Reviews

Introduction to methods for collecting human gametes in assisted reproduction

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Abstract

The first objective of IVF is the collection of oocytes and spermatozoa. This article reviews the methods of choice for each sex. Collecting oocytes involves laparoscopic puncture and ultrasonographic puncture via transabdominal/transvesical and periurethral/transvesical, transvaginal methods. Male gametes are collected by masturbation, or by aspirating spermatozoa from the epididymis or from the testicle. Sperm collections can be taken from patients with disorders of ejaculation. A brief review of each approach is presented, with a critical analysis of each method.

Keywords: *folliculogenesis, gamete collection, IVF, ovarian stimulation, spermatogenesis, spermiogenesis*

Introduction

Male gametes have been collected for artificial insemination or intrauterine insemination, often using a donor (IUD) over many years. Most men produce semen samples with numerous active spermatozoa. Fewer are available in cases of oligozoospermia. This had led to recent developments in the collection of epididymal spermatozoa, and even testicular spermatozoa and spermatids that are usually injected directly into ooplasm.

An obvious first priority for IVF and its derivatives is to collect sufficient mature oocytes to provide embryos for transfer. In the absence of knowledge on human ovulation, oocytes were traditionally collected by isolating them from their follicles in pieces of excised ovaries by aspirating the follicles *in vitro* (Pincus and Enzmann, 1935; Edwards, 1965). The correct interval needed for oocyte maturation was timed as 37 h, and this interval was confirmed by the visual examination of rupturing follicles, and collection of mature oocytes from their follicles by laparoscopy (Steptoe and Edwards, 1970). The collection technique had evolved to aspirating the follicular contents and subsequently identifying mature oocytes microscopically. Since the early 1980s, ultrasound has evolved and has become the method of choice for collecting female gametes (Lenz *et al.*, 1981). However, it is first appropriate to consider the physiological process of folliculogenesis leading to ovulation and luteinization in the natural cycles.

Collection of female gametes

Natural folliculogenesis

Formation of the ovary

Following several rounds of mitotic amplification within the genital ridge of the developing fetus, oogonia enter meiosis at around the beginning of the second trimester of pregnancy and after their last round of DNA synthesis (Gougeon *et al.*, 1994; Edwards and Brody, 1995). Unlike spermatogenesis, in which stem cells persist throughout adult life and replenish the pool of developing spermatozoa, oogenesis has to supply the ovary with all germ cells required in the reproductive lifetime of a woman. This has resulted in the requirement for follicles to remain dormant but viable for up to 50 years, awaiting the signal to continue to ovulation and fertilization.

Of the many unique features of follicle development, one of the best studied and most important is meiosis, in which reductions divisions of the oocyte produce a haploid partner of the male gamete. The first stage of meiosis consists of an

This review will also be published in a Spanish textbook entitled *Modern Assisted Conception.*

extended prophase I, subdivided into leptotene, zygotene, pachytene, diplotene and diakinesis. In leptotene, the chromosomes condense and become visible, although the chromatid bivalents do not appear until pachytene. Until the exclusion of the first polar body at metaphase II, the cells are diploid (2N) with a 4C DNA content. By early pachytene, the chromosomes are shorter and thicker and genetic recombination has begun, which synaptonemal complexes visible. In the fetal human ovary, prophase I progress over a period of several weeks to halt at early diplotene, by which time the chiasmata are clearly visible. By the time of birth, virtually all oocytes are arrested in diplotene and in intimate association with a shell of somatic pregranulosa cells forming a pool of quiescent primordial follicles. The recruitment of any particular primordial follicle from the resting into the growing pool is unpredictable and occurs by unknown mechanisms. This process continues throughout life until the oocyte store is almost exhausted at menopause.

Migration from the pool is accompanied by an increase in size and in the synthesis of mRNA and proteins. Major transcriptional activity is associated with the zona pellucida genes, and the formation of a store of proteins that are utilized during the maturational stages on oocyte, or by the embryo after fertilization. Considerable amounts of ribosomal mRNA also characterize these stages of growth (Edwards and Brody, 1995).

Growth of ovarian follicles

There are approximately 2×10^6 primordial follicles in the human ovary at birth. Most of these are destined to become atretic. In a fertile woman having regular monthly cycles for 35 years, only 400 or so ovulatory follicles would be expected to develop to ovulation. The most dramatic changes in follicle growth are seen in the 2 weeks prior to follicle rupture. Some women suffer from premature ovarian failure, with a lack of follicles often stemming from genetic causes (Edwards, 2001).

The human primordial follicle measures 0.05 mm in diameter. By day 1 of the menstrual cycle, the growing follicle has developed to antral stages and is 4 mm in size. It contains 1×10^6 granulosa cells. By the time of ovulation, the follicle has expanded to 20–25 mm and contains 50×10^6 granulosa cells.

After its formation in the fetal ovary, the precursory primary follicle contains an oocyte surrounded by a single layer of spindle-shaped cells. Primordial follicles grow to contain one ore more layers of recognizable granulosa cells, without any fluid-filled antrum. Secondary follicles expand, and contain variable volumes of antral fluid that increase markedly as the follicle enlarges to become a Graafian follicle and approaches ovulation.

Certain later characteristics of folliculogenesis are of essential importance in assisted human conception (Gougeon *et al.*, 1994). Follicle recruitment, which normally occurs during the first few days of the primate ovarian cycle, is the process whereby follicles continue to mature in a correct gonadotrophic environment. This hormonal support is essential for continued progress towards ovulation. Recruited follicles correspond to small antral follicles. Follicle selection involves physiological mechanisms by which a single follicle is chosen to develop further, avoid atresia and achieve ovulation. Dominance involves a series of processes by which the selected follicle maintains its pre-eminence over other follicles. This period occupies days 8–12 of a human ovarian cycle. Follicles expand to reach diameters approximating to 2 cm in human ovaries, when Graafian follicles are ready for an ovulating surge of LH. After ovulation, the corpus luteum functions until the end of the menstrual cycle.

Many growing follicles fail to survive, undergoing apoptosis via the action of several genes along this pathway. These include *bcl-2* and the caspase genes (Reed, 1997). Follicles that have been exposed to the optimum sequence of FSH and LH avoid atresia. New methods are approaching whereby the occurrence of apoptosis in follicles can be greatly reduced, delaying their utilization and so increasing the numbers persisting at the approach to menopause. This approach involves control of apoptotic pathways, e.g. by means of sphingosine-1-phosphate (S1P) to reduce levels of the apoptotic gene functional acid sphingophosphodiesterase (Perez *et al.*, 1999). This approach may one day lead to the delay of the menopause years.

During the luteal phase, gonadotrophin concentrations are low due to negative feedback effects of oestradiol and progesterone; at this stage all follicles >4 mm are atretic. Further follicular development can only occur following removal of the corpus luteum or the administration of exogenous gonadotrophin. Such evidence suggests that although small antral follicle development requires low concentrations of FSH, larger amounts are necessary to promote subsequent growth. This development may occur once a threshold level of FSH has been attained and a 20% increase in the concentration of FSH, as might occur in the early proliferative phase, is sufficient to stimulate follicular development beyond 4 mm. Granulosa aromatase activity results in high local concentrations of oestradiol-17β in the selected follicle and dominance is achieved by inhibiting FSH secretion with oestradiol and inhibin. In addition, the secretion of a follicle regulatory protein by the dominant follicle may impair the sensitivity of other follicles to gonadotrophin stimulation (de Ziegler and Frydman, 1990).

Although folliculogenesis is heavily dependent on FSH and LH, its early stages are probably independent of cyclical gonadotrophin concentrations until the follicle reaches the small antral stage. Thereafter, further development will only occur when FSH concentrations rise above threshold levels, permitting only those follicles at the appropriate stage of maturity to proceed. The interval during which FSH remains elevated above threshold value can be seen as a gateway through which a follicle must pass to avoid atresia on the path towards ovulation. The width of the gateway therefore determines the number of follicles that can be selected for ovulation.

In natural cycles, the pre-ovulatory LH surge is triggered by the positive feedback of oestradiol from the dominant follicle. This action has been reproduced using exogenous oestrogen in women. However, it is likely that other follicles also contribute to the LH triggering mechanism, as an LH surge is initiated even when serum oestradiol concentrations differ widely between women. The rate of change among various endocrine

parameters, e.g. rising oestrogen concentrations, and alterations in the synthesis of inhibitors such as inhibin, may also have unique roles in follicular formation and selection.

On the other hand, considerable variation exists in the time of ovulation in successive cycles even in the same patient. Most studies estimate that ovulation occurs 10–12 h after the LH peak and 24–36 h after the oestradiol peak. Steptoe and Edwards (1970) and Taymor *et al.* (1983) observed that follicle rupture and ovulation do not begin until 36–38 h after the onset of the LH surge. Subsequent to the LH surge, tissue concentrations of cAMP increase to initiate the completion of meiosis and maturation of the oocyte. These are the events triggering luteinization of granulosa cells. They overcome local non-steroidal inhibitors of meiosis. Together with progesterone, such ovulatory stimuli enhance the activity of proteolytic enzymes that digest collagen located in the follicular wall. The LH surge promotes the synthesis of progesterone by granulosa cell and the prostaglandins E and F, which may act to digest the follicular wall or to stimulate contraction of smooth muscle fibres located in the theca externa of the follicle, causing oocyte expulsion and ovulation.

In conclusion, the timing of oocyte retrieval in women undergoing IVF depends on a closely coordinated series of ovarian systems. Knowledge of the precise time interval between the luteinization stimulus and oocyte collection, typically 34–38 h to allow final maturation of the oocyte, enables the moment of aspiration of ovulatory follicles to be predicted well in advance of their rupture.

Stage of meiosis during oocyte growth and maturation

Throughout its growth up to the pre-ovulatory Graafian stage, the oocyte remains in diplotene, until it is stimulated to undergo meiotic maturation by the onset of the LH surge. The competence of oocytes to resume meiosis is indicated by a rim of chromatin around the prominent nucleolus in the germinal vesicle. This nucleus migrates to the oocyte periphery, indicative of the actions of polarizing factors at this stage of growth. It progresses through germinal vesicle breakdown, through diakinesis until metaphase I. The first meiotic spindle forms at the oocyte periphery and chromosomes align on the equatorial plate prior to separation of the centromeres and separation of the chromosomes. As a result of the eccentric location of the spindle and subsequent plane of cleavage, a small polar body containing a haploid set of chromosomes is expelled.

The second division of meiosis commences immediately without an intervening S phase. The cell proceeds to metaphase II, at which it arrests again. Fertilization or parthenogenetic activation triggers its movement to anaphase-I. Segregation of chromatids between oocyte and second polar body and telophase-I produces a haploid complement of chromatids on oocyte and polar body. The interval between the activation of the follicle by the gonadotrophin surge and metaphase II is consistently around 36 h, and approximately co-ordinated with the rupture of the follicle.

Meiotic disorders can result in anomalous forms of chromosome segregation into daughter cells. Two such consequential anomalies are the formation of hyperhaploid (i.e. >23 chromosomes) and hypohaploid complements in the sperm head (**Table 1**). Structural chromosomal anomalies have several causes, including the complex forms of inheritance of translocations.

Table 1. Chromosomal anomalies in human spermatozoa capacitated in two different media for 24 h (Benet *et al.*, 1991).

	BWW medium with HSA buffer data	*Test-yolk*	*Combined (%)*
No. of spermatozoa	126	138	264
Haploid (23)	99	102	201 (76)
<23 chromosomes	14	19	33 (13)
>23 chromosomes	2	4	6 (2)
Structural anomalies	11	13	24 (9)

HSA = human serum albumin.

Ovarian stimulation for IVF

After the clinical introduction of IVF, various forms of ovarian stimulation were utilized to stimulate the development of several follicles. Treatments included clomiphene or human menopausal gonadotrophins alone, or in combination. This situation changed with the introduction of the gonadotrophin-releasing hormone (GnRH) agonists and antagonists, and the availability of recombinant gonadotrophins.

Methods of ovarian stimulation

At present, the most commonly practiced ovarian stimulation regimen for IVF includes pituitary down-regulation with a GnRH agonist for at least 2 weeks followed by the co-administration of high-dose exogenous gonadotrophins (Perl *et al.*, 1987). This so-called 'long protocol' aims to induce ongoing growth of multiple dominant follicles in normo-ovulatory women. Despite intensive monitoring, a concomitant risk of complications remains.

Short-term risks include ovarian hyperstimulation syndrome and higher order multiple pregnancies arising from the transfer of multiple embryos. Potential detrimental effects of stimulating larger numbers of follicles on oocyte quality and fertilization rates cannot be ruled out. High plasma concentrations of oestradiol arising as a result of these regimens might possibly reduce the rate of embryo implantation, although this possibility seems unlikely after several years experience with these regimens. With regard to long-term complications, ovarian hyperstimulation could be associated with ovarian cancer, although epidemiological studies have yet to confirm such an association.

In parallel with development of GnRH agonist, other analogues were synthesized that also bind to the pituitary GnRH receptors but do not function to induce the release of gonadotrophins. These compounds are far more complex than GnRH agonists, with greater modifications to their molecular structure. The pharmacological mechanism by which GnRH antagonist suppresses the liberation of gonadotrophins differs from the action of GnRH agonists (Chillik and Acosta, 2001).

Agonists act through a release of hormones and down-regulation of receptors leading to the desensitization of the pituitary gonadotrophic cells. Antagonists, in comparison, bind competitively to the GnRH receptors and thereby prevent endogenous GnRH from exerting its stimulatory effects on the pituitary cells. Since antagonists posses no intrinsic activity, the initial 'flare-up' common with agonist treatments is completely avoided. Within hours, the amount of gonadotrophin secretion decreases sharply. This mechanism of action depends on the equilibrium established between endogenous GnRH and the applied antagonist. Consequently, antagonists have an action that is highly dose dependent, in contrast to the agonists.

It still too early to speculate about the possible end of the agonist 'era'. GnRH agonists are valuable, safe and well-tested pharmaceutical tools, fitting well within controlled ovarian stimulation protocols. Safety and efficacy of the antagonists remain to be proven in long-term studies.

No agreement exists regarding the optimal number of oocytes required for IVF. Individual responses to standard treatment vary greatly. In most patients, numbers vary between seven and 20 oocytes. Cryopreservation of supernumary embryos and later thaw and transfer in subsequent cycles is often considered to justify the stimulation of so many follicles. Nevertheless, the cryopreservation of excess embryos can give rise to complex ethical, religious and legal considerations, e.g. if the patients separate or disagree about their embryos. The possibility of cryopreserving supernumerary oocytes rather than embryos, has been proposed as a means of circumventing these issues. New freezing methods such as vitrification might prove to be effective in this respect. Maternal age is a major factor in a diminished ovarian reserve, a condition usually measured by the FSH test, i.e. measuring concentrations of this hormone in the early follicular phase. A diminished ovarian reserve is also associated with higher levels of pregnancy loss in older women (**Table 2**).

Follicular environment in women with endometriosis

An oocyte of reduced quality may be the consequence of an altered folliculogenesis. It is known that follicular fluid taken from patients with endometriosis contains soluble factors that influence the growth pattern of different tissues (Bahtiyar *et al.*, 1998). Considerable evidence from natural cycles supports the concept that patients with endometriosis differ to controls.

Table 2. Diminished ovarian reserve and pregnancy loss according to age among 9802 patients (Levi *et al.* 2001).

Age (years)	*Patients with DOR*			*Normal patients*
	Pregnant	*Loss (n)*	*% loss*	*% loss*
<35	7	4	57	16
35–45	11	7	64	14
>40	10	9	90	33

DOR = diminished ovarian reserve.

The duration of their follicular phase is longer in patients with endometriosis than in controls. Reductions in secretion of LH have been described in these patients. Recent studies have also shown that aromatase activity and progesterone accumulation *in vitro* are impaired in cells derived from patients with mild endometriosis, as compared with controls (Harlow *et al.*, 1996).

Impaired fertilization has been suggested as a possible mechanism of infertility associated with endometriosis. However, the most recent evidence suggests otherwise when only normozoospermic partners are considered. No significant differences were found in fertilization rates of oocytes from women with endometriosis versus those of patients with tubal factor or unexplained infertility (Geber *et al.*, 1995). On the other hand, a recent retrospective analysis (Hull *et al.*, 1998) revealed a small but significant reduction in fertilization rates in couples with endometriosis as compared with more with tubal infertility, these rates being similar with spermatozoa from either the husband or a donor. Furthermore, the presence of endometriosis does not affect fertilization in couples undergoing intracytoplasmic sperm injection (ICSI) due to severe male infertility (Minguez *et al.*, 1997).

Low oocyte quality in women with endometriosis is reflected in ovulation defects, lower response to IVF, poor quality embryos, and lower implantation rates. When good quality oocytes are fertilized or when a higher number of embryos are transferred, implantation rates return to control values. Thus, a good quality embryo may overcome the slight decrease observed in endometrial receptivity.

Aspiration of human oocytes from their follicles

Laparoscopic puncture

Laparoscopy was the classical method introduced by the pioneers of the IVF technique (Steptoe and Edwards, 1970). Today the indications of follicular aspiration by the laparoscopic route are practically limited to cases in which visualization of the pelvic organs is desired simultaneously with intratubal gamete transfer (GIFT) or intratubal zygote transfer (ZIFT), i.e. cases of sterility with no apparent cause or of possible pelvic endometriosis.

General anaesthesia with endotracheal intubation is frequently required, with pneumoperitoneum performed with CO_2 plus a triple puncture. The ovaries are grasped with special forceps, pulled and manipulated so that each follicle is punctured with a double lumen needle connected to a tube by Teflon tubing. The follicular content is aspirated and the follicular fluid collected is examined under the microscope for the identification of oocytes and of their degree of maturity. The patient is hospitalized for 24 h.

Laparoscopy presents inconveniences such as surgical and anaesthetic morbidity, requiring a considerable support apparatus for its execution.

Ultrasonographic puncture

In 1981, Lenz *et al.* used ultrasound with a guide for the

puncture needle and aspirated the ovarian follicles by the percutaneous and transvesical route. The needle used for follicular puncture penetrates the pelvis through the abdominal wall, crossing the anterior and posterior wall of the bladder previously filled with 300–400 ml of physiological saline. Patients can be managed on an ambulatory basis, since slight sedation and local anaesthesia were sufficient for the process of oocyte collection in most cases (Wikland *et al.*, 1983).

This technique represented a great advance in the simplification of oocyte collection, permitting follicular aspiration in cases of pelvic adhesions, a fact that, in principle, contraindicated the use of laparoscopy. However, the technique still presented some drawbacks such as the difficulty in visualizing the tip of the needle, which penetrated obliquely in relation to the transducer and to the ovarian axis. The technique was also painful, since it was not possible to anaesthetize the posterior bladder wall and the visceral peritoneum. There were also some complications, especially haematuria after puncture. At the time, rates of oocyte retrieval were about 57% per puncture and no serious complications were observed during the procedure, except for transitory haematuria in 10% of patients.

Perurethral/transvesical ultrasonographic puncture

In 1985 Parsons *et al.* initiated the collection of oocytes by the urethral route. In this procedure, the bladder is filled with saline solution and, after bladder intubation with a Foley catheter, a needle is introduced through the catheter itself into the bladder. This is transfixed, and the needle penetrates the ovarian follicles. The discomfort of bladder repletion and haematuria, as well as the pain occurring after the procedure, led to a decline in the use of this route after the advent of the vaginal transducer.

Ultrasonographic puncture: transvaginal

Finally, the introduction of the vaginal route was suggested by Gleicher *et al.* (1983) in order to reach the ovaries high in the pelvis or fixed behind the uterus by adhesions. Since stimulated ovaries are increased in size, this would cause them to be located more frequently on the bottom of the sac, to which transvaginal puncture would have better access. However, puncture with the free hand through the fundus of the vaginal sac did not prove to be easy in all cases.

In 1986, Feichtinger and Kemeter introduced the vaginal transducer, to be used as a support for the puncturing needle. Compression of the fundus of the lateral and posterior vaginal sac by the vaginal transducer permits the puncturing needle to approach the ovary, increasing the efficiency of the collection process. Vaginal transducers of 5–7.5 MHz permitted to obtain a better image of the pelvis, facilitating the visualization of the follicles and the identification of other structures, especially blood vessels. The bladder does not need to be full, with consequent reduced discomfort and easier access to the ovaries fixed in a superior position in the pelvis. The needle is coupled to the transducer, permitting the surgeon himself to direct it for follicle puncture, aspiration and washing. Several 17–18 gauge needles are used, with a single or a double lumen, and aspiration can be performed with a 10 ml syringe directly coupled to a Falcon tube or linked to it through Teflon tubing and to a controlled aspiration pump such as a Craft pump. Rates of oocyte retrieval are high, and anaesthesia can be given locally or preferably be accompanied by sedation.

Low-quantity vaginal haemorrhage is one of the most common complications of transvaginal oocyte recovery in IVF. It occurs in 8.6% of all punctures and is susceptible to local treatment such as simple compression. In some cases, this bleeding may be due to a lesion of the ovary originating in haematoma, while in other more serious cases it may cause haemoperitoneum. Cases have been described of lesions of iliac vessels following both methods of approach, transvesical and transvaginal, which may require laparoscopy or emergency laparotomy (Coroleu *et al.*, 1997).

Pelvic infection represents the second most common complication. It may be produced in various ways: by direct inoculation of vaginal micro-organisms, by the reactivation of an existing infectious focus, or by damage to an intestinal loop. One of the most difficult situations is the superinfection of an endometriotic cyst, a rich culture medium for pathogenic bacteria. Prophylactic administration of antibiotics to patients undergoing puncture is required in order to prevent this pathology.

This is currently the technique most frequently used, with reduced costs and execution on an ambulatory basis. Also, it is easy to learn and can be repeated more frequently.

Collection of male gametes

Spermatogenesis and spermiogenesis

Brief outline of spermatogenesis

This brief outline describes the basic aspects of spermatogenesis and spermiogenesis. The testis fulfils a double function similar to that of the ovary, producing both sex hormones and gametes in distinct parts of the gonad. The testis consists of seminiferous tubules embedded in connective tubule tissue, which contains interstitial cells. The seminiferous tubules are responsible for the production of gametes (spermatogenesis), while interstitial cells produce hormones within the tubules, Sertoli cells form and regulate the growth and differentiation of the germ cell and developing spermatid. The primary stages of testis formation, including germ cells, Sertoli cells and Leydig cells have been described by Josso and Picard (1986) and Edwards and Brody (1995).

Germ cells do not divide until after puberty. Spermatogonia are diploid, and situated close to the basement membrane. They multiply mitotically, either to produce other spermatogonia or enter differentiation to produce primary spermatocytes. Spermatogonia are divided overall into three types (A-A0 A1-A2 A3-A4, intermediate and B). Spermatogonial mitoses occur either at random (confined to A0) or in a synchronized manner where their divisions are coordinated to the spermatogenic cycle. In the latter divisions, cytoplasmic links persist between daughter cells after each division. Type B spermatogonia are the precursors of spermatocytes. Mitotic kinetics of these divisions in the human testes are complicated, and are still a matter of controversy.

Spermatocytes divide by meiosis to form four spermatids. Major genetic consequences arise during the random separation of homologous chromosome, and as a consequence of the crossing over of genetic material. These two events produce the genetic diversity required for the survival of the species.

Prophase is very protracted and divided into stages: leptotene, zygotene, pachytene, diplotene and diakinesis. As spermatocytes enter leptotene of the first meiotic prophase, they move from the basement membrane towards the lumen of the seminiferous tubule. During zygotene (where the homologous chromosome pair), they become separated from the basement membrane by the blood–testis barrier. Pachytene is the most prolonged stage of meiosis, and the time when spermatocytes are highly susceptible to physical or mutational damage during crossing over and gene recombination (de Rooij, 1989; Edwards and Brody, 1995). Each primary spermatocyte produces two secondary spermatocytes, which have a very short duration. Each of these in turn divides to form two haploid spermatids. The behaviour of the sex chromosomes during meiosis differs from that of the autosomes in the timing of DNA replication, condensation and transcription.

Numbers of Sertoli cells decline with increasing paternal age (**Table 3**). This relationship holds in relation to their unit numbers in parenchyma, and to total numbers in the testis. The same situation also applies to spermatocytes in varying stages of their development. Consequently, the ratios between numbers of Sertoli and meiotic cells remain fairly constant with increasing age (**Table 3**).

Table 3. Changing seminal characteristics in ageing men (Johnson *et al.*, 1984).

Seminal characteristics	*Age (years)* *20–40*	*50–55*
Sertoli cells		
Weight of parenchyma (g)	37	34
No. per g parenchyma ($\times 10^6$)	26.4	18.0
No. per testis ($\times 10^6$)	502.7	312.0
Germline cells		
Spermatocytes in pachytene/diplotene (*n*)	40.8	29.6
Spermatids with round nuclei (*n*)	55.4	41.1
Ratio germline/Sertoli cells		
Spermatocytes in pachytene/diplotene	1.6 ± 0.1	1.7 ± 0.1
Spermatids with round nuclei	2.2 ± 0.1	2.4 ± 0.2

Spermiogenesis

The stage includes the development of spermatids from meiosis to the detachment of the spermatozoa from the Sertoli cells (Eddy 1988; Eddy *et al.*, 1991). It is a truly remarkable phase, involving highly differentiated spermatozoa undergoing complex morphological, physiological and biochemical modifications. It requires approximately 22 days in humans.

This complex process of differentiation is poorly understood. It involves unique events that are tightly synchronized and integrated so that very small deviations are likely causes of infertility. These deviations are noted in mature spermatozoa and include variation in morphology, motility and function. In many animals, the young spermatids are associated in the seminiferous tubules with an older generation of spermatids formed one cycle earlier. Initially a simple cell, with major modifications in the basic cytoskeleton leads to the emergence of a highly differentiated spermatozoon (de Kretser and Kerr, 1988). Nuclear chromatin becomes highly condensed. Cisternae of the Golgi apparatus, which are more complex than in other cells, fuse to form an acrosome. This structure then migrates to the nucleus and covers the nuclear surface as it forms the acrosome system. The inner membrane of the acrosome is closely associated with the nuclear envelope. This is one of the few secretory structures which becomes intimately associated with a nucleus.

Two centrioles migrate to opposite poles of the developing acrosome. One assumes a radial alignment and develops into the axoneme of the future tail. The other, located at right angles, forms the connecting piece that joins the tail to the nucleus. The axoneme of the tail elongates, and becomes attached to the base of annulus. Mitochondria divide, although the mechanism is unknown, and then associate with the outer dense fibres to form a characteristic double helix. Several structures have a transitory expression during spermatogenesis including the manchette and the chromatoid body. The latter may be associated with the storage of genetic material.

When spermatogenesis is complete, cytoplasmic extensions to Sertoli cells are broken, and spermatozoa are released. In some cases, up to 70% of the cytoplasm of the mature spermatid is shed from the developing gamete and may be phagocytosed by the Sertoli cells.

Post-testicular sperm maturation and transport in the excurrent ducts

On release from the seminiferous epithelium of the testis, the spermatozoon has completed its striking morphological transformation from a round undifferentiated germ cell. It is near to the end of a developmental process which has already taken already about 64 days. It makes its final journey out of the testis and along the highly convoluted, epithelial-lined tubules of the vasa efferentia, epididymis and vas deferens before eventually passing out of the body (via the urethra) at ejaculation. This excurrent duct system is much more than a passive conduit between the testis and the outside (Cooper, 1986). Not only does it maintain the viability of spermatozoa for a sojourn that might last several weeks, but it also promotes the development of their fertilizing capacity, the acquisition of full motility and an ability to bind to and penetrate the ovum.

Protective functions of the epididymis in rats include an anti-microbial action encoded genetically and produced in the caput epididymis (Li *et al.*, 2001).

The passage of spermatozoa along the excurrent duct is associated with a number of distinct physiological and biochemical processes on the gamete and the tissues of the tract (Edwards and Brody, 1995). These include (in order from the testis) resorption of fluid secreted by the testis, the development of sperm fertilizing capacity and finally sperm storage and ejaculation. Many such processes are mediated by the secretory and absorptive activity of the epithelial lining of the excurrent duct, which, in turn, is under the control of androgens.

Perhaps the most conspicuous change to spermatozoa passing along the epididymal lumen is the potential for flagellar movement. This involves a sustained progressive motility in spermatozoa, induced by the appropriate medium. For men of proven fertility, the best assessment of this aspect of sperm maturation has been made by examining samples of luminal contents recovered from various regions of epididymis of volunteers undergoing vasectomy under general anaesthetic. In these cases, most caput spermatozoa recovered remain immotile or display weak twitching of the tail in a modified Tyrode's medium; just 3% display forward motility (Cooper *et al.*, 1991). By contrast, >60% of cauda spermatozoa are progressively motile. Equally important to changes in motility, epididymal spermatozoa also acquire the ability to recognize and bind to oocytes.

These critical functional alterations to spermatozoa during epididymal transit are accompanied by numerous physiological and biochemical modifications. Some of these maturational changes are probably 'housekeeping' to ensure that the cell remains viable during its stay in the excurrent ducts. For example, alterations in the lipid composition of the sperm membranes may help to stabilize them for prolonged storage. Likewise, the movement of the cytoplasmic droplet along the flagellum during maturation does not impart an obvious benefit on human spermatozoa in terms of fertility. Yet the sequestering of redundant enzymes in such vesicles may help to protect the cell. A number of biochemical changes may be directly associated with the development of full sperm fertility (Eddy *et al.*, 1991). Structural stabilization of the dense fibres of the tail with disulphide bonds probably leads to a more rigid movement of the tail that enables mature spermatozoon to become progressively motile A similar stabilization occurs in sperm chromatin, thereby hardening the nucleus for penetration through the zona pellucida and perhaps in preparation for pronucleus formation in the zygote.

Determinants on the plasmalemma are also modified. They may undoubtedly enable spermatozoa to recognize and bind to the zona surface of the egg, as a prelude to fertilization. It is now believed that spermatozoa acquire specific receptors on the plasma membrane overlying the acrosome during the final stages of epididymal maturation. These receptors engage with complementary ligands on the zona surface to initiate binding and probably the acrosome reaction of the fertilizing spermatozoon.

The acrosome reaction in relation to sperm transport

The acrosome reaction is an exocytotic process involving fusion of sperm plasma membrane and outer acrosomal membrane (Edwards and Brody, 1995). Only acrosome-reacted spermatozoa can penetrate the zona pellucida. Oehninger *et al.* (1994) studied the acrosome reaction and its prerequisite, a calcium influx, in spermatozoa of infertile men with a high incidence of abnormal sperm forms. They concluded that infertile patients with a high incidence of abnormal sperm forms as diagnosed by strict criteria have a low incidence of spontaneous acrosome reactions and a diminished progesterone-stimulated acrosome reaction. In contrast, the non-specific response to a calcium ionophore is conserved.

Liu and Baker reported on patients with severe teratozoospermia and the inability of their spermatozoa to undergo the acrosome reaction in the presence of zona pellucida, in contrast to those from controls (Liu and Baker, 1994). This observation is important and helps to explain the poor fertilization potential of spermatozoa taken from some of these patients *in vitro*.

Collection and isolation of spermatozoa for assisted conception

Sperm collection from the ejaculate

Semen is habitually collected for artificial insemination, IVF or ICSI, by masturbation into a sterile glass or plastic flask after a period of sexual abstinence ranging from 2 to 4 days. However, Gerris (1999) believes that semen collection into silicon media during sexual intercourse produces superior samples compared with those obtained by masturbation. In cases of retrograde ejaculation, diabetic neuropathy, and major pelvic surgery, for example, spermatozoa must be collected in urine. In this situation, sodium bicarbonate at a concentration of 2 g is taken at bedtime and a further 2 g is taken in the morning, so adjusting urinary pH to 7. After masturbation, urine is collected into a sterile and atoxic flask and spermatozoa are separated by centrifugation.

When this procedure is not possible, other methods of collection are used, including electrical or vibratory direct stimulation on the prostate or penis, puncture of the spermatic ducts, or testicle biopsy. Occasionally, the ejaculate can be collected in a fractionated manner into a dish containing culture medium, especially in cases where antibodies against spermatozoa are a risk factor to assisted conception.

The absence of spermatozoa in semen, i.e. azoospermia, may arise in 10% of men seeking an infertility clinic. A correct diagnosis of this condition requires the examination of at least two ejaculates. Spermatozoa are centrifuged at higher speeds than normal (i.e. 1800 *g* for 5 min), and the pellet must be examined in detail. In this way, cases of oligo- or asthenozoospermia can be identified. Typical characteristics of such patients are shown in **Table 4**. In severe cases of oligo/azoospermia, it is necessary to collect spermatozoa using surgical procedures such as testicular puncture or biopsy.

Table 4. Seminal characteristics in successive semen samples of azoospermic and asthenozoospermic men (Purvis *et al.*, 1989).

	Normal	*Oligo/azoospermia*		*Asthenozoospermia*	
		First	*Second*	*First*	*Second*
Sperm count ($\times 10^6$)	442	26	24	293	266
Living (%)	77	58	64	66	57
Abnormal (%)	32	73	76	52	53
Motile (%)	77	19	25	54	52
Progressive motility[a] (%)	36	8	9	21	18

[a]Velocity >15 µm/s.

Sperm collection from the epididymis

Several collection techniques are indicated in cases of azoospermia. These include microsurgical sperm aspiration from the epididymis (MESA), percutaneous sperm aspiration from the epididymis (PESA), biopsy and sperm extraction from the testicle (TESE), and percutaneous sperm aspiration from the testicle (TESA). The choice of method depends on various factors such as available facilities for cryopreservation and characteristics of each case, and especially on the experience of the surgeon with these techniques.

MESA is an invasive technique, indicated in principle for cases in which a possibility exists for the microsurgical correction of azoospermia. When vasoepididymostomy cannot be performed, MESA can be used to collect a sample containing sufficient spermatozoa for cryopreservation. In general, when obstructive azoospermia is due to vasectomy, the most frequent cause, the treatment of choice is a reversal of vasectomy. The rate of success of reversal is 50%.

In cases of epididymal obstruction due to infection by *Chlamydia trachomatis* species or *Neisseria gonorrhoea*, results of surgical treatment do not exceed a 20% pregnancy rate. In this situation, sperm collection from the epididymis followed by ICSI yields satisfactory results. In general, there is no difference between the incidences of fertilization or pregnancy after ICSI, whether performed with cryopreserved or fresh spermatozoa (Tournaye *et al.*, 1999).

When surgical reconstruction is not indicated, MESA can be performed, and a large number of spermatozoa can be collected for cryopreservation. A less invasive option such as PESA is indicated, since it requires local anaesthesia and patients can be discharged soon after collection. Collins *et al.* (1996) believe no difference in sperm recovery exists between MESA and PESA. Friedler *et al.* (1998) report a similar prognosis after ICSI for cases of obstructive azoospermia, in which cryopreserved spermatozoa are collected by PESA or MESA.

PESA can be executed several times in the same epididymis. Its major disadvantage is the possible occurrence of local fibrosis. The results of PESA followed by ICSI are satisfactory, and vary mainly as a function of the age of the women (**Table 5**).

Table 5. Clinical and laboratory results of sperm collection by TESE or PESA followed by ICSI at the Human Reproduction Centre, Sinhá Junqueira Maternity Foundation, Ribeirão Preto, Brazil.

	TESE	*PESA*
Cycles (*n*)	102	127
Patient age (years)	33.4 ± 5.1	31.8 ± 5.2
No. of aspirated oocytes	9.26 ± 6.0	10.9 ± 6.9
No. of MII oocytes used for ICSI	7.20 ± 4.5	8.6 ± 5.2
Fertilization rate (%)	44 ± 28.2	62.2 ± 24.6
Embryos transferred (mean)	2.5 ± 1.2	2.8 ± 1.2
No. of pregnancies	23	37
Pregnancy rate per aspiration (%)	22.5	29.1
Pregnancy rate per transfer (%)	28	30.1
Implantation rate per embryo (%)	8.7	14.9

Sperm collection from the testicle

TESE has been used for many years as part of the differential diagnosis of azoospermia. The observation of abnormal spermatogenesis during this procedure would lead to a diagnosis of non-obstructive azoospermia. Testicular biopsy is an efficient method for sperm collection for ICSI. The procedure is usually ambulatory, requiring the use of a local anaesthetic. However, in some cases, it can cause great discomfort in more sensitive patients. The major acute complications are scrotal haematoma and infection. On a long-term basis, patients should be monitored for the onset of antibodies against spermatozoa, and for testicular fibrosis and calcifications followed by testicular devascularization (Schlegel and Su, 1997).

TESA (puncture with 21-gauge needles) is a procedure more indicated than testicular biopsy in cases of normal spermatogenesis. In general, samples collected by TESA contain a sufficient quantity of testicular tissue for the ICSI procedure and subsequent preservation of excess spermatozoa. Tournaye *et al.* (1998) believe that in cases of normal spermatogenesis there is no difference in sperm collection by TESE or TESA.

Approximately 20% of the men searching for an infertility clinic have azoospermia due to faulty spermatogenesis or primary testicular insufficiency. Histology may reveal arrested maturation, germ cell aplasia (Sertoli cell-only syndrome) or tubular atrophy and sclerosis. Levin (1979) also showed that in some cases the histological pattern may not be uniform, i.e. the arrested maturation or the Sertoli syndrome may not involve all tubules, with the presence of focal spermatogenesis being observed in some of them. This fact has led many specialists to try to remove testicular samples from several regions in cases of primary testicular insufficiency.

Nevertheless, no decisive prognosis is possible with respect to the chances of collecting male gametes for ICSI in cases of non-obstructive azoospermia. Cases of Klinefelter syndrome (47, XXY) with sperm collection followed by births have even been reported (Palermo *et al.*, 1998). Today, the use of collagenase to dissolve testicular tissue may facilitate the identification of spermatozoa in the sample. The use of TESA in cases of non-obstructive azoospermia yields discrete results. Some investigators indicate a biopsy under the microscope so that the tubules with the best characteristics and therefore presenting the highest chance of sperm collection may be identified by dissection. In azoospermic patients, a bilateral testicular biopsy increased the detection of focal spermatogenesis in 68% of cases compared with a unilateral approach (Plas *et al.*, 1999). The rate of oocyte fertilization with spermatozoa obtained from the epididymis is usually slightly higher than that obtained with spermatozoa from the testicle (Watkins *et al.*, 1997), as confirmed by our data (**Table 1**).

Use of spermatids as gametes

Recently, a few reports in the literature have described ICSI with immature sperm cells, including round spermatids. Fertilization and pregnancy rates, however, generally remain far below those obtained with mature spermatozoa and elongated spermatids. The use of round spermatids in ICSI raises several concerns such as DNA immaturity, genomic imprinting, normality of the centrosome and presence of sperm-derived oocyte activation factor.

Furthermore, the correct identification of the round spermatids within a heterogeneous population of testicular cells has not received enough attention. Identification of round spermatids is problematic when conventional Hoffman modulation contrast systems were used on the inverted microscope. If appropriate phase-contrast optics are used on an inverted microscope reliable recognition of the round spermatids in a cell suspension smeared at the glass bottom of a dish is possible. However, exploration of several biopsies from patients with non-obstructive azoospermia has never revealed round spermatids despite extensive research (Silber and Johnson, 1998).

Sperm collection from patients with disorders of ejaculation

The absence of ejaculation can occur for different reasons such as spinal lesions, psychosexual dysfunctions, diabetes mellitus and multiple sclerosis. Traumatic injury to the medulla resulting from car accidents, falls, and sport or violent activities is a very frequent cause. Lesions of the spinal medulla affect a young male population (16–35 years), leading in most cases to the inability to ejaculate during intercourse. Thus, the partner will become pregnant only with the application of assisted ejaculation methods.

The use of penile vibratory stimulation (PVS) in combination with ICSI may solve these cases in a non-traumatic manner. In general, patients with a recent injury (<18 months) do not respond rapidly to PVS. Urine alkalinization is indicated, since the patient may ejaculate urine together with spermatozoa or have retrograde ejaculation. The bladder must be drained and 25–50 ml physiological saline is instilled about 10 min before PVS. Through a reflex pathway, PVS can cause increased arterial pressure, sudoresis, shivering and headache, usually in patients with lesions at the T6 level or above. Application can be performed at 5-min intervals; the condition of the glans skin is observed before restarting and usually a total of 15 min of PVS are applied per session.

Even when anterograde ejaculation is not observed, one cannot exclude the occurrence of retrograde ejaculation, which is always accompanied by somatic responses such as increased arterial pressure and contraction of the periurethral or abdominal muscles. An important fact is the absence of any somatic response, which predicts the absence of ejaculation. The success rate is about 50% (Brackett, 1999).

Conclusions

This paper describes the methods and outlines the gametogenic background for collecting the gametes of both sexes for assisted human reproduction. Highly novel approaches to the ovary were needed to collect mature oocytes from their Graafian follicles. Following a brief description of the formation and growth of follicles and the endocrine control of later follicle stages, details of the maturation process are outlined including the nature of its timing and its endocrine control. The rupture of the follicle and its timing after the LH surge or an injection of human chorionic gonadotrophin is discussed in relation to the successive stages of post-diplotene stages of meiosis and aspiration of the mature oocyte just before ovulation. Endocrine control is discussed in outline, and related to method used to stimulate follicle growth and ovulation. The effects of endometriosis on the follicular environment and fertilization are mentioned briefly. Details of the methods used to aspirate oocytes begin with a description of the classical method using laparoscopy. Collection by ultrasonography is discussed in detail. The different methods are discussed. Initially, the value of the perurethral/transvesicle approach is considered. Most procedures are now performed using the transvaginal approach, and this approach is discussed in some detail.

Consideration of methods used to collect male gametes is opened with a brief review of human spermatogenesis and spermiogenesis. Transport through the excurrent ducts, and its control are described briefly. The nature of physiological changes in the spermatozoon during this transport is described in some detail, together with the acrosome reaction, which is mentioned within the context of sperm transport. Sections on the collection and isolation of spermatozoa open with a brief comment on sperm collection from the ejaculate, then turn to

collections from the male reproductive tract and the testis. Recent methods of aspirating epididymal spermatozoa, using percutaneous aspiration (PESA) and its value in relation to testicular extraction are described. The two approaches to aspirating testicular spermatozoa are discussed in detail, i.e. using an open testicular biopsy (TESA) or methods of puncturing the testis (TESA). These methods are described and compared. Brief descriptions are given of the use of spermatid as gametes, and finally the collection of spermatozoa from patients suffering disorders of ejaculation. Details of the biochemistry of the follicle and its endocrine control are presented elsewhere (e.g. Ulloa-Aguirre and Timossi, 2000).

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