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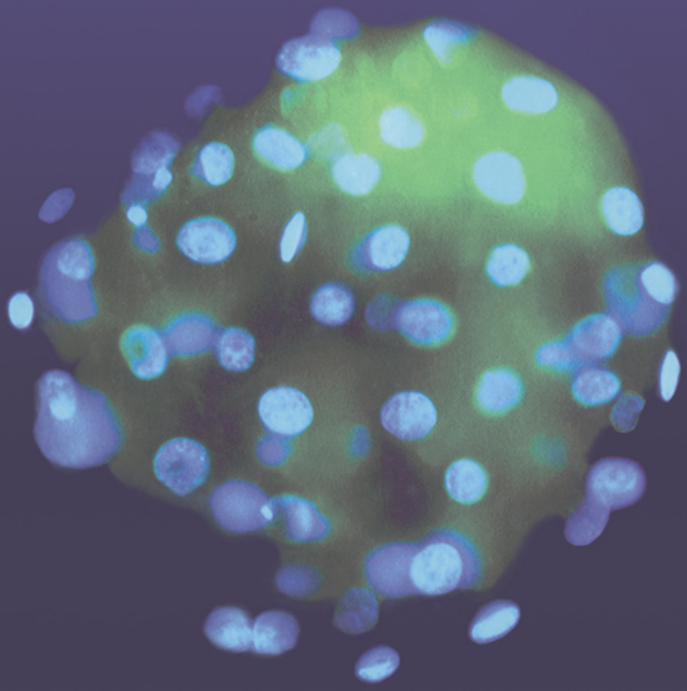
An Atlas of HUMAN BLASTOCYSTS



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Cover photo: Human blastocyst stained with fluorescence dyes: Bcl-2-FITC (green), DAPI (blue)
'Thumbnail' figures: Human frozen-thawed single-pronucleate conceptus donated for time-lapse studies: cleavage from the eight-cell stage of development through to the hatched blastocyst stage

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Foreword

Assisted reproductive technology has many intriguing aspects, but two are of surpassing fascination. First is the breathlessness of clinical result. With success, there is elation and presumed understanding of the physiological process; with failure, there is a frustration that leads the scientific community to strive for better understanding and more satisfactory results. Second is the moving experience of viewing microscopically the morphological processes that lead to an independent being—in this case a human being. The enigma is to distinguish the potential for viability from the many observed variations, which, according to our present understanding, are often expressions of the genetic misfits and misfires that are characteristic of human reproduction. Are we having a glimpse of the evolutionary process?

From its microscopic origin, the preembryo blooms into the human form, a process both wondrous and scientifically intriguing. Heeding biological commands, cells grow purposefully according to a predefined plan, endowing the new genetic entity with viability and function. Now, there is even more; as our experience extends beyond morphology. We are beginning to understand something about genes, gene products, enzymes, and proteins which drive these morphological characteristics.

The excitement and reality of clinical and laboratory work are captured by Lucinda Veeck, Nikica Zaninović, and their collaborators in *An Atlas of Human Blastocysts*. This is a book for those who wish to experience the satisfaction of being certain that they are up to date regarding extended culture procedures and the complexities of blastocyst development, considered key to achieving high pregnancy rates while minimizing the troublesome complication of multiple pregnancies.

In the following pages, the reader is given an opportunity to study the human, rhesus and murine blastocyst under optimal clinical and research conditions. The blastocyst's nutrient requirements during culture, its growth through various key stages, and its ability to survive freezing and thawing are all examined. We are guided through the early aspects of cell allocation and differentiation and are enlightened to the processes of hatching and programmed cell death. In sum, the reproductive process is demonstrated photographically from fertilization through to completion of implantation. Additionally, and of great interest, current scientific research applications are included.

Leaders in various clinical and scientific fields have come together to create this superb volume. *An Atlas of Human Blastocysts* is a dynamic and authoritative collection of microanatomical examples and definitively captures the earliest events of mammalian development *in vitro*. It is an absolute 'must-read' for clinicians and scientists working in the field of assisted reproduction.

Howard W.Jones, Jr, MD
Georgeanna Seegar Jones, MD

Preface

Why have those of us working in assisted reproductive laboratories become so suddenly fascinated with blastocysts? The answers are simple. First and foremost, never before in history have we had the opportunity to study closely human blastocyst development *in vitro*. Early descriptions of human morulae and blastocysts often relied on studying discarded material grown under suboptimal culture conditions after *in vitro* fertilization (IVF) trials. Investigating morphology, growth rate, metabolic requirements and genetic factors under these conditions probably led us to many misleading conclusions. Only with the development of sequential media have we been able routinely to grow viable blastocysts in our laboratories with some measure of confidence. Without doubt, *in vitro* culture techniques will continue to improve as additional knowledge is gained, enabling us to understand better the human reproductive process and ultimately provide our patients with tremendous benefit.

Second, we recognize that, through *in vitro* developmental investigations involving extended culture, we have been given the opportunity to offer a much improved and safer service to our patients by reducing the number of preembryos for transfer. How often in the past have we observed patients desperately desiring a healthy child, anxious to receive three or more pre-embryos for transfer, and then watched them agonize guiltily when forced to reduce selectively a high-order multiple pregnancy? This sad treatment option is all too often necessary because higher-order gestations, those involving more than two fetal hearts on ultrasound examination, are the largest single cause of poor obstetric outcome and subsequent neonatal difficulties. Triplet and quadruplet pregnancies are associated with high incidences of preeclampsia, gestational diabetes, pregnancy-induced, hypertension, preterm labor, low birth weight and extensive neonatal care[1]. Although multifetal pregnancy reduction to twins is an option, the procedure itself carries medical and emotional risks[2,3].

Clearly, the most efficient way to avoid any form of multiple pregnancy is to limit the number of preembryos for intrauterine transfer to a single conceptus. While straightforward in theory, the reality of this approach leaves much to be desired. Indeed, most IVF programs experience no greater than a 20–30% clinical pregnancy rate per transfer when a single 4–8-cell conceptus is replaced. With treatment costs of $5000–$15000 per IVF attempt in the United States, often not covered by insurance, this figure is too low to be cost-effective or desirable to the couple being treated. For this reason, more than one, and frequently more than three, day-2 or day-3 preembryos have been routinely replaced in an effort to optimize the chances for pregnancy. Therein lies the problem: *multiple transfer of early developing preembryos carries the risk of plural gestation, a risk that, until recently, could not be fully eliminated without decreasing the overall likelihood of pregnancy*. In the Cornell program, one in three young women under the age of 34 years will establish a multiple pregnancy if three preembryos are replaced on day 3, and 20% of women aged 34–39 years old will follow the same pattern. Because this trend is seen world-wide, it has become the recommended policy of many IVF

centers to replace no more than two conceptuses whenever possible, many countries mandating this by law.

The incidence of multiple pregnancy has risen throughout the world as a consequence of assisted reproductive technologies. It has been reported that the rate of triplet and higher-order gestation infants per 100 000 Caucasian live births in the United States increased by 191 % between 1972 and 1991, with 38% due to assisted conceptions and 30% to increased child-bearing among older women[4]. Another negative aspect to the rising rate of multiple pregnancy involves the associated economic burden resulting from preterm birth and increased hospital stays. Analysis of births at Brigham and Women's Hospital in Boston between 1986 and 1991 revealed that assisted reproductive technologies accounted for 2% of single, 35% of twin and 77% of triplet deliveries in that particular unit. Hospital charges per single baby averaged $9845, for twins they averaged $37947 ($18974 per baby) and for triplets they averaged $109765 ($36588 per baby)[5]. Since these figures are more than a decade old, one may assume that hospital costs are even higher today for couples experiencing multiple births.

Unfortunately, early demise of the human conceptus is a common event. Approximately 73% of *natural* single conceptions are lost before reaching 6 weeks of gestation, and, of the remainder, roughly 90% survive to term[6]. Although conceptions from IVF do nearly as well as natural pregnancies after clinical recognition, they result in higher losses between the onset of fertilization and completion of implantation, presumably due to developmental arrest or unrecognized abnormalities. Realization of this shortcoming prompts patients to ask for the replacement of multiple preembryos and allows us to agree in an effort to optimize ongoing pregnancy rates. Nevertheless, the necessity of replacing more than a single preembryo in order to establish good pregnancy rates would be moot if one could appropriately choose for transfer the healthiest and most viable conceptus from a cohort of growing preembryos. Imagine one day in the future when our patients will receive a single, healthy hatched blastocyst while having all other potentially viable ones frozen. The incidence of twin and greater gestations would be effectively eradicated!

It is not only reasonable, but prudent, to enquire which factors contribute to preembryo viability. Certainly, genetic stability is a major prerequisite for the implantation and delivery of a healthy child. At present, we know little about the genetic make-up of the preembryos within our incubators unless they are biopsied and examined, hardly a practical screening modality for every preembryo growing in the laboratory. Yet, when these examinations are performed, evidence comes to light that chromosomal abnormalities, both numerical and structural, are often associated with fragmented, multinucleated or poorly developing preembryos, and, conversely, many preembryos presenting good morphology possess lethal genetic aberrations. This leads us to recognize that, although morphological evaluations may furnish clues that minimally enhance our proficiency at choosing the best preembryos for transfer, these systems are severely limited in their ability to provide rock-hard evidence for subsequent normal development. Only by using new and very exciting non-invasive methods of assessment, such as amino acid profiling, will we enter a new era for diagnostic preembryo selection[7].

In further support of blastocyst transfer, it has been observed that genetically unhealthy preembryos often cease growth at very early cleavage stages. Almeida and Bolton proposed that there is a progressive loss of chromosomally abnormal preembryos

after pronuclear development to at least the 8-cell stage[8]. When preembryos of varying morphological grades were studied cytogenetically, these investigators found a 65% incidence of abnormality at the pronuclear stage, a 55% incidence at the 2–4-cell stage and a 27% incidence at the 5–8-cell stage. Preembryos with poor morphology demonstrated almost a three-fold increase in chromosomal anomalies as compared to those with good morphology. From these data, it is logical to deduce that some form of natural selection continues beyond the 8-cell stage, perhaps through early fetal development. Might we not expect progressive natural selection to occur if we extend culture times beyond the standard 2 or 3 days? Will blastocyst transfer allow us successfully to replace a single conceptus? The purpose of the following text and photographic collection is to demonstrate to the reader that extended culture to blastocyst stages of development is now indeed an achievable option in our laboratories.

Lucinda L.Veeck

'Faith' is a fine invention when
Gentlemen can see. But
Microscopes are prudent in an emergency
Emily Dickinson (contributed by Helen Maloney)

Nikica Zaninović

It will be found that everything depends on the composition of the forces with which these particles of matter act upon one another: and from these forces, as a matter of fact, all phenomena of Nature take their origin
Ruđer Bošković, Croatian scientist (The Theory of Natural Philosophy, 1758)

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1
Overview of early human preimplantation development *in vitro*

Ovulation induction for assisted reproductive procedures

The cornerstone of successful assisted reproductive technology (ART) has been the ability to replace several selected preembryos from a larger cohort obtained following recruitment, harvest and fertilization of multiple oocytes. Thus, although the first successful human *in vitro* fertilization (IVF) pregnancy followed the retrieval of a single oocyte in a spontaneous menstrual cycle[1], current standard practice in ART programs worldwide entails the use of controlled ovarian hyperstimulation in order to maximize pregnancy rates. This strategy, while maximizing pregnancy rates, has also been associated with the inherent increased risks of multiple pregnancies. A wide variety of ovulation-inducing agents have been employed in the practice of ART, including clomiphene citrate, human menopausal gonadotropins (hMG), and recombinant gonadotropin preparations, with and without the adjunctive use of gonadotropin releasing hormone (GnRH) agonists and antagonists. Currently, the dominant approach to ovulation induction for IVF combines exogenous gonadotropins (hMG, Puriffied follicle stimulating hormone (FSH) and recombinant FSH) with GnRH agonists.

Although clomiphene citrate was once extensively employed for ART, either as a single agent or in combination with gonadotropins, the current dominance of pure gonadotropin-based protocols was spurred by the premise that this approach is more physiological and might avoid the potentially detrimental effects of clomiphene on the oocytes and endometrium. Commercially available gonadotropin preparations include: hMG, formulated in ampules containing 75 IU each of FSH and luteinizing hormone (LH); purified FSH, containing 75 IU of FSH with less than 1 IU of LH and, more recently, recombinant FSH. Urinary-derived gonadotropins are heterogeneous with respect to glycosylation and the presence of degraded fragments, which can lead to varying biopotency among batches, whereas recombinant gonadotropins are uniform.

Over 2000 different GnRH agonists have been synthesized. Endogenous GnRH is rapidly degraded by cleavage at the Gly^6-Leu^7 and Pro^9-Gly^{10} positions, with a resultant half-life of less than 10 min. The selective substitution of amino acids at positions 6 and 10 of the GnRH molecule leads both to enhanced binding affinity to the GnRH receptor and decreased susceptibility to degradation by endopeptidases, thus prolonging the half-life and enhancing the biological activity of these agents. The pharmacological response to GnRH agonist administration is biphasic, with an initial surge of gonadotropin release from the adenohypophysis, but prolonged GnRH receptor occupancy results in desensitization and down-regulation of the gonadotropes, thus effecting reversible hypogonadism. The adjunctive use of GnRH agonists in IVF has several apparent

advantages, including a reduction in the incidence of untimely LH surges and premature luteinization, the ability to program the initiation of stimulations so as to permit a more even distribution of a clinic's workload, and, most significantly, an overall improvement in IVF success rates, a finding confirmed by a published meta-analysis of randomized, controlled trials[2].

When applied to IVF, GnRH agonists may be administered either in a long or a short protocol. In the long protocol, currently favored by most centers, GnRH agonist treatment is initiated in the mid-luteal phase of the preceding menstrual cycle; pituitary down-regulation ensues within 5–10 days, andis indicated by the onset of menses. Gonadotropin therapy is then undertaken concurrently, typically commencing on cycle day 3 or once adequate suppression of estradiol is documented. The dosage of gonadotropins ranges from two to four ampules per day, with higher doses occasionally employed in patients predicted to have a poor response. The cycle is monitored with daily estradiol determinations commencing after 2–3 days of therapy; serial sonographic follicular measurements are performed once the estradiol exceeds a threshold level, generally by the sixth or seventh day of the cycle. The daily dosage of gonadotropins may be adjusted according to the individual patient's response, e.g. with a step-down once follicular recruitment has been achieved, in an effort to attain greater synchronization of follicular maturation and a reduced risk for the development of ovarian hyperstimulation syndrome. Appropriate timing of the ovulatory dose of human chorionic gonadotropin (hCG) is critical for the retrieval of an adequate number of optimally mature oocytes, and is determined by parameters including the mean diameter of the lead follicles (typically >16 mm), the absolute estradiol level (e.g. >500 pg/ml) and the pattern of estradiol rise and follicular growth. The GnRH agonist is discontinued on procedure, performed transvaginally with ultrasound the day of hCG administration. The oocyte retrieval guidance, is typically undertaken 34–36 h following the administration of hCG.

In the short or 'flare' GnRH agonist protocols, the agonist is initiated in the early follicular phase, usually on cycle day 2 or 3. Concurrent therapy with gonadotropins commences 1–3 days later. This approach exploits the agonist phase of GnRH agonist treatment, thus reducing the total dosage requirement for gonadotropins and shortening the duration of stimulation. Although both long and short protocols have their adherents, the former approach is more prevalent. More recently, GnRH antagonists have been introduced, which allow for late follicular suppression of the LH surge, eliminating the need for prolonged pretreatment down-regulation.

The goal of all ovulation induction protocols for ART is to permit the recruitment and harvest of an optimal number of preovulatory oocytes, so as to maximize clinical efficiency. Pregnancy rates may thus be optimized through the selection and transfer of a few of the 'best quality' preembryos, with the option of cryopreserving potentially viable conceptuses in excess of that number.

Gametes

In most species, there are just two types of gametes, and they are radically different. Apart from motor neurons with their remarkably long axons, the oocyte is among the

largest cells of the human organism. Conversely, spermatozoa and red blood cells are two of the smallest.

The diameter of the mature human oocyte is approximately 110–115 μm, and it is bounded by a plasma membrane called the *oolemma*. Surrounding the oocyte/oolemma is a glycoprotein envelope called the *zona pellucida*, a structure approximately 15–20 μm wide (becoming a bit thinner after fertilization) that protects the oocyte during transport and fertilization. Between the oolemma and the zona pellucida is the fluid-filled *perivitelline space*. The use of this term persists despite its inaccuracy when describing the oocytes of humans or most other mammals; it acknowledges the word *vitellus*, a term traditionally used to describe the yolky substance of a hen's egg, which contains abundant nutrient reserves. The cytoplasm of the mammalian oocyte is usually referred to as the *ooplasm*, a more appropriate term for describing the living portion of the human gamete. The main organelles of the ooplasm are the mitochondria, the endoplasmic reticulum and the Golgi system.

When fully capable of undergoing a normal fertilization process, the secondary oocyte is briefly arrested in its course of maturation at metaphase II of meiosis. Nuclear maturation is usually closely attended by a general maturation of the cytoplasm, and is characterized by an increase in the number of organelles scattered throughout the ooplasm. The presence of a first polar body conveys that nuclear maturation has reached this stage. Along with the zona pellucida and perivitelline space, the total diameter of the mature human oocyte is approximately 150 μm.

An oocyte incubated with spermatozoa before reaching metaphase II may incorporate a spermatozoon into its ooplasm and yet fail to initiate events leading to sperm decondensation; such an oocyte ultimately lacks a functional male pronucleus[3]. One study examining 518 non-fertilized oocytes demonstrated that 22% had actually been penetrated by sperm, but without oocyte activation or pronuclear formation[4]. Many of these oocytes may have been immature when combined with spermatozoa.

Besides the requirement for nuclear maturation, it is believed that a brief period is necessary after extrusion of the first polar body for the oocyte to gain full cytoplasmic competence. An oocyte that is meiotically mature but slightly underdeveloped or overdeveloped with regard to its cytoplasm may be more apt to display one, three or more pronuclei. With immature cytoplasm, the cortical granule numbers and response may be inadequate; with postmature cytoplasm, cortical granule release may be inhibited owing to the inward migration of the granules towards the interior of the cell. In either instance, there is evidence that the zona reaction is also often poorly functional when the sperm-oocyte interaction is not appropriately timed with regard to oocyte nuclear and cytoplasmic maturity[5].

Oocytes collected for IVF are generally surrounded by several layers of cells, which define the *cumulus oophorus*. Cells of the cumulus are instrumental, via gap junctions, in nurturing the oocyte during growth and possibly in passing inhibiting factors (e.g. cyclic adenosine monophosphate (cAMP)) necessary for deterring the resumption of meiosis[6]. The innermost layer of cells is called the *corona* or *coronal layer*. This layer expands and presents a radiant pattern as oocytes mature in response to exogenous hCG or a mid-cycle surge of LH. Near ovulation, as they loosen and expand, cumulus cells are observed to retract from the zona pellucida of the oocyte, presumably cutting off the previously important cellular-oocyte communication. It has been proposed that oocytes not

associated with proliferative cellular changes near ovulation have very limited potential for implantation, despite fertilization and apparently normal development *in vitro*[7].

In most mammalian species studied *in vivo,* the oocyte arrives at the site of fertilization in the ampulla of the Fallopian tube still surrounded by the cumulus mass. The cumulus may play a role in assisting transport of the oocyte into the Fallopian tube through fimbrial cilia-cumulus cell contact. Another possible use of the cumulus after oocyte maturation is that its radially arranged cells help to guide spermatozoa towards the oocyte just before fertilization; however, there is no hard evidence for this speculation. Break-up of the cumulus mass is brought about by dissolution of its mucoid hyaluronic acid matrix by enzymes released by the spermatozoa.

Follicular *membrana granulosa cells* disassociated from cumulus cells are found in follicular aspirates collected for IVF. The number of cells collected will vary from follicle to follicle according to the extent of negative pressure exerted during suction, the size of the needle and the overall maturity of the follicle. As with cumulus cells, thecorrelation between morphological aspects of free granulosa cells and oocyte nuclear maturity is not exact, but mature-appearing cells (large, well-dispersed cells) are generally collected along with mature oocytes, and immature-appearing cells (smaller, tightly packed cells) along with immature oocytes. Follicular membrana granulosa cells may be assessed at the time of oocyte harvest to aid in the evaluation of follicular maturity. They are subsequently often used during *in vitro* studies to examine metabolic activity or steroid synthesis.

The oocyte observed while its chromosomes are at metaphase I of maturation requires some time in culture before attaining full meiotic competence[8]. More than 98% of these oocytes will complete their journey towards metaphase II and first polar body extrusion. Oocytes with chromosomes at prophase I of maturation are truly immature; more than 80% of these will continue through metaphase I to metaphase II if isolated and incubated in an appropriate medium for 24 h.

Assessment of maturity

Traditionally, evaluation of oocyte maturity has been based upon the expansion and radiance of the cumulus-corona complex which surrounds the harvested oocyte[9,10]. With this assessment, oocytes are rapidly categorized as mature (correlated to metaphase II of maturation) when they possess an expanded and luteinized cumulus matrix and a radiant or *sun-burst* corona radiata. A less-expanded cumulus-corona complex denotes an intermediate stage of maturity (correlated to metaphase I of maturation), and absence of expanded cumulus is generally associated with immaturity (correlated to prophase I of maturation). While this type of analysis usually closely approximates the true nuclear status of the oocyte, it is too often imprecise, and may lead to subsequent laboratory errors in the handling of gametes. In fact, nuclear maturation of the oocyte and cellular maturation of the cumulus are frequently disparate[11,15]. When disparity occurs, immature oocytes may be inseminated prematurely, and fail to produce a favorable outcome. As well as fertilization failure, other detrimental side-effects accompany combining sperm and eggs at suboptimal times; ovulation-induction protocols may not be suitably appraised and male factor issues become difficult to interpret, based on poor fertilization results.

Because of these pitfalls, techniques have been developed to assess more accurately the meiotic status of the oocyte. A systematic approach can be used to produce a *maturation score* by grading the size of the follicle, expansion of the cumulus mass, radiance of the corona cells, size/cohesiveness of associated membrana granulosa cells and shape/color of the oocyte itself, if visible within the mass of surrounding cellular investments. Alternatively, frank visualization of the oocyte and its germinal vesicle or first polar body can be attempted by spreading out the cumulus mass, or by removing it altogether with the aid of enzymes.

If clearly visible or denuded of cells, oocytes are classified according to the presence or absence of first polar bodies/germinal vesicles, and are inseminated/injected accordingly:

Metaphase II (MII) First polar body present, no germinal vesicle; inseminated or injected 3–5 h after collection;

Metaphase I (MI) No first polar body, no germinal vesicle; inseminated or injected 1–5 h after extrusion of the first polar body;

Prophase I (PI) Germinal vesicle present; inseminated or injected 26–29 h after collection.

Our experience has been that oocytes collected at more advanced stages of *in vivo* maturation demonstrate the greatest ability to form two pronuclei after insemination[8,9,11]. Fertilization rates drop only slightly when oocytes require a period of 5–15 h in culture before extruding the first polar body, but fertilization is markedly reduced when more than 15 h pass before the maturational process is completed. The reason for this is probably related to sperm functionality as well as oocyte maturity, since processed sperm may be more than 24 h old before being placed with an early MI or PI oocyte. Under these conditions, the precise cause of the lower incidence of fertilization of very immature oocytes is difficult to interpret[8].

If small follicles are punctured, approximately 20–30% of oocytes collected for IVF are meiotically immature at the time of harvest from the ovary. This is undoubtedly due to the stimulation of multiple follicles during clinical ovulation induction, some large and well-vascularized, and some small with late recruitment. If all oocytes are placed with sperm at the same time, a proportion slightly higher than this percentage will fail to become fertilized normally. Logically enough, when oocytes are placed with sperm only as they have reached full maturity, far better fertilization results are attained.

The incidence of abnormal fertilization (one pronucleus, three or more pronuclei) is not different between MII oocytes and MI or PI oocytes that have matured in culture before insemination or injection[8,10]. Pregnancy potential after the transfer of preembryos developed from MII and MI oocytes is similar, regardless of whether 0 or 20 h has been required for maturation before insemination or injection[16]. Only preembryos developing from PI oocytes demonstrate a significantly reduced potential for implantation and live birth, although such births are certainly within the realm of possibility[17,20].

Metaphase II oocyte

The MII oocyte (Figure 1.1) is often termed *mature*, *ripened* or *preovulatory*, vague descriptions that fail to specify the exact meiotic status of the gamete. This oocyte is at a resting stage of meiosis II after extrusion of the first polar body and direct passage to

MII. Chromosomes are divided between the oocyte and the polar body (23 chromosomes, 46 chromatids, 2n DNA in each), those in the oocyte being attached to spindle microtubules[3] (Figure 1.2).

For a while after its formation, the first polar body remains connected to the oocyte by the meiotic spindle, forming a cytoplasmic bridge. Chromosomes within the first polar body may remain clumped together, may undergo a second meiotic division or may scatter within the cytoplasm; generally a nucleus is not formed[3,21]. The first polar body contains cortical granules because of its extrusion before sperm penetration and cortical granule release; in the oocyte, 1–3 layers of cortical granules are present at the periphery. Under the microscope, the oocyte is characterized by its round, even shape and displays an ooplasm of light color and homogeneous granularity. It is usually associated with an expanded, luteinized cumulus and a *sun-burst* corona radiata. Membrana granulosa cells harvested along with the MII oocyte are loosely aggregated, with mature features[8,10,14,20].

Metaphase I oocyte

The MI oocyte (Figure 1.3) is considered *nearly mature* or *intermediate* in maturation. The oocyte has completed prophase of meiosis I; the germinal vesicle and its nucleolus have faded and disappeared. During this stage a spindle forms, and recombined maternal and paternal chromosomes line up randomly towards the poles. Later, at telophase, whole chromosomes sort independently to oocyte or first polar body.

An MI oocyte requires 1–24 h in culture before reaching full maturity. Those needing less than 15 h are considered *late* in maturity, while those requiring more than 15 h are defined as early[8–11,14,15].

Under the microscope, the MI oocyte is characterized by the absence of both germinal vesicle and first polar body. A late MI oocyte is round and even in form, with homogeneously granular and light-colored ooplasm. Early MI oocytes may display minor central granularity. Mature-appearing cumulus cells are usually associated with late stages.

Because first polar body extrusion can occur at any time after harvest, it is necessary to examine the oocyte at regular intervals to determine the correct timing for insemination. If sperm are placed with the oocyte before nuclear and cytoplasmic maturation are complete, they generally fail to decondense within the ooplasm, or abnormal fertilization occurs. If insemination is delayed too long, *in vitro* aging may follow, with similar undesired consequences[3,8] (Figures 1.4 and 1.5).

Prophase I oocyte

The PI oocyte (Figure 1.6) is often termed *immature* or *unripened*. It possesses a tetraploid amount of DNA owing to the presence of 46 double-stranded chromosomes. This oocyte begins to mature in response to gonadotropin surges and reduction in follicular maturation-inhibiting factors. The germinal vesicle, which persisted throughout earlier growth phases, begins its progression to germinal vesicle breakdown (GVBD) and the oocyte enlarges. Most PI oocytes collected for IVF have been stimulated to resume meiosis, are in the final stages of the first meiotic prophase and have already reached full size. If a spermatozoon penetrates this immature oocyte, it will fail to promote activation