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# Treatment of severe male infertility by micromanipulation-assisted fertilization: an update

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## 1. ABSTRACT

In the past 5-10 years the evolution of micromanipulation-assisted fertilization for the treatment of severe male infertility was marked by the introduction of new technical support, refinement of diagnostic methods for the evaluation of sperm developmental potential, and development of new treatment regimens for the newly discovered abnormalities. The new technical support involves the use of non-contact laser technology to assist micromanipulation for fertilization, the evolution of polarized microscopy-based optical systems to non-invasively detect the position of the meiotic spindle in living human oocytes, and the development of high-magnification optical systems for a better morphological selection of spermatozoa to be used for fertilization. Diagnostic approaches were enriched by commercial availability of kits for the analysis of sperm DNA integrity, leading to the definition of sperm nuclear DNA damage as a distinct cause of male infertility, and by the development of tests, based on heterologous ICSI, for detection of sperm failure to activate oocytes. Several treatment options for these conditions have been proposed and are currently being tested in larger-scale trials. Some technical improvement was also achieved in the field of *in vitro* maturation of germ cells from men with *in vivo* maturation arrest, but only a modest clinical improvement resulted from their application. As to the risk for the offspring, recent data are rather reassuring. Except for the risk of transmission of genetically based infertility, no straightforward evidence for a health risk derived from these techniques has been provided. Nevertheless, caution is necessary, particularly concerning the eventual increase in genomic-imprinting abnormalities.

## 2. INTRODUCTION

Micromanipulation-assisted fertilization has revolutionized the treatment of severe male infertility over the past 15 years. Not only has it made possible assisted reproduction with the patient's own gametes in cases of extremely severe male factor which previously required the recourse to sperm donation, but it also brought about conceptual changes in our understanding of fertilization-related biological events and opened new fields of research aimed at the treatment of cases in which mature spermatozoa are completely absent in the patient's testis. It was noted that fertilization often failed even when a spermatozoon was present in the oocyte cytoplasm. This observation drew the attention of researchers to the complex issue of oocyte activation and led to the definition of yet unknown pathological conditions in which infertility was due to the lack or insufficiency of oocyte-activating factors in spermatozoa or to a failed or abnormal response of the oocyte to sperm-derived activating factors.

The possibility of using immature (epididymal or testicular) spermatozoa, and even less mature germ cells for micromanipulation-assisted fertilization opened the question about the relationship between germ cell nuclear and cytoplasmic maturity, on the one hand, and fertilizing ability and developmental potential of the resulting embryo on the other hand. It has been shown that nuclear immaturity is often associated with nuclear DNA damage which, however, can be present in mature spermatozoa of some men, too. Clinical consequences of this condition and possibilities of their alleviation have been studied.

Finally, technical progress in the field of microscopic analysis and micromanipulation of living cells, namely the introduction of computer-assisted polarized microscopy (Polscope) systems, of non-contact laser micromanipulation devices, and of high-power optical systems, brought about new possibilities in the selection of gametes to be used for micromanipulation-assisted fertilization and in the management of some particular cases in which standard manipulation procedures failed.

This paper updates the current options for the treatment of severe male infertility by micromanipulation-assisted fertilization taking into account these new developments.

## 3. NEW DEVELOPMENTS IN MICROMANIPULATION-ASSISTED FERTILIZATION TECHNIQUES AND THEIR USE IN MALE INFERTILITY MANAGEMENT

### 3.1. New technical support

Since the publication of the last version of this review (1), new technical possibilities emerged, namely in the fields of laser-assisted manipulation, microscopy and *in vitro* culture. The introduction of non-contact laser-mediated manipulation devices has made it possible to perform ICSI through a pre-drilled opening in the zona pellucida, thus making the manipulation more oocyte-friendly, which is of importance in some special cases. New optical systems introduced to micromanipulation assisted fertilization include the Polscope and the high-power magnification Nomarski optics. The former enables avoidance of the oocyte chromosome region during injection, and the other facilitates the selection of spermatozoa to be injected according to finer structural analysis as provided by the conventional systems. Finally, new *in vitro* culture systems have been proposed for *in vitro* maturation of immature germ cells from patients with maturation arrest.

#### 3.1.1. Laser-assisted ICSI

Laser-assisted ICSI has been first described in 2001, when it was used in a couple with four previous ICSI failures because of poor oocyte survival resulting in a clinical pregnancy (2). A similar case report was published one year later (3). These pilot studies were followed by larger clinical series which confirmed the suitability of laser-assisted ICSI in cases of inherent oocyte fragility (4, 5, 6). Moreover, in addition to reducing the risk of post-ICSI oocyte death, an improvement of day 2 embryo quality, facilitation of blastocyst hatching and an increase in the clinical pregnancy rate were reported in sibling oocytes randomly allocated to conventional or laser-assisted ICSI (6).

In spite of these encouraging results the indication criteria for the use of laser-assisted ICSI still remain to be defined in a more precise and reproducible manner, since the use of the laser technique represents an additional workload associated with the ICSI procedure and the clinical benefit of this ICSI modification in the general patient population has not been demonstrated.

#### 3.1.2. The use of Polscope system for meiotic spindle visualization in living oocytes

Polscope is a computer-assisted polarization microscopy system with which the oocyte meiotic spindle can be visualized in living oocytes on the basis of its birefringence (7). It permits the analysis of the meiotic spindles of oocytes subjected to ICSI and was first described in this function in 2001 (7, 8). It was shown that the prognosis of oocytes in which meiotic spindle cannot be easily visualized by Polscope after ICSI is compromised (7, 8, 9, 10, 11). The ease with which the oocyte meiotic spindle can be visualized by Polscope may depend on the duration of *in vivo* action of HCG on the maturing oocyte, since a spindle was imaged in a significantly higher number of oocytes from ≥38 h after HCG administration compared with those in the <38 h group (11).

Further studies have addressed the potential predictive value of the actual position of the spindle with regard to the first polar body in ICSI-subjected oocytes (9, 10). The predictive value of the spindle position remains a controversial issue because no relationship was found in one of these studies (9), whereas the other showed that high degrees of misalignment between the meiotic spindle and the first polar body predict an increased risk of fertilization abnormalities (10).

Quite recently Polscope was used to evaluate optical qualities of the zona pellucida; this analysis suggested that high-quality oocytes, associated with

conception cycles in ICSI, show a higher magnitude of light retardation by the zona pellucida as compared to oocytes used in non-conception cycles (12).

### 3.1.3. The use of high-magnification sperm selection

In conventional ICSI both sperm selection and injection are performed with the use of Hofman modulation contrast optics and the objective of x20, leading to an overall optical magnification of about x400 (13). It was suggested recently that ICSI outcomes may be improved by selecting spermatozoa with the use of higher-powered optical systems (14). To this end a system using Nomarski interference contrast optics with a x100 oil-immersion objective and a secondary magnification system, leading to a total magnification of x6000, was developed (14).

A prospective controlled study, performed in couples with male infertility and at least two previous failed ICSI attempts, showed that ICSI with spermatozoa selected with this high-magnification optical system resulted in a significantly higher pregnancy rate as compared with conventional ICSI (15). A subsequent study, performed by the same group, showed that higher implantation and pregnancy rates were achieved by ICSI when at least some morphologically normal spermatozoa could be selected with this high-power system as compared to cases in which only morphologically abnormal spermatozoa were available (16).

These observations have suggested that high-magnification ICSI may be the treatment of choice in cases of repeated conventional ICSI failures caused by a paternal effect on embryo development. Two types of paternal effect have been recognized, an early paternal effect which becomes manifest as early as the pronuclear zygote stage and is usually not associated with increased sperm nuclear DNA fragmentation, and a late paternal effect which does not impair embryo growth and morphology during three days post-fertilization and is mostly associated with elevated sperm nuclear DNA fragmentation (17, 18, 19). Work is in progress to compare the benefits of high-magnification ICSI in each of the two types of paternal effect.

### 3.1.4. *In vitro* culture systems for germ cell *in vitro* maturation

At the time at which the first edition of this review (1) was written the first *in vitro* culture system with which human male germ cell maturation arrest could be overcome was developed (20, 21). The first childbirths after transfer of embryos conceived with *in vitro* matured late spermatids in patients with post-meiotic maturation arrest at the round spermatid stage and in one patient with meiotic maturation arrest at the primary spermatocyte stage were subsequently achieved with the use of this system (22). The feasibility of obtaining healthy offspring from oocytes fertilized with spermatids developed *in vitro* from primary spermatocytes has been confirmed in the mouse model (23). The system used in humans was a relatively simple one and was based on culture of isolated segments of dissected seminiferous tubules at 30°C in a commercial culture medium destined for incubation of human gametes supplemented with high concentrations of FSH and testosterone (20, 21). It was speculated that the maintenance of the original cellular associations between germ cells and their supporting Sertoli cells was a key to success because it allowed the Sertoli cells to support germ cell differentiation and to protect the differentiating germ cells from programmed cell death in response to stimuli received by FSH and testosterone, respectively (21). This avoided the use of an exogenous supporting cell layer which was demonstrated to be required in animal models if germ cells were separated from Sertoli cells at the outset of *in vitro* culture (24). However, it appears that some developmental controls, normally exerted by Sertoli cells upon germ cells, were released in this *in vitro* system because virtually all of the *in vitro* matured spermatids took atypical forms; the elimination of these control processes might be the main reason why the *in vitro* differentiation progressed at a markedly accelerated path (some primary spermatocytes developed to elongated spermatids within 48 hours of culture) as compared with *in vivo* conditions (20, 21).

Further study showed that the efficacy of *in vitro* maturation of germ cells from patients with maturation arrest can be increased by further increasing FSH concentration in culture medium up to 50 IU/l (25). However, the early belief that germ cell *in vitro* maturation will rapidly develop to a highly efficient and routinely used treatment option did not come true (reviewed in 26). The main problem hampering fertilization outcomes with germ cells from men with maturation arrest is not so much germ cell immaturity but rather the basic pathological condition underlying the maturation arrest. Germ cells from men with maturation arrest have often fragmented DNA in their nuclei, a condition resulting from programmed cell death (apoptosis) activated in the testis to remove these blocked cells from the seminiferous tubules (27). Even though *in vitro* culture exerts a negative pressure against apoptotic germ cells and decreases the risk of inadvertently injecting a cell with fragmented nuclear DNA into the oocyte (28), it may not overcome functional abnormalities disturbing early embryo development and implantation which are associated with maturation arrest.

Based on the observation that *in vitro* incubation of testicular biopsy samples facilitates the selection of late spermatids and spermatozoa devoid of nuclear DNA damage (28), this technique was tested with success in cases in which a limited number of spermatozoa could be found in the testis, but most testicular germ cells had fragmented DNA (29, 30). In fact, as comparing with different types of maturation arrest, it was this latter indication in which *in vitro* culture of testicular germ cells achieved the best clinical outcomes (31).

More recently, an alternative *in vitro* maturation system, in which isolated human germ cells, detached from their supporting Sertoli cells, are cultured on a monolayer of Vero cells or human fibroblasts (32). As compared with the previously described system, relying on the preservation of the original germ-Sertoli cell association, this new system appeared to be more efficient in terms of

the percentage of germ cells from men with *in vivo* maturation arrest that resumed meiosis and post-meiotic differentiation *in vitro*, but the clinical efficacy, in terms of ongoing pregnancy and delivery rates, remains to be determined (33).

## 4. NEW DEVELOPMENTS IN THE DIAGNOSIS AND TREATMENT OF GENETIC AND EPIGENETIC DEFICIENCIES OF THE MALE GAMETE

### 4.1. Sperm chromatin defects and DNA fragmentation

Elevated percentage of spermatozoa whose nuclear DNA is subject to fragmentation, similar to that resulting from programmed cell death in somatic cells, occurs in some men and is suspected to reduce fertility and to compromise assisted reproduction outcomes (18, 34, 35, 36, 37, 38, 39, 40 41). Different techniques have been developed to assess the degree of sperm nuclear damage. Sperm chromatin structure assay (SCSA) (42, 43), terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) assay (44, 45) and single cell gel electrophoresis (Comet assay) (37, 38) are currently the three most widely used ones.

To interpret and correlate data obtained with each of these three techniques, it is important to be aware of the existence of essential conceptual differences between SCSA, TUNEL and Comet assays, each of which reveals different aspects of sperm chromatin damage. Hence, according to the technique used, different cut-off values for clinically relevant extent of sperm DNA fragmentation have been proposed (reviewed in 46). Moreover, even with the same technique, results obtained in different laboratories may differ because of variability of laboratory factors influencing the assessment protocol and criteria of interpretation (47). Notwithstanding, with a few exceptions (48), most studies performed so far concord in that the establishment of a full-term pregnancy by assisted reproduction is compromised if the percentage of DNA-fragmented spermatozoa in the ejaculate, as detected by TUNEL, is high (reviewed in 49). A similar relationship has been reported for SCSA (50).

The first efficient treatment of male infertility due to sperm nuclear DNA fragmentation implied the use of surgically retrieved testicular spermatozoa for ICSI (46). The rationale of this approach was based on previous observations which showed that sperm DNA damage is basically produced after the release of spermatozoa from the seminiferous tubules, suggesting that testicular spermatozoa can be expected to have less damaged DNA as compared with ejaculated spermatozoa (51). More recently, oral antioxidant treatment with high doses of vitamins C and E (1 g of both daily during 2 months) has been shown to markedly reduce the percentage of DNA-fragmented spermatozoa in the ejaculate (52), and satisfactory clinical pregnancy rates were reported after ICSI with ejaculated spermatozoa in patients in whom this effect was observed (49). However, sperm DNA damage was insensitive to antioxidant treatment in some men for whom the recourse to testicular sperm retrieval for ICSI was thus indicated (52).

An alternative approach to the treatment of male infertility due to sperm nuclear DNA damage was suggested recently by a group that pioneered the use of ICSI with spermatozoa morphologically selected with the use of a high-magnification, computer-assisted optical system (14, 15, 16). With the use of this technique small vacuoles, not detectable with magnifications currently employed for conventional ICSI, can be detected in the head of some spermatozoa, and avoiding the injections of such spermatozoa to oocytes was reported to increase significantly clinical pregnancy rate (16). It can be hypothesized that these vacuoles represent regions of incompletely condensed sperm chromatin where sperm nuclear DNA is less protected against oxidative damage, which is the main cause of sperm nuclear DNA fragmentation (53), as compared with DNA embedded within highly condensed chromatin regions. Accordingly, spermatozoa with intra-nuclear vacuoles may still have intact DNA, but they would be at a higher risk of suffering DNA damage in pathological conditions leading to excessive production of reactive oxygen species in the male genital tract. Preliminary observations, indicating that the use of high-magnification ICSI improves significantly clinical pregnancy rate in couples with high sperm nuclear DNA fragmentation as compared with a previous attempt using conventional ICSI (54), corroborate this hypothesis.

### 4.2. Deficiency of oocyte-activating factors

Complete fertilization failure or severely impaired fertilization rate after ICSI is a rare condition if motile spermatozoa can be selected for injection. However, it occurs in 2–3% of ICSI cycles (55), repeatedly in most of them, and is mainly due to lack of oocyte activation (56, 57). This has been noted for the first time in patients with round-headed spermatozoa (globozoospermia) although spermatozoa of these men fertilize normally after ICSI in about one half of these cases (58). However, complete or nearly complete fertilization failure after ICSI can also occur in patients with morphologically normal spermatozoa (58, 59, 60, 61).

The observations that most of human oocytes that failed to fertilize after ICSI can be 'rescued' by an artificial increase of free intracellular calcium ion concentration with the use of a ionophore (62) and that a virtually normal pattern of calcium signals can be restored in such oocytes, possibly owing to the presence of sub-threshold quantities of oocyte activating factors in the spermatozoa injected (63), were at the origin of clinical attempts at boosting fertilization with calcium ionophores in patients with previous complete or near-complete fertilization failures. After the first report describing a successful pregnancy and delivery after intracytoplasmic sperm injection (ICSI) and assisted oocyte activation with ionophore A23187 after ICSI in a globozoospermic patient (64), this method was validated by other groups achieving ongoing pregnancies and births in patient with defective sperm oocyte-activating activity both associated and not associated with globozoospermia (58, 59, 60, 61, 65, 66).

Failed oocyte activation is mostly a consequence of defective calcium signalling after sperm deposition in

oocyte cytoplasm (67). Hence, other methods increasing calcium concentration in the oocyte cytoplasm during or after ICSI can also overcome oocyte-activation deficiencies. In addition to the use of ionophores, this can be achieved by electrical stimulation of sperm-injected oocytes (68) or by simple modifications of the ICSI techniques, increasing the intensity or duration of calcium influx from external medium to oocyte cytoplasm during ICSI (58, 61). All these methods have been reported to work in cases of oocyte-activation defects and led to the establishment of ongoing pregnancies. A larger, multi-centre prospective study is needed to compare the clinical efficacy and safety of these different assisted oocyte activation methods.

An animal test using heterologous ICSI with the patient's spermatozoa to mouse, hamster, rabbit or bovine oocytes (69, 70, 71) can be used to evaluate the oocyte-activating potential of spermatozoa and to decide whether the recourse to a special method to boost oocyte activation after ICSI is required.

**4.3. Deficiency of the sperm centriole**

In some cases repeated ICSI failures are suspected to be due to a defective function of sperm centriole (18, 72, 73, 74, 75). Defects of centriole function as nucleation sites for zygote's microtubule organizing regions can be suspected in cases in which oocytes become fertilized, but the two pronuclei fail to establish a close apposition and to enter syngamy. This functional abnormality can be revealed by a heterologous ICSI test using bovine oocytes and cytochemical visualization of microtubuli in newly formed asters with the use of antibodies against acetylated- and ß-tubulins (75).

Whether techniques relying on micromanipulation-assisted fertilization can be of help in these cases still remains to be evaluated. Centriolar abnormalities are often associated with subtle morphological alterations of the sperm head-tail junction, and pronuclear alignment followed by syngamy was achieved in a case of previous complete syngamy failure by carefully avoiding sperm with obvious anomalies of the connecting piece to be used for ICSI (75 ). However, out of three cycles performed in this way only two yielded a pregnancy and both pregnancies resulted in a pre-clinical abortion (75). A question arises whether the newly described technique of high-magnification ICSI (see the section 3.1.3) would be of help in these cases.

## 5. CLINICAL EFFICACY

The introduction of new diagnostic, microsopic and micromanipulation methods in the past years has improved clinical efficacy of assisted reproduction in some types of severe male infertility. Major improvements have been achieved in cases of severe teratozoospermia and high degrees of sperm nuclear DNA fragmentation. Some improvement was also made in the treatment of germ cell maturation arrest, although the efficacy remains low in comparison with the former two indications.

**5.1. Management of severe teratozoospermia**

Several studies have reported a lack of association between the degree of teratozoospermia and ICSI outcomes (76, 77, 78). This observation can be explained by the selection of normal-appearing spermatozoa to be used for ICSI which is a rule in most laboratories. In extreme cases, however, in which no morphologically normal spermatozoa could be found, lower pregnancy and implantation rates were reported as compared with those in which at least some morphologically normal spermatozoa were used for ICSI (13). Even though morphologically abnormal spermatozoa may fertilize oocytes, the resulting embryos often fail to implant or are aborted because of chromosomal abnormalities which are linked to sperm morphological deformities (79).

Moreover, some spermatozoa which would be considered as normal with the use of the current optical magnification employed in conventional ICSI would be classified as abnormal if higher magnifications were used. This can explain the beneficial effects on implantation and pregnancy rates reported with the use of high-magnification sperm selection (15, 16). Similar to conventional ICSI, clinical outcomes of this high-magnification ICSI are better when at least a few morphologically normal spermatozoa can be selected (16).

These data suggest that the use of high-magnification ICSI could hardly help those cases in which all spermatozoa available for ICSI are classified as morphologically abnormal already with the use of conventional magnifications. On the other hand, the high magnification can be expected to improve outcomes in cases in which many spermatozoa bear subtle morphological abnormalities which would not be recognized in the conventional ICSI setting. Further study is needed to determine the frequency of such cases in the population of infertile patients in which ICSI is indicated.

**5.2. Management of high degree of sperm DNA fragmentation**

Elevated percentages of spermatozoa with damaged DNA in the ejaculate do not necessarily predict assisted reproduction failure (48). However, most studies found a negative correlation between the degree of sperm nuclear DNA fragmentation and the chance of pregnancy although there is a lack of consensus as to the cut-off values (see Section 4.1). Recently it was reported that elevated percentages of TUNEL-positive (80) and SCSA-positive (81) spermatozoa are negatively correlated with *in vitro* blastocyst development. Furthermore, increased figures of sperm nuclear DNA fragmentation were found in couples with recurrent pregnancy loss (40) This is in agreement with the assumption that spermatozoa with DNA fragmentation can still fertilize an oocyte but that when paternal genes are "switched on," further embryonic development stops, resulting in failed pregnancy (37, 41, 82, 83, 84).

A recent study has reported high pregnancy (44.4%) and implantation (20.7%) rates in patients with at

least two previous non-conception ICSI cycles and elevated percentage of TUNEL-positive spermatozoa in the ejaculate in a subsequent ICSI attempt performed with testicular spermatozoa (46). Moreover, similar high success rates (48.2% pregnancy rate and 19.6% implantation rate) were achieved by ICSI with ejaculated spermatozoa after 2 months of oral antioxidant treatment (1 g vitamin C and 1 g vitamin E daily) in patients who showed a significant decrease in the percentage of TUNEL-positive spermatozoa after this treatment (49).

Preliminary data suggest that the application of high-magnification ICSI (see section 3.1.3) may also substantially improve clinical outcomes of ICSI in patients with sperm nuclear DNA damage (54). Further research is needed to define the patient groups for which each of the three above approaches would be the most suitable.

### 5.3. Management of maturation arrest

In spite of the improvement owing to the application of germ cell *in vitro* maturation systems with which *in vivo* maturation arrest can be overcome (see section 3.1.4), the chance of ongoing pregnancy and birth after injection of the resulting late spermatids to oocytes remains low (1-10% depending on the type of maturation arrest and on the female partner's age). These low success rates contrast with relatively high efficacy of fertilization with immature or *in vitro* matured germ cells reported in animal experiments (85). This difference can be explained by the fact that the animal experiments were performed with germ cells from healthy animals as opposed to the human clinical data obtained with germ cells recovered from patients suffering from different underlying pathological conditions, mostly of unexplained aetiology (31). Unless the pathological mechanisms leading to testiculopathies underlying maturation arrest in humans are defined, and appropriate therapies of the causative factors are discovered, no substantial progress can be expected.

## 6. HEALTH HAZARDS FOR THE OFFSPRING

In the last edition of this review (1) potential hazards of micromanipulation-assisted fertilization in cases of severe male infertility were outlined, but all of them were merely hypothetical. In the meantime some new data emerged, as nicely reviewed by Retzloff and Hornstein (86). However, the major conclusion of this review was that, although there remains some uncertainty, the overwhelming majority of studies are reassuring in their findings of no specific increased risk for the offspring.

In 1996, Reijo *et al.* (87) found that 13% of 89 men with nonobstructive azoospermia have microdeletions on the long arm of the Y chromosome in the DAZ cluster of genes. This suggests that these abnormalities may easily be incorporated into the genome of the male offspring of ICSI. It remains to be determined whether the sons of fathers with Y chromosome microdeletions will have the same "reproductive phenotype" and thus require these same procedures themselves once they reach maturity.

Transmission of chromosomal abnormalities, whose risk is two-fold to twelve-fold increased in infertile men (86) to offspring is another subject of concern. In fact, a significant increase in the incidence of sex chromosome abnormalities has been demonstrated in children conceived by ICSI as compared to general population (88, 89), presumably mainly due to transmission of paternal chromosomal aberration, whose frequency is known to be increased in infertile men (90, 91, 92, 93, 94) and to be negatively correlated with sperm concentration and particularly with the percentage of normal forms (95). De novo karyotypic abnormalities, unrelated to abnormalities in the male, are less frequent, but they exist (96) and may be produced by some factors related to the ICSI procedure (97).

In spite of isolated case reports on specific congenital malformation and developmental abnormalities after ICSI, there is probably no significant increase in these pathological conditions in the overall ICSI offspring population (86). However, one large, well-designed study on the risk of major birth defects after ICSI (98) as well as the reports of imprinting defects, mainly the cases of Beckwith-Wiedemann syndrome (99, 100, 101) and Angelman syndrome (102) are cautionary in this regard. It is reassuring that there appears to be no increase in malformations of any specific organ system (86).

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**Key Words:** Male Infertility, Micromanipulation-Assisted Fertilization, Sperm Dna Damage, Teratozoospermia, Maturation Arrest, Oocyte Activation Failure, Laser-Assisted Icsi, High-Magnification Icsi, Polscope, Review

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