Human in-vitro gamete interaction analysed by DNA-fluorescence

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PROEFSCHRIFT

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CONTENTS

SUMMARY

Fertilization after assisted reproductive technologies such as in vitro fertilization (IVF), subzonal insemination (SUZI) and intracytoplasmic insemination (ICSI) is rarely 100% successful. Therefore, in this study we focus on fertilization failure and abnormal embryo development after IVF. Unfertilized oocytes, delayed zygotes, zygotes which interrupted their development and embryos which were unsuitable for transfer, were generally obtained 48 h after in-vitro insemination. The intra- and extracellular DNA-containing structures (cell-nuclei and spermatozoa adherent to the zona pellucida) were studied in-vitro, after incubation with the DNA fluorescent dye Hoechst 33342. Some of the oocytes (n=26) observed by fluorescence microscopy were subsequently fixed and sectioned for classical light microscopy to allow parallel observations.

In Chapter II, the following observations were discussed: sperm penetration in both mature and immature oocytes, decondensation of spermheads, premature condensation of male chromatin, polyspermy, pronucleus formation and ageing processes of the oocyte such as the centripetal migration of the metaphase II chromosomes, the formation of a restitution nucleus and the lagging of chromosomes within the metaphase spindle. The clinical use of these findings was demonstrated in Chapter III. Four main causes contributed to the low fertilization and cleavage rate (0 to 20%) for 10 patients: (1) sperm incapacity, (2) oocyte immaturity, (3) delayed fertilization, and (4) oocyte abnormalities revealed by aberrations in the morphology of female chromatin. The number of spermatozoa tightly bound to or partly penetrated into the zona pellucida was positively related to the presence of sperm chromatin inside the ooplasm but without pronucleus formation. Furthermore, both were positively related to the incidence of fertilization of cohort oocytes. In some cases, sperm-zona binding and sperm penetration were dissociated, sperm-zona binding was successful but not penetration, and vice versa. This dissociation may be due to particular sperm or zona pellucida defects (Chapter IV).

Out of 440 unfertilized oocytes or zygotes analysed, the majority of oocytes was at metaphase II (63.4%), 14.8% were immature (at germinal vesicle or metaphase I stage), 7.0% were activated, 6.8% had pronuclei and 8.0% had an aberrant morphology of female chromatin or could not be analysed. Sperm penetration without pronucleus formation was frequently found in immature, mature and activated oocytes (23.6%). A subpopulation of functionally unfit oocytes was present when most cohort oocytes had cleaved; sperm chromatin was found inside the ooplasm without pronucleus formation. When none or few cohort oocytes had cleaved (with or without male infertility) and when infertility was of male origin, sperm penetration incapacity was demonstrated by the presence of post-meiotically aged oocytes without sperm chromatin inside the ooplasm (Chapter V). In general, these results indicate that oocyte and sperm quality can only be assessed after IVF if the fertilization outcome of cohort oocytes is taken into account.

The sequential transformations of human sperm nuclei in oocytes after SUZI (n=139) were compared to those of zygotes obtained after IVF with donor spermatozoa (D-IVF; n=220) by non-invasive observations. For most zygotes, pronuclear formation took place between 4.5 h and 10.5 h post-SUZI. They remained visible for ≈13 h and began to disappear 18.5 h post-SUZI. D-IVF zygotes had a similar rate of pronuclear disappearance but ≈4 h later. The second cell cycle was more rapid for zygotes obtained by D-IVF than by SUZI. The developmental rate of zygotes obtained by SUZI varied according to sperm phenotypes. For patients with unexplained fertilization failures and normal spermatozoa, the oocyte quality seemed implicated (Chapter VI).

From 33 out of 104 earlier described IVF cycles 67, 48 h old embryos were observed. Of these embryos 31.3% contained only single nucleated cells and 65.7% had at least one multinuclear blastomere (2 embryos were not analysable). The incidence of embryos with multinuclear blastomeres tended to be higher in cycles with mono- and polyspermic fertilization of cohort oocytes (1.7±1.7 versus 0.9±0.7 respectively). For these cycles, the number of oocytes retrieved and the monospermic fertilization rate were both significantly higher than for cycles with only monospermic fertilization. Multinucleation can result after the fertilization of oocytes which are unable to support normal nuclear and cell division because of immaturity or impaired quality. These oocytes are simultaneously retrieved with mature, fertilizable oocytes after ovarian stimulation (Chapter VII).

In conclusion, analysis of fertilization failures or abnormal embryo development after IVF as well as early embryo development after SUZI and D-IVF can give important information about the capacity of female and male gametes to interact in vitro and to generate viable embryos. This information allows to orient further infertility treatment toward the male, the female, or both partners.

SAMENVATTING

Bevruchting met behulp van in vitro technieken zoals in vitro fertilisatie (IVF), subzonale inseminatie (SUZI) en intracytoplasmatische inseminatie (ICSI), is zelden voor 100% succesvol. In deze studie hebben we ons zowel beziggehouden met de oorzaken voor het uitblijven van bevruchting als met abnormale bevruchtingsprocessen na IVF. Onbevruchte eicellen, vertraagde zygoten en zygoten gestopt in hun ontwikkeling evenals embryos die niet geschikt waren voor transplantatie, zijn meestal 48 uur na de in vitro inseminatie geobserveerd. De intra- en extracellulaire DNA bevattende structuren (celkernen en spermatozoïden gebonden aan de zona pellucida) zijn bestudeerd na in vitro incubatie met de DNA specifieke, fluorescerende kleurstof Hoechst 33342. Een aantal eicellen (n=26) is vervolgens gefixeerd en tot coupes verwerkt ten behoeve van klassieke licht-microscopie. Beide technieken leverden vergelijkbare waarnemingen op.

In Hoofdstuk II, worden de volgende bevindingen besproken: sperma penetratie in zowel rijpe als niet rijpe eicellen, decondensatie van spermakernen, vroegtijdige condensatie van spermachromatine, polyspermie, pronucleus formatie en verouderingsprocessen van de eicel zoals, centrale migratie van de metafase II chromosomen, de formatie van een restitutie nucleus en de dislocatie van individuele chromosomen. Van 10 patiënten (12 IVF cycli) met dergelijke waarnemingen zijn de klinische gevolgen beschreven. Vier oorzaken zijn gevonden voor de lage bevruchtings- en klievings-ratio (0-20%): (1) sperma van onvoldoende kwaliteit, (2) niet volledig gerijpte eicellen, (3) verlate bevruchting, (4) eicel afwijkingen die tot uiting komen in de morfologie van het chromatine (Hoofdstuk III).

Het aantal spermatozoa stevig gebonden aan of gedeeltelijk gepenetreerd in de zona pellucida, is gerelateerd aan de aanwezigheid van sperma chromatine in het eicel-cytoplasma (zonder pronucleus formatie). Bovendien waren beide factoren gerelateerd aan het wel of niet voorkomen van bevruchting in eicellen die tegelijk werden verkregen. In sommige gevallen zijn de sperma-zona adhesie en de sperma penetratie niet met elkaar gecorreleerd; zo was de sperma-zona adhesie successvol maar niet de sperma penetratie en vice versa. Deze dissociatie is mogelijk het gevolg van afwijkingen van de spermatozoa of van de zona-pellucida (Hoofdstuk IV).

In een serie van 440 onbevruchte eicellen en zygoten was de meerderheid van de eicellen in het metafase II stadium (63,4%), 14,8% was niet rijp (germinal vesicle stadium), 7,0% was geactiveerd, 6,8% had pronuclei en 8,0% had een abnormale morfologie van het eicelchromatine of was niet te analyseren. Sperma penetratie zonder pronucleus formatie werd vaak gevonden in niet rijpe, rijpe en geactiveerde eicellen (23,6%). Een subpopulatie van niet functionerende eicellen werd gevonden als de meerderheid van de tegelijk aanwezige eicellen bevrucht en na bevruchting gedeeld waren. Als geen, of slechts enkele van de tegelijk aanwezige eicellen bevrucht en gedeeld waren (met of zonder indicatie voor mannelijke onvruchtbaarheid) én als de onvruchtbaarheid van mannelijke oorsprong was, dan kan onvermogen tot sperma penetratie worden aangetoond door de aanwezigheid van post-

meiotisch verouderde eicellen, zonder sperma-chromatine in het eicel-cytoplasma (Hoofdstuk V). In het algemeen kan de eicel en sperma kwaliteit alleen worden geëvalueerd indien rekening gehouden wordt met de bevruchtingsresultaten na IVF van mede aanwezige eicellen.
De opeenvolgende transformaties van humane spermakernen in eicellen na SUZI (139 eicellen) werd vergeleken met die van zygoten verkregen na IVF met donor spermatozoïden (D-IVF, 220 eicellen) door middel van niet invasieve observaties. Voor de meeste zygoten vond pronucleus formatie tussen 4,5 uur en 10,5 uur post-SUZI plaats, de pronuclei bleven ongeveer 13 uur zichtbaar en begonnen vanaf 18,5 uur post-SUZI te verdwijnen. Pronuclei verdwenen met hetzelfde ritme in D-IVF zygoten maar dan ongeveer 4 uur later. De tweede cel-cyclus verliep sneller voor zygoten verkregen na D-IVF dan zygoten verkregen na SUZI. De ontwikkelingssnelheid van zygoten verkregen na SUZI hield verband met het sperma fenotype. Bij patiënten met niet te verklaren mislukkingen van de bevruchting met normaal sperma lijkt de eicel kwaliteit van invloed (Hoofdstuk VI).
Embryo's die niet geschikt waren voor transplantatie (67 embryo's), konden geobserveerd worden m.b.v. DNA fluorescentie. Deze embryo's waren afkomstig van 33 van de 104 eerder bestudeerde IVF cycli. Van de geobserveerde embryo's had 31,3% uitsluitend cellen met één enkele nucleus, 65,7% van de embryo's had minimaal één cel met meer dan één nucleus, en de twee resterende embryo's konden niet geanalyseerd worden. Embryo's met meerkernige cellen lijken vaker voor te komen als in eenzelfde cyclus mono- en polyspermie voorkomt dan wanneer alleen monospermie voorkomt ($1,7\pm1,7$ en $0,9\pm0,7$). In cycli met zowel mono- als polyspermische bevruchting worden hogere aantallen eicellen verkregen en een hogere monospermische bevruchtingsratio geconstateerd, dan in cycli met uitsluitend monospermische bevruchting. Meerkernigheid in cellen kan het gevolg zijn van de bevruchting van eicellen die ongeschikt zijn voor een normale kern- en celdeling veroorzaakt door onvoldoende rijping of onvoldoende kwaliteit en die gelijktijdig met rijpe, bevruchtbare eicellen zijn verkregen na een hormonale stimulatie van de ovaria (Hoofdstuk VII).
Tot conclusie; de analyse van onbevruchte eicellen en embryo's die na IVF ongeschikt zijn voor transplantatie evenals de analyse van vroege embryo-ontwikkeling na SUZI en D-IVF, kan belangrijke informatie leveren aangaande het vermogen van oocyten en spermatozoa tot interactie in vitro en om levensvatbare embryos te genereren. Deze informatie draagt ertoe bij dat de onvruchtbaarheidsbehandeling op de mannelijke of de vrouwelijke partner gericht kan worden.

ABBREVIATIONS

ART	Assisted Reproductive Technologies
CSF	Cytostatic factor
D-IVF	IVF with donor sperm
DNA	Deoxyribonucleic Acid
ET	Embryo Transfer
GnRHa	Gonadotrophin Releasing Hormone agonist
GV	Germinal Vesicle
GVBD	Germinal Vesicle Breakdown
HCG	Human Chorionic Gonadotrophin
ICSI	Intracytoplasmic Insemination
IVF	In Vitro Fertilization
LH	Luteinizing Hormone
MI	Metaphase I
MII	Metaphase II
MIII	Metaphase III
MNB	Multinuclear Blastomere
MPF	Maturation Promoting Factor
PB1	First Polar Body
PB2	Second Polar Body
PCC	Premature Condensed Male Chromosomes
PDS	Partially Decondensed Sperm
PN	Pronucleus or Pronuclei
PZD	Partial Zona Dissection
S-phase	Phase of DNA synthesis
SBZ	Sperm bound to or partly penetrated into the zona pellucida
SH	Sperm Head
SUZI	Subzonal Insemination

CHAPTER I

General Introduction and aims of the study

Contents chapter I:

Adapted from: B. van Wissen, O. Bomsel-Helmreich. "DNA fluorescence revealing oocyte quality, sperm behaviour during gamete interaction and embryo morphology" Published in: Assisted Reproduction Reviews 1994, Vol. 4, pp. 128-133.

INTRODUCTION

Human fertility has under natural conditions a low efficiency. The chance of conception during one menstrual cycle is approximately 25% (Mandelbaum et al, 1991). For an important part this low efficiency is due to embryonic or fetal loss, due to various causes such as chromosomal anomalies in early pregnancy or pathological complications (Plachot et al, 1991). Additionally, a certain number of couples suffer from sterility or infertility. The causes of infertility may be female, male, a combination of both or idiopathic (Table 1). Female infertility may be caused by defects occurring at different moments of the reproductive process, especially during follicular development including oocyte maturation.

Table 1. Sex distribution of causes of infertility, WHO (1993)

Causes of infertility	Percentage
Female factor only	41
Male factor only	24
Female and Male factors	24
No demonstrable cause	11

To bypass certain causes of infertility such as tubal obstruction, endometriosis, male infertility and infertility with unknown origin, which mainly hinder fertilization and early embryonic development, Assisted Reproductive Technologies (ART) are available. The first successfull in vitro fertilization in mammals, dates from 1952 and was performed in the rabbit (Chang, 1952). In vitro fertilization (IVF) and embryo transfer (ET), were first successfully applied in the human in 1978 when Louise Brown was born (Steptoe et al, 1980). Together with the development of many human IVF-ET programs, attention focused on cases where fertilization failed partly or completely after conventional IVF. For these cases, especially when infertility was due to male factors, more invasive technologies were developed, and subzonal insemination (SUZI; Ng et al, 1988) and especially intracytoplasmic insemination (ICSI; Palermo et al, 1992) are nowadays currently applied in IVF laboratories besides conventional IVF. Within the limits of ethical laws defined by each country these different ART opened the way for research on human oocytes which failed to fertilize, arrested zygotes and embryos not used for transfer to the uterine cavity of the patient. In this general introduction we focus on oocyte maturation, normal and abnormal fertilization processes and early embryo development, in vivo and in vitro. Many references are made to animal studies since these aspects of

the reproductive cycle have been extensively studied in non-human mammalians.

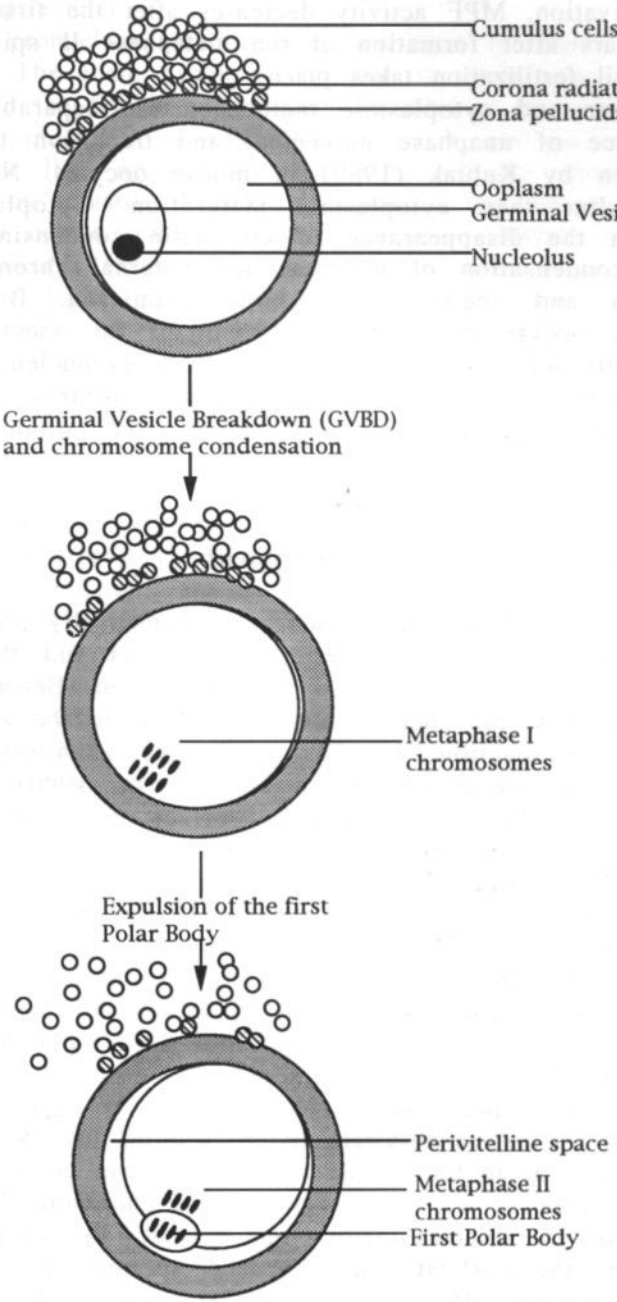
ASPECTS OF FERTILIZATION

Oocyte Maturation

To achieve fertilization, maturity of the oocyte at the time of sperm penetration is essential. In the majority of mammalian species, including the human, oocytes undergo the prophase stages of meiosis until diplotene, just before or shortly after birth. Chromatin decondenses into a dictyate state in which it remains for a long period until shortly before ovulation. The oocyte is characterized by the presence of a nucleus, called germinal vesicle (GV). Apparently this state is the most appropriate for "long conservation" of the DNA. During this period of apparent arrest, stage specific and coordinated modifications of nuclear and cytoplasmic components, characterize the transition to a meiotically competent state (Mattson et al, 1990; Wickramasinghe et al, 1991).

The oocyte in the GV-stage within the Graafian follicle resumes its meiosis after the luteinising hormone (LH) surge. In figure 1, stages of oocyte maturation are schematically presented. Nuclear maturation is characterized by a migration of the germinal vesicle towards the oocyte's periphery, the dissolution of the nuclear membrane (germinal vesicle breakdown GVBD) and the condensation of dictyate chromosomes into discrete bivalents. The homologous chromosomes separate and the first polar body containing a haploid set of chromosomes is released. The meiotic process stops at metaphase II until after fertilization. Meiotic inhibiting factors which may be responsible for the maintenance of the germinal vesicle stage were demonstrated in immature oocytes (Endo et al, 1990). Morphological changes in nuclear structures such as GVBD and chromosome condensation are induced by the Maturation Promoting Factor (MPF) first described by Masui and Markert (1971). MPF is a heterodimeric complex that contains a cyclin and the cdc 2 protein kinase. Phosphorylation and partial dephosphorylation of the protein kinase and a continuous equilibrium between cyclin synthesis and degeneration are responsible for the maintenance of high MPF activity during metaphase arrest (reviewed by Coleman and Dunphy, 1994). In the mouse, the mechanism maintaining the metaphase arrest is probably controlled by cytostatic factor (CSF), but is also dependent upon the three dimensional organisation of the spindle (Kubiak et al, 1993).

Figure 1: Schematic drawing of oocyte maturation



At the metaphase/anaphase transition, both the total dephosphorylation of the protein kinase and a rapid proteolysis of cyclin is associated with MPF inactivation. MPF activity decreases after the first meiotic division but reappears after formation of the metaphase II spindle and remains present until fertilization takes place.

Nuclear and cytoplasmic maturation are separable processes, the independence of anaphase movement and transition to interphase has been shown by Kubiak (1989) in mouse oocytes. Nuclear maturation occurs earlier than cytoplasmic maturation. Cytoplasmic maturation consists in the disappearance of chromatin condensing factors, which allows decondensation of maternal and paternal chromatin after sperm penetration and second polar body expulsion. Both nuclear and cytoplasmic oocyte maturation are important for oocyte activation after sperm penetration, and for female and male pronucleus formation. After in vivo oocyte maturation, the follicle ruptures and the mature, metaphase II -stage oocyte is expulsed into the fallopian tube: ovulation takes place.

Fertilization and early development

In figure 2, aspects of fertilization are schematically presented. Following meiotic maturation, the completion of meiosis and the release of the second polar body are triggered by fertilization. Before penetrating the oocyte vestments, the spermatozoon must bind to the zona and complete it's acrosomal reaction. In the mouse, the zona-glycoprotein ZP3 is responsible for sperm recognition (Wassarman, 1990). The sperm head immobilizes at the zona pellucida surface and subsequent action of acrosomal enzymes diminishes locally the resistance of the zona (reviewed by Tesarik, 1992). Both the sperm motility and the release of acrosomic enzymes facilitate the passage of the spermatozoon through the zona pellucida. Once in the perivitelline space the spermatozoon immobilizes on the oolemma after which fusion takes place. After sperm penetration the cortical granules situated in the oocyte periphery, are released preventing multiple spermatozoa to penetrate (Barros and Yanagimachi, 1971).

Inside the ooplasm of mature metaphase II stage oocytes, the sperm head starts successive re-arrangements leading to PN formation: in the mouse as well as in the human three phases can be described (Adenot et al, 1991; Lasalle et al, 1991). The paternal chromatin decondenses rapidly while the oocyte resumes meiosis and extrudes the second polar body. In the human, the earliest decondensed spermhead has been observed inside the ooplasm 45 minutes after insemination (Plachot et al, 1986). Meiosis is completed 3-4 hours after insemination (Lopata and Leung,

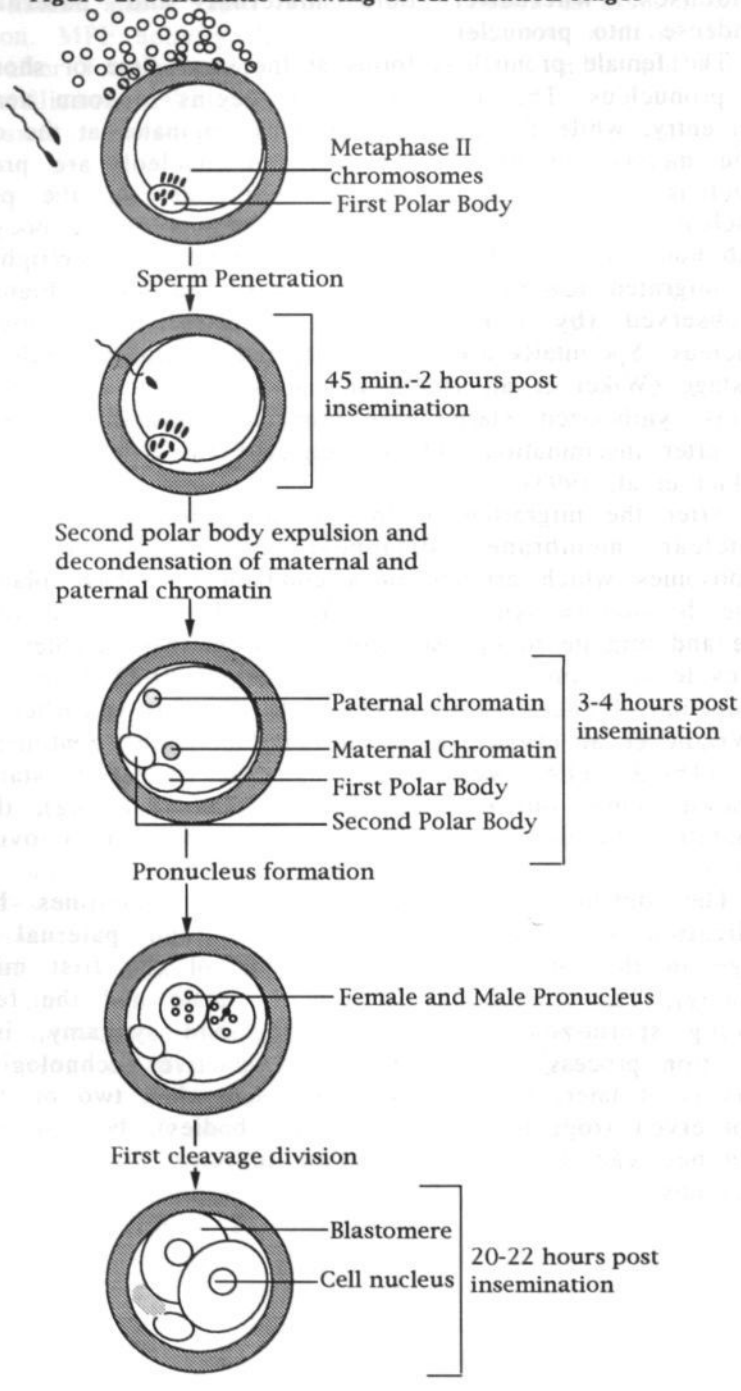
1988). After completion of meiosis, the decondensed spermhead recondenses, whereafter both maternal and paternal chromatin decondense into pronuclei.

The female pronucleus forms at the same time or shortly before the male pronucleus. The male pronucleus begins to form near the site of sperm entry, while the female pronucleus originates at the ooplasmic pole of the meiotic spindle. In average four nucleoli are present in each pronucleus, which line up on adjacent sides of the pronuclei once pronuclear migration towards the central region of the oocyte is finished i.e. 26 hours after oocyte collection and insemination (Wright et al, 1990). Once migrated towards the oocyte center, no morphological differences are observed (by light microscopy) between the male and female pronucleus. Spermtails are rarely observed within the male pronucleus at this stage (Wiker et al, 1990). In the human, the S-phase, during which DNA is synthesized, starts when pronucleus formation is ended i.e. 9-10 hours after insemination and is completed 14-17 hours after insemination (Balakier et al, 1993).

After the migration of the pronuclei towards the cell center, the pronuclear membranes disappear and two sets of condensed chromosomes which arrange in a common metaphase plate of the first mitotic division (= syngamy), are formed. The combined 46 chromosomes divide and migrate to opposite poles to form two daughter cells. The first cell cycle in human zygotes is completed 20-22 hours after in vitro insemination (Balakier et al, 1993). Note that this is earlier than described by Wright et al (1990). Early cleavage divisions continue until morula stage (16-32 cells) whereafter blastocyst formation starts. Once the blastocyst comes out of the zona pellucida (hatching), the process of implantation in the uterus can start (5-6 days after ovulation in the human).

The definition of fertilization may sometimes be confusing. Fertilization is achieved when maternal and paternal chromosomes arrange in the common metaphase plate of the first mitotic division (syngamy). The interaction between the male and the female gamete, including sperm-zona pellucida binding and syngamy, is called "the fertilization process". For Assisted Reproductive Technologies which will be discussed later, fertilization is assessed when two or more pronuclei are observed (together with two polar bodies), because the pronuclear membranes can be easily observed in vitro by simple bright field microscopy.

Figure 2: Schematic drawing of fertilization stages



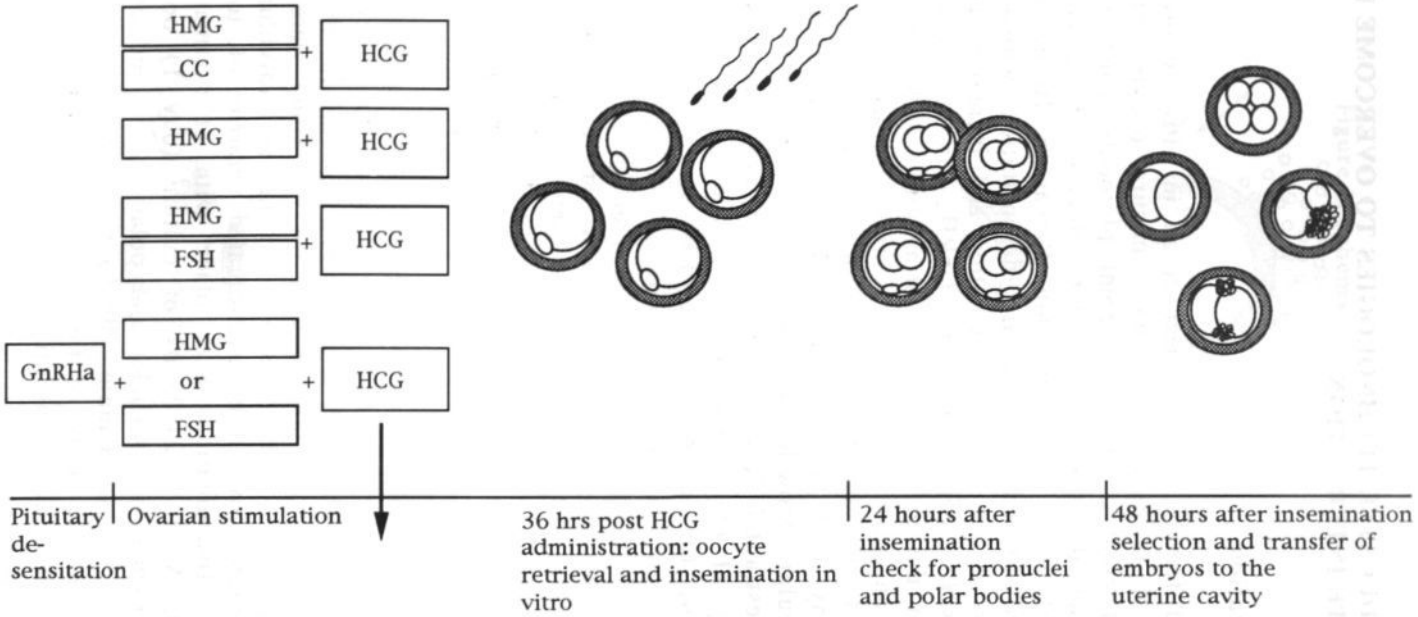
ASSISTED REPRODUCTIVE TECHNOLOGIES TO OVERCOME DEFECTS OF SPERM-OOCYTE INTERACTION

In Vitro Fertilization

For IVF, male and female gametes meet in an in vitro culture dish. In order to succeed it is essential to have mature oocytes and motile spermatozoa in sufficient numbers. Different protocols using exogeneous hormones to stimulate the growth of ovarian follicles can be used (Figure 3). In the present work, Gonadotrophin Releasing Hormone agonist (GnRHa) was used to achieve pituitary desensitation which prevents premature LH peaks before ovarian stimulation and increases the number of retrieved oocytes (Pellicer et al, 1989). GnRHa can be administered on day 1 or day 2 of the menstrual cycle. Stimulation with gonadotrophins (FSH or HMG) can start either after complete ovarian desensitation (around day 13 of the menstrual cycle but depending on individual responses) or on day 3 of the menstrual cycle when desensitation is still incomplete. Follicular growth is monitored by ultrasound and by measuring serum estradiol levels. GnRHa treatment continues in both cases until the day of ovulation induction (Frydman et al 1988). After ovarian stimulation, the action of the LH surge is simulated by administration of human Chorionic Gonadotrophin (HCG). Expulsion of the first polar body and metaphase II appear at 32 hours post HCG ; i.e. 6 to 8 hours before ovulation (Bomsel-Helmreich et al, 1987). These informations allow the calculation of the time needed for in vivo maturation before oocyte retrieval for in vitro fertilization. To obtain mature or almost mature oocytes, oocytes from the largest follicles are retrieved approximately 36 hours after hCG administration just before ovulation would occur.

During oocyte retrieval, semen is treated with a washing procedure to remove seminal plasma. Migration on a discontinuous percoll density gradient allows the selection of motile spermatozoa. In the Béclère University Hospital where most oocytes used for this study were obtained, oocytes are inseminated 3-5 hours after retrieval in individual 30 µl drops of culture medium covered by equillibrated paraffin oil, to maintain constant conditions. Insemination takes place with approximately 4000 motile spermatozoa. After 18-24 hours of culture (day 1) oocytes are checked for the presence of pronuclei and polar bodies and another 24 hours (day 2) later a maximum of four resulting embryos are transplanted to the uterine cavity of the patient. Supernumerary embryos were frozen 48 hours after insemination or co-cultured until the blastocyst stage and frozen for later transplantation if necessary (Olivennes et al, 1994).

Figure 3: Schematic overview of possible ovarian stimulation protocols and IVF



Abbreviations: GnRHa, Gonadotrophin Releasing Hormone agonist; HMG, human Menopausal Gonadotrophin; FSH, Follicle Stimulating Hormone; CC, Clomiphene Citrate; HCG, human Chorionic Gonadotrophin

Most zygotes with two pronuclei on day 1, are in 3-4 cell stage on day 2 (56%). Only a minority is at the 2-cell stage (29%) or 5-8 cell stage (15%) (Plachot and Popescu, 1991). Ongoing pregnancies have been achieved after the transfer of embryos of different stages varying from the 2-cell stage up to the blastocyst stage (Edwards et al, 1984; Bolton et al, 1991).

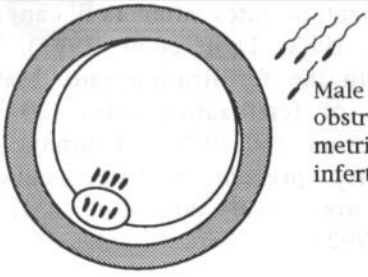
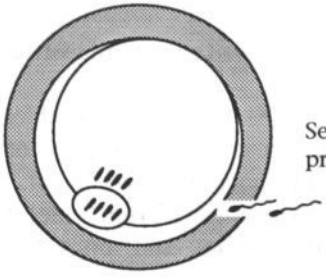
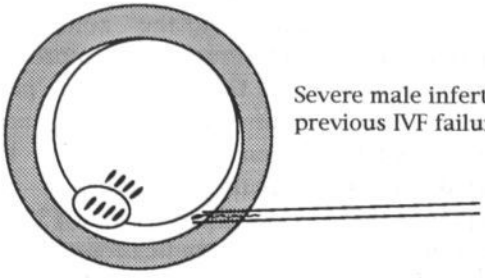
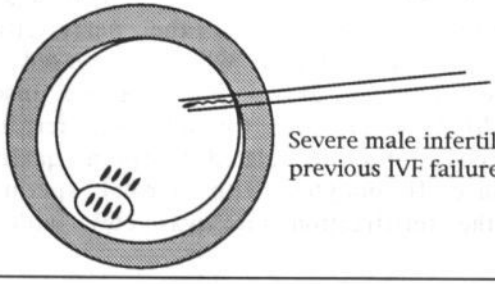
Overall fertilization rates after IVF can achieve 70 % (Pellicer et al, 1989; Veeck et al, 1983; Tarin et al, 1991). The cause of infertility plays an important role in the fertilization rate. Male infertility patients are less succesful in terms of fertilization rates (20-30 %) than patients suffering from tubal obstruction (60-70 %) (Tournaye et al, 1992). Patients with idiopathic infertility present a heterogeneous group but the overall fertilization rates are lower than for tubal patients (60.4% vs 87.3%; Mackenna et al, 1992).

Zonal-techniques and intracytoplasmic insemination

Patients with several IVF failures or with a severe male infertility factor are nowadays referred to new techniques of ART: partial zona dissection (PZD), subzonal insemination (SUZI) or intracytoplasmic insemination (ICSI). In Table 2 these different techniques are shown. For these techniques ovarian stimulation and oocyte retrieval are performed as for regular IVF. Partial zona dissection (PZD) (Malter et al, 1989) consists of making a small breach in the zona pellucida either chemically or mechanically. This technique can improve the chances of fertilization when spermatozoa lack the ability to penetrate the zona pellucida or when only a small number of sperm are available to fuse with the oolemma (Vanderzwalmen et al, 1992). However, in only half of the manipulated oocytes, sperm succeeds in entering the perivitelline space which explains the low fertilization rate (33 %; Levron et al, 1991).

With the same clinical indications SUZI gives better results than PZD (Ng et al, 1988; Fishel et al, 1990; Wolf et al, 1992) . For SUZI, spermatozoa are brought directly into the perivitelline space. The incidence of polyspermy is reduced when compared with PZD, because a small, controled number of spermatozoa are inseminated into the perivitelline space. SUZI is efficient for achieving fertilization in cases of IVF failures, but success rates are related to sperm quality (Wolf et al, 1992). SUZI of metaphase II oocytes with normal sperm resulted in 66% fertilization while the fertilization rate was 26% with subnormal sperm (Wolf et al, 1992).

Table 2: Different assisted reproductive technologies (ART)

Method	Abbreviation	Schematic view	Clinical indications
In vitro fertilization	IVF		Male infertility, tubal obstruction, endometriosis, idiopathic infertility
Partial zona dissection	PZD		Severe male infertility, previous IVF failures
Subzonal insemination	SUZI		Severe male infertility, previous IVF failures
Intracytoplasmic insemination	ICSI		Severe male infertility, previous IVF failures

With the most recent technique, intracytoplasmic sperm injection (ICSI) by-passing both penetration through the zona pellucida and the oolemma, success rates increase dramatically (Palermo et al, 1992; Van Steirteghem et al, 1993). A study comparing SUZI and ICSI, carried out in 300 treatment cycles, showed a higher monospermic fertilization rate after ICSI (51%) than after SUZI (14%) and high delivery rates of 22 % per cycle (Van Steirteghem et al, 1993). The indications for SUZI or ICSI, were total IVF failure (no pronuclei on day 1) and single or multiple sperm defects.

High success rates after SUZI and ICSI when compared to IVF for the same clinical indications suggest that a natural selection step which exists at the level of sperm-zona pellucida binding, zona penetration and fusion of sperm with the oolemma, is circumvented by these micromanipulation techniques. It is unknown whether this selection takes place at a later stage during development, even while it is known that most chromosomal anomalies result in spontaneous abortions. Hence, the quality assessment of gametes is particularly important for SUZI and ICSI.

WHEN ASSISTED REPRODUCTION TECHNOLOGIES FAIL

Fertilization failure and fertilization anomalies

Despite the elaborate assisted reproductive technologies in use, fertilization may fail. The succes of fertilization depends on oocyte and semen quality. When no pronuclei are observed within the expected time, 18-24 hours after IVF, the fertilization process failed. The different steps are involved in this process, all might go wrong.

To realise fertilization, the spermatozoon first must bind to the zona and complete its acrosomal reaction before penetrating the oocyte vestments. Sperm-zona binding can be defective due to the presence of antibodies against certain sperm antigens (Naz et al, 1992) or to abnormal sperm morphology (Liu and Baker, 1992). The number of spermatozoa bound to the oocyte's surface varies per oocyte (Sundström, 1984). This may be due to differences in the sperm-binding capacity of the zona pellucida from individual oocytes. Zona pellucida abnormalities have been suggested and they may hinder sperm-zona binding and penetration (Bongso et al, 1992; Benshushan et al, 1993). Even when sperm-zona binding is successful, penetration of the zona pellucida may fail (Bedford and Kim, 1993).

After in vitro fertilization as well as after subzonal insemination, it has been shown that the presence of spermheads in the perivitelline space or adjacent to the oolemma, not necessarily leads to fusion of the membranes (Hartshorne et al, 1989; Alikani et al, 1992). Besides other

elements, the influence of certain sperm parameters on sperm-oolemma fusion at this stage cannot be excluded. The fertilization rate after subzonal insemination is inversely correlated with the percentage of acrosome defects in the semen(Wolf et al, 1992).This strongly suggests the involvement of sperm factors in sperm-oolemma fusion.

Oocyte immaturity may also cause fertilization failure. After ovarian stimulation, the oocytes from the same cohort are known to differ in their maturational status. It has been demonstrated that the use of GnRHa in IVF protocols results in a higher incidence of meiotically and cytoplasmically immature oocytes (Pellicer et al, 1989; Tarin et al, 1990; Pieters et al, 1991). Sperm can penetrate the human oocyte at any stage of maturity (Lopata and Leung, 1988). However, experiments in other mammals showed that complete chromatin decondensation and pronucleus formation is only achieved if sperm penetrated meiotically mature oocytes (Usui and Yanagimachi, 1976; Szöllösi et al, 1990). Metaphase arrest of maternal chromatin after sperm penetration may be caused by cytoplasmic immaturity of meiotically mature oocytes (Kubiak, 1989; Schmiady and Kentenich, 1989; Zenzes et al, 1990).

After IVF, SUZI or ICSI, abnormal fertilization has occurred when more than two pronuclei per oocyte are observed. After IVF, polyspermic fertilization rates vary from 3.2% to 10.2% (Trounson et al, 1982; Diamond et al 1985; Plachot et al, 1988; Dandekar et al, 1990; Ho et al, 1994). Different causes for polyspermy have been suggested such as the fertilization of immature oocytes (Trounson et al, 1982), post-meiotically aged oocytes, or atretic oocytes (Wentz et al, 1983). Interestingly, also monospermic fertilization rates are higher in cycles with polyspermic fertilization of cohort oocytes (Golan et al, 1992). After SUZI multiple pronuclei can be observed as a result of the penetration of several spermatozoa. However, for ICSI a single spermatozoon is injected inside the ooplasm, so that polyspermy can be excluded. Non-expulsion of the second polar body can induce a third pronucleus by gynogenesis (Van Steirteghem et al, 1993). This observation suggests a triggering role of sperm-oolemma fusion on polar body expulsion. It is also possible that in some cases large pronuclear size vacuoles (pseudopronuclei; Van Blerkom et al, 1990), may mistakenly be regarded as real pronuclei.

Ageing female gametes and aspects of delayed fertilization

The live span of the mammalian oocyte after ovulation is limited. In almost all mammalian species, but not in the human, the time of ovulation and mating are synchronized. Hence, in the human, in vivo fertilization of a post-meiotically aged oocyte is possible. In human IVF procedures, overripeness of the oocyte at the time of fertilization may occur due to

late oocyte retrieval after hCG administration or when in vitro fertilization of the mature oocyte fails.

If fertilization does not take place in time, the secondary oocyte will undergo ageing processes. The cortical granules migrate towards the center of the oocyte and this will allow multiple spermatozoa to penetrate (Szöllösi, 1975). On the other hand, spontaneous "zona-hardening" due to prolonged culture may hinder or delay sperm penetration (DeFelici et al, 1986). Migration of the metaphase spindle towards the oocyte's center is a uniform feature of ageing mouse, rabbit and rat oocytes and is due to degenerative changes in the cytoskeleton (Szöllösi, 1975; Webb et al, 1986; Eichenlaub-Ritter, 1986). In mice, the centripetal migration of the spindle leads to non-expulsion of the second polar body resulting in digynic oocytes after mating (Szöllösi, 1975). A dislocation of one or several of the chromosomes from the degenerating spindle is frequently found in post ovulatory aged oocytes and can account for malsegregation of the chromosomes during the first mitotic division leading to aneuploidy (Szöllösi, 1975; Eichenlaub-Ritter et al, 1986; Eichenlaub-Ritter, 1988, Santalo et al, 1987). Spontaneous activation or parthenogenesis is also characteristic for aged oocytes and can be induced in fresh oocytes by artificial inhibition of protein synthesis (Webb et al, 1986; Pickering et al, 1988). This may indicate that in ageing oocytes diminishing protein synthesis can be responsible for spontaneous activation.

Embryo Morphology and Quality

An important issue in ART studies is the establishment of a selection method based on embryo morphology before transfer to maximize chances of implantation. Two morphological parameters are generally used to determine embryo quality, 1) size equality and number of blastomeres, 2) fragmentation degree. Blastomere fragments are in general anucleate, small and irregularly shaped when compared to intact blastomeres. Different authors showed the correlation between the transfer of one or more good quality embryos and pregnancy (Grillo et al, 1991; Erenus et al, 1991). The chromosomal regularity of embryos has been studied in healthy spare embryos, in morphologically poor embryos and in embryos resulting after polyspermic fertilization (Plachot et al, 1988; Ma et al, 1990; Bongso et al, 1991; Pellestor et al, 1994). It has been shown that grossly abnormal embryos (blastomeres uneven in shape and the extensive presence of anucleate fragments), have a high incidence (32%-90%) of chromosomal abnormalities (Bongso et al, 1991; Pellestor et al, 1994). This confirms the morphological assessment of the poor quality of these embryos and the uselessness of their transplantation. However, morphologically normal embryos may have abnormal chromosomal

complements as is the case in embryos from polypronuclear zygotes (reviewed by Zenzes et al, 1992).

A frequently found anomaly in human embryos after IVF is the presence of multinucleated blastomeres (MNBs). The developmental capacity of embryos with MNBs, is still uncertain as well as are the underlying causes leading to multinucleation. Besides normal single-nucleated cells multinucleated blastomeres are frequently found in human embryos varying from the 2-cell stage to 5 day old blastocysts (Sathananthan et al, 1982; Winston et al, 1991). However, only poor-quality embryos were analysed and it remains to be established whether these results can be extrapolated to morphologically normal embryos.

METHODS OF ANALYSIS

With low fertilization success or even more after complete absence of fertilization, it is necessary to explain this failure in order to reconsider further treatment of infertility. Different techniques to study unfertilized oocytes and embryos unsuitable for transfer to the uterine cavity, allow the exploration of the causes of the fertilization failure and of the different aspects of gamete-interaction involved.

Cytogenetics

Relatively easy to perform, cytogenetic analysis that was initially developed in mice (Tarkowski, 1966) is now widely used on human oocytes which failed to fertilize and on early embryos not suitable for transfer (Zenzes et al, 1985; Plachot et al, 1988; Schmiady and Kentenich, 1989; Pieters et al, 1989). It allows chromosome numbering and a rough classification. With this cytogenetic observation, the incidence of chromosomal abnormalities in unfertilized oocytes is estimated to vary from 20-35 % (Plachot et al, 1988; Pieters et al, 1989; Tejada et al, 1991; Zenzes and Casper, 1992). The abnormalities observed were diploidy, indicating unreduced oocytes, and aneuploidy. Also specific abnormalities such as premature condensation of male chromatin (PCC) (Schmiady and Kentenich, 1989; Tejada et al, 1992) and triploidy and polyploidy can be detected (Angell et al, 1986; Plachot et al, 1987; Pieters et al, 1989).

More advanced staining methods such as Giemsa (G) banding and other banding methods allow identification of individual metaphase chromosomes. A recent innovation is in situ hybridisation for identification of specific chromosomes not only in metaphase spreads but in interphase nuclei as well. Selection of oocytes with chromosomal

abnormalities based on morphological aspects would diminish chromosomal abnormalities in embryos. A correlation between cytoplasmic dysmorphism in metaphase II oocytes and the incidence of aneuploidy has been reported (Van Blerkom and Henry, 1992). However, for the moment this type of selection is not in use for IVF.

DNA fluorescence on oocytes and embryos in vitro

A disadvantage of classical cytogenetic techniques is that they alter the integrity of the oocyte so that only chromosomes are available for analysis. Furthermore, the metaphase spindle, pronuclei and sperm cannot be localized topographically because the cytoplasm has disappeared. Furthermore, a relatively important number of metaphases is not analysable (50% of the metaphases could not be analysed as reported by Plachot et al, 1988 and 17% by Tejada et al, 1991). A rapid fluorescent technique allows the observation of DNA containing structures in and around the oocyte. Conover and Gwatkin reported a preloading of mouse oocytes with the DNA specific fluorescent probe Hoechst 33342 (Conover and Gwatkin, 1988; Gwatkin et al, 1989). This bisbenzimidazole fluorochrome is a vital dye which diffuses passively into the nuclear structures of intact oocytes and embryos. It binds reversibly to sequences of three or more adenine-thymidine pairs of double stranded DNA and binding occurs externally rather than intercalating. The fluorochrome Hoechst 33342 has been used to label and analyse nuclei of preimplantation mouse embryos without loss of their viability, as demonstrated by the birth of live young (Debey et al, 1989).

Chromatin staining by DNA fluorescent dyes can be combined with staining of the cytoskeleton using for example anti-tubulin antibodies. Oocytes which remained unfertilized 48 hours after insemination and stained this way, showed a degeneration of the cytoskeleton causing a disruption of the meiotic spindle and the lagging of chromosomes (Eichenlaub-Ritter et al, 1988). A tripolar metaphase spindle after dispermic fertilization of human oocytes has also been observed by this technique (Eichenlaub-Ritter et al, 1988; Plachot and Crozet, 1992).

The observations using DNA fluorescence can be confirmed by simultaneous bright field microscopy and subsequent histological observations on thin sections (Figures 4a, b, c and d). While the presence of sperm chromatin inside the ooplasm has mainly been described after oocyte preparation for cytogenetic analysis, sperm chromatin can also be identified by DNA fluorescent observations. While undecondensed spermheads are easily recognized, the male origin of partially decondensed spermheads and PCC is difficult to certify by DNA fluorescence only. However, several indications are useful, a) the

localization within the ooplasm sometimes at a large distance from the oocyte chromatin, b) the presence of clearly identifiable oocyte chromatin, c) the difference in condensation grades between oocyte and sperm chromatin.

AIMS OF THIS STUDY

This study was mainly initiated to examine unfertilized oocytes, delayed or arrested zygotes and abnormal embryos after IVF. It was particularly focused on normal and abnormal aspects of the fertilization process. The aims of this study were:

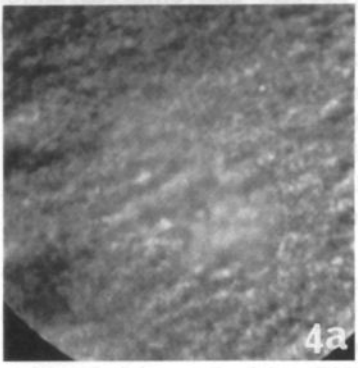
1. To study oocyte maturation, sperm penetration, aspects of fertilization and chromatin condensation stages in human oocytes considered to be unfertilized in an IVF program (Chapters II, IV, V)
2. To investigate the diagnostic use of such a detailed analysis of human oocytes which remained unfertilized after IVF especially for couples with total IVF failure or with very low fertilization and cleavage rates after IVF (Chapters III, V)
3. To analyse the prognostic value of sperm-zona binding and sperm penetration into unfertilized oocytes (Chapter IV)
4. To investigate the chronology of pronuclear development and the developmental rate after subzonal insemination (SUZI) with spermatozoa of different quality compared to IVF with donor sperm (D-IVF) (Chapter VI)
5. To study the incidence and origin of embryos containing multinucleated blastomeres in an IVF program (Chapter VII)

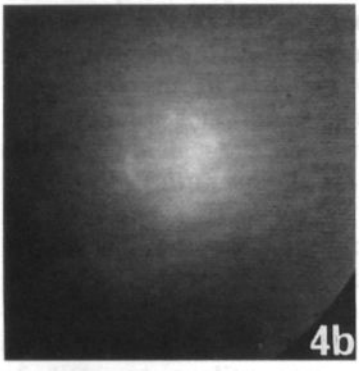
ORIGIN OF HUMAN GAMETES AND EMBRYOS STUDIED

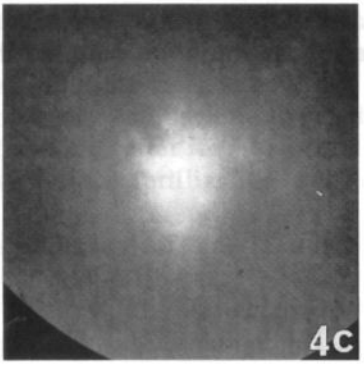
The first part of the study, from 1990 until 1991 (Chapters II and III), was performed on oocytes obtained from the Bichat University Hospital (Paris, France) with the agreement of the Ethical Committee of the hospital. Fluorescence observations took place at the INSERM Unity 310 (Paris, France). During this period oocytes were observed under culture conditions in modified petri dishes (Debey et al, 1989) within a thermostatic box using an inverted microscope equipped for fluorescence (Nikon). Images were recorded with an amplified camera (Lhesa Electronique), digitized and treated by the SAPHIRE processing system (Quantel micro consultants) and stored on cartridge discs (Bernouilli).

From 1991 to 1993 (Chapters IV, V, VII), oocytes and embryos were obtained and observed in the Béclère University Hospital (Clamart, France) with the agreement of the Ethical Committee of the hospital. The method of observation was simplified and adapted so that fluorescence observations could be performed in the IVF laboratory. Oocytes were no longer observed under culture conditions, but *in toto* on modified slides with a Zeiss (not inverted) microscope equipped for fluorescence. Pictures were taken with X-Tri-Pan films (Kodak). Different aspects of the same oocytes were described in different chapters (Chapter IV and V). The embryos in Chapter VII resulted from the same patients and IVF cycles as described in Chapters IV and V.

In Chapter VI, non-invasive observations were performed on zygotes and embryos obtained after SUZI and IVF with donor sperm for clinical indications. The SUZI programme performed in the Bicetre University Hospital received the agreement of the local Ethics Committee.







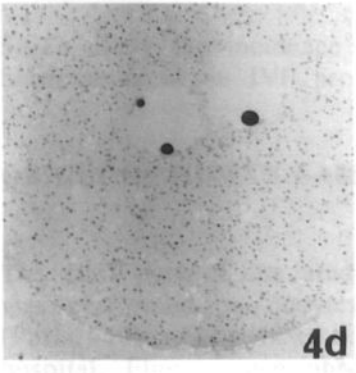


Figure 4: Observation of pronuclei in vitro by light microscopy, one pronucleus (PN) is visible, the other is not (a). Two pronuclei (PN) are visible using fluorescence microscopy after incubation with Hoechst 33342 (b and c). The same oocyte was fixed and sectioned (c), both pronuclei (PN) and their nucleoli (N) are visible. 1cm=10μ

REFERENCES

Adenot PG, Szollosi MS, Geze M, Renard JP, Debey P. Dynamics of paternal chromatin changes in live one-cell mouse embryo after natural fertilization. *Mol. Reprod. Dev.* 28: 23-34, 1991

Alikani M, Adler A, Reing AM, Malter HE, Cohen J. Subzonal sperm insertion and the frequency of gamete fusion. *J. of Ass. Reprod. and Genetics* 9: 97-101, 1992

Angell RR, Templeton AA, Messines IE. Consequences of polyspermy in man. Cytogenet. *Cell Genet.* 42: 1-7, 1986

Balakier H, MacLusky NJ, Casper R: Characterization of the first cell cycle in human zygotes: implications for cryopreservation. *Fertil Steril* 59: 359-365, 1993

Barros C, Yanagimachi R. Induction of zona reaction in golden hamster eggs by cortical granule material. *Nature* 233: 268-269, 1971

Benshushan A, Ezra Y, Simon A, Gordon JW, Laufer N: Subzonal insertion. A possible treatment for "defective oocytes". *J Ass Rep Gen* 10: 98-99, 1993

Bolton VN, Wren ME, Parsons JH. Pregnancies after in vitro fertilization and transfer of human blastocysts. *Fertil. Steril.* 55: 830-832, 1991

Bongso A, Ng SC, Lim J, Fong CY, Ratnam S. Preimplantation genetics: chromosomes of fragmented human embryos. *Fertil. Steril.* 56: 66-70, 1991

Bongso A, Yee Fong C, Ng SC, Ratnam S. Fertilization, cleavage, and cytogenetics of 48-hour zona-intact and zona-free human unfertilized oocytes reinseminated with donor sperm. *Fertil Steril* 57: 129-133, 1992

Bomsel-Helmreich O, Vu N Huyen L, Durand-Gasselin I, Salat-Baroux J, Antoine JM. Timing of nuclear maturation and cumulus dissociation in human oocytes stimulated by CC, hMG and hCG. *Fertil Steril* 48: 586-595, 1987

Chang MC. Fertilizability of rabbit ova and the effects of temperature in vitro on their subsequent fertilization and activation in vivo. *J. Exp. Zool.* 121: 351- 382 , 1952

Coleman TR, Dunphy WG. Cdc2 regulatory factors. *Current opinion in Cell Biology* 6: 877-882, 1994.

Conover JC, Gwatkin RBL. Pre-loading of mouse oocytes with DNA-specific fluorochrome (Hoechst 33342) permits rapid detection of oocyte-sperm fusion. *J. Reprod. Fertil.* 82: 681-690, 1988

Dandekar PV, Martin MC, Glass RH. Polypronuclear embryos after in vitro fertilization. *Fertil. Steril.* 53: 510-514, 1990

Debey P, Renard JP, Coppey-Moisan M, Monnot I, Geze M. Dynamics of chromatin changes in live one-cell mouse embryos: a continuous follow-up by fluorescence microscopy. *Exp. Cell Res.* 183: 413-433, 1989

De Felici M, Salustri A, Siracusa G. "Spontaneous" hardening of the zona pellucida of mouse oocytes during in vitro culture. II. The effect of follicular fluid and glycosaminoglycans. *Gamete Res.* 12: 227-235, 1985

Diamond MP, Rogers BJ, Webster BW, Vaughn WK, Colston Wentz AC. Polyspermy: effect of varying stimulation protocols and inseminating concentrations. *Fertil. Steril.* 43: 777-780, 1985

Edwards RG, Fishel SB, Cohen J, Fehilly CB, Purdy JM, Slater JM, Steptoe PC, Webster JM. Factors influencing the success of in vitro fertilization for alleviating human infertility. *J. in vitro Fert. Embryo Trans.* 1: 3-22, 1984

Eichenlaub-Ritter U, Chandley AC, Gosden RG. Alterations to the microtubular cytoskeleton and increased disorder of chromosome alignment in spontaneously ovulated mouse oocytes aged in vivo: an immunofluorescence study. *Chromosoma* 94: 337-345, 1986

Eichenlaub-Ritter U, Stahi A, Luciani JM. The microtubular cytoskeleton and chromosomes of unfertilized human oocytes aged in vitro. *Hum. Genet.* 80: 259-264, 1988

Endo Y, Komatsu S, Suzuki S. Demonstration of maturation-promoting and -inhibiting activities in mouse oocytes. *J. in vitro Fert. Embryo Transf.* 7: 271-274, 1990

Erenus M, Zouves C, Rajamahendran P, Leung S, Fluker M, Gomel V. The effect of embryo quality on subsequent pregnancy rates after in vitro fertilization. *Fertil Steril* 56: 707-710, 1991

Fishel S, Jackson P, Antinori S, Johnson J, Grossi S, Versaci C: Subzonal insemination for the alleviation of infertility. *Fertil Steril* 54: 828-835, 1990

Frydman R, Belaisch-Allart J, Parneix I, Forman R, Hazout A, Testart J. Comparison between flare up and down regulation effects of Luteinizing hormone-releasing hormone agonists in an in vitro fertilization program. *Fertil. Steril.* 50: 471-921

Golan A, Nachum H, Herman A, Ron-El R, Soffer Y, Caspi E. Increased fertilization and pregnancy rate in polypronuclear fertilization cycles in in vitro fertilization-embryo transfer. *Fertil. Steril.* 57: 139-141, 1992

Grillo JM, Gamerre M, LacroixO, Noizet A, Vitry G. Influence of the morphological aspect of embryos obtained by in vitro fertilization on their implantation rate. *J of in vitro Fert. and Embryo Transfer* 8: 317-321, 1991

Gwatkin RBL, Conover JC, Collins RL, Quigley MM: Failed fertilization in human in vitro fertilization analysed with the deoxyribonucleic acid-specific fluorochrome Hoechst 33342. *Am J Obstet Gyn* 160: 31-35, 1989

Hartshorne GM: A simple method for the morphological analysis of human ova: zona penetration in oocytes failing to fertilize or fertilizing abnormally in vitro. *Hum Reprod* 4: 588-594, 1989

Ho PC, Yeung WSB, Chan YFC, So WWK, Chan STH. Factors affecting the incidence of polyploidy in a human in vitro fertilization program. *Int. J. Fertil.* 1: 14-19, 1994

Kubiak J. Mouse oocytes gradually develop the capacity for activation during the metaphase II arrest. *Dev. Biol.* 136: 537-545, 1989.

Lasalle B, Testart J: Sequential transformations of human sperm nucleus in human egg. *J Reprod Fertil* 91: 393-402, 1991.

Kubiak JZ, Weber M, Pennart H de, Winston NJ, Maro B. The metaphase II arrest in mouse oocytes is controlled through microtubule-dependent destruction of cyclin B in the presence of CSF. *The EMBO Journal* 12: 3773-3778

Levron N, Stein DW, Bar-Ami S, Brandes JM, Itskovitz J: Presence of sperm in the perivitelline space (PVS) predicts fertilization rates after partial zona dissection (PZD). *Hum Reprod* 6, Suppl 1: 145, 1991

Liu DY, Baker HWG: Sperm nuclear chromatin normality: relationship with sperm morphology, sperm-zona pellucida binding, and fertilization rates in vitro. *Fertil Steril* 58: 1178-1184, 1992

Lopata A, Leung PC. The fertilizability of human oocytes at different stages of meiotic maturation. *Ann. N.Y. Acad. Sci.* 541: 324-336, 1988

Ma S, Kalousek DK, Zouves C, Yuen BH, Gomel V, Moon YS. The chromosomal complements of cleaved human embryos resulting from in vitro fertilization. *J. in vitro Fert. Embryo Transf.* 7: 16-21, 1990

Mackenna AI, Zegers-Hochschild F, Fernandez EO, Fabres CV, Huidobro CA, Prado JA, Roblero LS, Altieri EL, Guadarrama AR, Lopez TH: Fertilization rate in couples with unexplained infertility. *Hum Reprod* 7: 223-226, 1992

Malter EH, Cohen J: Partial zona dissection of the human oocyte: a non-traumatic method using micromanipulation to assist zona pellucida penetration. *Fertil Steril* 51: 139, 1989

Mandelbaum J, Salat-Baroux J, David G. Maîtrise de la fertilité humaine. In: La reproduction chez les mammifères et l'homme (Thibault C, Levasseur MC, eds.) Ellipses, 1991

Masui Y, Markert CL. Cytoplasmic control of nuclear behaviour during meiotic maturation of frog eggs. *J. Exp. Zool.* 177: 129-146, 1977

Mattson BA, Albertini DT. Oogenesis: Chromatin and microtubule dynamics during meiotic prophase. *Molec. Reprod. Dev.* 25: 374-383, 1990

Naz RK, Brazil C, Overstreet JW: Effects of antibodies to sperm surface fertilization antigen-1 on human sperm-zona pellucida interaction. *Fertil Steril* 57: 1304-1313, 1992

Ng SG, Bongso A, Ratnam SS, Sathananthan H, Chan CLK, Wong PC, Hagglund L, Anandakumar Wong YC, Goh VHH: Pregnancy after zona transfer of sperm under zona pellucida. *Lancet* 2: 790, 1988

Palermo G, Joris H, Devroey P, Van Steirteghem AC: Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 34: 17, 1992

Pellestor F, Dufour MC, Arnal F, Humeau C. Direct assessment of the rate of chromosomal abnormalities in grade IV human embryos produced by in-vitro fertilization procedure. *Hum. Reprod.* 9: 293-302, 1994

Pellicer A, Ruiz A, Castellvi RM, Calatayud C, Ruiz M, Tarin JJ, Miro F, Bonilla-Musoles F. Is the retrieval of high numbers of oocytes desirable in patients treated with gonadotrophin-releasing hormone analogues (GnRHa) and gonadotrophins? *Hum. Reprod.* 4: 536-540, 1989

Pickering SJ, Johnson MH, Braude PR. Cytoskeletal organisation in fresh,aged and spontaneously activated human oocytes. *Hum. Reprod.* 3: 978-979, 1988

Pieters MHEC, Geraedts JPM, Dumoulin JCM, Evers JLH, Bras M, Kornips FHAC, Menheere PPCA. Cytogenetic analysis of in vitro fertilization (IVF) failures. *Hum. Genet.* 81: 367-370, 1989

Pieters MHEC, Dumoulin JCM, Engelhart CM, Bras M, Evers JLH, Geraedts JPM. Immaturity and aneuploidy in human oocytes after different stimulation protocols. *Fertil. Steril.* 56: 306-310, 1991

Plachot M, Junca AM, Mandelbaum J, Cohen J, Salat-Baroux J, DaLage C. Timing of in-vitro fertilization of cumulus-free and cumulus-enclosed human oocytes. *Hum. Reprod.* 1: 237-242, 1986

Plachot M, Junca AM, Mandelbaum J, de Grouchy J, Salat-Baroux J, Cohen J. Chromosome investigations in early life. I. Human oocytes recovered in an IVF programme. *Hum. Reprod.* 1: 574-551, 1986

Plachot M, Veiga A, Montagut J, Grouchy J de, Calderon G, Lepretre S, Junca AM, Santalo J, Carles E, Mandelbaum J, Barri P, Degoy J, Cohen J, Egozcue J, Sabatier JC, Salat-Baroux J. Are clinical and biological IVF parameters correlated with chromosomal disorders in early life: a multicentric study. *Hum. Reprod.* 3: 627-635, 1988

Plachot M, Popescu P. Les anomalies chromosomiques et géniques, leurs conséquences sur le développement et la reproduction. In: La reproduction chez les mammifères et l'homme (Thibault C, Levasseur MC, eds.) Ellipses, 1991

Plachot M, Crozet N: Fertilization abnormalities in human in-vitro fertilization. *Hum Reprod* 7: 89-94, 1992

Sathananthan AH, Wood C, Leeton JF. Ultrastructural evaluation of 8-16 cell human embryos cultured in vitro. Micron 13: 193-203, 1982

Santalo J, Catala V, Egozcue J. Chromosomes of mouse embryos in vivo and in vitro: effect of manipulation, maternal age and gamete ageing. *Hum. Reprod.* 2: 717-719, 1987

Schmiady H, Kentenich H. Premature chromosome condensation after in-vitro fertilization. *Hum. Reprod.* 4: 689-695, 1989

Steptoe PC, Edwards RG, Purdy J. Clinical aspects of pregnancies establihed with cleaving embryos grown in vitro. *Br. J. Obstet. Gynaecol.* 87: 757-768, 1980

Sundstrom P. Interaction between human gametes in vitro: a scanning electron microscopic study. *Arch. Androl.* 13: 77-85, 1984

Szöllösi D: Mammalian eggs ageing in the fallopian tubes. In: Blandau RJ, (ed), Ageing gametes, Karger, International Symposium, Seattle, pp 98-121, 1975
Szöllösi D, Szollosi M, Czolowska R, Tarkowski A. Sperm penetration into immature mouse oocytes and nuclear changes during maturation: an EM study. *Biol. Cell.* 69: 53-64, 1990
Tarin JJ, Pellicer A. Consequences of high ovarian response to gonadotrophins: a cytogenetic analysis of unfertilized human oocytes. *Fertil. Steril.* 54: 665-670, 1990
Tarin JJ, Ruiz A, Miro F, Bonilla-Musoles F, Pellicer A. Failed in vitro fertilization of human oocytes: a cytogenetic analysis. *Fertil. Steril.* 56: 290-295, 1991
Tarkowski AK. An air-drying method for chromosome preparations from mouse eggs. *Cytogenetics* 5: 394-400, 1966
Tejada MI, Mendoza R, Corcostegui B, Benito JA. Chromosome studies in human unfertilized oocytes and uncleaved zygotes after treatment with gonadotrophin-releasing hormone analogs. *Fertil. Steril.* 56: 874-880, 1991
Tesarik J. Control of the fertilization process by the egg coat: How does it work in humans. *J. Ass. Reprod. Genetics* 9: 313-317
Tournaye H, Devroey P, Camus M, Staessen C, Bollen N, Smitz J, Van Steirteghem AC. Comparison of in-vitro fertilization in male and tubal infertility: a 3 year survey. *Hum. Reprod.* 7: 218-222, 1992
Trounson AO, Mohr LR, Wood C, Leeton JF. Effect of delayed insemination on in vitro fertilization, culture and transfer of human embryos. *J. Reprod. Fert.* 64: 285-294, 1982
Usui N, Yanagimachi R. Behaviour of hamster sperm nuclei incorporated into eggs at various stages of maturation, fertilization, and early development. *J. Ultrastructure Res.* 57: 276-288, 1976
Van Blerkom J, Bell H, Henry G. The occurrence, recognition and developmental fate of pseudo-multipronuclear eggs after in vitro fertilization of human oocytes. *Hum. Reprod.* 2: 217-225, 1987
Van Blerkom J, Henry G: Oocyte dysmorphism and aneuploidy in meiotically mature human oocytes after ovarian stimulation. *Hum Reprod* 7: 379-390, 1992
Vanderzwalmen P, Barlow P, Nijs M, Bertin G, Leroy F, Schoysman R. Usefulness of partial dissection of the zona pellucida in a human in-vitro fertilization programme. *Hum. Reprod.* 7: 537-544, 1992
Van Steirteghem AC, Liu J, Joris H, Nagy Z, Janssenswillen C, Tournaye H, Derde MP, Van Assche E, Devroey P: Higher success rate by intracytoplasmic sperm injection than by subzonal insemination. Report of a second series of 300 consecutive treatment cycles. *Hum Reprod* 8: 1055-1060, 1993
Veeck LL, Worthham JWE, Witmeyer J, Sandow BA, Acosta AA, Garcia JE, Jones GS, Jones HW. Maturation and fertilization of morphologically immature human oocytes in a program of in vitro fertilization. *Fertil. Steril.* 39: 594-602, 1983
Wassarman PM. Profile of a mammalian sperm receptor. *Development* 108, 1-17, 1990
Webb M, Howlett SK, Maro B. Parthenogenesis and cytoskeletal organization in ageing mouse eggs. *J. Embryol. exp. Morph.* 95: 131-145, 1986
Wentz AC, Repp JE, Maxson WS, Pittaway DE, Torbit CA. The problem of polyspermy in in vitro fertilization. *Fertil. Steril.* 40: 748-754, 1983
WHO manual for the standarized investigation and diagnosis of the infertile couple. Rowe PJ, HargreaveTB, mellows HJ, Comhaire FH (eds). Cambridge, Cambridge University Press, 1993
Wickramasinghe D, Ebert KM, Albertini D. Meiotic competence acquisition is associated with the appearance of M-phase characteristics in growing mouse oocytes. *Dev. Biol.* 143:162-172, 1991
Wiker S, Malter H, Wright G, Cohen J. Recognition of paternal pronuclei in human zygotes. *J. in vitro Fert. Embryo Transf.* 7: 33-37, 1990

Winston NJ, Braude PR, Pickering SJ, George MA, Cant A, Currie J, Johnson MH. The incidence of abnormal morphology and nucleocytoplasmic ratios in 2, 3, and 5 day human pre-embryos. *Hum Reprod* 6: 17-24, 1991.

Wolf JPh, Ducot B, Kunstmann JM, Frydman R, Jouannet P. Influence of sperm parameters on outcome of subzonal insemination in the case of previous IVF failure. *Hum. Reprod.* 7: 1407-1413, 1992

Wright G, Wiker S, Elsner C, Kort H, Massey J, Mitchell D, Toledo A, Cohen J. Observations on the morphology of pronuclei and nucleoli in human zygotes and implications for cryopreservation. *Hum. Reprod.* 5: 109-115, 1990

Zenzes MT, Belkien L, Bordt J, Kan I, Schneider HPG, Nieschlag E. Cytologic investigation of human in vitro fertilization failures. *Fertil. Steril.* 43: 883-891, 1985

Zenzes MT, de Geyter C, Bordt J, Schneider HPG, Nieschlag E. Abnormalities of sperm chromosome condensation in the cytoplasm of immature oocytes. *Hum. Reprod* 5: 842-845, 1990

Zenzes MT, Casper RF. Cytogenetics of human oocytes, zygotes, and embryos after in vitro fertilization. *Hum. Genet.* 88:367-375, 1992

CHAPTER II

Fertilization and ageing processes in non-divided human oocytes after GnRHa treatment: An analysis of individual oocytes

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ABSTRACT

Some human oocytes cultured together with spermatozoa for in-vitro fertilization (IVF) do not subsequently divide. The arrest of the fertilization process at different moments during development may provide information about the cause of fertilization failure. Oocytes which subsequently divide are transferred 48 h after insemination; when oocytes do not divide, ageing processes can be observed. Therefore these oocytes are interesting material in which to observe both fertilization and ageing. Our study concerns 72 undivided human oocytes 0, 48 or 72h post-insemination. DNA of the oocyte and spermatozoa was visualized by the DNA fluorescent dye Hoechst 33342. Living oocytes were observed *in toto* by fluorescence and bright field microscopy which allowed nuclear and pronuclear membranes to be discerned. Oocytes were subsequently fixed and sectioned for bright field microscopy. Both techniques allowed parallel observations. Oocytes at various stages of fertilization are described: sperm penetration in both mature and immature oocytes, decondensation of sperm-heads, premature condensation of male chromatin, polyspermy and pronucleus formation. Typical ageing processes such as the centripetal migration of the metaphase II chromosomes, the formation of a restitution nucleus and the lagging of chromosomes within a metaphase spindle are observed. DNA fluorescence appears to be a quick, easy and valuable means to analyse fertilization and its failure.

INTRODUCTION

A certain number of oocytes retrieved after ovarian stimulation for in-vitro fertilization (IVF) failed to fertilize after insemination with suitable spermatozoa. These oocytes are the subject of this study. The cleavage rate within 48 h post-insemination, of oocytes from an IVF programme using a gonadotrophin releasing hormone agonist (GnRHa) in a long protocol, ranges between 43 and 78% (Neveu et al., 1987; Frydman et al., 1988; Bryski et al., 1988; Testart et al., 1989). Furthermore only 2-3% of the retrieved oocytes give rise to babies (Pellicer et al., 1989). To know more about the fertilization process as well as about the origin of fertilization failure, we have used the DNA fluorescent dye Hoechst 33342 which allows observation of nuclear features in living oocytes. Together

with bright field microscopic observations of the same oocytes either *in toto* or after fixation and sectioning, a more complete image of the oocyte was obtained. This enables us to attribute the fertilization failure in some cases to either the male or the female gamete.

MATERIALS AND METHODS

Unfertilized oocytes were obtained from 18 selected patients and 19 cycles. The patients were being treated for infertility in an IVF programme of the CHU-Bichat Claude Bernard in Paris. The causes of infertility were tubal (nine patients), idiopathic (four patients), endometriosis (two patients), male factor (one patient) and endocrine (two patients). The mean age of the patients was 30 ± 1.7 years; one patient was 42. In 13 cycles, 1.80 mg GnRHa and in three cycles, 3.75 mg GnRHa Decapeptyl (IPSEN-BIOTECH) was associated with human menopausal gonadotrophin (HMG, 2-10 ampoules per day; Neopergonal, Serono or Humegon, Organon) in a long protocol. In one cycle, an ultrashort protocol was used (GnRHa/HMG) and in two cycles, HMG alone. Follicular diameters were measured by ultrasound. Serum oestradiol (sE2) levels ranged from 1380 to 3300 pg/ml (sE2 in one patient was 3600 pg/ml). A single injection of either 5000 or 10 000 U HCG (depending on sE2 levels) was given 36 h before oocyte retrieval. After recovery, oocytes were washed free of follicular fluid and within 2 h were incubated in B2 medium (INRA Menezo) with suitable spermatozoa for 48 h.

In this study, 72 unfertilized oocytes were observed 0, 48 or 72 h post-insemination (p.i.). The cumulus was removed (from 0-h oocytes) by incubation with 1000 IU/ml hyaluronidase (bovine testis type IVS, Sigma) in B2 medium. After washing with several changes of prewarmed B2 medium, oocytes were incubated for 20 min in 20 ng/ml Hoechst (Hoechst 33342) in B2 medium. They were then distributed individually in small drops of the same Hoechst-containing medium on a modified Petri dish (Debey et al., 1989) under paraffin oil (BDH). The dish was placed in the thermostatic box of an inverted microscope (Nikon) flushed with 5% CO2. Images were recorded with an amplified camera (type 4336, Lhesa Electronique). They were further digitized and treated by the SAPHIRE processing system (Quantel micro consultants) and were stored on a cartridge disc (Bernouilli). Technical details have been described previously (Debey et al, 1989). From the oocytes observed *in toto*, 26 were fixed and prepared for bright field microscopy, as previously described (Bomsel-Helmreich et al., 1987).

RESULTS

One-hundred-and-seventy-five oocytes were retrieved from 18 women during 19 cycles; 152 oocytes were incubated with spermatozoa and observations for IVF at 24 h p.i. showed 34 oocytes with two pronuclei and five oocytes which were probably immature. At 48 h post-insemination, 46 oocytes had cleaved and 106 had not. The mean number of oocytes retrieved per patient was 9.4 and the mean number of undivided or abnormal oocytes provided from each patient was 6.5. In all, 39 embryos (1-4 oocytes per women) were transferred to the uterus; three women had an ongoing pregnancy. From the general batch of 106 undivided oocytes, 70 undivided oocytes were the object of this study. Two control oocytes which were not incubated with sperm were obtained and observed directly after retrieval. These two control oocytes were obtained because of a problem in obtaining spermatozoa. In Table I, the nuclear features of 72 human oocytes observed *in vitro* with fluorescence microscopy are shown.

Table I. Chromatin visualized by fluorescence in living oocytes

Nuclear features	Number of oocytes		
	0 h	48 h	72 h
GV	1	3	3
GV + decondensed sperm	-	0	1
MI	0	1	1
PBI + MII	1	18	11[a]
PBI + clumped female chromatin	-	4	1
PBI + restitution nucleus	-	2	0
PBI + MII + sperm	-	3[b]	6[c]
PBI + 2 and 2 pronuclei	-	5	7
PBI + 2 and 3 pronuclei	-	0	1
PBI + 2, metaphase/telophase first mitotic division	-	0	2
3-Cell embryo	-	0	1

[a]In one oocyte of 72 h p.i. the transformation of the MII into a restitution nucleus was observed during 6 h of culture. [b]In two oocytes condensed male chromatin (PCC) was found and in one oocyte a decondensed sperm. [c]In six oocytes PCC were found and one of these oocytes extruded PB2.

Seven oocytes were in the germinal vesicle (GV) stage. One of these contained decondensed spermatozoa which showed the capability of spermatozoa to enter immature oocytes and to decondense (Figure la,b). The other six GV oocytes were surrounded by spermatozoa but none had penetrated. All the GV oocytes had a large nucleolus and one of them had two nucleoli, with surrounding heterochromatin; the formation of chromosomes had started. Only two oocytes in metaphase I (MI) were found. Twenty-nine oocytes were in metaphase II (MII); despite their maturity and the presence of spermatozoa around them, no sperm penetration took place. Four oocytes examined at 48 h (from one patient) showed a dense mass of chromatin surrounded by a membrane visible with bright field microscopy, instead of a MII, and one oocyte-from the same patient showed a restitution nucleus.

The transformation of a MII into a restitution nucleus within 6 h of observation was seen in an oocyte aged 72 h p. i. Nine oocytes in MII were penetrated by spermatozoa. Male chromatin inside the oocyte appeared in two forms: decondensed (in one oocyte, Figure le,f) or condensed in distinct chromosomes, i.e. premature condensed chromosomes (PCC) which may also appear as a dense chromatin mass (in eight oocytes). Two oocytes in MII were found which had extruded the second polar body after sperm penetration but remained in metaphase, which may be considered as MIII as defined by Kubiak (1989). In both oocytes, spermatozoa had condensed (PCC, Figure Ig). In seven oocytes two pronuclei were found. Besides these apparently normal pronuclei, small or clumped pronuclei were also found (in five oocytes). Oocytes in the pronuclear stage or in their first mitotic division at 48 or 72 h post-insemination are evidently delayed or stopped in their development. Two control oocytes from two different patients were observed directly after retrieval without insemination. One of them was in GV and one in MII.

Ageing processes

From 38 oocytes in MII aged 48 or 72 h, with or without penetrated spermatozoa, 28 showed a greater or lesser centripetal migration of the MII chromosomes in comparison with control oocytes (Figure lc,d); 10 were not migrated. The four dense chromatin masses observed in 48 h oocytes were also far from the polar body. Such migration in 72 h oocytes was more pronounced than in 48 h oocytes, however in both groups non-migrated MII chromosomes were found. The MII chromosomes in oocytes appeared in several forms, most of them as normal MII plates; four oocytes aged 48 h had clumped chromosomes, one had lagging chromosomes and one oocyte showed a restitution nucleus. Three oocytes aged 72 h showed lagging chromosomes and two had restitution nuclei.

Polyspermy was found in two cases, a MII oocyte penetrated by two spermatozoa and one oocyte containing three pronuclei.

Morphological features determined by light microscopy

Of the 72 intact oocytes observed by DNA fluorescence, 26 were cut in thin sections and observed by bright field microscopy. The observations with DNA fluorescence and bright field microscopy are highly complementary. The spermatozoon, because of its small size, is best visualized with fluorescence microscopy. In the six GV oocytes cut into thin sections (including the control oocyte in GV), the nucleus was in a peripheral position. In the oocyte observed directly after retrieval and in four oocytes aged 48 or 72 h p.i., chromosomes had condensed and perinucleolar chromatin had almost disappeared, which is a sign of proximity to resumption of meiosis. Only one of six oocytes in GV showed invagination of the nuclear membrane which is also a sign of resumption of meiosis. In 10 oocytes in MII, spermatozoa had partly penetrated into the zona pellucida but no fertilization occurred.

The cytoplasmic features of 21 oocytes are described. The cytoplasm of the six GV oocytes showed without exception, organelles clumped together and a large number of vacuoles regularly spread in the oocyte. Of 10 MII oocytes, nine showed organization of organelles around small vacuoles and one had regularly spread organelles. In one MIII oocyte, pronuclear size vacuoles were found (Figure 1h). Four oocytes at the pronuclear stage were observed, two showed regularly spread organelles and small vacuoles, the other two had organelles clumped together and small regularly spread vacuoles. It is interesting to mention that all the GV oocytes had clumped organelles which is probably a sign of atresia, whereas the MII oocytes showed different cytoplasmic features such as organization of organelles around small vacuoles. The cytoplasm of pronuclear oocytes may allow a determination of whether they are arrested (clumping organelles) or delayed (normal cytoplasmic organization).

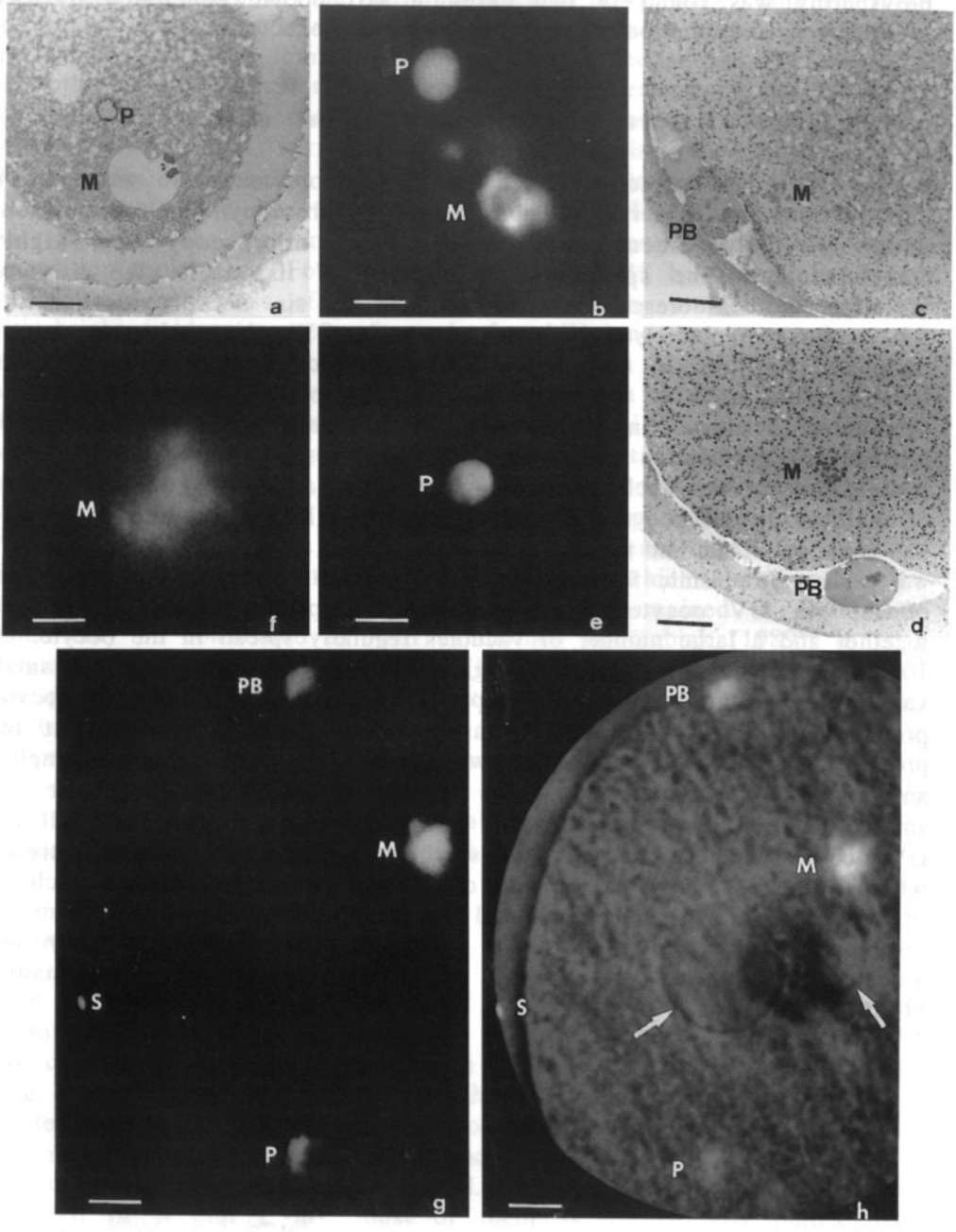
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Figure. 1. (**a**)Fixed oocyte observed by bright field microscopy; visible are maternal (M) germinal vesicle and decondensed paternal spermatozoon, (P); bar represents 4.7 µm. (**b**)The same oocyte observed *in toto* with fluorescence confirms the DNA contents of the male decondensed spermatozoon (P); the GV is visible (M); bar = 10 µm. (**c,d**)Oocytes fixed and observed by bright field microscopy; (**c**)shows the cortical position of MII chromosomes in a control oocyte (0 h p.i.); bar = 4.7 µm and (**d**)shows the centripetal migration of the MII chromosomes in an oocyte observed 48 h p.i.; bar = 4.7 µm. An oocyte observed *in toto* with fluorescence shows a MII, M (**f**)and a decondensed spermatozoon, (P); (**e**)bar = 0.4 µm. (**g**)Fluorescence of an oocyte observed *in toto,* shows one of the two polar bodies (PB), MIII (M) and PCC (P); a spermatozoon is outside the oocyte (S); bar = 10 µm. The superposition of the image obtained by fluorescence (**g**)and a bright field image gives the localization of the fluorescence (**h**); no fluorescence is seen in two pronuclear size vacuoles (arrows); bar = 10 µm.

DISCUSSION

For the present work, most of the oocytes were provided after GnRHa treatment. Oocytes when retrieved after ovarian stimulation, are known to differ in the maturational state of the nucleus. In our study, oocytes were observed in GV, MI and MII (Table II). The oocytes in GV (10%) appeared by cytoplasmic signs to be near the resumption of meiosis. This is not surprising as they were retrieved after HCG exposure, which affects oocytes even in small follicles as shown previously (Bomsel-Helmreich et al., 1987). Veeck et al. (1983) found 13.5% of the oocytes to be in GV 24-35 h after retrieval; Sathananthan (1985) found 11% GV oocytes 1-10h postretrieval and 5% 11-72 h after retrieval. It seems likely that if resumption of meiosis did not take place within 24 h, the oocytes remained in GV. So the 10% GV oocytes we found can be compared with the results of the other authors. Our results agree with those of Nayudu et al. (1989) who found that GV oocytes showed a high degree of vacuolization and organelle clustering which indicates atresia. Only a few MI oocytes were found, because this stage lasts a relatively short time. In contrast to previous observations (Tesarik et al., 1988), we show that sperm penetration can take place in immature as well as in mature oocytes. The spermatozoon penetrated into a GV oocyte, decondensed and seemed to be surrounded by a membrane. In this case it is clear that the oocyte and not the spermatozoon is responsible for the fertilization failure.

Our observation method using both fluorescence and bright field microscopy allows the DNA contents of the decondensed spermatozoon to be visualized and thus its presence to be ascertained. Lopata and Leung (1986) describe undecondensed sperm-heads in GV oocytes from small follicles (< 10 mm) not exposed to luteinizing hormone (LH) or HCG. This suggests the evolution of the capacity of the cytoplasm to decondense the

male chromatin. It is probable that this capacity increases with the approach of germinal vesicle breakdown (GVBD) when sperm decondensing factors accumulate (Usui and Yanagimachi, 1976; Clarke and Masui, 1983; Szollosi et al., 1990). The penetrability of the zona and vitellus is low in the GV stage and increases when the MII stage is reached (Iwamatsu and Chang, 1972; Lopata and Leung, 1988); this may be the reason why we found only one GV oocyte which had been penetrated by spermatozoa. The increase in zona penetrability is probably due to structural changes on the surface of the zona (Familiari et al., 1988; Motta et al., 1988).

The majority of unfertilized oocytes observed at 48 or 72 h post-insemination were in metaphase of the second meiotic division, the stage in which they can be fertilized; however, they remained unfertilized. Since oocyte maturation can take place in vitro, the oocytes found at 48 or 72 h post insemination in MII come possibly from two sources. The first source proposed for MII oocytes, are the oocytes which were in GV stage at retrieval and matured in culture either immediately or with delay. When these oocytes are subsequently found by us (48 or 72 h p.i.) in MII, ageing processes causing a centripetal migration of the MII chromosomes had not yet, or had just started so that the position of MII chromosomes would be still cortical (in the proximity of PBI) as is the case in MII oocytes just after retrieval (Bomsel-Helmreich et al., 1987). Oocytes in MII at retrieval, however, aged during the time of culture causing a centripetal migration which we expect to observe 48 or 72 h p.i. Migration of the MII chromosomes towards the centre of the oocyte was not observed in all cases. In other mammals, the centripetal migration of the MII chromosomes is an effect of oocyte ageing (Szollosi, 1975).

We showed the centripetal migration of the MII chromosomes which could only be ascertained in fixed and sectioned oocytes. Eichenlaub-Ritter et al. (1988) postulated a migration of the meiotic spindle in human oocytes, but because of the technique used (which disturbed the topography of the oocyte) this could not be confirmed. Pickering et al. (1988) did not observe any centripetal migration of MII chromosomes until 48 h (p.i.). Migration of the MII chromosomes prevents a normal expulsion of the second polar body and thus leads to abnormal fertilization after sperm penetration (Boerjan and de Boer, 1990).

In oocytes matured in vitro, hardening of the zona pellucida in culture as described by DeFelici et al. (1985) may prevent sperm penetration or delays it. Another effect of ageing is shown by lagging chromosomes in oocytes aged 48 and 72 h, this implies degeneration of the MII spindle, as also described by EichenlaubRitter et al. (1988). This degeneration is facilitated by the fact that no centrioles are present in the first and second meiotic spindle in mammals (Szollosi et al., 1972). The dispersion of chromosomes and spindle disruption leads to aneuploidy

(Szollosi, 1975; Santalo et al., 1987). In a multicentric study, Plachot et al. (1988) found an ≈ 20% incidence of chromosome abnormalities in non-transferred human embryos. Increasing maternal age or superovulation may equally cause spindle disruption. In four oocytes at 48 h (p.i.) from one patient, we found clumped chromatin masses instead of MII chromosomes. Probably this was not due to ageing but indicates a metabolic defect (ovopathy).

We observed the formation of a restitution nucleus in the human oocyte after 48 h as well as after 72-80 h in culture, which is earlier than the findings of Eichenlaub-Ritter et al. (1988). The formation of a restitution nucleus after ageing is also found in the mouse but not in the rat and in the rabbit. A restitution nucleus has the same characteristics as a pronucleus (Szollosi, 1971).

Table II. Possible causes of fertlizaton failure in individual oocytes

Nuclear status of oocyte	Cause of fertilization failure	Ovopathy	Gamete responsible
GV	Immaturity		Oocyte
MI	Immaturity		Oocyte
PB1+MII (not migrated)	Immaturity		Oocyte
PB1+MII (migrated)	Incapacity for penetration		Oocyte/sperm
PB1+clumped female chromatin		Metabolic defect	Oocyte
PB1+MII+decondensed sperm	Immaturity		Oocyte
PB1+MII+PCC	Immaturity		Oocyte
PB1+2+MII+PCC	Incapability of interphase transition		Oocyte
Two pronuclei	Immaturity or penetration incapacity		Oocyte/sperm
		Developmental arrest	Oocyte
Three pronuclei	Immaturity or overmaturity		Oocyte/sperm
Two pronuclei clumped chromatin		Developmental arrest, degeneration	Oocyte

We observed oocytes penetrated by spermatozoa. If a spermatozoon penetrates the oocyte in MII, activation of the oocyte marked by the

expulsion of the second polar body (PB2) and the formation of the male and the female pronucleus can be expected. However, mammalian oocytes acquire only gradually the possibility of responding to a penetrating spermatozoon or to parthenogenetic activation by completing the second meiotic division, extruding the second polar body and undergoing interphase transition (Kubiak, 1989). Therefore, three phases can be proposed.

The first phase concerns a spermatozoon penetrating into MI or MII oocytes which had matured too recently so the spermatozoon is not able to induce the oocyte to complete meiosis and to extrude the second polar body. The spermatozoon first decondenses, then recondenses to form distinct chromosomes (Clarke and Masui, 1986; Kubiak, 1989). This could explain why we found in one MII oocyte, a decondensed spermatozoon and in 10 MII oocytes PCCs. PCCs in human oocytes arrested in MII after sperm penetration were also observed at the same rate by Schmiady and Kentenich (1989). Observations of living oocytes incubated with the DNA fluorescent dye Hoechst 33342 allows the visualization of decondensed spermatozoa besides PCCs. The male origin of the PCCs was suggested in our study by their situation far away from MII and PB.

In the second phase, the MII oocyte reacts to a penetrating spermatozoon by resuming meiosis and extruding the second polar body, but no pronuclear formation takes place. The oocyte remains in metaphase (MIII), while the sperm nucleus first decondenses and then condenses into distinct chromosomes (Kubiak; 1989). We found two MIII oocytes with male chromosomes. The third phase would be the normal reaction of a MII oocyte to a penetrating spermatozoon by extruding the second polar body and forming the pronucleus.

The presence of two pronuclei at 48 or 72 h indicates that development is either arrested, which may be due to an ovopathy, or delayed. Normally pronuclei are found between 17 and 24 h p.i. (Trounson et al., 1982). Late fertilization may be caused by immaturity of the oocyte or by a reduced capacity of spermatozoa to penetrate the zona pellucida. The implantation of embryos obtained by reinsemination 24 h after the first insemination occurs only in 1-2% of the cases (Pampiglione et al., 1990). After a certain time, arrested pronuclei will degenerate as illustrated by two pronuclei containing clumped chromatin found at 72 h p.i. Large pronuclear size vacuoles, pseudopronuclei (PPN), as described by van Blerkom et al. (1987) can easily be distinguished from real pronuclei by the superposition of the fluorescence- and the bright field images of living oocytes, which shows their lack of DNA.

Our results show two cases of polyspermic penetration. In one MII oocyte, this may have been due to overmaturity causing a migration of the cortical granules into the cytoplasm (Szollosi, 1975). The oocyte with three pronuclei was indeed polyspermic because no digyny took place

(two polar bodies were found). While Trounson et al. (1982) observed multipronuclear embryos coming from oocytes which were classified as immature, Rudak et al. (1984) did not. In agreement with Rudak et al. (1984), Ben-Rafael et al. (1987) found a positive correlation between progesterone levels in the follicular fluid and polypronuclear fertilization. High progesterone levels may result from luteinization after prolonged maturation. Both overmaturity and immaturity may cause polyspermic penetration and subsequent polypronuclear development (Colston et al., 1983).

The different nuclear aspects found in oocytes which failed to cleave within 48 h gives information about the causes of fertilization failure (Table II) for individual oocytes. However, the complete set of unfertilized oocytes from one patient observed in toto and fixed, may give additional information about the clinical origins of the failure, especially for those patients with unexplained infertility (B.van Wissen, C.Eisenberg, P.Debey, G.Pennehouat, J.Auger and O.Bomsel-Helmreich, in preparation). We conclude that the use of three complementary methods, fluorescence and bright field microscopy in toto and observations on fixed and sectioned oocytes, allows a rapid observation of the status of the male and female chromatin and their eventual modifications in living oocytes. We show that a systematic analyis of uncleaved oocytes (48 h p.i.) can help to evaluate the causes of fertilization failure. Furthermore we show clearly that the technique of DNA fluorescence can easily be applied in clinical situations where oocytes (48 h p.i.) fail to fertilize after several attempts; thus in some cases it becomes possible to attribute this failure to either the male or the female gamete.

REFERENCES

Ben-Rafael,Z., Meloni,F., Strauss,J.F., Blasco,L., Mastroianni,L. and Flickinger,G.L. (1987) Relationships between polypronuclear fertilization and follicular fluid hormones in gonadotropin-treated women. *Fertil. Steril.*, 47, 284-288.

Boerjan,M. and de Boer,P. (1990) First cell cycle of zygotes of the mouse derived from oocytes aged postovulation *in vivo* and fertilized *in vivo*. *Mol. Reprod. Dev.*, 25, 155-163.

Bomsel-Helmreich,O., Huyen,L.V.N., Durand-Gasselin,I., SalatBaroux,J. and Antoine,J.M. (1987) Timing of nuclear maturation and cumulus dissociation in human oocytes stimulated with clomiphene citrate, human menopausal gonadotropin, and human chorionic gonadotropin. *Fertil. Steril.*, 48, 586-595.

Bryski,R.G., Muasher,S.J., Droesch,K., Simonetti,S., Jones,G.S. and Rosenwaks,Z. (1988) Follicular atresia associated with concurrent initiation of gonadotropin-releasing hormone agonist and folliclestimulating hormone for oocyte recruitment. *Fertil. Steril.*, 50. 917-921.

Clarke,H.J. and Masui,Y. (1983) The induction and irreversible chromosome decondensation by protein synthesis inhibition during meiotic maturation of mouse oocytes. *Dev. Biol.*, 97, 291-301.

Clarke,H.J. and Masui,Y. (1986) Transformation of sperm nuclei to metaphase chromosomes in the cytoplasm of maturing oocytes of the mouse. J. *Cell Biol.*, 102, 1039-1046.

Colston Wentz,A., Repp,J.E., Maxson,W.S., Pittaway,D.E. and Torbit,C.A. (1983) The problem of polyspermy in in vitro fertilization. *Fertil. Steril.*, 40, 748-754.

Debey,P., Renard,J.P., Coppey-Moisan,M., Monnot,l. and Geze,M. (1989) Dynamics of chromatin changes in live one-cell mouse embryos: a continuous follow-up by fluorescence microscopy. *Exp. Cell Res.*, 183, 413—433.

DeFelici,M., Salustri,A. and Siracusa,G. (1985) 'Spontaneous' hardening of the zona pellucida of mouse oocytes during in vitro culture. II. The effect of follicular fluid and glycosaminoglycans. *Gamete Res.*, 12, 227-235.

Eichenlaub-Ritter,U., Stahl,A. and Luciani,J.M. (1988) The microtubular cytoskeleton and chromosomes of unfertilized human oocytes aged in vitro. *Hum. Genet., 80,* 259-264.

Familiari,G., Nottola,S.A., Micara,G., Aragona,C. and Motta,P.M. (1988) Is the sperm-binding capability of the zona pellucida linked to its surface structure? A scanning electron microscopic study of human in vitro fertilization. J. *In vitro Fertil. Embryo Transfer, 5,* 134-143.

Frydman,R., Belaisch-Allart,J., Parneix,l., Forman,R., Hazout,A. and Testart,J. (1988) Comparison between flare-up and down-regulation effects of luteinising hormone-releasing hormone agonists in an in vitro fertilization program. *Fertil. Steril., 50,* 471-475.

Iwamatsu,T. and Chang,M.C. (1972) Sperm penetration in vitro of mouse oocytes at various times during maturation. J. *Reprod. Fertil.*, 31, 237-247.

Kubiak,J.Z. (1989) Mouse oocytes gradually develop the capacity for activation during the metaphase II arrest. *Dev. Biol.*, 136, 537-545.

Lopata,A. and Leung,P.C. (1988) The fertilizability of human oocytes at different stages of meiotic maturation. *Ann. N. Y. Acad. Sci.*, 541, 324-336.

Motta,P.M., Nottola,S.A., Micara,G. and Familiari,G. (1988) Ultrastructure of human unfertilized oocytes and polyspermic embryos in an IVF-ET program. *Ann. N.Y. Acad. Sci.*, 54, 367-383.

Nayudu,P.L., Lopata,A., Jones,G.M., Gook,D.A., Bourne,H.M., Sheather,S.J., Brown,T.C. and Johnston,W.H.I. (1989) An analysis of human oocytes and follicles from stimulated cycles: oocyte morphology and associated follicular fluid characteristics. *Hum. Reprod.*, 4, 558-567.

Neveu,S., Hedon,B., Bringer,J., Chinchole,J.M., Arnal,F., Humeau,C., Cristol,P. and Viala,J.L. (1987) Ovarian stimulation by a combination of a gonadotropin-releasing horrnone agonist and gonadotropins for in vitro fertilization. *Fertil. Steril.*, 47, 639-643.

Pampiglione,J.S., Mills,C., Campbell,S., Steer,C., Kingsland,C. and Mason,B.A. (1990) The clinical outcome of reinsemination of human oocytes fertilized in vitro. *Fertil. Steril.*, 53, 306-310.

Pellicer,A., Ruiz,A., Castellvi,R.M., Calatayud,C., Ruiz,M., Tarin,J.J., Miro,F. and Bonilla-Musoles,F. (1989) Is the retrieval of high numbers of oocytes desirable in patients treated with gonadotrophinreleasing hormone analogues (GnRHa) and gonadotrophins? *Hum. Reprod, 4,* 536-540.

Pickering,S.J., Johnson,M.H., Braude,P.R. and Houliston,E. (1988) Cytoskeletal organisation in fresh, aged and spontaneously activated human oocytes. *Hum. Reprod., 3,* 978-989.

Plachot,M., Veiga,A., Montagut,J., Grouchy,J.de, Calderon,G., Lepretre,S., Junca,A.M., Santalo,J., Carles,E., Mandelbaum,J., Barri,P., Degoy,J., Cohen,J., Egozcue,J., Sabatier,J.C. and Salat-Baroux,J. (1988) Are clinical and biological IVF

parameters correlated with chromosomal disorders in early life: a multicentric study. *Hum. Reprod.*, 3, 627-635.

Rudak,E., Dor,J., Mashiach,S., Nebel,L. and Goldman,B. (1984) Chromosome analysis of multipronuclear human oocytes fertilized in vitro. *Fertil. Steril., 41,* 538-545.

Santalo,J., Badenas,J., Català,V. and Egozcue,J. (1987) Chromosomes of mouse embryos in vivo and in vitro: effect of manipulation, maternal age and gamete ageing. *Hum. Reprod., 2,* 717-719.

Sathananthan,A.H. (1985) Maturation of the human oocyte in vitro: nuclear events during meiosis (an ultrastructural study). *Gamete Res.*, 12, 237-254.

Schmiady,H. and Kentenich,H. (1989) Premature chromosome condensation after in vitro fertilization. *Hum. Reprod., 4,* 689-695.

Szöllösi,D. (1971) Morphological changes in mouse eggs due to ageing in the fallopian tube. *Am. J. Anat.*, 130, 209-225.

Szöllösi,D. (1975) Mammalian eggs aging in the fallopian tubes. In Blandau,R.J. (ed.), *Aging Gametes,* Karger, International Symposium, Seattle, pp. 98-121.

Szöllösi,D., Calarco,P. and Donahue,R.P. (1972) Absence of centrioles in the first and second meiotic spindles of mouse oocytes. *J. Cell Sci.*, 11, 521-541.

Szöllösi,D., Szöllösi,M., Czolowska,R. and Tarkowski,A.K. (1990) Sperm penetration into immature mouse oocytes and nuclear changes during maturation: an EM study. *Biol. Cell., 69,* 53-64.

Tesarik,L., Pilka,L. and Travnik,P. (1988) Zona pellucida resistance to sperm penetration before the completion of human oocyte maturation. *J. Reprod Fertil.*, 83, 487-495.

Testart,J., Forman,R., Belaisch-Allart,J., Volante,M., Hazout,A., Strubb,N. and Frydman,R. (1989) Embryo quality and uterine receptivity in in-vitro fertilization cycles with or without agonists of gonadotrophin releasing hormone. *Hum. Reprod., 4,* 198-201.

Trounson,A.O., Mohr,L.R., Wood,C. and Leeton,J.F. (1982) Effect of delayed insemination on in-vitro fertilization, culture and transfer of human embryos. J. *Reprod. Fertil., 64,* 285-294.

Usui,N. and Yanagimachi,R. (1976) Behaviour of hamster sperm nuclei incorporated into eggs at various stages of maturation, fertilization, and early development. *J. Ultrastruct. Res., 57,* 276-288.

Van Blerkom,J., Bell,H. and Henry,G. (1987) The occurrence, recognition and developmental fate of pseudo-multipronuclear eggs after in vitro fertilization of human oocytes. *Hum. Reprod., 2.* 217-225.

Veeck,L.L., Wortham,J.W.E., Witmyer,J., Sandow,B.A., Acosta,A.A., Garcia,J.E., Jones,G.S. and Jones,H.W. (1983) Maturation and fertilization of morphologically immature human oocytes in a program of in vitro fertilization. *Fertil. Steril.*, 39, 594 -602.

CHAPTER III

In Vitro DNA Fluorescence after in Vitro Fertilization (IVF) failure

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CHAPTER VII

In Vitro DNA Fluorescence after In Vitro Fertilization (IVF) Failure

ABSTRACT

Purpose: A pilot study was performed to test the diagnostic value of in vitro DNA fluorescence in oocytes that failed to fertilize after IVF. Ten patients with a cleavage rate less than 20% after IVF were included.
Results: Uncleaved oocytes were observed by fluorescence microscopy after incubation with the DNA fluorescent dye Hoechst 33342. Four main causes which may have contributed to the low cleavage rate werefound: (I) sperm incapacity to penetrate the oocyte despite the absence of the usual criteria for male infertility, (2) oocyte immaturity, (3) delayed fertilization, and (4) oocyte abnormalities revealed by aberrations in the morphology of the female chromatin.
Conclusions: The possibility of a rapid and detailed analysis of the maturational status of unfertilized oocytes, the morphology of the female chromatin, the presence and quantity of spermatozoa tightly bound to the zona pellucida, and sperm penetration into the oocyte without subsequent pronucleus formation, using DNA fluorescence, allows us to clarify further the cause of fertilization failure and to orient infertility treatment toward the male, the female, or both partners.

INTRODUCTION

Complete or partial cleavage failure after in vitro fertilization (IVF) cannot always be explained. Observation of oocytes considered unfertilized 48 hr postinsemination may help to determine the causes of such counterperformances. In classical IVF procedures, oocytes are observed by phase-contrast microscopy. Yet this does not permit analysis of the nuclear status of the oocyte. Hence, other approaches have been proposed such as serial sectioning of fixed oocytes for light or electron microscopy (1-4) and cytogenetic analysis (5-9). These techniques are time-consuming and alter the integrity of the oocyte. In a previous study we analyzed intact, living oocytes obtained from IVF procedures using fluorescence microscopy, after exposure to a DNA fluorescent dye (Hoechst 33342). In that report, criteria for oocyte immaturity, oocyte aging, sperm penetration, and fertilization stages were described and discussed (4). The present pilot study was undertaken to test the practical diagnostic value of DNA fluorescence after IVF failure. Ten IVF patients who had a cleavage rate of less than 20% were included. Nondivided oocytes were

observed by DNA fluorescence to determine the causes of cleavage failure and, more specifically, to identify the responsible gamete.

MATERIALS AND METHODS

Patients and Protocol

Ten patients (12 IVF cycles) were included in this study. They had a total of 19 previous unsuccessful IVF attempts. All patients were between 27 and 38 years of age, except for one patient who was 42 years old. Reasons for entering the IVF program were tubal infertility (n = 5), tubal combined with male infertility (n = 1), endometriosis combined with male infertility (n = 1), male infertility exclusively (n = 1), and idiopathic infertility (n = 2). Sperm parameters were evaluated according to the WHO recommendations (10). In 11 of 12 cycles pituitary suppression was achieved by a gonadotropin releasing hormone agonist (GNRHa; DTRP6, Ipsen Biotech, Paris, France; 3.75 mg) (11). Follicular growth was stimulated with 2-5 vials (75 IU) of human menopausal gonadotropin (HMG; Neopergonal, Serono, Paris, France), administered for 8-13 days depending on the individual responses. In the remaining cycle, no GnRHa was used and ovarian stimulation was induced by a combination of follicle stimulating hormone (FSH; Metrodine, Serono) and hMG. A single injection of human chorionic gonadotropin (HCG) of 10,000 IU was given 36 hr before oocyte retrieval when at least two follicles exceeded 16 mm in diameter and plasma estradiol (pE2) was between 200 and 300 pg/ml per follicle larger than 16 mm. After recovery, oocytes were washed in B2 medium (Biomerieux, Montalieu-Vercieu, France), and each was inseminated with 20,000 motile sperm (prepared by centrifugation on discontinuous Percoll gradients) and cultured (37°C, 5% CO2) for 48 hr. Oocytes were examined between 17 and 24 hr postinsemination (pi) for the presence of pronuclei and 44-48 hr pi for cleavage.

Fluorescence Microscopy

Oocytes were incubated for 20 min (37°C in 5% CO2) with the DNA specific fluorescent dye Bisbenzimide H33342 (Coger Pharmaceuticals, Paris, France; 20 ng/ml in B2 medium) and subsequently observed by bright-field and fluorescence microscopy as described before (4,12).

Morphological Definitions

In a previous study (4) we discussed the nuclear features of nondivided oocytes analyzed by DNA fluorescence 48 hr pi. Oocytes that are immature at the time of retrieval may mature in vitro and appear 48 hr pi as mature metaphase II oocytes with a cortical location of the metaphase chromosomes, characteristic of newly matured oocytes. But mature oocytes, which are not penetrated by sperm, undergo aging processes characterized by a centripetal migration of the metaphase II chromosomes. Both immature and aged oocytes can be penetrated by sperm, but usually no developing embryo ensues. Fertilization of oocytes that resumed and finished their meiosis in vitro leads to delayed pronuclear zygotes but the resulting embryos rarely give pregnancies. Arrested pronuclear zygotes evidently do not cleave. The oocyte classification used in this study is illustrated in Table I. The number of spermatozoa bound to each zona pellucida was counted and a mean was calculated per patient. According to this mean, three categories were created: no spermatozoa, between one and three spermatozoa, and more than five spermatozoa attached to the zona.

RESULTS

During a 3-month study period, 56 IVF cycles were performed, with a mean cleavage rate of 44%. All the oocytes retrieved were counted including those atretic, fragmented, or with a fractured zona pellucida. Twenty of the 56 cycles had a cleavage rate less than 44%. Of these, 12 cycles (10 patients) were randomly chosen for this study. From 112 retrieved oocytes, 11 embryos ensued, of which 9 had two blastomeres, 1 had three blastomeres, and 1 had four blastomeres. All but a single two-cell embryo had irregular-sized blastomeres and multiple fragments. Ten embryos were transferred but no pregnancy resulted.

Twenty-two uncleaved oocytes were discarded because of atresia (8 oocytes), fragmentation (2 oocytes), polyspermy (1 oocyte), and fractured zona pellucida (1 oocyte), and 10 oocytes were lost. Finally, 68 uncleaved oocytes were observed by DNA fluorescence. The patients included in this study were divided into two groups, depending on whether no cleavage had occurred during the studied cycles (group 1) or cleavage of some of the inseminated oocytes (group 2) had occurred. In group 1, three couples had never developed embryos in previous IVF cycles and two couples had one embryo each in a previous cycle (cases 2 and 3). In group 2, 22 embryos were obtained and transferred from four couples during 10

previous IVF cycles (total cleavage rate, 13.9%). One couple (case 8) had no embryo in a previous IVF cycle.

Table I. Morphological Criteria Determining the Fertilizability of the Oocytes at the Time of Insemination

Classification	Observations 48 hr pi	Presumed stage at time of retrieval/insemination
Immature	GV or MI	Immature
Mature	PBI + MII cortical position : maturation in vitro	Immature
Overmature	PBI + MII centripetally migrated : aging processes in vitro	Mature
Abnormal chromatin morphology	PBI + clumped female chromatin	Abnormal chromatin morphology
	PBI + interphase nucleus	
Evidence of sperm penetration	GV + male chromatin	Immature
	PBI + MII + male chromatin	Cytoplasmic immaturity
	PBI + 2 + 2PN : delayed or arrested development	Immature or mature?

Abbreviations: GV, germinal vesicle; MI, metaphase I: MII, metaphase II; PB1, first polar body; PB1/2, first and second polar body; PN, pronuclei.

In group 1 (Table II), 37 oocytes from five patients (six cycles) were obtained: 11 were considered as immature at the time of retrieval, of which 10 matured in vitro; 22 were mature at the time of retrieval and overmatured (aged) in vitro; 2 showed an abnormal morphology of the female chromatin; and 2 were penetrated by sperm. Patient 1 had a majority of mature oocytes (Fig. 1c) and sperm was never found attached to or penetrated into these oocytes. Oocyte immaturity together with sperm incapacity was suggested as the cause for IVF failure. For cases 2, 3, and 4, there was a majority of overmature (Fig. 1d) oocytes, with between zero and three spermatozoa attached to the zona and none penetrated, thus indicating a sperm malfunction. In case 5 (two cycles), delayed fertilization due to oocyte immaturity was suggested by the presence of both meiotically immature and mature oocytes. Two oocytes were penetrated by sperm: one was in metaphase II stage with male chromatin prematurely condensed, while the other had two pronuclei

which were not observed the previous day. Since some of the oocytes were penetrated by sperm and more than five spermatozoa were attached to the zona, sperm did not appear to be implicated in the IVF failure.

In group 2 (Table II), at least one embryo was obtained from each of the five patients (six cycles). Thirty-one oocytes were observed, of which 4 oocytes were immature at the time of retrieval (2 matured in vitro), 18 were mature (all overmatured in vitro), and 5 oocytes originating from 1 single cycle had chromatin abnormalities (clumped or decondensed female chromatin), and in 4 oocytes from three patients (including the one showing chromatin abnormalities) sperm penetration was ascertained. For case 6, persistent immaturity was suggested since mostly immature or oocytes which matured in vitro were found. One germinal vesicle stage (GV) oocyte was penetrated by sperm and the male chromatin was partly decondensed. Cases 7 and 8 are comparable with cases 2, 3, and 4 since a majority of overmature oocytes was found, suggesting sperm incapacity to penetrate into the oocytes. Delayed fertilization in case 9 was due to impaired sperm quality since both overmature oocytes and pronuclear-stage oocytes (not observed the previous day) were found. Case 10 showed a heterogeneous oocyte population: mature and overmature oocytes, oocytes with an abnormal chromatin morphology (Figures 1b, e, and f), and one pronuclear stage oocyte (not observed the previous day) were found.

Table II. Nuclear Features by Fluorescence in Nondivided Oocytes

		Number of oocytes									
Nuclear features 48 hr pi	Presumed stage at retrieval	Group 1 : no cleavage						Group 2 : cleavage			
		1[a]	2	3	4	5	6	7	8	9	10
Immature	Immature					1	2				
Mature	Immature	7	2			1	1				1
Overmature	Mature		4	10	6	2		2	11	2	3
Abnormal chromatin morphology	Abnormal chromatin morphology	1		1							5
Evidence of sperm penetration	Immature/ mature					2[b,c]	1[d]			2[c]	1[c]

[a]Case number. [b]First polar body and metaphase II chromosomes, male chromatin observed in ooplasm, [c]First and second polar body and two pronuclei, [d]Germinal vesicle-stage oocyte, male chromatin observed in ooplasm.

A nonrecurrent abnormality of the chromatin morphology was observed in half of the oocytes during one of the two cycles studied from this patient. Four of five patients had mostly more than five spermatozoa attached to the zona pellucida; only one patient had between one and three spermatozoa attached to the zona (case 10).

DISCUSSION

When failed fertilization or cleavage occurs in IVF, its etiology most often remains unsolved. In this work, the analysis of nuclear features by DNA fluorescence in oocytes remaining uncleaved from a small number of patients provided additional information about the respective involvements of oocyte or sperm in IVF failure in 8 of 10 cases. In three cases, oocytes were immature, with one having total sperm incapacity. In three cases, sperm was found completely unable to penetrate mature and a priori fertilizable oocytes. In two cases, the sperm penetrating capacity was impaired. No conclusive information was obtained in one case because too few oocytes were available for observation and in another case because the population of oocytes was too heterogeneous and a nonrecurrent oocyte abnormality was present.

Sperm Incapacity

It has generally been suggested that in the case of complete fertilization failure, sperm is most often the suspected gamete (9,13,14). For the couples induded in this study, sperm defects are suggested when oocytes remained uncleaved 48 hr pi and were found to be overmature and not penetrated by sperm. Overmaturity of uncleaved oocytes signifies that oocytes were mature and proper to be fertilized at the time of retrieval (4). We postulate that cases where exclusively overmature oocytes were found (group 1), or overmature oocytes in association with only a few, morphologically poor embryos (cases 7 and 8, group 2), have a comparable etiology, as in both cases cleavage failure appears to have a male origin. In support of our observations, Ron-El *et al.* (15) have reported that certain sperm defects are correlated with poor embryo morphology. Failure of sperm to penetrate the oocytes can result from multiple factors. For sperm penetration to occur, sperm-zona binding, penetration of the zona pellucida, and fusion with the oolemma are requisite.

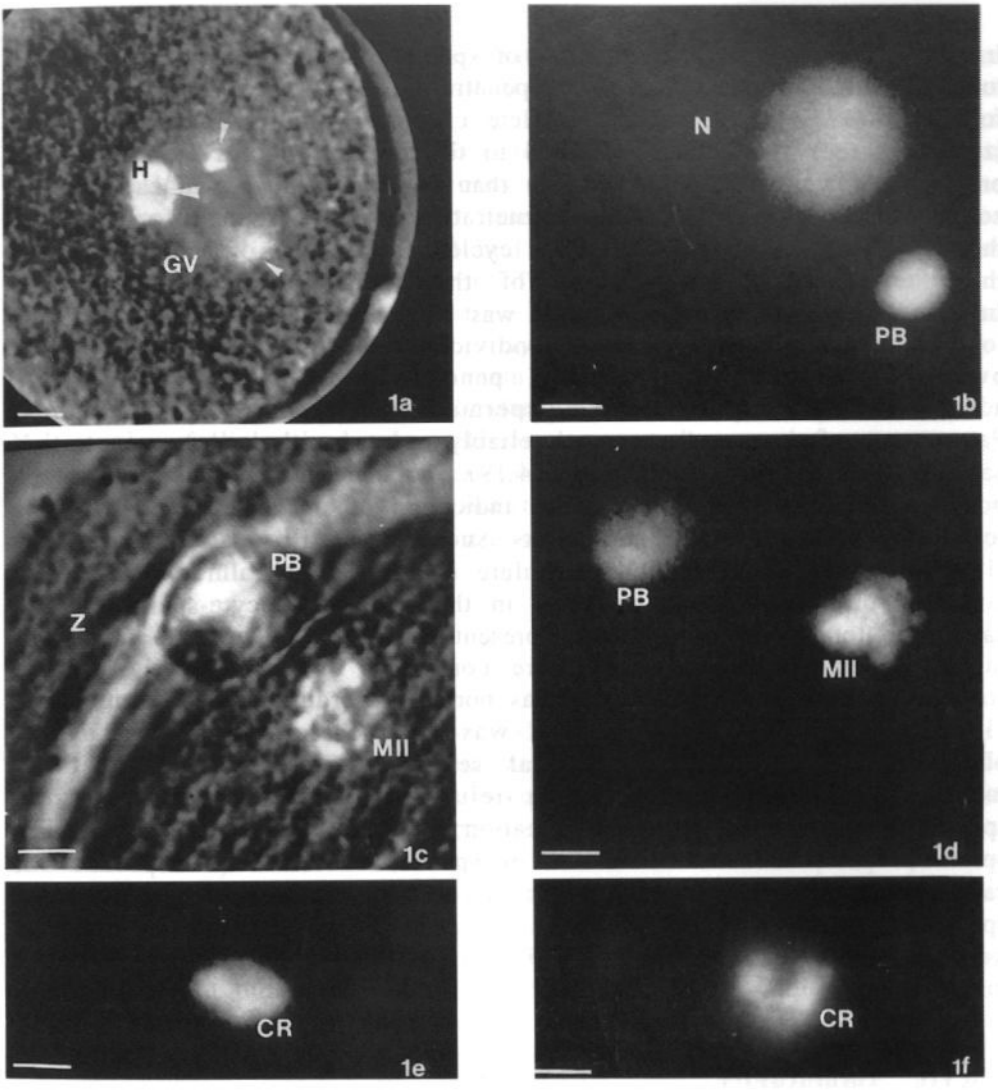

Figure 1. **(a)** Immature: oocyte in germinal vesicle (GV) stage. Nucleolus (arrowhead), surrounding heterochromatin (H). start of chromosome formation (small arrowheads). Superposition of image obtained by DNA fluorescence and bright-field microscopy in vitro. Bar = 10 µm. **(b)** Abnormal chromatin morphology: Oocyte with one polar body (PB). Instead of metaphase chromosomes a decondensed chromatin mass is present as a nucleus (N). Bar = 10 µm. **(c)** Mature: Zona pellucida (Z), polar body (PB), and metaphase II chromosomes in a cortical position (MII). Notice the clear presence of individual chromosomes. Superposition of DNA fluorescence and bright-field microscopy in vitro. Bar = 5 µm. **(d)** Overmature: Chromatin of polar body (PB) and centripetally migrated metaphase II (MII) chromosomes (aging process). Individual chromosomes are less clear since they are slightly out of focus. DNA fluorescence in vitro. Bar = 5 µm. **(e, f)** Abnormal chromatin morphology: Oocyte with a clumped mass of female chromatin (CR), instead of metaphase II chromosomes. DNA fluorescence in vitro. Bar = 5 µm.

It has been shown that the number of sperm bound to the zona is related to fertilization (16) and to sperm penetration with or without pronucleus formation (17). Couples with complete cleavage failure showed very rare (zero to three) spermatozoa attached to the zona of uncleaved oocytes. In one case, however, there were more than five spermatozoa attached to the zona, and here successful sperm penetration was ascertained for some of the oocytes. For most of the studied cycles, the number of sperm bound to the oocyte appeared predictive of the sperm penetration capacity. Impaired sperm penetration capacity was suggested when in the case of a low cleavage rate (group 2), nondivided oocytes were found to be overmature (48 hr pi) and not penetrated by sperm, despite the adherence of more than five spermatozoa to the zona pellucida. Parameters of sperm that most reliably relate with sperm penetration capacity are morphological ones (14,19). Yet the number of spermatozoa bound to the zona may also be an indication of oocyte maturity, besides reflecting normal sperm parameters such as motility and morphology (18). However, when fertilization failure is complete or almost-complete, a possible role of the zona pellucida in the impaired oocyte-sperm binding cannot be totally excluded. In our present study all known male factors of infertility diagnosed previously were confirmed by our analysis. Yet in some other cases in which sperm was normal according to WHO guidelines (10), actual sperm dysfunction was nevertheless suspected. This observation indicates that classical semen parameters are sometimes insufficient to predict fertilization reliably. In cases having impaired sperm penetration capacity, modification of the IVF method. concerning sperm preparation and the number of spermatozoa used for insemination, can be proposed (20). When sperm incapacity was complete, functional sperm tests are suggested, such as the hemizona assay (21) and the hamster test (22), before considering a new IVF attempt or a micromanipulation protocol.

Oocyte Immaturity

In the case of partial fertilization failure. oocyte immaturity has been reported to be the most likely cause for the failure (9,13). Our results are in agreement with this concept; oocytes that were immature at retrieval and matured in vitro were found in association with mature oocytes which aged in vitro (when no sperm penetration took place) or with embryos (group 2). This observation could have been predicted, as ovarian stimulation protocols fail to achieve perfect oocyte synchrony, thus yielding mature fertilizable oocytes together with immature oocytes. We also found, however, complete batches of immature oocytes despite the presence of large preovulatory follicles with corresponding plasma

estradiol levels, suggesting oocyte maturity. Oocytes from small follicles can be penetrated by sperm at all stages of in vitro meiotic maturation (23). However, immature or too recently matured oocytes, when penetrated by sperm, are not able to form pronuclei because of their meiotic and/or cytoplasmic immaturity (6,24). In our study, at least some of the oocytes which remained immature in culture or which were immature at the time of retrieval and matured in vitro would have been expected to be penetrated by sperm. Therefore when all the oocytes from one patient are immature at the time of retrieval but none is penetrated by sperm, a sperm dysfunction may coexist with oocyte immaturity. When oocyte immaturity was the main cause of poor IVF results, a more detailed analysis of the whole follicular phase including the growth rate of èhe ovarian follicles and a correlation between the number of maturing oocytes and the plasma estradiol rise is suggested as well as an adaptation of the stimulation protocol. For oocyte immaturity combined with sperm incapacity, functional sperm tests are suggested before a new IVF attempt is considered.

Delayed Fertilization

The extrusion of the second polar body in response to sperm penetration can take place only when the oocyte is mature, irrespective of whether maturation has been completed in vivo or in vitro. Thus, development of embryos derived from oocytes that are immature at the time of retrieval and subsequently matured in vitro will be delayed. In keeping with this concept, Zenzes *et al.* (25) have shown a delayed appearance of pronuclei and subsequent cleavage stages after fertilization of immature oocytes, which they have hypothesized might affect pre- and postimplantation development. In our study, when pronuclei were observed as much as 44-48 hr after insemination and not, as would be expected, 17-24 hr after insemination, they could indeed be considered a delayed occurrence. The finding of immature oocytes together with delayed pronuclear zygotes may indicate that delayed fertilization results from oocyte immaturity. In such cases. delayed fertilization may far exceed the time course (0-5 hr) allowed in some assisted conception protocols to complete the normal maturation process either in vivo (by increasing the time course between hCG administration and oocyte retrieval) or in vitro (by increasing the time course between retrieval and insemination), which has been shown to improve IVF results (26,27). There is no proof that in vitro resumption of meiosis in IVF procedures is comparable to the in vivo process and that subsequent development can yield viable embryos. Results following reinsemination (12-22 hr after the initial insemination) may indicate that complete maturation in vitro, among other factors such as overmaturation,

is not beneficial in IVF since it does not or only occasionally leads to fertilization and pregnancies (28,29). Practically, however, the problem is often compounded, as both oocyte immaturity and impaired semen quality can delay fertilization (19,30). Therefore, when. besides immature oocytes. an important part of the oocytes is found to be overmature associated with delayed fertilization, sperm quality may also be impaired.

Oocyte Abnormalities

Abnormalities of the female chromatin, such as the presence of a nucleus or clumped chromatin when metaphase II chromosomes are expected in the presence of the first polar body, were observed in uncleaved oocytes. Because of these chromatin condensation abnormalities, oocytes are not able to expulse the second polar body. While such abnormalities were sometimes observed, they did not appear to have played a major role in the IVF failure of the studied couples, except in one case where half of the observed oocytes showed chromatin anomalies. Failure of resumption of meiosis and the maintenance of germinal vesicle oocytes 48 hr postinsemination, as observed by several authors (31,32), may also indicate an oocyte abnormality since immature human oocytes are able to mature spontaneously in vitro when released from the follicular fluid (33). This is supported by the finding that germinal vesicle-stage oocytes found in IVF 48 hr pi are highly vacuolated and show clumping of organelles, which are probably signs of atresia (4,34), The presence of morphological abnormalities is to be taken into account only when a majority of the oocytes from one patient shows these features and when this observation is recurrent. For these cases oocyte donation can be proposed.

Conclusions

In the present pilot study, we analyzed oocytes obtained from couples with complete or partial cleavage failures. The outcome of present and past IVF cycles was combined with the results of the morphological analysis of uncleaved oocytes after chromatin staining. Cleavage failure of male origin was suggested when we observed an incapacity of sperm to attach to the zona and penetrate the oocyte despite maturity of the oocyte at the time of retrieval. On the contrary, cleavage failure of female origin was suggested when oocyte abnormalities were found, revealed by aberrations in the female chromatin morphology or a persistent germinal vesicle. Depending on the oocyte's maturational status, cleavage failure of either female or both female and male origin was suggested when delayed

fertilization was observed. Despite the absence of a recognized male factor for infertility, cleavage failure of male origin was, in some cases, nevertheless suspected. The possibility of a rapid and detailed analysis of the maturational status of the uncleaved oocytes, the morphology of female chromatin, the presence and number of spermatozoa bound to the zona pellucida, and sperm penetration into the oocyte allows orientation of infertility treatment toward the male, the female, or both partners. Based on the promising results of this pilot study, a prospective trial is currently implemented in our IVF program to determine precisely the practical value of this technique as a clinical means to attribute counterperformances of assisted Conception to either a female or a male origin.

REFERENCES

1. Bomsel.Helmreich O. Durand-Gasselin 1. Pennehouat G. Vu N Huyen L, Antoine JM, Salat-Baroux J: Sructure and ultrastructure of human preovulatory oocytes immediately before the administration of hCG. *In:* From Ovulation to Implantation, JLH Evers, MJ Heineman (eds). Exerpta Medica, Int. Congr. Ser. 917, 1990. pp 81-90
2. Sathananthan AH: Maturation of the human oocyte in vitro: Nuclear events during meiosis (an ultrastructural study). *Gamete Res* 1985; 12:237-254
3. Van Blerkom J, Henry G: Cytogenetic analysis of living human oocytes: Cellular basis and developmental consequences of pertubations in chromosomal organisation and complement. *Hum Reprod* 1988; 3: 777-790
4. Wissen AMB VAN. Bomsel-Helmreich O. Debey P, Eisenberg C, Vaulthier, D, Pennehouat G: Fertilization and ageing processes in non divided human oocytes after GnRHa treatment: An analysis of individual oocytes. *Hum Reprod* 1991; 6:879-884
5. Tarkowski AK: An air-drying method for chromsome preparation from mouse eggs. *Cytogenetics* 1966; 5:394-400
6. Schmiady H, Kentenich H: Premature chromosome condensation after in vitro fertilization. *Hum Reprod* 1989; 4:689-695
7. Pieters MHEC, Geraedts JPM, Dumoulin JCM, Evers JLH. BRAS M, Kornips FHAC. Menheere PPCA: Cytogenetic analysis of in vitro fertilization (IVF) failures. *Hum Genet* 1989;81:367-370
8. Selva J, Martin-Pont B, Hugues JN, Rince P. Fillion C, Herve F, Tamboise A, Tamboise E: Cytogenetic study of human oocytes uncleaved after in-vitro fertilization. *Hum Reprod* 1991;6:709-713
9. Tarin JJ, Ruiz A, Miro F, Bonilla-Musoles F, Pellicer A: Failed in vitro fertilization of human oocytes: a cytogenetic analysis. *Fertil Steril* 1991;56:290-295
10. World Health Organization: Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction, MA Belsey, R Eliasson, AJ Gallegos, KS Moghissi, CA Paulson (eds). Singapore. Press Concern. 1980
11. Frydman R, Parneix I, Belaisch-Allart J, Forman R, Hazout A, Fernandez H, Testart J: LHRH agonists in IVF: Different methods of utilization and comparison with previous ovulation stimulation treatments. *Hum Reprod* 1988;3:559-561

12. Debey P, Renard JP, Coppey-Moisan M. Monnot I. Geze M: Dynamics of chromatin changes in live one-cell mouse embryos: a continuous follow-up by fluorescence microscopy *Exp Cell Res* 1989;183:413-433
13. Gwatkin RBL, Conover JC. Collins RL, Quigley MM: Failed fertilization in human in vitro fertilization analysed with the deoxyribonucleic acid-specific fluorochrome Hoechst 33342. *Am J Obstet Gynecol* 1989;160:31-35
14. Liu DY, Lopata A, lohnston WIH, Balcer HWG: Human sperm-zona pellucida binding, sperm characeristics and in vitro fertilization. *Hum Reprod* 1989,4:696 701
15. Ron-EI R, Nachum H, Herman A. Golan A. Caspi E. Soffer Y: Delayed fertilization and poor embtyonic development associated with impaired semen quality. *Fertil Steril* 1991;55:338-344
16. Liu DY, Lopata A, Johnston WIH, Balcer HWG: A human sperm-zona pellucida binding test using oocytes that failed to fertilize in vitro. *Fertil Steril* 1988;50:782-788
17. Van Wissen B, Bomsel-Helmreich O, de Ziegler D. Pietri V, Frydman R: Examen morpho-fonctionnel des gamètes en cas d'echec de fécondation in vitro. Numéro spécial de *Fertilité Contraception Sexualité,* 1:1992;20:801-803
18. Mahadevan MM, Trounson AO, Wood C, Leeton JF: Effcct of oocyte quality and sperm characteristics on the number of spermatozoa bound to the zona pellucida of human oocytes inseminated in vitro. *J Vitro Fert Embryo Transfer* 1987;4: 223-227
19. Kruger TF, Acosta AA. Simmons KF, Swanson RJ, Matta JF, Oehninger S: Predictive value of abnormal sperm morphology in vitro fertilization. *Fertil Steril* 1988;49: 112-117
20. Liu DY, Du Plessis Y, Nayudu PL, Johnston WIH. Baker HWG: The use of in vitro fertilization to evaluate putative tests of human sperm function. *Fertil Steril* 1988:49:272-279
21. Burkman LJ, Coddington CC, Franken DR. Kruger T, Rosenwaks Z, Hodgen GD: The hemi-zona assay (HZA): Development of a diagnostic test for the binding of human spennatozoa to the human hemizona pellucida to predict fertilization potential. *Fertil Steril* 1988;49:688-697
22. Kruger TF. Swanson RJ, Hamilton M, Simmons KF, Acosta AA, Motta JF, Oehninger S, Morsedhi M: Abnormal sperm morphology and other semen parameters related to the outcome of the hamster oocyte human sperm penetration assay. *Int J Androl* 1988;11:107-113
23. Lopata A, Leung PC: The fertilizability of human oocytes at different stages of meiotic maturation. *In:*IVF and Other Assisted Reproduction. HW Jones, CS Schroeder (eds). *Ann NY Acad Sci,* 1988, pp 324-336
24. Kubiak J: Mouse oocytes gradually develop the capacity for activation during the metaphase II arrest. *Dev Biol* 1989; 136: 537-545
25. Zenzes MT, Belkien L, Bordt J, Kan 1, Schneider HPG, Nieschlag E: Cytologic investigation of human in vitro fertilization failures. *Fertil Steril* 1985;43:883-891
26. Jamieson ME, Fleming R, Kader S. Ross KS, Yates RWS. Coutts JRT: In vivo and in vitro maturation of human oocytes: Effects on embryo development and polyspermic fertilization. *Fertil Steril* 1991;56:93-97
27. Trounson AO, Mohr LR, Wood C. Leeton JF: Effect of delayed insemination on in-vitro fertilization, culture and transfer of human embryos. *J Reprod Fert* 1982;64:285-294
28. Trounson A, Webb J: Fertilization of human oocytes following reinsemination in vitro. *Fertil Steril* 1984;41:816-819
29. Pampiglione JS, Mills C, CampbeU S, Steer C, Kingsland C. Mason B: The clinical outcome of reinsemination of human oocytes fertilized in vitro. *Fertil Steril* 1990;53:306-310

30. Oehninger S, Acosta AA, Veeck LL. Simonetti S, Muasher SJ: Delayed fertilization during in vitro fertilization and embryo transfer cycles: Analysis of causes and impact on overall results. *Fertil Steril* 1989;52:991-997
31. Veeck LL, Wortham JWE. Witmeyer J, Sandow BA, Acosta AA, Garcia JE, Jones GS, Jones HW: Maturation and fertilization of morphologically immature human oocytes in a program of in vitro fertilization. *Fertil Steril* 1983;39:594-602
32. Rudak E, Dor J, Kimchi M, Goldman B, Levran D. Mashiach S: Anomalies of human oocytes from infertile women undergoing treatment by in vitro fertilization. *Fertil Steril* 1990;54:292-296
33. Edwards RG: Maturation in vitro of human ovarian oocytes. *Lancet* 1965;2:926-929
34. Nayudu PL, Lopata A, Jones GM. Gook DA, Bourne HM, Sheather SJ, Brown TC, Johnston WIH: An analysis of human oocytes and follicles from stimulated cycles: Oocyte morphology and associated follicular fluid characteristics. *Hum Reprod* 1989;4:558-567

CHAPTER IV

Sperm-Zona binding and sperm-oocyte penetration analysed by DNA Fluorescence: a test for gamete quality after IVF

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ABSTRACT

Sperm-zona binding, sperm-oocyte penetration and pronucleus formation in oocytes obtained after IVF failure were studied analysing DNA fluorescence. Oocytes were classified according to their maturational status, sperm-zona pellucida binding and sperm-oocyte penetration. Spermatozoa were classified as normal or abnormal according to conventional parameters. Sperm-zona binding and sperm-oocyte penetration into uncleaved oocytes were positively related to fertilization: 6.2 % of those oocytes from cohorts where none of the oocytes fertilized, had >10 spermatozoa bound to the zona, and 7.7 % of them were penetrated. When at least one oocyte per cohort fertilized, these rates were 37.1 % and 41.1 % respectively. Sperm-zona binding is related more strongly to sperm-oocyte penetration even without pronucleus formation, than to the maturational status of the oocyte: 26.9 % of the oocytes were penetrated by sperm of which 20.7 % had reached the pronuclear stage. For 25 out of 78 couples with a total IVF failure, whatever the spermogram, defective sperm-zona binding, sperm-oocyte penetration or pronucleus formation was observed. Sperm-zona binding and penetration were positively related especially when oocytes came from patients with partial IVF failure. Clinical information concerning the fertilizing capacity of oocytes and spermatozoa after IVF failure can be obtained by the simultaneous analysis of sperm-zona binding and sperm-oocyte penetration, and whether or not the latter results in in pronucleus formation.

INTRODUCTION

In vitro fertilization (IVF) has been shown to be successful for patients with different causes of infertility. However, for couples with male or unexplained infertility, fertilization rates are lower than for patients suffering from tubal occlusion (Mackenna et al., 1992; Tournaye et al., 1992). Within the male infertility group, variable fertilization rates suggest the heterogeneity of sperm quality (Tournaye et al., 1992). Indeed several authors have confirmed that conventional criteria for sperm quality such as sperm concentration, motility and morphology, are not necessarily related to the fertilizing capacity of spermatozoa (Aitken et al., 1982; Dunphy et al., 1989). Several functional tests have been developed to improve the evaluation of sperm quality (reviewed by Liu

and Baker 1992), such as the capacity of sperm to bind to the zona pellucida of immature human oocytes (Hemizona assay, Burkman et al., 1988) and to bind to and penetrate the oolemma of hamster or human oocytes (Yanagimachi et al., 1976; 1984; Liu and Baker., 1990). While it has been shown that pronuclear stage oocytes, obtained 48 h after insemination have more spermatozoa bound to the zona surface than unfertilized oocytes (Mahadevan et al., 1987), successful sperm penetration into the oocyte after IVF could only be confirmed non-invasively, by the presence of the female and male pronucleus 18-24 hour after insemination. While IVF can be considered to be a suitable sperm test (Yates and de Kretser, 1987), the result depends on the capacity of the oocyte to react normally to the penetrating spermatozoon by expulsion of the second polar body and pronucleus formation. The absence of pronuclei does not exclude the possibility that spermatozoa penetrated the oocyte, because male chromatin inside the ooplasm can also be found as undecondensed or partly decondensed spermheads (Van Wissen et al., 1991; Van Blerkom et al., 1994) or as premature condensed chromosomes (PCC, Schmiady and Kentenich, 1989; Tejada et al., 1992). The aim of the present work was to study sperm behaviour during gamete-interaction in IVF. We analysed sperm-zona binding, sperm-oocyte penetration and pronucleus formation in oocytes after incubation with the DNA fluorescent dye Hoechst 33342. We found that the number of spermatozoa bound to the zona pellucida, and the evidence of sperm-oocyte penetration in oocytes without pronuclei, are useful parameters to allow us to evaluate sperm functional quality and indirectly to reveal oocyte defects, especially for couples for whom fertilization fails completely or who have low fertilization rates after IVF.

MATERIALS AND METHODS

Patients and Protocols

A total of 78 patients (82 IVF cycles) from whom one or more oocytes failed to fertilize were included in this study. Indications for IVF were tubal occlusion or endometriosis (female infertility, n=39), infertility of unknown cause (n=17), male infertility (n=11) and combined female and male causes for infertility (n=11). Conventional sperm parameters were considered normal when sperm count was $>20x10^6$ spermatozoa/ml semen, motility was >40 % and normal morphology was >40 % (WHO, 1992). Of the 22 patients with abnormal sperm parameters, three were oligozoospermic (mean spermcount $14.1x10^6$ spermatozoa/ml semen, range $2.2x10^6$-$20x10^6$), five were asthenozoospermic (mean motility

34 %, range 10-40), four were teratozoospermic (mean normal morphology 25.5 %, range 20-39), six were astheno-teratozoospermic (mean motility 27.5 %, range 20-35; mean normal morphology 24 %, range 13-40 and four patients were oligo-astheno-teratozoospermic (mean $8.5x10^6$ spermatozoa/ml semen, range $2.9x10^6$-$18x10^6$; mean motility 32.5%, range 30-35; mean normal morphology 22.5 %, range 12-31). Ovarian stimulation included desensitization with Gonadotrophin releasing hormone agonist (GnRHa, Decapeptyl 3.75 mg; Ipsen-Biotech, Paris, France) and was performed as previously described (Frydman et al., 1988). Oocytes were inseminated 3-5 hours after retrieval with 4000-9000 motile spermatozoa (depending on the % of spermatozoa with normal morphology), and cultured in 30 µl microdrops of B2 medium (Biomerieux, Montalieu-Vercieu, France) under liquid paraffin oil. Spermatozoa were prepared on a discontinuous Percoll gradient. Oocytes were denuded mechanically from surrounding cumulus cells 18-24 h after insemination, checked for the presence of pronuclei, placed in a fresh drop of B2 medium and cultured for another 20-24 h, then cleaved zygotes were transferred to the uterine cavity.

Oocytes

A total of 305 oocytes were available for evaluation 48 hours after insemination and observed by phase-contrast and fluorescence microscopy after incubation with the DNA fluorescent dye Hoechst 33342 (Coger Pharmaceuticals, Paris, France). In all, 130 oocytes (42.6 %) came from cohorts whith total fertilization failure (group A, 25 patients) and 175 (57.4 %) from cohorts with at least one oocyte fertilized (group B, 53 patients). Fragmented oocytes, oocytes with fractured zona pellucida and atretic oocytes were discarded; only cytoplasmically normal oocytes (without vacuoli or granulation) were included in our study. Oocytes were incubated in a 100 ng Hoechst solution in B2 medium for 10-15 min under culture conditions, in small, 20 µl drops covered by liquid paraffin oil. After this time, they were transferred in fresh drops of B2 medium and kept under culture conditions until observation. An adhesive tape (0.15 mm thick) in which a small window was cut was put on the glass slide to create a chamber so as to avoid flattening the oocyte. For observation, oocytes were transferred in a drop of B2 medium within the window on prepared glass slides and covered by a coverslip. No contact occurred between the B2 medium containing the oocyte and the adherent tape. Observations took place with a Zeiss microscope equiped for fluorescence.

The number of spermatozoa bound to or partly penetrated into the zona pellucida was counted after at least two washes of each oocyte. According to the number of spermatozoa attached to the zona pellucida,

they were classified into four groups, allowing a quick evaluation of sperm-zona binding: no, one to five, six to 10 and >10 spermatozoa bound to the zona pellucida.

Statistics

The Chi-square test was used, as appropriate, for frequency comparisons. Significance was defined as $p<0.05$.

RESULTS

IVF Results

In 82 IVF cycles a mean of 10.4 oocytes per cycle were retrieved. For 25 patients none of the oocytes retrieved fertilized after IVF (Group A). For 53 patients the fertilization rate 18-24 hours after insemination was 35.6 % (Group B) and 14 pregnancies resulted after the transfer of one to three embryos. After insemination with normal spermatozoa 42.5 % of the oocytes were fertilized; after insemination with abnormal spermatozoa, 19.2 % of the oocytes were fertilized. The low fertilization rate in our study population was caused partly by the selection of a large number of couples for whom fertilization failed completely, and partly by the large number of oocytes inseminated with abnormal spermatozoa. The overall fertilization rate in our IVF center during the study period was 65%.

Oocyte Morphology

According to the nuclear status observed 48 hours after insemination, oocytes were classified as immature, mature or aged. Immature oocytes were either at the germinal vesicle stage (figures 1a, 1b, 2a), recognized by both the absence of polar bodies and the presence of a germinal vesicle with a more or less condensed chromatin mass surrounding a large single nucleolus (non-fluorescent), or at metaphase I (figure 3a), recognized by both the absence of the first polar body and the presence of fluorescent metaphase chromosomes. Mature oocytes were at metaphase II (figures 5a, b, c) and the metaphase chromosomes were situated close to the first polar body. Aged oocytes were also at metaphase II but with the chromosomes migrated towards the centre of the egg (figure 4), as we defined previously (Van Wissen et al, 1991). Sperm penetration was assessed by the presence of sperm chromatin in the cytoplasm (figures 1c,

2b, 3b, 5a, 5c). Pronuclear stage oocytes were recognized by the presence of two or more pronuclei and two DNA containing polar bodies, which distinguished them from activated oocytes.

Sperm binding and IVF outcome

Oocytes without spermatozoa bound to their zona pellucida were more frequent in Group A (complete fertilization failure) than in Group B (partial fertilization failure, table I) patients. Conversely, oocytes with >10 spermatozoa bound to their zona pellucida were frequent in group B and rare in group A. This shows that the capacity of spermatozoa to fertilize at least one oocyte in a cohort is positively related to the capacity of spermatozoa to bind to the zona pellucida. In both groups A and B, the majority of oocytes had one to five spermatozoa bound to their zona pellucida; oocytes with between six and 10 spermatozoa bound to their zona pellucida were equally frequent in both groups.

Table I: Sperm binding and penetration in relation to the IVF outcome

	Group A[a]		Group B[b]	
No of spermatozoa bound to zona	Total no of oocytes observed	No of penetrated oocytes (%)	Total no of oocytes observed	No of penetrated oocytes (%)
0	34*	1(2.9)	6*	1(16.7)
1-5	71§	4(5.6)¥	73§	25(34.3)¥
6-10	17	4(23.5)	31	10(32.3)
>10	8¶	1(12.5)¢	65¶	36(55.4)¢
Total	130	10(7.7)	175	72(41.1)

[a]Group A: oocytes from cohorts with complete fertilization failure 18-24 hours post insemination. [b]Group B: oocytes from cohorts with partial fertilization failure 18-24 hours post insemination.
Significant difference between values with the same symbols
* $p<0.001$, § $p<0.05$, ¥ $p<0.001$, ¶ $p<0.001$, ¢ $p<0.025$ respectively

Sperm-zona binding for oocytes from the same cohort

Of the 78 patients, 63 had two or more oocytes analysed. Of these 63, the majority (46 patients, 73.0 %) had the same number of spermatozoa bound to the zona pellucida for more than half of the observed oocytes from the same cohort. In addition, four patients had the same number of

spermatozoa bound to the zona pellucida for the majority of oocytes from subsequent IVF cycles. It appeared that sperm-zona binding could be considered as a patient-linked parameter.

Sperm binding and sperm penetration

Out of the 305 oocytes included in our study, 82 (26.9 %) oocytes were penetrated by spermatozoa. A total of 18 unfertilized oocytes (no pronuclei) were penetrated by more than one spermatozoon (between two and six). Five of these oocytes were in germinal vesicle stage, four were at metaphase I, and nine were at metaphase II. The incidence of penetrated oocytes was frequent among patients in our study: 44 out of 78 patients had one or more oocytes penetrated by spermatozoa. In group A, considerably fewer oocytes were penetrated than in group B (7.7 % versus 41.1 %, $p<0.001$). This suggested a positive association between the incidence of normally fertilized oocytes after IVF and the occurrence of penetrated oocytes but with abnormal or arrested fertilization processes. For both groups A and B, sperm-oocyte penetration was very rare when no spermatozoa were bound to the zona (2 out of 305 oocytes). Surprisingly, in group A, the number of penetrated oocytes increased only slightly when >10 spermatozoa were bound to the zona. Spermatozoa were apparently able to bind to the zona in some cases but not to penetrate it. In group B, the number of spermatozoa bound to the zona and sperm-oocyte penetration followed a more expected pattern and increased with increasing numbers of spermatozoa bound to the zona, 55.4% of the oocytes with >10 spermatozoa bound to their zona pellucida was penetrated.

For penetrated oocytes, the presence of spermatozoa inside the ooplasm did not necessarily result in pronuclear formation or syngamy within 48 hours after insemination. Only a few (17 out of 82) penetrated oocytes were at the pronuclear stage, but spermatozoa were nevertheless observed to bind and penetrate the zona and to fuse with the oolemma. Of the pronuclear stage oocytes, 12 had two pronuclei, 3 had three pronuclei and 2 had multiple pronuclei. For total fertilization failures (Group A), 4 out of 10 penetrated oocytes were at the pronuclear stage 48 h post insemination. Sperm-zona binding for pronuclear stage oocytes is shown in table 2. While no definite conclusions can be drawn because of the small number of pronuclear stage oocytes studied, it is noticeable that one of these oocytes had no spermatozoa bound to the zona at all. A low sperm-oocyte penetration capacity possibly delayed fertilization of this oocyte.

Sperm-oocyte interaction and oocyte maturity

Sperm-zona binding differed with the stages of nuclear maturity of the oocyte (table II). Again, for all groups, one to five spermatozoa bound to the zona pellucida was the most frequent. The absence of spermatozoa bound to the zona was relatively frequent for mature and aged oocytes. Of mature oocytes, 24.4 % had >10 spermatozoa bound to the zona, while11.3 % of aged oocytes had >10 spermatozoa bound.

Table II: Sperm binding and oocyte maturational and developmental stage

	Maturational status of the oocyte			Fertilized oocytes:
No of spermatozoa bound to zona	No of immature oocytes (%)[a]	No of mature oocytes (%)	No of aged oocytes (%)[b]	No of PN stage oocytes (%)[c]
0	3(5.8)*	22(14.1)	14(17.5)*	1(5.9)
1-5§	19(36.5)§	72(46.2)	45(56.3)§	8(47.1)
6-10	7(13.5)	24(15.4)	12(15.0)	5(29.4)
>10	23(44.2)¥¶	38(24.4)¥¢	9(11.3)¶¢	3(17.7)
Total	52	156	80	17

[a]Nine oocytes were in germinal vesicle stage of which 2 oocytes had 1-5 SBZ, 2 had 6-10 SBZ and 5 had >10 SBZ. Of forty-three oocytes in metaphase I stage, 3 oocytes had 0 SBZ, 17 had 1-5 SBZ, 5 had 6-10 SBZ and 18 had >10SBZ. [b]Aged oocytes as observed after 48 hours of culture. [c]Twelve oocytes had two pronuclei: 6 had 1-5 SBZ, 4 had 6-10 SBZ and 2 had >10 SBZ. Three oocytes had three pronuclei: 1 had 0 SBZ, 1 had 1-5 SBZ and 1 had >10 SBZ. Two oocytes had 4-6 pronuclei: 1 had 1-5 SBZ and 1 had 6-10 SBZ. *,§ Significant difference between immature and aged oocytes ($p<0.05$). ¥¶¢ Significant difference between immature and mature oocytes ($p<0.01$), between immature and aged oocytes ($p<0.001$) and between mature and aged oocytes ($p<0.025$).

On the contrary, almost half (44.2 %) of the immature oocytes had >10 spermatozoa bound to the zona. Sperm-oocyte penetration results agreed with sperm-zona binding for immature, mature and aged oocytes. Spermatozoa penetrated in 40.4 % of the observed immature oocytes (21 out of 52) and in 25.6 % (40 out of 156) of mature oocytes. Only 5.0% (4 out of 80) of the aged oocytes were penetrated. Spermatozoa inside the ooplasm were found in different configurations depending on the maturational status or the quality of the oocyte. Of 52 immature oocytes nine were at the germinal vesicle stage and 43 at metaphase I. Undecondensed or partly decondensed sperm heads were found mainly in germinal vesicle stage oocytes (figures 1c, 2b, 2c), but partially decondensed sperm heads were also found in two metaphase I oocytes

(figure 3b) and in two metaphase II oocytes. In the remaining metaphase I and II oocytes male chromatin was found as PCC (figures 5a, 5c).

Sperm-oocyte interaction and conventional sperm parameters

Sperm binding was positively related to sperm-oocyte penetration when oocytes were inseminated with normal spermatozoa (table III). On the contrary, 69 out of 123 (56.1 %) oocytes inseminated with abnormal spermatozoa had only one to five spermatozoa bound to the zona, and penetration remained occasional even when the number of spermatozoa bound to the zona increased. Sperm-zona binding and penetration were apparently dissociated. Sperm penetration was seen in 37.4 % of the observed oocytes inseminated with normal spermatozoa. For oocytes inseminated with abnormal spermatozoa, this rate was significantly lower (11.4 %, $p<0.001$). Hence, fertilization anomalies were detected more efficiently when normal (penetrating) spermatozoa were used. For 25 out of 78 couples fertilization failed completely whether oocytes were inseminated with normal or abnormal spermatozoa.

Table III: Sperm binding and penetration in relation to sperm parameters*

	Normal Sperm		Abnormal Sperm	
No of spermatozoa bound to zona	Total no of oocytes	No of penetrated oocytes (%)	Total no of oocytes	No of penetrated oocytes (%)
0 (%)	21	2 (9.5)	19	0
1-5	75§	23 (30.7)¥	69§	6 (8.7)¥
6-10	30	9 (30.0)	18	5 (27.8)
>10	56¶	34 (60.7)¢	17¶	3 (17.7)¢
Total	182	68(37.4)	123	14(11.4)

*Conventional sperm parameters were considered normal when: sperm count $>20*10^6$ spermatozoa/ml semen, >40% motility and >40% normal morphology.
Significant difference between values with the same symbols:
§ $p<0.025$, ¥ $p<0.01$, ¶ $p<0.001$, ¢ $p<0.01$ respectively.

Of these 25, 13 couples with total IVF failure (67 oocytes observed) had normal sperm parameters but sperm-zona binding was low: most oocytes had either no (18 out of 67 oocytes) or between one and five spermatozoa bound to the zona pellucida (36 out of 67 oocytes); only 10 oocytes had between six and 10 spermatozoa bound to their zona, and only three oocytes had >10 spermatozoa bound. From five of these couples, eight out of 23 oocytes were penetrated (three had pronuclei). For the remaining

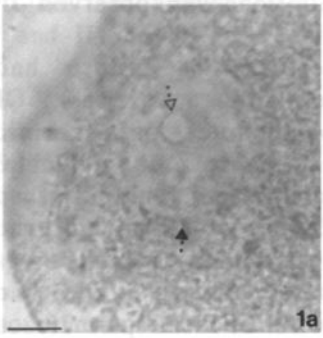
eight couples, no evidence of sperm-oocyte penetration was observed in any of the oocytes studied (44 oocytes).

The other 12 couples with complete fertilization failure had abnormal sperm parameters (see above for sperm characteristics). Of these 12 couples, 11 (including four oligoasthenoteratozoospermic patients) had low numbers of spermatozoa bound to their zona pellucida: of the 58 oocytes, 16 had no, 34 had between one and five, only five had six to 10 and three had >10 spermatozoa bound to the zona pellucida; no sperm-oocyte penetration was observed. The remaining patient was asthenoteratozoospermic, and from five inseminated oocytes, one had between one and five, two had six to 10 and two had >10 spermatozoa bound to the zona. For this patient, 2 oocytes were penetrated of which one had pronuclei. In total for this group, from 63 observed oocytes inseminated with abnormal spermatozoa, only two oocytes were penetrated.

However, despite abnormal sperm parameters, sperm-zona binding, sperm-oocyte penetration and fertilization can be successful. Out of 22 patients with abnormal sperm parameters, 10 achieved fertilization in at least one of the inseminated oocytes. For these patients, the absence of spermatozoa bound to the zona was rare (three out of 60 oocytes), and a large number of oocytes had either one to five (34 oocytes), six to 10, (11 oocytes) or >10 (12 oocytes) spermatozoa bound to their zona pellucida. A total of 12 oocytes (of which four oocytes were at the pronuclear stage) from six of the 10 patients were penetrated by spermatozoa, the remaining four patients had no penetrated oocytes despite some oocytes of the cohort being fertilized.

DISCUSSION

For the oocyte population studied, the absence of sperm-oocyte penetration in most of the 236 meiotically mature and aged oocytes was the main cause of fertilization failure (192/305, 63.0 %). In those oocytes which were penetrated, the most important cause of fertilization failure was fertilization anomalies characterized by sperm-oocyte penetration without pronucleus formation (65/305, 21.3 %). Oocyte immaturity (52/305, 17 %) was another cause of fertilization failure. Arrested or delayed pronuclear stage oocytes were rare (17/305, 5.6 %). Meiotically immature oocytes penetrated by spermatozoa were considered as both immature and fertilization anomalies. These results apply in particular to couples with low fertilization rates after IVF or couples with complete fertilization failure.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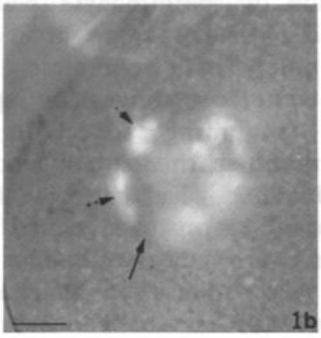
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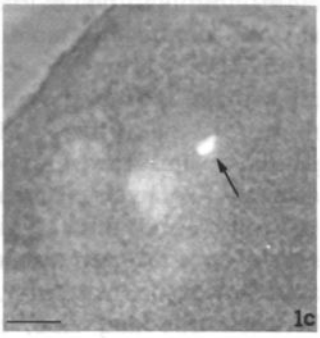
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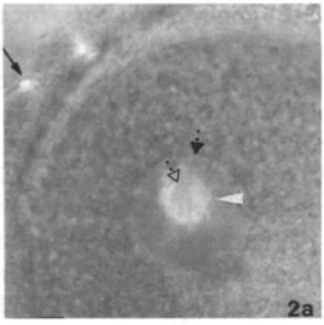
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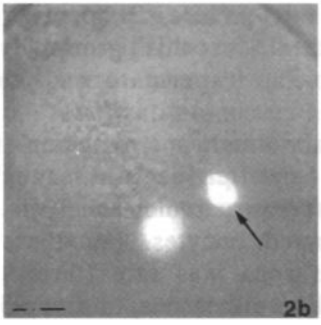
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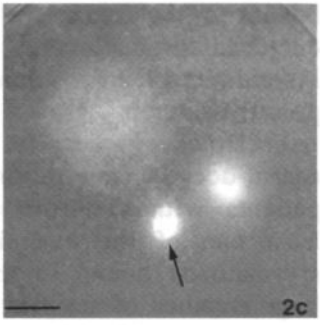
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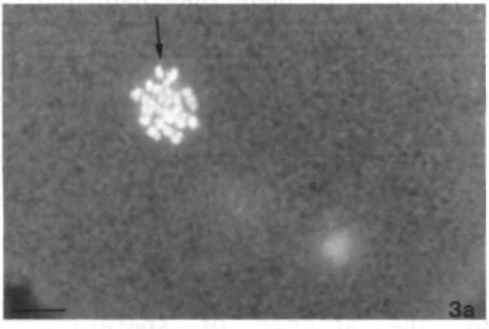
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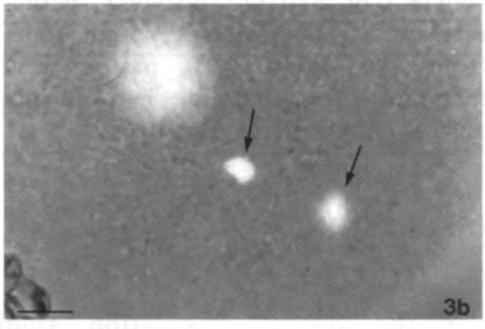
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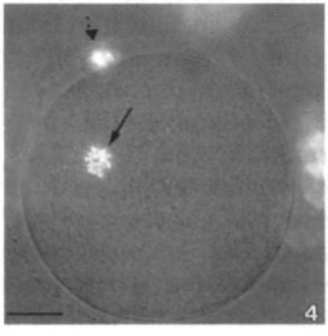
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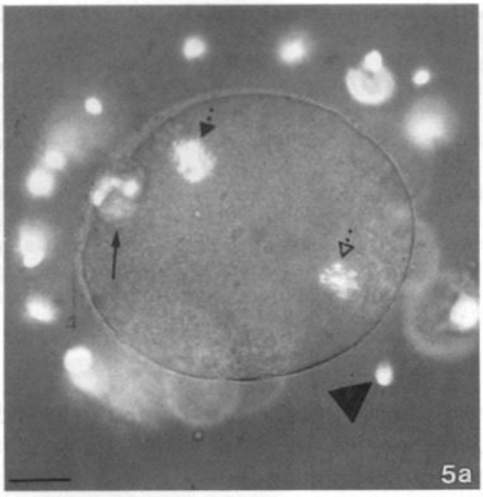
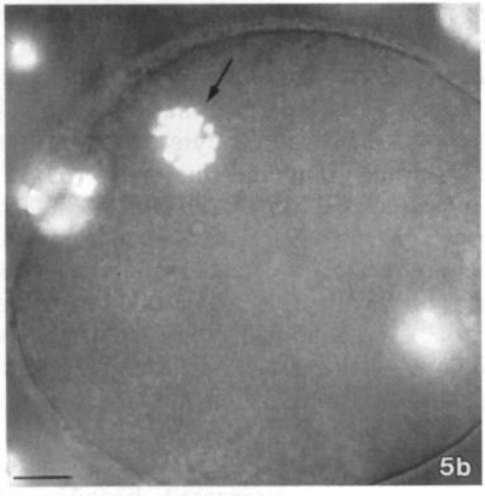
Figure 1: Germinal vesicle stage oocyte with intact germinal vesicle (a: arrowhead, b: arrow), a nucleolus (a: open arrowhead) and condensed chromatin (b: arrowheads). A single spermatozoon penetrated the oocyte and was found in the ooplasm as an undecondensed spermhead (c: arrow), bar=10μ. **Figure 2:** Germinal vesicle stage oocyte with intact germinal vesicle (a: arrowhead), a nucleolus (a: open arrowhead) and condensed chromatin surrounding the nucleolus (a: large arrowhead). Note spermatozoa outside the oocyte bound to the zona pellucida (a: arrow). Two spermatozoa penetrated the oocyte and were found in the ooplasm as partially decondensed spermheads (b and c: arrows), bar=10μ. **Figure 3:** Metaphase I stage oocyte with metaphase I chromosomes (a: arrow) and two partially decondensed spermheads inside the ooplasm (b: arrows), bar=10μ. **Figure 4:** Aged oocyte, the metaphase II chromosomes (arrow) are located far from the first polar body (arrowhead). No spermatozoa are bound to the zona, bar=17.8μ.

For couples with high fertilization rates, the incidence of immaturity and fertilization anomalies among unfertilized oocytes may be more important as spermatozoa are known to have fertilization potential.

A large number (47.0%) of oocytes had one to five spermatozoa bound to the zona independent of the IVF outcome and sperm quality. Similar results were reported by Mahadevan et al. (1987), who like us studied mostly unfertilized oocytes for which sperm-zona binding is expected to be lower than for successfully fertilized oocytes (which are not available for study-purposes). Our finding that the majority (51/82, 62.2%) of penetrated oocytes had six to ten or >10 spermatozoa bound to the zona was expected because sperm-zona binding and sperm-oocyte penetration were positively related. However this association was less evident in cases where all oocytes failed to fertilize or when oocytes were inseminated with abnormal spermatozoa. We suggest that sperm-zona binding and penetration can be dissociated because of particular sperm defects, such that sperm-zona binding but not penetration is allowed, or defects concerning the capacity of the zona pellucida to bind spermatozoa.

In 19 out of 25 patients with complete fertilization failure, a sperm defect as the cause of this failure was strongly suspected because of absent or low levels of sperm-zona binding and the total absence of sperm-oocyte penetration, despite a normal spermogram attributed to eight of the patients. For these couples, micromanipulation techniques circumventing the zona pellucida, such as subzonal insemination, or circumventing both the zona and the oolemma such as intracytoplasmic insemination, may be useful as spermatozoa were apparently not able to bind to and penetrate the zona pellucida. Indeed, it has been suggested that total fertilization failure combined with superficial or no sperm-zona binding is mostly related to sperm defects (Bedford and Kim, 1993; Liu and Baker, 1994a). A defective acrosome reaction on the zona pellucida surface may be responsible for the failure of zona penetration despite sperm-zona binding (Liu and Baker, 1994b).





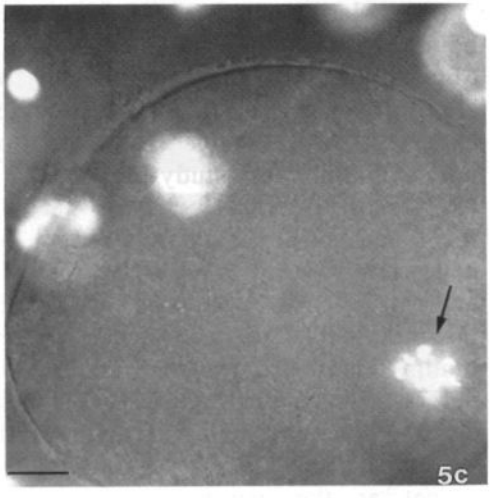


Figure 5: Metaphase II stage oocyte with metaphase II chromosomes (a: arrowhead, b: arrow) close to the first polar body (a: arrow) and premature condensed male chromatin (PCC, a: open arrowhead, c: arrow). Note spermatozoa bound to the zona (a: large arrowhead). a: bar=17.8µ, b and c: bar=10µ.

Complete fertilization failure can occur despite positive sperm-zona binding and sperm-oocyte penetration inside the ooplasm. In our study, this was the case for six out of 25 patients (of whom five had a normal spermogram). For these cases, in contrast to cases with complete sperm penetration failure, intracytoplasmic sperm injection may not be successful because either the penetrated oocytes were not capable of supporting pronucleus formation or pronucleus formation was delayed or arrested. Indeed, a new IVF attempt should only be carried out when the ovarian stimulation and the morphology of the retrieved and inseminated oocytes have been analysed carefully because in such cases oocyte quality and/or maturity are most probably implicated.

Both sperm-zona binding and the presence of sperm chromatin inside the ooplasm despite the absence of pronuclei were positively related to the occurrence of fertilization within the same cohort. Other authors have described a positive association between the IVF outcome and sperm-zona binding as determined by phase contrast observations (Liu et al., 1989) and by light microscopy after oocyte fixation and lacmoid staining (Bedford and Kim, 1993). While in both studies sperm penetration rates inside the zona pellucida were assessed, no simultaneous confirmation of sperm-oocyte penetration without fertilization was described. For total fertilization failures with normal sperm parameters when spermatozoa bound to and penetrated up to half-way through the zona, non-penetrability of the zona-pellucida caused by cryptic oocyte anomalies was suggested (Bedford and Kim, 1993). If pronucleus formation occurred in re-inseminated zona-free oocytes which had initially failed to fertilize after IVF, an impenetrability of the zona pellucida or of the oolemma was proposed (Bongso et al., 1992). However, in both studies evidence for sperm-oocyte penetration was obtained only by recording the presence of pronuclei. The suggestion of Bedford and Kim (1993) that sperm penetration without pronucleus formation occurrs only in a very small number of eggs which remain unfertilized after IVF is not confirmed by our results as we show that an important proportion of unfertilized oocytes was penetrated by sperm.

The high frequency of abnormally fertilized oocytes can be attributed to the patient selection for this study but may also be a commonly occurring phenomenon in IVF, and we demonstrate the importance of checking the ooplasm for the presence of spermatozoa even when no pronuclei are visible to distinguish either oocyte or sperm involvement in the fertilization failure.

Sperm-zona binding was related to the occurrence of sperm penetration rather than to the maturational status of the oocyte. Immature oocytes were penetrated relatively often when compared with mature and aged oocytes. In our study, 37 out of 52 immature oocytes came from partial fertilization failures which revealed the presence of

fertilizing sperm. This may explain why in our study almost half of the immature oocytes had >10 spermatozoa bound to the zona and sperm penetration was frequent. The penetrability of human oocytes at all stages of meiotic maturation has been reported by several authors (Lopata and Leung, 1988; Van Wissen et al., 1991; Van Blerkom et al., 1994). This suggests the capacity of the zona pellucida of immature oocytes to bind spermatozoa. In fact, similar numbers of spermatozoa have been found to bind to the zona pellucida of immature and mature oocytes (Bedford and Kim, 1993; Liu and Baker, 1994a). Oocyte quality and maturity are probably not relevant in the failure of sperm-zona binding and zona penetration associated with complete fertilization failure (Liu and Baker, 1994a). Because the immaturity of the oocyte is no limiting factor sperm binding and penetration is likely to depend on sperm quality.

Several cytogenetic studies have demonstrated the presence of PCC in metaphase I and II stage oocytes with frequencies varying from 7.4 % to 28 % (Reviewed by Tejada et al., 1992). In our study, 44 metaphase I and II oocytes had PCC out of 305 observed oocytes (14.4 %). These findings in different IVF laboratories seems to indicate that fertilization anomalies such as PCC are fairly frequent in IVF protocols.

Besides PCC in metaphase I and metaphase II oocytes, we also found four metaphase oocytes with partially decondensed spermheads inside the ooplasm. This may correspond to a stage of sperm decondensation preceding PCC formation, such as described in the mouse by Clarke and Masui (1986) and Kubiak (1989).

Of the 82 penetrated oocytes observed, 17 (20.7 %) were at the pronuclear stage 48 h post insemination. The presence of pronuclear stage oocytes at this time indicates delayed or arrested development (Zenzes et al., 1990). Both oocyte immaturity and sperm pathology can be responsible for delayed fertilization after IVF (Oehninger et al., 1989).

In conclusion, absence of sperm-oocyte penetration associated with no or low sperm-zona binding was found to be a frequent cause of fertilization failure, and a sperm defect was suspected for the majority of these cases. Sperm-oocyte penetration was rare when fertilization failed completely. Therefore, sperm quality, as defined by conventional parameters was not always relevant in our study. A quality assessment of oocytes is needed when fertilization rates are very low and embryonic development stops at a very early stage. The occurrence and frequency of fertilization anomalies, oocyte immaturity and arrested or delayed fertilization can assess the oocyte quality in relation to its capacity to support pronucleus formation and embryo development.

The simultaneous analysis of sperm-zona binding and sperm-oocyte penetration into the ooplasm by DNA fluorescence in intact oocytes obtained after IVF is easy to perform and valuable for obtaining the maximum information of IVF results. To improve the analysis of sperm-

zona binding and penetration as a functional test for sperm-oocyte interaction and oocyte quality, more comprehensive information is required concerning fertilization anomalies and their incidence relating to IVF parameters (cause of infertility, IVF outcome, pregnancy resulting after IVF).

REFERENCES

Aitken,R.J., Best,F.S.M., Richardson,D.W., Djahanbakhch,O., Mortimer,D., Templeton,A.A., (1982) An analysis of sperm function in cases of unexplained infertility: conventional criteria, movement characteristics, and fertilizing capacity. *Fertil. Steril.*, 38, 212-221.

Bedford,J.M. and Kim,H.H. (1993) Sperm/egg binding patterns and oocyte cytology in retrospective analysis of fertilization failure in vitro. *Hum. Reprod.*, 8, 453-463.

Bongso,A., Fong,C.Y.F., Ng,S.C., Ratnam,S. (1992) Fertilization, cleavage, and cytogenetics of 48-hour zona-intact and zona-free human unfertilized oocytes reinseminated with donor sperm. *Fertil. Steril.*, 57, 129-133.

Burkman,L.J., Coddington,C.C., Franken,D.R., Kruger,T.F., Rosenwaks,Z., Hodgen,G.D. (1988) The hemizona assay (HZA): development of a diagnostic test for the binding of human spermatozoa to the human hemizona pellucida to predict fertilization potential. *Fertil. Steril.*, 49, 688-697.

Clarke, H.J. and Masui, Y. (1986) Transformation of sperm nuclei to metaphase chromosomes in the cytoplasm of maturing oocytes of the mouse. *J. Cell Biol.*, 102, 1039-1046.

Dunphy,B., Neal,L.M., Cooke,I.D. (1989) The clinical value of conventional semen analysis. *Fertil. Steril.*, 51, 324-329.

Frydman,R., Belaisch-Allart,J., Parneix,I., Forman,R., Hazout,A., Testart,J. (1988) Comparison between flare-up and down regulation effects of luteinizing hormone-releasing hormone agonists in an in vitro fertilization program. *Fertil. Steril.*, 50, 471-475.

Kubiak, J.Z. (1989) Mouse oocytes gradually develop the capacity for activation during the metaphase II arrest. *Dev. Biol.*, 136, 537-545.

Liu,D.Y., Baker,H.W.G. (1992) Tests of human sperm function and fertilization in vitro. *Fertil. Steril.*,58, 465-483.

Liu,D.Y. and Baker,H.W.G. (1994a) A new test for the assessment of sperm-zona pellucida penetration: relationship with results of other sperm tests and fertilization in vitro. *Hum. Reprod.* 9, 489-496.

Liu,D.Y. and Baker,H.W.G. (1994b) Disordered acrosome reaction of spermatozoa bound to the zona pellucida: a newly discovered sperm defect causing infertility with reduced sperm-zona pellucida penetration and reduced fertilization in vitro. *Hum. Reprod.*, 9, 1694-1700.

Liu,D.Y., Lopata,A., Johnston,W.I.H., Baker,H.W.G. (1989) Human sperm-zona pellucida binding, sperm characteristics and in-vitro fertilization. *Hum. Reprod.* 4, 696-701.

Lopata,A., Leung,P.C. (1988) The fertilizability of human oocytes at different stages of meiotic maturation. *Ann. NY Acad. Sci.* 541, 324-336.

Mackenna,A.I., Zegers-Hochschild,F., Fernandez,E.O., Fabres,C.V., Huidobro,C.A., Prado,J.A., Roblero,L.S., Altieri,E.L., Guadarrama,A.R., Lopez,T.H. (1992) Fertilization rate in couples with unexplained infertility. *Hum. Reprod.*, 7, 223-226.

Mahadevan,M.M., Trounson,A.O., Wood,C., Leeton,J.F. (1987) Effect of oocyte quality and sperm characteristics on the number of spermatozoa bound to the zona pellucida of human oocytes inseminated in vitro. *J. in Vitro Fert. Embryo Trans.*, 4, 223-227.

Oehninger,S., Acosta,A.A., Veeck,L.L., Simonetti,S., Muasher,S.J. (1989) Delayed fertilization during in vitro fertilization and embryo transfer: analysis of causes and impact on overall results. *Fertil. Steril.*, 52, 991-997

Schmiady,H., Kentenich,H. (1989) Premature chromosome condensation after in-vitro fertilization. *Hum. Reprod.*, 4, 689-695.

Tejada,M.I., Mendoza,M.R., Corcostegui,B., Benito,J.A. (1992) Factors associated with premature chromosome condensation (PCC) following in vitro fertilization. *J. Ass. Reprod. Genet.*, 9, 61-67.

Tournaye,H., Devroey,P., Camus,M., Staessen,C., Bollen,N., Smitz,J., Van Steirteghem,A.C. (1992) Comparison of in-vitro fertilization in male and tubal infertility: a 3 year survey. *Hum. Reprod.*, 7, 218-222.

Van Blerkom,J., Davis,P.W., Merriam,J. (1994) The developmental ability of human oocytes penetrated at the germinale vesicle stage after insemination in vitro. *Hum. Reprod.* 9, 697-708.

Van Wissen,B., Bomsel-Helmreich,O., Debey,P., Eisenberg,C., Vautier,D., Pennehouat,G. (1991) Fertilization and ageing processes in non-divided human oocytes after GnRHa treatment. *Hum. Reprod.*, 6, 879-884.

World Health Organization. (1992) *Laboratory manual for examination of human semen and semen-cervical mucus interaction.* Cambridge University Press.

Yanagimachi,R., Yanagimachi,H., Rogers,B.J. (1976) The use of zona-free animal ova as a test-system for the assessment of fertilizing capacity of human spermatozoa. *Biol. Reprod.*,15, 471-476.

Yanagimachi,R. (1984) Zona free hamster eggs: their use in assessing fertilizing capacity and examining chromosomes of human spermatozoa. *Gamete Research*, 10, 187-232.

Yates,C.A., de Kretser,D.M. (1987) Male-factor infertility and in vitro fertilization. *J. In Vitro Fert. Embryo Transf.*, 4, 141-147.

Zenzes,M.T., de Geyter,C., Bordt,J., Schneider,H.P.G., Nieschlag,E. (1990) Abnormalities of sperm chromosome condensation in the cytoplasm of immature oocytes. *Hum. Reprod.*, 5, 842-845.

CHAPTER V

The use of morphological and functional oocyte parameters to determine causes of IVF failure

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ABSTRACT

The aim of the present study was to evaluate morphological and functional oocyte characteristics, such as maturity and fertilization anomalies in relation to IVF parameters such as indication for IVF, the fertilization and cleavage rate of cohort oocytes and the estabishment of pregnancy. Four-hundred-and-forty unfertilized oocytes and zygotes from 100 women entering the IVF program because of tubal obstruction, endometriosis, male or idiopathic infertility were analysed by DNA visualisation using the fluorescent dye Hoechst 33342. Forty-eight hours after in vitro insemination, the majority of oocytes was meiotically mature (63.4%). Meiotic immaturity occurred in 14.8% of the observed oocytes. Furthermore, 7.0% was activated and 6.8% had pronuclei. The remaining 8.0% had abnormal maternal chromatin or was not analysable. A high frequency of sperm penetration without pronucleus formation or cleavage was found in immature, mature and activated oocytes (23.6%). The total incidence of penetrated oocytes without pronuclei, increased with increasing cleavage rates and was low in cycles with a male IVF indication. The same trend was found for the incidence of cytoplasmic immaturity, manifested by the presence of premature male chromatin (PCC). The finding of sperm-oocyte penetration without fertilization in high cleavage rate groups defined a subpopulation of functionally unfit oocytes and is probably underestimated when fertilization failed or when fertilization rates were low. Different condensation stages of sperm chromatin found in the ooplasm of unfertilized oocytes were discussed. Post-meiotically aged oocytes were frequent when no cleavage occurred, when cleavage rates were low, and when infertility was of male origin, so that a sperm dysfunction could be suggested. A relatively small amount of oocytes activated by sperm and oocytes having reached the pronuclear stage but unable to develop further, showed arrested or delayed development. Hence, the population of unfertilized oocytes after IVF, is defined by the gamete quality of the couple which is expressed by the cleavage rate. IVF, even upon failure can thus provide a useful diagnostic mean in the treatment of infertility.

INTRODUCTION

In vitro fertilization (IVF) is now a well established treatment for certain forms of human infertility. However, failure of fertilization is still an unsolved problem affecting a relatively important number of the IVF patients. With low fertilization success or, even more importantly, after complete absence of fertilization, it is necessary to find causes for this failure to reconsider further infertility treatment. Several causes have been evoked. Sperm defects have been suggested when all the inseminated oocytes fail to fertilize and impaired oocyte quality or oocyte immaturity when part of the oocytes fail to fertilize. However, these results are ambiguous and information is lacking with respect to nuclear and cytologic morphology of oocytes which failed to fertilize after IVF.

Unfertilized oocytes have been investigated mainly by chromosomal studies (Selva et al, 1991). While there seems to be a relatively high incidence of chromosomal abnormalities in oocytes remaining unfertilized 48 hours after insemination (Plachot et al, 1988; Pieters et al, 1989; Edirishinge et al, 1992), no relation between the incidence of chromosomal abnormalities in unfertilized oocytes and the fertilization rate (Tarin et al, 1991; Almeida and Bolton, 1994) or pregnancy outcome of the cohort was found (Tarin et al, 1991).

Besides the direct cytogenetic analysis of oocytes which failed to fertilize after IVF, oocytes have been analysed after re-insemination either with intact zona pellucida or zona free (Bongso et al, 1992) or after subzonal insemination (Tesarik, 1993; Tesarik and Mendoza, 1994). This allowed to analyse gamete fusion and to evaluate the capacity of the oocyte to form pronuclei. Oocytes used for these tests were supposed to be unfertilized after initial IVF because of the presence of the first polar body and the absence of pronuclei and cleavage. However, these criteria do not exclude sperm penetration without fertilization (Van Wissen et al, 1991; Van Wissen et al, 1994; Van Blerkom et al, 1994a).

For the reasons mentioned above, it seems necessary to better characterize the population of oocytes, which remain unfertilized after IVF. The visualization of DNA containing structures using the fluorescent dye Hoechst 33342, has been reported to be efficient to analyse both mouse (Conover and Gwatkin, 1988; Debey et al, 1989) and human oocytes (Gwatkin, 1989; Van Wissen et al, 1991; 1992). When using this staining method on fresh unfertilized human oocytes in toto, detailed information concerning oocyte immaturity, maturity and post-meiotic ageing can be obtained. Ageing is an indication for the time span between the expulsion of the first polar body and observation if no fertilization has taken place. This information allows retrospective evaluation of the maturational status of the oocyte at the time of insemination. Also, sperm-

oocyte penetration can be observed in oocytes which were considered unfertilized by the absence of pronuclei (Van Wissen et al, 1991).

The aim of the present study was to evaluate these morphological and functional parameters in oocytes grouped according to IVF indication, the fertilization and cleavage rate and pregnancy. This allowed not only to analyse fertilization anomalies in a large number of oocytes and to define different causes of non-fertilization but also to evaluate the suitability of unfertilized oocytes for further gamete fusion tests.

MATERIALS AND METHODS

Patients and Protocols

This study consisted of 104 IVF cycles (100 patients, mean age 33.0±4.0 years) performed in the Department of Gynecology and Obstetrics of the Béclère University Hospital, in each of which at least one retrieved oocyte remained unfertilized or one zygote remained uncleaved after in vitro insemination. Indications for the IVF procedure in these cycles included tubal obstruction (n= 58), endometriosis (n=2), combined tubal factor and endometriosis (n=3), male infertility (n= 15) combined male and female infertility (n=5) and idiopathic infertility (n=21). Sperm parameters were evaluated according to WHO guidelines (Belsey et al, 1980). For ovarian stimulation, Gonadotrophin releasing hormone agonist (GnRHa, Decapeptyl, Ipsen-Biotech, Paris, France) was administrated in combination with human menopausal gonadotrophin (HMG, Humegon, Organon, Saint-Denis, France) and HCG as described before (Frydman et al, 1988).

In vitro fertilization

One-thousand-and-seventy oocytes were retrieved (between 2 and 16 oocytes per cycle). Each oocyte was inseminated 2-4 hours after retrieval with 4000 to 10000 motile spermatozoa prepared by centrifugation on a discontinuous percoll gradient. After insemination, oocytes were cultured in 30 µl microdrops of B2 medium (Biomerieux, Montalieu-Vercieu, France), under liquid paraffin oil (BDH) in petri dishes. Eighteen to 20 hours after insemination, 408 oocytes were fertilized. Fertilization was assessed when two or more pronuclei and two polar bodies were present. Of these, 368 oocytes had two pronuclei (34.4%) and 40 oocytes had more than two pronuclei (3.7%). Twenty-four hours later, 393 oocytes had cleaved and between one and four embryos were transferred to the uterus. Fragmented oocytes, oocytes without intact zona pellucida and

atretic oocytes were discarded and only cytoplasmically normal oocytes (without vacuoli or granulation) were included in the study. In total 197 oocytes (range per patient: 0-4) were excluded for these reasons between the time of retrieval and observation 48 hours after insemination. After excluding embryos, polyspermic zygotes, and morphologically abnormal oocytes, unfertilized oocytes and uncleaved zygotes (n=440) were included in our study.

The low cleavage rate in this study (36.7%) when compared with the overall cleavage rate in our IVF center during the same period (63.0%) is due to the inclusion of a high number of cycles without any cleavage and of cycles with low fertilization and cleavage rates which presented a clinical interest.

Fluorescence Microscopy

Four-hundred-and-forty unfertilized oocytes and uncleaved zygotes were obtained 48 hours after insemination and incubated for 10-15 minutes (37°C in 5%CO2) with the DNA specific fluorescent dye Bisbenzimide, Hoechst 33342 (Coger Pharmaceuticals, Paris, France) at a concentration of 100 ng/ml, in small 20 µl drops of B2 medium covered by liquid paraffin oil. After incubation oocytes were transferred to fresh B2 drops and kept under culture conditions until observation. An adhesive tape (0.15 mm thick) in which a small window was cut was put on the glass slide to create a chamber in order to avoid flattening of the oocyte. For observation, oocytes were transferred into a drop of B2 medium within the window on prepared glass slides and covered by a coverslip. No contact occurred between the B2 medium containing the oocyte and the adherent tape. Observations were made under a Zeiss microscope equipped for epi-fluorescence. Phase contrast and fluorescence pictures were taken with Kodak Tri-X-pan film.

Oocyte classification

Unfertilized oocytes and uncleaved zygotes were classified according to three parameters of clinical interest.

With regard to the IVF indication a division in three groups was made: female, male and idiopathic causes of infertility. IVF cycles with combined female and male causes for infertility (n=5), were left out for the indication analysis.

The second parameter was the cleavage rate of the oocyte cohort: Group 1, no cleavage (n=31), Group 2, cleavage rate between 1% and 33% (n=26), Group 3, cleavage between 34% and 63% (n=25) and group 4,

cleavage >63% (n=22). The average cleavage rate in our IVF center during the study period was 63%.

Thirdly a division was made based on the occurrence of pregnancy (n=16) or its absence (n=55) after the replacement of at least one embryo. Oocytes observed in the pregnant group were considered as controls since insemination resulted for at least one of the retrieved cohort-oocytes in a viable embryo. Two patients became pregnant in group 2, four in group 3 and ten in group 4.

Statistics

For the comparison of means, Student's t-test was applied. The Chi-square test was used for frequency comparisons as appropriate. Significance was defined as $p<0.05$.

RESULTS

In table I, IVF parameters for the different groups are shown. No differences, either in the patient's age or for the number of oocytes retrieved per cycle, were found between the defined groups. Fertilization and cleavage rates where highest when the IVF indication was female and lowest when the IVF indication was male. Evidently, fertilization and cleavage rates differed significantly between the four cleavage rate groups defined. Both fertilization and cleavage rates were significantly higher for the pregnant group than for the non-pregnant group.

In figures 1-10, some of the nuclear configurations found in 440 observed unfertilized oocytes and uncleaved zygotes are shown. Eighteen out of 440 oocytes had abnormal chromatin morphology, such as the absence of individually recognizable metaphase chromosomes or the presence of a single nucleus instead of metaphase II (MII) chromosomes. Despite abnormal chromatin morphology, no cytologic anomalies were observed in these oocytes. In 17 other oocytes chromatin was not analysable. Oocytes with abnormal chromatin morphology had clumped chromatin or a single nucleus instead of metaphase II chromosomes as described elsewhere (Van Wissen et al, 1991).

Oocyte Immaturity

Sixty-five out of 440 observed oocytes (14.8%) were meiotically immature, either in germinal vesicle stage (GV-stage,13 oocytes) or in

Table I: IVF results according to IVF indication, cleavage rates and pregnancy outcome

	IVF indication[a]			Cleavage rate after IVF of the oocyte cohorts				Pregnancy outcome	
	female	male	idiopathic	gr. 1 0%	gr. 2 1-33%	gr. 3 34-63%	gr. 4 >63%	yes	no[b]
IVF cycles	n=63	n=15	n=21	n=31	n=26	n=25	n=22	n=16	n=55
Oocytes retrieved	639	164	199	284	257	276	253	151	642
Fertilized oocytes (%)	302 (47.3)*	37 (22.3)	67 (33.7)	3 (1.1)	72 (28.0)	152 (55.1)	181 (71.5)	98 (64.9)¥	309 (48.1)
Cleaved zygotes (%)	289 (45.2)§	38 (23.2)	62 (31.2)	0	65 (25.3)	137 (49.6)	191 (75.5)	97 (64.2)¶	290 (45.2)

[a]Combined male and female IVF indications were not included for the indication analysis (5 IVF cycles); [b]In the non-pregnant group only cycles with at least one embryo transplanted were considered.
The fertilization rate (*) differed between female, male and idiopathic indications and the cleavage rate (§) between female and the other IVF indications ($p<0.05$). Fertilization (¥) and cleavage rate (¶) differed significantly between the pregnant and the non-pregnant group ($p<0.001$). Evidently, fertilization and cleavage rates differed significantly between the four cleavage rate groups.

metaphase I stage (MI-stage, 52 oocytes). Spermatozoa penetrated in 24 (36.9%) immature oocytes of which the majority was in MI-stage (18 oocytes) and six were in GV-stage. Five GV-stage oocytes and five MI-stage oocytes were penetrated by multiple spermatozoa. While sperm chromatin in GV-stage oocytes was found as undecondensed (figure 1) or partly decondensed spermheads (figures 2a and b), in MI-stage oocytes male chromatin was mostly found as premature condensed chromatin (PCC, figure 3), but sometimes as a partially decondensed head (figures 4a and b).

Cytoplasmic immaturity was observed not only in meiotically immature oocytes but also in mature, metaphase II-stage (MII), oocytes where it was evidenced by the presence of PCC. Oocytes were considered meiotically mature when one polar body and a single group of MII chromosomes were observed. PCC were found in 29 oocytes (10.4%, figures 5a and b) and partly decondensed spermheads in only two meiotically mature oocytes. Nine oocytes were penetrated by more than one sperm. Penetrated MII-stage oocytes mostly had chromosomes close to the oolemma (64.5%).

In total, 115 oocytes (26.1% of the observed oocytes) were immature, including both meiotic and cytoplasmic immaturity. While no

differences were found in the incidence of meiotically immature oocytes for the defined groups (Table 2), cytoplasmic immaturity was most frequent when the cleavage rate was high (groups 3 and 4), when pregnancy occurred and when the IVF indication was female (Tables 3 and 4). The combined incidence of meiotic and cytoplasmic immaturity (Table II), was especially high in cycles where only few oocytes failed to fertilize (groups 3 and 4) and when the IVF indication was female, compared to cohorts where all, or most oocytes failed to fertilize (groups 1 and 2) and when the IVF indication was male. The pregnancy group also had a high incidence of immature oocytes which corresponds to the high cleavage rate of this group.

Table II: Oocyte immaturity and IVF parameters

IVF indications, cleavage rates, pregnancy outcome	Total number of observed oocytes[d]	GV+MI-stage oocytes total (%)	GV+MI-stage oocytes penetrated (%)	Meiotic or Cytoplasmic Immature oocytes (%)[c]
Female[a]	202	32 (15.8)	15 (7.4)	63 (31.2)
Male	99	13 (13.1)	3 (3.0)	20 (20.2)*
Idiopathic	92	16 (17.4)	6 (6.5)	25 (27.2)
Group 1	197	26 (13.2)	4 (2.0)§	34 (17.3)§
Group 2	139	21 (15.1)	4 (2.9)[c]	32 (23.0)¥
Group 3	75	15 (20.0)	13 (17.3)	39 (52.0)
Group 4	29	3 (10.4)	3 (10.3)	10 (34.5)
Pregnant	30	8 (26.7)	7 (23.3)¶	14 (46.7)¶
Non-pregnant[b]	202	29 (14.4)	13 (6.4)	57 (28.2)

Abbreviations: GV, Germinal Vesicle; MI, Metaphase I

[a]Combined male and female IVF indications were not included for the indication analysis; [b]In the non-pregnant group only cycles with at least one embryo transplanted were considered; [c]The total incidence of cytoplasmic immaturity is obtained by adding penetrated MII-stage oocytes (Table 3) and oocytes activated by sperm (Table 4); [d]The total number of observed oocytes is composed of GV+MI stage oocytes (Table 2), MII stage oocytes (Table 3), activated and pronuclear oocytes (Table 4), spontaneously activated oocytes (12 out of 440 oocytes) and abnormal and non-analysable oocytes (35 out of 440 oocytes). *Significantly different from female indications; §Significantly different from groups 3 and 4; ¥Significantly different from group 3; ¶Significantly different from the non-pregnant group; $0.001<p<0.05$.

Oocyte Maturity and Ageing

The majority of unfertilized oocytes, 279 (63.4%) was mature with the metaphase chromosomes either in a cortical position (147 oocytes, figure 7) or with chromosomes centripetally migrated into the cytoplasm (101 oocytes, figure 8). In the remaining oocytes the position of the metaphase spindle was neither cortical nor clearly migrated.

The total incidence of MII oocytes was higher in group 1 (no cleavage) than in the high cleavage rate groups (groups 3 and 4; Table III), while no significant differences were found for the occurrence of pregnancy and the IVF indications. The incidence of post-meiotic aged oocytes decreased with increasing cleavage rates and was more frequent when the IVF indication was male than female (Table 3). On the contrary, penetration by sperm into MII-stage oocytes without activation, increased with cleavage rates and was highest in group 3.

Table III: Oocyte Maturity, Post-meiotic Ageing and IVF parameters

IVF indications, cleavage rates, pregnancy outcome	Total number of observed oocytes[c]	MII-stage oocytes total (%)	Aged oocytes(%)	MII-stage oocytes penetrated (%)
Female[a]	202	115 (56.9)	36 (17.8)	19 (9.4)
Male	99	67 (67.7)	35 (35.4)*	4 (4.0)
Idiopathic	92	60 (65.2)	21 (22.8)	8 (4.0)
Group 1	197	141 (71.6)§	60 (30.5)§	5 (2.5)
Group 2	139	87 (62.6)	31 (22.3)¥	5 (3.6)
Group 3	75	37 (49.3)	8 (10.7)	18 (24.0)¶
Group 4	29	14 (48.3)	2 (6.9)	3 (10.3)¢
Pregnant	30	19 (63.3)	6 (20.0)	5 (16.7)
Non-pregnant[b]	202	115 (56.9)	35 (17.3)	21 (10.4)

Abbreviations: MII: Metaphase II. [a]Combined male and female IVF indications were not included for the indication analysis; [b]In the non-pregnant group only cycles with at least one embryo transplanted were considered; [c]The total number of observed oocytes is composed of GV+MI stage oocytes (Table 2), MII stage oocytes (Table 3), activated and pronuclear oocytes (Table 4), spontaneously activated oocytes (12 out of 440 oocytes) and abnormal and non-analysable oocytes (35 out of 440 oocytes). *Significantly different from female indications; §Significantly different from groups 3 and 4; ¥Significantly different from group 3; ¶Significantly different from groups 1 and 2; ¢Significantly different from group 1; $0.001<p<0.05$.

Sperm penetration and Fertilization

Sperm penetration without fertilization (i.e. without pronuclei) or with arrested or delayed fertilization was observed in 55 out of 104 IVF cycles and 6 out of 16 cycles leading to pregnancy. Successful oocyte-sperm fusion was found in activated and pronuclear stage oocytes but also in meiotically immature and mature oocytes, in total 23.6% (104 out of 440 oocytes; Table IV). In total seventy-four oocytes (16.8%) were penetrated by sperm without pronucleus formation (immature, mature or activated oocytes).

Activated oocytes were defined by the presence of two polar bodies and a single metaphase plate (figures 6a and b). Eighteen oocytes had expulsed the second polar body after penetration of a single spermatozoon found as PCC inside the ooplasm, and one was penetrated by multiple spermatozoa which were present as partly decondensed spermheads. These activated oocytes were relatively frequent when the majority of cohort oocytes fertilized and cleaved (Table 4, groups 3 and 4). Besides the 19 oocytes which had expulsed the second polar body after sperm penetration without further development, 12 oocytes expulsed the second polar body spontaneously since no male chromatin was found inside the ooplasm (figures 6a and b).

Thirty (6.8%) pronuclear stage oocytes were observed, 19 oocytes had 2 pronuclei (figure 9) and 11 were polypronuclear (figures 10a and b). No significant differences were found between the defined groups in the incidence of delayed or arrested pronuclear stage oocytes.

The total number of oocytes penetrated by sperm without fertilization, was significantly less frequent when the IVF indication was male and for groups 1 and 2, corresponding to no cleavage or low cleavage rates of cohort oocytes (Table IV). Spermatozoa penetrated more often in both immature (Table 2) and mature (Table III) oocytes from cycles with a high cleavage rate (groups 3 and 4) than in oocytes from the other groups. In the pregnant group where fertilization and cleavage rates were high (table 1), immature oocytes were more often penetrated by sperm than in the non-pregnant group (Table II) but the total incidence of penetrated oocytes, did not significantly differ (Table IV). However, this may be due to the small number of oocytes observed from cycles resulting in pregnancy.

Oocyte populations

The above presented results concern 41.1% of the oocyte population originally retrieved for IVF (440 out of 1070 oocytes).

Table IV: Sperm Penetration and Fertilization

IVF indications, cleavage rates, pregnancy outcome	Total number of observed oocytes[e]	Oocytes penetrated by sperm total (%)[c]	Oocytes Activated by sperm(%)[d]	Pronuclear oocytes(%)
Female[a]	202	60 (29.7)	12 (5.9)	14 (6.9)
Male	99	15 (15.2)*	3 (3.0)	5 (5.1)
Idiopathic	92	25 (27.2)	1 (1.1)	10 (10.9)
Group 1	197	17 (8.6)§	3 (1.5)	5 (2.5)
Group 2	139	25 (18.0)¥	6 (4.3)	10 (7.2)
Group 3	75	49 (65.3)	6 (8.0)¶	12 (16.0)
Group 4	29	13 (44.8)	4 (13.8)¢	3 (10.3)
Pregnant	30	15 (50.0)	1 (3.3)	2 (6.7)
Non-pregnant[b]	202	69 (34.2)	15 (7.4)	20 (9.9)

[a]Combined male and female IVF indications were not included for the indication analysis; [b]In the non-pregnant group only cycles with at least one embryo transplanted were considered; [c]The number of oocytes penetrated by sperm was calculated by adding sperm penetration without fertilization and pronuclear stage oocytes; [d]Activation: expulsion of the second polar body but no pronucleus formation; [e]The total number of observed oocytes is composed of GV+MI stage oocytes (Table 2), MII stage oocytes (Table 3), activated and pronuclear oocytes (Table 4), spontaneously activated oocytes (12 out of 440 oocytes) and abnormal and non-analysable oocytes (35 out of 440 oocytes). *Significantly different from female indications; §Significantly different from groups 2, 3 and 4; ¥Significantly different from groups 3 and 4; ¶Significantly different from group 1; ¢Significantly different from groups 1 and 2; $0.001<p<0.05$.

The total sperm penetration rate for the different groups was estimated by adding the number of fertilized oocytes (Table I) to the number of penetrated oocytes observed by DNA fluorescence (Table III). Sperm penetration rates increased significantly ($p<0.05$) from 7% in group 1 to 37.7% in group 2 and reached peak values in groups 3 and 4 as expected (72.8% and 76.7%). Interestingly, no significant difference in sperm penetration capacity was found between groups 3 and 4 while the fertilization and cleavage rate in group 4 was significantly higher than in group 3 (Table I). This was due to a higher number of penetrated oocytes for which development stopped at different stages before syngamy and cleavage in group 3. Significant differences were found in sperm penetration rate between the indication groups (female, 56.7% vs male, 31.7% vs idiopathic 46.2%; $p<0.01$) and between the pregnant and the non-pregnant group (74.8% vs 58.9%, $p<0.001$).

DISCUSSION

The population of unfertilized oocytes after IVF comprise oocytes of different maturational status which are penetrated or not, its composition depending on the fertilization and cleavage rate of the cohort oocytes. In the studied oocyte population a high frequency of sperm penetration in meiotically and cytoplasmically immature oocytes without subsequent fertilization (16.8%), and post-meiotic ageing of mature oocytes (23%) was found. The present analysis of gamete interaction accorded with the morphological findings obtained from a small number of oocytes 48 or 72 hours post-insemination (Van Wissen et al, 1991).

Most inseminated oocytes were meiotically mature and remained unfertilized because of the absence of sperm penetration (56.4% of the oocytes analysed). Almost half of these oocytes showed post-meiotic ageing processes (40.7%) manifested by the loss of the cortical position of the metaphase chromosomes, previously described in other mammals (Szöllösi, 1976) and in the human (Van Wissen et al, 1991).

Post-meiotic aged oocytes were especially frequent when none or only few oocytes were fertilized or when the IVF indication was male. It can be expected that insemination with functional sperm results in fertilization of the mature oocytes present at the time of insemination. The observation of post-meiotic ageing processes 48 hours after insemination, in these mature oocytes may indicate the incapacity of the sperm to penetrate. Indeed, the total incidence of metaphase II oocytes was especially high when none of the cohort oocytes fertilized and cleaved. The low incidence of chromosomal abnormalities of unfertilized, metaphase II-stage oocytes after IVF with a male indication (Selva et al, 1991; DeSutter et al, 1991) suggests their potential developmental capacity after fertilization. The diagnosis of dysfunctional sperm by the presence of aged oocytes not penetrated by sperm, can be of specific importance for patients with spermatozoa previously diagnosed as normal by conventional criteria.

The incidence of oocytes remaining meiotically immature (Germinal Vesicle or Metaphase I stage) seemed independent of the IVF indication, the cleavage rate and the pregnancy outcome of the cohort but the patient population was heterogeneous. Indeed, in our study, all meiotically immature oocytes came from only 36 different patients (range: 1-4 per patient).

A high percentage of the meiotically immature oocytes was penetrated by sperm (36.9%) but no meiotic maturation or fertilization processes followed. Sperm chromatin in the ooplasm of germinal vesicle stage oocytes was mostly observed as condensed spermheads but sometimes partial decondensation had taken place.

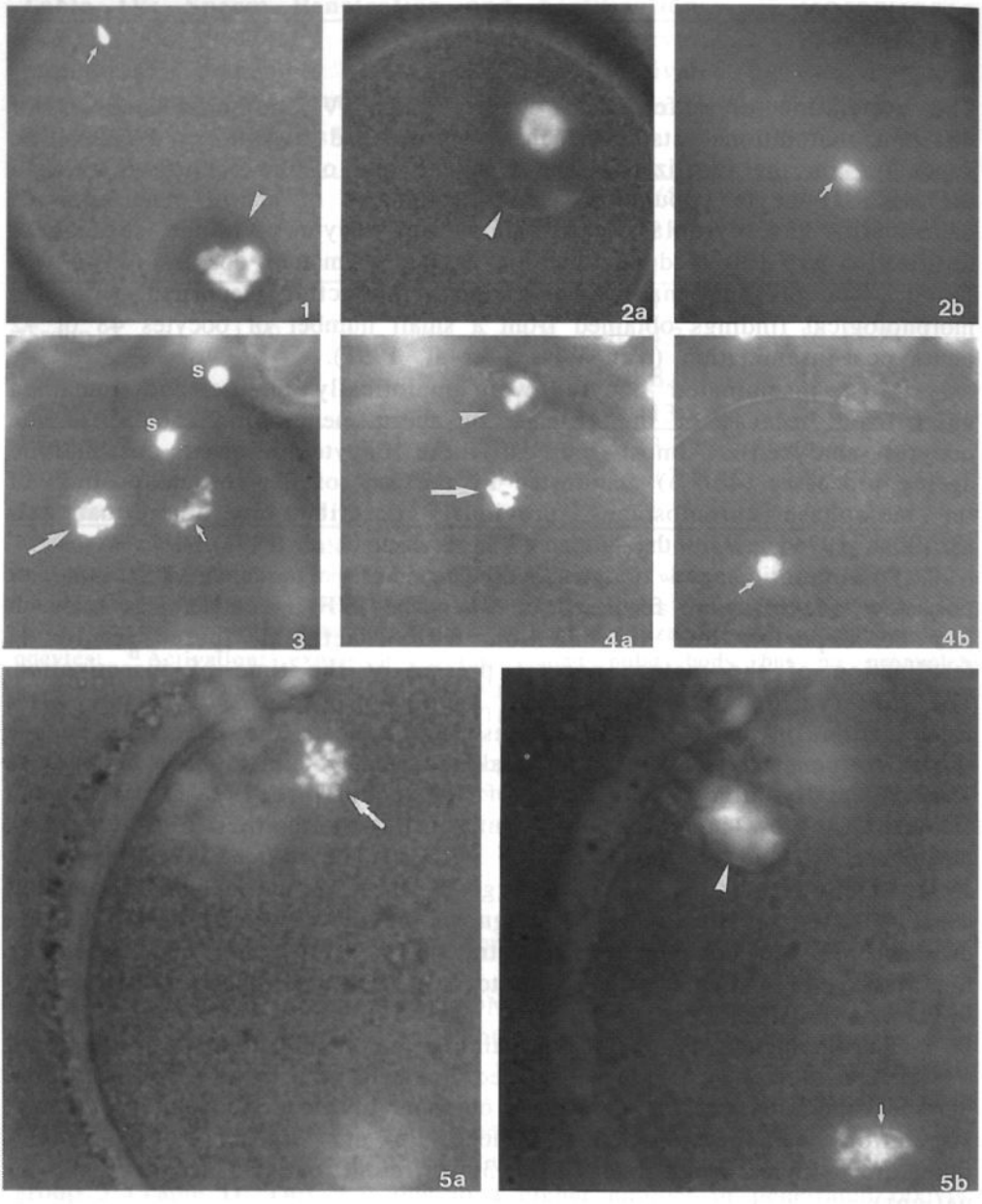
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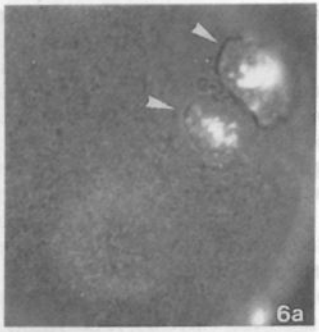
Figure 1: Oocyte with intact germinal vesicle (arrowhead) containing a large nucleolus surrounded by condensing chromatin. A single spermatozoon penetrated the oocyte and was observed as an condensed spermhead (arrow). 1cm=13.8µ. **Figure 2:** Oocyte with intact germinal vesicle (2a, arrowhead) containing a large nucleolus surrounded by condensing chromatin. A single spermatozoon penetrated the oocyte and was observed as a partially decondensed spermhead (2b, arrow). 1cm=13.8µ. **Figure 3:** Oocyte with metaphase I chromosomes (large arrow), no polar bodies were present. A second group of chromosomes represented premature condensed sperm chromatin (PCC, arrow), note the difference of chromatin condensation between the male and the oocyte chromatin. Two spermatozoa are localized outside the oocyte (S). 1cm=13.8µ. **Figure 4:** Oocyte with metaphase II chromosomes (4a, large arrow), and the first polar body (4a, arrowhead) were present. A partially decondensed spermhead was found inside the ooplasm (4b, arrow).1cm=13.8µ. **Figure 5:** Oocyte with metaphase II chromosomes (5a, arrow), the first polar body was present close to the metaphase (5b, arrowhead). A second group of chromosomes represented premature condensed sperm chromatin (PCC, arrow), note the difference of chromatin condensation between the sperm and the oocyte chromatin. 1cm=7.8µ.

In mice, a penetrated sperm attains its greatest decondensation in maturing germinal vesicle stage oocytes while the oocyte chromatin condenses (Szöllösi et al, 1990). So that it can be expected that germinal vesicle stage oocytes observed in our study, containing undecondensed spermheads, were not short before germinal vesicle breakdown even when condensation of chromatin within the germinal vesicle had sometimes started. It has been shown that sperm penetration occurs frequently in meiotically immature oocytes from small antral follicles, but this does not lead to pronuclear formation in the hamster (Usui and Yanagimachi; 1976), in mice (Szöllösi et al, 1990) and in the human (Lopata et al, 1988). However, recently Van Blerkom et al (1994b) described in the human, that some rare germinal vesicle stage oocytes accidently retrieved for IVF completed meiosis after sperm penetration and formed two pronuclei. We observed that oocytes which remained immature 48 hours after culture whether or not penetrated by sperm, never resumed meiosis. Some immature oocytes may complete meiotic maturation during culture before sperm penetration takes place but fertilization is likely to be delayed (Zenzes et al, 1990).

The presence of prematurely condensed sperm chromatin (PCC) in metaphase oocytes has been described previously in mouse (Kubiak, 1989) and human oocytes (Schmiady and Kentenich, 1989) and indicates the persistance of condensing factors which is probably due to cytoplasmic immaturity of meiotically mature oocytes (Kubiak, 1989; Calafell et al, 1991). PCC found in our study resulted from sperm penetration shortly after the expulsion of the first polar body i.e. 32 hours post hCG (Bomsel-Helmreich et al, 1987), as we observed the location of metaphase chromosomes near the first polar body indicating its recent expulsion and signaling the possibility of cytoplasmic immaturity.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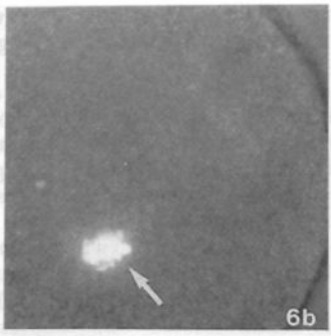
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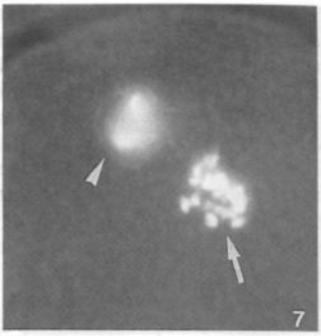
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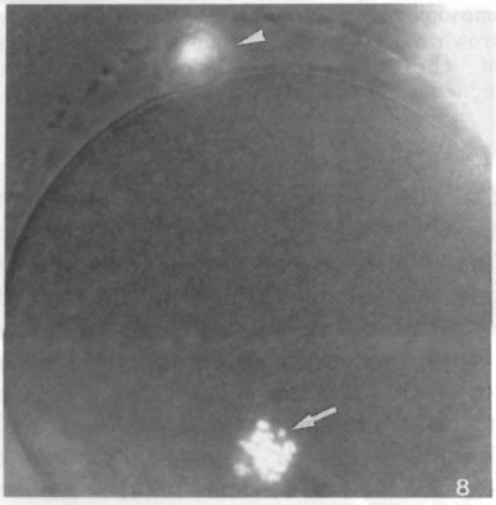
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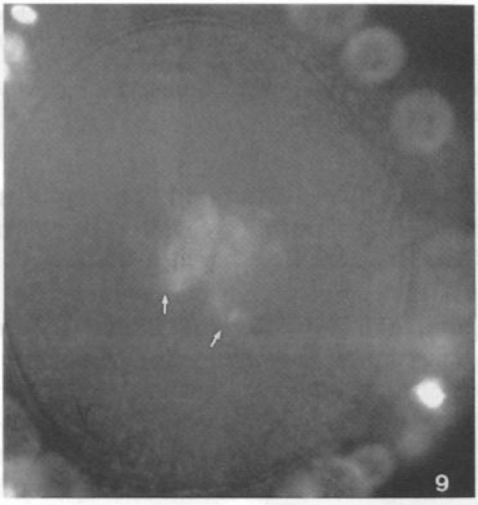
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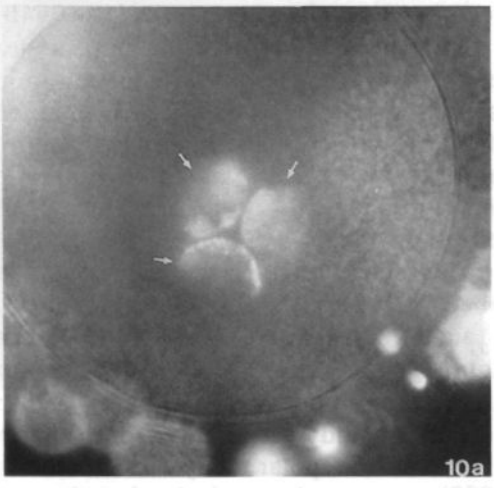
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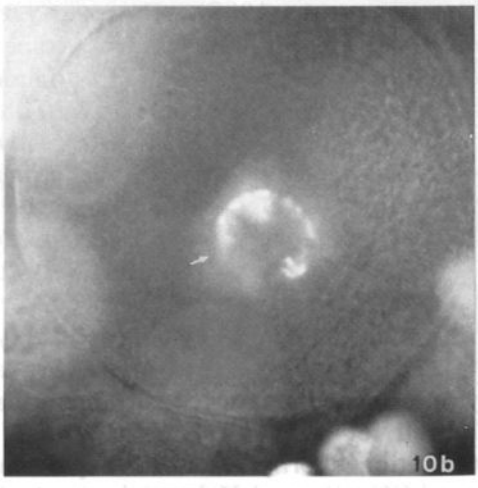
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Figure 6: Spontaneously activated oocyte, two polar bodies were present (6a, arrowheads) as well as a single metaphase plate (6b, arrow). No sperm chromatin was observed inside the ooplasm. 1cm=10μ. **Figure 7:** Oocyte with the metaphase II chromosomes (arrow) close to the first polar body (arrowhead) in a cortical position. 1cm=10μ. **Figure 8:** Post-meiotic aged oocyte with the metaphase II chromosomes migrated towards the oocyte center (arrow) far away from the first polar body (arrowhead). 1cm=10μ. **Figure 9:** Delayed zygote with two pronuclei (arrows) and two polar bodies (out of focus). 1cm=11μ. **Figure 10:** Arrested polypronuclear zygote, presence of two polar bodies (out of focus) and four pronuclei (10a and b, arrows) indicated polyspermic penetration. 1cm=11μ.

Tejada et al (1992) considered PCC to be a fertilization anomaly rather than a result of oocyte immaturity since all the PCC containing oocytes were in MII stage. However, they did not differentiate recently matured from post-meiotic aged oocytes. The frequency of PCC varies between different ovarian stimulation protocols influencing oocyte maturity and ploidy (Pieters et al, 1991; Edirishinge et al, 1992), suggesting the importance of the maturational status of oocytes at the time of sperm penetration for pronucleus formation. The presence of PCC has been proposed as a cause of idiopathic infertility (Selva et al, 1991). Our results as well as other studies (Tejada et al, 1992) do not confirm this but a high incidence of PCC may explain idiopathic infertility in some individual patients.

Cytoplasmic immaturity which was evidenced despite nuclear maturity, by the presence of PCC was found in 6.5% of the analysed oocytes and in 10.7% when activated oocytes are added. The incidence of PCC in our study corresponds with other studies using cytogenetic techniques (Schmiady and Kentenich, 1989; Zenzes et al, 1990; Tarin et al, 1991).

It is important to note that cytoplasmic immaturity of meiotically mature oocytes can only be revealed after sperm penetration and PCC formation. Hence, the increasing incidence of cytoplasmic immaturity with increasing cleavage rates is probably not due to a changing oocyte population but is demonstrated by an increased sperm penetration capacity. Consequently, the incidence of cytoplasmic immaturity when all the oocytes fail to cleave or when the cleavage rate is low (groups 1 and 2, male infertility, non-pregnancy) is likely to be underestimated. Only a relatively small amount of oocytes activated by sperm and oocytes having reached the pronuclear stage but unable to develop further show unfitness of oocytes or a delayed development.

While sperm penetration rates in the highest cleavage rate groups (groups 3 and 4), were equal, the incidence of functionally defective oocytes was higher in group 3. The detection of such abnormal oocytes may be important for couples with fertilization but without developing embryos. The present study shows that sperm penetration without

fertilization in meiotic or cytoplasmic immature oocytes occurs frequently in IVF, even when pregnancy resulted, and can be considered as an excellent parameter for the sperm penetration capacity especially when IVF failed. Additional information concerning sperm-oocyte interaction can be obtained when spermatozoa bound to or partly penetrated into the zona pellucida are counted. For the oocytes used in the present study, sperm-zona binding and penetration was described previously (Van Wissen et al, in press).

When oocytes were penetrated by sperm but none fertilized and cleaved, the oocyte was either not able to support pronucleus formation or syngamy and cleavage failed. For these cases an oocyte problem can be suspected. This important information should be taken into consideration for individual patients, even if this was rare in our study (8.6%, group 1).

In opposition to meiotic immaturity, cytoplasmic immaturity increased when the cleavage rate was high (groups 3 and 4). This results in an increased total incidence of immature oocytes for these groups. A high incidence of cytoplasmic immaturity results in lower fertilization rates since it represents a developmental blockage. While germinal vesicle stage oocytes if penetrated by sperm may possibly be able to complete maturation and form pronuclei (Van Blerkom et al, 1994b), a high number of cytoplasmically immature metaphase II stage oocytes when penetrated do not, since they remain in metaphase. We have no information regarding the development of the PCC containing oocytes since we did not keep them in vitro after observation.

Partially decondensed spermheads were only rarely found in metaphase oocytes and we never observed condensed spermheads in metaphase oocytes. This is contradictory to the findings of Van Blerkom et al (1994a) who observed almost exclusively condensed or partly decondensed spermheads in penetrated but unfertilized metaphase II-stage oocytes. In our study, oocytes were obtained 48 hours after insemination for observation whereas in the study of Van Blerkom et al (1994a) oocytes were obtained 28 hours after insemination i.e. 20 hours earlier which may explain the difference in sperm chromatin morphology inside the ooplasm. The results obtained in both studies confirm our previous hypothesis (Van Wissen et al, 1991) that partly decondensed spermheads in metaphase oocytes represent a stage of sperm nucleus transformation preceding PCC formation provided that the cytoplasmic factors needed are present.

Studies where the absence of sperm penetration was deduced from the absence of pronuclei (Bongso et al, 1992; Bedford and Kim, 1993) probably underestimated the proportion of oocytes penetrated by sperm and may have come to erronous conclusions concerning the incidence of sperm-oocyte fusion, since sperm penetration occurs frequently even without subsequent pronucleus formation. Our findings also suggest that

unfertilized oocytes from partial IVF failures are unsuitable to evaluate gamete fusion by reinsemination because they may already have been penetrated even when pronuclei are absent. It can not be excluded that these oocytes have an altered reaction to a second penetrating sperm.

In summary, our study shows that within the population of unfertilized oocytes after IVF, the incidence of post-meiotic aged oocytes, immature and mature oocytes penetrated by sperm without fertilization, is related with the fertilization and cleavage rate of cohort oocytes. The present results also indicate that oocytes which remained unfertilized after IVF should be carefully characterized especially with respect to sperm penetration before they can be used for research purposes. In clinical situations, analysis of unfertilized oocytes can give prognostic information concerning the fertilizing capacity of both gametes. On this basis one can predict the outcome of intracytoplasmic sperm insemination (ICSI).

REFERENCES

Almeida, PA, Bolton, V. (1994) The relationship between chromosomal abnormalities in the human oocyte and fertilization in vitro. *Hum. Reprod.* 9, 343-346.

Bedford, JM, Kim, HH. (1993) Sperm/egg binding patterns and oocyte cytology in retrospective analysis of fertilization failure in vitro. *Hum. Reprod.*, 8, 453-463.

Belsey, MA, Eliasson R., Gallegos AJ, Moghissi KS, Paulson CA, editors. (1980) World Health Organisation: Laboratory manual for the examination of human semen and semen-cervical mucus interaction. MNR Prasad. Singapore: Press Concern.

Bomsel-Helmreich O, Huyen LVN, Durand-Gasselin I, Salat-Baroux J, Antoine JM. (1987) Timing of nuclear maturation and cumulus dissociation in human oocytes stimulated with clomiphene citrate, human menopausal gonadotrophin, and human chorionic gonadotrophin. *Fertil. Steril.*, 48, 586-595.

Bongso A, Yee Fong C, Ng SC, Ratnam S. (1992) Fertilization, cleavage, and cytogenetics of 48-hour zona-intact and zona-free human unfertilized oocytes reinseminated with donor sperm. Fertil. Steril. 57, 129-133.

Calafell JM, Badenas J, Egozcue, Santalo J. (1991) Premature chromosome condensation as a sign of oocyte immaturity. *Hum. Reprod.* 6, 1017-1021.

Conover JC, Gwatkin RBL. (1988) Pre-loading of mouse oocytes with DNA-specific fluorochrome (Hoechst 33342) permits rapid detection of oocyte-sperm fusion. *J. Reprod. Fertil.* 82, 681-690.

Debey P, Renard JP, Coppey-Moisan M, Monnot I, Geze M. (1989) Dynamics of chromatin changes in live one-cell mouse embryos: a continuous follow-up by fluorescence microscopy. *Exp. Cell Res.* 1183, 413-433.

De Sutter P, Dhont M, Vanluchene E, Vandekerckhove D. (1991) Correlations between follicular fluid steroid analysis and maturity and cytogenetic analysis of human oocytes that remained unfertilized after in vitro fertilzation. *Fertil. Steril.* 55, 958-963.

Edirishinge WR, Murch AR, Yovich JL. (1992) Cytogenetic analysis of human oocytes and embryos in an in-vitro fertilization programme. *Hum. Reprod.* 7, 230-236.

Frydman R, Belaisch-Allart J, Parneix I, Forman R, Hazout A, Testart J. (1988) Comparison between flare-up and down regulation effects of luteinizing hormone-releasing hormone agonists in an in vitro fertilization program. *Fertil. Steril.* 50, 471-475.

Gwatkin RBL, Conover JC, Collins RL, Quigley MM. (1989) Failed fertilization in human in vitro fertilization analysed with the deoxyribonucleic acid-specific fluorochrome Hoechst 33342. *Am. J. Obstet. Gynecol.* 160, 31-35.

Kubiak J. (1989) Mouse oocytes gradually develop the capacity for activation during the metaphase II arrest. Dev. Biol. 136, 537-545.

Lopata A, Leung PC. (1988) The fertilizability of human oocytes at different stages of meiotic maturation. *Ann. N.Y. Acad. Sci.* 541, 324-336.

Pieters MHEC, Geraedts JPM, Dumoulin JCM, Evers JLH, Bras M, Kornips FHAC, Menheere PPCA. (1989) Cytogenetic analysis of in vitro fertilization (IVF) failures. *Hum. Genet.* 81, 367-370.

Pieters MHEC, Dumoulin JCM, Engelhart CM, Bras M, Evers JLH, Geraedts JPM. (1991) Immaturity and aneuploidy in human oocytes after different stimulation protocols. *Fertil. Steril.* 56, 306-310.

Plachot M, Veiga A, Montagut J, Grouchy J de, Calderon G, Lepretre S, Junca AM, Santalo J, Carles E, Mandelbaum J, Barri P, Degoy J, Cohen J, Egozcue J, Sabatier JC, Salat-Baroux J. (1988) Are clinical and biological IVF parameters correlated with chromosomal disorders in early life: a multicentric study. *Hum. Reprod.* 3, 627-635.

Schmiady H, Kentenich H. (1989) Premature chromosome condensation after in-vitro fertilization. *Hum. Reprod.* 4, 689-695.

Selva J, Martin-Pont B, Hugues JN, Rince P, Fillion C, Herve F, Tamboise A, Tamboise E. (1991) Cytogenetic study of human oocytes uncleaved after in-vitro fertilization. *Hum. Reprod.* 6, 709-713.

Szöllösi D. (1976) Morphological changes in mouse eggs due to ageing in the fallopian tube. *Am. J. Anat.* 130, 209-226.

Szöllösi D, Szöllösi MS, Czolowska R, Tarkowski AK. (1990) Sperm penetration into mouse oocytes and nuclear changes during maturation: an EM study. *Biol. Cell.* 69, 53-64.

Tarin JJ, Ruiz A, Miro F, Bonilla-Musoles F, Pellicer A. (1991) Failed in vitro fertilization of human oocytes: a cytogenetic analysis. *Fertil. Steril.* 56, 290-295.

Tejada MI, Mendoza MR, Corcostegui B, Benito JA. (1992) Factors associated with premature chromosome condensation (PCC) following in vitro fertilization. *J. Ass. Reprod. Genet.* 9, 61-67.

Tesarik J. (1993) Subzonal sperm insertion with aged human oocytes from failed in-vitro fertilization attempts: fertilization results and some applications. *Hum. Reprod.* 8, 1245-1250.

Tesarik JT, Mendoza C. (1994) Most living acrosome-reacted spermatozoa do not fuse with the oocyte when inserted into the perivitelline space. *Fertil. Steril.* 61, 529-535.

Usui, N., Yanagimachi, R. (1976) Behaviour of hamster sperm nuclei incorporated into eggs at various stages of maturation, fertilization, and early development. *J. of Ultrastructure Research* 57, 276-288.

Van Blerkom, J., Davis, P.W., Merriam, J. (1994a) A retrospective analysis of unfertilized and presumed parthenogenetically activated human oocytes demonstrates a high frequency of sperm penetration. *Hum. Reprod.* 9, 2381-2388.

Van Blerkom, J., Davis, P.W., Merriam, J. (1994b) The developmental ability of human oocytes penetrated at the germinal vesicle stage after insemination in vitro. *Hum. Reprod.* 9, 697-708.

Van Wissen B, Bomsel-Helmreich O, Debey P, Eisenberg C, Vautier D, Pennehouat G. (1991) Fertilization and ageing processes in non-divided human oocytes after GnRHa treatment. *Hum. Reprod.* 6, 879-884.

Van Wissen B, Eisenberg C, Debey P, Pennehouat G, Auger J, Bomsel-Helmreich O. (1992) In vitro DNA fluorescence after in vitro fertilization (IVF) failure. *J. Ass. Reprod. Genet.*, 9, 564-571.

Van Wissen B, Bomsel-Helmreich O. (1994) DNA fluorescence revealing oocyte quality, sperm behaviour during gamete interaction, and embryo morphology. *Ass. Reprod. Reviews* 4, 128-133.

Van Wissen B, Bomsel-Helmreich O, Frydman R. (In Press) Sperm-zona binding and sperm-oocyte penetration analysed by DNA fluorescence: a test for gamete quality after IVF. *Human Reproduction.*

Zenzes MT, de Geyter C, Bordt J, Schneider HPG, Nieschlag E. (1990) Abnormalities of sperm chromosome condensation in the cytoplasm of immature oocytes. *Hum. Reprod.* 5, 842-845.

CHAPTER VI

Timing of pronuclear development and first cleavages in human embryos after subzonal insemination: influence of sperm phenotype

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ABSTRACT

The sequential transformations of human sperm nuclei in human eggs after subzonal insemination (SUZI, n=104) and the influence of sperm defects on this timing were studied. This chronology was compared to that of two control series of zygotes obtained after SUZI with normal spermatozoa (n=35) and after in vitro fertilization (IVF) with normal donor spermatozoa (D-IVF, n=220). Pronuclear formation took place between 4.5 and 10.5 hours post-SUZI for 92.8 % of the zygotes. They remained visible for 13 hours and began to disappear 18.5 hours post-SUZI. The time span between PN disappearance and cleavage was 3 hours. Zygotes obtained after D-IVF had a similar rate of pronuclear disappearance but ≈4 hours later. The second cell cycle was more rapid for zygotes obtained by D-IVF than by SUZI, but the developmental rate of zygotes obtained by SUZI varied according to sperm phenotypes. For patients with previous unexplained IVF failures (control group with normal spermatozoa), the developmental rate was lower, suggesting the influence of oocyte quality. In conclusion, the end of the first cell cycle of zygotes obtained by insemination under the zona pellucida appears 4 hours earlier compared to zygotes obtained after insemination outside the zona pellucida.

INTRODUCTION

Subzonal insemination (SUZI) with spermatozoa is used to improve the possible reproductive outcome for patients having repeated in vitro fertilization (IVF) failures and for patients with sperm parameters that are so severely impaired that even standard IVF is impossible (Cohen et al, 1991; Ng et al, 1991). As it is not possible to know which spermatozoon is capable of fertilizing the egg, in most cases several spermatozoa are inseminated under the zona pellucida. Since in human oocytes hardening of the zona pellucida induced by fertilization, is the main block to polyspermic fertilization (Barros and Yanagimachi, 1971), there is an increased risk of polyspermy. Indeed, the monospermic fertilization rate varies from 12-30%, while the polyspermic fertilization rate varies from 5-30% after SUZI, depending on sperm quality and the number of spermatozoa micro-injected (Ng et al, 1991; Cohen et al, 1991; Wolf et al, 1993). This is much more than the 2-9%

rate seen after IVF (Diamond et al, 1985; Plachot et al, 1988; Tournaye et al, 1992). It has also been reported that ploidy scores performed 16-18 hours after SUZI, may be lower than the ploidy found after cytogenetic analysis (Selva et al, 1993), suggesting a possible asynchrony in the development of pronuclei after SUZI. Actually, it is not known whether by-passing the egg investments influences pronuclear appearance and disappearance or early embryo development. Furthermore, as SUZI is indicated for patients whose spermatozoa cannot fertilize eggs during routine IVF insemination, it may be possible that sperm defects couls also modify this development. These facts require that the sequential transformations of human sperm nuclei in human eggs after subzonal insemination be carefully determined for patients whose sperm characteristics require SUZI for fertilization. This study was done to determine the timing of pronuclear development during the first cell cycles of normally fertilized oocytes, and to evaluate the possible influence of gamete related factors on this chronology. Two control series were done. Firstly, SUZI was performed with normal spermatozoa to compare their sequential transformation into pronuclei to that of abnormal spermatozoa. However, as it is not ethically possible to include couples who do not need it in a programme of SUZI, only patients with normal spermatozoa but unexplained IVF failures were considered for this study. In any case, prior to their inclusion, the fertilization ability of their spermatozoa was assessed by a zona-free hamster egg penetration test (Wolf et al, 1992). In the second control series, oocytes were inseminated with donor spermatozoa in routine IVF (D-IVF) to compare the development of pronuclei after SUZI to development after IVF and to eliminate the influence of any sperm defects. The SUZI program received the agreement of the Ethical Committee of the Bicêtre Hospital.

MATERIALS AND METHODS

Patients

To study SUZI in our laboratory, the oocytes from every couple were divided into two groups to allow comparison between IVF and SUZI for all the possible indications for the technique (Wolf et al, 1992; 1993). Only patients presenting sperm defects which had been shown to induce IVF failures as well as patients with unexplained but repeated IVF failures were subsequently included in the SUZI programme. Between January 1991 and March 1993, 60 patients (69 cycles) with at least one cleaving diploid zygote after a SUZI attempt were included

according to the following criteria: group A (flagellar dyskinesia): patients with abnormal sperm movement parameters (n=24). These patients had a very poor cervical mucus sperm penetration assay. Sperm movement analysis using a computer assisted method showed flagellar dyskinesia.(Serres et al, 1986). Electron microscopy identified either a specific structural flagellar defect such as the absence of outer dynein arms (Jouannet et al, 1983; Wolf et al, 1993) or peri-axonemal abnormalities (Serres et al, 1986). For these patients the mean sperm concentration in the semen was $41.3\pm34x10^6$/ml with 28±15% motile spermatozoa and 30±16% spermatozoa with normal morphology. Group B (non-specific sperm defects): patients (n=16): with $<5x10^6$ spermatozoa/ml in the semen, and patients with antisperm antibodies (>60 % of spermatozoa recovered with immunoglobulin G; De Almeida et al, 1986). Group C (control group): in this group were included patients (n=20) with normal sperm parameters and normal sperm functions. They had normal fusiogenic properties as assessed by the zona-free hamster egg penetration assay. they were included in the SUZI programme because of repeated and unexplained IVF failures. Knowing the risk of polyspermy, it was actually not ethically acceptable to include patients with gametes that already fertilized during routine IVF insemination. The patients in this group had had at least two IVF attempts involving a minimum of 10 oocytes, with unexplained failures of fertilization. Their semen had normal sperm concentration, motility and morphology, according to World Health Organization guidelines (WHO, 1987; Wolf et al, 1992). Their mean sperm concentration in the semen was $50.3\pm33x10^6$/ml with 46±15% motile spermatozoa and 42±16% spermatozoa with normal morphology. Over the same period, 37 patients (46 cycles) underwent IVF with donor spermatozoa (D-IVF). Patients for whom donor spermatozoa were indicated (Czyglik, 1991) were included in the IVF programme (D-IVF) when at least six in-utero inseminations with donor spermatozoa did not result in pregnancy or when there was a female factor. The donor spermatozoa had normal parameters (concentration, motility, morphology) according to WHO guidelines (1987).

Sperm preparation

The patients' semen was collected by masturbation the day of SUZI and allowed to liquefy for 30 min at 37°C. It was layered over a two-density percoll gradient (47.5% and 90%; Pharmacia, Uppsala, Sweden) and spun for 20 min. at 300 g. The pellets were carefully removed and washed twice by centrifugation (5 min at 600 g) and resuspended in B2 medium (CCD, Paris, France). Motile spermatozoa were then kept for 4-6 hours at

room temperature in B2 medium. The donor spermatozoa came from men of proven fertilizing ability. They had normal sperm parameters according to WHO criteria. The freezing protocol included dilution of their semen with Ackerman Freezing medium to obtain a final concentration of 7% glycerol (David and Czyglik, 1977). This dilution was begun slowly to prevent osmotic shock before mixing with gentle rotation for 15 min. Then, 250 µl straws were filled with the sperm suspension before being frozen using an LC40 Minicool R (CFPO, France) and stored in liquid nitrogen (David and Czyglik, 1977). The straws were thawed by exposure at 37°C for 2 min. The spermatozoa were then progressively diluted with 2 ml of B2 medium at 37°C prior to selection using the same Percoll gradient. The subsequent handling of the thawed spermatozoa was similar to that of fresh spermatozoa.

Oocyte preparation

Follicular growth was stimulated by human menopausal gonadotropin (HMG) in conjunction with gonadotrophin releasing hormone agonist (GnRHa; DTRP6, Ipsen Biotech, France). Ovulation was induced with 5000 IU of human chorionic gonadotrophin and the oocytes were harvested 36 hours later using a transvaginal ultrasound procedure (Frydman et al, 1988). When ovulation induction was performed for SUZI, the release of endogenous gonadotropins was completely suppressed by GnRHa before HMG administration, whereas for D-IVF the flare-up effect was more frequently used (Frydman et al, 1988). When SUZI was performed, the cumulus was removed by incubation with hyaluronidase 0.1% in B2 medium (Wolf et al, 1992). The eggs were then washed and individually placed into 30 µl drops of B2 medium under equilibrated mineral oil. The oocytes were checked for their nuclear status based on the presence or absence of the first polar body or of the germinal vesicle (Veeck, 1986). SUZI took place 6-8 hours after retrieval for the oocytes in the metaphase I or metaphase II stage. When the oocytes were collected for D-IVF, the cumuli were placed into 30 µl drops of B2 medium under equilibrated mineral oil and inseminated with 5000 motile spermatozoa. The cumulus cells were removed between 16 and 24 hours post insemination and the oocytes were checked for the presence of pronuclei.

Subzonal insemination

SUZI was performed using two Narishige micromanipulators and an inverted Olympus IMT-2 microscope. Micropipettes were pulled on a vertical Narishige PB-7 puller and holding pipettes were prepared on a De

Fonbrune microforge (Alcatel, France). The zona pellucida was pierced and between five and eight spermatozoa delivered into the perivitelline space. The total procedure did not exceed 5 min. The technical details have been described previously (Wolf et al, 1992). A mean number of 1.8 embryos (1-4) were replaced into the uterus of the patients and the remaining embryos were frozen.

Pronuclei and cleavage scores

The oocytes were checked for pronuclei after SUZI and insemination with donor spermatozoa. Those presenting two pronuclei were considered normally fertilized. When a maximum of one or more than two pronuclei were seen, the oocytes were considered as unfertilized and polyspermic respectively (Veeck, 1986). Only diploid zygotes were considered in this study. For 139 embryos from SUZI, 450 observations took place between 3 and 30 hours after microinjection. Of the embryos, 131 zygotes resulted from the micro-injection of metaphase II oocytes and eight from metaphase I oocytes. A total of 220 embryos from D-IVF were also included in this study, for which 417 observations with precise timing took place between 16 and 30 hours after insemination. Each zygote was observed randomly at least once and at most six times, during the indicated time-span. The number of pronuclei (0, 1, 2) and the number of blastomeres when cleavage took place were recorded. All embryos were observed once more between 44 and 48 hours (day 2), before uterine transfer or cryopreservation. These non-invasive observations were done with low luminosity using a heated microscope platform (37°C).

Statistical analysis

Student's t-tests were used to compare means and Chi-square tests were applied to examine qualitative differences between groups of oocytes. The differences were considered significant at $P<0.05$. Regression and correlation coefficients were studied using the Statworks package.

RESULTS

The mean age of the patients having SUZI was 33 years (range 24-42), which did not differ from patients having D-IVF (mean 35 years; range 26-42). The SUZI and D-IVF results are reported in table I.

Table 1: Fertilization rates according to sperm pathology and after in vitro fertilization with donor spermatozoa (D-IVF).

	Patients (cycles)	Oocytes treated*	Mono-spermy (%)	Poly-spermy (%)	Trans fer-red em-bryos	Im-planted em-bryos (%)
Group A	24 (29)	245	67 (27.3)[a]	36 (14.7)[b]	58	5 (8.6)
Group B	16 (19)	182	37 (20.3)	9 (4.9)	33	5 (15.2)
Group C	20 (21)	182	35 (19.2)	11 (6.0)	35	2 (5.7)
Total SUZI	60 (69)	609	139 (22.8)[c]	56 (9.2)[c]	126	12 (9.5)
Total DIVF	37 (46)	320	220 (68.8)	11 (3.4)	123	19 (15.5)

Group A: patients with flagellar dyskinesia; group B: patients with non-specific sperm defects; group C: control group. *Oocytes which were microinjected by SUZI or inseminated for DIVF. Significantly different; [a]from group B ($P<0.05$); [b]from group B and C ($P<0.01$); [c]from DIVF ($P<0.001$).

In group A (flagellar dyskinesia), the monospermic fertilization rate was higher than in group C (controls; $P<0.05$) but did not differ from group B (non-specific sperm defects). The polyspermic fertilization rate was higher in group A than in groups B and C ($P<0.01$). After SUZI, the monospermic fertilization rate was significantly lower, and the polyspermic fertilization rate significantly higher than after D-IVF ($P<0.001$).

Chronology of the first cell cycle after SUZI and DIVF.

Several periods of pronuclear formation and disappearance and of zygote cleavage can be defined (Figure 1A). No pronuclei were seen before 4.5 hours post-SUZI. The first pronuclei were observed 5 hours post-SUZI and they regularly appeared in most zygotes between 5 and

10.5 hours post SUZI. Indeed at 10.5 hours post SUZI 92.8% of zygotes had two pronuclei. A single pronucleus was observed once. Between 10.5 and 18 hours post-SUZI little variation was observed for the stage of the zygotes. Most zygotes remained with two pronuclei (141 out of 147 observations). Zygotes which had not achieved pronuclear formation represented a group of 10 eggs (12 out of 248 observations between 12 and 21 hours post-SUZI), and 7.2 % of all zygotes.

At 18 hours post-SUZI, the pronuclei began to disappear. The rate of pronuclear disappearance was regular and rapidly progressive. Data analysis showed that this rate followed a regression line ($P<0.001$) with a slope of -11.2. Extrapolation suggested that the disappearance of pronuclei should be terminated 32.7 hours post-SUZI. The pronuclei remained visible for ≈13 hours, with this delay being quite constant for all zygotes. They began to disappear 18 hours post micro-injection and the delay between pronuclear disappearance and cleavage was 3 hours. Remarkably, the rate of cleavage seemed to follow a regression line ($P<0.001$) almost parallel (slope of -12.3) to that of pronuclear disappearance. Calculation suggested that all cleavages should have been completed 34.7 hours post-SUZI. Between 21 and 30 hours post-SUZI, 32 out of 155 observed zygotes had already cleaved. Six zygotes had one remaining pronucleus during this time span.

For the eight oocytes which were micro injected at the metaphase I stage, we did not find any statistically significant differences in their pronuclear development compared to the others. They showed, however a tendency to delay their further development as they had a mean of 2.7 ± 0.9 blastomeres on day 2 compared with 3.2 ± 1.0 for all SUZI zygotes. However, this series was too small to draw any definitive conclusions.

The observations following D-IVF (Figure 1B), started 16 hours after insemination so that none took place at the time of pronuclei formation. Between 16 and 22.5 hours post insemination, most zygotes had two pronuclei (251 out of 263 observations): only one had not yet formed a pronucleus, 11 zygotes had a single pronucleus and none had cleaved. The pronuclei began to disappear 23 hours post-insemination. After that, the rate of pronuclear disappearance was regular and rapidly progressive. Extrapolation according to the regression line ($P<001$) suggested that they all should have disappeared for 36.9 hours post-insemination. The first cleavage division was observed 25.5 hours post-insemination. Between 21 and 30 hours post-insemination only 19 cleaved zygotes were found out of 154 observations. Asynchronous pronuclear disappearance was rare (15 out of 417 observations, for eight different zygotes).

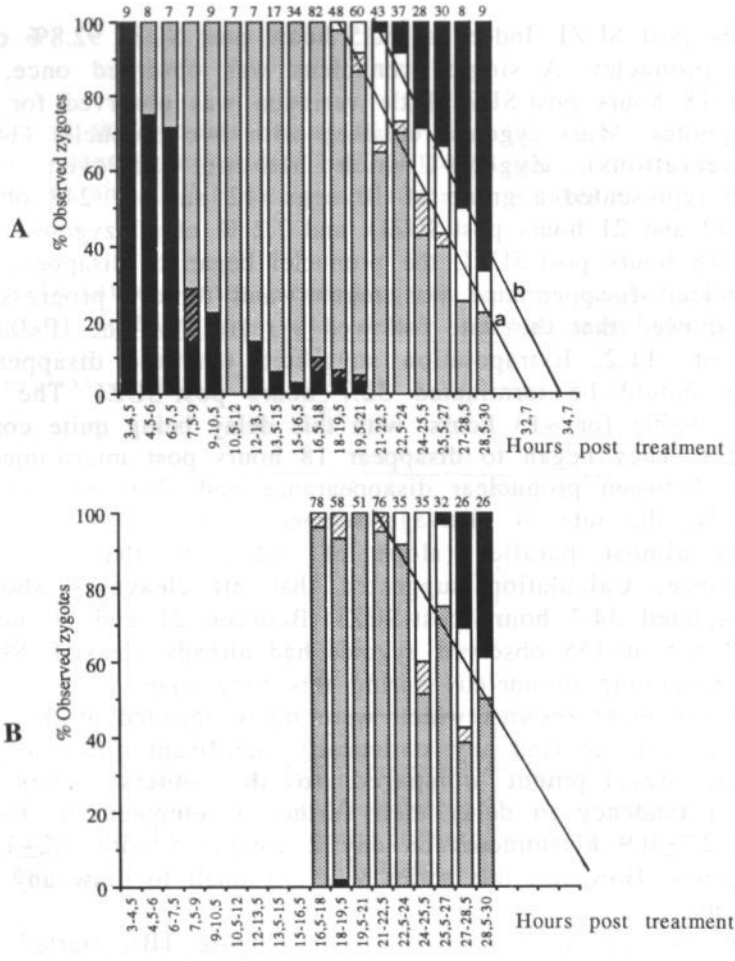

Figure 1A: Pronuclear development and cleavage of human zygotes after subzonal insemination (SUZI, n=139). The numbers of observations for each time interval are given above the bars. Zygotes for which pronuclei have not appeared are shown in the lower part of the chart, while those for which pronuclei have already disappeared are shown in the upper part. The diagonals represent the regression lines of the moments of pronuclear disappearance (a) and of cleavage (b). **Figure 1B**: Pronuclear development in human zygotes after in vitro fertilization with donor spermatozoa (D-IVF, n=220). The number of observations for each time interval are given above the bars. Zygotes for which pronuclei have not appeared are shown in the lower part of the chart, while those for which pronuclei had already disappeared are shown in the upper part. The diagonal represents the regression line of the moments of pronuclear disappearance. ■ percentage of observed zygotes without pronuclei before the appearance of two pronuclei, ▨ percentage with one pronucleus before the appearance of the two pronuclei: ▩ percentage with two pronuclei, ▨ percentage with one pronucleus after the disappearance of one pronucleus, □ percentage with no pronuclei after the disappearance of both pronuclei, ■ percentage of cleaved zygotes.

Influence of sperm phenotype on the chronology

In Figure 2, the percentage of zygotes with two pronuclei is shown according to sperm phenotype. In zygotes from group A (flagellar dyskinesia), the disappearance of the pronuclei which was completed 27 hours post-SUZI is more rapid than for zygotes from group B (non-specific sperm defects, $P<0.02$). An opposite tendency is seen for zygotes from group C (control group), where the pronuclei remained present for a longer time in a higher number of zygotes, but the difference with group A was not significant.

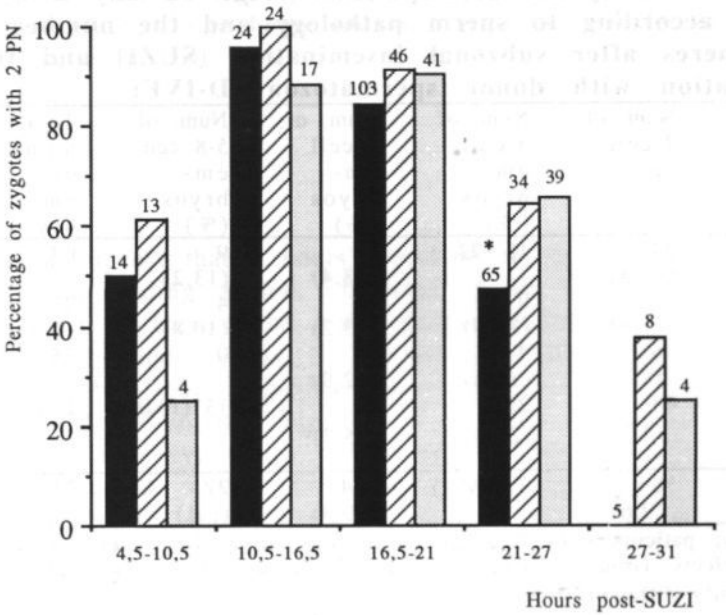

Figure 2: Appearance and disappearance of pronuclei in human zygotes resulting from subzonal insemination (SUZI) according to sperm pathology. No pronuclei were observed before 4.5 hours post-SUZI (n=9). The number of observations for each time interval are given above the bars. ■ group A patients with flagellar dyskinesia, ▩ group B non-specific sperm defects, ▨ group C control group. *: Significantly different from group C ($P<0.02$).

Developmental rates after SUZI and DIVF

In Table II, the developmental stages on day 2 are reported for the different groups. The developmental rate (mean number of blastomeres

per embryo) was significantly lower for SUZI (3.2±1.0) than for D-IVF (3.6±0.9, P<0.001). After D-IVF nearly 70 % of embryos were at the 4-cell stage or beyond on day 2 while there were <50 % after SUZI. No significant differences in the developmental rate, as measured by the mean number of blastomeres per embryo, were found between zygotes from groups A, B and C. However, patients from the control group C appeared to have a lower mean number of blastomeres per embryo than those from groups A and B (3.0±1.0 compared with 3.2±1.1 and 3.5±1.0 respectively).

Table II: Embyonic developmental stage on day 2 (44-48 hours) according to sperm pathology and the number of blastomeres after subzonal insemination (SUZI) and in vitro fertilization with donor spermatozoa (D-IVF)

	Num. of 2-cell em-bryos (%)	Num. of 3-cell em-bryos (%)	Num. of 4-cell em-bryos (%)	Num. of 5-8 cell em-bryos (%)	Total num. of em-bryos	Develop-mental rates*
GroupA	24 (35.3)	15 (22.1)	19 (28.4)	9 (13.2)	67	3.2±1.1[a]
Group B	8 (21.6)	9 (24.3)	16 (43.2)	4 (10.8)	37	3.5±1.0
Group C	16 (45.7)	4 (11.4)	15 (42.9)	0	35	3.0±1.0[c]
Total SUZI	48 (34.3)[a]	28 (20.0)[b]	50 (35.7)[c]	13 (10.0)	139	3.2±1.0[c]
DIVF	50 (20.6)	27 (11.1)	144 (59.3)	22 (9.1)	243	3.6±0.9

Group A: patients with flagellar dyskinesia. Group B: patients with non-specific sperm defects. Group C: control group. *Mean number of blasomeres per embryo. Significantly different from D-IVF group: [a]P<0.01; [b]P< 0.025; [c]P<0.001.

The developmental rate of embryos for which uterine transfer resulted in a pregnancy after SUZI was higher than when there was no implantation (3.7±1.1 compared with 3.2±1.1, P<0.01). The same was true for embryos resulting from D-IVF (4.4±1.4 versus 3.8±1.3, P<0.02). No significant differences were found between SUZI and D-IVF for transferred embryos leading to pregnancy. The 8 embryos resulting from oocytes that were microinjected at the metaphase 1 stage had a mean number of 2.7±0.9 blastomeres (range 2 to 4). No significant differences were found with the developmental rate of oocytes that were microinjected at the metaphase 2.

Influence of the time of PN formation and disappearance on the developmental rate after SUZI

The 16 zygotes which first showed two pronuclei (between 4.5 and 10.5 hours post-SUZI had altogether 53 blastomeres on day 2 (3.3 blastomeres/ embryo, range 2-6). The next 14 which had two pronuclei had 46 blastomeres (3.3 blastomeres/embryo, range 2-8). The embryos resulting from the group of 10 zygotes still without pronuclei after 10.5 hours post-SUZI, had 32 blastomeres (mean 3.2 ± 1.0 blastomeres/embryo, range 2-5). There was no statistically significant difference in mean number of blastomeres per embryo between "early" and "late" embryos. Concerning the patients from the latter subgroup, four pregnancies resulted, one being obtained after the transfer of only one of these "late" embryos.

DISCUSSION

The present data suggest that 5 hours seems to be the minimum delay for sperm-oolemma binding, fusion, expulsion of the second polar body and decondensation of female and male chromatin. Pronuclei appear in >90% of zygotes between 5 and 10.5 hours post micro-injection, and <10% of zygotes show their pronuclei later, with the last one appearing 20 hours post-SUZI. Computer analysis of the rate of appearance of pronuclei provides two hypothesis. Either two different populations of zygotes are represented, some developing slower than others, or the latest oocytes to fertilize may correspond to the end of an exponential curve representing the rate of pronuclear appearance for all zygotes. However, neither their nuclear status at the moment of SUZI, nor their rate of cleavage seem to differentiate these "late" zygotes from the others.

The development of D-IVF zygotes seems to be more homogeneous. Indeed, in contrast to what happens after SUZI, all the observed zygotes were in the pronuclear stage 16 to 18 hours post-insemination indicating that pronucleus formation was simultaneously completed in all zygotes before this time. Their rate of pronuclear disappearance seems to be comparable to that observed for SUZI zygotes (Figures 1 A and B), but it began 3-4 hours later (around 22.5 hours post insemination). The delay between disappearance of pronuclei and cleavage was also 3 hours, with the period of cleavage beginning earlier after SUZI than after D-IVF (21 compared with 25 hours respectively). Indeed, as shown on figures 1 A and B, the percentage of

cleaved zygotes between 21 and 30 hours afterSUZI is much greater than after D-IVF (20.9 % vs. 6.8 % respectively). These data suggest that the artificial passage of spermatozoa through the egg investments shortens the delay for the appearance of first cleavage by 3-4 hours. However, on day 2, only 45% of the SUZI zygotes were at the 4-cell stage or beyond, while the corresponding figure for the D-IVF zygotes was 70%. Taken together, these data suggest that the second cell cycle is quicker for D-IVF zygotes.

Sequential nuclear changes in human oocytes at fertilization after intracytoplasmic sperm injection (ICSI) have been recently reported (Nagy et al, 1993). The first pronucleus appeared 6 hours after ICSI for 17% of the fertilized oocytes, 80% of the oocytes had two pronuclei at 8 hours post-ICSI, 99% at 16 hours, 82% at 18 hours and 68% at 20 hours. Apparently, the first pronucleus observed after ICSI did not appear sooner than after SUZI, suggesting that the passage through the oolemma by human spermatozoa can be very quick. The time-span during which pronuclei were present after ICSI is obviously shortened as compared to that after SUZI as they began to disappear 2 hours earlier. Since during ICSI the spermatozoon is delivered to the centre of the ooplasm, the spread in the delay of pronuclear appearance after SUZI seems to indicate that the duration of oolemma passage and of migration of the sperm head toward the centre of the oocyte and decondensation may vary.

In SUZI, the spermatozoa interact directly with the oolemma, as they do in zona-free systems. It has been reported that decondensation of sperm chromatin in such a system, can be observed between 1 and 8 hours post-insemination in aged oocytes (Tesarik et al, 1989a; Lassalle et al, 1991). The earliest fully developed pronuclei was observed 12 hours post-insemination (Tesarik et al 1989a) whereas in our study most pronuclei had already appeared at 11 hours post-SUZI (Figure 1A). The delay of pronuclear formation in zona free systems seems to be spread over a larger period of time when compared with SUZI zygotes, but these chronological studies on zona-free human eggs (Tesarik et al, 1989a; Lassalle and Testart, 1991) may not be fully comparable to our results since aged oocytes which failed to fertilize after IVF may already be of impaired quality.

Balakier et al (1993) inseminated zona-intact human eggs and studied their DNA synthesis. This starts ≈9 to 10 hours and is completed 13-14 hours after insemination. As DNA synthesis begins after pronucleus formation, this timing corresponds to 4 hours more than our findings (starting 5 hours and completed 10.5 hours post-SUZI). Similarly, the first chromosome condensation appeared 18-19 hours post insemination in the zona-intact system (Balakier et al, 1993) while we had observed the first cleavage already 19 hours post-SUZI. As

chromosomes appear before cleavage, this timing corresponds also to a difference of a few hours. Such sequential transformation of human sperm nuclei corresponds to that reported for D-IVF in our study. This confirms that the developmental rate of SUZI zygotes seems comparable to the developmental rate of intact zygotes but with a difference of 3-4 hours. This delay probably partly corresponds to the passage through the zona-pellucida and the oolemma and to the completion of the second meiotic division.

The spread in time of pronuclear formation can be explained by gamete quality. Sperm phenotype has an influence on its transformation into a male pronucleus. Figure 2 shows that in zygotes from group A (flagellar dyskinesia) the pronuclei disappear significantly earlier than the pronuclei in group B (non-specific sperm defects) and group C (controls). Indeed, the influence of sperm quality on pronuclear development has been reported by Boerjan et al. (1990) who showed that development after fertilization of mouse oocytes with homologous irradiated spermatozoa is significantly arrested at the pronuclear stage. It has been shown in mice that mutation can adversely affect early development (Green, 1981). Similarly, very early mammalian development is under genetic control (Magnuson and Epstein, 1981). It is therefore possible that this spread in pronuclear formation could be related to the quality of the sperm genome. Besides sperm phenotype, oocyte immaturity may influence the timing of pronuclear development. as suggested by Oehninger et al (1989) and Yanagimachi (1994). In group C (control group with unexplained IVF failure), none of the zygotes reached a stage beyond the 4-cell stage on day 2, whereas ≈10 % of all the other zygotes did (Table II). This result may reflect some undetectable defect of the oocyte and supports our previous hypothesis that in the SUZI group with normal sperm parameters a female factor could be responsible for the sterility and of the previous IVF failures (Wolf et al. 1992).

The developmental stage on day 2 does not appear to depend on the time of pronuclear appearance. Actually, the chronology of the first cell cycle for zygotes which were all at the 4-cell stage on day 2 after SUZI presented a large variation in pronuclear appearance. Pronuclei formation took place over a period of 12 hours and pronuclear disappearance over 10 hours, with no consequences for the total duration of the first and second cell cycles.

The SUZI procedure does not favour asynchronous pronuclear development in normozoospermic zygotes since asynchronous disappearance of pronuclei was as rarely observed after SUZI as after D-IVF. In monospermic mouse eggs, though the male pronucleus is in the process of nuclear envelope assembly while the female one is already formed (Adenot et al, 1991), the asynchrony is also minimal. However,

in polyspermic mouse oocytes either all or only some of the spermatozoa complete pronuclear development while the remainder stop at an early stage (Witkowska, 1981). Similar results were observed in the human (Tesarik and Kopecny, 1989b). Thus the discrepancy between pronuclear observation and cytogenetic analysis after SUZI reported by Selva et al (1993) is most likely limited to the polyspermic eggs. However, knowing the risk of polyspermy, this emphasizes the importance of careful and repeated examination of micro-injected eggs to prevent the transferring of zygotes with asynchronous development which are possibly polyspermic.

It can be concluded that the time of pronuclear development and of the first cleavage division after SUZI is generally 4 hours ahead, but remains comparable to that of IVF. The time-span between pronuclear appearance and cleavage seems constant after SUZI, with the observed spread depending mostly on the delay of pronuclear formation. Furthermore, this chronology varies according to sperm phenotype and to oocyte quality. Most zygotes after D-IVF have a higher developmental rate and a shorter second cell cycle than those obtained after SUZI. Interestingly, for both, transferred embryos which resulted in pregnancy had a higher developmental rate at day 2 than non-implanted embryos.

REFERENCES

Adenot PG, Szöllösi MS, Geze M, Renard JP and Debey P (1991) Dynamics of paternal chromatin changes in live one-cell mouse embryo after natural fertilization *Molecular Reproduction and Development* **28** 23-34

Balakier H, MacLusky NJ and Casper RF (1993) Characterization of the first cell cycle in human zygotes: implications for cryopreservation *Fertility and Sterility* **59** 359-365

Barros C and Yanagimachi R (1971) Induction of zona reaction in golden hamster eggs by cortical granule material *Nature* **233** 268-269

Boerjan ML, Saris LA and van der Hoeven FA (1990a) The integrity of chromatin in cleavage stage mouse embryos derived from unaged oocytes and oocytes aged post-ovulation in vivo *Zygote development after delayed fertilization* PhD pp 49-67 Ed. M. Boerjan, Ponsen and Looijen, Wageningen

Boerjan ML and De Boer P (1990b) First cell cycle of zygotes of the mouse derived from oocytes aged postovulation in vivo and fertilized in vivo *Molecular Reproduction and Development* **25** 155-163

Cohen J, Alikani M, Malter HE, Adler A, Talansky BE and Rosenwaks Z (1991) Partial zona dissection or subzonal sperm insertion: microsurgical fertilization alternatives based on evaluation of sperm and embryo morphology *Fertility and Sterility* **56** 696-706

Czyglik F (1991) L'insémination artificielle avec sperme de donneur *L'insémination artificielle* pp 62-70 Eds. Cecos, Masson, Paris

David G and Czyglik F (1977) Tolérance à la congélation du sperme humain en fonction de la qualité initiale du sperme. *J. Gynecol. Obstet. Biol. Reprod.* **6** 601-608

De Almeida M, Soumah A and Jouannet P (1986) Incidence of sperm associated immunoglobulins in fertile men suspected of antisperm autoimmunity *International Journal of Andrology* **9** 321-326

Diamond MP, Rogers BJ, Webster BW, Vaughn WK and Wentz AC (1985) Polyspermy: effect of varying stimulation protocols and inseminating sperm concentrations *Fertility and Sterility* **43** 777-780

Frydman R, Forman RG, Belaisch-Allart J, Hazout A, Rainhorn JD, Fries N and Testart J (1988) Improvements in ovarian stimulation for in vitro fertilization *Annals New York Academy of Sciences* **541** 30-36

Green MC. (1981) *Genetic variants and strains of the laboratory mouse.* Gustav Fisher, Stuttgart, pp. 8-278

Jouannet P, Escalier D, Serres C, David G (1983) Motility of human sperm without outer dynein arms. *J Submicrosc Cytol* 15 : 67-71

Lassalle B and Testart J (1991) Sequential transformations of human sperm nucleus in human egg *Journal of Reproduction and Fertility* **91** 393-402

Magnuson T and Epstein CJ. (1981) Genetic control of very early mammalian development. *Biol. Rev.* **56**, 369-408

Nagy ZP, Joris H, Liu J, Devroey P, Van Steirteghem AC ((1993) Sequential nuclear changes in human oocytes at fertilization after intracytoplasmic sperm injection. *J. Reprod. Fertil. Abstract Ser.* **12** December 1993, p.39

Ng SC, Bongso A and Ratnam SS (1991) Microinjection of human oocytes: a technique for severe oligoasthenoteratozoospermia *Fertility and Sterility* **56** 1117-1123

Oehninger S, Acosta AA, Veeck LL, Simonetti S and Muasher SJ (1989) Delayed fertilization during in vitro fertilization and embryo transfer: analysis of causes and impact on overall results *Fertility and Sterility* **52** 991-997

Plachot M, Veiga A, Montagut J, De Grouchy J and Calderon G (1988) Are clinical and biological parameters correlated with chromosomal disorders in early life: a multicentric study *Human Reproduction* **3** 627-635

Selva J, Wolf JPh, Rince P, Rodriques D, Frydman R and Jouannet P (1993) Cytogenetic analysis of human oocytes after subzonal insemination *Prenatal Diagnosis* 13 : 311-321

Serres C, Feneux D and Jouannet P (1986) Abnormal distribution of the periaxonemal structures in a human sperm flagellar dyskinesia *Cell motility and the Cytoskeleton* 6: 68-76

Tesarik J and Kopecny V (1989a) Development of human male pronucleus: ultrastructure and timing *Gamete Research* **24** 135-149

Tesarik J and Kopecny V (1989b) Developmental control of the human male pronucleus by ooplasmic factors *Human Reproduction* **4** 962-968

Tournaye H, Devroey P, Camus M, Staessen C, Bollen N, Smitz J and Van Steirteghem AC (1992) Comparison of in-vitro fertilization in male and tubal infertility: a 3 year survey *Human Reproduction* **7** 218-222

Veeck L (1986) Human oocytes at the time of follicular harvest. In Jones H.W. (ed), *Atlas of the human oocyte and early conceptus.* Williams and Wilkins, Baltimore, pp. 5

Wassarman PM. (1990) Regulation of mammalian fertilization by zona pellucida glycoproteins. *J. Reprod. Fertil.* **42**, 79-87

Witkowska A. (1981) Pronuclear development and the first cleavage division in polyspermic mouse eggs. *J. Reprod. Fertil.* **62**, 493-498

Wolf JPh, Ducot B, Kunstmann JM, Frydman R and Jouannet P (1992) Influence of sperm parameters on outcome of subzonal insemination in the case of previous IVF failure *Human Reproduction* **7** 1407-1413

Wolf JPh, Feneux D, Escalier D, Rodriques D, Frydman R and Jouannet P (1993) Pregnancy after subzonal insemination with spermatozoa lacking outer dynein arms *Journal of Reproduction and Fertility* **97** 487-492

World Health Organization (1987) *WHO Laboratory Manual for the Examination Of Human Semen and Semen-Cervical Mucuc Interaction*, 2nd edn. The Press Syndicate of the University of Cambridge, Cambridge

Yanagimachi R. (1994) Mammalian fertilization. In Knobil E and Neill JD (eds), Raven Press, New York, pp. 189-318.

CHAPTER VII

Multinucleation in embryos resulting after IVF

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ABSTRACT

Cleaving mammalian embryos normally possess one nucleus per blastomere. However, multiple nuclei within the same blastomere can be observed in human embryos after in vitro fertilization. Thirty-three IVF cycles (32 patients) were divided into groups based on: a) the IVF indication, b) mono- and/or poly-spermic fertilization within the same oocyte cohort, c) establishment of pregnancy. Sixty-seven embryos unsuitable for transfer, 11 embryos from polypronuclear zygotes, 9 monospermic and 9 polyspermic zygotes and 80 unfertilized oocytes were analysed by DNA fluorescence. Twenty-one out of 67 (31.3%) embryos had only single nucleated cells, 44 embryos (65.7%) had at least one multinuclear blastomere and in two embryos multinucleation could not be assessed. Twenty-seven out of 80 (33.8%) oocytes were penetrated by sperm but had not formed pronuclei. The developmental rates of polyploid embryos (4.2±2.0 blastomeres/embryo) were comparable with the developmental rates of transferred embryos (3.8±1.5) but higher (not significant) than for analysed, embryos from monospermic zygotes (3.3±1.4). The morphological aspects of multinuclear blastomeres were discussed. Fertilization anomalies such as polyspermic fertilization and sperm-oocyte penetration without pronuclear formation were found simultaneously with multinucleation in embryos from two-pronuclear zygotes in 30% of the IVF cycles. The incidence of embryos with multinuclear blastomeres tends to be higher in cycles with mono-and polyspermic fertilization with a higher number of oocytes retrieved per cycle, than in cycles with only monospermic fertilization. No significant differences were found between the IVF indication and pregnancy groups. Multinucleation in early human embryos may be correlated with the incidence of abnormal fertilization processes such as polyspermy and sperm penetration without pronucleus formation within the same oocyte cohort. Multinucleation may be caused by the fertilization of oocytes which are unable to support normal nuclear and cell division because of immaturity or impaired quality.

INTRODUCTION

Cleaving mammalian embryos normally possess one nucleus per blastomere. However, multiple nuclei can be observed in some blastomeres of rat embryos developing from aged metaphase II stage oocytes (Blandau, 1952) and of human pre-implantation embryos developing in vivo (Hertig et al, 1954) and in vitro (Sathananthan et al, 1982; Munné and Cohen, 1993). In cleaved embryos which stopped their development, multinucleate cells were found in 30.4% of the analysed embryos (Munné and Cohen, 1993). The question arises whether blastomere multinucleation can cause a developmental arrest.

Several suggestions have been made as to the origin of blastomere multinucleation. It has been proposed that multinuclear blastomeres (MNBs) may arise from partial fragmentation of nuclei or from mitotic replication of nuclei without cytoplasmic division (Lopata et al, 1983) or after a defective migration of chromosomes at mitotic anaphase (Tesarik et al, 1987). Extrinsic factors have been evoked such as culture media which might be insufficient to support embryo development (Winston et al, 1991), and adverse effects of cooling on the cytoskeleton during manipulations for IVF (Pickering et al, 1990).

Oocyte quality at fertilization may play an important role during early cleavage divisions. In support of this, human gene expression has been found to occur between the four- and eight-cell stage, indicating that development before the four cell stage is regulated by maternally inherited information (Braude et al, 1988). However, oocyte characteristics, determining its quality in terms of its fertilizability and its capacity to support early embryonic development, still need to be established.

We report the incidence of MNBs in oocyte cohorts in relation to IVF parameters such as the IVF indication and the establishment of pregnancy. We also investigated the incidence of MNBs in relation to the incidence of abnormal fertilization processes in cohort oocytes such as polyspermic fertilization and sperm-oocyte penetration without pronucleus formation as described previously (Van Wissen et al, 1991; Van Wissen et al, in press). For the present study, embryos which were considered unsuitable for transfer to the uterine cavity of the patient, have been analysed.

MATERIALS AND METHODS

Patients and protocols

During a 1.5 year period, 104 IVF cycles were studied when at least one unfertilized oocyte, one zygote or one embryo could be obtained for DNA fluorescence, the oocytes analysed were described elsewhere (Van Wissen et al, In Press; Submitted). For this work, 33 out of 104 IVF cycles (32 patients) were included when at least one embryo was not transferred to the uterine cavity of the patient or was not frozen, and could be obtained for DNA fluorescence. The mean age of female partners was 33 years (range 24-40 years). The indications for IVF were tubal obstruction (n=22 cycles), endometriosis (n=2), combined tubal obstruction and endometriosis (n=2), male infertility (n=1) and idiopathic infertility (n=6). Sperm parameters were evaluated according to WHO guidelines (Belsey et al, 1980). After pituitary desensitation with GnRHa (Decapeptyl 3.75mg; Ipsen/Biotech, Paris, France), patients were stimulated with human menopausal gonadotrophin (HMG, Humegon; Organon, Saint Denis, France). Human chorionic gonadotrophin (HCG, ENDO, 10000IU; Organon) was used to initiate oocyte maturation. Oocytes were collected 36 hours after HCG administration and inseminated 3-5 hours later with approximately 4000 spermatozoa. Twenty-four hours after insemination, oocytes were checked for fertilization evidenced by the presence of two or more pronuclei and two polar bodies. Forty-eight hours after insemination between 1-4 cleaved embryos were transferred to the uterine cavity of the patient and supernumerary embryos were frozen on day 2 or cultured until the morula or blastocyst stage and cryopreserved for later transfer as described elsewhere (Olivennes et al, 1994).

Patients groups

For this study IVF cycles were divided into groups based on:
1) The IVF indication: female (n=26), male (n=1) and idiopathic (n=6)
2) The incidence of only monospermic fertilization (n=16), mono and polyspermic fertilization (n=15), only polyspermic fertilization (n=1) and no or one pronucleus and two polar bodies (n=1).
3) The occurrence of pregnancy (n=6) or not after transfer of at least one embryo (n=24) and no transfer (n=3)

Fluorescence Microscopy

Eighty unfertilized oocytes, 18 zygotes, 67 embryos originating from zygotes with two pronuclei on day 1 and 11 embryos from multipronuclear zygotes were analysed by DNA fluorescence 48 hours after insemination. Oocytes and embryos were incubated with the DNA fluorescent dye Hoechst 33342 (100 ng/ml; Coger Pharmaceuticals, France) under culture conditions and observed as we described previously (Van Wissen et al, in press). Fluorescence analysis was performed 48-52 hours after insemination and included:

1) The assessment of sperm penetration in unfertilized oocytes as we described previously (Van Wissen et al, 1991; Van Wissen et al, in press). Briefly, sperm which penetrated the oocyte was found inside the ooplasm as undecondensed spermheads, partially decondensed spermheads or premature condensed chromosomes (PCC).

2) Assessment of mono-or polyspermic penetration in zygotes. When two pronuclei and two polar bodies were present zygotes were considered mono-spermic and when more than two pronuclei and two polar bodies were present zygotes were considered polyspermic.

3) Embryo grading according to blastomere number, size, and fragmentation degree. Four grades were distinguished, grade 1: equal sized blastomeres without fragments, grade 2: equal or slightly unequal sized blastomeres and few or no fragments, grade 3: equal or unequal sized blastomeres and fragmentation up to 20-25%, grade 4: unequal sized blastomeres or blastomeres difficult to distinghuish and heavy fragmentation (>25%). Note that for grade 3 and grade 4 embryos in this study the number of blastomeres was determined by counting the non-fragmented cells. Completely fragmented embryos (<1 intact blastomere) were not included in our study.

4) Determination of number of nuclei per blastomere in each embryo, this parameter was independent from grading.

Statistics

Student's t-tests were used to compare means. Significance was defined as $p<0.05$.

RESULTS

IVF results

In tables I, II and III, IVF results for the patient groups are shown. In total 422 oocytes were retrieved for IVF with a mean per cycle of 12.8 oocytes (range 4-26). Twenty-four hours after insemination, 221 (52.4%) zygotes had two pronuclei and 27 (6.4%) zygotes had more than two pronuclei and were considered polyspermic. In 17 oocytes one pronucleus and two polar bodies were observed. In one oocyte no pronuclei were observed but two polar bodies were present. Forty-eight hours after insemination, 239 embryos resulted. Eighty-six embryos were transferred in 30 IVF cycles. Six IVF cycles resulted in pregnancy. No age differences of the female patients were found between the defined groups. The mean number of oocytes retrieved was significantly higher in cycles with mono- and polyspermic fertilization compared with monospermic fertilization cycles (Table II). The same was true for the incidence of monospermic fertilization and the number of resulting embryos per cycle (Table II). The developmental rates (mean number of blastomeres per embryo) did not differ between transferred embryos and embryos which transfer resulted in pregnancy (3.8±1.5 versus 3.8±0.7; the transferred blastocyst was excluded from this calculation). The number of retrieved oocytes, monospermic zygotes and embryos did not differ between the IVF indication and the pregnancy groups.

Table I: IVF indications and results

IVF indication groups (n=num.IVF cycles)	Num. of oocytes retrieved (mean±SD per cycle)	Num. of oocytes fertilized (mean±SD per cycle)		Num. of embryos[a] on day 2 (mean±SD per cycle)	Num. of embryos transferred (mean±SD per cycle)	Num. pregnancies
		2PN	>2PN			
Female n=26	325 (12.5±5.1)	181 (7.0±3.7)	15	196 (7.5±3.9)	72 (2.8±0.9)	5
Male n=1	21	11	1	10	3	0
Idiopathic n=6	76 (12.7±3.2)	29 (4.8±4.8)	11	33 (5.5±4.1)	11 (1.8±1.5)	1
Total n=33	422	221	27	239	86	6

[a]Embryos resulting from 2PN zygotes

DNA fluorescence observations 48 hours post insemination

Nine polypronuclear zygotes were observed of them 5 had three pronuclei and 4 zygotes had >3 pronuclei. Eleven embryos originated from polypronuclear zygotes and ten of them had at least one MNB (Figures 1a and 1b), one embryo had only single nucleated cells. Six embryos came from zygotes with three pronuclei, one from a zygote with four pronuclei and four from zygotes with more than four pronuclei. The mean cell number (4.2±2.0) seemed higher (not significant) than for embryos originating from zygotes with two pronuclei on day 1 but was comparable with the mean cell number of transferred embryos. The analysed embryos were graded 1, 2 or 3. Four embryos, from a single patient, were heavily fragmented (grade 4). This patient had only polyspermic fertilization and the resulting embryos all had MNBs. The zona pellucida of oocytes and embryos from this patient all showed structural anomalies. Instead of a regular and smooth zona pellucida, it was irregular and ruffled.

Table II: Mono-and polyspermic fertilization groups and IVF results

Fertilization groups (n=num. of IVF cycles)	Num. of oocytes retrieved (mean±SD per cycle)	Num. of oocytes fertilized (mean±SD per cycle)		Num. of embryos[a] on day 2 (mean±SD per cycle)	Num. of embryos transferred (mean±SD per cycle)	Num. of pregnancies
		2PN	>2PN			
Only monospermic fertilization n=16	166 (10.4±4.1)*	89 (5.5±3.4)§	0	97 (6.1±3.3)¥	39 (2.4±1.1)	3
Mono-and polyspermic fertilization n=15	229 (15.3±4.9)*	132 (8.8±3.2)§	18	139 (9.3±3.5)¥	45 (3.0±0.7)	3
Only polyspermic fertilization n=1	16	0	9	0	0	0
Other n=1[b]	11	0	0	3	2	0
total n=33	422	221	27	239	86	6

[a]Embryos resulting from 2PN zygotes, [b]On day 1, one oocyte with two polar bodies but no pronuclei and two oocytes with two polar bodies and one pronucleus were observed for this patient. Same symbols for two items indicate significant difference between the two items: *$p<0.01$, §$p<0.02$, ¥$p<0.05$

Table III: Pregnancy outcome and IVF results

Pregnancy groups (n=num. of IVF cycles)	Num. of oocytes retrieved (mean±SD)	Num. of oocytes fertilized (mean±SD)		Num. of embryos[a] on day 2 (mean±SD)	Num. of embryos transferred (mean±SD)
		2PN	>2PN		
pregnancy n=6	68 (11.3±3.3)	46 (7.2±3.6)	3	52 (8.7±2.3)	16 (2.7±0.8)
no pregnancy n=24	317 (13.2±5.4)	172 (7.7±3.9)	15	182 (7.9±3.7)	70 (3.0±0.5)
no transfer[b] n=3	37	3	9	5	0
total	422	221	27	239	86

[a]Embryos resulting from 2PN zygotes, [b]In these cycles no embryos were transferred because: only polyspermic fertilization (n=1), morphologically poor embryos, polynucleated (n=1), only one normal embryo resulted resulted which arrested further development in culture (n=1).

After IVF procedures, we obtained 9 zygotes with two pronuclei and 67 embryos originating from zygotes with two pronuclei on day 1. Figures 2-10 show embryos originating from two-pronuclear zygotes. Twenty-one embryos (31.3%) had only single nucleated cells (Figures 5 and 9), 44 (65.7%) had at least one multinuclear blastomere (Figures 2, 3, 4, 6a, 6b, 7, 8 and 10). In two embryos the number of nuclei could not be determined, one because of heavy fragmentation and one because of the incomplete (first) cellular division. In this last embryo, the chromosomes had just separated and were still condensed. In one 5-cell embryo (Figure 5), one large blastomere had condensed chromosomes which was considered as one nucleus since no division had taken place yet. Different types of MNBs were distinghuished based on the size and number of nuclei. 1) One "main" nucleus with one or several micronuclei (Figure 2). 2) Only micronuclei, all smaller in size than nuclei in single nucleated cells within the same embryo (Figure 4 and 7). 3) Two or more equally sized nuclei each with the same size as nuclei in single nucleated cells within the same embryo (Figure 6a). In table IV, characteristics of observed embryos are shown. From all observed embryos, 128 out of 217 (59%) blastomeres had one nucleus. Only one embryo was graded 1 while the majority of analysed embryos were graded 2(40%) or 3 (41.(%). Eleven embryos were graded 4 (16.9%). The mean number of blastomeres was 3.3±1.4 which is significantly ($p<0.05$) lower than for transferred embryos. For all IVF indications, embryos with MNBs were found varying from 50% to 71.4% of the analysed embryos per group. MNBs were found in cycles with only monospermic fertilization (12 out of 16 cycles) as well as in cycles with

both mono and polyspermic fertilization (12 out of 15 cycles). Embryos with MNBs were observed in all 6 pregnancy cycles. However, the mean number of embryos with MNBs per cycle tends to be higher in cycles with mono and polyspermic fertilization than in cycles with only monospermic fertilization (1.8±1.3 vs 1.1±0.8; no significant difference). Eighty unfertilized oocytes were analysed from 24 out of 33 IVF cycles. Twenty-seven oocytes (33.8%) showed abnormal fertilization processes since they were penetrated by sperm but had not formed pronuclei. Six of the analysed oocytes were atretic or fragmented and sperm penetration could not successfully be assessed.

Table IV: Analysis of embryos by DNA fluorescence

Cell number	Num. of embryos observed	Num. of cells with one nucleus	Embryo morphology[a]			
			grade1	grade2	grade3	grade4
2	23	15	0	5	14	4
3	15	35	0	5	8	2
4	17	42	0	10	4	3
5	4	14	0	3	1	0
6	4	22	1	3	0	0
7	2	0	0	0	0	2
total	65[b]	128	1	26	27	11

[a]For embryo grading definitions see M&M section. [b]In two embryos the number of nuclei could not be determined.

Abnormal fertilization and incidence of embryos with MNBs in patient groups

In table V, sperm penetration in unfertilized oocytes and multinucleation in embryos analysed on day 2 by DNA fluorescence, is shown for the different groups defined. Sperm penetration without fertilization was found in oocytes from all groups, except in one cycle with only monospermic fertilization and in one cycle without zygotes with pronuclei, and varied from 7.7% to 60% of the analysed oocytes, . The mean number of penetrated oocytes per cycle tends to be higher (not significant) in cycles with mono and polyspermic fertilization than in cycles with only monospermic fertilization (1.7±1.7 vs 0.9±0.7). For the other patient groups, the number of oocytes observed was too small for comparison. In table VI, the number of IVF cycles with a simultaneous incidence of fertilization anomalies and embryos with MNBs within the same oocyte cohort is shown. Abnormal fertilization of oocytes and embryos with MNBs on day 2, were found within the same cohort in approximately 30% of the IVF cycles. In 73.3 % of the mono- and polyspermic fertilization cycles MNB's were observed in the resulting embryos.

Table V: IVF parameter groups and fluorescence observations on day 2

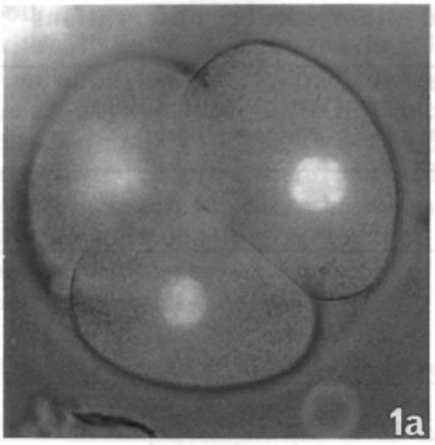
IVF parameter groups (n=num. of IVF cycles)	Num. of unfertilized oocytes penetrated by sperm/total number of oocytes observed	Num. of embryos with MNBs /total number of embryos observed
female (n=26)	22 /59	38 /54
male (n=1)	3 /5	1 /2
idiopathic (n=6)	2 /16	5 /7
only monospermic fertilization (n=16)	10 /31	17 /23
mono and polyspermic fertilization (n=15)	17 /39	23 /39
only polyspermic fertilization n=1	0 /6	--
other (n=1)[a]	0 /4	0 /1
pregnancy (n=6)	3 /6	10 /14
no pregnancy (n=24)	23 /61	30 /49
no transfer (n=3)	1 /13	4 /4

[a]On day 1, one oocyte with two polar bodies but no pronuclei and two oocytes with two polar bodies and one pronucleus were observed for this patient.

Table VI: IVF parameter groups and associated oocyte and embryo characteristics

IVF parameter groups (n=num. of IVF cycles)	Num. of cycles with unfertilized oocytes penetrated by sperm and embryos with MNBs on day 2(%)	Num. of cycles with polyspermic fertilization on day 1 and embryos with MNBs on day 2(%)
female (n=26)	8 (30.8)	8 (30.8)
male (n=1)	1	1
idiopathic (n=6)	1	2
only monospermic fertilization (n=16)	5 (31.3)	--
mono and polyspermic fertilization (n=15)	5 (33.3)	11 (73.3)
only polyspermic fertilization n=1	0	--
other (n=1)[a]	0	--
pregnancy (n=6)	3	3
no pregnancy (n=24)	6 (25.0)	8 (33.3)
no transfer (n=3)	1	0

Note that from 9 out of 33 IVF cycles no oocytes were observed by DNA fluorescence so that no sperm penetration could be assessed. [a]On day 1, one oocyte with two polar bodies but no pronuclei and one oocyte with two polar bodies and one pronucleus were observed for this patient.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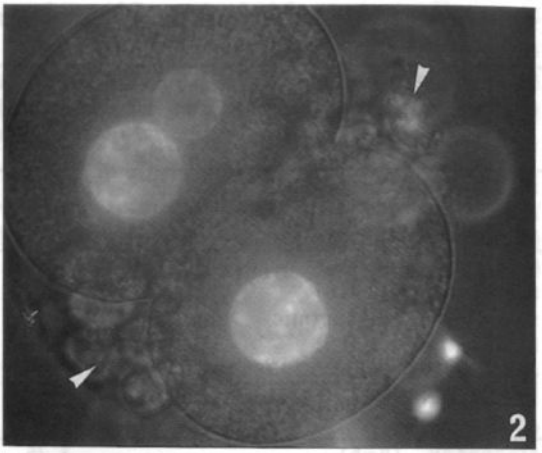
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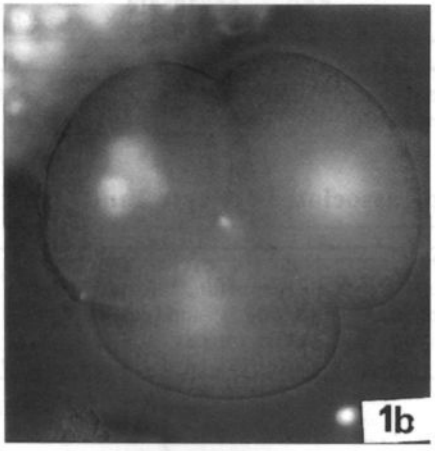
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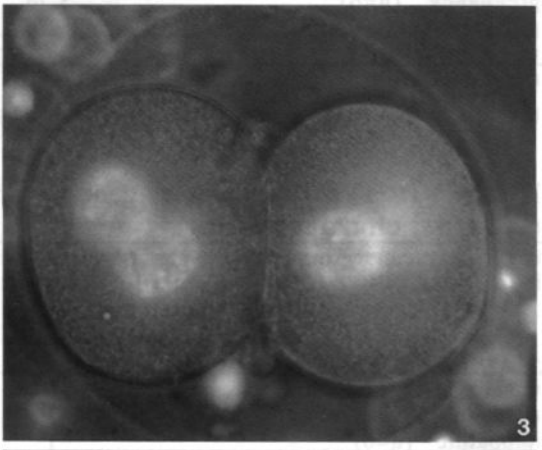
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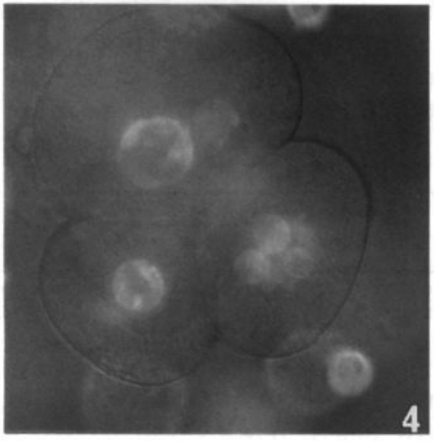
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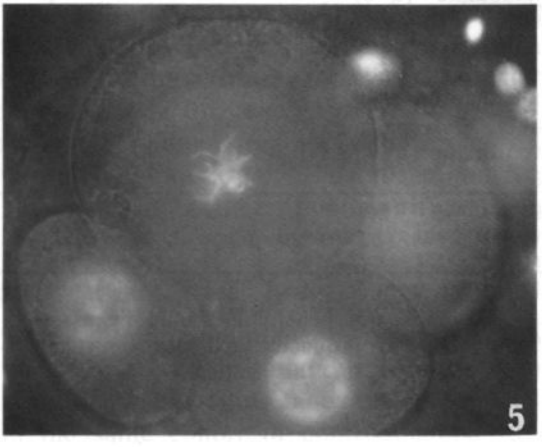
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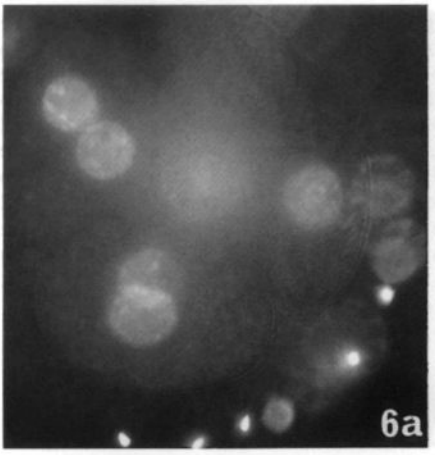
Figure 1: Three cell embryo (grade 1) originating from a zygote with three pronuclei on day 1, observed on day 2 by DNA fluorescence. Figure 1a shows two blastomeres each with one nucleus. In figure 1b (same embryo) the focus is on the third blastomere which has three small nuclei. 1cm=10μ. **Figure 2:** Two cell embryo (grade 2) originating from a zygote with two pronuclei on day 1, observed on day 2 by DNA fluorescence. One blastomere has two nuclei and one blastomere has one nucleus. The arrowheads points at fragments. 1cm=10μ. **Figure 3:** Two cell embryo (grade 1) originating from a zygote with two pronuclei on day 1, observed on day 2 by DNA fluorescence. Both blastomeres have two nuclei (one nucleus in one blastomere is out of focus). 1cm=10μ. **Figure 4:** Four cell embryo (grade 2) originating from a zygote with two pronuclei on day 1, observed on day 2 by DNA fluorescence. Tree blastomeres have MNBs and one blastomere has one nucleus (out of focus). 1cm=10μ.

DISCUSSION

The results suggest that multinucleation is frequently found in embryos resulting after IVF. However, only embryos unsuitable for transfer to the uterine cavity of the patient were analysed so that results may reflect the abnormality of these embryos. In total 67 out of 239 embryos (28%) which resulted after IVF were analysed by DNA fluorescence. Our data show the low developmental rate (number of blastomeres per embryos) of analysed embryos (23 out of 65 embryos had only two blastomeres) compared with transferred embryos. Besides selection of the best embryos for transfer, in general several causes can be evoked for a difference in developmental rate: delayed fertilization, arrested development during early cleavage divisions or a low developmental rate in analysed embryos. Delayed fertilization can be due to oocyte immaturity (Trounson et al, 1982) or to impaired sperm quality (Oehninger et al, 1989). However, it is unlikely that impaired sperm quality plays an important role in our study since the overall fertilization rate was >50%. In multinuclear blastomeres (MNBs), transcriptional activity of the embryonic genome was only exceptionally found (Tesarik et al, 1987). Early embryonic development, until the four cell stage depends on the maternal genome (Braude et al, 1988). This suggests that embryos with multiple MNBs in which the transition between maternal and embryonic gene activity fails, are likely to stop their development between the four- and eight-cell stage but not as early as the two-cell stage which was the stage of almost half of the observed embryos. A low developmental rate can be due to impaired oocyte quality at fertilization as we showed previously (Van Wissen et al, 1995).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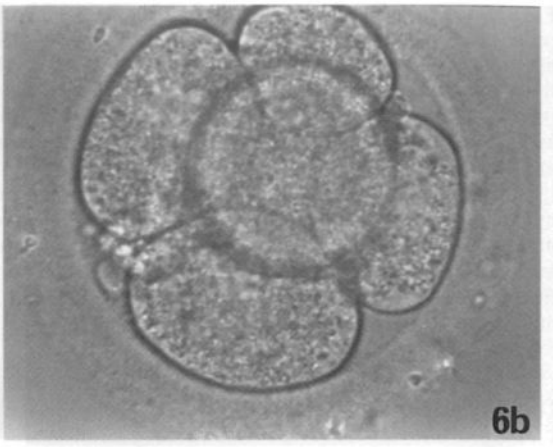
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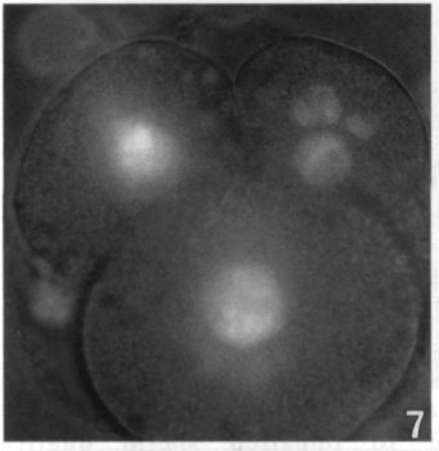
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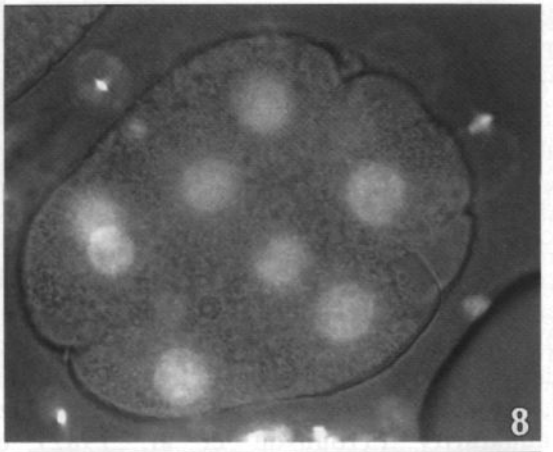
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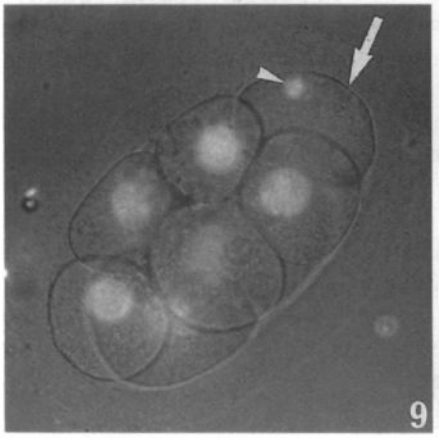
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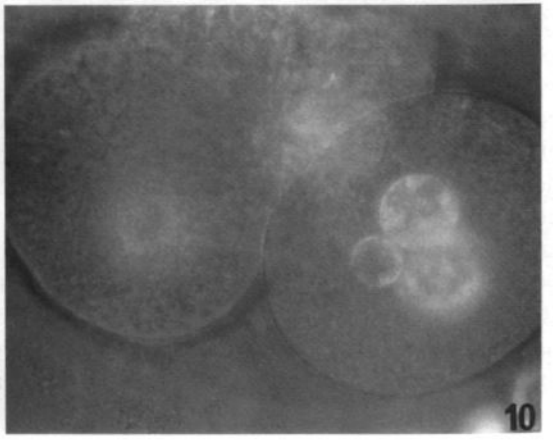
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Figure 5: Five cell embryo (grade 2) originating from a zygote with two pronuclei on day 1, observed on day 2 by DNA fluorescence. Two blastomeres each have one nucleus, two blastomeres each with one nucleus are out of focus and in one large blastomere a metaphase was present. 1cm=10µ. **Figure 6:** Five cell embryo (grade 2) originating from a zygote with two pronuclei on day 1, observed on day 2 by DNA fluorescence (6a). Two blastomeres both had two nuclei, three blastomeres each with one nucleus, are out of focus). Figure 6b shows the same embryo but observed by light microscopy. 1cm=10µ. **Figure 7:** Four cell embryo (grade 3) originating from a zygote with two pronuclei on day 1, observed on day 2 by DNA fluorescence. One blastomere has two nuclei (out of focus), one blastomere has one nucleus and two small nuclei (lower blastomere), one blastomere has one fragmented nucleus (upper left) and one blastomere has three small nuclei (upper right). 1cm=10µ. **Figure 8:** Seven cell embryo (grade 4), originating from a zygote with two pronuclei on day 1, observed on day 2 by DNA fluorescence. Individual blastomeres are difficult to distinghuish. Two nuclei were found in each others proximity (upper left). 1cm=10µ. **Figure 9:** Six cell embryo (grade 2) originating from a zygote with two pronuclei on day 1, observed on day 2 by DNA fluorescence. Arrow points at a large fragment or a "blastomere" without nucleus. The fluorescent spot (arrowhead) is the DNA of a polar body. 1cm=10µ. **Figure 10:** Two cell embryo (grade 4) originating from a zygote with two pronuclei on day 1, observed on day 2 by DNA fluorescence. One blastomere has one nucleus (nucleus is out of focus) and one blastomere has three nuclei. A mass of fragments is present (upper middle). 1cm=7.8µ

In support of this, the developmental rate of embryos of polypronuclear zygotes seemed higher than the developmental rate of the analysed embryos from two-pronuclear zygotes but comparable with the developmental rate of transferred embryos. Since polyploidy in human embryos can slow down early cleavage divisions (Balakier et al, 1993), a lower developmental rate could be expected. Hence, for the observed embryos from monospermic zygotes, other factors than polyploidy may be able to slow down early cleavage divisions.

The results suggest a positive correlation between the incidence of embryos from monospermic zygotes with MNBs and polyspermic fertilization of cohort oocytes. Polyspermy and multinucleation in embryos from both polypronuclear and two-pronuclear zygotes may have a common cause like the retrieval and fertilization of a high number of oocytes obtained after ovarian stimulation by exogenous hormones. We found the incidence of polyspermy correlated with higher numbers of oocytes retrieved per cycle and with higher monospermic fertilization rates. Accordingly, high rates of polyspermy have been found after ovarian stimulation with clomiphene citrate and the retrieval of a high number of oocytes (Colston Wentz et al, 1983). Polyspermy possibly resulted after sperm penetration in immature, postmature or atretic oocytes which were retrieved together with mature oocytes. Other authors reported polyspermic fertilization mostly in mature oocytes and found no correlation with the stimulation protocol (Diamond et al, 1985; Golan et al, 1992). Golan et al (1992) reported higher monospermic fertilization and

pregnancy rates in cycles with polyspermic fertilization and suggested an increased penetrability of these oocytes. Increased sperm penetration capacity was also associated with polyspermic cycles (Diamond et al, 1985; Dandekar et al, 1992). The incidence of polyspermy reveals aspects of individual oocyte quality concerning its capacity to prevent polyspermic penetration. A defective expulsion of the cortical granules (Sathananthan et al, 1985), inherent defects or fractures on the zona pellucida or mechanical damage caused during oocyte collection may cause failure to prevent polyspermic fertilization. The oocytes and embryos of one patient all showed structural zona-pellucida anomalies which may have caused polyspermic fertilization.

Multinucleation in early pre-implantation embryos was found both in embryos originating from polyspermic zygotes and in embryos from monospermic zygotes. For both types of embryos multinucleation did not occur systematically. The relative insufficiency of the spindle apparatus caused by the high number of chromosomes at syngamy may explain the high incidence of MNBs in human embryos developing from polypronuclear eggs after IVF (Van Blerkom et al, 1991; Tesarik et al, 1987). However, this does not explain the incidence of MNBs in embryos developing from diploid zygotes. In analogy, fertilization of oocytes with a degenerating cytoskeleton may cause multinucleation. The degeneration of the cytoskeleton may be a result of ageing, degeneration or cooling during manipulation (Pickering et al, 1988; Eichenlaub-Ritter et al, 1986).

Different types of MNBs could be morphologically distinghuised based on the number and the size of the nuclei. Accordingly we can speculate about their origin. The presence of one "main", normal sized nucleus and several micronuclei may result from separate chromosomes located outside the metaphase spindle during division. Nuclear fragmentation can be suggested when only micronuclei were found. The presence of two or more large equally sized nuclei suggests the possibility of polyploidy which may have resulted when no cell division followed after nuclear division.

The question arises wether MNBs are genetically diploid especially after one round of DNA-synthesis and nuclear and cellular division. It has been shown that the number of sex chromosomes and their distribution in each nucleus varies greatly (Munné et al, 1993). This makes embryos with MNBs unsuitable for preimplantation diagnosis. Besides, embryos of poor quality have a high incidence (up to 90%) of chromosomal anomalies (Pellestor et al, 1994). Approximately 30% of human morulae and blastocysts obtained after IVF and culture were found mixoploid (Benkhalifa et al, 1993). Polyploid cells found in the trophophoblast of preimplantation sheep blastocysts, appear to be part of normal developmental processes (Murray et al, 1986). However, in human embryos the normality of the presence of polyploid cells still needs to be

elucidated since embryos which are available for research are mostly of poor quality.

Sperm-penetration without pronucleus formation seemed associated both with polyspermic fertilization in cohort oocytes and with the incidence of embryos with MNBs. Previously we showed that this type of fertilization anomaly caused by the incapacity of the oocyte to support pronucleus formation can only be detected if penetrating sperm is present (Van Wissen et al, submitted). Both oocyte penetrability and sperm penetration capacity can explain the higher incidence of penetrated but unfertilized oocytes in cycles with mono- and poly-spermic fertilization than in cycles with only monospermic fertilization. Under these conditions not only mature oocytes are fertilized but also immature oocytes and oocytes of impaired quality which were simultaneously retrieved and inseminated with mature fertilizable oocytes.

When studying embryos with multinuclear blastomeres, it is important to consider IVF parameters such as the number of oocytes retrieved, mono-and poly-spermic fertilization rates but also the destiny of cohort oocytes. For ethical reasons, the nature of the observed material makes it difficult to obtain sufficient numbers of embryos. Hence, the absence of sufficient statistical power does not allow to draw definite conclusions but multinucleation seems a result of fertilization of oocytes unable to support normal nuclear and cell division because of immaturity or impaired oocyte quality, especially frequent when high numbers of oocytes are retrieved. The incidence of multinucleated blastomeres in early human embryos may be correlated with the incidence of abnormal fertilization processes such as polyspermy and sperm penetration without pronucleus formation. Their transfer should be avoided when morphologically normal embryos are available.

REFERENCES

BalakierH. (1993) Tripronuclear human zygotes: the first cell cycle and subsequent development. *Hum. Reprod.* 8, 1892-1897

Belsey MA, Eliasson R, Gallegos AJ, Moghissi KS, Paulson CA, eds. (1980) World Health Organisation: Laboratory manual for the examination of human semen and semen-cervical mucus interaction. MNR Prasad. Singapore: Press Concern.

Benkhalifa M, Janny L, Vye P, Malet P, Boucher D, Menezo Y. (1993) Assessment of polyploidy in human morulae and blastocysts using co-culture and fluorescent in-situ hybridisation. *Hum. Reprod.* 8, 895-902.

Blandau RJ. (1952) The female factor in fertility and infertility. I. Effects of delayed fertilization on the development of the pronuclei in rat ova. *Fertil. Steril.* 3, 349-365

Braude P, Bolton V, Moore S. (1988) Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* 332, 459-461.

Colston Wentz A, Repp JE, Maxson WS, Pittaway DE, Torbit CA. (1983) The problem of polyspermy in in vitro fertilization. *Fertil. Steril.* 40, 748-754.

Dandekar PV, Martin MC, Glass RH. (1990) Polypronuclear embryos after in vitro fertilization. *Fertil. Steril.* 53, 510-514.

Diamond MP, Rogers BJ, Webster BW, Vaughn WK, Colston Wentz A. (1985) Polyspermy: effect of varying stimulation protocols and inseminating sperm concentrations. *Fertil. Steril.* 43, 777-780.

Eichenlaub-Ritter U, Chandley AC, Gosden RG. (1986) Alterations to the microtubular cytoskeleton and increased disorder of chromosome alignment in spontaneously ovulated mouse oocytes aged in vivo: an immunofluorescent study. *Chromosoma* 94, 337-345

Golan A, Nachum H, Herman A, Ron-El R, Soffer Y, Caspi E. (1992) Increased fertilization and pregnancy rate in polypronuclear fertilization cycles in in vitro fertilization-embryo transfer. *Fertil. Steril.* 57, 139-141.

Hertig AT, Rock J, Adams EC, Mulligan WJ. (1954) On the preimplantation stages of the human ovum: A description of four normal and four abnormal specimen ranging from the second to the fifth day of development. *Contrib. Embryo.* 35, 201-220

Lopata A, Kohlman D, Johnston I. (1983) The fine structure of normal and abnormal human embryos developed in culture. In: Fertilization of the human egg in vitro, Beier HM, Lindner HR (eds). Springer Verlag, Heidelberg, 189-210.

Munné S, Cohen J. (1993) Unsuitability of multinucleated human blastomeres for preimplantation genetic diagnosis. *Hum. Reprod.* 8, 1120-1125.

Murray JD, Moran C, Boland MP, Nancarrow CD, Sutton R, Hoskinson RM, Scaramuzzi RJ. (1986) Polyploid cells in blastocysts and early fetuses from Australian Merino sheep. *J. Reprod. Fert.* 78, 439-446.

Oehninger S, Acosta AA, Veeck LL, Simonetti S, Muasher SJ. (1989) Delayed fertilization during in vitro fertilization and embryo transfer cycles: analysis of causes and impact on overall results. *Fertil. Steril.* 52: 991-997.

Olivennes F, Hazout A, Lelaidier C, Freitas S, Fanchin R, de Ziegler D, Frydman R. (1994) Four indications for embryo transfer at the blastocyst stage. *Hum. Reprod.* 9, 2367-2373.

Pellestor F, Dufour MC, Arnal F, Humeau C. (1994) Direct assessment of the rate of chromosomal abnormalities in grade IV human embryos produced by in-vitro fertilization procedure. *Hum. Reprod.* 9, 293-302

Pickering SJ, Johnson MH, Braude PR, Houliston E. (1988) Cytoskeletal organization in fresh, aged and spontaneously activated human oocytes. *Hum. Reprod.* 3, 978-989.

Sathananthan AH, Wood C, Leeton JF. (1982) Ultrastructural evaluation of 8-16 cell human embryos cultured in vitro. Micron 13, 193-203.

Sathananthan AH, NG SC, Chia CM, Lay HY, Edirisinghe WR, Ratnam SS. (1984) The origin and distribution of cortical granules in human oocytes with reference to Golgi, nucleolar, and microfilament activity. *Ann. NY Acad. Sci.* 442, 251-264

Tesarik J, Kopecny V, Plachot M, Mandelbaum J. (1987) Ultrastructural and audiographic observations on multinucleated blastomeres of human cleaving embryos obtained by in-vitro fertilization. *Hum. Reprod.* 2, 127-136.

Trounson AO, Mohr LR, Wood C, Leeton JF. (1982) Effect of delayed insemination on in vitro fertilization, culture and transfer of human embryos. *J. Reprod. Fert.* 64: 285-294.

Van Blerkom J, Henry G, Porreco R. (1984) Preimplantation development from polypronuclear eggs after in vitro fertilization. *Fertil. Steril.* 41, 686-696

Van Wissen B, Bomsel-Helmreich O, Debey P, Eisenberg C, Vautier D, Pennehouat G. (1991) Fertilization and ageing processes in non-divided human oocytes after GnRHa treatment: An analysis of individual oocytes. *Hum. Reprod.* 6, 879-884

Van Wissen B, Wolf JP, Bomsel-Helmreich O, Frydman R, Jouannet P. (1995) Timing of pronuclear development and first cleavages in human embryos after subzonal insemination: influence of sperm phenotype. *Hum. Reprod.* 10, 642-648

Van Wissen B, Bomsel-Helmreich O, Frydman R. (in press) Sperm-zona binding and sperm-oocyte penetration analysed by DNA fluorescence: a test for gamete quality after IVF. *Human Reproduction.*

Van Wissen B, Bomsel-Helmreich O, Frydman R. (submitted) The use of morphological and functional oocyte parameters to determine causes of IVF failure.

Winston NJ, Braude PR, Pickering SJ, George MA, Cant A, Currie J, Johnson MH. (1991) The incidence of abnormal morphology and nucleocytoplasmic ratios in 2-, 3- and 5-day human pre-embryos. *Hum. Reprod.* 6, 17-24.

Winston NJ, Johnson MH. (1992) Can the mouse embryo provide a good model for the study of abnormal cellular development seen in human embryos? *Hum. Reprod.* 7, 1291-1296

CHAPTER VIII

General Discussion

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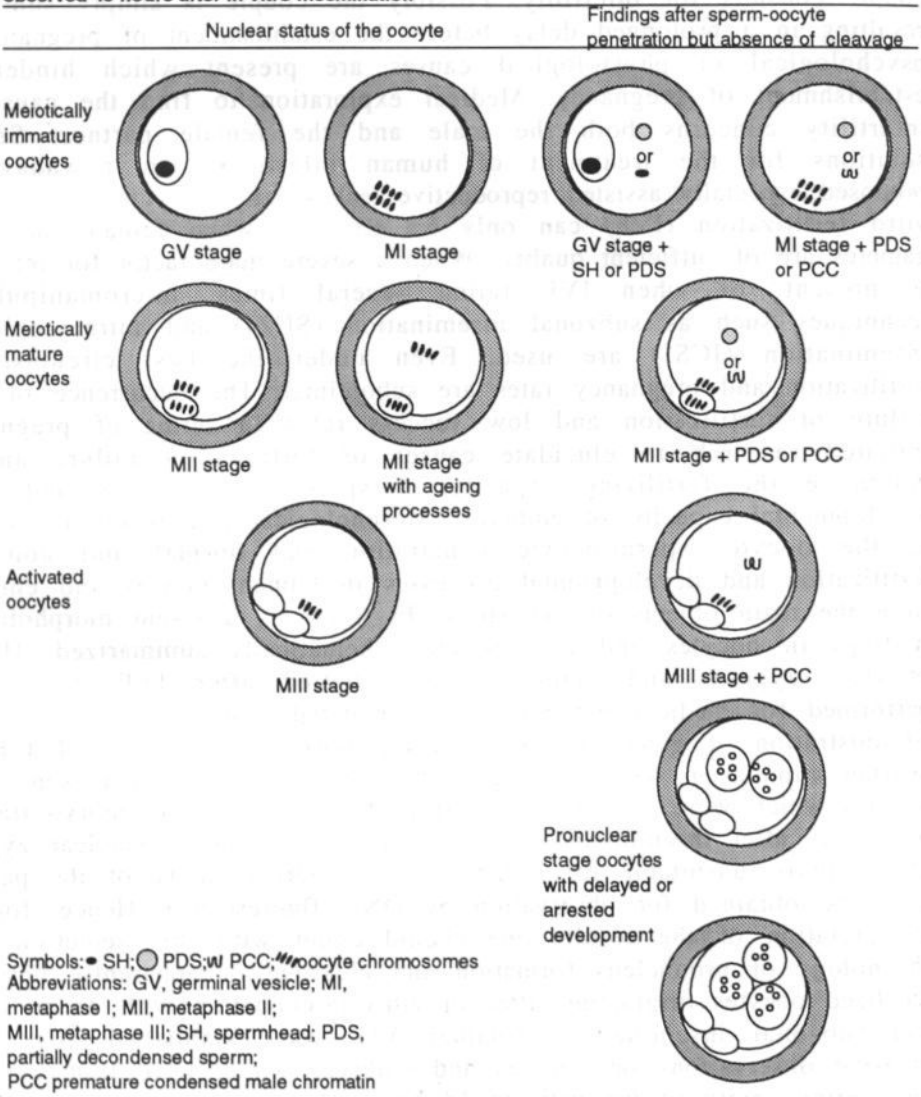
General Discussion

INTRODUCTION

The wish to control human fertility has mainly socio-demographic reasons. On one hand, contraceptive methods are used in order to plan the moment of pregnancy. If a pregnancy does not occur when wished for, a couple consults for infertility. Possibly the couple is simply subfertile, resulting in a prolonged delay before the establishment of pregnancy or psychological or physiological causes are present which hinder the establishment of pregnancy. Medical exploration to find the cause for infertility concerns both the male and the female partner. Several solutions for the treatment of human infertility can nowadays be proposed especially assisted reproductive technologies (ART). "Classical" in vitro fertilization (IVF) can only be effective when female and male gametes are of sufficient quality. When a severe male factor for infertility is present, or when IVF failed several times, micromanipulation techniques such as subzonal insemination (SUZI) and intracytoplasmic insemination (ICSI) are used. Even under the best circumstances fertilization and pregnancy rates are suboptimal. The occurrence of total failure of fertilization and low success rates in terms of pregnancy, initiated research to elucidate causes of fertilization failure and to determine the fertilizing capacity of sperm and oocytes and the developmental capacity of embryos. Morphological and functional aspects of the oocyte, sperm-oocyte penetration and normal and abnormal fertilization and developmental processes in human oocytes and embryos were the main objects of our study. In figure 1, different morphological findings in oocytes and zygotes are schematically summarized. Human oocytes, zygotes and embryos were studied after IVF which was performed for medical indications and included pituitary desensitation by administration of gonadotrophin releasing hormone agonist (GnRHa) and ovarian stimulation with exogeneous hormones. For evident reasons, only oocytes which were considered unfertilized at the time of embryo transfer (48 hours after insemination), delayed, arrested or polypronuclear zygotes and embryos unsuitable for transfer to the uterine cavity of the patient, could be obtained for observation by DNA fluorescence. Hence, for the interpretation of the results one should count with this selection. The chronology of pronucleus formation and cleavage of apparently normally fertilized oocytes originating after in vitro insemination with donor sperm and subzonal insemination (Chapter VI), was established after non-invasive observations on zygotes and embryos which were transferred to the uterine cavity of the patients 48 hours after subzonal insemination or in vitro insemination with donor spermatozoa (D-IVF).

Figure 1: Schematic overview of morphological findings in human oocytes observed 48 hours after in vitro insemination



OOCYTE MATURITY AND QUALITY

In natural menstrual cycles only one oocyte attains the fully mature stage and ovulates, whereas after ovarian stimulation for IVF or other ART, a certain number of oocytes are retrieved. These oocytes are known to differ in their maturational status at the time of retrieval even when the bolus injection of human chorionic gonadotrophin (HCG) synchronizes their nuclear maturation (Bomsel-Helmreich et al, 1987). It would be expected that oocytes not fully mature at retrieval, mature spontaneously after retrieval during culture as in all mammals studied sofar including the human (Edwards, 1965). However, part of the immature oocytes do not since approximately 15% of the oocytes are still meiotically immature and in the germinal vesicle (Figures 2a, 2b, 3) or metaphase I stages, 48 hours after insemination (Chapters II and V). One of the reasons for non-resumption of meiosis may be the beginning of atresia as evidenced by a high degree of vacuolization and organelle clustering in the cytoplasm of some of these oocytes (Chapter II). Nuclear degeneration has also been observed in a minority (22%) of germinal vesicle oocytes (Racowski and Kaufman, 1992). The possibility of a patient specific phenomenon can not be excluded. Patients with a high incidence of immature oocytes not able to resume meiosis, have lower numbers of follicles recruted in ovarian stimulation and show an altered oestradiol response (Bar-Ami et al, 1994). Besides sperm defects, the incidence of immature oocytes may account for delayed fertilization (Zenzes et al, 1985; Oehninger et al, 1989; Ron-El et al, 1991). Delayed fertilization has a negative impact on IVF results, because of often related oocyte and sperm defects or because of insufficient development of the embryo before implantation. A short period of culture before insemination (5 hours, Trounson et al, 1982) or a prolonged interval between hCG administration and oocyte retrieval to allow in vivo maturation (Jamieson et al, 1991) has been shown to increase fertilization rates. In our study, the IVF protocol allowed for 3-5 hours of oocyte in vitro maturation before insemination. Veeck et al (1983) reported that 82% of the immature oocytes resumed meiosis in vitro before insemination. This corresponds with our findings that only a small part of the immature oocytes remain immature and that most of them mature in vitro. Oocytes which continued maturation in vitro had metaphase II chromosomes in the proximity of the first polar body since no ageing processes took place yet. Most oocytes obtained for analysis 48 hours after insemination were mature in metaphase II stage (40-63%) and unfertilized. These findings are comparable to cytogenetic reports where mature oocytes are recognized as haploid (Tarin et al, 1991; Selva et al, 1991). If oocytes are mature at the time of retrieval and fertilization fails, they age in vitro. In our study ageing processes evidenced by a

central drift of the metaphase spindle were found 48 hours after insemination in almost half of the meiotically mature oocytes (Chapters II and V). Pickering et al (1988) did not observe spindle migration as early as 48 hours after insemination. However, they reported abnormal or disrupted spindle structures when compared with fresh oocytes observed 4-6 hours after insemination (Pickering et al, 1988). Abnormal nuclear morphology such as a restitution nucleus, instead of metaphase II chromosomes or clumped chromatin of condensed metaphase chromosomes was observed which are the result of degenerative processes commonly due to ageing or to atresia.

Certain functional oocyte characteristics can be determined after insemination with fertilizing sperm. Firstly, the capacity of the oocyte to avoid polyspermic penetration. Secondly, the capacity of the oocyte to resume meiosis and expulse the second polar body after sperm penetration and thirdly the capacity of the oocyte to support pronucleus formation and cleavage, but as shown in our study, all of these processes may go wrong. It has been shown that the early stages of development are mainly controlled by the oocyte and that cleavage is not sensitive to transcriptional inhibition until after the four cell stage (Braude et al, 1988). Hence, the initial quality of the oocyte seems to be an essential factor for successful fertilization and early development. We found the number of blastomeres (the developmental rate) of embryos to be an indication for the functional oocyte quality. Indeed, embryos originating after SUZI from patients with unexplained IVF failures and showing normal sperm parameters had lower developmental rates than embryos from patients with known severe male factor for infertility (Chapter VI). In the same way, the incidence of abnormal nuclear and cell divisions resulting in embryos with multinuclear blastomeres may result after the fertilization of oocytes of impaired quality (Chapter VII).

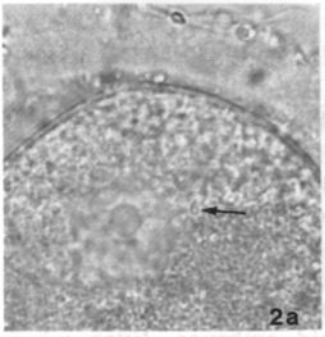
GAMETE FUSION TESTS WITH AGED OOCYTES

Oocytes which fail to fertilize 48 hours after insemination can be used for reinsemination either by in vitro fertilization or by subzonal or intracytoplasmic sperm injection. The incidence and quality of gamete fusion in terms of pronucleus formation can be determined this way which can be of prognostic value for further infertility treatment. It has been shown that aged oocytes may fertilize after reinsemination but implantation of the resulting embryos rarely occurs (Trounson et al, 1984; Pampiglione et al, 1990). This questions the clinical use of reinsemination. After subzonal insemination of aged oocytes which previously failed to fertilize, a higher fertilization rate was observed than after in vitro

reinsemination of intact oocytes (Imoedemhe et al, 1994) but embryo replacement did not result in pregnancy. These findings suggest that besides an effect of zona hardening, the oocyte loses its developmental capacity after prolonged culture. Indeed the negative effect of ageing on embryo development is known in other mammalian species (mouse and rabbit; reviewed by Szollosi, 1975). After subzonal insemination of aged oocytes the number of pronuclei was often higher than the number of spermatozoa injected (Tesarik, 1993). Similar findings were reported after subzonal insemination of fresh oocytes for clinical purposes (Selva et al, 1993). The difficulties of interpreting the frequency of sperm-oocyte fusion, may increase the risk of transfer of polyploid embryos. Even when most of these genetically abnormal embryos abort spontaneously, their transfer should be limited. A reason for the discrepancy between the number of spermatozoa injected and the number of pronuclei observed, can be asynchronous development of different pronuclei. However, we did not observe an important asynchrony neither after SUZI nor after IVF with donor sperm (Chapter VI). However, we only studied monospermic zygotes. Asynchrony possibly exists between the different pronuclei of a polyspermic zygote. Asynchronous sperm decondensation was indeed observed in polyspermic, zona-free human oocytes which failed to fertilize in vitro (Lasalle and Testart, 1991).

NORMAL AND ABNORMAL FERTILIZATION PROCESSES

At fertilization, the spermatozoon must bind to and penetrate into the zona pellucida. Several studies showed that high numbers of sperm bound to the zona of unfertilized oocytes are related with the incidence of fertilization of cohort oocytes (Mahadevan et al, 1987; Liu et al, 1989). More recently sperm-zona binding and partial penetration into the zona pellucida was found to be positively linked to the fertilization rate of cohort oocytes (Bedford and Kim, 1993; Liu and Baker, 1994). The direct relationship between the number of spermatozoa bound to or partially penetrated into the zona and sperm penetration into the ooplasm without fertilization was studied by DNA visualisation (Chapter IV). In accordance with the studies mentioned, unfertilized oocytes from patients with complete fertilization failure after IVF, had less sperm bound to their zona pellucida than oocytes from patients with partial fertilization failure. But also, sperm-zona binding and sperm-oocyte penetration without pronucleus formation were closely related. These findings indirectly suggest that the capacity of the oocyte to resume meiosis and to form pronuclei after sperm penetration does not interfere with its capacity to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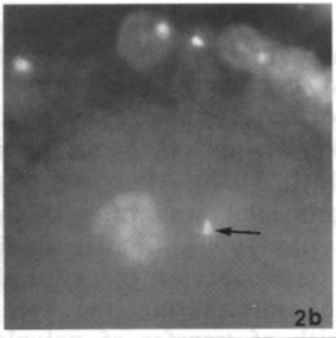
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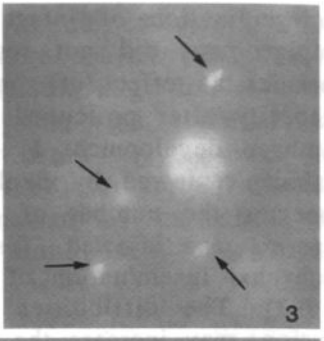
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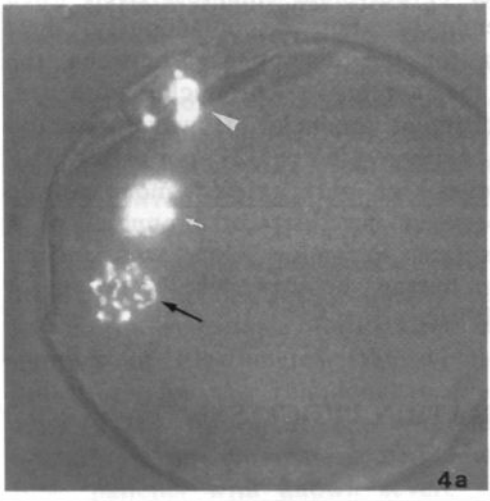
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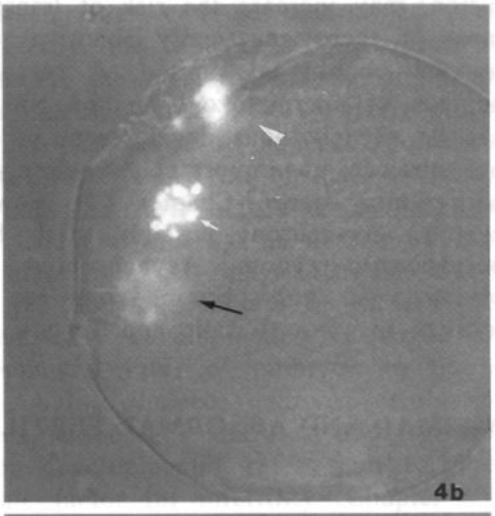
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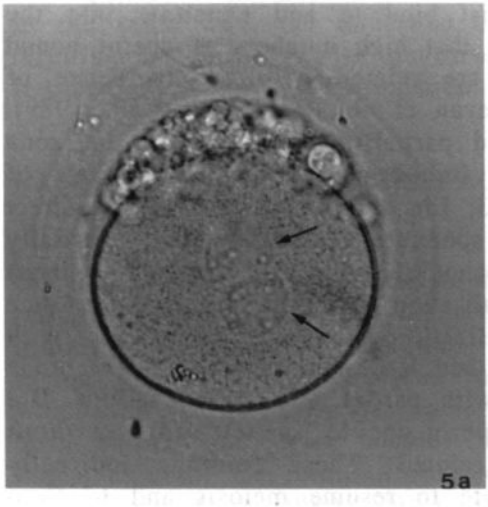
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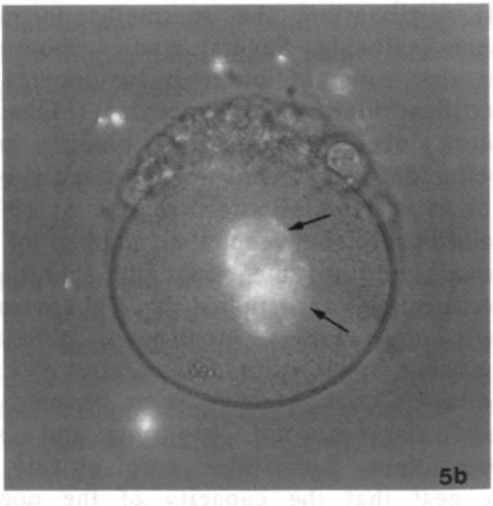
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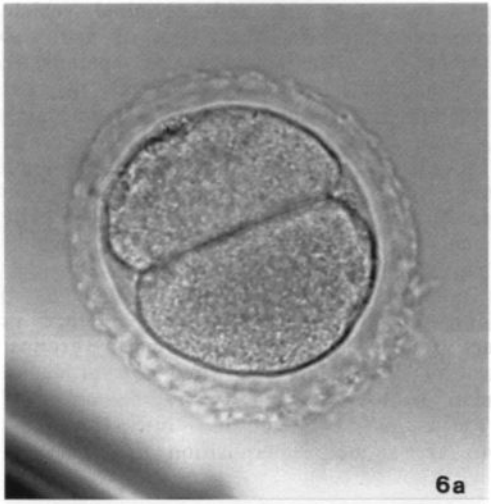
Figure 2: Oocyte in vitro observed by light microscopy (a) with germinal vesicle and a single large nucleolus, a single spermatozoon penetrated the oocyte and was found as an undecondensed head (arrow). Note the difference between spermhead, without tail, inside ooplasm (arrow) and those outside. Same oocyte as (a) but observed by fluorescence microscopy (b). Within the germinal vesicle condensing heterochromatin surrounds the nucleolus, the spermhead. 1cm=10μ. **Figure 3:** Oocyte observed in vitro by fluorescence microscopy: within the germinal vesicle condensing heterochromatin surrounds the nucleolus but the focus is on spermheads. Multiple (4) spermheads penetrated this immature oocyte, the spermheads are indicated by the arrows. 1cm=10μ. **Figure 4:** Oocyte observed in vitro by fluorescence microscopy. Focus on the first polar body (arrowhead) and PCC (arrow) metaphase II chromosomes are slightly out of focus (small arrow) (a). Same oocyte as (a) but focus on metaphase II chromosomes (b). Note the difference in chromatin condensation between the distinct female and male chromatin groups. 1cm=10μ. **Figure 5:** Zygote observed in vitro by light microscopy (a), two pronuclei are present (arrows) containing multiple small nucleoli. Note the fragmentation of this arrested zygote. Spermheads bound to or partly penetrated into the zona pellucida can be observed. The same oocyte as (a) but superposition of light and fluorescence microscopy (b). 1cm=14.5μ.

bind spermatozoa and the penetrability of the zona pellucida and oolemma.

Immature oocytes from small antral follicles can be penetrated by sperm (Lopata and Leung, 1988). Meiotically immature oocytes were frequently found penetrated by sperm (Chapters II, V, Figures 2a, 2b and 3) but these oocytes did not complete maturation and no pronucleus formation had followed. It has been shown that maturation can continue after sperm penetration in zona-free mouse oocytes but no activation followed (Clarke and Masui, 1986). Recently the same observations were made in human germinal vesicle stage oocytes maturing after sperm penetration but here pronuclei were formed (Van Blerkom et al, 1994a). This indicates that the oocyte somehow became activated, by a second penetrating sperm, spontaneously or by the sperm already present inside the ooplasm by an unknown mechanism. However, the developmental capacity of resulting zygotes remains to be established.

In contradiction to the finding that germinal vesicle stage oocytes can continue maturation after sperm penetration in some cases, in metaphase I oocytes sperm penetration results in premature condensed sperm chromatin (PCC) in mouse (Clarke and Masui, 1986) and in human oocytes (Chapters II, V, Figure 4a and 4b). After IVF, PCC are mostly observed in metaphase II oocytes (Schmiady and Kentenich, 1989; Tejada et al, 1992; Chapters II, IV and V). The occurrence of PCC is due to the persistence of chromatin condensing factors (MPF) in metaphase II stage oocytes. In the human both cytoplasmic immaturity of the oocyte and asynchronous development between the male and the female pronucleus are associated with PCC (Schmiady and Kentenich, 1989; Zenzes et al, 1990).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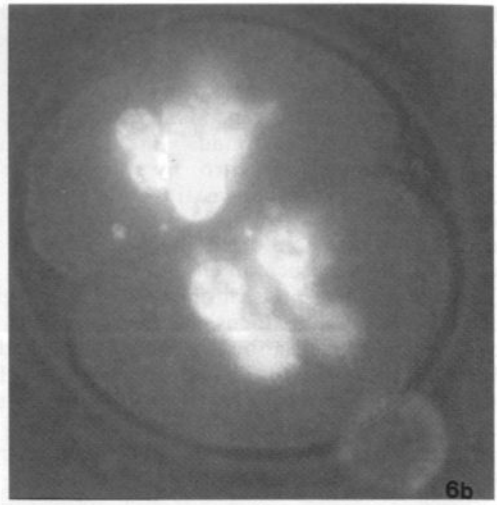
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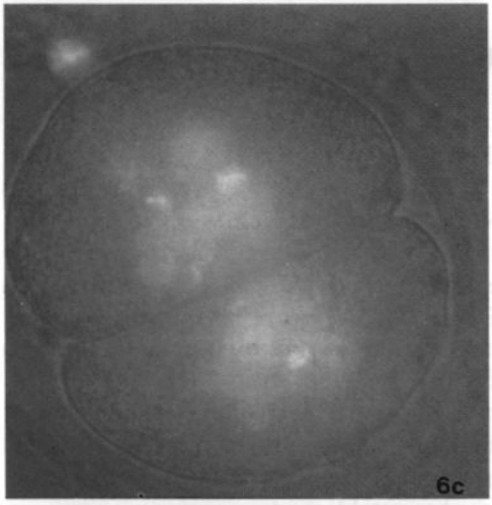
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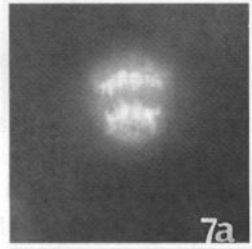
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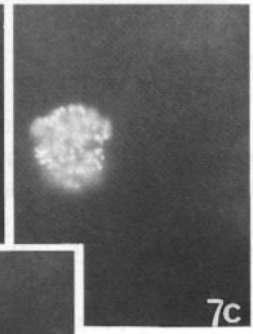
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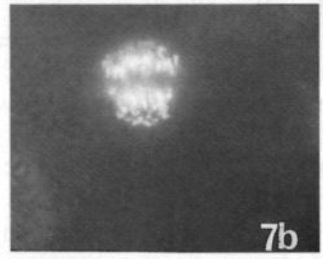
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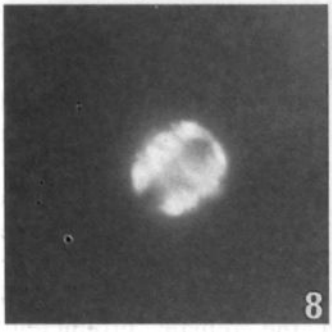
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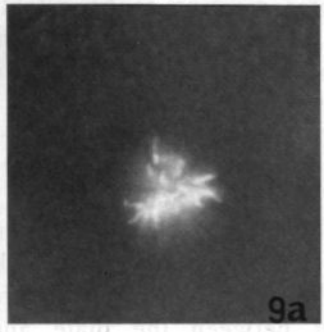
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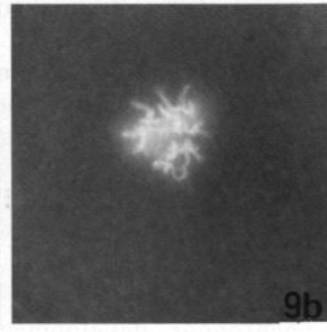
9b

Figure 6: Two-cell embryo observed in vitro by light microscopy (a, 1cm= 14.5µ). Note the abnormal structure of the zona when compared with the previous figures. Figures (b) and (c) show the same embryo but observed by fluorescence microscopy: multiple nuclei are found within each blastomere. 1cm=10µ. **Figures 7a, 7b, 7c, 8, 9a and 9b:** Tripronuclear zygotes (day 1) during the first cell division observed by DNA fluorescence on day 2. 1cm=7.8µ. **Figure 1 is adapted from:** B. Van Wissen, O. Bomsel-Helmreich. "DNA fluorescence revealing oocyte quality, sperm behaviour during gamete interaction and embryo morphology" Published in: Assisted Reproduction Reviews 1994, Vol. 4, pp. 128-133. **Figures 2, 4 and 6 were published in:** Van Wissen B, Bomsel-Helmreich O, De Ziegler D, Pietri V and Frydman R. (1992) "Examen morpho-fonctionnel des gametes en cas d'échec de fécondation in vitro" Contraception Fertil. Sex., 20, 801-803.

Partial decondensation of sperm chromatin was rarely found in metaphase I and II oocytes, probably since oocytes were obtained after IVF failure 48 hours after insemination and sperm chromatin decondensation preceded PCC formation. This hypothesis is confirmed by the presence of partial decondensed spermheads in the majority of penetrated metaphase II stage oocytes observed 28 hours after insemination (Van Blerkom et al, 1994b). In contrast to the findings after IVF, in oocytes without pronuclei after ICSI, only a minority of PCC are found and mostly intact or swollen spermheads were observed (Dozortzev et al, 1994). The reasons for this difference may be related to the capacity of the oocyte to break down the nuclear sperm envelope and to decondense the DNA of the completely intact spermhead after ICSI. Interestingly, the earliest time when pronuclei were observed was 6 hours after ICSI (Nagy et al, 1994) and 4.5 hours after SUZI (Chapter VI). This may suggest that structural changes in sperm occur after passage of the oolemma facilitating male pronucleus formation, which could explain the earlier occurrence of pronuclei after SUZI. It can not be excluded that PCC containing metaphase II oocytes will continue maturation and form pronuclei in the same way as penetrated germinal vesicle stage oocytes. The results of Dozortzev et al (1994) may suggest such a continuation since PCC and partially decondensed spermheads were rarely found after artificial activation by puromycin, possibly because the sperm chromatin decondensed together with the female chromatin. However, whether metaphase II oocytes with PCC actually can continue maturation, become activated and form pronuclei still needs to be elucidated.

DIAGNOSTIC VALUE OF OOCYTE AND EMBRYO ANALYSIS BY DNA FLUORESCENCE

The use of the DNA fluorescent dye Hoechst 33342 is a valuable mean to analyse sperm-oocyte interaction after in vitro fertilization. More

particularly, sperm-zona pellucida binding and penetration, the nuclear and cytoplasmic maturity of the oocyte as well as its capacity to support the different stages of fertilization and development, brings important informations about the quality of the interacting gametes. Moreover, it allows the prognostic evaluation of the usefulness of SUZI or ICSI for a given patient.

The finding that sperm-zona binding is positively related both with sperm-oocyte penetration without fertilization and with the incidence of fertilization of cohort oocytes, makes the assessment of these parameters useful to evaluate sperm penetration capacity. This is especially important when fertilization had failed, and for patients with impaired sperm quality defined by conventional criteria.

Cytoplasmic immaturity as evidenced by the incidence of PCC and in general failure of fertilization after sperm penetration, can only be evidenced if sperm is penetrating and can therefore be underestimated for patients with impaired sperm quality irrespective of whether sperm defects were observed by conventional criteria. On the other hand, fertilization failure after sperm penetration can be a criterion for oocyte quality concerning its capacity of pronucleus formation and syngamy. Also, zygotes which arrested development before syngamy and abnormally cleaved zygotes can be detected and analysed (Figures 5a, 5b, 6a, 6b and 6c). The interpretation of gamete fusion tests using oocytes which were considered unfertilized on the basis of the absence of pronuclei, may be erroneous since oocytes may already be penetrated.

The chronology of pronuclear formation after SUZI and after IVF with donor spermatozoa is comparable, even while after SUZI pronuclear development and the first cleavage division is 4 hours ahead. This means that the time for fertilization assessment after SUZI can be maintained at 16-18 hours after insemination. It seems that the chronology of pronucleus formation and cleavage depends on the sperm phenotype and on the quality of the oocyte. In support of this, both after SUZI and after IVF with donor spermatozoa, implantation rates for transplanted embryos resulting in pregnancy had higher developmental rates than non-implanting embryos.

Multinucleation in human embryos was frequently found after IVF and may be a result of fertilization of oocytes unable to support normal nuclear and cell division because of immaturity or impaired quality. The incidence of multinucleated blastomeres in early human embryos may also be correlated with the incidence of abnormal fertilization processes such as polyspermy and sperm penetration without pronucleus formation. These phenomena are especially frequent when high numbers of oocytes are retrieved after ovarian stimulation with exogenous hormones for IVF. The transplantation of embryos with multinuclear blastomeres, should be avoided when morphologically normal embryos are available.

SUGGESTIONS FOR FURTHER RESEARCH

The technique of DNA fluorescence has proven useful to evaluate gamete interaction after IVF. It may also serve to analyse characteristics of fertilization processes in oocytes which failed to form pronuclei after SUZI (unpublished results) and ICSI and to evaluate the incidence of sperm-oocyte fusion. Also, visualization of DNA after reinsemination of aged, unfertilized oocytes, may help to elucidate differences of early fertilization processes after IVF, SUZI and ICSI. Recently, aged zona-pellucida free human oocytes were used to test the fertilizing potential of sperm after reinsemination by fluorescence observations (Rufas et al, 1994). Because of recent bioethical laws in France (Annex 1) and in other countries prohibiting reinsemination for research purposes (even of oocytes which remained unfertilized after IVF) and restricting research on zygotes and embryos, observation of unfertilized oocytes after IVF, SUZI and ICSI may be the only mean in the future to explore fertilization and developmental abnormalities and to obtain detailed information about sperm-oocyte interaction.

The combination of DNA fluorescence with other techniques should also be developed further. In vitro DNA fluorescence can be combined with histological observations on thin sections as we showed in chapter II but also with cytogenetics (Van Wissen et al, 1993). Also, early developmental processes such as the first nuclear division can be observed in vitro in polypronuclear zygotes (Figures 7a, 7b, 7c, 8, 9a and 9b). The use of complementary techniques seem promising to further explore fertilization and its failure.

REFERENCES

Bar-Ami S, Zlotkin E, Brandes JM, Itskovitz-Eldor J. Failure of meiotic competence in human oocytes. *Biol. Reprod.* 50: 1100-1107, 1994

Bedford JM, Kim HH. Sperm/egg binding patterns and oocyte cytology in retrospective analysis of fertilization failure in vitro. *Hum. Reprod.* 8: 453-463, 1993

Bomsel-Helmreich O, Vu N Huyen L, Durand-Gasselin I, Salat-Baroux J, Antoine JM. Timing of nuclear maturation and cumulus dissociation in human oocytes stimulated by CC, hMG and hCG. *Fertil Steril* 48: 586-595, 1987

Braude P, Bolton V, Moore S. Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* 332: 459-461, 1988

Clarke HJ, Masui Y. Transformations of sperm nuclei to metaphase chromosomes in the cytoplasm of maturing oocytes of the mouse. *J. Cell Biol.* 102: 1039-1046, 1986

Dozortzev D, De Sutter P, Dhont M. Behaviour of spermatozoa in human oocytes displaying no or one pronucleus after intracytoplasmic sperm injection. *Hum. Reprod.* 9: 2139-2144, 1994

Edwards RG. Maturation in vitro of human ovarian oocytes. *Lancet* 6: 926-929, 1965
Imoedemhe DAG, Sigue AB. The influence of subzonal microinsemination of oocytes failing to fertilize in scheduled routine in-vitro fertilization cycles. *Hum. Reprod.* 9: 669-672, 1994
Jamieson ME, Fleming R, Kader S, Ross KS, Yates RWS, Coutts JRT. In vivo and in vitro maturation of human oocytes: effects on embryo development and polyspermic fertilization. *Fertil. Steril.* 56: 93-97, 1991
Lasalle B, Testart J: Sequential transformations of human sperm nucleus in human egg. *J Reprod Fertil* 91: 393-402, 1991
Liu DY, Lopata A, Johnston WIH, Baker HWG. Human sperm-zona binding, sperm characteristics and in-vitro fertilization. *Hum. Reprod.* 4: 696-701, 1989
Liu DY, Baker HWG. A new test for the assessment of sperm-zona pellucida penetration: relationship with results of other sperm tests and fertilization in vitro. *Hum. Reprod.* 9: 489-496, 1994
Lopata A, Leung PC. The fertilizability of human oocytes at different stages of meiotic maturation. *Ann. N.Y. Acad. Sci.* 541: 324-336, 1988
Mahadevan MM, Trounson AO, Wood C, Leeton JF. Effect of oocyte quality and sperm characteristics on the number of spermatozoa bound to the zona pellucida of human oocytes inseminated in vitro. *J. in Vitro Fert. Embryo Trans.* 4: 223-227, 1987
Nagy ZP, Liu J, Joris H, Devroey P, Van Steirteghem A. Time-course of oocyte activation, pronucleus formation and cleavage in human oocytes fertilized by intracytoplasmic sperm injection. *Hum. Reprod.* 9: 1743-1748, 1994
Oehninger S, Acosta AA, Veeck LL, Simonetti S, Muasher SJ. Delayed fertilization during in vitro fertilization and embryo transfer cycles: analysis of causes and impact on overall results. *Fertil. Steril.* 52: 991-997, 1989
Pampiglione JS, Mills C, Campbell S, Steer C, Kingsland C, Mason BA. The clinical outcome of reinsemination of human oocytes fertilized in vitro. *Fertil. Steril.* 53: 306-310, 1990
Pickering SJ, Johnson MH, Braude PR. Cytoskeletal organisation in fresh,aged and spontaneously activated human oocytes . *Hum. Reprod.* 3: 978-979, 1988
Racowsky C, Kaufman ML. Nuclear degeneration and meiotic aberations observed in human oocytes matured in vitro: analysis by light microscopy. *Fertil. Steril.* 58: 750-755,1992
Ron-El R, Nachum H, Herman A, Golan A, Caspi E, Soffer Y. Delayed fertilization and poor embryonic development associated with impaired semen quality. *Fertil. Steril.* 55: 338-344, 1991
Rufas O, Fish B, Phillips DM, Shalgi R. Human gamete fusion test: an experimental model for assessing the fusion potential of sperm from nonfertilizing normospermic patients. *Fertil. Steril.* 62: 786-792, 1994
Schmiady H, Kentenich H. Premature chromosome condensation after in vitro fertilization. *Hum. Reprod.* 4: 689-695, 1989
Selva J, Martin-Pont B, Hugues JN, Rince P, Fillion C, Herve F, Tamboise A, Tamboise E. Cytogenetic study of human oocytes uncleaved after in vitro fertilization. *Hum. Reprod.* 6: 709-713, 1991
Selva J, Wolf JP, Rince P, Rodriques D, Frydman R, Jouannet P. Cytogenetic analysis of human oocytes after subzonal insemination. *Prenatal Diagn.* 13: 311-321, 1993
Szollosi D: Mammalian eggs ageing in the fallopian tubes. In: Blandau RJ, (ed), Ageing gametes, Karger, International Symposium, Seattle, pp 98-121, 1975
Tarin JJ, Ruiz A, Miro F, Bonilla-Musoles F, Pellicer A. Failed in vitro fertilization of human oocytes: a cytogenetic analysis. *Fertil. Steril.* 56: 290-295, 1991
Tesarik J. Subzonal sperm insertion with aged human oocytes from failed in-vitro fertilization attempts: fertilization results and some applications. *Hum. Reprod.* 8: 1245-1250, 1993

Trounson AO, Mohr LR, Wood C, Leeton JF. Effect of delayed insemination on in vitro fertilization, culture and transfer of human embryos. *J. Reprod. Fert.* 64: 285-294, 1982

Trounson A, Webb J. Fertilization of human oocytes following reinsemination in vitro. *Fertil. Steril.* 41: 816-819, 1984

Usui N, Yanagimachi R. Behaviour of hamster sperm nuclei incorporated into eggs at various stages of maturation, fertilization, and early development. *J. Ultrastructure Res.* 57: 276-288, 1976

Van Blerkom J, Davis PW, Merriam J. The developmental ability of human oocytes penetrated at the germinal vesicle stage after insemination in vitro. *Hum. Reprod.* 9: 697-708, 1994a

Van Blerkom, J., Davis, P.W., Merriam, J. A retrospective analysis of unfertilized and presumed parthenogenetically activated human oocytes demonstrates a high frequency of sperm penetration. *Hum. Reprod.* 9: 2381-2388, 1994b.

Van Wissen B, Bomsel-Helmreich O, Bergere M, Volante M, Selva J, Frydman R. (1993) Oocyte maturity, sperm behaviour during gamete interaction and embryo morphology evidenced by DNA fluorescence. *J. of Ass. Reprod Genet.* Supplement, Abstracts, 10: pp 107

Veeck LL, Wortham JWE, Witmeyer J, Sandow BA, Acosta AA, Garcia JE, Jones GS, Jones HWJ. Maturation and fertilization of morphologically immature human oocytes in a program of in vitro fertilization. *Fertil. Steril.* 39: 594-602, 1983

Zenzes MT, Belkien L, Bordt J, Kan I, Schneider HPG, Nieschlag E. Cytologic investigation of human in vitro fertilization failures. *Fertil. Steril.* 43: 883-891, 1985

Zenzes MT, de Geyter C, Bordt J, Schneider HPG, Nieschlag E. Abnormalities of sperm chromosome condensation in the cytoplasm of immature oocytes. *Hum. Reprod* 5: 842-845, 1990.

ANNEX I: French laws relative to artificial reproductive technologies, prohibiting reinsemination for research and research on human zygotes and embryos

Loi no. 94-653 du 29 juillet relative au respect du corps humain (1).
Loi no. 94-654 du 29 juillet 1994 relative au don et à l'utilisation des éléments et produits du corps humain, à l'assistance médicale à la procréation et au diagnostic prénatal.
Chapitre II bis:
Art. L. 152-1-L'assistance médicale à la procréation s'entend des pratiques cliniques et biologiques permettant la conception in vitro, le transfert d'embryons et l'insémination artificielle, ainsi que de toute technique d'effet équivalent permettent la procréation en dehors du processus naturel.
Art. L. 152-8-La conception in vitro d'embryons humains à des fins d'étude, de recherche ou d'expérimentation est interdite.
Toute expérimentation sur l'embryon est interdite.
A titre exceptionnel, l'homme et la femme formant le couple peuvent accepter que soient menées des études sur leurs embryons. Leur décision est exprimée par écrit. Ces études doivent avoir une finalité médicale et ne peuvent porter atteinte à l'embryon. Elles ne peuvent être entreprises qu'après avis conforme de la commission mentionnée à l'article L. 184-3 ci-dessous dans des conditions définies par décret en Conseil d'Etat. La commission rend publique chaque année la liste des établissements où s'effectuent ces études ainsi que leur objet.

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CURRICULUM VITAE

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PUBLICATIONS

Van Wissen B, Bomsel-Helmreich O, Debey P, Eisenberg C, Vautier D, Pennehouat G (1991). Fertilization and ageing processes in non-divided human oocytes after GnRHa treatment: An analysis of individual oocytes. Human Reproduction 6, 879-884.

Van Wissen B, Bomsel-Helmreich O, De Ziegler D, Pietri V, Frydman R (1992). Examen morpho-fonctionnel des gametes en cas d'echec de fécondation in vitro. Contraception-Fertilité-Sexualité 20, 801-803.

Van Wissen B, Eisenberg C, Debey P, Pennehouat G, Auger J, Bomsel-Helmreich O (1992). Rapid analysis of fertilization failure after IVF by the use of in vitro DNA fluorescence. Journal of Assisted Reproduction and Genetics 9, 564-571.

Van Wissen B, Bomsel-Helmreich O,Frydman R (1994) .DNA fluorescence revealing oocyte quality, sperm behaviour during gamete interaction and Embryo morphology. Assisted Reproduction Reviews 4, 128-133.*

Van Wissen B, Wolf JP, Bomsel-Helmreich O, Frydman R, Jouannet P (1995). Timing of pronuclear development and first cleavages in human embryos after subzonal insemination: influence of sperm phenotype. Human Reproduction 10, 642-648**

Van Wissen B, Bomsel-Helmreich O,Frydman R (In Press). Sperm-zona binding and sperm-oocyte penetration analysed by DNA fluorescence: a test for gamete quality after IVF. Human Reproduction.

Van Wissen B, Bomsel-Helmreich O, DeZiegler D, Frydman R. The use of morphological and functional oocyte parameters to determine causes of IVF failure. Submitted.

Van Wissen B, Bomsel-Helmreich O, Frydman R. Multinucleation in embryos after IVF. Submitted.

COMMUNICATIONS

Van Wissen B, Bomsel-Helmreich O, Debey P, Eisenberg C, Vautier D, Pennehouat G (1990). Fertilization and ageing processes in non-divided human oocytes. Human Reproduction, Abstracts of the second joint meeting of ESHRE and ESCO. Abstract 212.

Van Wissen B, Bomsel-Helmreich O, Eisenberg C, Debey P, Jouannet P, Frydman R (1991). Therapeutic advise after observation of fertilization processes in non-divided human oocytes. Journal of Reproduction and Fertility, Abstract series 7, Abstract 51.

Van Wissen B, Bomsel-Helmreich O, Debey P, Eisenberg C, Durand-Gasselin I, Vu N Huyen L, Pennehouat G (1991). Human sperm and oocyte interaction in fertilization processes. Human Reproduction, Abstracts from the 7th meeting of ESHRE and the 7th World Congress on IVF and Assisted Procreation, Abstract 86.

Van Wissen B, Eisenberg C, Debey P, Pennehouat G, Madelenat P, Feldmann G, Bomsel-Helmreich O (1991). Fertilization in IVF: which partner is responsible. Abstracts from the 7th meeting of ESHRE and the 7th World Congress on IVF and Assisted Procreation, Abstract P78.

Van Wissen B, Bomsel-Helmreich O, Bergere M, Volante M, Selva J, Frydman R (1993). Oocyte maturity, sperm behaviour during gamete interaction and embryo morphology evidenced by DNA fluorescence. Journal of Assisted Reproduction and Genetics 10, 267.*

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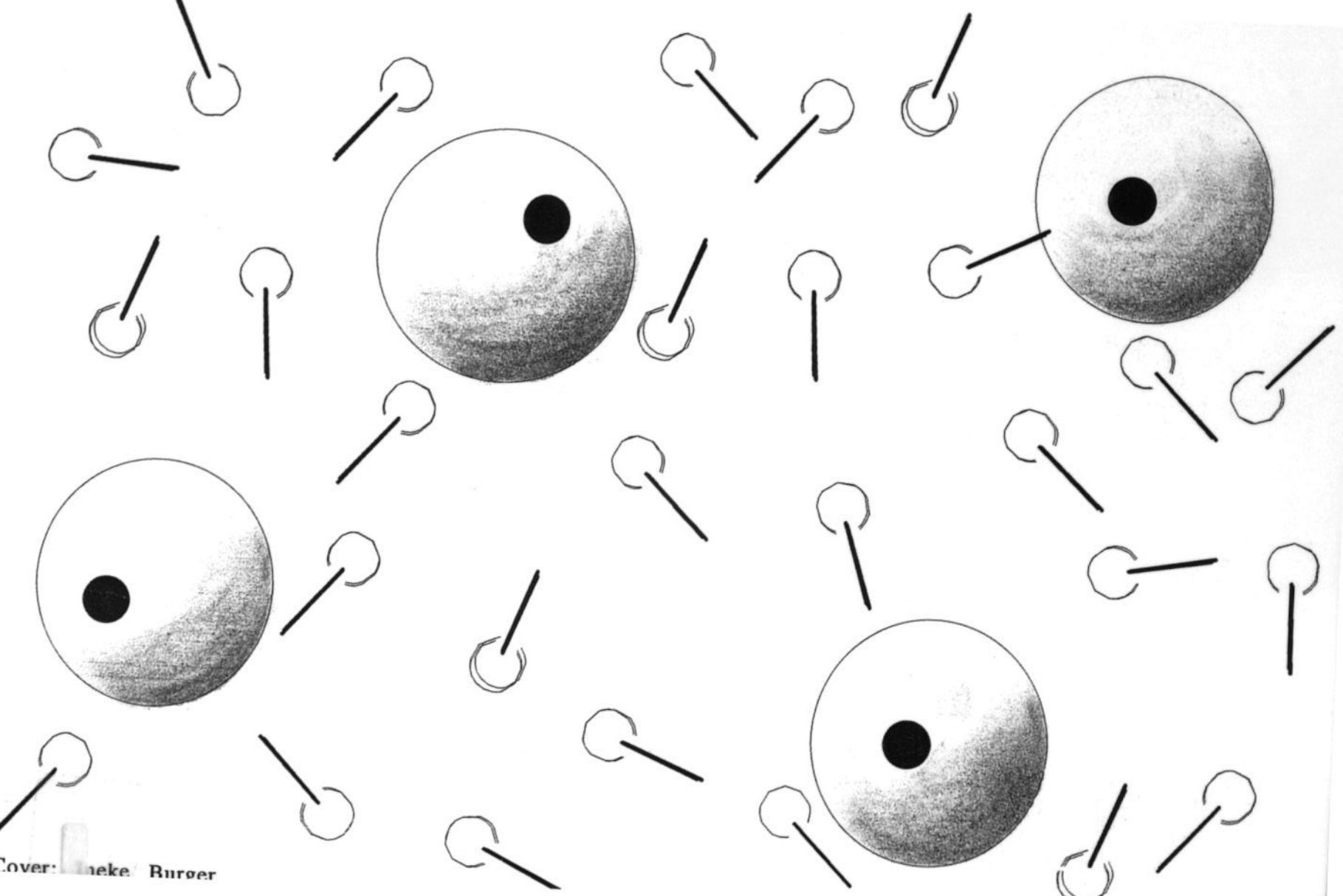
** Presented at the ESHRE congress 1994 (Bruxelles, Belgium)



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