European Society of Human Reproduction and Embryology

Course 2

Special Interest Group Andrology

"Treating Male Subfertility: Controversies in the State-of-the-ART"

29 June 2003

Madrid - Spain

Evaluation Form

Treating male subfertility: Controversies in the "State-of-the-ART"

29 June 2003 Madrid, Spain

DEMOGRAPHICS

Are you a physician? ___ Yes ___ No

Years since completion of training: ___ less than 5 ___ 5-9 ___ 10-19 ___ 20/over ___ currently in training

GENERAL INFORMATION	*Strongly* Agree	*Moderately* Agree	*Moderately* Disagree	*Strongly* Disagree
1. The course objectives were clearly stated.	_____	_____	_____	_____
2. The course objectives were clearly met.	_____	_____	_____	_____
3. Faculty/participant interaction was satisfactory.	_____	_____	_____	_____
4. The course was well organized.	_____	_____	_____	_____
5. Site accessibility was not a problem.	_____	_____	_____	_____
6. Meeting facilities were adequate.	_____	_____	_____	_____
7. Course director conducted program well.	_____	_____	_____	_____
8. Overall course grade (circle one)	A	B	C	D
9. Overall syllabus grade (circle one)	A	B	C	D

EDUCATIONAL VALUE

1. I learned something new that was important	_____	_____	_____	_____
2. I verified some important information	_____	_____	_____	_____
3. I plan to discuss some of this information with my colleagues.	_____	_____	_____	_____
4. I plan to seek more information on this topic	_____	_____	_____	_____
5. My attitude about this topic changed in some way	_____	_____	_____	_____
6. This course is likely to have an impact on my teaching	_____	_____	_____	_____

FACULTY EVALUATIONS

Strongly Agree = 4 Moderately Agree = 3
Moderately Disagree = 2 Strongly Disagree = 1

	Aboulghar	Alvarez	Mortimer	Ombelet	Sakkas	Sofikitis	Templeton	Tournaye
1. Sufficient depth								
2. Concepts clearly explained								
3. Enhanced understanding of key matters								
4. Without commercial bias								
5. Slides readable								
6. Learning objectives clear								
7. Helpful for future reference								

COMMENTS

1. What change(s), if any, do you plan to make in your teaching as a result of this course?_____
   _____
2. Future suggested meeting topics and speakers? _____
   _____
3. Additional comments _____
   _____

OPTIONAL Would you be willing to be contacted in the future regarding how this course influenced your practice?

YES **NO**

Name _____ Phone _____

E-mail _____

Content

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Program

SUNDAY, 29 June 2003
Room "Berlin" (1st floor – left wing)

SIG Andrology Course on "Treating Male Subfertility: Controversies in the State-of-the ART"

Course Coordinator/ D. Mortimer (CND) & J. Alvarez (USA)

08.45-09.00:	Introduction
09.00-10.30:	Selection of Spermatozoa: Nature vs. Nurture
09.00-09.30:	Nature - D. Sakkas (USA)
09.30-10.00:	Nurture - J.Alvarez (USA)
10.00-10.30:	H. Tournaye (B) + Discussion
10.30-11.00:	Coffee break
11.00-12.30:	Is IUI Effective in Cases with Male Factor Subfertility?
11.00-11.30:	Scientific Aspects - D. Mortimer (CND)
11.30-12.00:	Medical Aspects - W. Ombelet (B)
12.00-12.30:	A. Templeton (UK) + Discussion
12.30-13.30:	Lunch break
13.30-15.00	Spermatids as Gametes
13.30-14.00:	The ART of using spermatids as gametes - N. Sofikitis (JPN)
14.00-14.30:	Spermatozoa vs spermatids as gametes - H. Tournaye (B)
14.30-15.00:	J. Alvarez (USA) + Discussion
15.00-15.30:	Coffee break
15.30-17.00:	Will ICSI Replace IVF?
15.30-16.00:	Yes - M. Aboulghar (EG)
16.00-16.30:	No - A. Templeton (UK)
16.30-17.00:	D. Mortimer (CND) + Discussion
17.00-18.00:	Andrology SIG Business Meeting

Selection of Spermatozoa: Nature versus Nurture

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USA

Objectives:

1. To understand how the process of spermatogenesis deals with the production of normal and abnormal spermatozoa?
2. To describe the key apoptotic proteins involved in normal spermatogenesis
3. To describe the remodeling of the nuclear DNA during normal spermiogenesis
4. To understand the anomalies observed in ejaculated spermatozoa
5. To understand how abnormal apoptosis and nuclear remodeling impacts on the production of the ejaculated spermatozoon

Introduction

During spermatogenesis, a complex and dynamic process of proliferation and differentiation occurs as spermatogonia are transformed into mature spermatozoa. This unique process involves a series of meioses and mitoses, changes in cytoplasmic architecture, replacement of somatic cell-like histones with transition proteins and the final addition of protamines, leading to a highly packaged nuclear DNA.
The main question concerning ART clinics is how can we be sure that we are utilizing normal spermatozoon. To comprehend and answer this question better we must also understand how the process of spermatogenesis deals with the production of normal and abnormal spermatozoa?

Apoptosis

Apoptosis or programmed cell death is a mechanism that allows cells to self-destruct when stimulated by the appropriate trigger. It may be initiated when a cell is no longer needed, when a cell becomes a threat to the organism's health, or for other reasons. In a number of animal models, overproliferation of early germ cells during spermatogenesis is tempered by selective apoptosis of their progeny. Testicular germ cell apoptosis occurs normally and continuously throughout life. Numerous apoptotic proteins have been discovered that have specific roles in either promoting or inhibiting apoptosis. One factor postulated to be implicated in sperm apoptosis is the cell surface protein, Fas. Fas is a type I membrane protein that belongs to the tumour necrosis factor / nerve growth factor receptor family and mediates apoptosis. Binding of Fas ligand (FasL) to Fas kills cells by apoptosis. In mice and rats it has been shown that, in the normal state, Sertoli cells express FasL and signal the killing of Fas positive germ cells limiting the size of the germ cells population to numbers they can support. In addition, after injury, Sertoli cells increase FasL expression to reach a new equilibrium state that matches the reduced capacity of the dysfunctional Sertoli cells with fewer germ cells. Thus upregulation of Fas in germ cells is seen as a self- elimination process for cells that are destined to die because of inadequate support. As in other mammalian systems it appears that the Fas mediated system and its related pathways are also responsible during the germ cell stages for controlling spermatogenesis in the human (Pentikainen et al., 1999). In addition to the evidence that the Fas mediated pathway plays a role, it is believed that members of the Bcl-2 protein family, in particular Bcl-x_L, which promotes apoptotic cell death, may be more prevalent in spermatogonia.
Therefore apoptosis is believed to act as it does in many other tissues throughout the body in that it acts to deal with the programmed cell death of abnormal cells. We therefore believe that it is one of the main mechanisms nature provides to eliminate abnormal spermatozoa produced during spermatogenesis.

Nuclear remodelling during spermiogenesis

During spermiogenesis, the transition from round to elongated spermatids, the sperm cell nucleus undergoes an incredible compaction whereby it is reduced nearly 40 fold in size. This process is brought about by the removal of histones and the incorporation of protamines as the core nuclear proteins. To facilitate the replacement of the histones and restructuring of the nuclear DNA it has been proposed that the endogenous nuclease, topoisomerase II, may play a role in both creating and ligating DNA nicks during spermiogenesis and that these nicks may provide relief of torsional stress and aid chromatin rearrangement during the displacement of histones by the protamines

Anomalies observed in ejaculated spermatozoa

Numerous studies have now shown that men with poor semen parameters are more likely to show a higher percentage of sperm nuclear DNA damage than men with normal semen parameters (Figure 1). A number of studies also indicate that a percentage of men with normal semen parameters also display nuclear DNA anomalies.

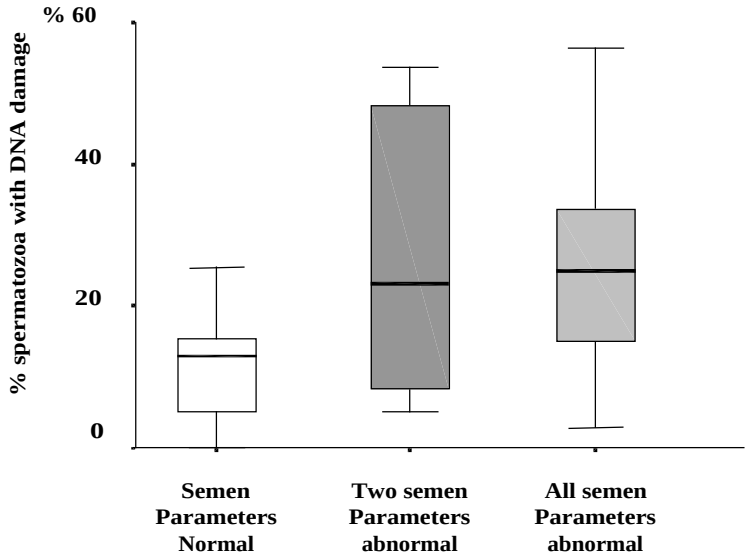

Figure 1. The percentage of ejaculated human spermatozoa per sample (shown as box plots) containing DNA damaged nuclei in relation to the assessment of the patient's semen parameters. Patient's with two or all parameters showing abnormalities have combinations of oligozoospermia, asthenozoospermia and teratozoospermia.

In addition to the abnormalities in the nucleus it is also becoming apparent that ejaculated spermatozoa retain the presence of apoptotic marker proteins. In a previous study we have found that men with abnormal sperm parameters display higher levels of the apoptotic protein Fas on their ejaculated spermatozoa. The presence of Fas on ejaculated spermatozoa correlates strongly with a decreased sperm concentration and sperm with abnormal morphology. More recently, other apoptotic markers such as Bcl-X_L, p53 and Annexin V have also been shown to be present on ejaculated human spermatozoa and show distinct relationships with abnormal semen parameters.

The identification of apoptotic marker proteins, such as Fas, on ejaculated spermatozoa leads to an interesting dilemma:

How do Fas labeled sperm escape apoptosis?

We have previously hypothesized that the presence of Fas on ejaculated human spermatozoa may be indicative of sperm cells that are 'earmarked' for apoptosis but they escape apoptosis because there are too many for the available Fas-L to induce apoptosis or the signaling through Fas is not functional. We have called this "abortive apoptosis".

How does abnormal apoptosis and nuclear remodeling impact on the production of the ejaculated sepmatozoon?

Above we have discussed two critical mechanisms involved in sperm production. A number of studies have shown that ejaculated spermatozoa possess abnormal nuclear DNA and / or the persistence of apoptotic marker proteins. We believe that abnormal ejaculated spermatozoa may arise due to anomalies in either one or both of the above systems.

The mechanisms responsible for producing abnormal spermatozoa in the human ejaculate have been poorly understood. The nuclear DNA damage may arise due to anomalies in nuclear remodeling and could be a direct result of faulty ligation of the nuclear DNA during spermiogenesis, possibly due to problems in the function of topoisomerase or a similar nuclear enzyme. A different population of sperm could exist that have escaped programmed cell death and express various apoptotic markers, a procedure we have previously called “abortive apoptosis”. This may not be classical apoptosis and could be due to defects in the remodeling of the cytoplasm that take place during spermatogenesis.

The end product of faulty nuclear remodeling and/or discrepancies in apoptosis lead to a heterogeneous population of ejaculated sperm, and it appears that this heterogeneity becomes greater as the quality of the semen parameters (ie. concentration, motility and morphology) decrease.

We would propose the following preliminary model to explain the presence of nuclear DNA damage and apparent anomalies in apoptosis during human spermatogenesis (see Figure 2a,b). As in other mammalian systems it appears that the Fas mediated system and its related pathways are responsible during the germ cell stages for controlling spermatogenesis. In addition, the Bcl-2 family of proteins, in particular Bcl-x_L, would also be candidates in the selection process of which cells proceed to maturity. These systems would have their greatest influence pre-spermiogenesis, prior to the remodeling of the nucleus and the exclusion of the cytoplasm. During spermiogenesis DNA breaks appear normally in the early spermatids and we would predict that the ejaculated spermatozoa possessing nuclear DNA breaks are those in which the DNA breaks have not been repaired. Furthermore, the nuclear remodeling that takes place during spermiogenesis could also change or derail the classic programmed cell death pathway(s) which were activated in the earlier stages. The extensive cytoplasmic remodeling that occurs during the later stages of spermatogenesis may also disrupt the apoptotic pathways that are functional prior to spermiogenesis. Taking this model in mind, the apparition of defective spermatozoa in the ejaculate could be related to anomalies in the repair of the DNA breaks that appear in spermatids and / or an increased apoptosis during early spermatogenesis.

The two processes may be operating independently but they also may obstruct the function of each other.

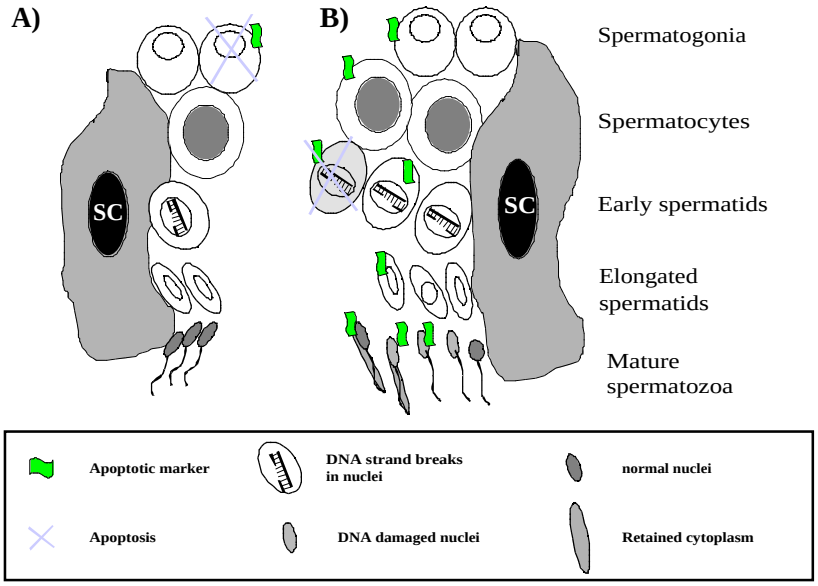

Figure 2. A hypothetical model of the relationship between apoptosis and nuclear remodeling during a) normal and b) abnormal spermatogenesis.

Conclusion

There are mechanisms in place that nature has provided to allow for the production of the best spermatozoa after spermatogenesis. Unfortunately, the nature of spermatogenesis in the human sometimes leads to a heterogeneous population of spermatozoa in the ejaculate. It is the overall number and relative proportions of abnormal spermatozoa in the ejaculate that defines the fertility potential of males.

Suggested references

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Nurture versus Nature: how can we optimize sperm quality?

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Introduction

The number and quality of sperm produced by the testis is determined to a great extent by the interaction of three main factors: (i) the genetic makeup of the spermatogonia; (ii) normal Sertoli and Leydig cell function; and (iii) the interaction of Sertoli, Leydig, and germ cells with endogenous factors and the environment. Even if the genetic endowment of Sertoli and germ cells were to be optimal, sperm quality and number can be greatly compromised by environmental factors. The importance of germ cell genetics in sperm production and quality is exemplified by the sperm produced by Holstein bulls compared to their predecessor bulls in the wild. Through more than 100 years of successful breeding, those genes that optimize spermatogenesis and spermiogenesis have been selected in the testis of these bulls while those genes that have a negative impact on these processes have not been incorporated into their genome. However, if we were to place these "superbreeders" in a hostile environment, those attributes that make them such a wonder of nature would be greatly compromised. On the other extreme of the spectrum are infertile males with severely compromised testicular function. In these males, sperm production and quality is decreased due to constitutive genetic deficiencies that affect both primary genes and gene modifiers involved in sperm production. In these males, "sperm nurturing" becomes limiting in terms of maintaining adequate sperm production and quality.

In this review, we describe two main strategies directed to optimize or "nurture" sperm production and quality:

(i) Pre-production nurturing, directed to optimize sperm quality before ejaculation; and
(ii) Post-production nurturing, directed to preserve and optimize sperm quality and function after ejaculation.

A. Pre-Production Nurturing

A number of environmental factors, including high temperature, proinflammatory factors, infectious disease, social habits, drugs, radiation therapy, and xenobiotics, have been shown to have a negative impact on testicular function. Interventions directed to minimize exposure to these factors could significantly improve sperm production and quality.

1. High temperature

It has long been recognized the importance of scrotal temperature in the regulation of sperm production and quality. Differences in the temperature of the male gonads have been proposed as a possible determinant of sperm quality (Bedford, 1991; Spira, 1991; Mieusset and Bujan, 1995; Setchell, 1998). Several studies in human populations have found a higher scrotal temperature in infertile men compared with fertile controls, regardless of the cause of impaired fertility, e.g. varicocele (Mieusset et al., 1987; Zorgniotti and Sealfon, 1988; Mieusset, 1991). Likewise, in population based samples, a negative association between scrotal temperature and sperm concentration has been demonstrated (Hjollund et al., 2000). The reason for the variation in scrotal temperature is not well understood although determinants could be of genetic nature. In a recent study, a correlation in median scrotal temperature has been found among monozygotic twins, but not in dizygotic twins and single-born brother (Hjollund et al., 2002). The results of this study suggest a genetic component to the variation in scrotal temperature. A hereditary element in male fecundity may be expressed through scrotal temperature, which constitutes a mechanism independent of those

responsible for the development of the sperm producing epithelium.

An increase in scrotal temperature can be brought about by an increase in environmental temperature (workers of glass and ceramic industry, taxi drivers, exposure to hot baths, Jacuzzis, saunas, type of underwear, etc.) or by an increase in the endogenous temperature (varicocele, fever). An increase in scrotal temperature not only can result in disruption of the process of spermatogenesis and spermiogenesis, but also has been shown to induce sperm DNA fragmentation (Evenson, 2000). Therefore, one way of optimizing sperm quality would be to maintain scrotal temperature at physiological levels by either avoiding exposure to risk factors, treating episodes of fever with antipyretics, or performing varicocelectomy in cases on varicocele grades II or III, especially in adolescents where the presence of a varicocele could significantly compromise their long-term testicular function (Romeo et al., 2003).

2. Proinflammatory factors

Chronic inflammatory disease has been shown to affect male reproductive function and fertility. Relevant inflammatory diseases include general and chronic infectious diseases, as well as localized acute or chronic infections of the male genitourinary tract. Male accessory gland infections account for almost 15% of all cases of male infertility seen in infertility clinics while fertility usually is not a clinical objective among patients with acute systemic infections such as Gram-negative sepsis (Hales et al., 1999). Infections of the male accessory glands frequently are associated with increased counts of white blood cells in semen and elevated levels of proinflammatory cytokines in semen and the testis. There is a mounting body of evidence that demonstrates the importance of cytokines and chemokines in the regulation of testicular and glandular function during pathophysiological states as well as under normal physiological conditions when cytokines act as growth and differentiation factors. Cytokines have been shown to affect Sertoli cell function and alterations in cytokine levels in the testis could affect spermatogenesis (Cohen and Pollard, 1995). Therefore, prompt diagnosis and treatment of these conditions could protect the testis against the negative effect of these proinflammatory factors.

3. Infectious disease

It is well recognized the effect(s) of infectious disease on testicular function. Perhaps the most prominent infectious condition is pubertal mumps that leads to testicular atrophy and infertility when it affects both testicles. Orchitis and epididymitis caused by a number of microorganisms can also affect testicular and epididymal function, respectively. Prompt diagnosis and treatment of these conditions can contribute to optimize sperm production and quality.

4. Social habits

Alcohol consumption and cigarette smoking have been shown to affect sperm production and quality. Cigarette smoking has been associated with reduced sperm count and motility (Kunzle et al., 2002), increased oxidative stress (Saleh et al., 2002) and oxidative DNA damage (Fraga et al., 1991). Chronic ethanol exposure in the peripubertal fathers decreases fecundity and that this may be mediated by testicular oxidative injury, perhaps leading to accelerated germ cell apoptosis (Emanuele et al., 2001). In addition, testes of rats fed an ethanol-containing liquid diet had more testicular DNA fragmentation than mice fed an isocaloric control diet. Ethanol increases the number of apoptotic spermatogonia as well as spermatocytes. Direct intratesticular injections of ethanol solution enhanced testicular DNA fragmentation, suggesting an increase in apoptosis. Moreover, Fas ligand levels are increased within the testes of rats that were chronically fed ethanol. In vitro, ethanol treatment of cultured Sertoli cells enhanced the production of Fas ligand. In addition, testicular levels of p53 messenger ribonucleic acid are increased in rats chronically fed ethanol. All of these observations suggest that ethanol enhances testicular germ cell apoptosis (Zhu et al., 2000). Therefore, males should be aware of the potential damaging effects of alcohol consumption on testicular function. The interplay of constitutive genes and disease modifier genes in males exposed to alcohol would determine whether some of these males develop or not any testicular pathology.

5. Drugs

A number of prescribed and recreational drugs have been shown to affect testicular and sperm function. Some of these include, alkylating agents, cocaine, marijuana, calcium channel blockers, cimetidine, colchicine, cyclosporine, erythromycin, gentamicin, neomycin, nitrofurantoin, spironolactone, sulfasalazine, glucocorticoids, and tetracyclines. In a thorough fertility evaluation of the male, the physician should determine what medication the patient is taking and recreational drug use (Nudell et al., 2002). Most adverse effects from drugs and medications can be reversed by simply discontinuing the offending agents. However, in some instances, such as

chemotherapeutic regimens, the medications cannot be discontinued, and pretreatment sperm cryopreservation remains critical. Treatment with cytotoxic chemotherapy is associated with significant gonadal damage in men. The likelihood of gonadal failure following cytotoxic chemotherapy is dependent on the drug and dose. At present, sperm banking remains the only proven method in men, although hormonal manipulation to enhance recovery of spermatogenesis and cryopreservation of testicular germ cells are possibilities for the future. Patients with testicular cancer should be informed of the effects of chemotherapy on testicular function and offered the option of sperm banking prior to the initiation of the therapy.

6. Radiation therapy

Ionizing radiation has been shown to affect testicular function and sperm production. Radioiodine treatment for thyroid cancer may result in transient impairment of gonadal function. The radiation dose absorbed by the testis after a single ablative dose of radioiodine is well below that associated with permanent damage to germinal epithelium and the risk of infertility in these patients is minimal. Patients requiring multiple administrations for persistent or metastatic thyroid cancer may be at greater risk of gonadal damage. Therefore, patients exposed to radiation therapy should be informed of the potential effects of this therapy on testicular function and offered the option of semen cryopreservation prior to the initiation of the therapy.

7. Xenobiotics

Xenobiotics and, in particular, xenoestrogens, have been implicated in the decline of semen quality observed in the last decades. Some of these include pesticides, insecticides, PVC plastics, polychlorinated biphenyls (PCB), phthalate esters (PE), 1,2-dibromo-3-chloropropane, and bisphenols. Post-exposure testicular atrophy has been observed in rodents after a variety of chemical toxicants. In a recent study, it has been shown that ejaculate volume, sperm count, progressive motility, normal morphology, and fertilizing capacity were significantly lower in infertile men compared with controls. The highest average PCB and PE concentrations were found in urban fish eaters, followed by rural fish eaters, urban vegetarians, and rural vegetarians (Rozati et al., 2002). The total motile sperm counts in infertile men were inversely proportional to their xenoestrogen concentrations and were significantly lower than those in the respective controls. PCBs and PEs may be instrumental in the deterioration of semen quality in infertile men (Rozati et al., 2002; Dallinga et al., 2002).

Xenoestrogens have recently been identified as endocrine disruptors that not only may affect the process of spermatogensis and spermiogenesis but also may alter meiosis at different stages of development. In fact, cumulative exposure to xenoestrogens has been proposed as a mechanism leading to meiotic alterations in human spermatozoa.

B. Post-production nurturing

1. Sperm Processing

1.1. Density gradient centrifugation

Because seminal plasma contains factors that inhibit the fertilizing ability of spermatozoa, it is essential that spermatozoa be separated from it quickly and efficiently. In addition, separation of mature sperm from ROS-producing immature sperm and leukocytes during sperm processing is of paramount importance in preventing ROS-induced damage to the sperm membranes and DNA (Lopes et al., 1998). It has been previously reported that defective spermiogenesis is associated with the release of high ROS-producing immature spermatozoa into the ejaculate (Aitken and Clarkson, 1987). It has recently been reported that sperm subsets in the ejaculate at different stages of maturation, isolated by a three-step density gradient, produce different levels of ROS (Gil-Guzman et al., 2001). The interface between 50% and 70% ISolate, containing mostly immature sperm with proximal cytoplasmic retention, produced the highest levels of ROS. ROS levels in this fraction were inversely correlated with percent motility in the sample (Gil-Guzman et al., 2001) and directly correlated with DNA damage, as measured by the SCSA test (Ollero et al., 2001). Recently, Sakkas et al., have reported that sperm isolated from semen by density gradient centrifugation showed a significant improvement in nuclear integrity, as assessed by CMA_3 positivity and DNA strand breakage (Sakkas et al., 2000). When prepared using the swim-up technique, the spermatozoa recovered showed no significant improvement in nuclear integrity. Therefore, density gradient centrifugation techniques can enrich the sperm population by separating out those with nicked DNA and with poorly condensed chromatin.

1.2. Swim-up

Unlike density gradient centrifugation techniques, which mainly rely on the separation of sperm by cell density (precluding, therefore, the migration to the gradient pellet of the low-density immature sperm), swim-up techniques rely solely on the ability of motile sperm to migrate to the upper culture media layer. Therefore, motile, low-density, ROS-producing immature sperm may be harvested in this fraction. In addition, standard swim-up techniques involve a semen washing centrifugation step, which has been shown to result in iatrogenic DNA fragmentation (Twigg et al., 1998). Since oxygen radicals have a short lifetime (of the order of nano- to microseconds), bringing immature, ROS-producing spermatozoa and/or leukocytes in close proximity to mature spermatozoa (e.g., sperm concentration in semen above 100 million/ml, pellet obtained after the centrifugation of semen, sperm packing in the seminiferous tubules and epididymis, etc.) places these sperm at high risk of ROS-induced damage. This can be minimized by (i) diluting semen samples of high sperm concentration; (ii) avoiding centrifugation of semen and using density gradient centrifugation instead (where the pellet obtained is comprised, for the most part, of mature sperm); and (iii) use in ART of testicular sperm obtained by TESE instead of epididymal sperm. Concerning the latter, it has been shown that DNA fragmentation is significantly higher in epididymal sperm compared to testicular sperm (Steel et al., 1999). Centrifugation of sperm suspensions *per se* is not harmful to sperm, unless mechanical damage is induced due to the use of high centrifugal forces. What might be really harmfull is the co-centrifugation of mature and immature sperm and/or leukocytes. Therefore, centrifugation of unprocessed semen should be discouraged and the use of swim-up techniques that bypass a semen centrifugation step recommended. Although seminal plasma is known to contain antioxidant enzymes, centrifugation of semen has the dual effect of bringing mature and immature sperm in close proximity. In addition, it will significantly reduce sperm exposure to seminal plasma which, for the most part, will be in the supernatant, leaving a relatively dehydrated pellet devoid of antioxidant enzyme protection. However, these limitations may not apply to semen samples that are devoid of leukocytes or contain relatively low levels of ROS-producing immature sperm.

2. Treatment with pentoxifylline and 2-deoxyadenosine

Previous studies showed that sperm treatment with pentoxifylline increases the curvilinear velocity, path velocity, straight-line velocity, lateral head displacement, beat cross frequency and sperm hyperactivation in both normozoospermic and asthenozoospermic specimens (Tesarik et al., 1992). However, pentoxifylline does not modify the percentage of motile spermatozoa. In a separate study, it was concluded that an unselective use of pentoxifylline and 2-deoxyadenosine or both compounds together, may restore sperm function in some patients, and perhaps improve fertilization in vitro, but in others it may produce no change or may even be detrimental to sperm function (Tournaye et al., 1994).

3. Sertoli/germ cell co-culture

In-vitro culture systems capable of supporting human early germ cell differentiation have been developed for treatment of azoospermic patients or patients with germ cell maturational defects. Sertoli cells, spermatogonia and spermatocytes are isolated from testicular biopsies of azoospermic patients, and co-cultured using Vero cell conditioned medium only or supplemented with recombinant (r)FSH or rFSH plus testosterone. Best results are achieved with both hormones. In-vitro matured spermatids microinjected into oocytes elicit 37.5% of fertilization and 28.6% blastocyst rates. Abnormal elongating and elongated spermatids enabled 8.3 and 27.3% fertilization rates respectively, but none achieved the blastocyst stage. Normal elongating and elongated spermatids elicit 30.5% fertilization and 42.9% of blastocyst rates (Souza et al., 2002). FISH analysis showed sex chromosome anomalies in all embryos, except in the case of morulae from normal late spermatids. These results suggest that meiosis and spermiogenesis can be resumed *in vitro*, with normal differentiated spermatids showing a low fertilization potential but regular rates of blastocyst formation. However, most of the embryos do not reach the morula stage and show major sex chromosome abnormalities.

4. Supplementation of culture media with antioxidants

Supplementation of culture media with antioxidants has been used to minimize sperm lipid peroxidation (Alvarez and Storey, 1983). More recently, oxidative DNA damage induced by 30 Gy X-irradiation was reported to be prevented by ascorbic acid (600 μM), alpha tocopherol (30 and 60 μM) and urate (400 μM). These antioxidants provided protection from subsequent DNA damage by X-ray irradiation. In contrast, acetyl cysteine or ascorbate and alpha tocopherol together induced further DNA damage. In contrast, supplementation in vitro with the antioxidants ascorbate, urate and alpha tocopherol separately has beneficial effects for sperm DNA integrity (Hughes et al.,

1998). These results underscore the importance of careful antioxidant and dose selection in preventing oxidative stress.

Incubation of sperm from oligoasthenozoospermic samples at 5% O_2 has been shown to result in a significant improvement in motility parameters, the percentage of hyperactivated motility and of induced-acrosome reaction compared with those observed under an atmosphere of 20% O_2. Exposure to 5% rather than 20% oxygen tension also induced a significant increase in the percentage of penetration of zona-free hamster eggs after capacitation for 17 h. After incubation for 24 h, a significantly higher survival rate is observed under 5% compared with 20% oxygen tension (Griveau et al., 1998), confirming previous reports (Alvarez and Storey, 1985; Alvarez et al., 1987). These results strongly suggest that use of low oxygen tension might improve sperm function by minimizing oxidative damage to sperm.

5. Use of hyaluronic acid

A relationship has been found between diminished cellular maturity of human spermatozoa and low-level expression of the testis-specific chaperone protein, HspA2 (formerly known as CK-MM). Because HspA2 is a component of the synaptonemal complex in rodents, and assuming that this is also the case in men, it has been postulated that the frequency of chromosomal aneuploidies would be higher in immature versus mature spermatozoa (Kovancic et al., 2001). Hyaluronic acid (HA) has been shown to improve the proportion of high HspA2, mature spermatozoa in a given sample (Sbracia et al., 1997). Therefore, the use of HA could be beneficial in assisted reproduction. Because HA is a physiological component of the cumulus and of the female and male reproductive tracts, use of HA should not cause ethical concerns. However, it should be emphasized that the release of mature sperm, with a normal HspA2 profile, into the seminiferous tubules does not ensure that these spermatozoa retain their fertilizing potential before they leave the testis. Mature sperm may undergo damage, e.g, DNA damage (Steel et al., 1999), during migration through the seminiferous tubules and the epididymis. This damage may not be compatible with the initiation and maintenance of a term pregnancy, and yet these sperm retain a normal HspA2 profile.

6. Intracytoplasmic sperm injection (ICSI)

ICSI could be considered as an effective tool in the treatment of certain cases of male infertility where sperm are unable to undergo capacitation, bind to the zona pellucida, undergo the acrosome reaction, or fuse with the oocyte's plasma membrane. Microinjection of spermatozoa directly into the oocyte's cytoplasm would bypass defects in these sperm functions.

7. Calcium ionophore-induced egg activation

Oocyte activation is a series of events triggered by the fertilizing spermatozoon and necessary for the beginning of the embryonic development. Calcium plays a pivotal role in this process. There is an initial considerable but short (< 2 min) increase in $[Ca^{2+}]i$ that is detected immediately after the penetration of the microinjection needle into the ooplasm. This rise by itself does not provoke oocyte activation and is also obtained after the injection of medium without spermatozoa. After a lag period of 4-12 h, oocytes that are subsequently activated initiated a second period of $[Ca^{2+}]i$ changes. These changes are sperm-dependent and follows one of two alternative patterns, a non-oscillatory one and an oscillatory one. The non-oscillatory pattern resembled the changes described previously during parthenogenetic activation of mammalian oocytes. The oscillatory pattern is similar to the changes accompanying normal fertilization in different mammalian species. It is concluded that the initial $[Ca^{2+}]i$ rise provoked by the ICSI procedure is not responsible for oocyte activation, and that a release of a sperm factor(s) is required to initiate this process (Tesarik et al., 1994).

Oocytes can be activated *in vitro* following treatment with calcium ionophore A23187 and 6-dimethylaminopurine (6-DMAP) (Tesarik et al., 2000). In this procedure, oocytes are exposed for 10 min at 37°C to a solution of 10 µmol/l ionophore A23187 in Gamete-100 medium (prepared from a 2 mmol/l stock solution in dimethylsulphoxide), washed three times in fresh Gamete-100 medium and incubated for 3 h in IVF-50 medium supplemented with 2 mmol/l 6-DMAP (37°C, 5% CO_2 in air). Oocytes were easily activated by the combined treatment with ionophore A23187 and 6-DMAP. Therefore, in those cases of failed fertilization due to sperm failure to induce oocyte activation, calcium ionophore and 6-dimethylaminopurine could be used.

Recently, a novel sperm-specific phospholipase C (PLC) has been identified as the sperm factor that triggers Ca^{2+} oscillations in mouse eggs indistinguishable from those observed at fertilization. PLC removal from sperm extracts

abolishes Ca^{2+} release in eggs. Moreover, the PLC content of a single sperm is sufficient to produce Ca^{2+} oscillations as well as normal embryo development to blastocyst (Saunders et al., 2002). Testing of this phospholipase C in *in vitro* fertilization could allow in the future its use to induce egg activation.

8. Selection of non-apoptotic sperm

Apoptotic sperm, expressing phosphatidylserine (PS) on the outer leaflet of the membrane, can be separated by magnetic-activated cell sorting (MACS) after binding to superparamagnetic annexin V-conjugated microbeads (ANMBs) (Paasch et al., 2003). Spermatozoa from donors show lower levels of bound annexin V and activated caspases than spermatozoa from infertile patients. MACS results in a significant decrease of spermatozoa with activated caspases in both donors and infertile patients. Separation effects of the MACS technique have been confirmed with flow cytometry using anti-annexin V antibodies and with electron microscopy. Therefore, ANMB-MACS removes spermatozoa with PS-bound annexin V and produces a higher quality sperm fraction (Paasch et al., 2003).

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Is IUI effective in cases with male factor subfertility? Scientific aspects

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Learning Objectives

1.To be able to employ laboratory assessments of spermatozoa and sperm preparation methods to identify correctly those couples with male factor infertility where IUI would be expected to have a good chance of success.

2.To be able to recognize when IUI is precluded due to impaired sperm quality or functional potential and other forms of assisted reproduction treatment should be pursued.

Background

Intra-uterine insemination (IUI), originally intended as a mean of bypassing cervical factor infertility, has been used in the treatment of infertility for many years. More recently its application has been extended to include male factor infertility, of varying degrees of severity, and its effectiveness in this regard remains controversial (Ombelet et al., 1997, Duran et al., 2002). The purpose of this presentation is to consider evidence as to the effectiveness of IUI in this regard, what factors appear to govern its success and/or the nature and significance of any contraindications; it will not consider IUI as a treatment for idiopathic infertility.

As a starting point, the analysis will take the recent systematic review on the determinants of success for IUI (Duran et al., 2002), and modern concepts concerning the management and the treatment of male factor infertility (Mortimer, 1999, 2000a; Oehninger, 2000; Rowe et al., 2000).

Expectations of Success

In general, the reported success rates for IUI treatment vary widely between centres and have been considered to be dependent upon many factors, although different studies have reported variable conclusions with regards to particular measurements. One generalized conclusion, however, is that the use of controlled ovarian hyperstimulation (COH) in conjunction with IUI leads to an increased probability of pregnancy by a factor of between 1.7 and 2.0, although the odds ratios varied between studies and the confidence intervals were quite wide (Duran et al., 2002).

Published pregnancy rates for various centres' success with IUI vary from 5% to 70% per patient/couple, although a pregnancy rate of 10 to 20% per cycle is generally considered to be an "acceptable" range. Inspection of studies included in the review by Duran et al. (2002) has shown success rates for IUI+COH varying between 7.5 and 20.0% (global mean = 11.85%). Considering only those studies reporting on at least 100 cases, the success rates ranged between 8.7 and 16.2% (global average = 11.3%).

The really important consideration here is not the "acceptable" or "expected" success rate for IUI treatment, but what the actual likelihood of achieving a pregnancy is for couples with male factor infertility attending any particular centre. Clearly it makes a great difference to these individual couples whether their chances of success are below, within or above the "acceptable" range, this being dependent not only upon their specific aetiology and the woman's age, but also upon the centre's actual achieved success rates. The latter are, of course, dependent upon the accuracy and reliability of their diagnostic work-up (determination of semen characteristics) as well as the efficacy of the sperm preparation technique(s) used and the particular COH protocol employed for the couple. A reasonable goal for any centre would, therefore, be to achieve a fecundity rate for IUI treatment at least equal to the current average.

Notwithstanding the recommendation to employ COH in conjunction with IUI, it must be remembered that more aggressive COH protocols carry increased risks of multiple pregnancy, which is more of a liability than a benefit. Indeed, some centres in the USA have described treatment protocols that aim to recruit large numbers of follicles in order to maximize the chance of achieving a pregnancy in each treatment cycle. Fetal reduction is then applied as a standard means of reducing high order multiple pregnancies. The idea that couples who have been trying to get pregnant for years, and who have just received the happy news of their pregnancy, should be asked to decide which fetus(es) should be reduced is, to many, morally repugnant. No more will be said here of this approach, which is typically considered poor medical practice in Europe, except to remind readers again that great care must always be taken when using COH in conjunction with IUI treatment.

Diagnostic Issues: Semen analysis and other sperm assessments

The typical criteria for male factor infertility are one or more semen characteristics below the WHO reference values (World Health Organization, 1999), i.e. "diagnoses" of oligozoospermia, asthenozoospermia or teratozoospermia, or combinations thereof (e.g. oligoasthenoteratozoospermia a.k.a. "triple defect" or OAT syndrome). These are not defined aetiologies or pathological entities, but descriptive classifications that are used to select patients for IUI or assign them to IVF (presumably not merely ICSI). However, even when centres state that they are performing semen analyses to "WHO standards" (World Health Organization, 1999), there is often little standardization or comparability of results (Björndahl et al., 2002). Consequently, the identification of male factor infertility patients or their sub-classification based upon basic semen analysis characteristics is not robust and is a major source of discrepancy between centres. As a corollary, this variability should, in fact, preclude meta-analysis of treatment regimens based upon such criteria.

The correct classification of male factor infertility cases and their assignment to IUI, IVF or ICSI treatment modalities will also have an impact on a centre's success rates. In two centres of comparable competence, the one that does not perform IUI will likely have a higher success rate for IVF treatment than the other centre where IUI is offered for appropriately selected couples. When many of the better prognosis patients (i.e. those with minimal sperm dysfunction) receive IVF instead of IUI the apparent success rate for IVF will be elevated. Of course, similar considerations must also be taken into account for centres that take the perceived "simplest" path and just treat all infertility patients using ICSI (this topic is considered in depth in another section of this pre-Congress course).

So, who are the sub-population of patients who would have the best chance of achieving a pregnancy by IUI? This is where the concept of structured management comes into play, in conjunction with (i) a clear understanding of the pathophysiology of getting the male partner's sperm to the site of fertilization (gamete approximation), (ii) the pathophysiology of sperm function and fertilization biology, and (iii) the practical and cost-effectiveness aspects of sperm function testing.

Structured management is a scientific approach to the management of infertile couples, especially those with a contributory male factor, whereby information from diagnostic testing is used in a progressive manner so that couples for whom simple insemination-based treatment has a good prognosis for conception do not proceed directly to assisted conception treatments such as IVF and ICSI. However, couples with poor prognosis due to problems with gamete approximation and/or sperm dysfunction do proceed directly to IVF, or to ICSI if indicated, without wasting time with artificial insemination. Several workers have elaborated upon this general concept (e.g. Mortimer, 1999, 2000a; Oehninger, 2000) and it was embraced in the WHO Manual for the Standardized Investigation, Diagnosis and Management of the Infertile Male (World Health Organization, 2000).
Structured management protocols for infertile couples determine the appropriate level of medical intervention required to achieve a reasonable chance of pregnancy according to available diagnostic information and the female partner

Structured management protocols for infertile couples determine the appropriate level of medical intervention required to achieve a reasonable chance of pregnancy according to available diagnostic information and the female partner's age (and tubal status). "Appropriate" is judged in terms of cost, the likelihood of a successful outcome (birth of a healthy baby) and all associated risk factors, thereby allowing more effective use of healthcare funds (either federal, insurers' or personal funds) by restricting the application of the most invasive techniques until they have been shown to be necessary.

Rigorously standardized and quality-controlled semen analysis, especially careful assessments of sperm morphology (Mortimer and Menkveld, 2001), remains the basis for identifying male factor infertility. However, other aspects of sperm function and fertilizing ability must also be included, and local protocols will vary according to the availability and experience with such testing. While the following investigations are currently the most commonly used, they are rarely employed as a comprehensive investigation:

- Antisperm antibodies using Immunobeads or the MAR test.
- Computer-aided sperm analysis (CASA) of sperm kinematics for: (i) likely ability to be able to penetrate cervical mucus; and (ii) hyperactivation (Mortimer, 2000c).
- Acrosome reaction testing: (i) premature acrosome reaction (high levels of spontaneous acrosome reactions); and (ii) acrosome reaction deficiency using the ionophore challenge "ARIC" test (e.g. ESHRE Andrology Special Interest Group, 1996).
- Sperm-zona binding, e.g. hemi-zona assay (e.g. ESHRE Andrology Special Interest Group, 1996; Oehninger et al., 2000).

Sperm Preparation Techniques

Duran et al. (2002) concluded that the impact of sperm preparation methods on the outcome of IUI treatment was inconclusive, due to variations in the techniques used, which included simple centrifugal washing with culture medium, swim-up and density gradient centrifugation. Nonetheless, it has long been established that some sperm preparation methods can have deleterious effects upon the spermatozoa, particularly simple centrifugal washing – and especially with abnormal semen samples. A recent review confirmed this conclusion and, based on considerations of safety, efficacy and efficiency, recommended that all sperm preparations for IUI, IVF or ICSI should employ density gradient centrifugation followed by a single washing step only (Mortimer, 2000b).

Sperm Preparation Assessments

Traditionally the prepared sperm population to be used for IUI has been evaluated in terms of its total (presumably progressive) motile sperm count ("TMSC") and sperm morphology (summarized in Duran et al., 2002). For example, higher pregnancy rates have been reported when the TMSC has exceeded various thresholds of, for example, 1, 2 or 5 H106 per inseminate. Higher sperm motility has also been reported to generate higher pregnancy rates, and a positive relationship between sperm morphology and IUI pregnancy rates has been reported by some – but not all – centres. Obviously, such positive relationships would be expected on the grounds of sperm physiology, but their robustness will depend heavily on the accuracy and objectivity of the quantitative assessments,.

Recently, we have become increasingly aware of the role of sperm DNA or chromatin damage in determining a man's fertility potential (Evenson et al., 2002). In this regard only the sperm chromatin structure assay (SCSA) is well-standardized and has a large, and growing, coherent clinical dataset to support its use – and also facilitate its clinical interpretation in prognostic terms. Methodological variations between laboratories using the TUNEL or COMET assays, as well as the much lower statistical robustness of these assays due to their being assessed typically on only 100 or 200 cells per test, greatly diminish their clinical potential. It is expected that the SCSA will see widespread use in the future and allow for a further dimension of patient management based upon the recognition of high levels of sperm DNA damage as a powerful predictor of impaired outcome (lower pregnancy rates and increased early pregnancy loss), or even a contraindication for treatment.

Sperm Stimulation Treatments

Although many compounds have been described that stimulate sperm motility or other aspects of sperm function in vitro (e.g. xanthine derivatives such as caffeine or pentoxifylline, adenosine derivatives and analogues including 2-deoxyadenosine and cAMP, kinin-enhancing drugs, human follicular fluid, prostaglandins, and platelet activating factor or "PAF"), their application in IUI therapy has "not yielded expected results" (Duran et al., 2002).

However, it was reported more than 20 years ago that caffeine did not improve the efficacy of cryopreserved spermatozoa (Vandeweghe et al., 1981), and many workers concluded then that pharmacological stimulation of spermatozoa for in-vivo therapeutic use was fraught with risks of sperm "burn out" and other inappropriate changes in their physiology. For example, after a wave of popularity in the late 1980s and early 1990s, pentoxifylline treatment of spermatozoa was recognized as having little real clinical benefit and its use has largely disappeared (Tournaye, 1994).

Great caution must therefore be taken when considering any pharmacological treatment – or even putative "physiological" treatment (e.g. homologous follicular fluid or PAF) – of spermatozoa. Promotion of sperm functional characteristics in vitro will not necessarily lead to automatic benefits when treated sperm are inseminated in vivo. Problems with metabolic "burn out", premature hyperactivation or premature acrosome reaction will all reduce the fertility of a prepared sperm population rather than confer clinical benefit.

The Way Forward

Following the recommendations of the WHO (Rowe et al., 2000) IUI should be available in all serious infertility treatment centres and offered to appropriately selected couples. Careful exclusion of couples where the male partner has severely compromised semen quality, or where specific aspects of sperm dysfunction can be identified, will:

- prevent those couples where there is an "occult" male factor wasting time on IUI. These couples should be directed to IVF (or even ICSI) treatment as necessary according to the severity of the lesion(s) identified; and

- increase the success rates that can be achieved using IUI treatment by only treating those couples who have a genuine good prognosis for success using this therapy.

Sperm populations for IUI should be prepared using standardized density gradient methods, and inseminations should be performed in cycles with mild COH to maximize the fecundity rate.

As evidence of the validity of this approach, a recent study employed screening of the male partners using comprehensive semen analysis (with detailed sperm morphology including assessment of the teratozoospermia index and antisperm antibody testing using Immunobeads), trial sperm preparation using PureSperm density gradients and CASA assessments of sperm mucus penetrating ability and hyperactivation (Mortimer et al., 2002). The authors reported a fecundity rate of 22% per IUI cycle for couples where IUI was recommended as a result of the pre-treatment work-up, compared to only 11% in couples who did not have the benefit of such pre-screening ($P < 0.01$). Moreover, these results were achieved with only 28% of cycles having used clomiphene of gonadotrophins.

Clearly, when sperm factors are identified that demonstrate impaired functional potential (abnormal sperm function tests) or competence (sperm DNA damage), alternative management strategies should be pursued, but for many couples IUI represents an efficacious start to their infertility treatment. In this way infertile couples will receive the most cost-effective treatment and achieve their goal of a healthy baby expeditiously.

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The ART of using spermatids as gametes

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Learning objectives

This lecture will enable the participant to describe

a) the major changes the male gamete undergoes during spermiogenesis,
b) the evolution of ooplasmic injections of round spermatids, secondary spermatocytes, or primary spermatocytes in animals,
c) the candidates for ooplasmic injections of human round spermatids and the results of these procedures,
d) the contributions of the early haploid male gamete to the zygote,
e) the guidelines for ooplasmic injections of human round spermatids, and
f) the genetic risks related to spermless conception.

Spermatogenesis / Spermiogenesis

Spermatogenesis is the sequence of cytological events that result in the formation of mature spermatozoa from precursor cells. In most of the mammals, this process takes place within the seminiferous tubule throughout the reproductive life span of the male. The process of spermatogenesis involves a continuous replication of precursor stem cells to produce cells that can undergo successfully the subsequent changes. A reduction of the number of chromosomes to the haploid state occurs in spermatogenesis. The diploid state is restored on syngamy.
There are three fascinating events that together constitute spermatogenesis: (i) stem cell renewal by the process of mitosis, (ii) reduction of chromosomal number by meiosis, and (iii) the transformation of a conventional cell into the spermatozoon (spermiogenesis). During spermiogenesis no cell division is involved.This process is a metamorphosis in which a round cell is converted into a highly motile structure. The changes can be grouped into (i) formation of the acrosome, (ii) nuclear changes, (iii) development of the flagellum, (iv) reorganization of the cytoplasm and cell organelles (De Kretser and Kerr, 1988).The acrosome structure arises from the Golgi complex. Pro-acrosomal granules are established in the Golgi complex. They coalesce to form a single, large granule that comes into contact with the nuclear membrane and spreads over-25-60% of the nuclear surface. The caudal region of the acrosome is partly attenuated and is termed the equatorial segment. Although the reason for this specialization is unknown, this region of the acrosome persists after the acrosome reaction and it represents the region in which the spermatozoon binds to the plasma membrane ofthe oocyte during the normal fertilization process. Alterations in the final structure of the spermatid head occur during spermiogenesis. Coalescence of chromatin granules is accompanied by chemical changes in the DNA, which is stabilized and becomes resistant to digestion by the enzyme DNase. This stabilization occurs at a time when lysine-rich histones are being replaced by arginine-rich, testis-specific histones in the spermatid nuclei. During the chromatin condensation, there is a progressive reduction in nuclear volume and dramatic changes in cellular shape. At the stage of the elongated spermatid the histones of round spermatids are replaced by protamines. The transition proteins determined in elongated spermatids consist of two major types (Fawcett et al., 1971). They occupy the nucleus between the removal of histones and their replacement by protamines. After fertilization the sperm DNA becomes devoid of protamines again and associated with histones of oocyte origin (Perreault and Zirkin, 1982; Perreault et al., 1987).
The central core of the sperm tail, the axial filament, develops from the pair of centrioles lodged at the periphery of the spermatid cytoplasm and subsequently moves centrally to be lodged at the caudal pole of the nucleus.
Generation of spermatid flagella in vitro has been observed (Gerton and Millette, 1984). The basic structure of the axial filament is common to flagella and cilia and consists of nine peripheral double microtubules. The last steps in spermiogenesis are characterized by additional changes in the relationship of the nucleus and cytoplasm and movement of organelles within spermatids (De Kretser and Kerr, 1988; Sofikitis et al., 1997a).

Evolution of round spermatid nuclei injections (ROSNI) and intactround spermatid injections (ROSI)

Ogura and Yanagimachi (1993) have shown that round spermatid nuclei injected into hamster oocytes form pronuclei and participate in syngamy. DNA synthesis was found in these pronuclei. However, the developmental potential of the obtained zygotes was not evaluated in this study. In another study, Ogura et al. (1993) injected intact round spermatids into the perivitelline space of mature hamster or mouse oocytes and applied a fusion pulse attempting to fuse the intact spermatids with the oocytes. They also studied the behaviour of hamster and mouse round spermatid nuclei incorporated into mature oocytes by the above electrofusion process. It was found that the spermatid nuclei commonly failed to develop into large pronuclei. In one additional study Ogura et al. (1994) confirmed that it is difficult to fuse successfully intact round spermatids with mature oocytes via the above electrical pulse-fusion method. However, they showed that when mouse intact round spermatids are successfully fused with oocytes, some of the resulting zygotes develop into normal offspring. The overall success rate of the electrofusion of intact spermatids with oocytes was low, attributable to the difficulty to fuse large cells like oocytes with small cells like spermatids without lysis of the larger cells (Bates, 1987; Ogura et al., 1993).

To avoid oocyte damage due to the fusion process we chose a microsurgical approach to transfer round spermatid nuclei into rabbit ooplasm. By this method we achieved three pregnancies after ROSNI in rabbit oocytes (Sofikitis et al., 1994a). In that study the proportion of implanted embryos to the number of injected oocytes and the ratio of offspring to the number of injected oocytes were low. The low values of these parameters may be attributable to the low developmental potential of the injected oocytes due to inadequate mechanical stimulation applied to activate oocytes prior to ROSNI. For this reason we designed another study the objective of which was to evaluate the effects of electrical stimulation of oocytes before ooplasmic ROSNI on oocyte activation and subsequent embryonic development (Sofikitis et al., 1996a). That study provided information on the optimal stimulation necessary for oocyte activation, fertilization, and normal embryonic development when ooplasmic ROSNI-embryo transfer procedures are scheduled. We showed that electrical stimulation of oocytes prior to ooplasmic ROSNI and embryo transfer procedures has beneficial effects on oocyte activation, fertilization, and subsequent embryonic development and results in 13% live birth rate per activated oocyte (Sofikitis et al., 1996a). We also assessed the possibility of achieving fertilization and embryonic development in vitro after ROSNI into the perivitelline space, speculating that ooplasmic injections may occasionally damage the oocytes and evaluating the possibility of increasing fertilization rates by avoiding injuries of the oocytes. However, injections of two round spermatid nuclei into the perivitelline space of non-stimulated oocytes did not result in fertilization. The proportion of 2-4-cell stage embryos to the successfully injected oocytes after ROSNI was much lower in electrically stimulated oocytes treated with perivitelline space injections than in electrically stimulated oocytes treated with ooplasmic injections. Therefore, it was concluded that the entrance of the round spermatid nucleus into the ooplasm is more effective than nuclear injections into the perivitelline space for achieving fertilization. After the above successful trials to produce offspring by microsurgical transferring round spermatid nuclei into rabbit oocytes, a question was raised: could round spermatid nuclei selected from subjects with various testicular disorders have similar fertilizing capacity? That question was of great concern because in our two previous studies the round spermatids had been harvested from healthy male rabbits. Furthermore, the probability that humans/animals with primary testicular failure may not have anatomically and physiologically normal spermatids cannot be excluded. To answer the above question we induced an experimental varicocele model in the rabbit, isolated round spermatid nuclei from the testicles of varicocelized rabbits, injected the nuclei into healthy mature oocytes, and proved that these nuclei had fertilizing potential. The overall fertilization rate was 23%. However, embryo transfer procedures did not result in pregnancies (Sofikitis et al., 1996b). In contrast, Sasagawa and Yanagimachi (1997) achieved delivery of normal offspring after ooplasmic injections of round spermatid nuclei recovered from cryptorchid mice. Delivery of healthy offspring after ooplasmic injections of round spermatid nuclei in the mouse has also been reported by Kimura and Yanagimachi (1995a). However, healthy mice were used in that study.

Goto et al. (1996) examined the possibility of utilizing in-vitro derived spermatids for intracytoplasmic injection. There were no significant differences in the development of bovine oocytes injected with various types of male gametes (testicular spermatozoa, spermatids, or spermatids obtained after in-vitro divisions of secondary spermatocytes). It was demonstrated that bovine oocytes injected with in-vitro derived spermatids were capable of developing to blastocyst stage. In another study, fertilization and embryo development in vitro has been achieved after ooplasmic injections of nuclei extracted from frozen-thawed rabbit round spermatids (Ono et al., 1996). Yamamoto and co-workers (1999) have proven that rabbit ROSNI procedures result in significantly larger fertilization and pregnancy rate compared with ROSI techniques. Furthermore, Yamamoto and co-workers (1999) have succeeded in achieving pregnancies after rabbit ROSNI techniques using frozen/thawed spermatids. Weiss et

al. (1997) demonstrated generation of round spermatids from primary spermatocytes in vitro. Goto and co-workers (1996) achieved induction of the second meiotic division in vitro.

Clinical application of ROSNI / ROSI technicques

After the encouraging message from the above animal investigations an attractive challenge was to apply ooplasmic injections of round spermatid nuclei selected from testicular biopsy material for the treatment of non-obstructed azoospermic men (Edwards et al., 1994; Sofikitis et al., 1994a). The first pregnancies in the international literature via ROSNI techniques were achieved in 1994 and reported in 1995 (Sofikitis et al., 1995a; Hannay, 1995). However, these pregnancies resulted in abortions. A few months later, Tesarik et al. (1995) reported delivery of two healthy children after round spermatid injections into oocytes. The mean fertilization rate was 45% in that study. Fishel et al. (1995) reported a pregnancy and birth after elongated spermatid injections into oocytes. Vanderzwalmen et al. (1995) and Chen et al. (1996) reported successful fertilization of human oocytes by intracytoplasmic injections of late-stage spermatids or round spermatids, respectively. The first ROSNI procedures in the USA were performed in California, Louisiana and Florida (Sofikitis et al., 1995b). Fertilization and development up to 10-cell stage embryos was achieved in non-obstructed American couples. The overall fertilization rate per injected oocyte was 31% in that study The peak of the two-pronuclei (2PN) appearance curve in the group of oocytes injected with round spermatid nuclei was 9 h post-injections. At that time all normally fertilized oocytes revealed 2PN, whereas 2 h later both pronuclei disappeared in 20% of the oocytes. Considering that the peak of 2PN appearance after intracytoplasmic sperm injection (ICSI) is 16 h post-injection (Nagy et al., 1994) it appears that the speed of human embryo development after ROSNI is faster compared with ICSI and that oocytes injected with round spermatid nuclei should be checked for pronuclei earlier than oocytes injected with spermatozoa. This difference in the speed of embryo development is compatible with previous studies in the hamster (Ogura and Yanagimachi, 1993) and the rabbit (Sofikitis et al., 1996a) and may be attributable to differences in the protein status of the nucleus (Perreault and Zirkin, 1982; Ogura and Yanagimachi, 1993).

Yamanaka et al. (1997) reported an oocyte cleavage rate of 61% after ROSNI procedures and confirmed that the appropriate time for assessment of fertilization after human ROSNI techniques is 9 h post-injection. Additional pregnancies achieved by ROSNI or ROSI techniques have been reported by Tanaka et al. (1996), Mansour et al. (1996a,b), Antinori et al. (1997a and 1997b), Vanderzwalmen et al. (1997), Amer et al. (1997) and Sofikitis et al. (1997b). Average fertilization rates were >25% in all the above studies.

Other pregnancies after ooplasmic injections of human spermatids have been reported by Sousa and co-workers (1999) and Barak and co-workers (1999). Zech and co-workers (2000) have reported human congenital malformations after intracytoplasmic injections of spermatids. Several studies have shown that culture of human primary spermatocytes in vitro occasionally results in the generation of round spermatids (Tesarik et al., 2000a; Tesarik et al., 2000b) that can be subsequently processed for ooplasmic injections.

Non-obstructive azoospermia and indications for ROSNI / ROSI procedures

Non-obstructive azoospermia may be due to secondary testicular damage or primary testicular damage. Secondary endocrine and exocrine testicular dysfunction may be due to (i) defects in the hypothalamic-pituitary-testicular axis, or (ii) systemic organic disease (i.e. chronic renal failure, liver insufficiency, sickle cell anaemia, diabetes mellitus). Primary testicular damage may be due to chromosomal abnormalities, orchitis, trauma, varicocele, cryptorchidism, gonadotoxins, radiation or it may be congenital (i.e. Sertoli cell-only syndrome, myotonic dystrophy). Furthermore, genetic abnormalities affecting the function of germ cells or Sertoli cells may be among the causes of animal or human non-obstructive azoospermia. Thus, mutations in the white spotting locus of the mouse (Chabot et al., 1988), the Si locus encoding the c-kit ligand (Anderson et al., 1990), and genes encoding retinoic acid receptor a (Akmal et al., 1997) may impair spermatogenesis and result in azoospermia. Sex or autosomal chromosomal deletions are also involved in the aetiology of non-obstructive azoospermia. Involvement of at least three Y-linked genes in spermatogenesis has been suggested (Chai et al., 1997). Several studies suggest that two gene families, RBM(RNA binding motif) and DAZ are present in Y-chromosomal regions that are deleted in some non-obstructed azoospermic men (Chai et al., 1997). Both gene families show specific testicular expression and encode proteins with RNA binding motifs. There is also increasing evidence for a putative human male infertility DAZ-like autosomal gene (Chai et al., 1997) related to human male infertility.

Recent studies have shown that a significant percentage of men with non-obstructive azoospermia have testicular foci of active spermatogenesis up to the stage of round spermatid, elongating spermatid, or spermatozoon (Sofikitis et al., 1995a, 1997b, 1998a; Silber et al., 1995a, b; Tesarik et al., 1995; Silber, 1996; Mansour et al., 1996a; Antinori et al., 1997a; Vanderzwalmen et al., 1997; Yamanaka et al., 1997). Ooplasmic injections of spermatozoa offer a solution for men positive for spermatozoa in the therapeutic testicular biopsy material (Palermo et al., 1992; Silber et al., 1995b; Silber, 1996). When spermatozoa are not present, ROSNI/ROSI techniques represent the only hope for treatment (Edwards et al., 1994; Sofikitis et al., 1994a).

Yamanaka et al. (1997) emphasized the need to collect an adequate amount of testicular tissue (>200 mg) for accurate demonstration of round spermatids by therapeutic testicular biopsy procedures in men who are negative for round spermatids in the routine diagnostic testicular biopsy specimen. It appears that some of the patients with a diagnosis of spermatogenic arrest at the primary spermatocyte stage or Sertoli cell-only syndrome may have rare foci of round spermatids somewhere in the testicles. Amer et al. (1997) used the term 'complete spermiogenesis failure' for men in whom the most advanced germ cell present in the testicular biopsy material is the round spermatid and the term 'incomplete spermiogenesis failure' for non-obstructed azoospermic men with a very limited number of elongated spermatids in testicular biopsy material. Several studies have shown clearly that in men with spermatogenic arrest at the primary spermatocyte stage or Sertoli cell-only syndrome a number of germ cells in a limited number of seminiferous tubules can break the barrier of the premeiotic spermatogenic block and differentiate up to the stage of the round or elongating spermatid (Mansour et al., 1996 ; Tesarik et al., 1996; Amer et al., 1997; Antinori et al.,1997a; Vanderzwalmen et al., 1997; Yamanaka et al., 1997; Sofikitis et al., 1998a). Defects in the secretory function of the Leydig and Sertoli cells or other factors may not allow the round or elongating spermatids to complete the spermiogenesis. Silber et al. (1997) have demonstrated that non-obstructed azoospermic men have a mean of 0-6 mature spermatids per seminiferous tubule seen on a diagnostic testicular biopsy, whereas, 4-6 mature spermatids per tubule must be present for any spermatozoa to reach the ejaculate. In that study the authors claimed that there were no round spermatids in the therapeutic testicular biopsy material of men with maturation arrest if there was absence of elongated spermatids or spermatozoa. Thus, Silber et al. have objections to the ROSNI/ROSI techniques since they support the thesis that when spermatozoa are absent in the therapeutic testicular biopsy specimen, round spermatids are also absent. However, the results of the latter study cannot be unequivocally adopted because: (i) a limited number of participants was evaluated, (ii) the authors attempted to identify round spermatids via Nomarski or Hoffman lens although they admit that they have difficulties in identifying round spermatids using this methodology, and (iii) the most reliable methodology for round spermatid identification [i.e. transmission electron microscopy (TEM)] or another objective method [i.e. confocal scanning laser microscopy (CSLM) or fluorescent in-situ hybridization (FISH) methods] were not applied. To exclude the presence of round spermatids in testicular tissue, a great number of droplets of minced testicular tissue should be processed for the above microscopical techniques and the vast majority of the round germ cells should be examined.

Several biochemical mechanisms may be responsible for the inability of the round spermatids to undergo the elongation process. O'Donnell et al. (1996) have shown that intratesticular testosterone concentration (ITC) suppression may be one of these mechanisms. Additional studies are necessary to clarify whether values of ITC below a threshold cause failure of elongation of round spermatids. If this hypothesis is correct, testicular pathophysiologies affecting optimal ITC may result in complete spermiogenetic failure. It should be emphasized that varicocele, the most frequent cause of male infertility, known to cause azoospermia occasionally, is accompanied by reduced lTC (Rajfer et al., 1987). A diagnostic testicular biopsy negative for round spermatids does not rule out the probability that few or many round spermatids will be found in the therapeutic testicular biopsy material. Furthermore, peripheral serum FSH levels and testicular size do not predict the presence/absence of round spermatids in the therapeutic testicular biopsy material. It should be emphasized that the diagnostic testicular biopsy refers to the evaluation of a limited amount of tissue recovered from one testicular location. Diagnostic testicular biopsy material is exposed to various detergents during fixation and subsequently a number of cells are degenerated and their identity cannot be defined. In contrast, therapeutic testicular biopsy refers to the isolation of a larger amount of tissue from several areas of testicular tissue.

Combined analysis of our studies in the the management of non-obstructed azoospermic men (Sofikitis et al.,1995a, 1995b, 1998a; Yamanaka et al., 1997) showed that among the men who participate for first time in an assisted reproduction programme (regardless of positive or negative results for spennatozoa in the diagnostic testicular biopsy), a percentage of 39% have spermatozoa in their therapeutic testicular biopsy material. For a percentage of 43% the most advanced spennatogenic cells are spermatids in the therapeutic testicular biopsy specimen. These men are candidates for ROSNI/ROSI techniques. It appears that nowadays a small subpopulation only of non-obstructed azoospermic men are excluded from assisted reproductive techniques.

Round spermatids are occasionally present in the seminal plasma of non-obstructed azoospermic men. These ejaculated round spermatids can be used for ooplasmic injections (Tesarik et al., 1996). Mendoza and Tesarik (1996) reported that 69% of non-obstructed azoospermic men have round spermatids in the ejaculate. However, the interesting results of that study may be criticized because the authors did not use standard, reliable methods for round spermatid identification [i.e. transmission electron microscopy (TEM), confocal scanning laser microscopy (CSLM) or fluorescent in-situ hybridization (FISH) techniques]. A current study by Tottori University International Research Group applying CSLM, FISH and TEM in semen samples of >200 non-obstructed azoospermic men indicates that round spermatids are present in >20% of the latter men (Y.Yamamoto et al., unpublished observations). A landmark study by O'Donnell (1997) provides strong evidence that testosterone withdrawal promotes stage- specific detachment of round spermatids from the seminiferous epithelium. It should be emphasized that even if round spematids are present in the ejaculate of a non-obstructed azoospermic man, therapeutic testicular biopsy is indicated for the following reasons: (i) if spermatids are present in the ejaculate, spermatozoa may be found in the biopsy specimen, (ii) the percentage of alive spermatids in the biopsy specimen is significantly larger than that in the ejaculate of the same individual (Y. Yamamoto, unpublished observations), (iii) round spermatids from testicular biopsy have larger fertilizing capacity than round spermatids from the respective ejaculate (Fishel et al., 1997), and (iv) primary testicular damage is often progressive.

Criteria for identification / isolation of human round spermatids

The gold standard for identification of round spermatids is TEM (Sofikitis et al.,1994a). Recently, Mendoza and Tesank (1996) attempted to identify round spermatids by selective staining of the acrosin contained in the acrosomal granules. Another approach is to visualize proacrosin with the use of a monoclonal antibody (Mendoza et al., 1996). A drawback to all the above techniques is that application of these methods results in cell death. Therefore, observed spermatids cannot be used in assisted reproduction programs. Indeed, one of the most perplexing problems in the laboratories applying human ROSNI/ROSI procedures is the identification of alive, undisturbed, non-fixed, non-stained round spermatids. The following approaches are suggested for identification of undisturbed round spermatids in therapeutic testicular biopsy material or in cellular populations isolated from semen samples.

Observation of samples by confocal scanning laser microscopy

Observation of testicular or semen samples via CSLM allows identification of round spermatids. The CSLM is a relatively new instrument in the field of microscopy (Sofikitis et al., 1994b). Unlike the conventional light microscope the CSLM produces sharp images free of out-of-focus artefacts that can be observed on a television monitor. Human and rabbit round spermatids are easily identified via laser microscopy by the presence of multiple or a single, large (acrosomic) granule adjacent to the nucleus (Sofikitis et al., 1994a, 1996b; Yamanaka et al., 1997). New models of CSLM computer-assisted systems (GAS) allow identification of round spermatids of stage 1 of spermiogenesis. These cells are negative for acrosomal granules. GAS analysis of images provided by CSLM differentiates between round spermatids of stage 1 and secondary spermatocytes. The size of round spennatids of stage 1 is <75% of the size of secondary spermatocytes (Sofikitis et al., 1997d). In addition, nuclei of stage 1 round sperinatids show a finely granular texture as opposed to a cloud-like texture exhibited by secondary spermatocytes.

Inverted microscope-computer assisted system (IM-CAS)

Application of quantitative criteria based on computer-assisted image analysis allows identification of round spermatids. 'Round cells' with minimum diameter between 6 and 10 mm that satisfy additional specific quantitative and qualitative criteria are considered to be spermatids (Yamanaka et al., 1997). The minimal cellular diameter of round spermatids is approximately equal to its average diameter (Yamanaka et al., 1997). In preliminary experiments human cadaveric germ cells with morphometric parameters satisfying Yamanaka's quantitative criteria were recovered and processed for TEM. It was found that 63 and 16% of the cells isolated were round spermatids of stages 2-5 and stages 6-8 of spermiogenesis, respectively (Yamanaka et al., 1997). These results were further confirmed by another study suggesting that cells <7.5 mm in diameter should be selected in assisted reproduction programmes using round spermatids (Angelopoulos et al., 1997). However, the last approach excludes round spermatids of average diameter of>7.5 mm.

Qualitative criteria

IM-CAS and CSLM-CAS are not available in most IVF centres. Human round spermatids (Mansour et al., 1996a; Tesarik et al., 1996; Antinori et al., 1997a; Vanderzwalmen et al., 1997; Yamanaka et al., 1997) can be distinguished from other cell types according to the cellular shape, size, and the form of the nucleus. A developing acrosomal granule can be recognized in the round spermatid as a bright/dark spot adjacent to the cell nucleus.

ROSI versus ROSNI

ROSI procedures ensure the transfer of all the cytoplasmic components of the male gamete into the maternal gamete and are less time-consuming than round spermatid nucleus injection. Furthermore, manipulations of the nuclear matrix and envelope are avoided when ROSI techniques are applied. In contrast, ROSI procedures have two disadvantages: (i) injecting micropipettes of larger diameter are necessary, and consequently the probability of injuring oocytes during injections is larger, and (ii) persistence of a large amount of cytoplasm around the round spermatid nucleus may impede its transformation into male pronucleus. In the mouse (Ogura et al., 1993; Kimura and Yanagimachi, 1995a; Kimura and Yanagimachi, 1995b) and the rabbit (Yamamoto et al., 1997), transferring the round spermatid nucleus into the oocyte is a far more efficient procedure in achieving fertilization and embyonic development than transferring the intact round spermatid cell. Embryonic development is faster after ROSNI than ROSI techniques (Ogura et al., 1993; Yamamoto et al., 1997).

The number of pregnancies achieved via ROSI techniques (Tesarik et al., 1995; Mansour et al., 1996a; Antinori et al., 1997a,b; Vanderzwalmen et al., 1997) is larger than the number of ROSNI-pregnancies (Hannay et al., 1995; Sofikitis et al., 1995a, 1997b). This difference in favour of ROSI techniques is false, however, for two reasons: (i) ROSI techniques are relatively simple and applied by a large number of centres internationally. In contrast, ROSNI techniques are applied by Japanese centres oniy; (ii) most of the Japanese centres applying ROSNI techniques cannot publish achieved ROSNI pregnancies because of recommendations by Japanese ethical committees.

Contributions of the round spermatid to the zygote

The male gamete contributes several components important for the fertilization process and early embryo development to the zygote: the genetic material, the reproducing element of the centrosome (Schatten et al., 1986; Schatten, 1994; Simerly et al., 1995), the microtubule organizer component of centrosome (Schatten, 1994), the oocyte-activating substance in spermatozoon/spermatid (OASIS; Swan, 1990; Parrington et al., 1996), nuclear proteins (Ogura and Yanagimachi, 1993), and factors affecting early embryonic development and capacity for implantation.

Genetic material

The deliveries of normal mouse and rabbit offspring (Sofikitis et al., 1994a; Ogura et al., 1994; Kimura and Yanagimachi, 1995a) and healthy human newborns (Tesarik et al., 1995; Mansour et al., 1996a; Vanderzwalmen et al., 1997; Antinori et al., 1997; Sofikitis et al., 1998b) after ROSNI/ROSI indicate the maturity of the genetic material of the early haploid male gamete (i.e. the chromosomes of the round spermatid are capable of pairing with those of the oocyte and participate in syngamy, fertilization, and subsequent embryonic and fetal development).

OASIS

The male gamete-induced cascade of biochemical ooplasmic events that results in resumption of meiosis of the female gamete is referred to as oocyte activation. Oocyte activation is a prerequisite for male pronucleus development and fertilization. Therefore, an anatomical or functional defect of the OASIS may cause fertilization failure ICSI/ROSNI/ROSI procedures. It is generally agreed that the spermatozoon or spermatid triggers the embryonic development by increasing the Ca ion concentration in the oocyte cytoplasm (Vitullo and Ozil, 1992; Sousa et al., 1996; Yamanaka et al., 1997; Sofikitis et al., 1998a). These transient oscillatory or wave-form increases in Ca2+ ion concentration have been observed both after normal fertilization (Taylor et al., 1993) and ICSI/ROSI techniques (Tesarik et al., 1994; Sousa et al., 1996).

Injections of mouse round spermatids into oocytes do not result in oocyte activation suggesting that the mouse OASIS has not been expressed at the round spermatid stage (Kimura and Yanagimachi, 1995a,b). In contrast, ooplasmic injections of rabbit round spermatids lead to oocyte activation in a significant percentage (Sofikitis et al.,

1994a). Electrical stimulation of the rabbit oocyte enhances the OASIS and benefits the activation process (Sofikitis et al., 1996a). Although electrical stimulation usually results in a monophasic ooplasmic Ca2+ response, it appears that there is a synergistic action of electrical stimulation and round spermatid OASIS which eventually produces Ca2+ oscillations. There is strong evidence that the human OASIS is activated at/before the round spermatid stage (Yamanaka et al., 1997; Sousa et al., 1996). The achievement of human pregnancies via ROSNI/ROSI without application of an exogenous electrical or chemical stimulation supports the above thesis. The human oocyte activation after ROSI/ROSNI may not be attributed to parthenogenetic activation of the oocytes since ooplasmic injections of medium only have not resulted in activation (Fishel et al., 1996b; Yamanaka et al., 1997; Sofikitis et al., 1998a). Dozortsev et al. (1995) and Meng and Wolf (1997) have emphasized that human or monkey mechanical ooplasmic stimulation and/or oocyte exposure to a low or a relatively high extracellular calcium concentration of medium can alter intracellular Ca but not, alone, cause activation. The human round spermatid OASIS should be nucleus-associated since nuclear injections are sufficient to cause activation (Yamanaka et al., 1997).
Human oocyte activation is faster after ROSNI techniques than ROSI procedures (Yamamoto et al., 1997). The faster speed of oocyte activation after ROSNI techniques may be due to the presence of a smaller amount of male gamete cytoplasm facilitating the closer contact of the male gamete OASIS-nucleus complex with the cytoplasm of the oocyte.

Similar levels of OASIS activity have been demonstrated in human round spermatids and testicular spermatozoa using a quantitative assay (Sofikitis et al., 1997c). Whether the technique applied for ooplasmic injections of spermatids or spermatozoa influences the oocyte activation process is controversial. Remarkably high oocyte activation rates after ROSNI/ROSI techniques have been demonstrated after injections with minimal (Yamanaka et al.,1997; Sofikitis et al., 1998a) or vigorous ooplasmic stimulation (Tesarik et al., 1996). Tesarik et al. (1994) have supported the idea of a positive role for vigorous ooplasmic stimulation during ICSI techniques in the oocyte activation process. In contrast, Mansour et al. (1996b) have recommended a minimal ooplasmic stimulation. Recent studies have suggested that there may be an OASIS deficiency in selected subpopulations of non-obstructed azoospermic men (Sofikitis et al., 1996c). In the latter men application of electrical (Sofikitis et at., 1995a) or chemical (Vanderzwalmen et al., 1997) stimulation prior, during, or immediately after ROSNI/ROSI techniques may (i) support the action of OASIS, or (ii) act synergetically with OASIS. Mouse OASIS has not been expressed at the secondary spermatocyte stage (Kimura and Yanagimachi, 1995b). We have recently shown that ooplasmic injections of human secondary spermatocytes with minimal ooplasmic stimulation do not activate oocytes and result in premature condensation of the chromosomes of the male gamete (Sofikitis et al., 1998b). However, when a second vigorous mechanical ooplasmic stimulation is applied 1-2 h after the secondary spermatocyte injection, human oocytes are activated in a significant percentage and both the oocyte and the secondary sperinatocyte complete the second meiotic division (Sofikitis et al., 1998b).

Centrosomic components: a challenge to the theory of centrosomes

The zygote's centrosome is a blend of paternal and maternal components. The restoration of the zygotic centrosome at fertilization requires the attraction of maternal centrosomal components to the paternal reproducing element (Schatten et at., 1994; Simerly et al., 1995). The male gamete contributes to the zygote centrosome by transferring the reproducing element of the centrosome, the microtubule organizing centre and a y-tubulin binding protein. However, the maternal y-tubulin is necessary for the function of the zygote centrosome (Schatten et al., 1994).
The delivery of healthy babies after human ROSI/ROSNI tends to suggest that the centrosomic components of the human round spermatid are normal, functional, and mature. Additional studies are necessary on the development of aster and the ooplasmic microtubule organization after ROSNI/ROSI procedures. Several studies have suggested that mammalian oocytes lose their centrosomes when they mature and that centrosomic material is introduced into oocytes by the spermatozoa (see for review Palermo et al., 1994; Schatten, 1994). However, the normal embryonic and fetal development after ROSNI plus embryo transfer procedures in the rabbit (nuclei were proven to be free of cytoplasmic and subsequently centrosomic material; Sofikitis et al., 1994a, 1996a,b), the artificial parthenogenesis in several mammalian female gametes (Schatten et al., 1994), and the development of parthenogenetic rabbit fetuses up to day 10 of pregnancy (Ozil, 1990) can be interpreted as a challenge to the theory of centrosomes and raise the probability that, when paternal centrosomic material is absent, novel maternal spindle organizing centres can develop and previously denatured/non-functional/inactive female centrosomic material can undergo renaturation/ activation. However, the probability that paternal centrosomic material is transferred during spermatid nuclear injections cannot be excluded (Navara et al., 1994) since the centrosomic material is tightly anchored to the nuclear envelope in most of the cells (Schatten, 1994).

The consideration of the centrosome as a cellular organ may be out of date (Mazia, 1984). Observations of Mazia (1984) on the centrosomic cycle refute any notion of the centrosome as an entity that is either present or non-present and is always the same when it is present, and suggest that the centrosomes should be considered as flexible cyclical structures altering their shape and form. The flexible centrosome hypothesis has been further supported by Schatten et al. (1986). Studies suggesting the absence of centrosomic material within mammalian oocytes depend on immunological recognition studies or TEM studies. However, when female gamete centrosomic material cannot be identified by immunological antibody studies, these negative results may be due to the lower concentration of the antigen (Schatten et al., 1986). If unitary centrosomes can exist in a linear form, auto-immune methods might only detect nodes of higher concentration of the antigen. Furthermore, negative results on oocyte centrosomic material obtained by TEM do not exclude the presence of centrosomic material within mature oocytes, because of difficulties locating centrioles and centrosomes at spindle poles by this method (Sathananthan et al., 1991) and the ability of centrosomic material to change shape.

Nuclear proteins

Spermiogenesis is characterized by alterations in the protein composition of the nucleus. Testis-specific histones are replaced by spermatid-specific basic proteins. The latter are gradually replaced byprotamines (Perreault et al., 1987). Following ROSNI/ROSI and disintegration of the round spermatid nuclear membrane within the ooplasm, the round spermatid DNA-nuclear protein complex is exposed to ooplasmic factors. Since the histones are proteins containing a reduced number of disulphide bonds, questions may be raised as to (i) how the round spermatid DNA that is not associated with disulphide bond proteins can survive within the ooplasm and how it is protected against an immediate action of ooplasmic factors, and (ii) how a male gamete nucleus that has not undergone removal of spermatid-specific histones has the capacity to undergo the cascade of events that leads to normal male pronucleus development.

The answer to the first question is that the activation of the oocyte can rescue the chromosomes of the round spermatid from premature condensation (Kimura and Yanagimachi, 1995a,b). Therefore, in non-obstructed azoospermic men whose spermatids expose an impaired capacity for oocyte activation, application of an exogenous stimulus for ooplasmic activation is of paramount importance. In addition, it may possible that spermatid-specific histone removal is not a prerequisite for the formation of the male pronucleus.

Factors affecting early development and capacity for implantation of ROSNI/ROSI embryos

Janny and Menezo (1994) have shown that the mission of the male gamete is not only to activate and fertilize the oocyte but also to contribute to the zygote potential to undergo the first mitotic divisions. It appears that there is a paternal effect on early embryonic development. This thesis has been further supported by Sofikitis et al. (1996b) and Ono and et al. (1997). The latter studies have shown a defect in the capacity for early development and implantation of embryos generated from the fertilization of oocytes by round spermatids or spermatozoa isolated from animals with varicoceles. Thus, embryos derived from the fertilization of human oocytes by spermatids recovered from men with primary testicular damage may have an impaired potential for further development and implantation. In addition, the round spermatid/elongating spermatid factors mediating the paternal influence on the embryonic development may be deficient since the round or elongating spermatid I~presents an immature stage of the male gamete.

Guidelines / prerequisites for ROSNI / ROSI techniques

The Tottori University International Research Group achieved the first spermless pregnancies in 1994 (Hanay, 1995; Sofikitis et al., 1995a). Later a number of other investigators achieved additional pregnancies. However, it is obvious that there is limited experience on human ROSNI/ROSI techniques. We consider the following issues important for successful performance of ROSNI/ROSI techniques.

Quality control for identification of round spermatids

It should be emphasized that training is necessary for the staff of assisted reproduction centres applying ROSNI/ ROSI. Even if a centre has an excellent ICSI programme, ROSNI/ROSI will result in poor outcome if the staff of that centre have not spent many hours observing animal testicular tissue specimens attempting to identify round spermatids via an inverted microscope. Technicians/embryologists/physicians performing ROSNI/ROSI should also confirm via TEM, FISH or CSLM that the cells that are considered as human round spermatids are indeed round spermatids.

Quality control for viability of round spermatids

An occasional finding in ROSNI!ROSI programmes is the absence or a reduced number of live spermatids. Fractions of round spermatids retrieved from testicular tissue should be processed for assessment of viability (Sofikitis et al., 1996a). Men with a percentage of live round spermatids <10% have a poor ROSNI outcome. It should be mentioned that the Trypan Blue stain assesses the plasma membrane and cytoplasmic viability but it does not evaluate the nuclear viability. Theoretically, a live nucleus of a round spermatid with partially degenerated cytoplasmic content may have the capacity to fertilize oocytes. Therefore, nuclear staining techniques are recommended for assessment of round spermatid viability.

Quality control for the capacity of round spermatids to activate oocytes

A previous study has shown that ICSI or ROSNI failure in a selected subpopulation of infertile men is attributable to subnormal OASIS profiles (Sofikitis et al., 1997c). Application of a recently reported quantitative assay to appreciate OASIS activity is recommended (Sofikitis et al., 1997c; two round spermatids are injected into a hamster oocyte). If the percentage of activated hamster oocytes in the latter assay is <8%, fertilization is not anticipated after human ROSNI/ROSI. Alternatively, when OASIS deficiency is suspected, an exogenous stimulus (i.e. chemical or electrical) may be applied to support human oocyte activation and subsequently facilitate fertilization.

Stage of the round spermatid and fertilization.

When round spermatids are observed via an inverted microscope the cells with the larger acrosomal (Golgi) bright dark spots should be preferred for ooplasmic injections because they represent the most mature forms of the male gamete. A recent study has clearly indicated that round spermatids of stages 1 and 2 have smaller reproductive potential than round spermatids of stages 3-5 (Sofikitis et al., 1997d).
When both elongating and round spermatids are present in the testicular biopsy specimen, ooplasmic injections of elongating spermatids are considered preferable because they result in a higher fertilization rate (Fishel et al., 1997).

Media for maintenance of round spermatids

Most of the popular media in assisted reproduction programmes have been devised to maintain spennatozoa rather than spermatids. However, there are several anatomical and biochemical differences between the round spermatid and the spermatozoon. A new medium (SOF medium) has been developed to prolong the viability of round and elongating spermatids (Sofikitis et al., 1998a). It has been already used for maintenance of human and rabbit round spennatids (Sofikitis et al., 1997d; Yamanaka et al., 1997). It contains lactate and glucose as energy substrates. Previous studies have demonstrated that lactate is the preferable energy substrate for round spennatids (Nakamura et al., 1978). Round spermatids have a larger amount of cytoplasm than spennatozoa. To protect round spermatids against environmental shock and to stabilize the spermatid membrane, cholesterol has been added to the SOF medium in a small concentration.

Media for culture of oocytes injected with round spermatids

Previous studies have shown that the addition of antioxidants to media used for culture of embryos generated from the fertilization of oocytes by spermatids has beneficial effects on embryonic development (Sofikitis et al., 1996a, 1997d).

The importance of preserving a cytoplasmic blanket around the round spermatid nucleus

During human ROSNI techniques round spermatids are treated with a variety of detergents to isolate nuclei surrounded by a thin cytoplasmic layer (cytoplasmic blanket; Yamanaka et al., 1997). Although ooplasmic injections of rabbit nude nuclei have resulted in delivery of healthy offspring, when human ROSNI techniques are scheduled, maintenance of a thin cytoplasmic blanket arround the nucleus is preferred to avoid exposure of the male gamete nuclear material to chemical and mechanical stimuli.

Time to observe pronuclei

Pronuclei should be observed at 9 h after human ROSNI techniques (Sofikitis et al., 1995b; Yamanaka et al., 1997). When human ooplasmic injections of elongating spermatids are performed, appropriate time for pronuclei observation is 13 h post-injection (Sofikitis et al., 1998a).

How many ROSNI/ROSI embryos to transfer?

As we discussed in a previous paragraph the implantation potential of human ROSNI/ROSI embryos is small and, therefore, we recommend transfer of all the normally fertilized oocytes that subsequently cleave (Yamanaka et al., 1997; Sofikitis et al., 1998a).

Cryopreservation of round spermatids

Antinori et al. (1997b) achieved the first human pregnancy via ooplasmic injections of frozen-thawed round spermatids. That study indicates the importance of cryopreserving round spermatids in all ROSNI/ROSI cycles.

Genetic implications of ROSNI/ROSI procedures

To evaluate the genetic risk of assisted reproductive technologies, one has to consider the genetic risk inherent to the treatment population and the genetic risk inherent to the procedure performed (Baschat et al., 1996). Considering the limited number of full-term pregnancies achieved by ROSNI/ROSI procedures to date, we can only speculate on the safety/risks of these procedures.

Genetic risks inherent to ROSNI/ROSI procedures may involve (i) centrosomic abnormalities resulting in aberrant spindle formation and subsequently in an increased risk of mosaicism, (ii) injection of disomic/diploid genetic material which could give rise at fertilization to a trisomic/triploid embryo and fetus, (iii) genomic imprinting abnormalities (see below), and (iv) abnormalities due to the out-of-phase cycles of the round spermatid and the oocyte. The round spermatid is at the G-1 stage, whereas the oocyte in the metaphase of the second meiotic division is in its M phase. However, the results of the studies of Kimura and Yanagimachi (1995a and 1995b), Sofikitis et al. (1994a, 1996a, 1997b), Fishel et al. (1996), and Tesarik et al. (1996) indicate that the cell cycle imbalance between the oocyte and the round spermatid does not affect fertilization, embryonic development, and fetal development. It must also be emphasized that the cell cycles of the spermatozoon and the oocyte are out of phase (Fishel et al., 1996). When spermatids are injected into oocytes the metaphase promoting factor which maintains the oocyte in the metaphase of the second meiotic division may also drive the spermatid nuclei to the metaphase (Fishel et al., 1996). Genetic risks of ROSNI/ROSI inherent to a population of men with primary testicular damage are the same with the genetic risks of ICSI procedures (transferring sex chromosomal abnormalities or reciprocal translocations associated with spermatogenic impairment). Inheritance of gene mutations/deletions of DNA sequences in specific regions of the Y-chromosome long arm represent additional risks.

Genomic imprinting abnormalities

Most genes are expressed equally from the two parental alleles, but a small subgroup of mammalian genes are differentially expressed depending on whether they have been inherited from the mother or the father. The process which differentially marks the DNA in the parental gametes is termed genomic imprinting. Genes whose expression is inhibited after passage through the mother's germline are called maternally imprinted, whereas genes whose expression is inhibited when transmitted by fathers are called paternally imprinted (Tycko, 1997). Imprinted genes have been identified in mice and humans.

Several studies have shown that imprinted genes regulate the development of the embryo/fetus. It has been also suggested that DNA methylation maintains the imprinting of some genes. Abnormalities in genomic imprinting are associated with genetic diseases. Furthermore, abnormal functional imprinting is implicated in tumorigenesis. Although previous studies (Ogura et al., 1994; Sofikitis et al., 1994a; Kimura and Yanagimachi, 1995a) suggest that genomic imprinting is complete at the rabbit and mouse round spermatid stage, additional studies are necessary in the human. If genomic imprinting is incomplete in subpopulations of men with primary testicular damage, abnormalities may not become manifest at the early embryonic development but they maybe detectable in the fetus or during the postnatal life. A question of great clinical importance is whether genomic imprinting has been completed at the human round spermatid stage. To attempt to answer this question the imprinting of a gene should

be divided into three stages: (i) erasure of the previous imprint, (ii) re-imprinting, and (iii) consolidation of the new imprint There is strong evidence that erasure of the previous imprint occurs prior to meiosis and that re-establishment of the new imprint begins prior to the pachytene stage of meiosis (see for review Tycko, 1997). In contrast, the fact that DNA methyltransferase enzyme is present in spermatids may be an argument against the thesis that genomic imprinting is complete at the round spermatid stage. However, it should be emphasized that waves of DNA methylation have been demonstrated during early embryonic development, the blastocyst stage, and the time ofimplantation (Fishel et al., 1996). These observations tend to suggest that even if genomic imprinting is not complete at the round spermatid stage, genomic imprinting may be completed after the transfer of the round spermatid within the ooplasm. The work of Kimura and Yanagimachi (1995a,b) supports the latter thesis. Fishel et al. (1996) claim that the genomic imprinting of mouse spermatogenic cells is complete in the testis prior to the male second meiotic division.

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Spermatozoa versus spermatids as gametes

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Learning objectives

- studies dealing with ART in "non-obstructive azoospermia" must support this diagnosis by testicular histopathology
- studies dealing with ART in "non-obstructive azoospermia" do not always discriminate between elongated spermatids and spermatozoa
- although ICSI with testicular spermatozoa has become a routine treatment, it is not an highly efficient treatment in men with "non-obstructive azoospermia"
- only in a small proportion of patients in whom no spermatozoa or late elongated spermatids can be recovered, less immature spermatids may be observed
- to date, only a very limited number of pregnancies have been reported, mainly after injection of elongated spermatids
- the viable pregnancy rate after the use of round spermatids from men without elongated spermatids or spermatozoa in their testes is limited to 1.6%
- 8 years after the introduction of ELSI and ROSI, there is still a lot of uncertainty whether these techniques can be considered as a safe treatment option

Non-obstructive azoospermia: what's in a name?

Most azoospermic patients suffer from primary testicular failure. They show either a germ cell aplasia (Sertoli cell-only) either a maturation arrest or tubular sclerosis and atrophy at their testicular histopathology.

Germ cell aplasia may be caused by irradiation or chemotherapy or may be the result of a genetic disorder such as 47,XXY Klinefelter's syndrome or a deletion on the long arm of the Y-chromosome. Also cryptorchidism may be at cause. In many cases, however, the cause of germ cell aplasia remains unknown. Causes of maturation arrest are viral orchitis, cryptorchidism, irradiation and/or chemotherapy and Yq-deletions. Other causes include systemic illness or exposure to gonadotoxins, but here too, idiopathic maturation arrest is most common. Tubular sclerosis and atrophy may be the result of testicular torsion, vasular injuries or infections, but is also a common finding in Klinefelter's patients. Because these patients do not show any clinical sign of obstruction, often they are referred to as patients with non-obstructive azoospermia.

However, in a few cases, azoospermia is caused by an hypogonadotrophic hypogonadism, i.e. a lack of adequate hormonal stimulation to support spermatogenesis. These patients too show a maturation arrest. Treatment with follicle stimulating hormone (FSH) and luteinizing hormone (LH) or pulsatile gonadotrophin releasing hormone (GnRH) will restore spermatogenesis and they do not, in the first instance, need assisted reproduction.
Many studies on ART with testicular spermatozoa or spermatids use inadequate definitions often based on the absence or presence of clinical signs of obstruction. The diagnosis of "nonobstructive azoospermia" should be made according to the histopathological findings, rather than on the basis of clinical indicators such as FSH levels or

testicular size. Testicular failure is found in a third of normogonadotropic azoospermic men with normally sized testes, on the other hand, small testicular size or elevated FSH does not preclude normal spermatogenesis!

ART with testicular spermatozoa

Azoospermic men with primary testicular failure may, however, still show occasional foci of active spermatogenesis in a few seminiferous tubules.

The terminology used in these cases are “incomplete Sertoli cell-only syndrome” or “incomplete maturation arrest”. Hypospermatogenesis is diagnosis that covers a wide range of conditions in which active spermatogenesis is present in all tubules, but with only a few spermatozoa developing. Some men with hypospermatogenesis may have an obstruction as the main cause of their azoospermia, rather than a severe testicular dysfunction
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In these azoospermic patients with foci of active spermatogenesis, testicular spermatozoa or elongated spermatids (type Sd 1 and 2 according to Clermont’s classification) may be recovered by testicular biopsy and wet preparation. Elongated spermatids may be differentiated from testicular spermatozoa by the presence or absence of cytoplasm continuity from the equatorial region towards the midpiece region (Sousa et al. 2002). Differentiating an elongated spermatid in its final stage of spermiation from a testicular spermatozoon requires a detailed microscopic examination (Verheyen et al., 1998). After routine wet preparation of testicular tissue and after using enzymatic digestion, it remains difficult to differentiate between spermatozoa that were released “naturally” during spermiation into the lumen of the seminiferous tubule and the less mature late elongated spermatids recovered after “artificial” loss of the tubulolobular complex during preparation. As a result, not all studies discriminate between these late elongated spermatids and spermatozoa.

The sperm recovery rates in men with “nonobstructive azoospermia” varies between 40% and 70% according to the recovery technique used and differences in the patient populations studied. Some studies tend to include a majority of patients showing hypospermatogenesis. In these patients spermatozoa will invariably be observed.
Even in about half the patients with a nonmosaic 47,XXY Klinefelter syndrome, mature spermatozoa can be recovered from multiple testicular biopsies.

Despite these acceptable recovery rates, our large consecutive study using a correct definition of non-obstructive azoospermia and excluding patients with hypospermatogenesis, shows that the fertilizing potential of these sperm is decreased compared to men with normal spermatogenesis. Furthermore, the implantation rates of embryos obtained from testicular sperm from men with “non-obstructive spermatogenesis” are significantly lower compared to men with normal spermatogenesis (Vernaeve et al., 2003).
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Apart from a higher chromosomal aneuploidy, chromatin packaging abnormalities (Hammadeh et al. 1999) and a higher rate of apoptosis (Palermo et al. 1999) in testicular spermatozoa from men with “non-obstructive azoospermia”may explain not only the reduction in fertilization rate, but also the lower implantation rate and clinical pregnancy rate observed in this study.

ART with spermatids: is there any target group?

In about half of azoospermic men with primary testicular failure, no spermatozoa or late elongated spermatids can be recovered. When no testicular spermatozoa can be recovered, more immature testicular spermatogenetic cells can be used for ICSI (Tesarik and Greco, 1999)

Shulze et al. (1999) reviewed 1418 biopsies from 766 azoospermic men and observed a late maturation arrest, i.e. round spermatid arrest, in only 7 men (0.9%). Silber and Johson (1998) reviewed 125 biopsies of idiopathic non-obstructive azoospermic men and found no evidence of an arrest at the round spermatid stage. When they observed round spermatids, elongated and even mature testicular spermatozoa too were observed !

Thus probably only in a minute proportion of patients in whom no spermatozoa or late elongated spermatids can be recovered, less immature spermatids may be observed. There have been reports on the use of elongating spermatids (Sc and Sb2 according to Clermont’s classification), and although often no discrimination is done between elongating and elongated spermatid injection (ELSI), in most studies elongated spermatids were used.

ART with spermatids: a critical appraisal?

There have been several reports on the use of round spermatids to treat male infertility because of a late maturation arrest. Either round spermatids as a whole (ROSI) or round spermatid nuclei (ROSNI) are injected into the oocyte. Although both ELSI and ROSI/ROSNI were introduced more than five years ago, the number of pregnancies reported after the use of spermatids is still limited.

A recent review of small case-series shows that results after ICSI with elongated spermatids are comparable or even better (!) than those of ICSI where testicular spermatozoa were used (Sousa et al. 2002). This may be explained by the retrospective character of these studies, patient selection and even publication bias. Some ELSI studies even include men with normal spermatogenesis! According to Sousa's review, even 7 years after its introduction only 48 viable pregnancies after ELSI were reported in the literature.

The results after ROSI are even more puzzling. The first publications (Tesarik et al. 1995 and 1996) reported 2 viable pregnancies out of 6 ROSI cycles (33%) in which ejaculated round spermatids were injected. But since then only 5 more viable pregnancies were reported (Sousa et al. 2002). If larger consecutive studies are added to Sousa's review, only 7 viable pregnancies are obtained in 266 reported ROSI cycles (2.6% pregnancy rate). Three of these pregnancies were obtained in patients with elongated spermatids or even spermatozoa in their testes. Thus eventually 4 viable pregnancies were obtained out of 251 cycles in which allegedly round spermatids from men without elongated spermatids or spermatozoa in their testes were used! (1.6% pregnancy rate).

From the above it may thus be assumed that spermatid ICSI is not a very successful approach for treating infertility in azoospermic men with a primary
testicular dysfunction.

A pregnancy has been reported after ICSI with secondary spermatocytes, a spermatogenetic cell with a diploid DNA content undergoing the second meiotic division (Sofikitis et al. 1998). If spermatogenesis is arrested at the primary spermatocyte stage, then only diploid cells are available. ICSI with spermatids obtained after in vitro culture of primary spermatocytes has been proposed to overcome sterility because of this early maturation arrest (Tesarik et al. 1999). But more than 3 years after these publications, these results await general confirmation.

ART with spermatids: a safe option?

Since only few pregnancies have been reported after the use of elongated and round spermatids, the use of these immature haploid germ cells still gives rise to a lot of concern and confusion. There are concerns relating to genomic imprinting. In a mouse model it has been shown that genomic imprinting is completed at the spermatid stage (Shamanski et al., 1999) and that normal viable young with normal behaviour can be obtained after round spermatid injection up to the fifth generation (Tamashiro et al., 1999). Since mouse offspring obtained after injection of secondary spermatocytes were found to be fertile, it was concluded that imprinting may be completed by the second meiotic division (Kimura et al. 1995). Although methylation of imprinted genes is thus assumed to occur between the spermatogonium and spermatocyte stage, there is still uncertainty whether round spermatids have acquired a stable paternal imprinting (Maning et al., 2001).

In a knock-out mouse model, round spermatids showed increased levels of DNA damage (Jurisicova et al. 1999). Whether spermatids from azoospermic men with maturation arrest have increased DNA-damage remains to be proven. Abnormal chromatin packaging too has been reported in elongated spermatids (Francavilla et al. 2001). Finally there has been a report of 2 major congenital malformations (trisomy 9 and Arnold Chiari syndrome) in a series of 4 pregnancies obtained after ELSI (Zech et al., 2000).

Thus, 8 years after the introduction of ELSI and ROSI, there is still a lot of uncertainty whether these techniques can be considered as a safe treatment option.

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Will ICSI Replace IVF?

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Learning objectives

- To improve the knowledge of participants on available data on the value of ICSI in different indications.
- To add to the knowledge of participants on risks of total failure of fertilization if ICSI is not used.
- To improve the skills of participants to choose the indication for ICSI or IVF.

Summary

There is ongoing debate among reproductive endocrinologists and embryologists about the indications for ICSI in the management of the infertile couple. Soon after the development of ICSI, it became the standard treatment of severe male factor, and azoospermic patients for whom sperms are retrieved surgically. However, a debate is continued concerning other indications for ICSI particularly when the male has normal or border line semen parameters.

Several randomized studies on sibling oocytes between ICSI and IVF revealed that in patients with unexplained infertility ICSI results in higher fertilization and pregnancy rates. In one randomized study, subgroup analysis revealed that there is no significant difference in pregnancy rate between IVF and ICSI.

In patients with border line semen, a meta analysis of eight randomized trials of IVF versus ICSI revealed that there is a significantly higher pregnancy rate in the ICSI group.

ICSI should be considered as the first line of treatment in any case of previous poor fertilization or complete fertilization failure.

There is no improvement of pregnancy rate in tubal factor patients by performing ICSI and the possibility of total failure of fertilization was small in conventional IVF in this group of patients. This supports the current practice of performing only conventional IVF in tubal factor patients with normal semen parameters in many centers. However an unexpected total failure of fertilization can always occur if only IVF is performed.

Concerning risks for the babies, there are no data suggesting that ICSI should not be performed in all cases of in-vitro conception. In all cases, female factor or male factor (normal or abnormal sperm) the use of ICSI bypasses most dysfunctions eliminating the majority of barriers to fertilization. So far, no large-scale well-documented data on the outcome of children after ICSI with normozoospermic semen are available.

Introduction

As early as 1995 Hamberger et al. (1) suggested that ICSI should completely replace IVF in the future and he anticipated that better pregnancy rate might be expected if normal spermatozoa are injected. They concluded that, until more knowledge is gained concerning the outcome of pregnancies, most centers have taken the attitude that ICSI should be applied when conventional IVF fails (7).

There is ongoing debate among reproductive endocrinologists and embryologists about the indications for ICSI in the management of the infertile couple. Although ICSI is relatively new (2), no other technique since IVF itself has had such a positive and almost explosive impact in helping infertile patients to achieve conception (3).

Soon after the development of ICSI, it became the standard treatment of severe male factor (4), and azoospermic patients for whom sperms are retrieved surgically (5,6). However, a debate is continued concerning other indications for ICSI particularly when the male has normal or border line semen parameters (7).

There is a general trend showing an increase of the ratio of ICSI as compared to conventional IVF and in some centers ICSI is used exclusively for all cases of IVF. The Human Fertilization and embryology authority (HFEA) reported a 14% increase in the last report compared to the previous year (8). The national registry of assisted reproduction in the United States showed that the percentage of ICSI procedure increased from 35% in 1997 to 43% in 1999 (9).

An important question arises as to whether ICSI will become the procedure of choice for all couples requiring assisted reproduction treatment. Without doubt, the ICSI procedure itself is more invasive. Therefore, questions with regard safety issues have to be raised (10).

There are several situations in which there is a continuing debate about the value of ICSI. They include patients with normal semen parameters as in cases of tubal factors, unexplained infertility and endometriosis. Another example is patients with borderline semen parameters with slightly subfertile criteria. Each of these items will be discussed separately followed by a general discussion.

ICSI for unexplained infertility

Regarding patients with unexplained infertility there are more conflicting data in the literature. In the randomized study by Bhattacharya et al. (11) subgroup analysis in couples with unexplained infertility revealed no significant difference in pregnancy rate between IVF 32% and ICSI 38%. Among 48 patients in the IVF arm, there was only one patient with total failure of fertilization. The authors' final conclusion was that ICSI offers no advantage over IVF in case of unexplained infertility. However, the authors also stated that a potential advantage of ICSI in this group could be the avoidance of total failure of fertilization. The results showed that the risk can be decreased but not entirely eliminated and the number needed to treat is 33 to avoid a single total fertilization failure in this study if only IVF is performed.

It was reported previously that there was a significantly lower fertilization rate in patients with unexplained infertility compared to patients with tubal-factor infertility. In a small study on 22 patients, ICSI and conventional IVF were performed on sibling oocytes. Total failure of fertilization with conventional IVF oocytes occurred in 5 patients (22.8%) and none in the ICSI oocytes (12). Ruiz et al. (13) in a controlled study on patients with unexplained infertility and endometriosis showed 11% total failure of fertilization in the IVF group and zero failure of fertilization in the ICSI group. In a larger study on 131 patients with the diagnosis of unexplained infertility, who failed to become pregnant on controlled ovarian hyperstimulation and intrauterine insemination; ICSI and IVF were performed on sibling oocytes. In 23 cycles (17.6%), patients had total failure of fertilization in the IVF oocytes. Those patients would have lost their chance for embryo transfer if the oocytes have not been divided between IVF and ICSI (14).

Khan et al. (15) reported total failure of fertilization in 30% of cases of unexplained infertility, which were subjected to IVF after failure of COH and IUI. Audibert et al. (16) reported that in cases of unexplained infertility with previous normal semen, several abnormalities in sperm analysis were reported, affecting at least one parameter on the day of retrieval. Their results suggested that there was a small proportion in the unexplained infertility group with undiscovered male abnormalities, which behaved in IVF line patients with male infertility.

In conclusion, we believe that ICSI at least on sibling oocytes should be done in the first cycles of unexplained infertility treatment.

ICSI for border-line semen

Assisted reproduction is proposed to couples suffering from male subfertility when no specific treatment is available or when specific treatment has failed to improve the husband's fertility potential. However, for many couples in whom the husband presents with moderate oligozoospermia, a difficult choice between IVF and ICSI has to be made (17).

In the group of 53 patients that had borderline semen, the oocytes were randomly divided between IVF and ICSI (18); a total of 560 oocytes were recovered and 361 were allocated for ICSI and 199 oocytes for conventional IVF. The fertilization rate in ICSI oocytes was 60%, while in conventionally inseminated oocytes, it was 18% ($P<0.001$). There was complete failure of fertilization in the conventional IVF of oocytes in 26 patients (49%). In the remaining 27 patients, fertilization was successful in both ICSI and conventional IVF. The authors believed that performing ICSI in patients with borderline semen offers several advantages. It resulted in a significantly higher fertilization rate in the ICSI oocytes and saved 49% of the patients from the possibility of total failure of fertilization with conventional IVF. Another advantage is that patients with borderline semen who fertilize with ICSI and fail to fertilize with IVF should be offered ICSI only in future treatment. On the other hand, patients who fertilize with both ICSI and IVF should be offered conventional IVF in their next trials (18).

In the subgroup analysis of Bhattacharya et al. (11), among couples diagnosed as having a mild male-factor problem, pregnancies occurred in six (26%) after IVF and five (22%) after ICSI (relative risk 1·2 [95% CI 0·42-3·38]). There was one case of failed fertilization in each group. The fertilization rate per oocyte retrieved was 55% in both groups (95% CI for difference -9 to 10), whereas the fertilization rate per oocyte injected was higher in the ICSI group (71% vs. 55% [-26 to -5]).

A study was conducted (19) in which half the oocytes from each of 58 couples with moderate oligo ± astheno ± teratozoospermia were inseminated (conventional IVF) and the other half microinjected (ICSI). Nineteen of the 58 IVF/ICSI attempts resulted in fertilization after ICSI only (32.8%) and 39 in fertilization after IVF and ICSI (67.2%) (19). This strategy enabled the authors to avoid 32.8% of complete fertilization failures after IVF, but not to decrease significantly the number of ICSI attempts in subsequent cycles. However, the uncertainties concerning the safety of ICSI suggest that ICSI should be used cautiously and judiciously (19). This study confirms that performing conventional IVF and ICSI in sibling oocytes in the first cycle for couples with borderline semen quality decreases the risk of transfer cancellation over that for conventional IVF alone. It is also an excellent test of sperm fertilizing ability, to be used as a guideline for the management of possible future cycles (19).

In a prospective randomized controlled trial on seventy-three couples undergoing ART with borderline and moderate male factor, a standard insemination concentration of 0.2X 106/mL was used in one IVF group, whereas in the other group a high insemination concentration (HIC) of 0.8 X 106/mL was used. Each protocol was compared with ICSI on sibling oocytes. The overall fertilization rate was significantly lower after standard IVF than after ICSI: 37.4% vs. 64.3%. Where HIC IVF was used, the overall fertilization rate was not significantly different from that after ICSI: 59.6% vs. 67.6% (17).

A meta analysis of eight randomized controlled trials was performed together with the present randomized controlled trial. The risk ratio for an oocyte to become fertilized was 1.9 (95% confidence interval of 1.4 to 2.5) in favor of ICSI, and 3.1 ICSI cycles may be needed to avoid one complete fertilization failure after conventional IVF (95% CI of 1.7 to 12.4) (17).

In all randomized controlled trials except one, more oocytes were fertilized after ICSI than after conventional IVF: 689 of 1932 oocytes were fertilized after IVF (35.7%) vs. 1423 of 2267 (62.8%). The RR (calculated on a per-oocyte basis) was 1.9, with a 95% CI of 1.4 to 2.5. Overall, complete fertilization failure occurred in 112 of 332 IVF cycles (33.7%), vs. 10 of 332 ICSI cycles (3.0%). The RR (calculated on a per-cycle basis) was 7.5 (95% CI, 2.7-20.5), and NNT was 3.1 (95% CI, 1.7-12.4).

It is concluded that either ICSI for all oocytes or IVF/ICSI on sibling oocytes should be performed in infertility associated with mild or border line semen.

ICSI in previous failed fertilization with IVF

In a study carried out by Hariprashad et al. (20) to determine if ICSI is an effective method for improving pregnancy rates among patients who had previously unsuccessful IVF cycles resulting from poor or total fertilization failure, it was found that fertilization, clinical pregnancy and implantation rates were all significantly higher after the use of ICSI. The ongoing pregnancy rate between the ICSI and insemination group were significantly different; 34.1% and 10.7% respectively. It was concluded that ICSI can overcome certain factors that may cause abnormally low or no fertilization, and that even in cases where semen parameters are normal, ICSI can be useful and give a positive result

The study that most supports this argument, is that of Liu and Baker (21), in which 563 couples were included. The couples underwent standard IVF, resulting in 369 with zero fertilization and 194 with a fertilization rate of 1-25%. Out of these couples 180 were subsequently treated with ICSI, resulting in an average fertilization rate of 58%. In summary, these authors suggest that IVF can be bypassed by ICSI in order to reduce the incidence of fertilization failure in standard IVF, and this includes cases of defective sperm and normozoospermia.

In 37 patients with unexplained infertility and idiopathic failed conventional IVF, 206 sibling MII oocytes were inseminated using HIC with husband's spermatozoa. Out of these oocytes, 69 fertilized normally (33%). On the other hand, out of 212 sibling MII oocytes which were microinjected with husband's spermatozoa, 128 fertilized normally (60%) ($P<0.001$) (22).

Similarly in patients with a previous idiopathic failed HIC cycle, an improved incidence of fertilization was seen after ICSI with donor spermatozoa as compared with conventional IVF with donor spermatozoa ($P<0.015$) (22). In another study with sibling MII oocytes retrieved from patients with total fertilization failure in a previous IVF attempt, ICSI appeared to be far superior to an additional IVF attempt with elevated HIC, as of the 143 injected (ICSI) oocytes, 62.9% fertilized normally, whereas none of the 85 IVF inseminated sibling oocytes was fertilized (23).

Two studies were performed to assess ICSI in potential oocyte-related failure of IVF, viz. when fertilization occurred in >50% of oocytes for one group of patients, and in <50% of oocytes in a second group. In both of these studies a significant proportion of the oocytes that failed to fertilize with conventional IVF eventually fertilized after ICSI. The overall conclusion was that ICSI as a first option offers a higher incidence of fertilization, maximizes the number of embryos and minimizes the risk of complete failure of fertilization for all cases requiring in vitro conception (22).

Non-human primate data suggest that the technique of ICSI itself may be responsible for alterations in the process of fertilization that could potentially have an impact on later embryogenesis stages (24).

Therefore ICSI should be considered as the first line of treatment in any case of poor fertilization or complete fertilization failure (20), and will consequently save time, money and most importantly stress to the patients (10). Cases with previous fertilization failure in IVF therapy usually benefit from ICSI, suggesting the presence of 'occult' male and/or female factors that can be bypassed by the technique. Therefore, and because of concerns about potential negative effects on the technique itself and the use of gametes of poor quality, efforts should be geared toward identification of the aetiology and pathophysiology of sperm and oocyte lesions/dysfunctions responsible for fertilization impairment and their potential contributions to defective embryogenesis (3).

ICSI for tubal factor infertility

In the tubal factor group, a prospective randomized trial of IVF versus ICSI in 58 patients in each arm with normal semen parameters showed similar implantation and pregnancy rates and there was no total failure of fertilization in both groups of the study (25). Staessen et al. (26) in a study on tubal factor patients with normal spermograms using IVF and ICSI on sibling oocytes showed that total failure of fertilization is 3.6% for ICSI and 12.5% in IVF. In the ART unit, Birmingham women's hospital UK, the total failure of fertilization in IVF for all indications was only 2.1% during an 18-months period (27).

Bhattacharya et al. (11) performed a large multicenter randomized controlled trial of conventional IVF versus ICSI for the treatment of non-male factor patients. They showed that the implantation rate was higher in the IVF group as compared to the ICSI group. There was no significant difference between PR between the two groups. There was a 5% total failure of fertilization in the IVF group as compared to 2% in the ICSI group.

The above mentioned studies showed that there is no improvement of pregnancy rate in tubal factor patients by performing ICSI and the possibility of total failure of fertilization was small in conventional IVF in this group of patients. This supports the current practice of performing only conventional IVF in tubal factor patients with normal semen parameters in many centers. However an unexpected total failure of fertilization can always occur if only IVF is performed (7).

There are a growing number of scientists who favour ICSI for all patients (10). They have the following argument: With ICSI we can have direct vision of oocytes and evaluate their maturation state, thereby determining female factors. Worry over germinal arrest or metaphase I has been eliminated because this technique allows us to see these conditions, along with the quality of the oocytes (10).

Concerning the safety of ICSI, in a recent publication (28) no statistical differences were observed between the rates of abnormalities among embryos generated by ICSI or conventional IVF. On the other hand, a follow-up study involving 1082 karyotypes of ICSI children demonstrated a slightly increased incidence of chromosomal abnormalities (29-30). It remains to be seen whether these findings are linked to the technique itself or to the severe andrological infertility of the treated patients. So far, no large-scale well-documented data on the outcome of children after ICSI with normozoospermic semen are available (26).

How can we be more worried over genetic abnormalities in normozoospermia than of those in cases of severe male factor cases, i.e. Cases where there is a much greater risk of genetic abnormalities (10). Bonduelle et al. (31), in their recent study using pre-natal testing in ICSI pregnancies, concluded that there is a higher risk of de-novo chromosomal anomalies that is mainly related to a higher level of sex chromosomal anomalies and also to a higher level of de-novo structural anomalies, and not to the actual procedure of ICSI (10).

Finally, the financial aspect of ICSI. The answer to the argument that ICSI is more expensive than IVF, resulting in worries over the practice of ICSI by clinics with profit rather than the patient in mind, is quite simple; make the cost of ICSI the same as that of IVF. This is feasible, and will be implemented in Germany next year (10).

There are no data suggesting that ICSI should not be performed in all cases of in-vitro conception. In all cases, female factor or male factor (normal or abnormal sperm) the use of ICSI bypasses most dysfunctions eliminating the majority of barriers to fertilization. If fertilization still does not occur, then there is a greater chance of it being a genetic reason, and the risk of genetic abnormalities in normal sperm should not be of greater concern than those in abnormal sperm (10).

The impact of an unexpected total failure on the psychological and economic status of the patient is severe. The financial loss is particularly emphasized in countries with low national per capita income and are not covered by insurance.

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Suggested readings:

1. Aboulghar MA, Mansour RT, Serour GI, Amin YA, Ramzy AM, Sattar M. (1999) Management of long standing unexplained infertility: a prospective study. Am. J. Obstet. Gynecol.,181, 371-5
This is a large study on the treatment of unexplained infertility. Patietns who failed to become pregnant by COH/IUI were treated by IVF and ICSI on sibling oocytes. Total failure of fertilization occurred in 17.6% of the IVF oocytes.

2. Bhattacharya, S., Hamilton, M.P.R., Shaaban, M., Khalaf, Y., Seddler, M., Ghobara, T., Braude, P., Kennedy, R., Rutherford, A., Hartshorne, G., Templeton, A. (2001) Conventional in-vitro fertilisation versus intracytoplasmic sperm injection for the treatment of non-male-factor infertility: a randomised controlled trial. Lancet,357,2075-9
This is the largest multicenter prospective randomized study which compares IVF and ICSI in non male factor infertility. There is subgroup analysis for patients with unexplained infertility and mild male factor. The conclusion of the study is that there is no advantage of ICSI over IVF in non-male factor infertility.

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A comprehensive debate on the role of ICSI in patients with normozoospermic males.

4. Ruiz A, Remohi J, Minguez Y, et al. (1997) The role of in vitro fertilization and intracytoplasmic sperm injection in couples with unexplained infertility after failed intrauterine insemination. Fertil. Steril., 68, 171-173
This is a randomized study for ICSI versus IVF on sibling oocytes in patients with unexplained infertility. The study shows an advantage of ICSI in the treatment of unexplained infertility.

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This is a prospective randomized trial of IVF versus ICSI on sibling oocytes in patients with no definite male factor infertility. ICSI results in a significantly higher fertilization rate. The study also includeda meta analysis of randomized trials comparing IVF and ICSI.

Considering the use of ICSI for all cases of in vitro fertilisation

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Learning Objectives

1. To be made aware of the evidence comparing the use of ICSI with conventional IVF.
2. To review the evidence in relation to the use of ICSI for non-male factor infertility.
3. To understand which aspects of current clinical practice are supported by evidence.
4. To identify areas for future investigation, including the need for randomised clinical trials.

Introduction

Two major developments have shaped the provision of infertility treatment in the last 20 years. The first was the introduction of IVF 25 years ago and the second has been the gradual development of an evidence-based approach to clinical management. IVF was originally introduced as a treatment for women with tubal disease, but is now acknowledged as an effective treatment for subfertility of other causes, and also even when the cause is unclear (unexplained infertility).

With the introduction of intracytoplasmic sperm injection (ICSI) more than 10 years ago, IVF treatment could be extended to overcome male infertility. Thus ICSI has the potential to overcome fertilisation problems due to poor semen quality or when fertilisation rates in previous IVF cycles have been low. So successful has ICSI been in this respect, that it has even been suggested that it could be used in all cases of IVF, thus minimising the risk of fertilisation failure. This view has been supported by observations from a number of studies (reviewed in Bhattacharya et al 2001), suggesting that ICSI is associated with a higher rate of fertilisation and embryo implantation. On the other hand ICSI is a more invasive technique and there are other considerations including safety and cost.

This review will explain why current evidence does not support the routine use of ICSI, whether on grounds of rationale, efficacy, safety or economics.

Rationale

There is no doubt that ICSI is superior to all other micromanipulation methods at overcoming the zona pellucida barrier between the sperm and the egg. Indeed the development and use of ICSI has helped a clearer understanding of the essential functions of a spermatozoon in undertaking human fertilisation. For example capacitation is an essential for a sperm to penetrate the zona and complete fertilisation in vitro, but not when the sperm is injected directly into the egg cytoplasm. A comparison of ICSI with other micromanipulation techniques is given in the Cochrane Review last updated in 1999 (van Rumste et al 2003).

It has also been said the ICSI is superior to conventional IVF and should be offered to all patients because of a significantly higher fertilisation rate. However the limitations of the studies supporting this view, and particularly the limitations of studies using sibling oocytes to compare fertilisation rates, are now accepted (van Rumste et al 1999). The issues are clearly discussed in the review by Ola et al (2001).

Another rationale for using ICSI routinely is that fertilisation failure is often unpredictable, and can be associated with “occult male factor” problems or undiagnosed egg and egg investment defects. For example capacitation problems may inhibit sperm hyperactivation and sperm-egg fusion. Sperm dysfunction may also inhibit egg activation because of failure of sperm head decondensation and pronuclear formation. Thus problems may arise both pre – and – post – gamete fusion. Similarly developmental defects in apparently mature oocytes can occur due

to aneuploidism. Structural defects can occur in the zona pellucida and cytoplasm and may lead to defective fertilisation and embryogenesis. (For review see Oehninger and Gosden, 2002)

One of the great difficulties is in predicting individual cases of fertilisation failure, which can occur in up to 20% of apparently mature eggs, fertilised with normal spermatozoa. However because most of the time, most of the available eggs will fertilise in vitro conventionally, the problem only becomes clinically relevant when an undiagnosed defect in either the sperm or the egg causes complete and repeated fertilisation failure or early embryogenic arrest in all the eggs and embryos on each occasion. The frequency of this problem is unknown, but is certainly uncommon and may not be sufficient to justify the routine use of ICSI, particularly considering other issues. Furthermore it is not at all clear that when such a problem occurs, that ICSI will overcome failure of fertilisation in all cases, although it has to be accepted that it will clearly bypass some of the steps.

Clinical Trials

There is evidence from systematic review of the literature that fertilisation rates are significantly improved where ICSI rather than IVF is used in couples with deficient and borderline semen parameters (van Rumste et al 2003). It remains a matter of clinical judgement, when to use ICSI in cases of borderline semen, but any past evidence should probably also be taken into consideration including previous failed fertilisation, failed intrauterine insemination, and possibly long duration of infertility with no previous conceptions. However the Cochrane Review found insufficient evidence of a difference in effectiveness between ICSI and IVF when the semen parameters were normal and the unit of randomisation was retrieved oocytes. Thus until 1999 at least there was no evidence to support the suggestion that ICSI should be used except where specific male indications justified its use. The review did not however address previous cases of fertilisation failure. It is likely that a further update of this review will take place soon and is likely to include recent trials now referred to.

Tournaye et al (2002) carried out a meta-analysis of the available literature reporting randomised controlled trials comparing IVF and ICSI for the treatment of male subfertility. Seven studies dealt with couples with borderline semen characteristics. All studies compared fertilisation rates in sibling oocytes, allocated to either IVF or ICSI. Some studies also compared the effect of conventional IVF using a high concentration of sperm, which was very effective. The risk ratio was 1.9 (1.4 – 2.5 95% confidence interval) in favour of ICSI and it was suggested that three ICSI cycles might be needed to avoid one complete fertilisation failure with IVF. Fertilisation failed completely in 33% of IVF cycles and only 3% of ICSI cycles. Tournaye et al (2002) also reported the results of their own trial which drew similar conclusions. Overall the fertilisation rate was lower with IVF than ICSI (37% versus 64%), although when conventional IVF was used with a high concentration of sperm, the fertilisation rate was not significantly different. The overall conclusion was that ICSI is more efficient than conventional IVF, but not in comparison with a high insemination concentration. Thus it appears that the case for the exclusive use of ICSI, even in the presence of borderline semen characteristics, requires further consideration.

Poehl et al (2001) compared IVF and ICSI in 91 couples with tubal infertility or hostile cervical mucus. The patients were randomised to undergo either IVF or ICSI, and although the study has limitations, particularly in size, there were similar pregnancy rates in both groups with a suggestion of a higher implantation rate in the IVF group. The authors concluded that ICSI should be applied only when conventional IVF fails, that is for male factor patients and for patients with unexplained infertility. The justification for the last statement is unclear in the paper.

Bhattacharya et al (2001) compared IVF and ICSI in 415 couples undergoing 435 treatment cycles for non-male-factor infertility. This was a multicentre trial involving four UK centres. Nearly half the couples had tubal infertility, around a third unexplained or endometriosis and a fifth either ovulatory or mild male factor. Couples were randomised using a centralised telephone system incorporating minimisation of key prognostic variables (age, parity, previous treatment). The fertilisation rates per oocyte inseminated/injected were for IVF 58% and for ICSI 65%, and the failed fertilisation cycles were respectively 5% and 2%. However the important issue with this trial is that women rather than oocytes were randomised, and further analysis revealed clinical pregnancy rates of 33% (IVF) and 26% (ICSI), and implantation rates of 30% and 22%, the latter difference being statistically significant. Thus it appears that ICSI may result in an apparently higher fertilisation rate (see discussion of pitfalls in Cochrane Review) but a poorer implantation rate, which in a larger study may have translated into a significantly poorer pregnancy and live birth rate. The strengths and weaknesses of this study are frankly addressed in its Discussion, but it is relevant that the study is in accord with the conclusions of the systematic review and the Poehl trial mentioned above, namely that ICSI was associated with a higher fertilisation rate per oocyte injected, although it did not affect overall fertilisation rate. This is probably due to the fact that only mature oocytes are selected for

microinjection with ICSI, whereas all oocytes are inseminated with IVF.

An important additional finding was that subgroup analysis did not find any increased rate of failed fertilisation among the unexplained group having IVF.

Thus the overall conclusion of the study was that, as the clinical outcomes were similar, the factors likely to influence the choice of treatment are not effectiveness, but cost, convenience and safety. These issues are addressed in the next two sections.

Safety

As with many aspects of assisted reproduction, the long term consequences of ICSI must remain a matter of concern and constant review. Although the results from studies form the health of children born following ICSI are broadly reassuring, there is enough concern to keep the situation under surveillance and to keep clinical and laboratory techniques under scrutiny. A slightly higher frequency of sex-chromosome anomalies in the offspring has been suggested, although the incidence of this problem in children born following non-male indications for ICSI requires clarification. There is still a lack of clarity about the longer term consequences of ICSI. As yet there is no information about children followed through to adolescence and adulthood. Many of the published studies are reassuring in the genetic sense, but limited in terms of size and follow up, and often with a high incidence of loss to follow up.

Notwithstanding that certain congenital abnormalities may be associated with the male gametes, we are as yet unclear whether the technique itself is contributory. The clinical studies already referred to suggest a reduced implantation rate, perhaps associated with reduced embryogenesis. Some have suggested that the incorporation of foreign DNA combined with possible enhancement of defects by bypassing the natural selection mechanisms may by an issue that requires further study in the human. Where there is still uncertainty, there must be responsibility to confine the intervention to situations where it is clinically justified in the light of the available evidence.

Economic Appraisal

It goes without saying that case for case, ICSI is more expensive than IVF. Besides the initial cost of the equipment and materials, the most significant cost is the additional laboratory handling, particularly the time of the embryologist, carrying out the micromanipulation and related activities. Certainly from the patients' point of view the price of ICSI is considerably greater. The cost difference estimated in 1999 was about £600 more than IVF per fresh cycle. Ola and colleagues (2001) have carried out a cost benefit analysis suggesting that the additional cost of each live birth conceived by ICSI is £2,000. Furthermore incremental cost effectiveness suggests that £60,000 will be needed to gain one additional live birth when ICSI is advocated for all patients requiring IVF. That money could be used to treat an additional 29 cycles of conventional IVF. They conclude that recommending ICSI for all those in need of IVF is unlikely to be considered a judicious use of scarce resources.

Conclusion

It is accepted that ICSI has been a welcome and substantial development, possibly having the greatest impact on clinical practice, since the advent of IVF itself. There is no dispute that for severe male factor infertility, where spermatozoa can be recovered either from the ejaculate or surgically, that it offers couples their only hope of having their own child. The situation regarding mild male factor infertility has become less clear since the publication of the Tournaye study, suggesting that a high insemination concentration may be as effective as ICSI. As far as non-male-factor infertility goes there is absolutely no evidence from randomised trials to suggest that ICSI should be used routinely, indeed there is the suggestion of a disadvantage in so doing, particularly with respect to reduced implantation rates.

Failed fertilisation is difficult to predict, although it has been suggested that where a sperm or egg lesion exists, then in some cases ICSI may be able to overcome this. However there are no randomised studies comparing IVF and ICSI in the next cycle following a case of poor or absent fertilisation in a previous cycle. This study urgently needs to be done.

Leaving aside issues of efficacy, there are enough continuing concerns about safety, particularly in the longer term, to be prudent in the use of ICSI at the present time. In cost effectiveness terms there is absolutely no justification for extending the use of ICSI beyond current indications, particularly as this may limit the availability of IVF to deserving patients in certain circumstances.

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Recent randomised studies comparing IVF and ICSI.

Notes

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