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New Advances in Intracytoplasmic Sperm Injection (ICSI)

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1. Introduction

In these last twenty years, intracytoplasmic sperm injection (ICSI) has efficiently permitted the treatment of male factor infertility (Van Steirteghem et al., 1993); the direct injection of spermatozoa into ooplasm has allowed the embryologist to overcome low sperm motility, poor sperm-Zona Pellucida (ZP) binding, and defective acrosome reaction. Although ICSI has been successfully applied worldwide for several years, nevertheless we have no real knowledge regarding the hypothetical long term side effects on ICSI adults. In fact, some doubts about the safety of this technique can arise (Oehninger, 2011) due to the fact that with ICSI some check points of natural fertilization are bypassed and that some steps differ considerably from the physiological process; For instance, the introduction of the sperm tail into the ooplasm may cause sperm nuclear decondensation problems (Dozortsev et al., 1995; Markoulaki et al., 2007). It should be considered that ICSI may increase the risk of injecting spermatozoa with genetic or functional anomalies (Sakkas et al., 1997; Bonduelle et al., 2002; Marchesi & Feng, 2007; Schatten & Sun, 2009; Heytens et al., 2009; Navarro-Costa et al., 2010). For these reasons and to minimize any risk related to ICSI, any new advance in this procedure which can help the operator to restore some of the basic physiological checkpoints and to simulate the natural fertilization process should be welcome (Parmegiani et al., 2010a).

2. Hyaluronic acid (HA) and Zona Pellucida: Two important human fertilization checkpoints

In nature, human oocytes are surrounded by:

- the cumulus oophorus-corona radiata complex (COC), made up of cells and an extracellular matrix of polymerized hyaluronic acid (HA) and proteins
- the Zona Pellucida (ZP), a thick elastic coat of glycoproteins located immediately next to the oocyte (Yanagimachi, 1994)

These layers have to be penetrated by spermatozoa before they fuse with the oolemma.

In the human testis, during spermiogenesis, the elongated spermatids undergo cytoplasmic extrusion and plasma membrane remodelling which determines the formation of the HA and ZP receptors, essential for sperm penetration into oocyte.

At the end of spermiogenesis, different expression levels of two specific proteins seem to be related to sperm maturity, DNA integrity, chromosomal aneuploidy frequency and fertilizing potential. These two proteins are:

- the heat shock protein HspA2 chaperone, involved in meiosis
- creatine kinase (CK), abundant in the sperm cytoplasm (Cayli et al., 2003)

Mature spermatozoa have high HspA2 (Huszar et al., 2000) and low CK (Cayli et al., 2003). In contrast, spermatozoa with arrested maturity have low HspA2 expression, which may cause meiotic defects and probably chromosomal aneuploidies, in fact, mature spermatozoa show a reduction of more than five fold in aneupliody rate than immature ones (Jakab et al., 2005). Immature spermatozoa also have higher levels of CK (Huszar & Vigue, 1993); this high level of CK in immature spermatozoa is due to a sperm defect in terminal spermiogenesis when in normal development the surplus cytoplasm is extruded from the elongating spermatid as 'residual bodies' (Cayli et al., 2003). In contrast, arrested/ diminished maturity spermatozoa show a higher retention of cytoplasm with CK and other cytoplasmic enzymes, increased levels of lipid peroxidation and consequent DNA fragmentation, and abnormal sperm morphology. Due to the lack of their membrane remodelling, these immature spermatozoa have deficiency in the ZP and HA binding sites and for this reason they are not able to fertilize the oocyte naturally.

3. Physiologic HA ICSI

In nature hyaluronic acid (HA), is involved in the mechanism of sperm selection because only mature spermatozoa which have extruded their specific receptors to bind to HA are able to reach the oocyte and fertilize it. The role of HA as "physiological selector" is also well recognized in-vitro. It has been demonstrated that the spermatozoa able to bind to HA in-vitro are those which have completed their plasma membrane remodelling, cytoplasmic extrusion and nuclear maturation (Cayli et al., 2003; Huszar et al., 2003; 2007). Furthermore, HA-bound spermatozoa have a better morphology (Prinosilova et al, 2009; Parmegiani et al., 2010a) and they show a reduced risk of being aneuploid (Jakab et al., 2005) or having fragmented DNA (Parmegiani et al., 2010a). Because of this, selection of spermatozoa by HA prior to ICSI helps to optimize the outcome of the treatment (Parmegiani et al., 2010 a, b) and also has a number of other advantages:

- in practical terms, HA-bound spermatozoa can be easily recovered using an injecting pipette (Balaban et al., 2003)
- HA-containing culture medium have no negative effects on post-injection zygote development (Van den Bergh et al., 2010)
- Because of its natural origin HA can be metabolized by the oocyte (Balaban et al., 2003; Barak et al., 2001; Van den Bergh et al., 2010)

At very least, HA represents a more natural alternative for handling spermatozoa prior to ICSI than the synthetic plastic polyvinylpyrrolidone (PVP), which is routinely used to reduce sperm motility during ICSI procedure in the majority of AR centres and has been hypothesized to have toxic effects on oocytes (Jean et al., 1996; 2001).

A "home made" HA-sperm selection system can be simply produced in any IVF lab (Huszar et al., 2003, Nasr-Esfahani et al. 2008). However at the present time, two ready-to-use systems specially designed for sperm-HA binding selection are currently available:

- a plastic culture dish with microdots of HA hydrogel attached to the bottom of the dish (PICSI® Sperm Selection Device, MidAtlantic Diagnostic - Origio, Måløv, Denmark), Figure 1.
- a viscous medium containing HA (Sperm Slow™, MediCult – Origio)

This new approach to ICSI with HA-bound spermatozoa, when using HA-viscous medium or HA-culture dishes, has been defined as "Physiologic ICSI" (Parmegiani et al., 2010a).

Since both these sperm-HA binding selection systems are easily available, efficient and approved for IVF use (Parmegiani et al., 2010 a; 2010 b; Mènèzo & Nicollet, 2004; Worrilow et al., 2007; 2010) IVF centres can choose the one best suited to their needs. The viscous medium requires a specific procedure of droplet preparation to optimize the selection of HA-bound spermatozoa (Parmegiani et al., 2010b); conversely, it is more versatile than PICSI as it can be used also on a glass-bottom culture dish for high magnification sperm evaluation: "physiologic IMSI" (Parmegiani et al., 2010 a) *see also paragraph 5, IMSI*. On the other hand, PICSI HA-bound spermatozoa can be easily recognized even by non-trained embryologists.

3.1 PICSI procedure

PICSI dishes are conventional plastic culture dishes pre-prepared with 3 microdots of powdered HA. The powdered HA is re-hydrated by adding a 5 µL droplets of fresh culture medium to each of the three microdots. A 2 µL droplet with suspension of treated spermatozoa is then connected with a pipette tip to these culture medium droplets. The PICSI dish is incubated under oil; within 5 minutes the bound spermatozoa are attached by their head to the surface of the HA-microdots and are spinning around their head (Figure 1).
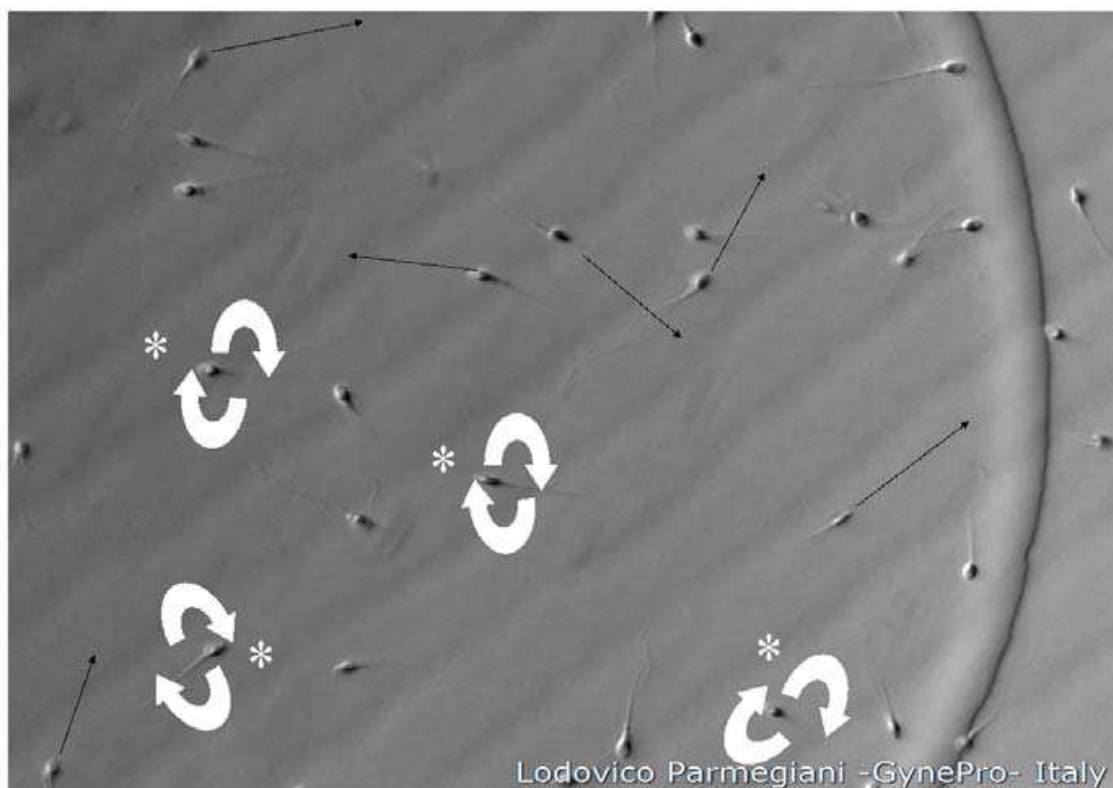

Fig. 1. Spermatozoa in PICSI dish (magnification 400 X)

An ICSI injecting pipette is used to pick the best motile HA-bound sperm up and inject them one by one into an oocyte. The ICSI injecting pipette can be previously loaded with viscous medium (PVP or Sperm Slow) to facilitate sperm micromanipulation.

In PICSI, HA-sperm (*) are bound by the head to the bottom of the dish and have vigorous motility with the tail spinning around their head. HA-unbound spermatozoa, in contrast, swim free all around the droplet of culture medium with varied motility.

3.2 Sperm slow procedure (*Parmegiani et al., 2010b*)

On a culture dish (plastic or glass bottomed), a 2 µL droplet with suspension of treated spermatozoa is connected with a pipette tip to a 5 µL droplet of fresh culture medium. Simultaneously, a 5 µL droplet of Sperm Slow is connected with a pipette tip to the 5 µL droplet of fresh culture medium (Figure 2). The spermatozoa on this culture dish are incubated for 5 min at 37°C under oil. Spermatozoa bound to HA are slowed (as if trapped in a net) in the junction zone of the 2 droplets, these spermatozoa are selected and detached by injecting pipette and subsequently injected into oocytes. In Sperm Slow, HA-bound sperm tail appears stretched, its motility is dramatically slowed and its beats have narrow amplitude. HA non-bound spermatozoa swim all around the medium droplet, they are less slowed by the viscosity of the medium and their tail-beats have wider amplitude.
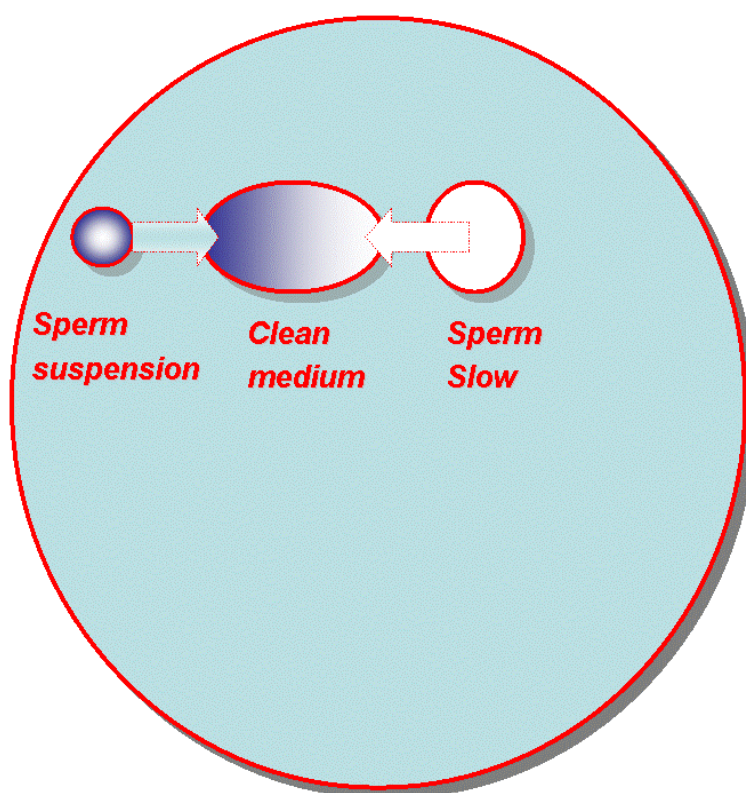

Fig. 2. Sperm Slow droplet preparation

A 2 µL droplet with suspension of treated spermatozoa is connected with a pipette tip to a 5 µL droplet of fresh culture medium. Simultaneously, a 5 µL droplet of Sperm Slow is connected with a pipette tip to the 5 µL droplet of fresh culture medium.

3.3 Clinical efficiency of "physiologic ICSI"

It has been demonstrated that the injection of HA-bound spermatozoa improves embryo quality and development by favouring selection of spermatozoa with normal nucleus and intact DNA: in fact, top-quality embryo rate is higher in HA–ICSI than in conventional PVP-ICSI and embryo development rate has also been found to be significantly increased (Parmegiani et al., 2010 a). Furthermore, HA-ICSI may speed up the time-consuming IMSI (Parmegiani et al., 2010 a). The largest study published to date as full article (428 patients) comparing physiologic HA-ICSI to conventional PVP-ICSI (Parmegiani et al., 2010 b) revealed that injection of HA-bound spermatozoa determines a statistically significant improvement in embryo quality and implantation.

A positive trend in fertilization and pregnancy rates - when injecting HA-bound spermatozoa – has been reported (Mènèzo & Nicollet, 2004). Nasr-Esfahani et al. (2008) have also published a study showing a higher fertilization rate when injecting oocytes with HA-selected spermatozoa.

A statistically significant improvement in fertilization rate and embryo quality and a reduction in the number of miscarriages were found by Worrilow et al. (2007) performing PICSI versus conventional ICSI. In a subsequent study, the same authors demonstrated that PICSI significantly improves embryo quality, significantly reducing embryo fragmentation rate on day 3 and favours good blastocyst formation and clinical pregnancy rate (Worrilow et al., 2010).

In contrast, one report found no differences in fertilization, pregnancy and implantation rates (Sanchez et al., 2005); this lack of significant clinical improvements after the injection of HA-bound spermatozoa may be due to the small number of patients studied (18). Recently, a historical comparison between 2014 HA-ICSI and 1920 PVP-ICSI showed no statistically significant increase in embryo quality and pregnancy rate for physiologic ICSI (Mènèzo et al., 2010).

Van den Berg et al., (2010) found no difference in zygote score when injecting, in a prospective randomized way, 407 sibling metaphase II oocytes, with either HA bound (HA+) or non-bound (HA-) spermatozoa. Our group (Parmegiani et al., 2010 c) questioned the ethical aspect of this study, which was based on the injection of HA non–bound spermatozoa, due to the risk of transmission of chromosomal anomalies.

In conclusion, most of the studies cited above showed an improved clinical outcome of physiologic ICSI using HA-viscous medium or HA-dish (Parmegiani et al., 2010 a; 2010b; Worrilow et al., 2007; 2010; Nasr-Esfahani et al., 2008). At the very least, in all the studies physiologic ICSI never caused a detrimental effect on ICSI outcome parameters (Table 1). If larger multi-centre prospective-randomized studies confirm the suggested beneficial effects on ICSI outcome, HA should be considered the first choice for "physiologic" sperm selection prior to ICSI because of its capacity to reduce genetic complications and for its total lack of toxicity (Parmegiani et al., 2010 c).

FR: fertilization rate; EQ: embryo quality; PR: pregnancy rate; IR: implantation rate; MR: miscarriage rate; ND: not described.

Authors	HA-System	N° of treatments or patients	HA-ICSI determines :
Menezo et Nicollet, 2004	Sperm Slow	92 HA-ICSI vs 110 PVP-ICSI	No differences
Sanchez et al, 2005	N.D.	18 HA-ICSI versus control group	No differences
Worrilow et al, 2007	PICSI	240 couples: PICSI vs PVP-ICSI	Improvement in FR, EQ, MR
Nasr-Esfahani et al, 2008	home-made	50 couples: sibling oocytes; HA-ICSI vs PVP-ICSI	Improvement FR
Van Den Berg et al, 2009	Sperm Slow	44 couples: sibling oocytes; HA+ vs HA- sperms	No differences
Parmegiani et al, 2010 a	Sperm Slow	125 HA-ICSI vs 107 PVP-ICSI	Improvement in EQ
Parmegiani et al, 2010 b	Sperm Slow	331 HA-ICSI vs 97 PVP-ICSI	Improvement in EQ, IR
Worrilow et al, 2010	PICSI	215 couples: PICSI vs PVP-ICSI	Improvement in EQ
Menezo et al, 2010	Sperm Slow	2014 HA-ICSI vs 1920 PVP-ICSI	No differences

Table 1. Studies on injection of HA-bound spermatozoa

4. Zona - Bound spermatozoa

Immature spermatozoa have a low density of ZP binding sites as well as HA receptors (Huszar et al., 2003). Human sperm bound to ZP exhibit attributes similar to those of HA-bound sperm, including minimal DNA fragmentation, normal shape, and low frequency of chromosomal aneuploidies (Yagcy et al., 2010). Furthermore, in some mammals, the same sperm membrane protein is involved firstly in hyaluronidase activity and subsequently in ZP binding (Hunnicutt et al., 1996). These findings suggest that the spermatozoa–ZP binding process plays an important role in the natural selection of spermatozoa as well as HA.

A spermatozoa-ZP binding test can be performed by culturing spermatozoa for a couple of hours with immature metaphase I oocytes; the spermatozoa bound to ZP can be recovered with an injecting pipette and used for ICSI: when using this system Paes de Almeida Ferreira Braga et al. (2009) found that the injection of ZP-bound spermatozoa increases embryo quality. Black et al. (2010) observed a trend in implantation and clinical pregnancy rates when injecting ZP-bound spermatozoa in a study on ZP-ICSI versus conventional ICSI. Liu et al. (2011) observed a significant improvement in top embryo quality rate comparing ZP-ICSI with conventional ICSI.

Even though at the present time there is little information regarding all the factors involved in sperm-ZP binding and its mechanism, these last studies suggest that the spermatozoa–ZP binding test may be an efficient method for identifying competent spermatozoa for ICSI. ZP selection could then be coupled to HA selection in order to replicate the natural path of the spermatozoa towards the oocyte.

5. Intracytoplasmic morphologically selected sperm injection (IMSI)

The conventional magnification for sperm evaluation at ICSI is a maximum 400 X. Some studies demonstrate that sperm morphology according to strict criteria (Kruger et al., 1986; 1988):

- has little prognostic value in ICSI cycle outcomes (Svalander et al., 1996; French et al., 2010)
- does not influence embryo development or morphology (French et al., 2010)

But, it seem logical that the goal of obtaining the most viable embryo and reducing diseases in newborns is dependent on the selection of ideal gametes, both oocytes and spermatozoa (Parmegiani et al., 2010 c). Unfortunately, when observed at 400-1000 magnification, sperm dimension and shape are no reliable attributes for predicting chromatin integrity or presence of numerical chromosomal aberrations (Celik-Ozenci et al., 2004). To improve "imaging" sperm selection, the group of Bartoov (1994, 2001, 2002) developed a method of unstained, real-time, high magnification evaluation of spermatozoa (MSOME: motile sperm organelle morphology examination). MSOME is performed using an inverted light microscope equipped with high-power Nomarski optic enhanced by digital imaging to achieve a magnification of up to 6300 X (Figure 3). Application of MSOME selection in patients undergoing ICSI demonstrated that morphological integrity of the human sperm nucleus is an important parameter associated with pregnancy rate (Bartoov et al., 2003, Berkovitz et al., 2005). The modified ICSI procedure based on MSOME criteria was defined as IMSI: intracytoplasmic morphologically selected sperm injection (Bartoov et al., 2003). A matched study (Bartoov et al., 2003) revealed that pregnancy rate was significantly increased in IMSI as compared with routine ICSI, and implantation rate was even the 3-fold higher. Berkovitz et al., 2005 found an increase in abortion rate from 10% (no spermatozoa with normal nuclei) to 57% if no normal sperm for ICSI was available. In fact, ICSI outcome is significantly improved by the exclusive microinjection into the oocyte of spermatozoa with a strictly defined, morphologically normal nucleus, in couples with previous ICSI failures (Bartoov et al., 2003; Berkovitz et al., 2005; Hazout et al., 2006; Antinori et al., 2008; Franco et al., 2008; Mauri et al., 2010; Souza Setti et al., 2010) or with severe male factor (Balaban et al., 2011, Souza Setti et al., 2011). IMSI positive effect is not evident on day 2 embryos (Mauri et al., 2010) but, conversely, the injection of spermatozoa with abnormal sperm head or with nuclear vacuoles negatively affects embryo development in day 5-6 (Vanderzwalmen et al., 2008) and ICSI outcome (Berkovitz et al., 2006a; 2006b; Cassuto et al., 2009; Nadalini et al., 2009). The positive effect on ICSI outcome given by the injection may be due to the significantly better mitochondrial function, chromatin status and reduced aneuploidy rate of spermatozoa without nuclear vacuoles when compared with vacuolized spermatozoa (Garolla et al., 2008; Boitrelle, et al., 2011). In addition, spermatozoa free of nuclear morphological malformations are related with lower incidence of aneuploidy in derived embryos (Figueira et al., 2011).

It should be mentioned that IMSI is a time-consuming procedure: selecting a "normal" MSOME spermatozoon requires 60-120 minutes (Antinori et al., 2008). Furthermore, the process of searching for spermatozoa at high magnification may itself damage sperm cytoplasm: sperm nucleus vacuolization significantly increases after 2 hours on the microscope's heated stage (Peer et al., 2007). IMSI procedure can be speeded up by merging

of high magnification microscopy together with HA-sperm selection. In fact, a HA-medium may help to select a sub-population of spermatozoa with normal nucleus according to MSOME criteria: Parmegiani et al (2010a) found that nucleus normalcy rate was significantly higher in HA-bound spermatozoa than in spermatozoa in PVP.

It can be concluded that, despite the time consuming procedure and the cost of the instrument for high magnification microscopy, IMSI has proved itself a valid tool for safe, non-invasive sperm selection and it can be widely applied in the near future.
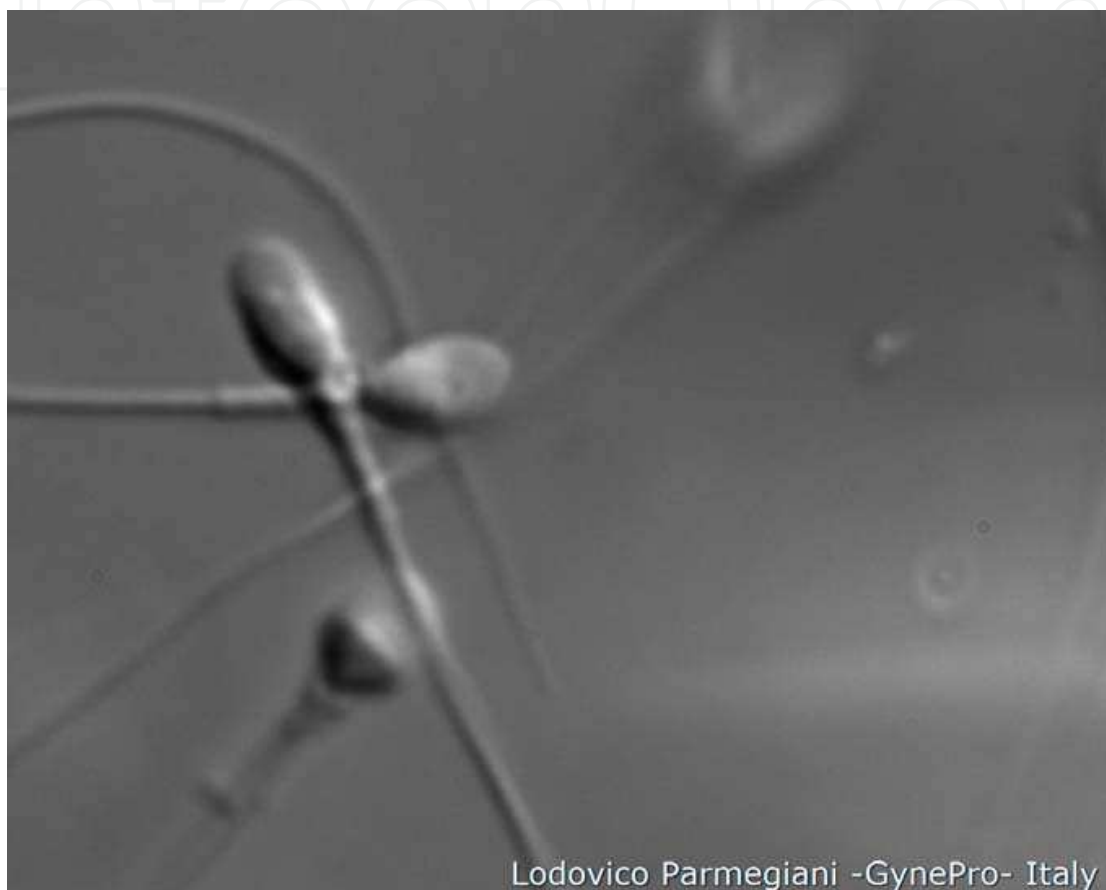

Fig. 3. Human Spermatozoa (magnification >6300 X)

5.1 IMSI procedure

Spermatozoa are generally first treated with a density gradient system. Then, the prepared sperm suspension is put into a PVP (or Sperm Slow in case of "Physiologic IMSI", *Parmegiani et al., 2010 a*) droplet, on a glass-bottom culture dish under oil. In order to choose the best spermatozoa to inject, sperm "nucleus normalcy" is evaluated. The nucleus normalcy is assessed in real time according to Motile Sperm Organelle Morphology Examination (MSOME) criteria. According to MSOME criteria, normally-shaped sperm nucleus is smooth, symmetric, and with oval configuration. Average lengths and widths (± Standard Deviation) must be 4.75±0.28 μm and 3.28 ±0.20 μm, respectively. Nuclear chromatin content is considered abnormal if sperm head contains one or more vacuoles (diameter of 0.78±0.18) that occupy more than 4% of the normal nuclear area. To be considered morphologically normal, a sperm nucleus has to have both

normal shape and normal chromatin content. For rapid evaluation of nuclear normalcy, a fixed, transparent, celluloid form of a sperm nucleus fitting MSOME criteria for length and widths can be superimposed on the examined cell on the screen: the nuclear shape is considered abnormal if it differs in length or width by 2 standard deviations from the normal mean axes values; vacuoles can be examined using a similar celluloid form (Figure 4). Alternatively, spermatozoa can be measured for nuclear length, width and vacuoles with specific digital imaging softwares

Fig. 4. IMSI procedure. Human Spermatozoa (magnification >10'000 X)

For evaluation of nuclear normalcy, a fixed, transparent, celluloid form of a sperm nucleus fitting MSOME criteria for length and widths is superimposed on the examined cell on the screen: the nuclear shape is considered abnormal if it differs in length or width by 2 standard deviations from the normal mean axes values; vacuoles are examined using a similar celluloid form (Bartoov et al., 2003).

6. Sperm head birefringence

A new tool for sperm selection is the application of polarization microscopy to ICSI (Baccetti, 2004). This method is based on the birefringence characteristics of the sperm protoplasmic texture. In the mature sperm nucleus, there is a strong intrinsic birefringence associated with nucleoprotein filaments that are ordered in rods and longitudinally oriented. An inverted microscope specifically equipped with polarizing lenses allows for the real-time selection of birefringent spermatozoa for ICSI. The localization of the birefringence in the postacrosomial region indicates that the acrosomial reaction has already occurred; the

injection of acrosome reacted spermatozoa seems to favour the development of viable ICSI embryos (Gianaroli et al., 2010). The injection of birefringent spermatozoa seems to be useful, especially in cases of oligoasthenoteratozoospermia or testicular spermatozoa (Gianaroli et al., 2008; 2010).

7. Conclusions

The introduction of ICSI (Palermo et al., 1992) has changed in a revolutionary way the world of assisted reproduction technology allowing us to efficiently treat patients with:

- oligoasthenoteratozoospermia
- testicular spermatozoa
- limited number of oocytes
- previous IVF failures

In these situations, suboptimal spermatozoa could by-pass the physiological check-points of natural fertilization and generate embryos, and subsequently babies. Conventional ICSI has the hypothetical risk of injecting immature, DNA damaged, aneuploid, low motile, morphologically abnormal, zona binding deficient, poor acrosome reacted, spermatozoa. Nowadays, we have no real knowledge of the effects of suboptimal sperm selection on ICSI adults in the long term, at least for humans. A potentially worrying aspect of the injection of DNA damaged spermatozoa for example, has been suggested by studies performed on animals which showed not only a negative effect on pregnancy and birth, but also later side effects on the health of adult animals such as aberrant growth, premature ageing, abnormal behaviour, and mesenchymal tumours (Fernandez-Gonzales et al., 2008).

Fortunately, in humans, the risk of injecting DNA damaged spermatozoa seems to be minimized by classical sperm preparation techniques prior to ICSI (Zini et al. 2000; Younglai et al., 2001; Donnelly et al., 2000; Ahmad et al., 2007; Jackson et al., 2010; Marchesi et al., 2010; Castillo et al., 2011; Ebner et al, 2011) and follow-up studies on ICSI children have demonstrated the safety of this technique (Van Steirteghem et al., 2002, Leunens et al., 2008; Belva et al., 2011; Woldringh et al., 2011) although a slight increase of chromosome aberration seems to be caused by the injection of aneuploid spermatozoa (Bonduelle et al., 2002).

The recent refinements of the ICSI procedure described in this chapter, are reliable, easy-to-do, non-invasive and in some cases "closest to the nature" than the conventional procedure. For example, selecting spermatozoa prior to ICSI by their maturation markers such as HA-ZP receptors (Huszar et al., 2003; Paes de Almeida Ferreira Braga et al., 2009) it is possible at very least to mimic nature in order to restore physiological selection and prevent hypothetical fertilization by DNA damaged and chromosomal unbalanced spermatozoa. In addition, non-invasive imaging sperm selection techniques such as IMSI (Bartoov et al., 2003) or sperm head birefringence (Gianaroli et al., 2008) can be valid tools for helping in selection of the ideal spermatozoa.

In fact, sperm selection based on non invasive morphology or maturity markers helps the embryologist in selection of the "ideal" spermatozoa to inject. These new advances in ICSI may allow the selection of the spermatozoa contributing to the improve:

- fertilization
- embryo quality
- blastocyst formation
- pregnancy
- reduction in abortion.

Furthermore, some of these new technologies also help the standardization of ICSI, reducing intra-operator and inter-operator variability in choosing the spermatozoon to inject. For example, HA-ICSI offers to the embryologist the possibility to recognize the spermatozoa which have completed the maturation process. On the other hand, IMSI allows a precise sperm evaluation and measurement. In particular, these two techniques may also be merged together, pre-selecting HA-bound spermatozoa before High –magnification evaluation. This combined procedure (Physiologic IMSI) speeds up the "time consuming" sperm selection according to MSOME criteria (Parmegiani et al, 2010 a)

The easy reproducibility of these new advances in ICSI should encourage the embryologists and clinicians to automatically offer these technical improvements to all ICSI patients, not only to optimize clinical results but most of all to restore some basic check-points of natural fertilization which are bypassed in the conventional ICSI.

8. Acknowledgment

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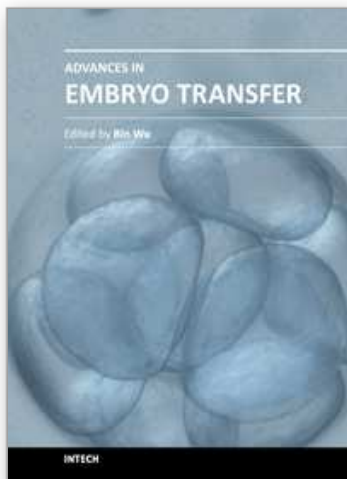
Advances in Embryo Transfer

Edited by Dr. Bin Wu

Embryo transfer has become one of the prominent high businesses worldwide. This book updates and reviews some new developed theories and technologies in the human embryo transfer and mainly focus on discussing some encountered problems during embryo transfer, which gives some examples how to improve pregnancy rate by innovated techniques so that readers, especially embryologists and physicians for human IVF programs, may acquire some new and usable information as well as some key practice techniques. Major contents include the optimal stimulation scheme for ovaries, advance in insemination technology, improved embryo transfer technology and endometrial receptivity and embryo implantation mechanism. Thus, this book will greatly add new information for readers to improve human embryo transfer pregnancy rate.

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