOPMENT AND PROTEIN SYNTHETIC CHANGES IN ZYGOTES OF THE MOUSE DERIVED FROM OOCYTES AGED POST-OVULATION *IN VIVO* AND FERTILIZED *IN VIVO*

M.L. Boerjan, C.W. op het Veld and M.B.H. Koopman

SUMMARY

The pattern of proteins synthesized by 1-cell embryos derived from unaged oocytes and oocytes aged post-ovulation *in vivo* was analyzed by means of ^{35}S-methionine labelling and gel electrophoresis. The oocytes were obtained after ovulation induction by an injection of luteinizing hormone releasing hormone (LHRH) at pro-oestrus, or after a superovulation procedure. The analysis was performed in unfertilized aged and unaged secondary oocytes and in zygotes derived from them.

The patterns of proteins synthesized by aged and unaged LHRH induced secondary oocytes and unaged superovulated secondary oocytes were very similar: these oocytes showed a predominant synthesis of 35 kDa proteins. Zygotes from aged as well as unaged LHRH-induced oocytes also showed a predominant synthesis of one group of polypeptides with a relative molecular weight of about 35 kDa. The proteins of the 35 kDa protein complex migrated in an upper (u), middle (m) or lower (l) band in 10% polyacrylamide SDS-gels, as has been found before (Howlett and Bolton, 1985). The (u)- and (m)-band 35 kDa proteins were shown to be synthesized by secondary oocytes and during the first cleavage division. Early pronuclear zygotes from unaged LHRH-induced oocytes synthesized (u)- and (m)-, but no (l)- band 35 kDa proteins. In contrast, part (38%, n=47) of the early pronuclear zygotes from aged LHRH induced oocytes did synthesize the (l)-band 35 kDa proteins. Late pronuclear zygotes from aged as well as unaged oocytes synthesized predominantly the (l)-band 35 kDa proteins. However, 25% (n=24) of late pronuclear zygotes from aged oocytes synthesized (m)- and (l)-band 35 kDa proteins, in late pronuclear zygotes from unaged oocytes this percentage was 5.8% (n=51). Thus, in zygotes from aged oocytes at least part of the fertilization dependent changes in the 35 kDa protein synthetic pattern are advanced with reference to the morphological stage of pronuclear progression.

Most (80.6%, n=36) of the early pronuclear zygotes from unaged superovulated oocytes synthesized (u)- and (m)- band 35 kDa proteins, like early pronuclear zygotes from unaged LHRH-induced oocytes. However, 16.7% did not synthesize any of the 35 kDa proteins at all. Thus, with respect to their protein synthetic pattern of 35 kDa proteins, zygotes from unaged superovulated oocytes are deviant from zygotes derived from unaged LHRH-induced oocytes.

Key words: post-ovulatory ageing, zygotes, protein synthesis, mouse.

INTRODUCTION

In a previous paper we have shown that major morphological changes induced by fertilization are accelerated in mouse zygotes derived from

oocytes aged post-ovulation *in vivo* (Boerjan and de Boer, in press). These processes include sperm penetration, formation of pronuclei and progression to the first cleavage division. In the present study we have analysed whether *in vivo* post-ovulatory ageing had any effect on the pattern of proteins synthesized by secondary oocytes and zygotes.

In oocytes that were obtained by superovulation, fertilization was correlated with changes in the pattern of synthesis (Howlett and Bolton, 1985) as well as modification of proteins (Howlett, 1986). This was particularly clear for a group of proteins with relative molecular weights of about 35 kDa, the 35 kDa protein complex (Howlett and Bolton, 1985). In one-dimensional polyacrylamide-SDS gels the proteins of this complex can be resolved into three bands, namely an upper (u), middle (m) and lower (l) band; these bands probably contain the same polypeptide (Howlett and Bolton, 1985), phosphorylated to different extents, with the l-band proteins being the less phosphorylated form (Howlett, 1986). The (u)- and (m)- band 35 kDa proteins are synthesized in unfertilized oocytes and during metaphase of the first cleavage division. Fertilization triggers the resumption of the second meiotic division and a transition from the synthesis of (u)- and (m)- band 35 kDa proteins to the synthesis of (l)-band 35 kDa proteins during the pronuclear stage (Howlett and Bolton, 1985).

Aged superovulated oocytes synthesize predominantly (u)-band proteins, although the synthesis of (l)-band 35 kDa proteins starts 24 hrs post-ovulation (Howlett and Bolton, 1985). Thus, some of the protein synthetic changes triggered by fertilization also occur after *in vivo* ageing of oocytes, although they take place later than the corresponding changes detected in fertilized oocytes (Howlett and Bolton, 1985). Other changes are entirely dependent on fertilization and never occur in aged oocytes (Howlett and Bolton, 1985). Protein synthesis has not been studied in zygotes derived from aged oocytes. We therefore decided to analyse which changes occur in the pattern of polypeptides synthesized by fertilized and unfertilized aged oocytes that were obtained under conditions resembling more closely the natural ovulation conditions than in superovulation procedures used in previous studies on protein synthetic activity in zygotes of the mouse (Cullen, 1980; Van Blerkom, 1981; Howlett and Bolton, 1985). For this purpose we made use of luteinizing hormone releasing hormone (LHRH) induced ovulation and artificial insemination (AI) (Boerjan and de Boer, in press). In contrast to superovulation procedures (mouse:

Fowler and Edwards, 1957; Beaumont and Smith, 1975), LHRH induction of ovulation does not influence detectably the number of ovulated oocytes or the frequency of embryonic death (Boerjan and de Boer, in press). Because superovulation itself may be correlated with changes in the protein synthetic pattern, at least in maturing sheep oocytes (Moor et al., 1985), we have included series of superovulated unaged oocytes and the zygotes derived from them, as a control.

We analyzed the protein synthetic pattern in early and late pronuclear zygotes. In zygotes from unaged oocytes we found a transition from the synthesis of exclusively (u)- and (m)- band 35 kDa proteins in early pronuclear zygotes to the synthesis of predominantly (l)-band proteins in late pronuclear zygotes. This is in agreement with Howlett and Bolton (1985). In contrast, several early pronuclear zygotes from aged oocytes synthesize predominantly the (l)-band 35 kDa proteins. Apparently, post-ovulatory ageing prior to fertilization causes an asynchrony between pronuclear development and modifications of the polypeptides of the 35 kDa complex.

In most superovulated oocytes and early pronuclear zygotes derived from them the protein synthetic pattern resembled that of LHRH-induced unaged oocytes before and after fertilization respectively. However, part of the zygotes from superovulated oocytes did not synthesize any protein of the 35 kDa complex.

MATERIALS AND METHODS

Animals and housing conditions

Females were random bred Swiss CpbSE(S))mice and males were F_1(CpbSE(S) x LIII), with LIII being a linkage testing stock from the MRC Radiobiology Unit, Chilton, UK

Housing conditions of animals have been described in detail in a previous paper (Boerjan de Boer, in press). Briefly, mice were kept in two separate animal rooms (20 °C, 60% relative humidity) in a 4 hrs dark/20hrs light schedule in which the onset of the dark periods differed by 11 hrs. In this dark/light schedule ovulation occurs between 3 hrs 45 min and 5 hrs 15 min after the midpoint of the dark period (Boerjan and de Boer, in press). The day of ovulation could be predicted by evaluation of vaginal smears (Thung et al. 1956).

Ovulation induction and superovulation

Ovulation was induced by an intraperitoneal injection of LHRH (200 ng/female, Sigma no: L 7134) 8-12 hrs before the expected endogenous LH-surge (Boerjan and de Boer, in press). Superovulation was induced by an intraperitoneal injection with 5 IU PMSG (pregnant mare serum gonadotrophin; Gestyl, Organon) at di-oestrus; 48 hrs later these females were injected with 7 IU hCG (human chorionic gonadotrophin; Pregnyl, Organon). In both systems, ovulation follows 12 hrs after the last injection (LHRH; Boerjan and de Boer, in press; hCG; Marston and Chang, 1964).

Collection and fertilization in vivo of unaged oocytes and oocytes aged post-ovulation for 12 hrs

Unaged oocytes were collected 13 hrs after LHRH or hCG injection and aged secondary oocytes were recovered 24 hrs after LHRH administration. *In vivo* fertilization was performed by artificial insemination (AI) exactly as described previously (Boerjan and de Boer, in press). In order to obtain embryos derived from aged and unaged oocytes respectively, we inseminated females 24 hrs (12 hrs post-ovulation, aged) and 13 hrs (1 hr post-ovulation, unaged) after LHRH administration. Superovulated females were inseminated 13 hrs (unaged) after the hCG injection.

We collected early pronuclear zygotes (EPZ) from aged and unaged oocytes respectively 5 and 7 hrs after insemination; late pronuclear zygotes (LPZ) from aged and unaged oocytes were recovered 13 and 16 hrs post-insemination respectively. These differences in times of collection were introduced to correct for the accelerated penetration and progression through the first cell cycle in zygotes derived from aged oocytes (Boerjan and de Boer, in press). The zygotes from superovulated oocytes were collected 7 hrs after AI.

The zygotes were recovered from the ampullae and cumulus cells were removed by incubation for 1-2 min in medium M2 with 300 U/ml hyaluronidase (Sigma, type I-S bovine testis, no. H3506; M2 according to Fulton and Whittingham, 1978). Eggs were then washed 3 times in 1 ml medium M2 and cultured under paraffin oil (Fisher O-119) in medium M16 (Whittingham, 1971) plus 4 mg/ml BSA (Sigma, fraction V) at 37 °C in humidified air with 5% CO_2.

Labelling with ^{35}S-methionine

Secondary oocytes and zygotes were labelled for 1 hr in medium M16+BSA containing 1 mCi/ml ^{35}S-methionine (1128 Ci mmol^{-1}, Amersham), washed 3 times in 2 ml protein free medium M2, and then transferred individually to 20 μl of sample buffer (Laemmli, 1970). The samples were then heated in a boiling waterbath for 3 min.

Measurement of ^{35}S-methionine incorporation into proteins by TCA precipitation

Incorporation of ^{35}S-methionine into trichloroacetic acid (TCA) insoluble proteins was determined in each of the samples as follows: 1.5 μl aliquots of sample were brought onto a piece 1 cm^2 Whatman no. 1 filterpaper and dried. The filterpaper was boiled in a 5% TCA solution for 10 min, washed in 5% TCA (2x), 100% ethanol (5 min), diethylether (1x) and dried. The radioactivity bound to the paper was counted in 1 ml scintillation fluid (Instagel II;Hewlett Packard) in a Beckman scintillation counter.

Electrophoresis and fluorography

The proteins synthesized by individual eggs were separated on 10% SDS polyacrylamide gels according to Laemmli (1970). Per single lane per gel, the same amount of TCA precipitable counts was loaded. A mixture of protein molecular weight markers was co-electrophoresed on each gel (^{14}C-methylated protein mixture, CFA.645; Amersham). Fluorography and radiography was performed according to Laskey (1977).

Statistical analysis

The samples loaded on a particular gel were selected such that we could expect (u)-, (m) or (l)-bands to be present in at least one lane of the gel. This enabled us to identify the proteins of the 35 kDa protein complex in individual oocytes and zygotes. The composition of the 35 kDa protein complex was recorded in early pronuclear zygotes (EPZ) and late

pronuclear zygotes (LPZ) from aged and unaged oocytes as well as in the unfertilized oocytes. This resulted into the classification of zygotes into 12 classes depending on the composition of the 35 kDa protein complex (Fig. 2). This classification was made for all the experimental groups of embryos and tested by 2xC contingency tables. The groups were pooled prior to analysis if necessary.

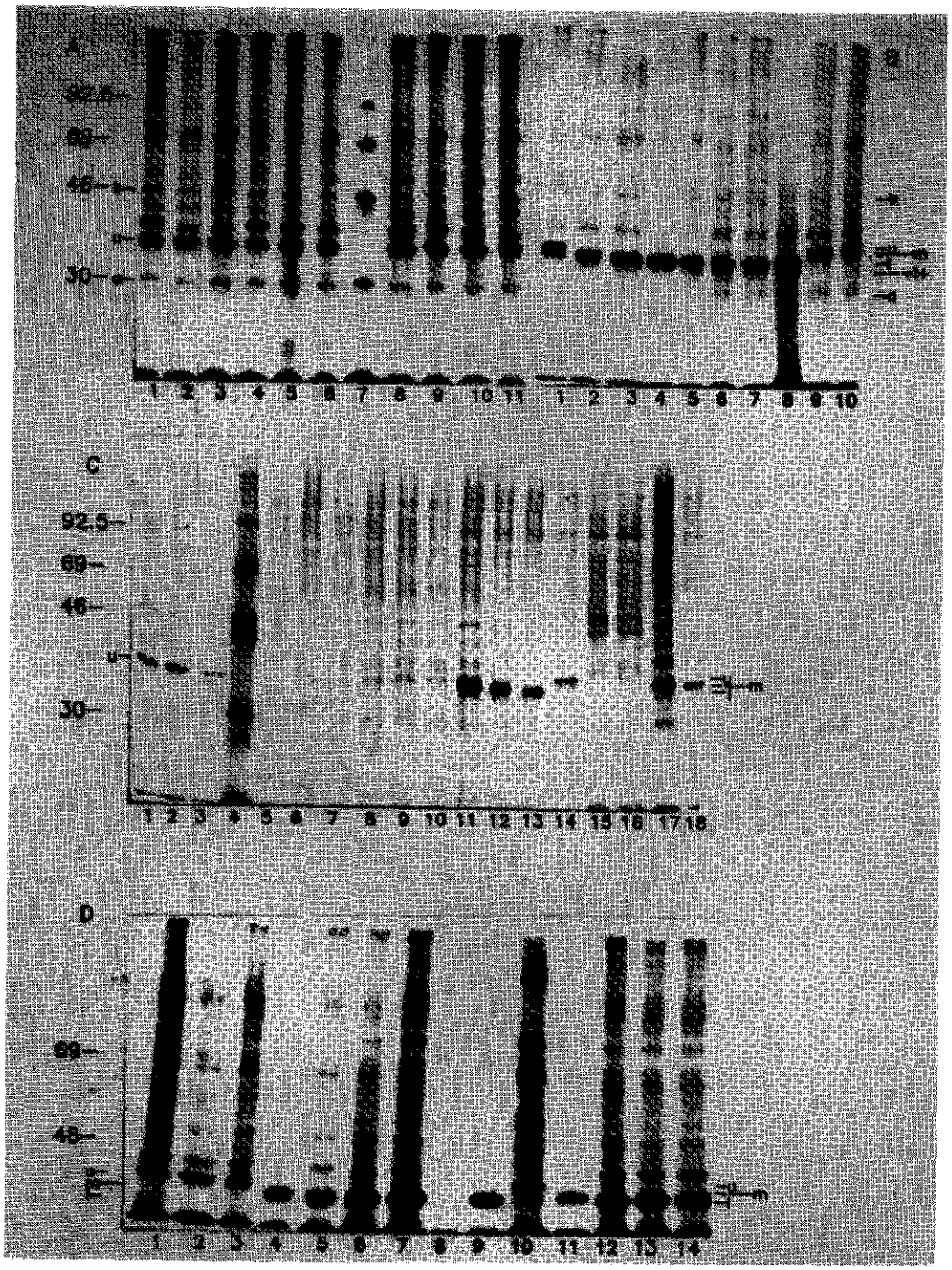
RESULTS

^{35}S-methionine labelled proteins from individual oocytes and zygotes were separated on 10% polyacrylamide SDS gels. Fig. 1 shows representative fluorographs of these gels.

The protein synthetic patterns in aged and unaged LHRH-induced secondary oocytes, as well as in unaged superovulated secondary oocytes, are similar in most respects (Fig. 1A, aged oocytes, lanes 1-3; unaged superovulated oocytes, lanes 4-6 and 8-9; unaged oocytes, lanes 10-11). Oocytes and zygotes from all experimental groups synthesized predominantly proteins of the 35 kDa protein complex, and smaller amounts of several other proteins, including a 30 and a 46 kDa polypeptide (cf. Howlett and Bolton, 1985). The synthesis of the 30 kDa and 46 kDa proteins was very similar in oocytes and zygotes from aged and unaged oocytes (Fig. 1A and 1B, bands a and b).

All aged as well as unaged LHRH induced, and unaged superovulated secondary oocytes synthesized predominantly (u)- and (m)- band 35 kDa proteins, and no (l)-band proteins (Fig. 1A; 1C, lanes 1-3 and lanes 5-10). In some of the experiments we found little protein synthetic activity in unaged LHRH induced (Fig. 1C, lanes 8-10) and superovulated oocytes (Fig. 1C, lanes 5-7). Aged oocytes analyzed at the same day showed higher levels of protein synthetic activity (Fig. 1C, lanes 1-3). We have no explanation for this difference in protein synthetic activity between unaged and aged secondary oocytes.

In zygotes, we analyzed the protein synthetic patterns at the early and late pronuclear stage. The major 35 kDa proteins synthesized by early pronuclear zygotes (EPZ) from LHRH induced unaged oocytes were still the (u)- and (m)- band 35 kDa proteins (Fig. 1B, lanes 9-10; Fig. 1C, lanes 17-18; Fig. 1D, lanes 1-3); 8.9% (n=45) of EPZ from unaged LHRH induced oocytes synthesized exclusively (u)-band proteins, whereas 91.1% synthesized both (u)- and (m)- band 35 kDa proteins. These zygotes showed no synthesis of l-band 35 kDa protein (Fig. 2a). In contrast, part of the early pronuclear zygotes from aged LHRH induced oocytes synthesized (l)-band 35 kDa proteins: 38.3% (n=47) of these zygotes synthesized only (l)-


A
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1 2 3 4 5 6 7 8 9 10 11 12 13 14

band 35 kDa proteins (Fig. 1C, lane 13 and Fig. 1D, lane 9; Fig. 2c); 8.5% showed the synthesis of both (m)- and (l)- band 35 kDa proteins and 42.6% synthesized (u) and (m)-band 35 kDa proteins (Fig. 1B, lane 1; Fig. 1C, lanes 11-12; Fig. 1D, lane 10-11; Fig. 2c). Furthermore, 8.5% synthesized the (l)-band 35 kDa proteins plus a polypeptide with a mobility slightly higher than that of the (l)-band proteins. We called these the (l+)-band 35 kDa proteins (Fig. 1B, lanes 3-5; l+-band). Two percent (2.1 %) of the EPZ from aged LHRH induced oocytes synthesized none of the 35 kDa proteins at all (Fig. 2c). The differences between EPZ from aged and unaged oocytes with respect to the synthesis of particular combinations of the polypeptides of the 35 kDa complex were significant (X^2_2= 35.9, P<0.001).

Most (92.2%, n=51) of the late pronuclear zygotes (LPZ) from unaged oocytes (Fig. 1B, lanes 6-8; Fig. 1D, lanes 4-7; Fig. 2b) synthesized predominantly (l)- or (l) and (l+)- band 35 kDa proteins. The remainder of these LPZ synthesized (u) and (m)- band (2%) or (m)- and (l)-band (5.8%) kDa proteins (Fig. 2b). Seventy five (75%, n=24) of LPZ from aged oocytes synthesized (l)- or (l) and (l+)- band 35 kDa proteins (Fig. 2d) and 25% synthesized (m)- and (l)-band proteins (Fig. 1D, lanes 12-14). The differences between LPZ from aged and unaged oocytes with respect to the synthesis of particular combinations of 35 kDa proteins were significant (X^2_2=7.4, P<0.025).

Of the EPZ from unaged superovulated oocytes, collected 7 hrs after AI, 80.6% (n=36) synthesized (u)- and (m)- band 35 kDa proteins (Fig. 1C, lane 14); 2.7% of these zygotes synthesized only (l)-band 35 kDa proteins and 16.7% did not synthesize 35 kDa proteins at all (Fig. 1C, lanes 15 and 16; Fig. 2e).

Figure 1. Protein synthetic patterns in aged and unaged LHRH induced or superovulated oocytes and in zygotes derived from them. After ^{35}S-methionine labelling of the secondary oocytes or zygotes their proteins were separated on 10% polyacrylamide SDS-gels. This figure shows autoradiographs of these gels. Each lane shows the proteins synthesized by one oocyte or zygote.

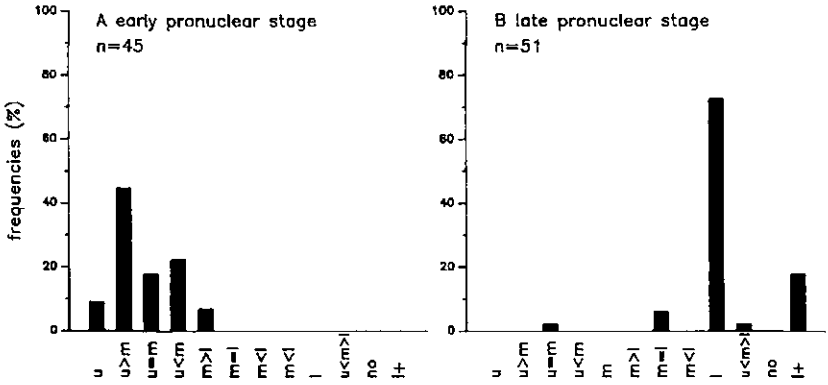
A. lanes 1-3, unfertilized aged LHRH induced oocytes; lanes 4-6, unfertilized unaged superovulated oocytes; lane 7, marker proteins; lanes 8-9, unfertilized unaged oocytes superovulated oocytes; lanes 10-11, unfertilized unaged LHRH induced oocytes.

B. lanes 1-5, early pronuclear zygotes (EPZ) from aged LHRH induced oocytes; lanes 6-8, late pronuclear zygotes (LPZ) from unaged LHRH induced oocytes; lanes 9-10, early pronuclear zygotes (EPZ) from unaged LHRH induced oocytes.

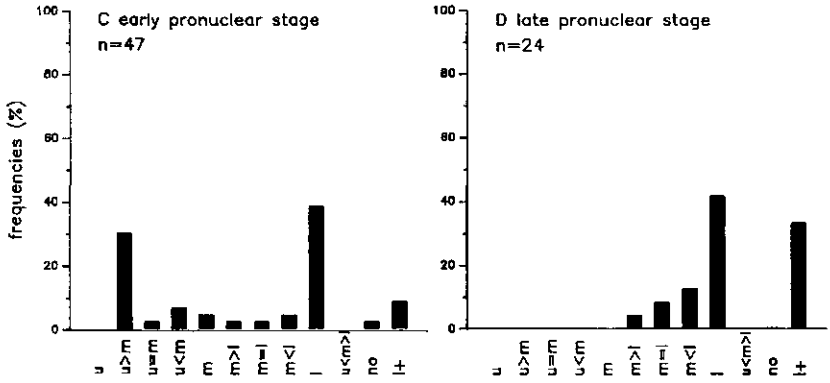
C. lanes 1-3, unfertilized LHRH induced aged oocytes; lane 4, marker proteins, lanes 5-7 unfertilized unaged superovulated oocytes; lanes 8-10 unfertilized LHRH induced unaged oocytes; lanes 11-13, EPZ from aged LHRH induced oocytes; lanes 14-16, EPZ from unaged superovulated oocytes; lanes 17-18, EPZ from unaged LHRH induced oocytes.

D. lanes 1-3, EPZ from unaged LHRH induced oocytes; lanes 4-7, LPZ from unaged LHRH induced oocytes; lanes 9-11, EPZ from aged LHRH induced oocytes; lanes 12-14, LPZ from aged LHRH induced oocytes.

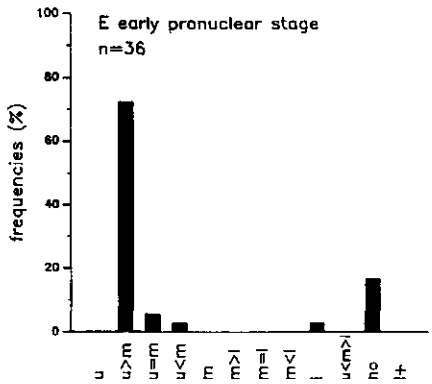
zygotes derived from unaged oocytes



zygotes derived from aged oocytes



zygotes derived from superovulated oocytes



DISCUSSION

In the present study we analyzed the pattern of proteins synthesized by aged and unaged LHRH induced oocytes and by zygotes derived from them. The overall patterns of polypeptides synthesized by oocytes and zygotes from aged as well as from unaged oocytes are comparable to those synthesized by unaged superovulated oocytes and zygotes derived from them (Howlett and Bolton, 1985). These authors have described the synthesis of three fertilization dependent protein complexes with relative molecular weights of 30, 35 and 46 kDa respectively. The electrophoretic mobilities of these polypeptides change after fertilization and during progression of the first cell cycle as a result of post-translational modifications (Howlett and Bolton, 1985; Howlett, 1986). We could recognize the synthesis of 30 kDa and 46 kDa polypeptides and a predominant synthesis of 35 kDa proteins. The synthesis of the 30 kDa and 46 kDa proteins was comparable in zygotes from aged and unaged oocytes and we therefore did not analyze the synthesis of these proteins in more detail.

In one-dimensional polyacrylamide-SDS gels the proteins of the 35 kDa protein complex could be resolved into four bands, namely an upper (u), middle (m), lower (l) band and a (l+)-band. We assume that the (u)-, (m)- and (l)- band 35 kDa proteins synthesized by oocytes and zygotes were similar to the (u)-, (m)- and (l)-band 35 kDa proteins described by Howlett and Bolton (1985). In that case, (u)-, (m)- and (l)-band 35 kDa proteins are translational products from one mRNA, phosphorylated to different levels, with (l)-band proteins being the less phosphorylated form. The nature of the (l+)-band protein synthesized by some of the zygotes is not clear. A more detailed study should be performed to determine whether this protein is a less phosphorylated form of the (l)-band 35 kDa proteins.

Figure 2. Histograms of frequencies of zygotes that synthesized particular combinations of the 35 kDa proteins. The newly synthesized proteins were analyzed as described in the materials and methods section. The classes have been placed from left to right according to their most likely order of appearance during the progression of the first cell cycle as described by Howlett and Bolton (1985).

u, (u)-band 35 kDa proteins; m, (m)-band 35 kDa proteins; l, (l)-band 35 kDa proteins; l+, (l)-band 35 kDa proteins are synthesized plus a band with a somewhat higher relative mobility than the l-band; no, these zygotes synthesized no proteins of the 35 kDa complex.

We did not observe synthesis of (l)-band 35 kDa proteins by fertilized LHRH induced and superovulated oocytes: (l)-band 35 kDa proteins were synthesized by fertilized oocytes from all experimental groups. We therefore conclude that synthesis of the (l)-band 35 kDa proteins is a fertilization dependent event, at least in zygotes from random-bred Swiss females. Howlett and Bolton (1985) did observe the synthesis of (l)-band 35 kDa proteins by secondary oocytes from (C57BL.10 x CBA)F_1 mice. The discrepancy between our observations and those describe by Howlett and Bolton (1985) could be ascribed to the use of different mouse strains.

The overall patterns of proteins synthesized by early pronuclear zygotes (EPZ) from aged and unaged LHRH induced oocytes were very similar; the same holds for the proteins synthesized by late pronuclear zygotes (LPZ) from aged and unaged oocytes. Thus, post-ovulatory ageing prior to fertilization had no detectable effect on detectable patterns of proteins synthesized by 1-cell embryos. The main differences between zygotes from aged and unaged oocytes was observed in the time, with respect to the development of the pronucleus, when modifications of 35 kDa proteins occur.

We found, like Howlett and Bolton (1985), a transition from a predominant synthesis of (u)- and (m)-band 35 kDa proteins in EPZ to a predominant synthesis of (l)-band 35 kDa proteins in LPZ. EPZ from unaged oocytes, collected 7 hrs post-insemination, synthesized exclusively (u)- and (m)- band 35 kDa proteins. However, a large proportion (38%) of EPZ from aged oocytes, collected 5 hrs post-insemination, synthesized already (l)-band 35 kDa proteins. Thus, in zygotes from aged oocytes an asynchrony occurred between pronuclear progression and modifications of the 35 kDa protein complex.

(u)- and (m)-band 35 kDa proteins are so-called M-phase proteins: the synthesis of these proteins is predominant during metaphase of the second meiotic division and the first cleavage division in zygotes from unaged superovulated oocytes (Howlett and Bolton, 1985; Howlett, 1986). One LPZ (2%) from an unaged oocyte synthesized (u)- and (m)- band 35 kDa proteins. Possibly this embryo developed at a slower rate than other remaining LPZ from unaged oocytes. 25% of the LPZ from aged oocytes synthesized, besides the 1-band 35 kDa proteins, m-band proteins, whereas only 5.8% of the LPZ from unaged oocytes did so. Apparently, in some LPZ from aged oocytes the synthesis of the metaphase associated (u)- and (m)-band 35 kDa proteins had already started, while LPZ from unaged oocytes still synthesized

predominantly the interphase associated 1-band 35 kDa protein.

In summary, in zygotes from aged oocytes some fertilization induced changes in the protein synthetic pattern of the 35 kDa protein complex are advanced with reference to the developmental stage of the pronucleus.

It has been suggested that the control of the timing of synthesis and modifications of proteins during progression of the first cell cycle is regulated by an oocyte programme and a fertilization programme (Van Blerkom, 1981; Johnson et al., 1984), and that the oocyte programme can proceed independently from fertilization (Howlett and Bolton, 1985). Synthesis of the (u)- and (m)-band 35 kDa proteins by aged LHRH induced oocytes (this paper) and by aged superovulated oocytes (Howlett and Bolton, 1985) could be controlled by the oocyte programme. This would explain why synthesis is continued after ovulation. It would be of interest to investigate if the (u)- and (m)- 35 kDa proteins play a role in the maintenance of meiotic arrest of the ovulated oocyte and chromosome condensation during the first cleavage division. The proteins of the 35 kDa complex are probably not a member of the histone H1-family, because the majority of the 35 kDa proteins were not acid soluble (data not shown) which is a characteristic of histone-like proteins (O.H.J. Destrée, pers. comm.; Smith et al., 1988).

The precocious synthesis of (l)-band 35 kDa proteins by early pronuclear zygotes from aged oocytes could be explained as follows: factors involved in modifications of (u)- and (m)-band 35 kDa proteins are progressively activated after ovulation. Therefore, the modification of (u)- and (m)-band 35 kDa proteins to (l)-band 35 kDa proteins can occur in early pronuclear zygotes from aged oocytes, but not in those from unaged oocytes.

The premature transition to the synthesis of (l)-band 35 kDa protein and the early synthesis of M-phase proteins in LPZ from aged oocytes may be the cause of a shorter first cell cycle in zygotes from aged oocytes (Boerjan and de Boer, in press). This premature protein synthetic transition also supports the notion that oocytes aged post-ovulation try to catch up with the normal developmental schedule as has been suggested by Eichenlaub-Ritter (1986). The study of the first cell cycle in zygotes from aged oocytes could provide a model to get more insight into oocyte and fertilization programmes and the regulation of the first cleavage division.

Protein synthesis in oocytes and zygotes after superovulation

The protein synthetic patterns of the superovulated unaged unfertilized oocytes were similar to those of LHRH induced oocytes.

Most of the EPZ from superovulated unaged oocytes, collected 7 hrs after AI, synthesized predominantly the (u)- and (m)-band 35 kDa proteins. However, 16.8% of these EPZ did not synthesize any of the proteins of the 35 kDa complex. This is remarkable since the 35 kDa complex proteins were synthesized in all superovulated unfertilized oocytes analyzed. Apparently, fertilization of some superovulated oocytes triggers the morphological changes but not the protein synthetic changes of the 35 kDa proteins. This observation supports the notion that superovulation procedures affect the quality of ovulated oocytes, which is reflected in (1) poor embryonic development of embryos derived from them (reviewed by Foote and Ellington, 1988; mouse; Beaumont and Smith, 1975); (2) increased frequencies of sister-chromatid exchanges (mouse; Elbling and Colot, 1985) or (3) premature activation of follicular oocytes (ovine; Moor et al., 1985).

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CHAPTER IV

THE INTEGRITY OF CHROMATIN IN CLEAVAGE STAGE MOUSE EMBRYOS DERIVED FROM UNAGED OOCYTES AND OOCYTES AGED POST-OVULATION *IN VIVO*.

M.L. Boerjan, L.A. Saris and F.A. van der Hoeven

SUMMARY

This paper gives the results of two studies concerning the integrity of chromatin in cleavage stage embryos from unaged oocytes and oocytes aged post-ovulation *in vivo* for 12 hrs. In these experiments ovulation was induced by an injection of luteinizing hormone releasing hormone (LHRH) at pro-oestrus. Artificial insemination was performed at 13 hrs and 24 hrs after LHRH in order to obtain embryos derived from unaged and aged (12 hrs post-ovulation) oocytes respectively.

In a first study we compared chromosome damage in zygotes from aged and unaged oocytes after fertilization with spermatozoa irradiated with 2 Gy of X-rays.

In a second study we analyzed the number of sister-chromatid exchanges (SCEs) during the 4th cleavage division. Two-cell embryos collected 36 hrs post-insemination were exposed to 10^{-6} M 5-Bromodeoxyuridine (BrdU) for two cell cycles (24 hrs) and chromosome preparations from these embryos were stained for sister-chromatid differentiation by means of the fluorescence plus Giemsa technique. In a control experiment 2-cell embryos collected 30 and 36 hrs post-insemination were cultured in the absence of BrdU for 66 and 24 hrs respectively.

Post-ovulatory ageing prior to fertilization affected neither the percentage of zygotes with chromosome abnormalities nor the number of SCEs per metaphase significantly, although both parameters gave a higher reading in embryos from aged oocytes.

Post-ovulatory ageing had an effect on the morphology of male as well as female pronuclear chromosomes of the first cleavage metaphase. After fertilization with X-irradiated sperm a larger fraction of zygotes from aged oocytes (27%) than from unaged oocytes (7%) was arrested at interphase. Also, the morphology of male and female pronuclei was affected more in zygotes from aged than from unaged oocytes after fertilization with X-irradiated sperm. This effect was particularly observed for the male pronuclear chromosomes of zygotes from aged oocytes. We suggest that post-ovulatory alterations in secondary oocytes affect the male and female pronuclear chromatin structure after fertilization. These chromatin alterations could interact with DNA-lesions induced in the spermatozoa prior to fertilization, such that development to first cleavage is blocked.

Delayed fertilization did not disturb the second to fourth cleavage divisions *in vitro* in the absence of BrdU, thereafter development *in vitro* ceased. Cell division was clearly retarded and asynchronous when late 2-cell stages from aged oocytes were cultured in the presence of 10^{-6} M BrdU.

In summary, irradiation and BrdU affected adversely the developmental capacity of zygotes and of late 2-cell embryos respectively. The developmental capacity of embryos derived from aged oocytes was affected more than of embryos from unaged oocytes. This could be an indication of an increased sensitivity of cleavage stage embryos from aged oocytes to DNA insults.

Key words: zygotes, post-ovulatory ageing, embryonic development, X-rays, BrdU, SCEs, mouse.

INTRODUCTION

Delayed fertilization of mouse oocytes results in poor embryonic development (Marston and Chang, 1964). Cytological studies of mouse oocytes have revealed that post-ovulatory ageing affects: (1) the duration of the first cell cycle after *in vitro* (Fraser, 1979) and *in vivo* (Boerjan and de Boer, in press) fertilization; (2) the block to polyspermy (reviewed by Szollosi, 1975); (3) the organization of both the cytoskeleton and the second meiotic spindle (Szollosi, 1975; Eichenlaub-Ritter et al., 1986; Webb et al., 1986) and (4) the formation of the second polar body (Webb et al., 1986; O'Neill and Kaufman, 1988). Triploidy has been demonstrated in embryos after delayed fertilization in the mouse (Marston and Chang, 1964; Vickers, 1969) and rabbit (Austin, 1967). Trisomy and monosomy have not been reported after post-ovulatory ageing in the mouse (Vickers, 1969; O'Neill and Kaufman, 1988; Zackowski and Martin-Deleon, 1988).

To our knowledge, the effect of post-ovulatory age of the oocyte on the post-fertilization sensitivity to DNA-damage has not been investigated. One-cell mouse embryos exhibit unscheduled DNA synthesis (UDS) in response to irradiation with ultraviolet light (Brazill and Masui, 1978; Brandriff and Pedersen, 1981). Cytogenetical analyses have revealed the influence of the oocyte on damage in sperm induced by X-rays prior to fertilization (Katoh et al., 1983; Matsuda et al., 1985). In this paper, we describe an experiment to compare chromosome damage in zygotes from aged and unaged oocytes after fertilization with X-irradiated spermatozoa. Males were irradiated with 2 Gy of X-rays and sperm was collected from the cauda epididymis within one week after treatment. This time interval was chosen for two reasons: (1) in male germ cells there is no UDS during the week prior to ejaculation after induction of DNA lesions by X-rays (Sega et al., 1978) and (2) the number of dominant lethal mutations that are induced by X-irradiation during this period is constant (Bateman, 1958).

The integrity of chromosomal DNA in preimplantation mouse embryos can also be analyzed by studying the number of sister-chromatid exchanges (SCEs) during early cleavage divisions (Bennett and Pedersen, 1984; Elbling and Colot, 1985; Vogel and Spielmann, 1988). Bennett and Pedersen (1984) suggested that, after *in vitro* fertilization with UV irradiated sperm, strain-specific differences in DNA repair by zygotes are reflected in variations between strains of the frequency of SCEs. This suggestion made us decide to study DNA repair in embryos from aged oocytes by means of a comparison of the frequencies of SCEs in embryos derived from aged

and unaged oocytes. To visualize sister-chromatid exchanges, we cultured late 2-cell stages for two cell cycles (24 hrs) in the presence of 5-Bromodeoxyuridine (BrdU).

The experiments carried out in this study are based on a method that was recently developed and that enables us to obtain embryos from aged and unaged oocytes in a strictly controlled manner; the method implies treatment with luteinizing hormone releasing hormone (LHRH) to induce ovulation, and artificial insemination (AI) to time fertilization *in vivo* (Boerjan and de Boer, in press).

Our findings indicate that post-ovulatory ageing before fertilization neither significantly affects the percentage of zygotes with chromosome abnormalities nor the number of SCEs per metaphase during early cleavage, although both parameters gave a higher reading in embryos from aged oocytes. We found however, that both irradiation and BrdU affected the developmental capacity of zygotes and 2-cell embryos respectively, particularly of those derived from aged oocytes. We consider this as an indication of an increased sensitivity of cleavage stage embryos from aged oocytes to DNA insults.

MATERIALS AND METHODS

Animals

Female mice were random bred Swiss (CpbSE(S)) and male mice were F_1(CpbSE(S)*LIII), with LIII being a linkage testing stock from the MRC Radiobiology Unit, Chilton UK.

Housing conditions and ovulation induction

The method of ovulation induction has been described elsewhere (Boerjan and de Boer, in press). Briefly, mice were housed in a 20 hrs light/4 hrs dark schedule. We used two air-conditioned animal rooms (20 °C, 60% relative humidity) in which the onset of the dark periods differed by 11 hrs. The day of ovulation was predicted by evaluation of vaginal smears and ovulation was induced by an intraperitoneal injection of 200 ng LHRH (luteinizing hormone releasing hormone, Sigma no. L 7134)/female 8-12 hrs before the expected endogenous luteinizing hormone (LH) surge, which occurs 7-8 hrs before the midpoint of the dark period (Boerjan and de Boer, in press).

Delayed fertilization in vivo

Fertilization was performed by means of artificial insemination (AI) with 50 μl of an epididymal sperm suspension collected from X-irradiated or from untreated males. Artificial insemination was performed as described by Boerjan and de Boer (in press). The cauda epididymis from untreated males were cut into 3-4 pieces in 0.3 ml Dulbeccos medium (Dulbecco and Vogt, 1954) supplemented with bovine serum albumin (BSA, BDH, no. 44004, 3 mg/ml). Epididymal sperm from X-irradiated males was collected in 0.25 ml Dulbeccos medium. This was necessary to obtain 30.10^6 moving spermatozoa/-ml, a prerequisite to achieve appropriate rates of fertilization (90-100%).

Ovulation takes place 12 hrs after LHRH administration (Boerjan and de Boer, in press). We inseminated females 13 and 24 hrs after LHRH administration, in order to obtain embryos derived from unaged and aged (for 12 hrs post-ovulation) oocytes respectively. In a typical experiment, two females carrying unaged and two females carrying aged oocytes were inseminated with sperm collected from one male.

X-irradiation of males

X-irradiated spermatozoa were obtained 2- months old males. The males were irradiated with 2 Gy of X-rays (Philips X-ray machine; 250 kV, 10 mA; internal filter 1.95 mm Al equivalent; external filter 0.6 mm Sn + 0.25 mm Cu + 1 mm Al) at a rate of 0.16 Gy/min. Males were used within one week of irradiation, because during this period the frequency of dominant lethals due to chromosome damage in embryos fertilized with X-irradiated sperm is constant (Bateman, 1958; see also Sega and Sotomayor, 1982).

Collection and culture of zygotes

We collected the zygotes derived from aged and unaged oocytes in medium M2 (Fulton and Whittingham, 1978), 5-6 and 7-8 hrs after artificial insemination respectively. This time difference was introduced as fertilization and pronuclear development is faster in aged oocytes than in unaged oocytes (Boerjan and de Boer, in press). The cumulus oocyte complexes were released from the ampullae, cultured in droplets of medium M16 (Whittingham, 1971) in an atmosphere of 5% CO_2 in air at 37 °C. Medium M16 was supplemented with 4 mg /ml BSA (BDH, no. 44004) and 0.03 µg/ml colchicine (Sigma, no. C 9754), to achieve arrest of the zygotes at metaphase of the first cleavage division. The droplets were covered with paraffin oil (Fisher Scientific Company, O-119), that had been equilibrated with medium M16 without BSA and colchicine.

Chromosome preparations

After a culture period of 19-20 hrs the zygotes from aged oocytes had lost their cumulus cells and could be used without further treatment. However, the zygotes from unaged oocytes still had some cumulus cells which were removed by a brief treatment in hyaluronidase (300 U/ml medium M2, Sigma, type I-S bovine testis, no. H 3506). The naked zygotes were incubated on ice in a 1% sodium citrate hypotonic solution for 15 min. Subsequently chromosome preparations of the first cleavage metaphases were made according to Tarkowski (1966). The preparations were preferentially stained for constitutive centric heterochromatin (C-banding) by the following technique, based on that of Sumner (1972). After slide preparation, the chromosomes were post-fixed for 10 min in Carnoy's fixative and hydrolysed for 30 min in 0.2 N HCl. Within 3 days of storage at 4 °C, the slides were dipped in a 4% solution of $Ba(OH)_2$ for 15 sec at 37 °C, thoroughly rinsed in aqua dest. and incubated in 2xSSC for 90-100 min at 60 °C. The chromosomes were stained in 5% Giemsa in Gurr's buffer pH 6.8 (BDH, no. 33193) for 20 min. Because of the great variation in chromosome morphology and the degree of spreading of metaphases (chromosome overlap, stickiness), we subjectively classified the preparations for both characteristics as good, fair or poor. Classification was performed independently by two persons after agreement about criteria.

Chromosome abnormalities were classified according to Evans and O'Riordan (1975).

Analysis of sister-chromatid exchange (SCE)

Females that carried embryos from unaged or aged oocytes, were killed by cervical dislocation 36 hrs after artificial insemination (AI). The embryos were recovered by flushing the oviducts in medium M2 supplemented with 4 mg BSA/ml. The numbers of unfertilized, 2-cell (2c), 3-cell (3c) and 4-cell (4c) embryos were recorded. The embryos were transferred to droplets of medium M16 containing 4 mg BSA/ml and 10^{-6} M 5-Bromodeoxyuridine (BrdU, Sigma no. B 5002) and cultured for 24 hrs in an atmosphere of 5% CO_2 in air at 37 °C as described previously. As a control, we cultured 2-cell embryos collected 36 hrs after AI without BrdU for 24 hrs. Colchicine (1 µg/ml) was added 3-4 hrs before the end of culture.
Chromosome preparations were made according to Tarkowski (1966). Inspection of the flattening specimen in Carnoy's fixative under a phase-contrast microscope enabled us to prevent cell loss of these multi-cellular embryos. The slides were stored in the dark for two weeks at 37 °C before further treatment. Sister-chromatid differentiation in embryonic mitoses was achieved according to the method of Elbling and Colot (1985), a variant of the Fluorescence Plus Giemsa (FPG) method: the slides were immersed in 50 µg 33258 Hoechst/ml in Dulbeccos medium:water (1:3, pH 7.4), exposed to UV-light (366 nm; Blak-Ray lamp model UVL-56; Ultraviolet-Prod. Inc, San Gabriel, Calif. USA) at a distance of 12 cm for 7-8 hrs, rinsed in double distilled water, incubated in 2xSSC pH 7 at 60 °C (20 min), rinsed again and stained in 5% Giemsa dissolved in Gurrs' buffer pH 6.8 for 20 min. For each embryo we recorded:(1) the number of interphase nuclei, (2) the number of metaphases and (3) in those mitoses that showed sister-chromatid differentiation the number of SCEs.

Culture of two-cell embryos without BrdU

Two cell embryos were collected 30 after AI and cultured in medium M16 supplemented with 4 mg/ml BSA under paraffin oil (Fisher Scientific Company, O-119, equilibrated with culture medium) for 66 hrs. Numbers of nuclei/embryo were determined in spread preparations made according to Tarkowski (1966).

RESULTS

Development to the first cleavage division in zygotes after fertilization with X-irradiated sperm

First cell cycle progression

A proportion of zygotes derived from the fertilization of aged and unaged oocytes with X-irradiated sperm, exhibited an arrest at pronuclear interphase, such an arrest was not shown by control zygotes of either age (Table 1). Of the zygotes from aged oocytes and irradiated sperm, 27% arrested in interphase, whereas 7% of zygotes from unaged oocytes and irradiated sperm were blocked during this phase (X^2_1=18.8, P<0.001, Table 1). The male and female pronuclei behaved synchronously with respect to progression to metaphase or to pronuclear arrest (Fig. 1).

TABLE 1: Development *in vitro* of aged and unaged secondary oocytes after *in vivo* fertilization with untreated and X-irradiated epididymal sperm.

	no. of females	no. of oocytes	no. of fertilized oocytes diploid (%)	triploid (%)	After 20 hrs culture no. of zygotes in interphase (%)	metaphase (%)
1) Fertilization with untreated sperm						
Embryos from unaged oocytes	10	85	80 (94)	1 (1)	0 (0)	81 (100)
Embryos from aged oocytes	9	97	84 (87)	5 (5)	1 (1)	88 (99)
2) Fertilization with X-irradiated sperm						
Embryos from unaged oocytes	19	140	121 (86)	0 (0)	9 (7)	112 (93)
Embryos from aged oocytes	20	187	153 (82)	3 (2)	42 (27)	114 (73)

Metaphase preparations of the first cleavage division

The metaphase preparations of the separate experimental groups appeared to differ with respect to chromosome morphology and the spreading of the metaphases. To analyze this further, we categorized the preparations into 3 classes with respect to chromosome morphology or metaphase spreading: good, fair and poor (Figs. 2 and 3). We analyzed the outcome of these classifications in 2x3 contingency tables. Table 2 shows the chi-square values for tests on independence for the variables "aged", "unaged", "X-ray" and "control" (= unirradiated) of the male and female pronuclear chromosomes.

The results obtained for zygotes from aged and unaged oocytes differed in the following aspects: (1) after fertilization with control sperm the morphology of both male and female pronuclear chromosomes was better in zygotes from unaged than from aged oocytes (X^2_2=10.7 and X^2_2=12.5 respectively, $P<0.005$). After fertilization with X-irradiated sperm such a difference was only demonstrable for male pronuclear chromosomes (X^2_2= 16.4, $P<0.001$,Fig. 1). (2) After fertilization with X-irradiated sperm,

male pronuclear metaphase chromosomes in zygotes from unaged oocytes spread better than those in zygotes from aged oocytes (X^2_2=10.6, P<0.005). X-irradiation of sperm prior to fertilization had an effect on chromosome morphology in both male and female pronuclei of zygotes derived from unaged oocytes (X^2_2=12.1 and X_2^2=11.2 respectively, P<0.005). Post-ovulatory ageing and fertilization with X-irradiated sperm had a cumulative effect on the morphology of the male pronuclear chromosomes (X^2_2=16.4, P<0.001, Fig. 1).

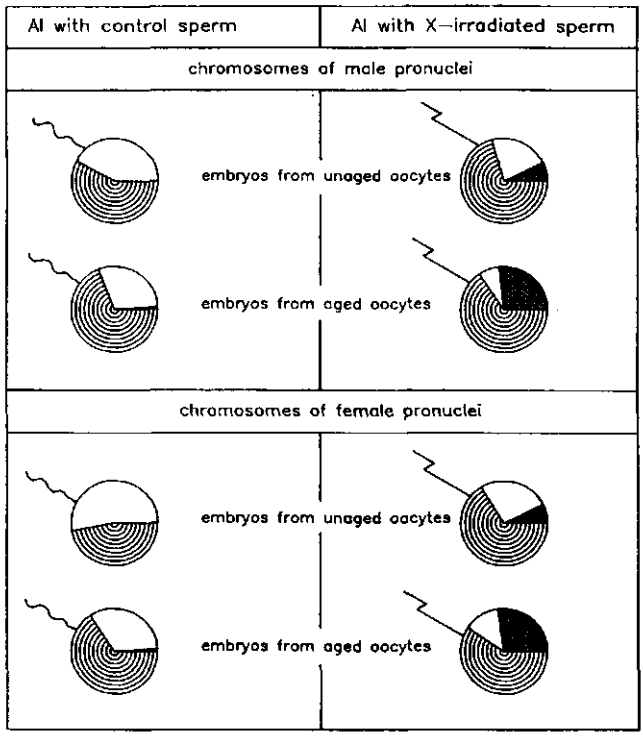

Figure 1: Diagrams showing the effect of X-irradiated spermatozoa on the development to the mitosis of the first cleavage division in unaged and aged oocytes. (■) zygotes in pronuclear arrest (%); (□) preparations classified as good (%); (▨) preparations classified as poor (%).

Chromosome aberrations at metaphase of the first cleavage division

As discussed in the previous paragraph, a fraction of the metaphase preparations from zygotes fertilized with X-irradiated sperm were of poor quality and could therefore not be analyzed for chromosome aberrations. Thus, 72 (64.3%) first cleavage metaphases of zygotes from unaged oocytes

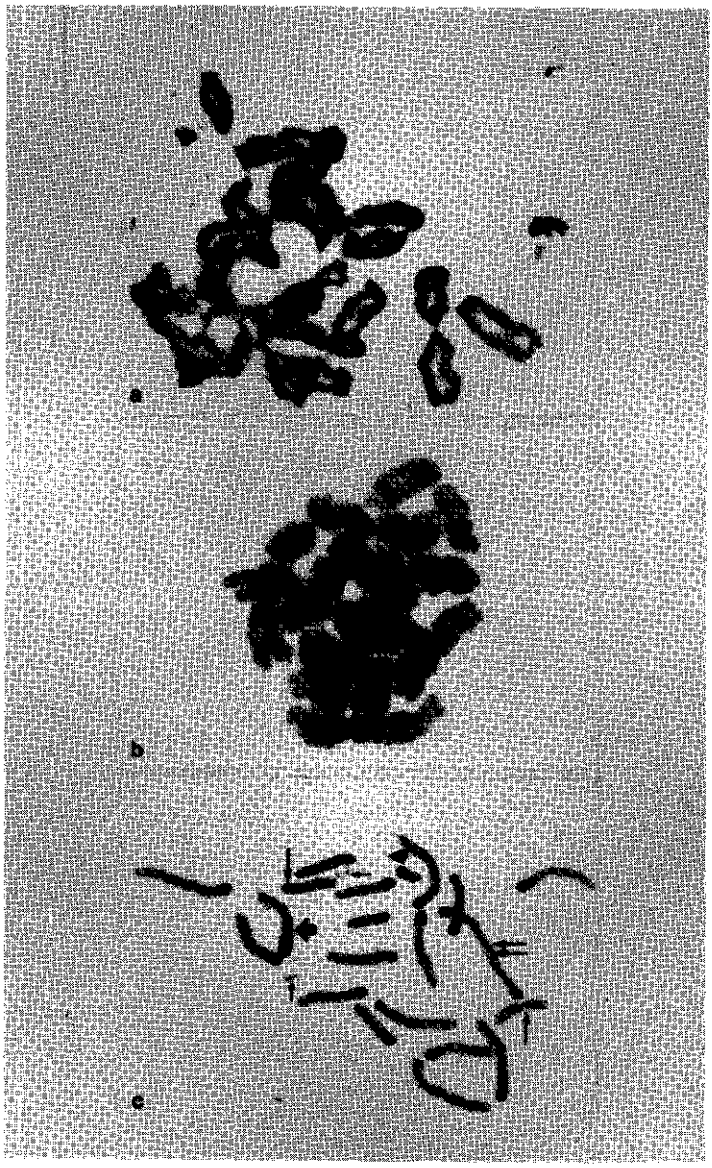

Figure 2: Chromosome spreads of male pronuclei at metaphase of the first mitotic cleavage division of embryos derived from oocytes aged post-ovulation for 12 hrs and fertilized with X-irradiated spermatozoa.

a-b. The chromosomes in these preparations were scored as poor.

c. The chromosomes in these preparations were scored as good and chromosome aberrations could be observed: 2 fragments (thin arrows); 1 dicentric chromosome (double arrows); 1 marker chromosome (arrow head); 1 presumptive translocation chromosome with 2 C-bands (thick arrow); 2 double minutes (small arrows).

TABLE 2: Chi-square values of tests on independence between the variables "aged", "unaged", "irradiated", "control" [1] for the morphology of the chromosomes and the spreading of the metaphases.

	Male pronuclei		Female pronuclei	
Morphology of the chromosomes				
	"unaged"	"aged"	"unaged"	"aged"
"X-ray"	16.4***		n.s.	
	12.1**	n.s.	11.2**	n.s.
"Control"	10.7**		12.5**	
Spreading of the metaphases				
	"unaged"	"aged"	"unaged"	"aged"
"X-ray"	10.6**		n.s.	
	7.6*	11.4**	n.s.	n.s.
"Control"	n.s.		n.s.	

Footnotes:1). "aged": zygotes from aged oocytes; "unaged": zygotes from unaged oocytes; "X-ray": oocytes fertilized with X-irradiated sperm; "control": oocytes fertilized with unirradiated sperm.
*** P< 0.001; ** P< 0.005; * P< 0.025.

and 46 (40.3%) metaphases of zygotes from aged oocytes were of sufficient quality to determine frequencies of chromosome aberrations. 22% of the analyzed metaphases from unaged oocytes exhibited aberrations, whereas 28% of the analyzed metaphases from aged oocytes did so (Table 3, $X^2_1 = 0.6$, n.s.). In both embryos from aged and unaged oocytes the observed aberrations were mainly of chromosome type (Table 3). One zygote from an aged oocyte fertilized with X-irradiated sperm showed 7 chromatid-type aberrations. This cell was omitted from the calculation of the mean number of aberrations/metaphase which was 0.28 for both the zygotes from unaged and aged oocytes.

Chromosome breaks were observed in the male pronucleus of one embryo from an aged oocyte fertilized with control sperm.

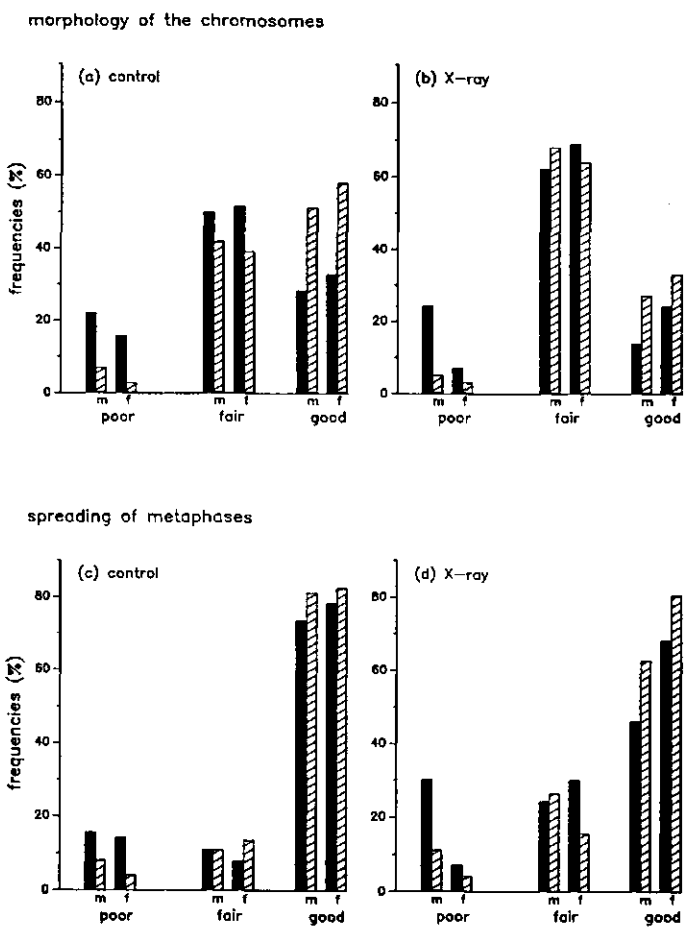

Figure 3: Quality of chromosome morphology (a-b) and spreading (c-d) in chromosome preparations of first cleavage metaphases. Zygotes derived from unaged (▨) and aged (■) oocytes, fertilized with control (a, c) or X-irradiated (b, d) sperm. f= female pronucleus; m= male pronucleus.

TABLE 3: Frequencies of chromosome aberrations in the first cleavage metaphases of unaged and post-ovulatory aged oocytes after fertilization with X-irradiated sperm.

	no. of zygotes	no. of zygotes with chromosome aberrations (%)	Chromatid type		Chromosome type				
			gap	fragment	fragment	di-centric	mi-nute	ex-chan-ge	iso-chroma-tid gap
Zygotes from unaged oocytes	72	16 (22)	4	0	4	2	1	4	5
Zygotes from aged oocytes	46	13 (28)	7	1	5	3	0	3	0

Rate of fertilization

For both unaged and aged oocytes the rate of fertilization was 10% lower after AI with X-irradiated sperm than with untreated (control) sperm (Table 1), although we used a concentration of irradiated sperm which was 1.2 times that of control sperm, in order to achieve a sperm count of at least 30.10^6 healthy looking spermatozoa/ml. Probably, irradiation of the male affected the fertilization capacity of sperm in the AI procedure.

Development of 2-cell embryos in vitro and the analysis of sister-chromatid exchanges

The *in vivo* stage of development 36 hrs after artificial insemination AI

At the time of collection (36 hrs after AI) a greater proportion of embryos from aged oocytes had developed beyond the 2-cell stage than those derived from unaged oocytes. Besides the expected 2-cell stages, we found 3.3% 3-cell and 3.7% 4-cell embryos from unaged oocytes. For embryos from aged oocytes these percentages were 11.2 and 2.8 respectively (Table 4, X^2_2=12.4, P<0.005).

Post-ovulatory ageing and *in vitro* development in the absence of BrdU

After a culture period of 24 hrs in the absence of BrdU, 83% of the embryos from aged oocytes and 72% of the embryos from unaged oocytes consisted of more than 8 blastomeres (Table 4, X^2_1=3.3 n.s). Thus, embryos from aged and unaged oocytes develop at the same rate during a 24 hrs culture period without BrdU. However, 2-cell embryos collected 30 hrs after AI and cultured without BrdU for 66 hrs showed poor development when derived from aged oocytes (Table 5, Mann-Whitney U test, P<0.05). Embryos from unaged oocytes divided at a rate only slightly slower than *in vivo* rates of development (mean number of cells ± s.d./embryo, collected from the same stock of mice on afternoon day 4, with day 1 the day of the vaginal plug is 46.2 ± 16.6, De Boer et al., submitted).

Post-ovulatory ageing and *in vitro* development in the presence of BrdU

After a culture period of 24 hrs in the presence of 10^{-6} M BrdU, we observed a heterogeneity in cell number/embryo which was greater among embryos from aged oocytes than among embryos from unaged oocytes (Table 4): about 51% of the embryos from aged oocytes had more than 8 blastomeres,

whereas 86% of embryos from unaged oocytes had more than 8 cells ($X^2_2=45.8$ $P<0.001$). Some nuclei in embryos derived from aged oocytes had a heteropycnotic appearance.

We conclude that BrdU and post-ovulatory altered factors interact such that the following cleavage divisions of late two-cell embryos are impaired.

TABLE 4: In vitro **development of embryos derived from aged and unaged oocytes collected at 36 hrs after AI and cultured for 24 hrs.**

	start of the culture period						end of the culture period (60 hrs after AI)			
	no. of			no. of embryos with						
	females	oocytes	fertilized (%)	2-C[1] (%)	3-C[1] (%)	4-C[1] (%)	no. of cells[2] ± s.d.	mitosis ± s.d.	cell no. <8 (%)	cell no ≥8 (%)
Embryos derived from:										
unaged oocytes	24	242	241 (99)	224 (93)	8 (3)	9 (4)				
aged oocytes	25	269	246 (91)	211 (86)	28 (11)	7 (3)				
Embryos cultured in the presence of BrdU										
Embryos derived from:										
unaged oocytes	15	160	159 (99)	149 (94)	5 (3)	5 (3)	10.4±3.7	1.9±2.5	22 (14)	136 (86)
aged oocytes	16	177	161 (93)	139 (86)	16 (10)	6 (4)	7.0±3.2	1.3±1.8	75 (49)	79 (51)
Embryos cultured in the absence of BrdU										
Embryos derived from:										
unaged oocytes	9	82	82 (100)	75 (91)	3 (4)	4 (5)	9.6±4.2	1.4±1.9	23 (28)	58 (72)
aged oocytes	9	92	85 (92)	72 (85)	12 (14)	1 (1)	9.8±3.5	1.5±1.8	14 (17)	70 (83)

Footnotes: 1) 2-C; 3-C and 4-C is respectively: 2, 3 and 4 blastomeres/embryo.
2) total cells=interphases + mitoses

The number of sister-chromatid exchanges in embryos from unaged and aged oocytes

Although the majority of the embryos from unaged oocytes developed beyond the 8-cell stage and thus had passed two DNA-synthetic phases in the presence of BrdU, the number of metaphases which demonstrated chromatid differentiation was low (Table 6). Sister-chromatid differentiation was observed in 28 metaphases of embryos derived from unaged oocytes and

TABLE 5: In vitro development of embryos derived from aged and unaged oocytes collected at 30 hrs after artificial insemination and cultured for 66 hrs in the absence of BrdU.

	no. of		Blastomeres/embryo
	females	*embryos*	*after a culture period of 66 hrs*
Embryos from unaged oocytes	4	43	70 ± 9
Embryos from aged oocytes	3	27	14 ± 15

in 13 metaphases of aged oocytes (Table 6). The SCE count of broken cells has been corrected for the chromosomes missing, under the assumption that these chromosomes were of average length and had the average number of SCEs per unit length. We found a slightly higher number of SCEs per mitosis in embryos from aged oocytes (32.5 ± 11.4) than in embryos from unaged oocytes (25.8 ± 11.0), but this difference is not significant at the 5% level (Mann-Whitney U test, $0.05<P<0.1$).

The results of this experiment confirm to some extent the outcome of the experiment described in the previous paragraph: post-ovulatory ageing does not result in an obvious increase in chromosome damage. However, development *in vitro* of two cell embryos from aged oocytes is inhibited in the presence of BrdU and, therefore, the number of SCEs could be underestimated in metaphases of these embryos.

TABLE 6: The number of sister chromatid exchanges in cleavage stage embryos derived from unaged and aged oocytes cultured for 24 hrs in the presence of BrdU.

	no. of		chromatid differentiation in no. of			SCE/
	females	fertilized oocytes	females	embryos	metaphases	*mitosis* ± s.d.
embryos from unaged oocytes	15	159	7	12	28	25.8 ± 11.0
embryos from aged oocytes	16	155	4	7	13	32.5 ± 11.4

DISCUSSION

In the present study several comparisons were made between embryos from aged and unaged oocytes. Firstly, we investigated the behaviour *in vitro* of aged and unaged oocytes that were fertilized *in vivo* with control and X-irradiated sperm. Secondly, we studied embryonic development *in vitro* of late two-cell embryos in the presence and absence of BrdU to determine the level of SCEs and the kinetics of the cleavage divisions.

The first cell cycle with and without irradiated sperm

First cell cycle progression

We observed no effect of post-ovulatory ageing on the transition of zygotes to metaphase of the first cleavage division when females were inseminated with non-irradiated sperm. However, fertilization with spermatozoa irradiated *in vivo* with 2 Gy showed an arrest in interphase in 27% and 7% of zygotes from aged and unaged oocytes respectively. We do not know whether this arrest occurs at G_1, S- or G_2-phase of the first cell cycle. To our knowledge, this is the first indication of a developmental arrest that might be the result of DNA-lesions present in sperm prior to fertilization.

A delay in the development to the 2-cell stage has been demonstrated in zygotes exposed to X-rays 5 hrs after *in vitro* fertilization (Matsuda et al., 1983) or after irradiation *in vivo* at the early pronuclear stage with a peak of sensitivity at the beginning of S phase (Grinfeld and Jacquet, 1987). BALB/c zygotes X-irradiated at the beginning of S-phase showed an arrest in G_2 of the first cell cycle and cleaved for the first time when normally the second cleavage division takes place (Grinfeld et al., 1987). In these retarded zygotes more chromosomal aberrations were found than in irradiated zygotes which cleaved at the right time (Grinfeld and Jacquet, 1988). Chromosomal aberrations were probably not the only cause of developmental delay, because embryos from F_1 hybrids (BALB/c x C57BL) were not susceptible to G_2 arrest when irradiated, despite the high levels of irradiation damage shown by these zygotes (Grinsfeld and Jacquet, 1988). These authors suggested that modifications in first cell cycle dependent protein synthesis and protein phosphorylation could be related to the observed G_2 arrest (Grinfeld et al., 1988).

Metaphase preparations of the first cleavage division

Post-ovulatory ageing had an effect on the morphology of the chromosomes

of the male as well as the female pronucleus. Possibly, factors involved in chromosome condensation have been changed during the post-ovulatory ageing period. We think it likely that these factors are related to the meiotic chromosome condensation factors whose condensation activity decreases with the post-ovulatory age of the secondary oocyte (Czolowska et al., 1986).

We also found a detrimental effect of fertilization with X-irradiated sperm on the morphology of male and female pronuclear chromosomes in zygotes from unaged oocytes. The deteriorated chromosome morphology shown in both pronuclei cannot be explained from X-ray induced DNA lesions alone, since DNA-damage was present in the male pronucleus and not in the female pronucleus. Matsuda et al. (1988) found that accumulation of UV-induced DNA-damage resulted in a pulverized appearance of chromosomes, but this effect on chromosome morphology only concerned the UV irradiated genome. In the present study, the effects of 'ageing' and 'irradiation' on chromosome morphology seem to be additive as especially shown in the male pronucleus (Fig. 1).

From our results we suggest that post-ovulatory alterations in the secondary oocyte affect the chromatin structure in the male and female pronucleus after fertilization. These chromatin alterations could interact with DNA-lesions induced in the spermatozoon prior to fertilization, such that development to first cleavage is blocked.

Chromosome aberrations at metaphase of the first cleavage division

We found no clear effect of post-ovulatory ageing on the frequency of zygotes with aberrations after fertilization with X-irradiated sperm. However, we could have underestimated the frequency of chromosome aberrations because a greater fraction of metaphase preparations from zygotes derived from aged oocytes than from unaged oocytes exhibited a fair or poor quality.

The percentages of zygotes from aged and unaged oocytes with chromosome aberrations (28 % and 22% respectively) were somewhat higher than the 15.6% given by Matsuda et al. (1985). These authors probably underestimated the number of di-centric chromosomes and fragments since they did not apply the C-banding technique, which was used in our study. The percentage found by us is in agreement with an estimate of dominant lethality (25%) of Searle and Beechey (1974) who used a comparable irradiation protocol.

Development of 2-cell embryos in vitro and the analysis of sister chromatid exchanges

Post-ovulatory ageing and *in vitro* development

Our aim was to collect late 2-cell embryos 36 hrs after AI. At this time we found a higher percentage of 3-cell embryos from aged oocytes than from unaged oocytes (Table 4). In a previous study we have shown that *in vivo* fertilization and progression through the first cell cycle of oocytes post-ovulatory aged for 12 hrs was accelerated (Boerjan and de Boer, in press). The relative high percentage of 3-cell embryos could be an indication of a more asynchronous cleavage of individual blastomeres within a 2-cell embryo from an aged oocyte than from an unaged oocyte.

Late 2-cell embryos from aged and unaged oocytes developed beyond the 8-cell stage after a 24 hrs culture period in the absence of BrdU. This is in agreement with the rate of development *in vitro* described for embryos from other mouse strains (Smith and Johnson, 1986). Embryonic development *in vitro* of 2-cell embryos after delayed insemination was strongly impaired during a 66 hrs culture period which started 30 hrs after AI (Table 5). Apparently, delayed fertilization does not disturb the first 3-4 cleavage divisions, but thereafter development ceases.

Cell division was clearly retarded and asynchronous in embryos from aged oocytes when cultured in the presence of 10^{-6} M BrdU. This could be an indication of disturbed DNA replication and transcription on a BrdU substituted template, in analogy with the retarded DNA replication on BrdU substituted templates shown in proliferating somatic cells (O'Neill et al., 1984). Possibly, chromatin structure changes during the post-ovulatory ageing period (see first part of this discussion) and thereby influences replication. In embryos from unaged oocytes we found no inhibitory effect of BrdU on development during the 24 hrs culture period.

From the literature it can be concluded that cell proliferation in pre-implantation embryos is very sensitive to BrdU (Garner, 1974; Pollard et al., 1976; Vogel and Spielmann, 1988). Garner (1974) found that 2-cell embryos undergo at least two cell divisions in the presence of 10^{-7} to 10^{-6} M BrdU, but these embryos died between the 8-cell and late morula stages.

The number of sister-chromatid exchanges in embryos from unaged and aged oocytes

The mean number of SCEs/metaphase is not significantly different at the 5% level in embryos from aged and unaged oocytes. However, the number of SCEs could have been underestimated, because chromatid differentiation was not shown in all metaphase preparations. Chromatid differentiation can only be shown in cells which passed two cycles of DNA replication in the presence of BrdU (Perry and Wolff, 1974) and this had not happened in all embryos examined. Particularly embryos from aged oocytes developed poorly in the presence of BrdU.

The number of SCEs/metaphase is comparable with that found in a previous study on SCEs in morulae and blastocysts of the mouse (Vogel and Spielmann, 1988) but much higher than the data presented by especially Elbling and Colot (1985) and by Bennett and Pedersen (1984). The overall conclusion from this and the previous paragraph can be formulated as follows: mouse oocytes change during the post-ovulatory ageing period such that embryos from aged oocytes are more sensitive to DNA-affecting agents like, X-rays and BrdU than embryos from unaged oocytes. This sensitivity can be linked to the impaired chromosome morphology during first cleavage that occurs after post-ovulatory ageing. We suggest that the study of post-ovulatory ageing prior to fertilization could give more insight into the relation between chromatin structure and early embryonic development.

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CHAPTER V

A CYTOCHEMICAL STAINING PROCEDURE FOR SUCCINATE DEHYDROGENASE ACTIVITY IN PREOVULATORY MOUSE OOCYTES EMBEDDED IN LOW GELLING TEMPERATURE AGAROSE

M.L. Boerjan, W.M. Baarends and H-J.T. Ruven,

SUMMARY

This report describes a cytochemical staining procedure for SDH (succinate dehydrogenase) activity in preovulatory oocytes of the mouse. The oocytes were embedded in low gelling temperature agarose and treated with caffeine prior to the cytochemical staining in the presence of NBT (nitro blue tetrazolium), PMS (phenazinemethosulphate) and succinate. This procedure resulted in an intense staining of the oocytes by formazan precipitate. The level of aspecific formazan production in the absence of succinate was very low. We applied the procedure to oocytes matured *in vitro* and found that the location of the formazan precipitate, as a result of SDH activity, correlated well with the location of mitochondria.

The chromatin of the cytochemically stained oocytes could subsequently be analyzed by means of the DNA specific fluorochrome DAPI. In preovulatory oocytes, we found a correlation between chromatin organization and the location of mitochondria: in oocytes with an intact germinal vesicle the mitochondria were uniformly distributed in the cytoplasm as shown by fine grains of formazan precipitate. In oocytes with condensed chromatin the mitochondria apparently had clustered, because the formazan precipitate was more coarse in these cells.

Key words: oocyte, cytochemical staining, succinate dehydrogenase, caffeine, low gelling temperature agarose.

INTRODUCTION

Mammalian oocytes require nuclear and cytoplasmic maturation for the acquisition of full developmental competence. Nuclear maturation or resumption of meiosis is characterized by germinal vesicle breakdown and development to metaphase II (Austin and Short, 1982). Cytoplasmic maturation is characterized by a spatial rearrangement of cellular organelles. In the mouse preovulatory oocyte, mitochondria move to the region of spindle formation of the first meiotic division (Van Blerkom and Runner, 1984); in bovine preovulatory oocytes, mitochondria move to the site of polar body formation (Hyttel et al., 1986). It has been suggested that mitochondria are located or moved to regions of the maturing oocyte which are energy (ATP) demanding (Van Blerkom and Runner, 1984). However, little is known about the activities of mitochondrial enzymes involved in energy metabolism of preovulatory oocytes. In this paper we describe a cytochemical method to determine in individual oocytes the distribution of the activity of SDH (succinate dehydrogenase), an enzyme which is located on the inner membrane of mitochondria.

SDH activity can be localized in tissues by incubation of tissue sections in a medium with a tetrazoliumsalt and succinate as substrate (Pearse, 1972). This procedure cannot be applied to whole cells, however, because the permeability for NBT (nitro blue tetrazolium) and PMS (phenazine methosulphate) of the intact inner mitochondrial membrane is limited (Hale and Wenzel, 1978). Certain drugs can induce injury of mitochondrial membranes and thereby increase the permeability for NBT and PMS. This can be measured by an increased rate at which mitochondria stain for SDH activity (Acosta and Wenzel, 1975). In this paper we show that treatment of oocytes with the drug caffeine prior to cytochemical staining results in intensely stained cells by formazan precipitates.

We applied the procedure to preovulatory oocytes of mice. In a maturation experiment *in vitro* we found that the location of formazan correlated well with the location of mitochondria in subsequent stages of maturation.

MATERIALS AND METHODS

Collection of primary mouse oocytes

Female Swiss CpbSE(S) mice were kept in air-conditioned rooms (20 °C, 60% relative humidity) with a 4 hrs dark (1:00-5:00 p.m.)/20 hrs light cycle. Females were killed by cervical dislocation and the ovaries were rapidly removed and freed from adhering tissue in cold (4 °C) 100 mM potassium phosphate buffer (Pi buffer, pH 7.4). We isolated primary oocytes as follows: the ovaries were rubbed through a nylon-filter (100 μm mesh, Stokvis-Smith BV, Haarlem, The Netherlands) in Pi buffer (4 °C) and oocytes with an intact germinal vesicle, free from follicular cells and a diameter of 85-100 μm were collected; these oocytes are probably fully grown (Pedersen and Peters, 1968). The isolated oocytes were washed in fresh Pi buffer (4 °C).

In vitro maturation of mouse oocytes

The preovulatory mouse oocytes were collected in Medium-2 (Fulton and Whittingham, 1978) supplemented with 4 mg/ml bovine serum albumin (BSA fraction V, Sigma; ST. Louis, MO) and cultured in droplets of Medium-16 (Whittingham, 1971) supplemented with 4 mg/ml BSA under paraffin oil (O-119, Fisher Scientific; New Jersey, USA) at 37 °C in an atmosphere of 5% CO_2 in air. The mouse oocytes were cultured for 6 hrs.
The cultured cells were washed in Pi buffer (4 °C) before embedding in low gelling temperature agarose.

Embedding of oocytes in low gelling temperature agarose

Droplets of 8-10 μl 0.5 % low gelling temperature agarose (Sea plaque agarose, FMC Bioproducts; Rockland, USA) in Pi buffer at 28-30 °C, were placed on small cover glasses coated with agar. The coating of cover glasses is necessary to fix droplets of low gelling temperature agarose to the cover glass. The coating was performed as follows: a 0.5% agar (Bacto-agar, Difco laboratories; Detroit Michigan, USA) solution in water was pipetted onto a cover glass (1 ml/cover glass of 24 x 40 mm) and after gelling the agar was dried in an incubator at 50 °C.

The oocytes were placed in the droplets of low gelling temperature agarose, the cover glasses with the oocytes were subsequently transferred to 4 °C until the agarose had set. The cover glasses with the cells embedded in the agarose droplets can be incubated in the incubation media used in the cytochemical staining protocol.

Protocol for cytochemical staining of SDH activity

1. Incubation of agarose droplets with the cells in freshly prepared 10 mM caffeine (BDH ltd; no. 275774, Poole, UK) in Pi-buffer pH 7.4 at 4 °C for 45 min.
2. Preincubation in Pi buffer at 37 °C for 10 min.
3. The enzyme reaction is carried out at 37 °C for 20 min. The composition of the reaction media is summarized in Table 1. Succinate is replaced by NaCl in the control medium, to obtain the appropriate osmolarity (280-300 mosmol). The incubation medium should be kept in the dark to avoid non-enzymatic staining of the oocytes.
4. The cover glasses with the agarose droplets are rinsed overnight in ice-cold Pi buffer in the dark. This washing step is crucial and should last at least 10-15 hrs. During this step excess of NBT and PMS diffuse out of the cells and agarose.

TABLE 1: Composition of the reaction mixtures for cytochemical staining of SDH-activity in primary mouse oocytes

	control	cytochemical staining of SDH activity	inhibition of SDH with malonate
NBT[1)]	1 mg/ml	1 mg/ml	1 mg/ml
Sodium succinate[2)]	-	50 mM	50 mM
Sodium malonate[2)]	-	-	50 mM
NaCl	130 mM	55 mM	-
HEPES buffer[3)]	10 mM	10 mM	10 mM
Calcium chloride[3)]	1 mM	1 mM	1 mM
Potassium cyanide[3)]	1 mM	1 mM	1 mM
PMS[4)]	50 µg/ml	50 µg/ml	50 µg/ml

Footnotes: 1) NBT (nitro blue tetrazolium, Sigma; grade III, N-6876) was added from a stock solution of 5 mg NBT/ml in a mixture of 1 part dimethylformamide and 1 part absolute ethanol or pure dimethylformamide.

2) Sodium succinate (BDH; no. 30219) and sodium malonate (Merck; no. 6527) were added from stock solutions in deionized water

3) 20 mM Calcium chloride ($CaCl_2$, Merck; no. 2382) is dissolved in 100 mM 2-(4(2hydroxyethyl)-1-piperazinyl)-ethansulfonacid (HEPES, Merck; no. 10110) pH 7.4 in deionized water; 20 mM potassium cyanide (KCN, Merck; no. 4967) is dissolved in 100 mM HEPES pH 7.4. The $CaCl_2$ solution and the KCN solution are mixed (1:1) before adding to the incubation mixture. A precipitate is formed in otherwise prepared Hepes buffers.

4) PMS (N'-methylphenazonium methosulphate, BDH; no. 24014) was added from a stock solution of 5 mg/ml in deionized water.

All stock solutions were prepared shortly before use.

After cytochemical staining oocytes were fixed in 0.1 % glutaraldehyde (Merck; no. 820603, Darmstadt, FRG) in Pi buffer for 20 min at 4 °C. Thus stained and fixed oocytes were then collected from the agarose, transferred to slides and mounted in DAPI (4,6-diamidino-2-phenylindole, Sigma; D-1388) 10 µg/ml in Pi buffer, pH 7.4. DAPI fluorescence was observed with a Zeiss fluorescence microscope (filter combination 487701).

Preparation of cryosections

Mouse oocytes cytochemically stained for SDH activity were embedded in 15% gelatine (Merck; 4078) in Pi buffer; gelatine blocks containing oocytes were frozen in liquid N_2 and 8 µm sections were cut on a cryostat (Reichert-Jung, Heidelberg, FRG) at -20 °C.

RESULTS

Fig. 1a through Fig. 1j show representative micrographs of oocytes cytochemically stained for SDH activity in preovulatory mouse oocytes. The reduction of NBT in the absence of substrate (succinate) was nihil if the oocytes were incubated in 10 mM caffeine prior to staining (Fig. 1a). In the presence of succinate the cytoplasm of the oocyte was stained intensely (Fig. 1e) and the dehydrogenase activity was inhibited in the presence of the competitive inhibitor of SDH, malonate (Fig. 1b). A preincubation in 10 mM caffeine was chosen because in oocytes incubated without caffeine a formazan precipitate was formed in the absence of succinate (Fig. 1c) and less formazan was produced in the presence of succinate (Fig. 1d) compared to the amount of formazan produced in oocytes treated with caffeine before the cytochemical staining (Figs. 1e-j). The caffeine treatment gave better results with respect to nonspecific reduction of tetrazolium than freezing and thawing of the cells (not shown).

Preovulatory oocytes were heterogeneous with respect to the distribution of formazan precipitate after staining for SDH activity: in some oocytes the precipitate was formed in fine grains (Fig. 1e), in others the grains were coarser (Figs. 1g and 1i). The formation of coarser grains was correlated with an altered chromatin organization (Figs. 1f, 1h and 1j), namely disappearance of the nucleolus and/or condensation of chromatin. This correlation is shown in detail in Table 2. We classified the cytochemically stained oocytes with respect to the clustering of formazan precipitate (class 1 = fine grains to class 4 = coarse grains) and also scored the chromatin organization of each oocyte (class I = chromatin organized into an intact germinal vesicle and class II = disappearance of the nucleolus and/or chromosome condensation). The mean class for formazan clustering was 1.9 ± 0.7 for oocytes with intact germinal vesicles

(class I), and 2.6 ± 0.8 for oocytes which showed chromatin condensation (class II). This difference is significant at the 0.1% level (t = 5.9, Student's t test, Snedecor and Cochran, 1967).

In order to find out whether the distribution of formazan precipitate correlates with the distribution of mitochondria, we also performed the cytochemical staining for SDH activity after maturation *in vitro* of mouse oocytes with intact germinal vesicles. Before maturation, the formazan precipitate is formed throughout the cytoplasm as shown in cryosections of cytochemically stained oocytes (Fig 2a). After 6 hrs of maturation *in vitro* most of the oocytes have proceeded to metaphase I (Fig. 2d). In a small fraction of these cells the SDH activity is concentrated in a sphere around the condensed chromosomes (Fig. 2d). These observations are in good agreement with those described by Van Blerkom and Runner (1984) with respect to the distribution of mitochondria in maturing oocytes.

Thus in maturing follicular oocytes an altered chromatin organization is accompanied with at least one aspect of cytoplasmic organization, namely the distribution of SDH activity, and thus of mitochondria.

TABLE 2: The relation between chromatin organization and the clustering of formazan precipitate in mouse oocytes, cytochemically stained for SDH activity.

		Formazan clustering				
	no. of oocytes	class 1 (fine) n (%)	class 2 n (%)	class 3 n (%)	class 4 (coarse) n (%)	mean ± s.d.
Class I: oocytes with an intact germinal vesicle	102	30 (29)	55 (54)	14 (14)	3 (3)	1.9 ± 0.7
Class II: oocytes with altered chromatin organization[1)]	87	3 (3)	40 (46)	31 (36)	13 (15)	2.6 ± 0.8

Footnote: 1. oocytes which showed chromatin condensation and/or disappearance of the nucleolus.

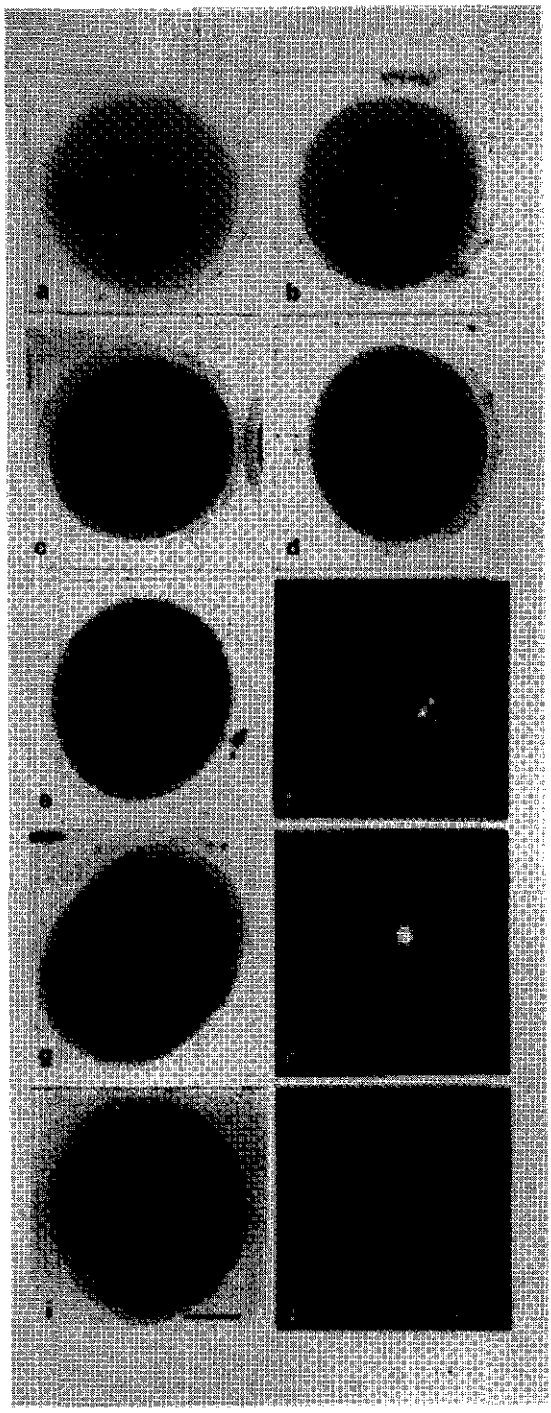

Figure 1. Representative micrographs of preovulatory oocytes of the mouse with intact germinal vesicles after cytochemical staining for succinate dehydrogenase activity. The oocytes were embedded in low gelling temperature agarose and treated with or without caffeine (a-b and e-j: 10 mM in Pi buffer and c-d: Pi buffer only) before the cytochemical staining. (a) This oocyte was treated with 10 mM caffeine prior to the cytochemical staining. No formazan is formed during the incubation in absence of substrate (succinate) for SDH. (b) This oocyte was treated with 10 mM caffeine prior to cytochemical staining in the presence of substrate (succinate) and a competitive inhibitor (malonate). (c) This oocyte was treated with Pi buffer only prior to cytochemical staining in the absence of substrate for SDH. Formazan is formed as a result of non-SDH activity. (d) This oocyte was treated with Pi buffer only prior to cytochemical staining in the presence of substrate. (e-f) Caffeine treatment was performed prior to cytochemical staining in the presence of succinate. The oocyte is intensely stained with fine grains of formazan (e) and demonstrates an intact germinal vesicle after DAPI staining (f). (g-j) These oocytes were treated with caffeine prior to the cytochemical staining in presence of succinate. Chromatin clustering (h) or chromosome condensation (j) is accompanied with the formation of coarse grains of formazan precipitate (g-i). Bars represent 30 μm.

DISCUSSION

In this report we present a cytochemical procedure for the demonstration of SDH activity in intact oocytes from the mouse. The procedure involves embedding of unfixed intact oocytes in an isotonic matrix of agarose, and permeabilization of cellular and mitochondrial membranes with caffeine prior to cytochemical staining. The embedding in isotonic agarose has the advantage that several oocytes can be carried together through the staining procedure, and that fixation, which interferes with SDH cytochemistry (Hansen and Andersen, 1983), is not necessary. However, if fixation is omitted, the oocyte and mitochondrial membranes are impermeable to NBT as well as PMS (Hale and Wenzel, 1978). We found that in oocytes treated with caffeine prior to the cytochemical staining the reduction of tetrazolium to formazan occurred at a higher level than in oocytes pre-incubated in phosphate buffer only. Also, caffeine treatment of the oocytes resulted in a very low level of reduction of tetrazolium in the absence of succinate. Caffeine inhibits, among other effects, Ca^{2+}-dependent ATPase (Nath and Rebhun, 1976) and probably affects the permeability of the inner mitochondrial membrane (GM Alink, personal communication). We have also tried to permeabilize the oocyte membranes by freezing on dry ice. However, in contrast to Vivarelli et al. (1976) we found that frozen and thawed oocytes had a high level of apparent dehydrogenase activity in the absence of added substrate (not shown). Another procedure to permeabilize oocyte membranes is the incorporation of oocytes in a polyacrylamide matrix. This method has been used successfully to determine cytochemically the activity of cytoplasmic enzymes involved in carbohydrate metabolism (De Schepper et al., 1985). However, because of the high osmolarity of the monomer acrylamide solution, this procedure can only be applied after fixation of the oocytes (Van Noorden et al., 1982).

Under our experimental conditions of SDH staining the oocytes are well preserved. The conformation of chromatin could still be analyzed in cells cytochemically stained for SDH activity, and the location of formazan precipitate was in good agreement with the location of mitochondria in matured oocytes (Van Blerkom and Runner, 1984).

The intrafollicular environment of atretic antral follicles does not sustain meiotic arrest in oocytes. Oocytes in these follicles could undergo premature nuclear maturation as shown by the disappearance of the nucleolus and chromosome condensation (Centola, 1983 and Himelstein-Braw, 1976). However, it is not known if this premature nuclear maturation is

accompanied by premature cytoplasmic maturation. Our results demonstrate a coarser formazan precipitate and therefore clustering of mitochondria in mouse oocytes which showed premature chromatin clustering (Figs. 1g and 1h) or chromosome condensation (Fig. 1i and 1j) in presumed pyknotic nuclei. Apparently in preovulatory mouse oocytes clustering of mitochondria is accompanied by an altered chromatin organization.

In conclusion, preovulatory mouse oocytes could be cytochemically stained for SDH activity after incorporation into low gelling temperature agarose and treatment with caffeine. This method offers a cytochemical procedure to study mitochondrial enzyme activity in individual oocytes in relation to the organization of chromatin.

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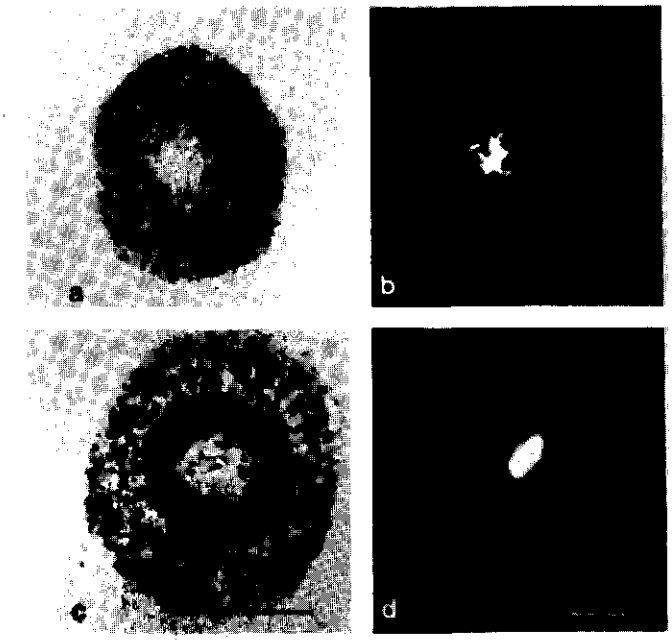

Figure 2. Cryosections of mouse oocytes before (a-b) and after (c-d) 6 hrs maturation in vitro. The oocytes were cytochemically stained for SDH activity, subsequently sectioned and stained with DAPI for chromatin localization. (a-b) A primary oocyte with an intact germinal vesicle. The formazan is distributed uniformly over the cytoplasm. After maturation to metaphase of the first meiotic division, the formazan is located in the perinuclear region (c-d). Bars represent 30 μm.

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CHAPTER VI

GENERAL DISCUSSION

The present study deals with developmental aspects of the first cell cycle as a function of the post-ovulation age of mouse oocytes prior to fertilization. The use of LHRH to induce ovulation and artificial insemination provided us with tools to study the effect of delayed insemination on the timing of fertilization events.

Several molecular and structural alterations occur in post-ovulatory aged oocytes and zygotes derived from them. In mouse secondary oocytes aged for 24 hrs a change in the carbohydrate composition of the plasma-membrane has been noted (Longo, 1981). Also, the organization of the cytoskeleton changes after post-ovulatory ageing (Webb et al., 1986; Eichenlaub-Ritter et al., 1986). Two examples of structural alterations are shown in Fig. 1: Figs. 1a and b are light micrographs, taken with Nomarski optics, of zygotes from unaged oocytes (Fig. 1a) and oocytes aged post-ovulation *in vivo* for 12 hrs (Fig. 1b). The zygotes from aged oocytes have several cytoplasmic bubbles on their surface (Fig. 1b, arrows), whereas those from unaged oocytes have not (Fig. 1a). In a preliminary electron microscopical study, we found an ageing related accumulation of granular material within the perivitelline space (Fig. 1d), whereas the perivitelline space of zygotes from unaged oocytes was clear (Fig. 1c). The nature of this granular material is not known. It has been suggested that cortical granules are discharged prematurely in the perivitelline space during post-ovulatory ageing of hamster oocytes (Longo, 1974). To our knowledge, premature discharge of cortical granules has not been described earlier in oocytes of the mouse.

In the present study, the consequences of delayed fertilization *in vivo* for embryonic development were our main interests. Oocytes post-ovulatory aged for 12 hrs could be fertilized *in* vivo, but we found a clear effect on the timing of several processes activated by fertilization. The timing of the morphological changes after fertilization was analyzed in Chapter II and, of the changes in protein synthetic patterns in Chapter III. Several of these changes appear to be accelerated after delayed fertilization, whereas retardation was not found in any of the processes studied. After delayed insemination, sperm penetration was accelerated by

1 hr 30 min compared with sperm penetration in unaged oocytes (Chapter II). The rate of sperm penetration can be influenced by: (1) the oviductal environment, (2) the cumulus mass, (3) the zona pellucida and (4) the perivitelline space and the oocyte membrane. For reasons discussed in Chapter II, it is unlikely to expect that post-ovulation alterations in the oviduct, cumulus mass or zona pellucida are the first responsible causes for acceleration of sperm penetration after delayed fertilization. Post-ovulation changes in the composition of the oocyte membrane and\or the perivitelline space could influence the rate of sperm penetration. It would be of interest to investigate the role of the post-ovulatory changed oocyte membrane and perivitelline space in the fusion of sperm and oocyte membranes during fertilization.

The rate of progression to the first cleavage division was also accelerated by the post-ovulation age of mouse oocytes prior to fertilization: penetrated aged oocytes needed less time (1 hr 30 min) to reach the 2-cell stage than zygotes from unaged oocytes. This could be an indication that oocytes aged post-ovulatory have lost their control over the meiotic arrest and the regulation of the first cell cycle. This is supported by the notion of the easy parthenogenetic activation of aged oocytes (O'Neill and Kaufman, 1988). Evidence for the accelerated development of zygotes from aged oocytes came also from the study of fertilization dependent changes in the protein synthetic patterns (Chapter III). This was particularly clear for the synthesis of a group of proteins with molecular weights of about 35 kDa, which represent the major newly synthesized polypeptides in secondary oocytes and zygotes. The individual proteins of the 35 kDa complex are probably the same polypeptide, phosphorylated to different extends (Howlett, 1986). One distinguishes upper (u)-, middle (m)- and lower (l)- band 35 kDa proteins. We found that a large fraction (38.3%) of the early pronuclear zygotes from aged oocytes synthesized (l)-band 35 kDa proteins; normally these proteins appear in mid- to late pronuclear zygotes from unaged oocytes. We conclude that first cell cycle dependent modifications of the 35 kDa complex appear to become separated from the major morphological changes triggered by fertilization. Thus, post-ovulatory ageing prior to fertilization results in an accelerated development and decoupling of the sequence of some fertilization events.

For several reasons we assume that the proteins of the 35 kDa protein complex play an important role in processes involved in the progression of the first cell cycle of mouse oocytes: (1) the 35 kDa proteins are

phosphorylated during the metaphases of the second meiotic division and the first cleavage division. (2) Early pronuclear zygotes from aged oocytes, which showed an accelerated progression to the first cleavage division, synthesized (1)-band 35 kDa proteins. These proteins are synthesized later in the pronuclear stage of zygotes from unaged oocytes (Chapter III and Howlett and Bolton, 1985). (3) Phosphorylation of (1)-band 35 kDa proteins is absent in zygotes from BALB/c females arrested in G_2-phase after X-irradiation of early pronuclear zygotes (Grinfeld et al., 1988). We suggest that studies concerning post-ovulatory ageing could give more insight in the functional role of the 35 kDa proteins.

Most studies concerning protein synthesis by cleavage stage embryos were performed with fertilized superovulated oocytes (Cullen, 1980; Van Blerkom, 1981; Howlett and Bolton, 1985). As a control we studied the pattern of proteins synthesized by superovulated oocytes and early pronuclear zygotes derived from them. The overall pattern of proteins synthesized by LHRH induced and superovulated oocytes was similar. However, a fraction (16.7%) of morphologically normal zygotes from unaged superovulated oocytes did not synthesize the 35 kDa protein complex at all. In view of the possible role of 35 kDa proteins during the progression of the first cell cycle, care should be taken when interpreting studies of timing of development in zygotes derived from superovulated oocytes.

Zygotes and embryos from aged oocytes showed a higher sensitivity to DNA insults than embryos from unaged oocytes (Chapter IV). Fertilization with X-irradiated spermatozoa led to an arrest at interphase in 27% and 7% of zygotes from aged and unaged oocytes respectively (Chapter IV). From the experiments in Chapter IV we tentatively concluded that post-ovulatory ageing affected the chromatin structure of the male as well as the female pronuclear chromosomes. The results suggest that factors involved in the formation of first cleavage metaphase chromosomes alter during the post-ovulatory ageing period. The ageing related alterations of these factors could interact with DNA-lesions induced by X-rays in the spermatozoon prior to fertilization, such that the progression to the first cleavage division is blocked. It would be of interest to investigate (1) if the 35 kDa phosphoprotein complex plays a role in the condensation of first cleavage chromosomes and (2) if the premature modifications of the 35 kDa proteins in zygotes from aged oocytes influence the chromatin structure.

Cell proliferation of late 2-cell embryos from aged oocytes, collected 36 hrs post-insemination, was clearly retarded and asynchronous during the 24 hrs culture period in the presence of 10^{-6} M BrdU (Chapter IV). Apparently, the development of cleavage stage embryos from aged oocytes is more sensitive to DNA insults (X-ray induced modifications, BrdU substitution) than the development of embryos from unaged oocytes. We would like to suggest that this sensitivity concerns the chromatin structure and not the integrity of the DNA helix, because post-ovulatory ageing prior to fertilization affected neither the percentage of chromosome abnormalities in oocytes fertilized with X-irradiated spermatozoa, nor the number of SCEs per metaphase during the 4th cleavage division (Chapter IV). The post-ovulation altered chromatin structure could influence DNA replication and transcription.

In summary, in the present study we have shown that zygotes from aged oocytes seem to catch up with the normal developmental schedule, with respect to major fertilization dependent morphological and protein synthetic changes. This accelerated development of cleavage stage embryos from aged oocytes had no significant effect on embryonic development up to the 8-cell stage in the absence of DNA insults. However, in the presence of DNA insults the developmental capacity of embryos from aged oocytes was impaired. It would be of interest to analyze the timing of embryonic genome activation in embryos from aged oocytes. Translational activity in 2-cell embryos appears to be essential for normal development up to the 8-cell stage including events concerned with the cell-contact-induced polarization of blastomeres (Johnson et al., 1984; Kidder and McLachlin, 1985). Studies of timing of DNA replication activity and of embryonic genome activation could possibly provide an insight into the causes of poor embryonic development after delayed fertilization.

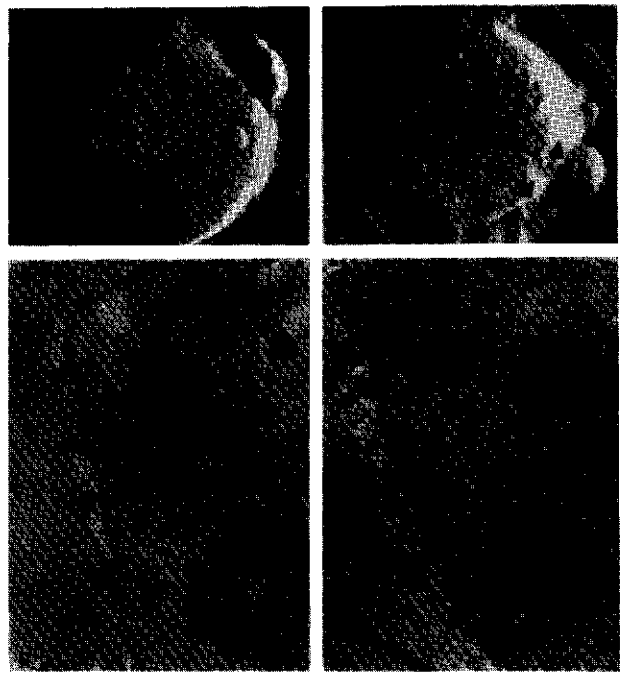

Figure 1.

a. Light micrograph of an unfixed early pronuclear zygote from an unaged oocyte collected 7 hrs post-insemination (Nomarski optics, bar represents 30 µm). Arrow head indicates the second polar body.

b. Light micrograph of an unfixed pronuclear zygote from an aged oocyte collected 5 hrs post-insemination (Nomarski optics, bar represents 30 µm). Cytoplasmic bubbles can be seen (arrow). Arrow head indicates the second polar body.

1c. Electron micrograph of pronuclear zygote from an unaged oocyte collected 7 hrs post-insemination (10440x). The perivitelline space is clear (arrow).

1d. Electron micrograph of pronuclear zygote from an aged oocyte collected 5 hrs post-insemination (10440x). The perivitelline space is filled with granular material (arrow).

Frans de Loos, Dept. of Herd Health and Reproduction, Utrecht University, kindly produced the electron micrographs from the zygotes from aged and unaged oocytes. Methodology according to: de Loos F et al., 1989.

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SAMENVATTING

Dit proefschrift beschrijft de resultaten van experimenten uitgevoerd met bevruchte verouderde eicellen. In de natuurlijke situatie zal het sperma reeds voor de ovulatie in de eileider aanwezig zijn, omdat zoogdieren, behalve de mens, een duidelijk bronstgedrag rondom het moment van ovulatie vertonen. De eicel zal dan ook spoedig na ovulatie in de eileider bevrucht worden, waarna de embryonale ontwikkeling start. Als het sperma te laat in de eileider aankomt veroudert de eicel voordat bevruchting plaats vindt. De mens vertoont geen bronstgedrag, het sperma zal in sommige gevallen de eileider pas na de ovulatie bereiken, met als gevolg dat de de eicel te laat bevrucht wordt. Door het toenemend gebruik van kunstmatige inseminatie in de landbouwkundige en de humane praktijk, is de kans dat verouderde eicellen bevrucht worden toegenomen. Toepassing van kunstmatige inseminatie vereist namelijk een nauwkeurige kennis van het ovulatie moment, zodat het sperma de eileider bereikt voordat ovulatie plaatsvindt. De verouderde eicel, mits niet te oud, kan nog wel bevrucht worden maar de embryonale ontwikkeling verloopt afwijkend, zoals uit het vervolg van deze samenvatting zal blijken.

De in dit proefschrift beschreven experimenten zijn steeds vergelijkingen tussen bevruchte niet verouderde eicellen en eicellen die na de ovulatie zijn verouderd voordat ze bevrucht werden. Een voorwaarde voor de bestudering van aspecten van eicelveroudering is een exacte kennis van het ovulatiemoment. Dit doel werd na enig ontwikkelingswerk bereikt en de resultaten hiervan zijn in hoofdstuk II terug te vinden. Bij de muis is de dag van ovulatie door middel van vaginale uitstrijkjes vast te stellen. Bij de muis ligt het moment van ovulatie vast ten opzichte van het middelpunt van de nacht (donkerperiode): bij Swiss 'outbred' vrouwelijke muizen vindt de ovulatie plaats tussen 3 uur 45 min en 5 uur 15 min na het middelpunt van de nacht (hoofdstuk II). De piek van het ovulatie inducerende luteiniserende hormoon (LH), die 12 uur aan de ovulatie vooraf gaat, valt dan 7-8 uur voor het middelpunt van de nacht. Van deze kennis is gebruik gemaakt bij het opstellen van een werkzaam model voor de bestudering van eicelveroudering: een injectie met luteinizerend hormoon releasing hormoon (LHRH, luteinizerend hormoon vrijmakend hormoom) 8-12 uur voor de verwachte endogene LH piek zorgde ervoor dat in ons systeem de ovulatie plaatsvond op een door ons gekozen moment. De methode van ovulatie inductie met behulp van LHRH blijkt geen significant nadelig

effect te hebben op de embryonale ontwikkeling na de implantatie, gemeten op dag 13 van de dracht (dag van dekking is dag 1, hoofdstuk II). Het gemiddeld aantal geovuleerde eicellen is vergelijkbaar met dat na spontane ovulatie (8-12 per vrouwtje, hoofdstuk II).

De ovulatie vindt tussen 12 en 13 uur na de LHRH injectie plaats. Wij hebben daarom het moment van de kunstmatige inseminatie als volg gekozen: 13 uur na de LHRH injectie voor de bevruchting van niet verouderde, en 24 uur na de injectie voor de bevruchting van verouderde eicellen. Toepassing van kunstmatige inseminatie was om twee redenen noodzakelijk: (1) om het moment van introduceren van het sperma nauwkeurig te bepalen en (2) omdat de vrouwtjes 12 uur na de ovulatie geen bronstgedrag meer vertonen en daarom niet meer door mannetjes worden gedekt.

Definitie: embryo's van niet verouderde eicellen zijn verzameld uit vrouwtjes die 13 uur na de LHRH injectie zijn geinsemineerd en embryo's van verouderde eicellen zijn verzameld uit vrouwtjes die 24 uur na de LHRH injectie zijn geinsemineerd.

De resultaten van de studie naar de gevolgen van eicelveroudering voor de vroeg embryonale ontwikkeling kunnen als volgt puntsgewijs worden samengevat:

1. Een-cellige embryo's van 12 uur verouderde eicellen hebben, na fixatie met Heidenhain's fixatief en kleuring met aceto-orcein, licht microscopisch een uiterlijk dat vergelijkbaar is met dat van een-cellige embryo's van niet verouderde eicellen (hoofdstuk II). Cytoplasma blaasjes zijn zichtbaar als zygoten van 12 uur verouderde eicellen met de Nomarski optiek bekeken worden, deze blaasjes zijn niet zichtbaar in embryo's van niet verouderde eicellen.
2. Is de periode tussen ovulatie en inseminatie langer dan 12 uur, dan zien bevruchte verouderde eicellen er niet meer normaal uit. Zeer oude eicellen hebben na bevruchting vaak een vergroot tweede poollichaampje (hoofdstuk II).
3. Bevruchte verouderde eicellen ontwikkelen zich *in vivo* tot twee-cellige embryo's (hoofdstukken II en IV).
4. In embryo's van verouderde eicellen is de morfologie van de metafase chromosomen van de eerste klievingsdeling significant minder mooi dan de morfologie van de metafase chromosomen van embryo's van niet verouderde eicellen (hoofdstuk IV).
5. Twee-cellige embryo's van verouderde eicellen, verzameld 36 uur na de

inseminatie, groeien gedurende een kweek van 24 uur in een tempo dat vergelijkbaar is met dat van 2-cellige embryo's van niet verouderde eicellen (hoofdstuk IV).

6. Embryo's van verouderde eicellen, verzameld 30 uur na de inseminatie, ontwikkelen zich slecht gedurende een 66-urige kweek, terwijl de embryo's van niet verouderde eicellen, ook 30 uur na de KI verzameld, zich goed ontwikkelen gedurende een 66-urige kweek (hoofdstuk IV).
7. Verouderde eicellen worden sneller bevrucht dan niet verouderde eicellen: 1 uur en 45 min na de inseminatie is 50% van de verouderde eicellen reeds bevrucht. Voor de niet verouderde eicellen is dit percentage bereikt 3 uur en 15 min na de inseminatie (hoofdstuk II).
8. De verouderde eicellen maken na de bevruchting de eerste celcyclus sneller af: 17 uur en 30 min na de inseminatie van de vrouwtjes die verouderde eicellen dragen is 50% van de zygoten reeds in het 2-cel stadium. In de groep van niet verouderde bevruchte eicellen wordt dit percentage bereikt na 20 uur en 30 min (hoofdstuk II). Deze versnelde ontwikkeling van bevruchte verouderde eicellen wordt bevestigd door het tweemaal hogere percentage (14% vs 7%) 3- en 4-cellige embryo's 36 uur na de inseminatie verzameld, vergeleken met het percentage 3- en 4-cellige embryo's van niet verouderde eicellen, eveneens 36 uur na de inseminatie verzameld (hoofdstuk IV).
9. Bevruchte eicellen van zowel verouderde als niet verouderde eicellen synthetiseren eiwitten. Na gelelektroforese is het patroon van deze gesynthetiseerde eiwitten voor beide proefgroepen vergelijkbaar. De beide proefgroepen synthetiseren relatief veel eiwitten met een molecuul gewicht van ongeveer 35 kDa (het 35 kDa complex) (hoofdstuk III).
10. Het 35 kDa eiwitcomplex bestaat uit een upper (u)-, middle (m)- en een lower (l)-band: de (u)- en (m)- band eiwitten worden door onbevruchte en pas bevruchte eicellen gesynthetiseerd. In een later stadium van de ontwikkeling, maar voordat eerste klievingsdeling plaats vindt, maken een-cellige embryo's de (l)-band eiwitten (hoofdstuk III).
11. 38% van de een-cellige embryo's van verouderde eicellen, die zich vroeg in het pronucleus stadium bevinden (5 uur na de inseminatie verzameld) synthetiseren de (l)-band van het 35 kDa eiwitcomplex. Deze vorm van het 35 kDa complex wordt door jonge zygoten (7 uur na de inseminatie verzameld) van niet verouderde eicellen nog niet gemaakt (hoofdstuk III).

12. Na bevruchting met X-ray bestraald sperma, groeit 27% van de een-cellige embryo's van verouderde eicellen niet door tot het twee-cel stadium. Voor embryo's van niet verouderde eicellen is dit percentage 7% (hoofdstuk IV).
13. De mannelijke pronucleus chromosomen in een-cellige embryo's van verouderde eicellen bevrucht met Röntgen bestraald sperma hebben een slechte morfologie (hoofdstuk IV).
14. In één-cellige embryo's van verouderde en niet verouderde eicellen, bevrucht met X-ray bestraald sperma, is het aantal chromosomen met zichtbare schade niet significant verschillend (hoofdstuk IV).
15. In een 24 uurs kweek, in aanwezigheid van de thymidine analoog 5-Bromodeoxyuridine (BrdU), groeien 2- cellige embryo's van verouderde eicellen, verzameld 36 uur na de inseminatie, slechter dan twee-cellige embryo's van niet verouderde eicellen (hoofdstuk IV).
16. Het aantal zuster chromatide uitwisselingen tijdens de 4de klievings-deling is niet significant verschillend in embryo's van verouderde en niet verouderde embryo's (hoofdstuk IV).
17. Er is een cytochemische kleuringsmethode voor het aantonen van de activiteit van het mitochondriale enzym succinaat dehydrogenase in individuele eicellen uit het ovarium is ontwikkeld (hoofdstuk V). Met behulp van deze methode kan de localisatie van de mitochondrien in ovariele eicellen aangetoond worden. Helaas bleek deze methode niet bruikbaar voor het aantonen van SDH activiteit in geovuleerde en bevruchte eicellen.

DANKWOORD

Het onderzoek waarvan de resultaten in dit proefschrift zijn samengevat is uitgevoerd op de Vakgroep Erfelijkheidsleer in het kader van de werkgroep Vroege Dracht van de Landbouwuniversiteit Wageningen. Velen hebben hun steentje bijgedragen aan het uitvoeren van de experimenten en interpretatie van de resultaten.

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Frans de Loos, Universiteit van Utrecht, bedankt voor de stimulerende discussies over ons beider werk en het uitvoeren van een beperkte aanvullende electronenmicroscopische studie.

De gastvrijheid van de medewerkers van de sectie Ontwikkelingsbiologie van de vakgroep Experimentele Diermorfologie en Celbiologie en de aanwezigheid van een juist uitgerust isotopenlaboratorium hebben het mogelijk gemaakt dat ik embryokweek experimenten met radioisotopen uit kon

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Christa Heyting is in laatste instantie bij het nu gepresenteerde onderzoek betrokken. Samen met Peter de Boer heeft zij veel tijd besteed aan de redactie van dit proefschrift. Ik ben hen beide hiervoor zeer erkentelijk.

Tot slot wil ik alle medewerkers van de Vakgroep Erfelijkheidsleer bedanken voor de plezierige werksfeer.

CURRICULUM VITAE

Marleen Boerjan werd op 13 juni 1951 geboren in Oostburg, West-Zeeuws Vlaanderen. Na het behalen van het HBS-B diploma in 1968 werd een opleiding tot klinisch-chemisch analiste begonnen in Brugge, Belgie. In 1971 werd deze opleiding met lof afgesloten. Van 1971 tot 1977 was zij als analiste in dienst van de Landbouwhogeschool, nu Landbouwuniversiteit Wageningen geheten. In 1975 werd aangevangen met de studie moleculaire wetenschappen aan deze hogeschool. In 1983 werd deze studie met lof afgesloten, de doctoraalvakken waren: entomologie, biochemie, erfelijkheidsleer en wiskundige statistiek.

In 1983 kwam zij in tijdelijke dienst van de vakgroep Erfelijkheidsleer van de Landbouwuniversiteit Wageningen. Tot 1986 werd onderzoek naar kriteria voor de beschrijving van de kwaliteit van muize-embryo's verricht in het kader van kortlopende projekten. In 1986 werd een aanvraag voor een promotieonderzoek, getiteld "Een onderzoek naar kwaliteitskriteria voor peri-ovulatoire oocyten en jonge embryonen" gehonoreerd.

Sinds november 1989 is zij in tijdelijke dienst van de sectie Ontwikkelingsbiologie van de vakgroep Experimentele Diermorfologie en Celbiologie van de Landbouwuniversiteit Wageningen.