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The ICSI procedure from past to future: a systematic review of the more controversial aspects

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BACKGROUND: ICSI is currently the most commonly used assisted reproductive technology, accounting for 70–80% of the cycles performed. This extensive use, even excessive, is partly due to the high level of standardization reached by the procedure. There are, however, some aspects that deserve attention and can still be ameliorated. The aim of this systematic review was to evaluate the results of available publications dealing with the management of specific situations during ICSI in order to support embryologists in trying to offer the best laboratory individualized treatment.

METHODS: This systematic review is based on material obtained by searching PUBMED between January 1996 and March 2015. We included peer-reviewed, English-language journal articles that have evaluated ICSI outcomes in the case of (i) immature oocytes, (ii) oocyte degeneration, (iii) timing of the various phases, (iv) polar body position during injection, (v) zona-free oocytes, (vi) fertilization deficiency, (vii) round-headed sperm, (viii) immotile sperm and (ix) semen samples with high DNA fragmentation.

RESULTS: More than 1770 articles were obtained, from which only 90 were specifically related to the issues developed for female gametes and 55 for the issues developed for male gametes. The studies selected for this review were organized in order to provide a guide to overcome road-blocks. According to these studies, the injection of rescue metaphase I oocytes should be discouraged due to poor clinical outcomes and a high aneuploidy rates; laser-assisted ICSI represents an efficient method to solve the high oocyte degeneration rate; the optimal ICSI timing and the best polar body position during the injection have not been clarified; injected zona-free oocytes, if handled carefully, can develop up to blastocyst stage

and implant; efficient options can be offered to patients who suffered fertilization failure in previous conventional ICSI cycles. Most controversial and inconclusive are data on the best method to select a viable spermatozoa when only immotile spermatozoa are available for ICSI and, to date, there is no reliable approach to completely filter out spermatozoa with fragmented DNA from an ejaculate. However, most of the studies do not report essential clinical outcomes, such as live birth, miscarriage and fetal abnormality rate, which are essential to establish the safety of a procedure.

CONCLUSIONS: This review provides the current knowledge on some controversial technical aspects of the ICSI procedures in order to improve its efficacy in specific contexts. Notwithstanding that embryologists might benefit from the approaches presented herein in order to improve ICSI outcomes, this area of expertise still demands a greater number of well-designed studies, especially in order to solve open issues about the safety of these procedures.

Key words: ICSI / fertilization failure / sperm / oocyte activation / globozoospermia / zona-free oocyte / metaphase I oocyte

Introduction

ICSI was introduced in the early 1990s as one of the most dramatic technological breakthroughs in assisted reproductive technology (ART). After its introduction, the technique was rapidly incorporated into the routine clinical practice of fertility centers throughout the world. Based on data from National and Regional registers worldwide, the use of ICSI increased from 39.6% of ART cycles in 1997 to 58.9% in 2004. To date, 10s of 1000s of children have been born around the world as the result of ICSI (Nyboe Andersen *et al.*, 2008). These figures suggest the trend of ICSI being used increasingly also in couples without a diagnosis of severe male factor and underlines that the procedure is practiced today as a medical adaptation and development to the benefit of the infertile couples. Indeed, the common thought in support of ICSI efficiency has favored a shift toward an increased use also for mild and borderline male factor infertility, for unexplained infertility and women of advanced age, although evidence supporting these indications is limited (Harper *et al.*, 2012; Babayev *et al.*, 2014; Sanchez-Calabuig *et al.*, 2014). This extensive use, even excessive, is partly due to the high level of standardization and the popularity reached by the procedure and by the tremendous increased efficacy demonstrated during recent years.

The various steps of the conventional ICSI, from oocyte denudation to sperm immobilization to sperm injection into the egg, seem well established considering the 1000s of highly trained ICSI technicians/embryologists in the world performing the procedure. This is, however, only partially true. As a matter of fact, there are still potential weaknesses in established ICSI protocols and what is common in performing micromanipulation procedures is the lack of attention to details other than the mechanical aspects. Specific situations and aspects responsible for poor performance or failure of the procedure mostly related to severely dysfunctional gametes are only partially unraveled.

The purpose of this systematic review was to evaluate the results of available publications dealing with the more controversial technical aspects of various steps in human ICSI. Issues still unclear related to methods used to limit the failure of the various steps, timing of the various phases and procedures to circumvent severe sperm alterations have been investigated in depth. A special attention has been paid to aspects for which systematic reviews or meta-analysis have not already defined the more scientifically correct strategy or that unexplainably has received only limited attention, with the ultimate aim to suggest possible refinements of the ICSI procedure to the 1000s of ICSI technicians/embryologists and provide them with a pragmatic tool for potential improvements of ART outcomes. Based on this analysis, safety concerns have emerged and will be discussed.

Methods

The PRISMA statement was used for reporting the Methods, Results and Discussion sections of the current review (Moher *et al.*, 2009). No institutional review board approval was required because only published and de-identified data were analyzed. The search strategy, inclusion and exclusion criteria were specific for each of the two main aims of this systematic review (issues related to female and male gametes) and described below.

Literature search

General criteria

A systematic computerized search of the literature, from January 1996 until March 2015, was performed in two electronic databases (PubMed and MEDLINE) in order to identify relevant articles to be included for the purpose of this systematic review. All pertinent articles were examined and their reference lists were systematically reviewed in order to identify other studies for potential inclusion in this article.

Studies were reviewed by two independent reviewers (P.R. and A.L.) and discrepancies were resolved by consensus including a third author (P.V.). The reviewers were not blinded to the names of investigators or sources of publication. First, eligibility was assessed based on titles and abstracts. Full manuscripts were obtained for all selected studies and decision for final inclusion was made after detailed examination of the papers.

Specific criteria

Two independent authors (P.R. and A.L.) run a specific literature search for each of the two main aims of this article (Fig. 1). The search included the combination of the following keywords and medical subjects heading terms alone or in combination:

(i) Metaphase I, oocyte degeneration, timing, polar body position, zona-free oocyte, fertilization failure, oocyte activation, ICSI, intracytoplasmic sperm injection.
(ii) Sperm, spermatozoa, globozoospermia, absolute asthenozoospermia, immotile, DNA fragmentation, ICSI, intracytoplasmic sperm injection.

Selection criteria

Peer-reviewed, English-language journal articles were included in this systematic review. At the first screening, titles were investigated and studies with lack of any relevance were excluded. The second screen was performed by reading the abstract of retained publications (Fig. 1). Analyses not reporting ICSI outcomes were excluded, as well as studies not reporting original data including reviews, meta-analyses or comments. Full papers of all remaining items were collected and for each study, the following information was collected: first author's last name, year of publication, research objective, design of the study, outcomes investigated and conclusions. Specifically,

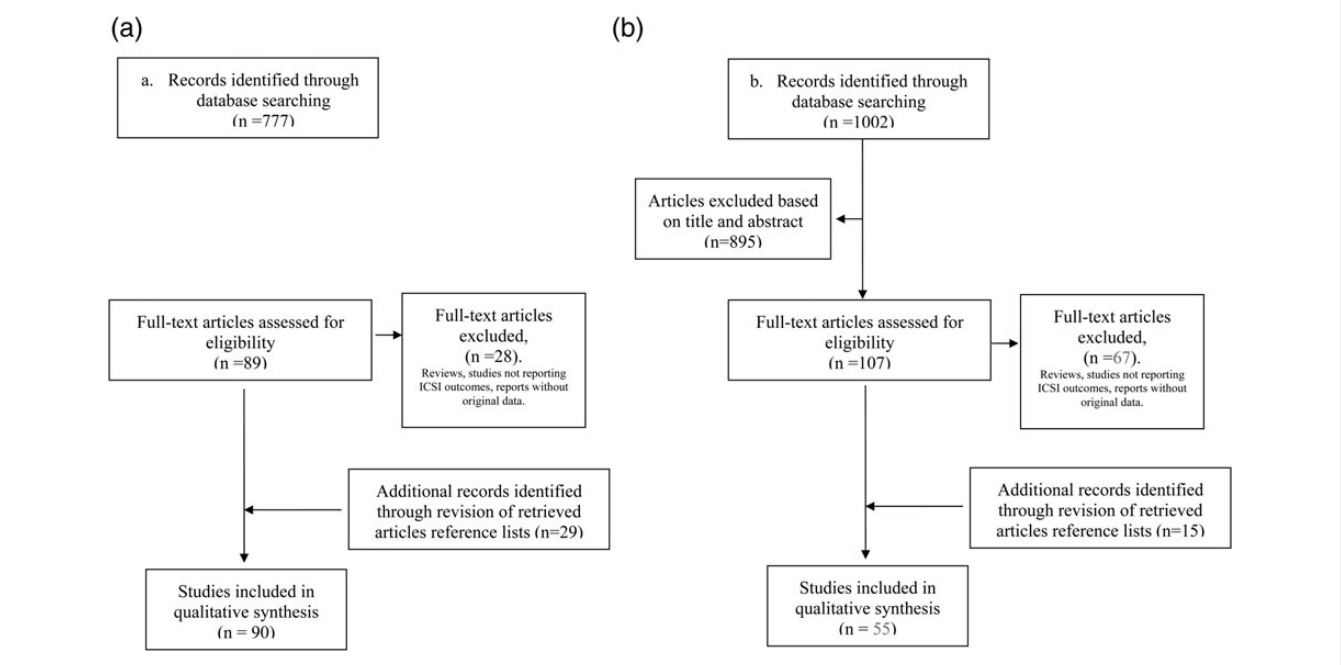

Figure 1 Flow diagram of the literature search: (**a**) search for issues related to human female gametes and (**b**) search for issues related to human male gametes.

the following outcomes were extracted: fertilization and cleavage rates, Day 3 embryo quality, blastocyst development, implantation, clinical pregnancy, abortion and live birth rates. Randomized controlled trials (RCTs), prospective cohort studies, case–control studies, retrospective cohort studies, case series and case reports were screened where available.

ICSI and female gametes

How to manage metaphase I oocytes

Biological basis

The majority of the oocytes recovered following administration of high doses of FSH and a high dose of human chorionic gonadotrophin (hCG) is at the metaphase II (MII) stage and ready to be fertilized. However, there are some that do not mature and remain in the germinal vesicle (GV) or metaphase I (MI) stage. The maintenance of meiotic arrest as well as meiotic resumption is the result of a highly coordinated system between the follicle cells and the oocytes. Chromatin condensation, organelle redistribution and cytoskeletal changes are only a few examples of the events taking place during oocyte maturation. After the acquisition of the capacity to resume meiosis (named nuclear competence), the ability to be fertilized and develop further into an embryo (named developmental competence) is also acquired during follicular growth and after the oocyte has reached nuclear competence. Developmental competence includes the relocation of ribosomes and mitochondria, the reorganization of the endoplasmic reticulum (ER), the storage of essential transcripts required during early embryo development and the ability to modulate calcium release (Coticchio and Brambillasca, 2013; Jones *et al.*, 2013). On the other hand, maturation *in vitro*, intended as meiotic resumption and progression to the MII stage, can be achieved. Oocyte *in vitro* maturation (IVM) has been developed in order to provide culture conditions that allow acquisition of developmental competence encompassing nuclear competence. However, collective evidence indicates that *in vitro*-matured oocytes have a reduced developmental potential in comparison to their *in vivo* counterparts (Coticchio *et al.*, 2012). Thus, IVM remains an approach that few clinics currently apply on a routine basis, needing a high clinical and technical expertise. But apart from the clinical IVM approach, in the daily IVF laboratory practice, ~15% of the oocytes obtained are not at the MII stage but remain at prophase I (~10%) and MI (~5%) and their potential clinical use represents a matter of debate (Cha and Chian, 1998; De Vos *et al.*, 1999; Schultz, 2002; Nogueira *et al.*, 2004). The clinical application of a short-term culture of MI oocytes in a conventional IVF program has been evaluated with controversial and confused results. It should be considered in this context that while both oocyte-derived and follicular-derived factors regulate both meiotic arrest and resumption, the timing of meiosis I is governed by the oocyte itself (Jones *et al.*, 2013). Following GV breakdown, increasing activity of the cyclin-dependent protein kinase 1 (CDK1) is essential for this dynamic period of the oocyte maturation as it promotes chromosome condensation and spindle formation. Once homologous chromosomes are aligned on the metaphase spindle, anaphase and meiotic exit can proceed, a process requiring a decline in CDK1 activity which is achieved principally by degradation of the cyclin B1. The ligase anaphase-promoting complex (APC) is critical in this step as it favors anaphase progression by promoting the degradation of cyclin B1 to lower CDK1 activity, thus allowing homolog separation and ultimately extrusion of the first polar body. Another mechanism important for completion of meiosis I implies the physical interaction between CDK1 and separase, the protein that allows the homologous chromosomes to be pulled to opposite poles of the meiotic spindle. Overall, these passages highlight a very sophisticated process that may reflect the need for large size of the oocyte. Moreover,

accumulation of cytoplasmic components occurs while the oocyte is progressing through maturation such that by time MII stage is reached, the bulk of cytoplasmic events are completed. Thus, there are several molecular candidates that may be perturbed during this process causing a maturation arrest, among which spindle organization defects or chromosomal aberrations represent the most significant (Coticchio and Brambillasca, 2013; Jones *et al.*, 2013).

Interventions

Based on the biological principles of oocyte development, only mature oocytes are injected by ICSI on a regular basis, whereas the immature oocytes are usually discarded. However, the use of immature oocytes could increase the number of embryos available. This issue is differently managed in the various clinical laboratories, but the possibility to use the MI oocytes in the daily practice might represent an advantage. Moreover, this topic has received a renewed interest from the diffusion of the pre-implantation genetic screening. From 1999, 10 papers have evaluated the clinical outcomes derived from short-term (<8 h) *in vitro* cultured MI oocytes that progressed to MII oocytes (Table I). Seven are retrospective (De Vos *et al.*, 1999; Huang *et al.*, 1999; Balakier *et al.*, 2004; Vanhoutte *et al.*, 2005; Shu *et al.*, 2007; De Vincentiis *et al.*, 2013; Shin *et al.*, 2013), one is a prospective cohort study (Strassburger *et al.*, 2010) and two have randomized the oocyte treatment according to time of culture (Strassburger *et al.*, 2004; Li *et al.*, 2011). All the studies have used the same culture medium for both mature and immature oocytes. Fertilization rate was shown to be significantly reduced for *in vitro* rescued MI oocytes compared with MII oocytes in eight studies (De Vos *et al.*, 1999; Huang *et al.*, 1999; Balakier *et al.*, 2004; Strassburger *et al.*, 2004; Vanhoutte *et al.*, 2005; Strassburger *et al.*, 2010; De Vincentiis *et al.*, 2013; Shin *et al.*, 2013), while no significant difference was found in two studies (Shu *et al.*, 2007; Li *et al.*, 2011). Interestingly, in five studies, fertilization rate of rescued oocytes was higher than 60% (Huang *et al.*, 1999; Shu *et al.*, 2007; Strassburger *et al.*, 2010; Li *et al.*, 2011; De Vincentiis *et al.*, 2013). Cleavage rate was significantly reduced for *in vitro*-matured oocytes in two studies (Balakier *et al.*, 2004; Strassburger *et al.*, 2004), not different in four studies (De Vos *et al.*, 1999; Huang *et al.*, 1999; Shu *et al.*, 2007; Li *et al.*, 2011) and not evaluated in the others (Vanhoutte *et al.*, 2005; Strassburger *et al.*, 2010; De Vincentiis *et al.*, 2013; Shin *et al.*, 2013). Unfortunately, most of the studies failed to document embryo transfer procedures derived from MI–II-derived embryos only. This was probably due to the fact that transfers were mostly performed mixing embryos derived from both mature and rescued oocytes or rather there was a tendency to avoid the transfer of MI–II-derived embryos. However, the two studies that reported the highest number of embryo transfers from rescued MI oocytes ($n = 15$ and 10) (De Vos *et al.*, 1999; De Vincentiis *et al.*, 2013) showed a clinical pregnancy rate of 6.6 and 10%. Overall, 33 embryo transfer procedures from short-term *in vitro*-matured oocytes have been reported and 4 clinical pregnancies derived, 2 ending in abortion.

The paper by Shin *et al.* (2013) reported the outcome of 24 ICSI cycles with no mature oocytes at oocyte denudation. Thirty-six MI oocytes matured to MII stage after *in vitro* culture of 3–4 h. The fertilization rate of this group of oocytes was 37% and five transfer procedures were performed with no clinical pregnancies. Importantly, the prospective study by Strassburger *et al.* (2010) has examined the fate and the chromosomal status of embryos achieved from immature oocytes arrested or rescued after various periods of *in vitro* culture. Embryo euploidy was evaluated by fluorescence *in situ* hybridization using probes for chromosomes X,Y and 18. In general, a high proportion (80.6%) of embryos from MI oocytes was genetically abnormal. However, whereas the aneuploidy rate was 100% for MI oocytes rescued after 24 h of incubation, this rate was reduced to 66.6% when oocytes were rescued after 4–8 h and to 40% after a short incubation period (2 h) and this latter figure was similar to the rate of abnormal embryos (40.3%) routinely found in the authors' PGD program (Strassburger *et al.*, 2010).

Based on the available publications, the widespread use of MI oocytes cannot be recommended.

The poor clinical outcomes and the generally higher aneuploidy rates would suggest that an additional patient counseling is advisable in case no mature oocytes are available, in order to discuss the potential risk of transferring embryos derived from MI oocytes. Aneuploidy screening would be useful in this regard. Although the use of immature oocyte could increase the number of embryos available to ensure the embryo transfer, the safety of patients and fetus should always represent a priority.

How to manage oocyte degeneration

Oocyte degeneration is a common phenomenon experienced by all technicians performing ICSI, ranging from 5 to 19% (Palermo *et al.*, 1992, 1996; Van Steirteghem *et al.*, 1993; Nagy *et al.*, 1995; Ebner *et al.*, 2001; Yavas *et al.*, 2001). The degeneration can be observed immediately after withdrawal of the injection needle, coexisting with sudden breakage upon needle entry and leakage of cytoplasm. However, the degeneration may also follow normal injection, being identified the following day by a retracted and/or darkened ooplasm (Rosen *et al.*, 2006).

Biological basis

Limited investigation has addressed the causes of oocyte degeneration.

Degeneration has been suggested to be dependent on the inherent oocyte quality in women undergoing ovarian stimulation and possibly related to the stimulation itself. Day 3 FSH levels, number of mature oocytes retrieved and estradiol levels on the day of hCG triggering have been shown to be significant independent predictors of degeneration rate (Rosen *et al.*, 2006). Sudden breakage of the oolemma (Nagy *et al.*, 1995; Palermo *et al.*, 1996), absence of persistence of funnel after the withdrawal of the injection pipette (Ebner *et al.*, 2003) and difficult breakage of the oolemma (Ebner *et al.*, 2001) have been reported as events possibly favoring oocyte degeneration during ICSI related to an intrinsic poor oocyte quality.

Although the injection technique requires experience by the laboratory technician and implies a learning curve, there are no publications sustaining the idea that oocyte degeneration rate may be a function of technician skills. In a prospective study on sibling oocytes, blastocyst development rate of surplus embryos was found to be significantly different among four ICSI technicians according to the volume of cytoplasm aspirated by each of them during the procedure. When >6 pl of cytoplasm was aspirated into the injection pipette, the development to the blastocyst stage was found to be compromised. Nevertheless, the degeneration seemed to be independent from the volume of cytoplasm aspirated (Dumoulin *et al.*, 2001). In line, in a prospective study regarding the influence of the polar body position during the injection, a difference between two operators in terms of fertilization rates was observed,

Table I Summary of the 10 papers identified in a systematic review of the literature in order to investigate the approaches used to manage the metaphase I oocytes in human ICSI cycles.

	Oocytes (*n*)	FR (%)	CR (%)	Good EQ rate (%)	Embryo transfer (*n*)	CPR	Incubation time	Culture medium	P/PR/R study
Huang *et al.* (1999)	239 (720)	62 (81) $P < 0.001$	90 (94) NS	58 (87) $P < 0.001$	—	—	<8 h	M2 (Medicult)	R
De Vos *et al.* (1999)	1210 (8803)	52.7 (70.8) $P < 0.001$	82.5 (83.4) NS	53.4 (59.8) $P < 0.01$	15	6.6%	4 h	B2 (SIGMA)	R
Strassburger *et al.* (2004)	116 (4668)	44 (68) $P < 0.01$	86 (95) $P < 0.01$	2.0 (1.9)[a] NS	1	One clinical pregnancy reported[b]	<4 h	G1.2 (Vitrolife)	PR
Vanhoutte *et al.* (2005)	117 (339)	48.7 (68) $P < 0.001$	—	40.3 (30.6) NS	—	—	2–7 h	Fertil medium (COOK) Early cleavage medium (Irvine Scientific)	R
Shu *et al.* (2007)	259 (1740)	62.2 (69.5) NS	98.8 (99.4) NS	49.7 (68.2) $P < 0.01$	—	—	<4–6 h	Cleavage medium (SAGE) + SSS (Irvine)	R
Balakier *et al.* (2004)	468 (3293)	42 (77) $P < 0.0001$	53 (74) $P < 0.0001$	55 (61) NS	4	0	<3 h	IVC-one medium (*In vitro* Care) + SSS (Somagen)	R
Strassburger *et al.* (2010)	96 (2500)	61.4 (71.5) $P < 0.05$	96.6 (—)	2.4–2.6[a] (—)	—	—	<4–8 h	G1.2 + HSA (Vitrolife)	P
Li *et al.* (2011)	51 (1674)	68.6 (74.5) NS	91.4 (90.5) NS	51.4 (59.4) NS	3	Twin ongoing pregnancy reported[b]	3–5 h	G-IVF (Vitrolife)	PR
De Vincentiis *et al.* (2013)	669 (3044)	65.2 (76.1) $P < 0.0001$	—	—	10 (306)	10%	<2–5 h	Early cleavage medium + HSA (Irvine)	R
Shin *et al.* (2013)	775 (3482)	37 (72.3) $P < 0.05$	—	—	—	—	<3–4 h	IVF (GIII series) (Vitrolife)	R

Numbers in parentheses refer to corresponding figures for control MII mature oocytes.
MII, metaphase II; CR, cleavage rate; FR, fertilization rate; EQ, embryo quality; CPR, clinical pregnancy rate; P, prospective; PR, prospective randomized; R, retrospective; HSA, human serum albumin; SSS, serum supplement substitute, NS, not significant.
[a]Morphology score.
[b]Ended in abortion.

whereas no significant differences were found with regard to the number of degenerated oocytes (Van der Westerlaken *et al.*, 1999).

On the other hand, degeneration of oocytes seems closely related to certain unexpected random technical problems. Among these, the excessive manipulation inside the oocyte due to the spermatozoon remaining attached to the injection pipette at the moment of deposition, or the inadvertent oolemma damage at the opposite site of the injection hole as a result of a too deep penetration by the injection pipette. Another critical issue is represented by the oocyte deformation during the various steps for the possible damage to the cytoskeleton. If the diameter of the cumulus-removal pipette is too small, then oocytes can be very strongly compressed when aspirated into the pipette, possibly favoring the leaking away of the cytoplasm after injection, resulting in degeneration (Nagy *et al.*, 1995).

The mechanical removal of cumulus and corona cells may also play a role in the etiology of oocyte degeneration and of the sudden breakage of oolemma during ICSI. Interestingly, in two different publications, Ebner *et al.* (2001, 2006) reported that an incomplete oocyte denudation, while allowing to improve Day 2 and 3 embryo morphology as well as blastocyst formation, also produced a significantly higher degeneration rate. In this case, the residual surrounding somatic cells may hinder optimal injection and, consequently, be the cause of an increased damage rate or, alternatively, some hydraulic fixation problems on the holding pipette may occur, resulting in a highly invasive injection process.

Interventions

In 2009, Evison *et al*. reported a significant decrement of oocyte damage when they switched from a traditional bovine-derived hyaluronidase to the recombinant human hyaluronidase for the removal of cumulus–corona–oocyte complex before ICSI (Evison *et al.*, 2009). The study had however some important limitations, including its retrospective nature. Moreover, the equipment used for ICSI was updated during the study, probably contributing to the improvement observed.

When the zona pellucida is hard and/or the injection pipette is relatively large and/or blunt, then the oocyte is compressed into a figure of eight shape before the microinjection pipette can penetrate the zona pellucida (Fig. 2), possibly causing oocyte degeneration (Ebner *et al.*, 2001). In line with this observation, in a retrospective study, Yavas *et al.* (2001) reported a significant decrease in the degeneration incidence after ICSI using injection pipette with a smaller inner diameter and a longer taper: 3–5 μm inner diameter (which is the smallest possible inner diameter according to the width of the normal spermatozoon head) instead of 5–7 μm. The positive effect of a thinner injection pipette was explained with at least three benefits: minimized degree of mechanical trauma exerted on the oolemma, minimized amount of ooplasm aspirated into the injection pipette prior to injection, and minimized amount of PVP (polyvinylpyrrolidone) injected into the oocyte along with the spermatozoon (Yavas *et al.*, 2001; Table II).

In order to minimize the oocyte deformation related to the difficulty of passing the zona pellucida, a new approach, the laser-mediated ICSI, was described in early 2000s. This technique allows to perform a micro-hole (diameter 5–10 μm) in the zona pellucida by a laser beam just prior to ICSI and the injection pipette is introduced through the hole permitting penetration of the micro-needle without any trauma. The first evidence of the efficacy of this technique was published in two distinct case reports (Rienzi *et al.*, 2001; Nagy *et al.*, 2002). Both described a clinical pregnancy obtained in cases with repeated ICSI failure due to oocyte degeneration

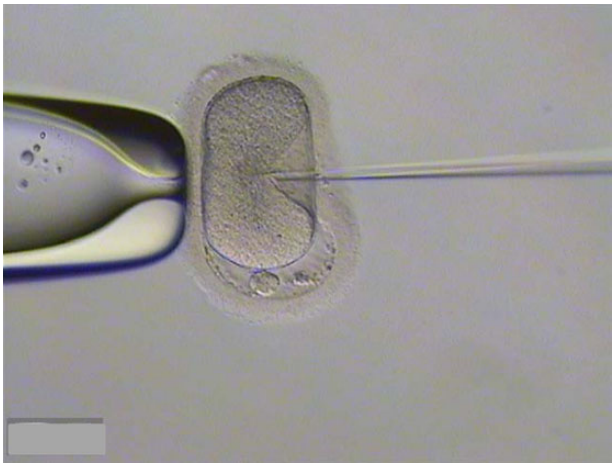

Figure 2 Oocyte deformation related to the difficulty of passing the zona pellucida (200× magnification).

(Table II). Afterwards, three prospective studies on sibling oocytes were published (Abdelmassih *et al.*, 2002; Rienzi *et al.*, 2004; Demirol *et al.*, 2006). Rienzi *et al*. included patients with at least six MII oocytes in whom the first three oocytes subjected to ICSI showed a sudden oolemma breakage reaction, while Abdelmassih *et al.* (2002) and Demirol *et al.* (2006) included patients with one or more previous ICSI cycles with a high degeneration rate (>20%). All three groups agreed that a significantly lower degeneration rate could be observed in the group of oocytes treated by laser-assisted ICSI compared with conventional ICSI. A significantly higher fertilization rate and a significantly higher embryo quality on Day 3 were reported after laser-assisted ICSI, thus leading to the idea that the technique may decrease also damage to subcellular components of oocytes that could affect embryo development (Table II).

The impossibility of localizing the laser-generated hole at a later developmental stage represents the major concern in the matter of laser-mediated ICSI (Abdelmassih *et al.*, 2002). The small hole created in the zona pellucida may be associated with a certain risk of embryo trapping leading to degeneration of the blastocyst if it cannot escape from the outer shell, or of monozygotic twinning if the emerging embryo is restricted and subsequent splitting occurs (Mantoudis *et al.*, 2001). Although no cases of monozygotic twinning have been reported for laser-assisted ICSI, Moser *et al*. described a different approach to laser-assisted ICSI in a prospective study on sibling oocytes of unselected population. The zona pellucida was simply thinned and not opened by means of laser immediately prior to injection. They reported a significantly lower degeneration rate in the study group, a better embryo quality on Day 2 and an increased clinical pregnancy rate following Day 3 embryo transfer. An increased herniation of Day 5 blastocysts was also described (Moser *et al.*, 2004).

More recently, in a prospective randomized study in a general patient population, Choi *et al*. (2011) observed higher fertilization, and higher blastulation and clinical pregnancy rates in the laser-assisted thinning ICSI group compared with the conventional ICSI group (Choi *et al.*, 2011). However, not all the studies agree on the positive effects of laser ICSI. In a prospective study on sibling oocytes, Richter *et al.* (2006) observed that the use of laser-assisted ICSI in unselected patients does not have any beneficial effect in terms of oocyte fertilization, degeneration rate,

Table II Summary of the 10 papers identified in a systematic review of the literature in order to investigate the approaches used to reduce oocyte degeneration rate after ICSI.

	Issue	Approach	Indications	DR	FR	Good EQ rate	BL rate	CPR	IR	AbR	P/PR/R/CR study
Yavas *et al.* (2001)	Oocyte injection	Injection pipette with ID of 3–5 μm	Non-selected population	Decreased	Improved	Improved	—	—	—	—	R
Rienzi *et al.* (2001)	Oocyte injection	Laser-assisted ICSI	High DR after ICSI	Decreased	—	—	—	One clinical pregnancy reported	—	—	CR
Nagy *et al.* (2002)	Oocyte injection	Laser-assisted ICSI	High DR after ICSI	Decreased	—	—	—	One twin clinical pregnancy reported	—	—	CR
Abdelmassih *et al.* (2002)	Oocyte injection	Laser-assisted ICSI	High DR after ICSI	Decreased	Unchanged	Improved	—	—	—	—	PR
Moser *et al.* (2004)	Oocyte injection	Laser-assisted ZT before ICSI	Non-selected population	Decreased	Unchanged	Improved	Unchanged	Improved[a]	—	—	PR
Rienzi *et al.* (2004)	Oocyte injection	Laser-assisted ICSI	Oocyte with sudden oolemma breakage	Decreased	Unchanged	Unchanged	—	—	—	—	P
Demirol *et al.* (2006)	Oocyte injection	Laser-assisted ICSI	Recurrent ICSI failure with limited MII oocytes	Decreased	Improved	Improved	—	—	—	—	PR
Richter *et al.* (2006)	Oocyte injection	Laser-assisted ICSI	Non-selected population	Unchanged	Unchanged	Unchanged	Unchanged	—	—	—	PR
Evison *et al.* (2009)	Oocyte denudation	Recombinant hyaluronidase	Non-selected population	Decreased	Improved	—	—	—	—	—	R
Choi *et al.* (2011)	Oocyte injection	Laser-assisted ZT before ICSI	Non-selected population	Unchanged	Improved	—	Improved	Improved	Unchanged	—	PR

Improved or decreased results are indicated only when statistically significant.
ID, inner diameter; ZT, zona thinning; DR, degeneration rate; BL, blastocyst formation; IR, implantation rate; AbR, abortion rate; CR, case report.
[a]Exclusive transfer of the study embryos on Day 3 resulted in a significantly better pregnancy rate.

embryo cell numbers and fragmentation rate on Day 2 and 3, as well as in terms of compaction and blastocyst formation rate (Richter *et al.*, 2006) (Table II). It is however reasonable to consider the laser-assisted ICSI as an efficient and easy approach to prevent oocyte damage in patients with a history of high oocyte degeneration rate in previous ICSI cycles or for oocytes with a sudden breakage feature.

How to manage choice of ICSI timing

Biological basis

Nuclear maturity can easily be assessed before ICSI as it is evidenced by the expulsion of the first polar body. However, the complete oocyte maturation does not only depend on nuclear meiotic progression and DNA remodeling. Cytoplasmic maturity process is thought to involve the synthesis of various maternal mRNAs and proteins with very specific and precise mechanisms, reorganization of the cytoplasmic organelles in the cytoskeleton and acquirement of a full complement of Ca^{2+} signaling molecules (Coticchio *et al.*, 2015).

For normal fertilization to occur, both nuclear and cytoplasmic maturity are required independently.

In natural cycles, nuclear and cytoplasmic maturity are highly coordinated, whereas in stimulated cycles, the two phenomena appear to be asynchronous (Sundstrom and Nilsson, 1988; Eppig *et al.*, 1994; Balakier *et al.*, 2004); therefore, pre-incubation of oocytes prior to IVF or ICSI may favor the cytoplasmic maturation that could eventually increase fertilization and also pregnancy rates. According to this idea, evidence has been provided indicating that human oocytes progressively develop the ability for normal activation and development during the MII arrest stage (Balakier *et al.*, 2004). On the other hand, when arrested at MII stage, if not fertilized in time, the ovulated oocytes undergo a time-dependent process of aging that can result in a significant decrease in embryonic development and pregnancy rate following *in vitro* procedures (Miao *et al.*, 2009). Moreover, contrary to *in vivo*-matured oocytes, the *in vitro*-matured oocytes stay in contact with their cumulus cells until artificially removed. If it is true that the surrounding cumulus cells have a role in oocyte maturation, ovulation and fertilization (Tanghe *et al.*, 2002), based on mouse models, cumulus cells seem to accelerate the aging process of oocytes in a way that increases with the density and duration of culture (Miao *et al.*, 2009). A significant culture time-dependent increase in the apoptotic rates of cumulus cells has been recently described, which is associated with the apoptotic cumulus cell release of a soluble FAS ligand interacting with FAS on oocytes and mediating oocyte aging (Zhu *et al.*, 2015). For all these reasons, ICSI timing represents a critical aspect of the overall procedure.

Although it has been shown that incubation of oocytes for 2–6 h prior to IVF improves fertilization and pregnancy rates (Trounson *et al.*, 1982; Harrison *et al.*, 1988; Veeck, 1988; Khan *et al.*, 1989), the results regarding the timing of ICSI are still conflicting, probably owing to the retrospective design of the studies and the different strategies proposed (Table III). Given the conceptually very different approach used by those that examined the pre-incubation period effect in the presence and absence of cumulus–corona cells, this part will be divided according to this aspect.

Interventions

Pre-incubation of oocytes with cumulus–corona cells before denudation and ICSI. Various authors examined the effects of different pre-incubation times in ICSI cycles in which the oocyte denudation was performed just before the injection of sperm (Rienzi *et al.*, 1998; Yanagida *et al.*, 1998; Hassan, 2001; Ho *et al.*, 2003; Isiklar *et al.*, 2004; Aletebi, 2011). Hence, the oocytes were incubated *in vitro* with their cumulus and corona cells. With the ovum retrieval performed at 35 h after hCG administration, Yanagida *et al.* (1998) did not find any statistical difference in survival, fertilization, cleavage and pregnancy rates when oocytes were cultured between 1 and 11 h before ICSI, with 3 h time intervals. At 9–11 h of pre-incubation time, the proportion of good-quality embryos was significantly lower (Yanagida *et al.*, 1998). In contrast, others consistently found a beneficial effect of a pre-incubation time ≥2–3 h on the fertilization rate (Rienzi *et al.*, 1998; Hassan, 2001; Ho *et al.*, 2003; Isiklar *et al.*, 2004; Aletebi, 2011; Table III). Three groups reported also a higher good-quality embryo rate, and Aletebi (2011) even a significantly higher clinical pregnancy rate when a pre-incubation time of ≥2–3 h was applied. The optimal time range after oocyte retrieval for a more successful ICSI varied from 2 h according to Aletebi (2011) to 3–12 h for Rienzi *et al.* (1998). The ovum retrieval was performed at 36 h from hCG administration in all these studies except for the study by Ho *et al.* (2003) who performed the ovum retrieval at 35 h.

Pre-incubation of oocytes without cumulus–corona cells before ICSI. Two retrospective studies have examined the effects of different pre-incubation times without the cumulus–corona cells surrounding the oocytes in ICSI cycles, reporting that a limited pre-incubation time has no effect on outcomes (Andrews *et al.*, 2001; Falcone *et al.*, 2008; Table III). Hence, in the two studies, cumulus and corona cells were removed within 1 h from oocyte retrieval (Andrews *et al.*, 2001) and after 2 h (Falcone *et al.*, 2008). Pre-incubation times without cumulus–corona cells from 2 to 6 h did not compromise outcomes parameters, while a longer period (>5 h) had negative effects on fertilization, cleavage and pregnancy rates. In both studies, the ovum retrieval was performed at 36 h from hCG administration.

Pre-incubation of oocytes with or without cumulus–corona cells before ICSI. The only two prospective studies focusing on the influence of a pre-incubation time with or without cumulus–corona cells showed opposite results (Table III). Van de Velde *et al.* (1998) did not find any statistical difference in terms of survival, fertilization and embryo cleavage rates between early (1–2 h after retrieval) and late (5–6 h after retrieval) injection, both when oocytes were pre-incubated with and without cumulus–corona cells. The oocyte retrieval was performed at 36 h from hCG administration (Van de Velde *et al.*, 1998). In contrast, Patrat *et al.* (2012) reported significant improvements in the fertilization rates when the denudation timing was delayed (optimal results at 3 h), while fertilization rate significantly decreased increasing the time between oocyte denudation and microinjection.

Among the many variables considered in the papers, time of hCG administration may vary from 35 to 36 h before ovum retrieval. Therefore, the different time lapses between retrieval and ICSI may have different effect depending on this period. Dozortsev *et al.* (2004) have described for the first time the ICSI timing in terms of time interval between hCG administration and ICSI and reported that implantation and ongoing implantation rates reached the highest level when ICSI was performed between 37 and 39 h after hCG administration, showing for the first time that human oocytes have a relatively narrow optimal insemination window, as happens in mice. Finally, in a recent retrospective cohort study, Garor *et al.* (2015) have evaluated the effect of manipulating

Table III Summary of the 13 papers identified in a systematic review of the literature in order to investigate the effectiveness of various pre-incubation times of oocytes before ICSI on embryo development and clinical outcomes.

	Incubation with/without cumulus (1/0)	Optimal hCG–OPU interval	Optimal OPU–ICSI interval	Optimal hCG–ICSI interval	Optimal OPU–denudation interval	Optimal denudation–ICSI interval	Proportion of MII oocytes	FR	Good EQ rate	BL rate	CPR	IR	AbR	P/PR/R study
Yanagida *et al.* (1998)	1	—	1–9	—	—	—	Unchanged	Unchanged	Improved	—	Unchanged	Unchanged	—	R
Rienzi *et al.* (1998)	1	—	3–12 h	—	—	—		Improved	Improved	—	Unchanged	Unchanged	—	R
Van de Velde *et al.* (1998)	1 + 0	—	—	—	None	None	Unchanged	Unchanged	Unchanged	—	—	—	—	PR
Hassan (2001)	1 + 0	—	—	—	4 h	0 h	Improved	Improved	Improved	Improved	Nv	Nv	—	PR
Jacobs *et al.* (2001)	1	—	None	—	—	—	—	Unchanged	Unchanged	—	Unchanged	Unchanged	Unchanged	R
Andrews *et al.* (2001)	0	—	<5 h	—	—	—	—	Unchanged	Unchanged	—	—	Improved	—	R
Ho *et al.* (2003)	1	—	2.5–5.5 h	—	—	—	Improved	Improved	Unchanged	—	Unchanged	—	—	R
Isiklar *et al.* (2004)	1	—	—	—	2–4 h	—	Improved	Improved	Improved	—	Unchanged	Unchanged	—	R
Dozortsev *et al.* (2004)	—	—	—	37–39 h	—	—	—	Improved	Unchanged	—	Improved	Improved	Unchanged	R
Falcone *et al.* (2008)	0	—	5–6 h	—	—	—		Improved	Improved	—	Improved	—	—	R
Aletebi (2011)	1 + 0	—	—	—	1–2 h	1–2 h	Improved	Improved	—	—	Improved	—	—	P
Patrat *et al.* (2012)	1 + 0	—	—	—	2–3 h	0 h	Unchanged	Improved	Unchanged	—	Unchanged	Improved	—	R
Garor *et al.* (2015)	1 + 0	>36 h	None	None	Unchanged	Unchanged	Unchanged	Improved	Unchanged	—	Improved	—	—	R

Improved or decreased results are indicated only when statistically significant. OPU, oocyte pick up.

oocyte denudation and injection timing in relation to hCG–ovum retrieval interval. Considering hCG–ovum retrieval intervals of more/less than 36 h, ovum retrieval–denudation intervals of more/less than 2 h and denudation–ICSI intervals of more/less than 1 h, it was shown that late ovum retrieval (>36 h) was associated with more available embryos, significantly higher fertilization and clinical pregnancy rates than early ovum retrieval (<36 h). Conversely, the length of incubation before or after denudation had no effect, regardless of ovum retrieval timing (Garor *et al.*, 2015).

In conclusion, the optimal ICSI timing is still unclear and the existing results are not fully conclusive (Table III). Most articles are concordant that a pre-incubation time before ICSI is beneficial on ICSI results, and cumulus–corona cells may have a positive effect during this pre-incubation. However, the data regarding the extension of the pre-incubation time are not concordant.

How to manage sperm and polar body position during injection

Biological basis

The oocytes provide almost the totality of the cytoplasmic determinants and membranes required for the formation and development of the pre-implantation embryo. An extreme asymmetric cytokinesis ensures the retention of these vital maternal stores. Indeed, the sequential emission of two polar bodies, whose sole purpose is to house the chromosomes, ensures the formation of a genetically competent gamete with minimal loss of cytoplasmic mass (Gitlin *et al.*, 2003). From a microtubule network with low dynamics during prophase arrest, the oocyte has to form the meiotic spindle, a structure built of mostly highly dynamic microtubules. The spindle must orchestrate the alignment and proper segregation of homologous chromosomes and sister chromatids in the two subsequent meiotic divisions. It forms in the center of the cell with no connection to the cortex and no constraint on its long axis, which takes a random orientation. After entry into meiosis I, the spindle always migrates toward the closest cortex and the first polar body is extruded at anaphase I while the spindle keeps moving, imposing a dynamic definition of the cleavage plan (Brunet and Verlhac, 2011; Chaigne *et al.*, 2012; Li, 2013). A prominent actin cap forms at the cortex overlying the spindle at the site of polar body extrusion upon anaphase I onset. In meiosis II, this cap seems to promote anchoring of the spindle parallel to the oocyte cortex via activation of an actin flow until fertilization triggers the emission of the second polar body (Longo and Chen, 1984; Maro *et al.*, 1986; Deng *et al.*, 2007; Azoury *et al.*, 2009; Larson *et al.*, 2010; Maddox *et al.*, 2012; Yi and Li, 2012). Thus, theoretically, the spindle should reform with the chromosomes aligned on the MII plate, directly below the first polar body. Historically, since the introduction of ICSI on human oocytes, it has been assumed that the second meiotic MII spindle lies in close proximity to the first polar body. This assumption has led to the accepted practice of injecting the spermatozoon headfirst at the 3 o' clock position, with the polar body at the 12 or 6 o' clock position, in order to avoid damage to the MII spindle. Interestingly, it has been published that the direction (headfirst or tailfirst) of the sperm exit from the injection pipette during ICSI would not influence the fertilization and good-quality embryo rate (Woodward *et al.*, 2008), while possible consequences of inserting the ICSI needle into or close to the MII spindle have been suggested to include partial or total disruption of the spindle or displacement of the spindle from the oolemma with consequent perturbation of chromosomal segregation and subsequent aneuploidy or even cell death. However, when the injection needle is entering the oocytes at the 3 o'clock position, the opening of the bevel of the needle is facing the 6 o'clock position. During the aspiration of the cytoplasm, different structures may be damaged or lost and it might be different if the oocytes are injected with the polar body at the 6 o'clock or 12 o' clock position, since oocyte chromosomes lie near the first polar body (Fig. 3).

Interventions

Few publications examined the optimal polar body orientation during ICSI with quite discordant results (Table IV). Two prospective studies reported better results when the injection was performed with polar bodies at 6 o'clock position compared with 12 o'clock position (Van Der Westerlaken *et al.*, 1999; Stoddart and Fleming, 2000). Of these, Stoddart and Fleming (2000) reported a lower proportion of poorest grade embryos for the injection with polar body at 6 o'clock position but underlined that clinical results were not influenced by the position of the polar body during ICSI. Van der Westerlaken *et al.* (1999) reported higher fertilization and pregnancy rates for the injection with polar body at 6 o'clock position, although based on logistic regression analysis, a confounding factor based on intra-individual differences was present. These results were justified

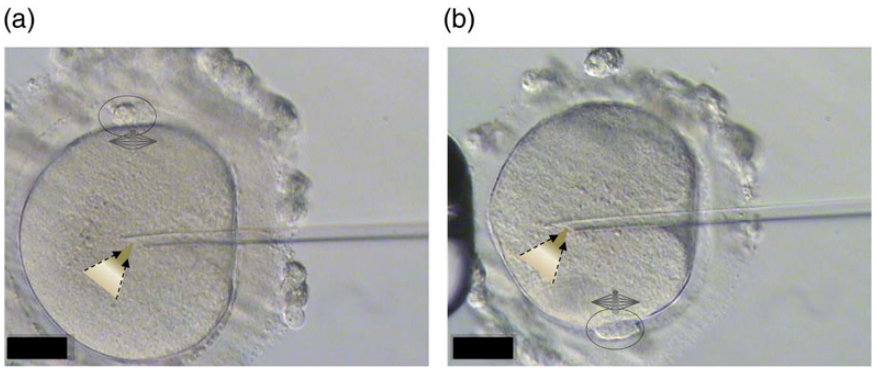

Figure 3 The injection pipette is inserted from the 3 o'clock position, that means that the opening is always facing down toward the 6 o'clock position. (**a**) The polar body is in the 12 o'clock position so that the cytoplasm is aspirated from a zona situated far from the meiotic spindle. (**b**) The polar body is in the 6 o'clock position so that the cytoplasm is aspirated from a zona situated near the meiotic spindle (400× magnification).

Table IV Summary of the five papers identified in a systematic review of the literature in order to investigate the effect of position of the polar body during ICSI on embryo development and clinical outcomes.

	Polar body position	DR	FR	Good EQ rate	BL rate	CPR	IR	AbR	P/PR/R
Van der Westerlaken et al. (1999)	6 and 12 o'clock	—	Improved for 6 o'clock position	Unchanged	—	Improved for 6 o'clock position[a]	—	Unchanged	PR
Blake et al. (2000)	1, 5, 6, 7, 8, 9, 10, 11, 12 o'clock	—	Unchanged	Improved for 7 and 11 o'clock position	—	—	Unchanged	—	R
Stoddart and Fleming (2000)	6 and 12 o'clock	Unchanged	Unchanged	Unchanged	—	Unchanged	Unchanged	—	PR
Dumoulin et al. (2001)	6 and 12 o'clock	Unchanged	Unchanged	Unchanged	Unchanged	—	—	—	
Anifandis et al. (2010)	6, 7, 11, 12 o'clock	—	Improved for 11 o'clock position	Improved for 11 o'clock position	—	Unchanged	—	—	PR

Improved or decreased results are indicated only when statistically significant.
[a]This difference was the result of significant interaction between polar body position and the operator (see the text).

by the shorter distance between the sperm and the spindle that could be obtained with the 6 o' clock polar body orientation and the injection pipette facing downward 6 o'clock, suggesting a certain polarization of oocyte structure in such a way that fertilization and cleavage would be facilitated when the spermatozoon is deposited closer to the spindle.

While Dumoulin et al. (2001) did not find any differences in terms of fertilization rate and embryo quality between 12 or 6 o'clock polar body orientation, in the same period, Blake et al. (2000) investigated in a retrospective manner all possible polar body orientations in order to find the 'ideal' area within the oocyte to deposit the sperm. They found a significantly higher quality embryo in the 7 and 11 o'clock orientation groups compared with the 6 and 12 o'clock orientation groups, whereas the 9 o'clock orientation was the most deleterious in terms of fertilization rate (Blake et al., 2000). A single RCT was performed by Anifandis et al. (2010) in which the polar body orientation at 11 o' clock during ICSI resulted in higher fertilization, embryo quality and clinical pregnancy rate compared with other orientations (6, 7, 11 and 12 o'clock). The shorter distance between sperm and the meiotic spindle that can be obtained with 7 and/or 11 o' clock polar body orientation compared with 6 and 12 o' clock orientations has been claimed as the potential explanation for these results (Anifandis et al., 2010).

We herein also mention the retrospective study on 1545 oocytes by Yanaihara et al. (2006) that showed that when the spermatozoa remained near the injection site after ICSI, the fertilization rate was significantly lower, although the embryo quality was not affected. Once again, the better results in terms of fertilization rate obtained when injected spermatozoa remained in the center or in the left of the oocytes have been explained by the smaller distance between the sperm and the meiotic spindle (Yanaihara et al., 2006).

When spindle imaging by polarization microscopy became available in IVF laboratories, it seemed that this technique would have been the answer to the questions about ICSI and spindle damage, making the previous studies obsolete. It was immediately clear that the MII spindle may not always be located directly below the first polar body and the spindle could be displaced from the polar body position by more than 90° (Hardarson et al., 2000; Rienzi et al., 2003). Manipulation and stress caused during oocyte denudation are thought to represent the main factors responsible for the spindle deviation but also intrinsic factors, such as oocyte ageing, as in the mouse (Eichenlaub-Ritter et al., 1986; Taylor et al., 2008), may also play a role in polar body displacement. The displacement of the spindle away from the polar body could make the spindle and the cytoplasm more susceptible to disturbances caused by the ICSI needle by causing an alteration of the position of the polarized molecules in the cytoplasm and, consequently, the course of embryo development (Garello et al., 1999; Cooke et al., 2003).

The possibility to visualize the meiotic spindle in oocytes has been the object of several contributions. This topic is beyond the purpose of our paper and a meta-analysis tends to suggest that spindle presence investigation may be useful in selecting gametes with a higher chance of fertilization, higher cleavage rate, Day 3 top quality embryo and blastocyst formation rate, though no improvements in implantation and clinical pregnancy rate per transfer were reported (Petersen et al., 2009). Moreover, studies that have investigated the relationship between the meiotic spindle location with regard to polar body position have demonstrated that displacements that exceed 90° are very rare (4.5%; Rienzi et al., 2003), so that by choosing an injection angle of 90° (corresponding to 3 o'clock position), it is still theoretically possible to disturb the spindle apparatus, but this is highly unlikely.

However, we must comment that, nowadays, the polarization microscopy is not widely available in IVF laboratories and the optimal polar body or spindle position during ICSI remains unclear. Differences in the technical procedure, such as the amount of cytoplasm sucked into the injection pipette or the force with which the injection pipette is pushed through the oocyte membrane, may explain the poor scientific consistency on this topic.

How to manage zona-free oocyte injection

During the denudation procedure, the zona pellucida occasionally can be damaged, resulting in the partial or total extrusion of the ooplasm out of the zona pellucida.

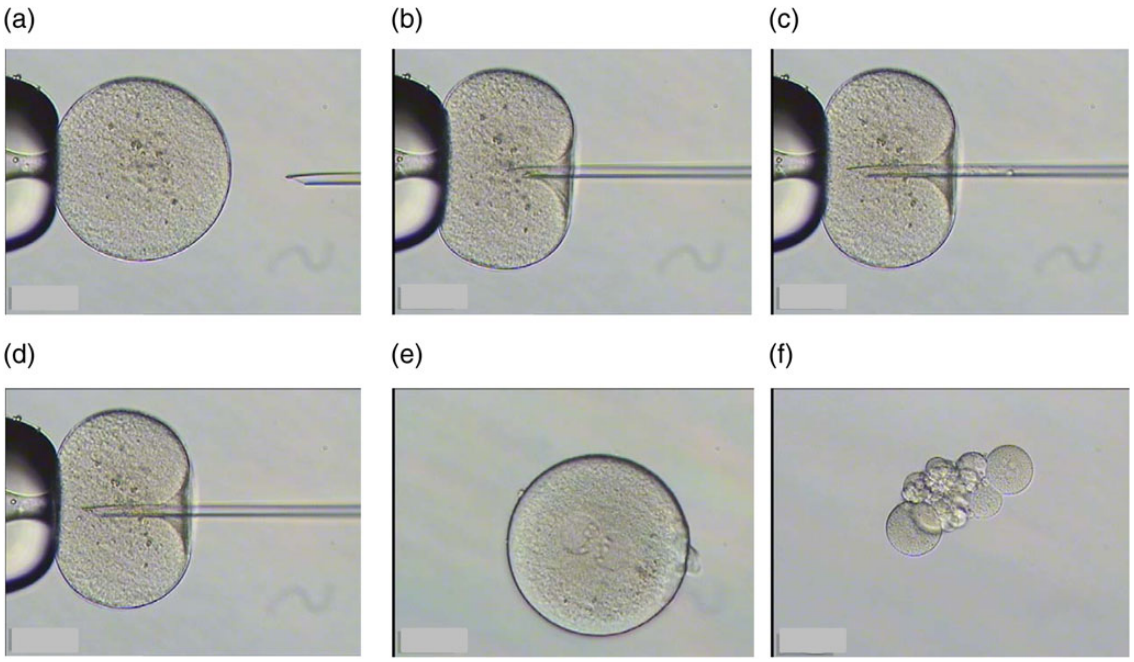

Figure 4 ICSI of zona-free oocyte (**a**–**d**). The polar body is not detectable. The oocyte fertilized (**e**) but resulted in one bad-quality embryo in Day 2 (**f**) that arrested in Day 3. (a–e, 400× magnification; f, 200× magnification.)

Biological basis

The zona pellucida plays an important, protective role during fertilization and early embryonic development. It prevents cross-species and polyspermic fertilization and ensures maximum contact between blastomeres before the compaction occurs. Indeed, early embryos that cleave inside the zona pellucida have blastomeres that are loosely attached to each other and can easily be dissociated. During compaction, tight junctions develop between outside cells of the embryo, sealing off the inside of the sphere and forming a compact morula. At this stage, even zona-free embryos may survive the transfer procedure (Vajta *et al.*, 2010). In most ART centers, eggs with damaged zona pellucida are generally discarded. However, especially when few oocytes are retrieved, it is imperative to fully utilize the developmental potential of each harvested egg to avoid the physical and psychological burden of cycle cancellation for the individual patient.

Interventions

Five case reports and one retrospective study reported the injection of zona-free oocytes (Fig. 4). Ding *et al.* (1999) described the cases of two couples in which one zona-free oocyte for each couple was injected. The polar bodies were not detectable. In both cases, the zona-free oocytes fertilized, cleaved and developed to an early blastocyst successfully even though in the first case, one blastomere degenerated *in vitro* at 4-cell stage. In one case, the early blastocyst was cryopreserved at Day 6, while in the second case, it was transferred together with a zona-intact, expanded blastocyst to the recipient but implantation did not occur. In both cases, the culture medium was renewed by removing the old medium from the culture drop and replacing with fresh in order to avoid the disintegration of the early embryos (Ding *et al.*, 1999).

Takahashi *et al.* (1999) reported the case of a low-quality blastocyst obtained from a zona-free injected MII oocyte which had been rescued from a GV oocyte after an overnight *in vitro* culture. No information was reported about the destiny of the blastocyst (Takahashi *et al.*, 1999). Stanger *et al.* (2001) reported the case of a couple who presented oocytes lacking a zona pellucida in two consecutive treatment cycles. Since in the first cycle, the injection of two zona-free oocytes resulted in two arrested 4-cell stage embryos (probably due to the disassembling of embryos during manipulation), they decided to inject the oocytes without the removal of the coronal cells. Polar bodies were discernible through the coronal cells. Two oocytes fertilized and were transferred at Day 3 and a clinical pregnancy was established (Stanger *et al.*, 2001). Subsequently, Hsieh *et al.* (2001) reported the injection of a zona-free MII oocyte that fertilized, developed to the 8-cell stage and was transferred with other zona-intact embryos. However, the implantation failed (Hsieh *et al.*, 2001).

More recently, Shu *et al.* (2010) reported two clinical pregnancies following the transfer of vitrified blastocysts developed from oocytes without zona pellucida. In one of the two cases, the polar body was not attached and on Day 2, all the four blastomeres of the early embryo were inserted back into ruptured zona in order to prevent the embryo from falling apart because there were loose contacts between blastomeres. The same case resulted in an abortion at 22 weeks. In the second case, the first polar body was attached to the oocyte, tight contacts between blastomeres were present on Day 2, and a healthy baby was born (Shu *et al.*, 2010).

Finally, Ueno *et al.* (2014) published a retrospective internally controlled study in which the reproductive potential of 135 zona-free eggs from minimally stimulated or natural cycles was compared with the reproductive potential of 216 zona-intact oocytes. Meiotic spindles of

zona-free oocytes were confirmed with polarized light microscopy. No significant differences in fertilization, cleavage, blastocyst formation, and good-quality blastocyst rate were observed between zona-free and zona-intact oocytes. Survival after thawing and live birth rate per thawed blastocyst were also similar. Newborns originating from all zona-free and zona-intact oocytes had a similar gestational age at delivery and birthweight (Ueno et al., 2014).

Based on this evidence, it can be concluded that zona-free mature oocytes may be successfully fertilized with the use of ICSI, cultured until the blastocyst stage and deriving blastocysts can be vitrified for further use. The basic neonatal outcome of resulting offspring is quite reassuring. Appropriated care in the handling of zona-free oocytes and subsequent early embryos should be used, namely the renewing of medium leaving the embryo undisturbed. The transfer of zona-free cleavage embryos should be avoided in order to prevent the blastomere falling apart during manipulation and/or after transfer and blastocyst transfer should be preferred (Ding et al., 1999; Stanger et al., 2001).

How to manage fertilization failure

Biological basis

Complete fertilization failure after ICSI is a rare event (1–3%) but does happen even in the presence of apparently normal spermatozoa (Sousa and Tesarik, 1994; Liu et al., 1995; Ludwig et al., 1999; Esfandiari et al., 2005). Moreover, low or moderate fertilization (<30%) can be observed in repeated ICSI cycles for some patients (Montag et al., 2012).

The cause of fertilization failure after ICSI is different from that of conventional IVF. In conventional IVF, 60–90% of the oocytes that failed to fertilize do not contain sperm nuclei, suggesting penetration failure or sperm ejection as the main cause of fertilization failure (Mahutte and Arici, 2003). In contrast, 60–70% of unfertilized MII oocytes after ICSI contain a swollen sperm head, indicating that oocyte was correctly injected and that the principal cause of failed fertilization should have been attributed to an oocyte-activation deficiency. Failure of oocyte activation has been reported in 40–70% of unfertilized oocytes exposed to ICSI (Sousa and Tesarik, 1994; Flaherty et al., 1995, 1998; Rawe et al., 2000; Yamano et al., 2000; Yanagida, 2004). Other causes include failed sperm head decondensation, premature sperm chromatin condensation, spindle defects or sperm aster defects or simply incorrect sperm injection (Swain and Pool, 2008). However, those causes are not considered to contribute substantially to fertilization failure.

Oocyte activation consists of the release of the meiotic arrest converting the oocyte into a totipotent zygote able to form all types of body cells. These events include cortical granule exocytosis, extrusion of the second polar body, maternal RNA recruitment, formation of pronuclei and initiation of embryonic gene expression (Horner and Wolfner, 2008). There is a general consensus that the master key to initiate all the cytological changes in fertilized oocytes is represented by a series of intracellular Ca^{2+} rises that start shortly following spermatozoon–oocyte fusion (Miyazaki and Ito, 2006; Ramadan et al., 2012). The binding of the male gamete to the oolemma results in intracellular Ca^{2+} release within the ooplasm, with an initial surge starting from the site of sperm penetration and expanding as a wave through the oocyte. While one Ca^{2+} transient wave is registered in echinoderm, fish, and frog oocytes, repetitive calcium oscillations that last up to several hours are observed in mammals. The nature, amplitude, duration and frequency of the Ca^{2+} signals are species-specific and are still a matter of debate. Moreover, it is known that Ca^{2+} oscillations affect embryonic development (Amdani et al., 2013).

The success of ICSI represents the proof that contact of sperm and egg plasma membranes is not a critical step for egg activation. However, egg activation and subsequent development following ICSI are sperm-specific and cannot be substituted by an artificial introduction of Ca^{2+} into the egg from the outside medium, and neither can insertion of a glass pipette with or without the injection of culture medium sustain an intracellular release of Ca^{2+} to activate the injected oocytes (Neri et al., 2014). Substantial evidence suggests that oocyte activation is triggered by a sperm factor, the phospholipase C ζ (PLCζ). Once in the oocyte's cytosol, this physiological agent causes the production of inositol-triphosphate (IP3) from the internal phospholipid phosphatidylinositol biphosphate stores, which binds to its receptor at the ER. Ca^{2+} is then released from these stores in an oscillatory mode. Indeed, sperm-induced Ca^{2+} oscillations stimulate mitochondrial respiration and, in turn, the resulting in ATP production required to maintain the Ca^{2+} waves. Recently, a close relationship between abnormal PLCζ expression in the spermatozoa and failed oocyte activation and fertilization was demonstrated in humans (Kashir et al., 2010; Amdani et al., 2013). Of course, several oocyte elements that transduce the sperm-derived signal to oocyte cell-cycle controlling systems via specific mechanism are necessary. Indeed, cytoplasmic as well as nuclear maturation are crucial steps for oocytes to obtain the ability to respond properly to the sperm PLCζ (reorganization of the ER, increase in the number of IP3 receptors, increase in the concentration of Ca^{2+} ions stored in ER etc.) (Kashir et al., 2010; Amdani et al., 2013).

Following ICSI fertilization failure, the issue is to establish whether the fertilization failure should be attributed to a sperm- or an oocyte-related fertilization deficiency. Cases of oocyte-activation failure can be currently treated by artificial oocyte activation (AOA) methods. Despite the fact that treatment options in terms of AOA are the same for both male and female deficiency, this represents crucial information both for the physician and for the patients while discussing the prognosis and the transmission risk when a genetic cause is involved. The diagnostic test, usually offered to patients following total fertilization failure or very low fertilization following ICSI, is the mouse oocyte-activation test (MOAT; Rybouchkin et al., 1996; Heindryckx et al., 2005). MOAT compares the activation percentage of mouse oocytes between ICSI using spermatozoa from the patient and heterologous ICSI using control spermatozoa (with proven activation capacity). According to the percentage of activated oocytes after MOAT, patients can be divided into three groups: low-activation group (≤20%); intermediate-activation group (21–84%); high-activation group (≥85%). A sperm-related deficiency is very likely in the low-activation group, while it can be refuted in most cases in the high-activation group. An inconclusive test should be attributed to the intermediate-activation group results, since both spermatozoa and oocyte deficiency may have contributed to the previous ICSI fertilization failure (Heindryckx et al., 2005, 2008). The best results in terms of AOA can be theoretically expected when the MOAT test indicates a deficiency of the sperm oocyte-activating ability, as in the case of globozoospermia. Indeed, poor or absent fertilization during ICSI of the high-activation group could also be the result of some transient biological reasons or technical imperfections. For this reason, in the case of suspected oocyte-related fertilization failure, it is advisable to seek confirmation of actual fertilization failure by applying AOA only on some oocytes (Heindryckx et al., 2008).

Interventions

As mentioned, oocyte-activation failure can be compensated by approaches that aim for an artificial cell Ca^{2+} entrance or release. A variety of artificial activating methods is used in human ART, including physical, mechanical or chemical stimuli (Table V).

Case reports related to this section are numerous. We herein discuss the more relevant and a complete list is provided in Table V.

Mechanical method. A modified ICSI technique has been successfully applied to obtain fertilization and pregnancy in patients with previous total fertilization failure in two different studies, although limited to few case reports (Table V). Tesarik *et al.* (2002) presented six cases, half with sperm-born and half with oocyte-born oocyte-activation deficiency in which normal fertilization and development of embryos were restored by a modified ICSI technique. In five of them, normal children were born. The modified ICSI consisted of a vigorous cytoplasmic aspiration from the central area of oocyte and its re-injection along with the spermatozoa as close as possible to the oocyte periphery opposite to the puncture site. The entire procedure has to be repeated two times. That vigorous aspiration and repeated in-and-out movements of the microinjection needle within the human oocyte could produce a considerable influx of Ca^{2+} from the surrounding culture medium and/or a mechanical disruption of ER and consequent releasing of the Ca^{2+} stored in this organelle, probably causing the higher fertilization. Modified ICSI characterized by aspiration close to the opposite site of injection, followed by central deposition of the sperm, has been reported by Ebner *et al.* (2004). The rationale was to accumulate the mitochondria with a high inner membrane potential from the pericortical region to the center of the oocyte, thus, theoretically, supplying more energy (ATP) directly to the place where the spermatozoon was injected. The application of this technique restored an adequate fertilization and pregnancy rate (53 and 33.3%, respectively) in 14 patients with previous ICSI failure with conventional ICSI. In the latter study, however, the causes of previous fertilization failure were not provided (Ebner *et al.*, 2004).

Physical method. The most popular physical stimulus is electrical activation. Animal studies have shown that an electrical field can generate micropores by moving charged proteins in the lipid bilayer of the cell membrane of gametes and somatic cells. This would induce sufficient Ca^{2+} influx through the pores to activate cytoplasm by means of Ca^{2+}-dependent mechanisms. It has been demonstrated that after the peak induced by electrical activation, the Ca^{2+} decreases to the original level in ~300 s. Yanagida *et al.* (1999) reported for the first time the use of combined ICSI with electrical stimulation in two couples with prior total failed fertilization after ICSI, despite normal semen parameters. The oocytes were stimulated with a single, square direct-current (DC) pulse (1.5 kV/cm, 100 μs) ~30 min after ICSI. A 100% fertilization rate and a dizygotic twin birth in one of the couples were reported. Another successful outcome was reported by Egashira *et al.* (2009). A healthy female was born by using electrical oocyte activation in a couple whose male partner was affected by globozoospermia. In this study, in order to prevent the generation of reactive oxygen species (ROS), as demonstrated in pigs, the intensity and the length of the DC pulse applied for activation were half of those previously described (Egashira *et al.*, 2009).

A different activation protocol was applied by Zhang *et al.* (1999) in a prospective way on discarded oocytes that showed no evidence of fertilization 16–24 h after ICSI. After an alternating current electrical pulse of 8 V for 6 s, the oocytes were assigned to three treatment groups: Group I (one DC pulse 1.36–1.50 kV/cm for 40–60 μs); Group II (3 pulses every 15–20 min) and Group III (no DC pulse). Although the fertilization rate was similar regardless of the number of the electrical pulses applied, subsequent embryo development was dramatically improved in those oocytes receiving three electrical pulses (Zhang *et al.*, 1999). Finally, two prospective randomized studies have been published more recently. Applying the same protocol of oocyte activation published by Yanagida *et al.* (1999) to sibling oocytes from patients with total fertilization failure in a previous ICSI, Baltaci *et al.* (2010) found that electrically activated oocytes were capable of developing into good-quality, healthy embryos, competent to establish ongoing pregnancies. Degeneration rate due to electric stimulation was reported as 12% (Baltaci *et al.*, 2010). Mansour *et al.* (2009) randomized sibling oocytes from couples with severe oligoasthenoteratospermia and non-obstructive azoospermia between electroactivation and no electroactivation protocols. A double square DC pulse to achieve the field strength of 2.6–2.8 kV/cm was used in this study and the fertilization rate was reported as significantly improved in the electroactivated group of oocytes. Degeneration rate in electroactivated oocytes was 5.9% and was not significantly different between the two protocols. In all published protocols, the electrical stimulation was applied 20–30 min after ICSI (Mansour *et al.*, 2009).

Chemical method. One of the chemical artificial activating agents which has been described in human ART is strontium chloride ($SrCl_2$). Unlike electrical stimulation and Ca^{2+} ionophore treatment resulting in a single transient increase in intracellular Ca^{2+}, $SrCl_2$ can induce Ca^{2+} oscillations in mice more similar to the pattern seen with spontaneous fertilization through a so far unclear mechanism. No oscillations are observed in human oocytes after $SrCl_2$ exposure. $SrCl_2$ is thought to move into the oocyte down the concentration gradient, causing Ca^{2+} to be released from the ER. It has been demonstrated that Ca^{2+} oscillations induced by $SrCl_2$ are mediated by the IP3 receptor and require PLCζ activation and the synergistic action of IP3.

In the literature, few case reports using this compound can be found (Table V). Yanagida *et al.* (2006) and Kyono *et al.* (2008) reported the birth of seven healthy babies in patients with previous low or total fertilization failure. In both papers, oocyte activation was performed incubating the oocyte, 30 min after ICSI, in a 10 mM $SrCl_2$ solution for 60 min. Kyono *et al.* (2008) also reported a normal mental and physical follow-up of children from birth to 12 years old.

Ionophores, such as ionomycin or calmicyn (A23187), are the most commonly used chemical artificial activating agents in ART practice. An ionophore is a lipid-soluble molecule, usually synthetized by microorganism, which transports ions across the lipid bilayer of the cell membrane, allowing extracellular Ca^{2+} to flow into the cell. Ca^{2+} is released also from its intracellular Ca^{2+} stores and in *Xenopus* ionomycin was shown to act on the same intracellular Ca^{2+} stores as IP_3. The artificially induced Ca^{2+} rises do not mimic the physiologically sperm-induced Ca^{2+} oscillations, as the exposure to any Ca^{2+} ionophore causes a singular prolonged Ca^{2+} rise.

There is a certain variability in the protocols used in the various studies and there are only few reports on AOA by means of ionophores used for large number of ICSI patients (Table V).

We have described below the five main ionophore activation protocols together with the different publications using them: (a) a

Table V Summary of the 47 papers identified in a systematic review of the literature in order to investigate the effectiveness of assisted oocyte activation during ICSI on embryo development and clinical outcomes.

	Type of AOA	Pro	Study population	MOAT	'Rescue' procedure	FR	Cleavage rate/good EQ rate	BL rate	CPR	IR	AbR	LBR	P/PR R/CR/CS study
Rybouchkin *et al.* (1997)	Calcium ionophore	(d)	Globozoospermia + previuos fertilization failure	Yes	No	—	—	—	One ongoing clinical pregnancy reported	—	—	—	CR
Battaglia *et al.* (1997)	Calcium ionophore	(a)	Globozoospermia + previuos fertilization failure	No	No	—	—	—	One biochemical pregnancy reported	—	—	—	CR
Yanagida *et al.* (1999)	Electrical		Previous fertilization failure	Yes	No	—	—	—	—	—	—	Two live births reported	CS
Zhang *et al.* (1999)	Electrical		Discarded unfertilized oocytes after ICSI	No	Yes	Reactivated	Reactivated	Reactivated	—	—	—	—	R
Tesarik *et al.* (2000)	Calcium ionophore	(a)	Round spermatids and their isolated nuclei	No	No	Normal fertilization reported	Normal cleavage reported only for round spermatids	—	—	—	—	—	CS
Nakagawa *et al.* (2001)	Calcium ionophore	(e)	Discarded unfertilized oocytes after ICSI	No	Yes	Reactivated	Reactivated	—	—	—	—	—	R
Kim *et al.* (2001)	Calcium ionophore	(a)	Globozoospermia	No	No	—	—	—	—	—	—	One live birth reported	CR
Tesarik *et al.* (2002)	Mechanical		Globozoospermia + previous fertilization failure	Yes	No	—	—	—	—	—	—	Six live births reported	CR
Eldar-Geva *et al.* (2003)	Calcium ionophore		Previous fertilization failure	No	No	—	—	—	One ongoing clinical pregnancy reported		One miscarriage reported	One live birth reported	CR
Ebner *et al.* (2004)	Mechanical		Previous fertilization failure, no indication	No	No	Improved only in fertilization failure group	—	Unchanged	Improved only in fertilization failure group	Improved only in fertilization failure group	Unchanged	—	PR
Murase *et al.* (2004)	Calcium ionophore	(e)	Previous fertilization failure	No	No	—	—	—	—	—	—	One live birth reported	CR
Chi *et al.* (2004)	Calcium ionophore	(a)	Previous fertilization failure	Yes	No	—	—	—	—	—	—	Twin live births reported	CR
Heindryckx *et al.* (2005)	Calcium ionophore	(d)	Previous fertilization failure	Yes	No	Improved	—	—	Improved	—	—	—	R
Moaz *et al.* (2006)	Calcium ionophore	(b)	100% abnormal sperm morphology + previous fertilization failure	No	No	Improved	Unchanged	—	—	—	—	—	P
Lu *et al.* (2006)	Calcium ionophore	(e)	Discarded unfertilized oocytes after ICSI	No	Yes	Reactivated	Reactivated	—	—	—	—	—	R
Yanagida *et al.* (2006)	Strontium		Previous fertilization failure	No	No							Twin live births reported	CR

Check *et al.* (2007)	Calcium ionophore	(a)	Globozoospermia + previous fertilization failure	No	No	Failed fertilization after AOA	—	—	—	—	—	One live birth from sperm donation	CR
Ahmady and Michael (2007)	Calcium ionophore	(a)	Non-viable testicular sperm	No	No	—	—	—	—	—	—	One live birth reported	CR
Nasr-Esfahani *et al.* (2008a, b)	Calcium ionophore	(b)	Abnormal sperm morphology	No	No	Improved	Improved	—	—	—	—	—	PR
Heindryckx *et al.* (2008)	Calcium ionophore	d)	Previous fertilization failure	Yes	No	Improved	—	—	Improved	—	—	—	R
Tejera *et al.* (2008)	Calcium ionophore	(d)	Globozoospermia + previous fertilization failure	No	No	—	—	—	—	—	—	One live birth reported	CR
Kyono *et al.* (2008)	Strontium		Previous fertilization failure	No	No	Improved	Improved	—	Improved	Improved	—	Five live births reported	CS
Dirican *et al.* (2008a, b)	Mechanical		Globozoospermia	No	No	—	—	—	—	—	—	Two live births reported	CS
Borges *et al.* (2009a, b)	Calcium ionophore	(a)	General infertile population	No	No	Unchanged	Improved in women <36 and ejaculated or epididymal spz	—	Unchanged	Improved in women <36 and ejaculated spz	Unchanged	—	PR
Terada *et al.* (2009)	Calcium ionophore	(b)	Previous fertilization failure	Yes* *bovine oocytes	No	—	—	—	—	—	—	One live birth reported	CR
Borges *et al.* (2009a, b)	Calcium ionophore	(a)	Surgically retrieved sperm	No	No	Unchanged	Improved with epididymal spz	—	Unchanged	Unchanged	—	—	PR
Mansour *et al.* (2009)	Electrical		Oligoasthenospermia, non-obstructive azoospermia with total teratozospermia or immotile sperm, previous fertilization failure	No	No	Improved	—	—	—	—	—	Fifteen live births reported	PR
Kyono *et al.* (2009)	Calcium ionophore	(a)	Globozoospermia	Yes	No	—	—	—	—	—	—	One live birth reported	CR
Egashira *et al.* (2009)	Electrical		Globozoospermia + previous fertilization failure	Yes	No	—	—	—	—	—	—	One live birth reported	CR
Combelles *et al.* (2010)	Calcium ionophore	(c)	Previous fertilization failure	Yes	No	Failed fertilization after AOA	—	—	—	—	—	—	CR
Baltaci *et al.* (2010)	Electrical		Previous fertilization failure	No	No	Improved	Improved	—	Improved	Improved	—	—	PR
Isachenko *et al.* (2010)	Calcium ionophore	(a)	Previous fertilization failure	No	No	—	—	—	One clinical pregnancy reported	—	—	—	CR
Taylor *et al.* (2010)	Calcium ionophore	(a)	Globozoospermia	No	No	—	—	—	One ongoing clinical pregnancy reported	—	—	—	CR
Chen *et al.* (2010)	Strontium		Previous fertilization failure	No	No	—	—	—	—	—	—	Four live births reported	CS

Continued

Table V *Continued*

	Type of AOA	Pro	Study population	MOAT	'Rescue' procedure	FR	Cleavage rate/good EQ rate	BL rate	CPR	IR	AbR	LBR	P/PR R/CR/CS study
Sugaya (2010)	Calcium ionophore	(a)	Previous fertilization failure	No	No	—	—	—	—	—	—	One live birth reported	CR
Vanden Meerschaut *et al.* (2012)	Calcium ionophore	(d)	Previous fertilization failure + oocyte-related activation deficiency	Yes	No	Improved	—	—	—	—	—	—	PR
Ebner *et al.* (2012)	Calcium ionophore	(a)	Azoospermia, criptozoospermia + previous fertilization failure	No	No	Improved	Unchanged	Improved	Improved	Improved	—	Improved	P
Montag *et al.* (2012)	Calcium ionophore	(a)	Previous fertilization failure	No	No	Improved	—	—	Improved	Improved in previous total fertilization failure group	Decreased in previous total fertilization failure group	Improved in previous total fertilization failure group	P
Kim *et al.* (2012)	Strontium		Previous fertilization failure	No	No	—	—	—	—	—	—	One live birth reported	CR
Lu *et al.* (2012)	Calcium ionophore		Previous fertilization failure	No	Yes	—	—	—	—	—	—	One live birth reported	CR
Kuentz *et al.* (2013)	Calcium ionophore	(d)	Globozoospermia	No	No	Improved	—	—	Unchanged		—	Unchanged	R
Yoon *et al.* (2013)	Calcium ionophore	(a)	Previous fertilization failure	No	No	Improved	Improved	—	Improved	Improved	—	Thirty-eight live births reported	R
Vanden Meerschaut *et al.* (2014)	Calcium ionophore	(d)	—	—	—	—	—	—	—	—	—	Normal neonatal and neurodevelopmental outcome of 21 children ≥3 years born following AOA	R
Karaca *et al.* (2014)	Calcium ionophore	(a)	Globozoospermia	No	No	—	—	—	—	—	—	One live birth reported	CR
Kim *et al.* (2014)	Strontium		Previous fertilization failure with and without calcium ionophore	No	No	—	—	—	Five ongoing clinical pregnancies reported	—	—	Eight live births reported. Normal physical and mental development up to 60 months	
Ebner *et al.* (2015)	Calcium ionophore	(a)	Previous fertilization failure without severe male factor	No	No	Improved	Unchanged	Unchanged	Unchanged	Unchanged	Unchanged	Unchanged	P
Kim *et al.* (2015)	Calcium ionophore	(a)	Previous fertilization failure of *in vitro*-matured oocytes	No	No	—	—	—	—	—	—	Twin live births reported	CR

AOA, artificial oocyte activation; MOAT, mouse oocyte activation test; LBR, live birth rate; CS, case series; Pro = protocol (only for calcium ionophore) (a) a single exposure of the oocytes, within 30–60 min from the injection, to 5–10 μM Ca^{2+} ionophore A23187 for 5–30 min; (b) a single exposure of the oocytes, within 30 min from the injection, to 10 μM Ca^{2+} ionophore ionomycin for 10 min; (c) two consecutive exposures to 10 μM ionomycin for 10 min, 30 min apart (the time after ICSI was not specified); (d) the injection with the spermatoon of 5 pl $CaCl_2$, 0.1 mM, followed after 30 min of incubation in 5–6% CO_2 atmosphere, by two consecutive exposures to 10 μM ionomycin for 10 min, 30 min apart; (e) a single exposure of the oocytes, after 30 min from the injection, to 5–10 μM Ca^{2+} ionophore A23187 for 5 min, followed by an incubation with 10 μg/ml puromycin for 4–5 h.
'+' symbol states the coexistence of both indications; previous fertilization failure include total failure, very low and low fertilization.

single exposure of the oocytes, within 30–60 min from the ICSI injection, to 5–10 μM Ca^{2+} ionophore A23187 for 5 to 30 min.

(b) a single exposure of the oocytes within 30 min from the injection, to 10 μM Ca^{2+} ionophore ionomycin for 10 min; (c) two consecutive exposures to 10 μM ionomycin for 10 min, 30 min apart (the time after ICSI was not specified); (d) the injection with the spermatozoan of 5 pl of 0.1 mM $CaCl_2$ followed after 30 min of incubation in 5–6% CO_2 atmosphere, by two consecutive exposures to ionomycin, 10 μM, for 10 min, 30 min apart; (e) a single exposure of the oocytes, 30 min from the injection, to 5–10 μM Ca^{2+} ionophore A23187 for 5 min, followed by an incubation with Puromycin, 10 μg/ml, for 4–5 h.

Protocol a. Borges *et al*. published two RCTs in 2009 (Table V; Borges *et al.*, 2009a, b). In the first study, the effects of AOA were evaluated on 314 ICSI cycles using spermatozoa from different sources (ejaculated, epididymal and testicular). No significant differences were observed between conventional cycles and cycles with AOA. When the subgroup of couples with women younger than 36 years and ejaculated spermatozoa was analyzed, the authors observed an increase in high-quality embryos and implantation rate in cycles with AOA. In the same subgroup, when epididymal spermatozoa were used, the percentage of high-quality embryos was improved in cycles with AOA. In the second RCT, the same authors considered only couples ($n = 204$) undergoing surgical sperm retrieval for ICSI. For patients undergoing testicular sperm aspiration (TESA), AOA did not improve ICSI outcomes. A significant increased rate of high-quality embryos was observed with AOA only with injected sperm retrieved from the epididymis, and no other advantages were reported.

Montag *et al*. (2012) reported the results of using AOA in patient cohorts with a history of no fertilization (0%—27 patients), fertilization between 1 and 29% (38 patients) and fertilization between 30 and 50% (24 patients) in initial ICSI cycles. The first two groups of patients brought the greatest advantage from the AOA, with a satisfactory fertilization (41.6 and 44.4%, respectively), clinical pregnancy (18.8 and 31.4%, respectively) and take-home baby rates (12.8and 24.1%, respectively). In the third group, however, a significantly higher fertilization rate was also reported (Montag *et al.*, 2012).

Ebner *et al*. (2012, 2015) reported the results obtained with a ready-to-use, commercially available Ca^{2+} ionophore A23187 in two different multicentric prospective studies (Table V). In the first study, only couples with azoospermic or cryptozoospermic male partners and previous low fertilization rates in previous cycles were included (88 patients): higher fertilization, blastocyst formation, implantation and take-home baby rates were reported for this group of patients (Ebner *et al.*, 2012). In the second multicenter study, patients with a history of low fertilization (<50%) in previous ICSI cycles and without severe male factor indications (101 patients) were considered: higher fertilization rates compared with previous cycles were reported and fewer patients had their embryo transfer cancelled compared with their preceding treatment. All children ($n = 35$) were born healthy except for one male newborn who was diagnosed with anal atresia (Ebner *et al.*, 2015). These studies, however, might have the bias that they have used previous cycles as control group.

Finally, it is worth mentioning the several case reports that have been published (Table V). Four case reports have reported one biochemical pregnancy, one ongoing pregnancy and two live births in couples with a globozoospermic male partner (Battaglia *et al.*, 1997; Kim *et al.*, 2001; Kyono *et al.*, 2009; Taylor *et al.*, 2010); two case reports have described five live births in two couples with normozoospermic male partners with repeated failed or very low fertilization rate (Eldar-Geva *et al.*, 2003; Chi *et al.*, 2004); one clinical pregnancy in a couple with an asthenozoospermic patient with low fertilization rate has been reported (Isachenko *et al.*, 2010); one full term delivery from a patient with only non-viable testicular spermatozoa (Ahmady and Michael, 2007) and a twin pregnancy in a cycle of *in vitro*-matured oocyte (Kim *et al.*, 2015) have been also described. Notably, a report of a fertilization failure has also been reported in a globozoospermic patient, although sibling oocytes injected with donor sperm did fertilize (Check *et al.*, 2007). Finally, we believe that we should mention the article of Tesarik *et al.* (2000), who reported a normal fertilization rate of oocytes injected with round spermatids after oocyte activation with Ca^{2+} ionophore. The authors did not report if the embryos obtained implanted and we did not find any similar study in the literature, but their data should encourage further research on this topic.

Protocol b. A higher fertilization rate was reported in a prospective study on sibling oocytes in selected cases ($n = 56$) with a previous history of failed or low (<40%) fertilization rate and 100% sperm abnormality (Moaz *et al.*, 2006). Similar results were reported also from another group in a prospective study on sibling oocytes in which 87 couples with severe teratozoospermia were involved. In this study, however, a significant increment of fertilization rate was reported only for the study groups of oocytes that had poor (<33%) or moderate (34–65%) fertilization rate in their respective control groups. Greater benefits were also found on fertilization rates for the semen samples with immotile sperm compared with those with motile sperm (Nasr-Esfahani *et al.*, 2008a). It is worth mentioning for this protocol a case report of a clinical pregnancy obtained in a couple with total fertilization failure in previous cycles due to sperm centrosomal dysfunction (Terada *et al.*, 2009; Table V).

Protocol c. Only a case report is available in the literature, in which a fertilization failure even after AOA is described. The oocytes were fixed for cytological examination and multiple polar bodies and two disorganized spindle structures were predominantly observed, pointing toward a cytoplasmic defect in the oocytes as the primary cause of the couple's infertility (Combelles *et al.*, 2010).

Protocol d. Heindryckx *et al*. (2005, 2008) published two studies both reporting the restoring of normal fertilization and pregnancy rates after AOA in a group of patients with failed or low fertilization rate (Table V). MOAT was used to distinguish between sperm-related and oocyte-related activation deficiency. In the first study on 17 patients (including 6 patients with globozoospermia), a fertilization rate of 77 and 71% was reported in the sperm- and oocyte-related deficiency groups, respectively, with 5 pregnancies in the globozoospermia group and 3 in cases of oocyte-related fertilization failure (Heindryckx *et al.*, 2005). In the second study (30 patients), fertilization and pregnancy rates were significantly increased for all the three groups of patients identified with MOAT (respectively, 75 and 34% for low-activation group; 73 and 43% for intermediate-activation group and 75 and 17% for high-activation group). All pregnancies gave rise to healthy infants with no major or minor malformations (Heindryckx *et al.*, 2008). These studies highlighted how even when MOAT reveals no activation deficiency in spermatozoa, assisted AOA still allows for high fertilization rate. Further evidence on the efficacy of AOA also for patients with an

oocyte-related activation deficiency was published by Vanden Meerschaut et al. (2012). In a prospective study on sibling oocytes, ICSI followed by AOA was able to improve fertilization rate (74.2 versus 43.5%) in couples with previous total fertilization failure or low fertilization and with a suspected oocyte-related fertilization problem. However, at least, in cases from other ART centers, a split of oocytes between conventional ICSI and ICSI followed by AOA should be proposed as a first option, in order to distinguish between a molecular oocyte-related activation deficiency and previous technical or other biological failures (Vanden Meerschaut et al., 2012).

Finally, Tejera et al. (2008) reported a healthy newborn with the application of this AOA protocol after ICSI in a couple with a globozoospermic male partner (Tejera et al., 2008).

Recently, Vanden Meerschaut et al. (2014) reported the neonatal, behavioral and neurodevelopmental outcomes of 21 children aged ≥3 years who had been born following AOA. The data obtained were reassuring because, for all the tests, the mean outcomes lay within the expected ranges (Vanden Meerschaut et al., 2014).

For the description of the study by Kuentz et al. (2013), we refer to the section 'How to manage globozoospermia'.

Protocol e. Two studies reported the rescue of the oocytes that were unfertilized after ICSI (Table V). Both studies reported a high activation rate (>80%), although it was differently defined in the two studies. Lu et al. (2006) considered activated oocytes as oocytes that showed at least one pronucleus or oocytes that immediately cleaved, while Nakagawa et al. (2001) considered the activation rate as the proportion of the oocytes that showed two pronuclei with the second polar body.

Chromosomal analysis applied to a subset of embryos derived from activated oocytes showed in both studies a normal diploid set of chromosomes. In particular, Lu et al. (2006) reported the presence of the Y chromosome in cleaving embryos. This would suggest that one pronucleus in the fertilized oocytes after activation originated from an injected spermatozoon and that embryos were not parthenogenones. The authors observed that the best results in terms of oocyte activation and development potential of activated oocytes were obtained when the oocyte activation was performed 20 h after ICSI and not later, suggesting that the function of organelles in oocytes is still maintained after this period. These results were encouraging for the possibility to rescue the oocytes in cases of total fertilization failure, especially if the oocytes are activated as soon as the total fertilization failure is recorded. However, the fate of embryos obtained from rescued oocytes was not described.

Finally, Murase et al. (2004) reported the delivery of a healthy male infant in an ICSI cycle with AOA in a couple with a normozoospermic male partner and previous repeated failed fertilizations.

Although the single study on the neurodevelopmental outcomes in children aged between 3 and 10 years is reassuring, prospective multicenter long-term studies are needed to draw definite conclusions about the safety of this technique. We can comment that this is not the only technique routinely applied in human IVF missing prospective multicentric long-term studies (Gleicher et al., 2013). However, the marketing of a ready-to-use ionophore solution has been viewed as an imprimatur of safety not well accepted by some authors (van Blerkom et al., 2015). It is true however that nowadays, a standardization of procedure is still missing, too many different protocols have been published and the literature reports mainly cases based on few patients. While molecular research is conducted on the effects of the altered pattern of Ca^{2+} oscillation caused by AOA, clinicians should aim for standardization of the AOA procedure, and a strict follow-up of children should be established. Hence, ICSI combined with AOA should be still performed only in cases where a clear indication is present and should be considered an experimental procedure.

At present, there is a great interest in PLCζ as the activating agent because of its physiologic activity and we expect significant progresses in the near future (Amdani et al., 2013).

ICSI and male gametes

As ICSI is the gold standard technique for male factor infertility, a great effort has been directed over the years toward the improvement of the selection of euploid viable spermatozoa, with intact DNA (Oehninger, 2011). This has resulted in a huge amount of papers addressing these topics, which will not be discussed extensively here. The aim of this section is to review and discuss only the studies that have evaluated the effectiveness of advanced sperm selection methods in terms of ICSI results.

How to manage globozoospermia

Biological basis

Globozoospermia was first described in 1971 as a very rare type of monomorphic teratozoospermia observed in <0.1% of infertile males. It is characterized by round-headed spermatozoa with an absent acrosome, an aberrant nuclear membrane and midpiece defects (Singh, 1992; Dam et al., 2007a, b).

During spermiogenesis, the acrosome is formed by the Golgi apparatus of the developing spermatocyte. After positioning of the prospective acrosomal cap, the sperm head is remodeled by the cytoskeleton to a species-specific shape. In patients with globozoospermia, this process does not occur, leaving spermatozoa with a characteristic round-head appearance (Kang-Decker et al., 2001; Yao et al., 2002; Xiao et al., 2009). The acrosomeless spermatozoon is unable to go through the zona pellucida and fuse with the oolemma of the oocyte (Aitken et al., 1990; Dale et al., 1994; Rybouchkin et al., 1996).

There is a group of patients whose semen samples show only a fraction of spermatozoa with this typical head shape. This condition was described 2 years after the first description of globozoospermia and the term 'partial' globozoospermia is often used in patients with <100% of the sperm cells showing a round-headed form without an acrosome (Holstein et al., 1973). These cases are more common in daily clinical practice than total globozoospermia. Although there is no defined threshold for the normal proportion of round-headed spermatozoa in an ejaculate, it is well known that even fertile men can have up to 3% round-headed sperm cells in the ejaculate (Kalahanis et al., 2002). The presence of consanguineous marriage in families affected with globozoospermia and reports of two or more sibs in several families suggested a genetic contribution to globozoospermia in humans with autosomal recessive mode of inheritance. Three genes with potential involvement in globozoospermia [*SPATA16* (spermatogenesis associated 16), *PICK1* (protein interacting with PRKCA 1) and *DPY19L2* (*one of a* transmembrane gene family)] segregating on an autosomal recessive mode have now been identified (Florke-Gerloff et al., 1984; Carrell et al., 1999; Kilani et al., 2004; Dam et al., 2007b; Dirican et al., 2008a; Liu et al., 2010; Harbuz et al., 2011; Koscinski et al., 2011; Perrin et al., 2013).

These findings reinforce the idea of a multigenic disorder in which several etiologies may cause the same morphologic phenotype.

Globozoospermic men had a sperm fragmentation index statistically significantly higher than fertile men but just a slight increase in aneuploidy rate compared with fertile men (Perrin *et al.*, 2013). Until the advent of ICSI in 1992, globozoospermia was considered intractable.

However, unfortunately, the fertilization and birth rates obtained following conventional ICSI are extremely low in globozoospermic patients. Recent reports suggest that globozoospermia is associated with a significant reduction in, or even the absence of, the sperm factor PLCζ and of perinuclear theca proteins (Heytens *et al.*, 2009; Oko and Sutovsky, 2009; Alvarez Sedo *et al.*, 2012). This might explain the very low or absent fertilization in globozoospermic patients because, as reported above, PLCζ is thought to be the main physiologic factor responsible for oocyte activation (Oko and Sutovsky, 2009).

Interventions

Although spontaneous activation of the oocyte after ICSI has been reported in some cases of globozoospermia (Lundin *et al.*, 1994; Kilani *et al.*, 1998; Stone *et al.*, 2000; Nardo *et al.*, 2002; Zeyneloglu *et al.*, 2002; Banker *et al.*, 2009; Bechoua *et al.*, 2009; Sahu *et al.*, 2010; Sermondade *et al.*, 2011; Zhang *et al.*, 2016), Zhang *et al.* (2016) have suggested that ICSI without AOA may be useful especially in cases of partial globozoospermia, because partial globozoospermia implies the presence of normal sperm cells that may be used in a clinical setting. On the other hand, Rybouchkin *et al.* (1997) have demonstrated that the rates of fertilization and embryo development in ICSI using round-headed sperm improved when a calcium ionophore was used for oocyte activation after microinjection. Activations of the oocytes by chemical agents (Rybouchkin *et al.*, 1997; Battaglia *et al.*, 1997; Kim *et al.*, 2001; Check *et al.*, 2007; Tejera *et al.*, 2008; Kyono *et al.*, 2009; Taylor *et al.*, 2010; Kuentz *et al.*, 2013), mechanical methods (Tesarik *et al.*, 2002; Dirican *et al.*, 2008a) and electrical stimuli (Egashira *et al.*, 2009) have been suggested and we refer to the section 'How to manage fertilization failure' for further details.

Interestingly, Kuentz *et al.* (2013) observed, in a retrospective study on 34 globozoospermic patients, that the fertilization rate with AOA, by means of calcium ionophore, was restored to normal when compared with conventional ICSI, regardless of the presence of a DPY19L2 mutation. In any case, when using conventional ICSI, globozoospermic DPY19L2-mutated patients had a better, although very low, fertilization rate than globozoospermic DPY19L2 non-mutated patients (Kuentz *et al.*, 2013). Probably, in the case of globozoospermia and especially in partial globozoospermia, it is advisable to apply AOA only to some of the oocytes in order to check the oocyte-activation ability of spermatozoa.

How to manage absolute asthenozoospermia

Biological basis

Absolute asthenozoospermia (the condition in which only immotile spermatozoa can be retrieved) is reported at a frequency of 1 in 5000 men and it implies a very poor fertility prognosis. Diagnosis, etiology and treatment of absolute asthenozoospermia have been already very well reviewed by Ortega *et al.* (2011) who reported that the injection of a completely immotile spermatozoon has an overall negative impact on fertilization and pregnancy rates. The obvious reason lies in the fact that immotile viable spermatozoa cannot be distinguished from immotile non-viable spermatozoa. Indeed, beyond the defects of the motor apparatus that can be found in genetic defects, such as Kartagener's syndrome or dysplasia of fibrous sheath or defective centrosome, a major condition associated with immotile sperm is necrozoospermia (Nagy, 2000), which is a passive, catabolic and degenerative process that ends in cell death. Only one birth resulting from injection of killed spermatozoa by freezing without cryoprotectant was reported (Hoshi *et al.*, 1994). However, viability staining following the 'killing' process was not performed, raising the possibility that the sperm used might have been immobilized but were not dead. Early experiments with proved non-viable spermatozoa demonstrated that they were able to fertilize but with very poor results (16.2–17.5%) and embryo development was heavily compromised (Poe-Zeigler *et al.*, 1997; Ahmadi and Ng, 1999).

Interestingly, based on experiments in animals, fertilization and blastocyst formation rate increased to normal level when a cytosolic sperm factor, obtained by lysing and centrifuging a sperm suspension, was co-injected with non-viable spermatozoa, suggesting that poor fertilization could be due to the inability of non-viable spermatozoa to trigger the activation process of the oocytes (Ahmadi and Ng, 1999). Indeed, in all mammalian species studied to date, normal oocyte fertilization and development follow a specific intracellular Ca^{2+} oscillation (see the Fertilization Failure, *Biological basis* section). Experiments with human spermatozoa killed by sonication and injected into mouse oocytes show that embryo development to blastocyst stage and the rate of normal Ca^{2+} oscillation declined after a short maintenance interval between sonication and injection, with the Ca^{2+} response as the most sensitive parameter affected, directly demonstrating the inability of non-viable spermatozoa to activate the oocytes (Yazawa *et al.*, 2009).

Interventions

In daily laboratory routine, selection methods currently used to choose a viable sperm for ICSI in the case of absolute asthenozoospermia are based on chemical substances added to the sample before the procedure, or biophysical approaches accomplished during the procedure. In the case of 100% necrozoospermia in the ejaculate, ICSI should be performed in combination with TESE using methods for selecting viable testicular spermatozoa (Ortega *et al.*, 2011).

Improvement of the sperm sample before ICSI procedure: pentoxifylline treatment. Pentoxifylline is a methylxanthine derivative that causes a non-selective inhibition of phosphodiesterase (PDE) leading to augmented generation of cyclic nucleotides such as cAMP and cyclic guanosine monophosphate. The compound is much in demand as a cardiovascular drug and has been widely used to ameliorate peripheral vascular diseases since it enhances blood cell flow and acts as a vasodilator. On sperm, the drug acts via the cyclic AMP pathway to induce downstream sperm tail protein phosphorylation, thus stimulating motility (Yovich, 1993). The effectiveness on ICSI outcome of pentoxyfylline, as a selective inhibitor of PDE able to stimulate sperm motility in cases of absolute asthenozoospermia, has been investigated by a total of four clinical studies and three case reports (Table VI).

A retrospective analysis of 47 ICSI cycles using immotile testicular sperm treated with pentoxifylline has demonstrated a significantly higher fertilization rate compared with 30 ICSI cycles using unselected immotile sperm, but the other outcomes were similar (Kovacic *et al.*, 2006). In this study, the final concentration of pentoxyfylline used was

Table VI Summary of 25 papers identified in a systematic review of the literature in order to investigate the effects of selection methods to manage absolute asthenozoospermia before ICSI on embryo development and clinical outcomes.

			Sperm sample	Notes	FR	Good EQ rate	Cleavage rate	BL rate	CPR	IR	AbR	LBR	P/PR/R/CR/CS study
Before ICSI procedure	Chemical substances	Nodar *et al.* (1999)	Ejaculated	Klinefelter's syndrome	—	—	—	—	—	—	—	Twin live births	CR
		Terriou *et al.* (2000)	Fresh–frozen epididimal and TESE	Immotile PF treated versus motile untreated	Unchanged	—	Unchanged	—	Unchanged	—	—	—	P
		Griveau *et al.* (2006)	Frozen–thawed TESE	Immotile PF treated versus motile untreated	Reduced	—	—	—	Unchanged	Unchanged	Unchanged	Unchanged	P
		Kovacic *et al.* (2006)	TESA/TESE	Immotile PF treated versus untreated	Improved	—	—	Unchanged	Unchanged	—	Unchanged	—	R
		Yildirim *et al.* (2009)	Ejaculated	Primary ciliary dyskinesia	—	—	—	—	—	—	—	Triplet live births	CR
		Ebner *et al.* (2011)	Frozen–thawed TESE	Theophylline treated versus untreated	Improved	Unchanged	Improved	Improved	Improved	Improved	—	Improved	PR
		Hattori *et al.* (2011)	Ejaculated	Kartagener's syndrome	—	—	—	—	—	—	—	One live birth	CR
		Mangoli *et al.* (2011)	TESE	PF versus HOS	Improved	—	Unchanged	—	Improved	—	—		PR
		Ebner *et al.* (2014)	Ejaculated	Immotile theophylline treated	—	—	—	—	—	—	—	One live birth	CR
During ICSI procedure	STFT	de Oliveira *et al.* (2004)	TESE	Motile versus immotile (fresh and frozen–thawed)	Unchanged	—	Unchanged	—	Unchanged	Unchanged	Unchanged	Unchanged	R
	HOS	Casper *et al.* (1996)	Ejaculated-thawed epididymal	HOS versus random selection	Improved	—	Improved	—	Improved	—	—	Three pregnancies in eight cycles	P
		Liu *et al.* (1997)	Ejaculated and TESE	HOS selection	—	—	—	—	One ongoing clinical pregnancy	—	—	—	CS
		Barros *et al.* (1997a, b)	Ejaculated	Total immotile after penthoxyphilline	—	—	—	—	Two ongoing clinical pregnancies	—	—	Two healthy children	CS
During ICSI procedure	HOS	Ved *et al.* (1997)	Ejaculated	—	—	—	—	—	One clinical pregnancy	—	—	—	CR
		El-Nour *et al.* (2001)	Ejaculated	HOS versus random selection	Unchanged	Unchanged	—	—	Unchanged	Unchanged	—	—	P
		Sallam *et al.* (2001)	Ejaculated and TESE	HOS ejaculated versus HOS TESE	Unchanged		Unchanged		Unchanged	Unchanged	Unchanged	—	R
		Westlander *et al.* (2003)	Ejaculated and TESE	Kartagener's syndrome; HOS ejaculated versus HOS TESE	—	—	—	—	Two clinical pregnancies	—	—	—	CS
		Peeraer *et al.* (2004)	Ejaculated	Immotile cilia syndrome	—	—	—	—	One clinical pregnancy	—	One miscarriage	—	CR
		Sallam *et al.* (2005)	TESE	HOS versus morphology	Improved	Improved	—	—	Improved	Unchanged	—	—	PR

Kordus *et al.* (2008)	Ejaculated	Immotile cilia syndrome	—	—	—	—	—	—	Twin live births	CR
Mangoli *et al.* (2011)	TESE	HOS versus PF	Decreased	Unchanged		Decreased	—	—	—	PR
Geber *et al.* (2012)	Ejaculated	Kartagener's syndrome	—	—	—	—	—	—	Twin live births	CR
Aktan *et al.* (2004)	Ejaculated totally immotile and TESE	LAISS versus morphology (fresh and frozen–thawed)	Improved	—	Improved	—	Unchanged	—	Unchanged	P
Gerber *et al.* (2008)	Ejaculated	Primary cilia dyskinesia	—	—	—	—	—	—	A singleton pregnancy	CR
Nordhoff *et al.* (2013)	TESE	LAISS versus morphology	Improved	—	—	—	—	—	—	R

LAISS

Improved or decreased results are indicated only when statistically significant.
STFT, sperm tail flexibility test; HOS, hypo-osmotic swelling test; LAISS, laser-assisted immotile sperm selection; PF, penthoxyphilline; TESE, testicular sperm extraction.

1.76 mM and the identification and isolation of motile spermatozoa started after a 20 min incubation. Two prospective studies showed that ICSI cycles performed using fresh or frozen epididymal and testicular pentoxifylline-treated immotile spermatozoa have similar outcomes in terms of clinical pregnancy rates compared with ICSI cycles using spontaneously motile spermatozoa, indirectly supporting the efficacy of the compound treatment (Terriou *et al.*, 2000; Griveau *et al.*, 2006). Pentoxyfylline was used at a final concentration of 3.6 and 1.5 mM with an incubation time of 10 min. As previously reported, a single randomized prospective study has demonstrated that pentoxifylline treatment (0.6 mM for 15 min) would be superior to hypo-osmotic swelling test (HOS) selection in TESE-recovered sperm for ICSI in terms of fertilization and pregnancy rates (Mangoli *et al.*, 2011). The sperm treatment with pentoxifylline is thought to be currently used, or to have been used in the past, in hundreds of laboratory worldwide: therefore, the limited evidence available for evaluating its usefulness is as unexpected as the lack of standardization of the protocols. Serious concerns have been raised regarding the potential embryotoxicity of pentoxifylline because of the controversial results derived from the analysis of this compound on animal embryo development (Tournaye *et al.*, 1993; Ain and Seshagiri, 1997). Thus, surprising is also the paucity of data regarding the babies born from this procedure, leaving a tremendous gap of knowledge in relation to its safety. Further studies are needed in this context.

Finally, three case reports described the occurrence of a pregnancy after ICSI performed by using pentoxyfylline-selected sperms in complete asthenozoospermic patients diagnosed with genetic syndromes such as Kartagener's syndrome (Hattori *et al.*, 2011), Klinefelter's syndrome (Nodar *et al.*, 1999) and primary ciliary dyskinesia (Yildirim *et al.*, 2009).

Improvement of the sperm sample before ICSI procedure: theophilline treatment. Theophylline is a weak non-selective inhibitor of PDE isoenzymes leading to increased intracellular concentrations of cAMP and cyclic 3′,5′ guanosine monophosphate. Theophylline would appear to be superior to pentoxifylline because of its increased half-life. A single group has investigated the usefulness of theophylline treatment instead of pentoxifylline (Ebner *et al.*, 2011) in a prospective study on thawed testicular sperm selected for ICSI in which a ready-to-use commercially available compound was used. Theophylline-treated thawed sperm compared with untreated sperm showed a significant improvement in searching time, increased fertilization and blastulation rates and higher implantation and clinical pregnancy rates. In a recent case report (Ebner *et al.*, 2014), theophylline has been demonstrated to be an efficient agent for stimulating immotile spermatozoa also in a patient with retrograde ejaculation and total sperm asthenozoospermia, and a healthy live birth was reported. It should however be considered that also for theophylline, a teratogenic effect has been reported in rodents and reproductive studies are recommended in the evaluation of the compound (National Toxicology Program, 1998).

Selection of sperm during ICSI procedure: the sperm tail flexibility test. The sperm tail flexibility test (STFT) represents a procedure developed to select viable immotile spermatozoa based on the observation that immotile viable sperms have a flexible tail (Soares *et al.*, 2003). If the tail bends and recovers its original position, the sperm is considered viable, while sperm rigidity and incapacity to recover to the initial tail position is considered a sign of non-viability. Others consider the sperm viable if the tail moves up and down independently of the head movement and non-

viable when head and tail move together when touched. There are concerns over the clinical routine use of this test. First of all, STFT needs laboratory staff with a high level of experience and skills. Furthermore, concerns have been raised about the use of this procedure for cryopreserved and thawed sperms, because of their, if any, spontaneous tail curling (Nordhoff, 2015). Only one retrospective study has investigated the clinical usefulness of STFT in ICSI practice (Table VI). de Oliveira et al. (2004) reported no significant differences in fertilization, pregnancy rates and take-home baby percentages achieved using motile versus immotile STFT-selected sperms. It should be considered that no prospective study has been published comparing STFT with others procedures used in ICSI practice, thus the benefits of the method in terms of reproductive outcome improvements remain to be proved.

Selection of sperm during ICSI procedure: HOS. The HOS is a simple, safe and repeatable alternative method allowing the identification of live and intact spermatozoa; exposure to hypo-osmotic conditions (75 mM fructose, 25 mM sodium citrate dehydrate) induces in cells different tail swelling patterns, due to water influx, which are classified from *a*-sperm to *g*-sperm (Casper et al., 1996; WHO, 2010). Pattern *a* spermatozoa have no expansion and are considered non-viable and non-functional and their use should be avoided during ICSI (Stanger et al., 2010; Bassiri et al., 2012); pattern *b* to *g* sperm have different tail morphologies as described by the World Health Organization (WHO, 2010) and are considered viable spermatozoa. The main disadvantages of this procedure are related to the possible steric problems arising from hypo-osmotic exposure and to false-positive as well as false-negative results (Barros et al., 1997a, b; Bollendorf et al., 2012). Although this technique is certainly time-consuming, it has been proven to work also for spermatozoa extracted from testicular biopsies (Sallam et al., 2001, 2005; Bollendorf et al., 2012). However, HOS is not suitable for spermatozoa that have been processed, specifically cryopreserved then thawed, as these cells spontaneously develop tail swelling (Hossain et al., 2010).

Apart from sporadic reports of successful results (Barros et al., 1997a, b; Ved et al., 1997; Liu et al., 1997), the effectiveness of this procedure in terms of ICSI outcome improvement has been evaluated by five clinical studies (Table VI). Casper et al. (1996) reported in a limited number of cycles an increased fertilization and cleavage rate after injection of HOS-selected sperm (to 43 and 39%, respectively) in comparison with random sperm injection where the fertilization and cleavage rates were lower (26 and 23%, respectively). A total of three pregnancies in the eight cycles with HOS-selected spermatozoa, including two from frozen epididymal sperms, were obtained. The effectiveness of HOS-selected 'live' sperm versus non-selected sperm on ICSI outcomes was, however, not confirmed in a prospective study involving 30 ICSI cycles by El-Nour et al. (2001). Sallam et al. (2001) retrospectively analyzed ICSI outcomes of ejaculated versus testicular sperms when sperm selection was performed according to a modified HOS procedure (50% culture medium + 50% Milli-Q grade water). There were no statistically significant differences between the ejaculated and the testicular sperm group in fertilization, cleavage, pregnancy and implantation rates or in the delivery/ongoing pregnancy rates. Two RCTs have been performed. The same group in 2005 (Sallam et al., 2005) carried out an RCT by comparing the use of the HOS procedure with the morphology selection alone for testicular sperm samples: higher fertilization, embryo quality, pregnancy and ongoing pregnancy rates were demonstrated in the HOS group. Nevertheless, Mangoli et al. (2011) analyzed use of the HOS method in comparison to pentoxifylline treatment in TESE-recovered sperms for ICSI: lower fertilization and pregnancy rates were observed for HOS-selected samples.

The HOS selection strategy has been successfully applied in cases of both Kartagener's syndrome and immotile cilia syndrome (Peeraer et al., 2004; Kordus et al., 2008; Geber et al., 2012). Interestingly, Westlander et al. (2003) compared HOS selection of ejaculated or testicular recovered sperms in two patients affected by Kartagener's syndrome, obtaining fertilization, good-quality embryos and two clinical pregnancies by using TESE together with HOS selection.

Selection of sperm during ICSI procedure: laser shot system. An alternative method for the identification of immotile sperm that are viable is based on a single laser shot through a non-contact 1.48 μm diode laser system, directed to the tip of the flagellum, which in a live immotile sperm causes a coiling of the tail at the site of impact (Aktan et al., 2004). The method appears to be quick, easy, repeatable and, even if a specific expertise is needed, its specificity in identifying viable sperms seems comparable to that of the HOS system (Nordhoff et al., 2013). The use of the laser-selected spermatozoa for ICSI in ejaculated absolute asthenozoospermic samples has been investigated in two clinical studies and in a case report (Table VI). In a prospective randomized study, Aktan et al. (2004) have achieved significantly higher fertilization and cleavage rates selecting immotile sperm from both TESE and ejaculated samples using the laser versus random selection. A trend toward a higher take-home baby rate was also reported, but the number of patients was too low to define the statistical significance. A significant increase in fertilization rate in TESE–ICSI cycles was also obtained when laser-assisted sperm selection was applied in comparison to the morphologic method (Nordhoff et al., 2013). A case report has described the achievement of pregnancy in a couple with an infertile man with primary ciliary dyskinesia by using laser-assisted sperm selection before ICSI (Gerber et al., 2008).

How to manage DNA fragmentation

Biological basis

An increasingly popular topic is the study of sperm DNA fragmentation. The main pathway leading to sperm DNA breaks is a process of apoptosis triggered by testicular conditions and by oxidative stress during the transit in the male genital tract (Muratori et al., 2015). Once the sperm nucleus has been delivered into the ooplasm, the condensed nucleus must decondense rapidly to release the DNA for formation of a paternal pronucleus. Any abnormal change in the structural organization can cause delays or defects in the delivery of the paternal DNA. Moreover, any damage to the DNA during the transition from the testicle to the oocyte cannot be repaired until the DNA is accessible for DNA repair systems in the ooplasm. The risk of error during the repair process increases with the number of DNA strand breaks in an individual sperm nucleus. Experiments in animals have demonstrated that when sperm induced to undergo to chromatin fragmentation by divalent cations were used to fertilized oocytes, the replication of the paternal pronucleus was severely delayed and if the damage is severe enough embryo development can be arrested (Gawecka et al., 2013). This phenomenon suggests that there might be a mechanism that stops replication forks in the presence of double-stranded breaks probably allowing the DNA to be repaired (Gawecka et al., 2015). Conversely, when the DNA damage is less severe (prevalence of single-stranded breaks), there is no detectable delay in the DNA synthesis but chromosomal

breaks are detected at mitosis demonstrating that DNA synthesis is possible in the zygote with some breaks (Gawecka et al., 2013). In both cases, embryo development might be compromised. These are the reasons why the injection of a spermatozoa with fragmented DNA can be detrimental.

The causes of DNA fragmentation have already been extensively reviewed (Lewis and Aitken, 2005; González-Marín et al., 2012; Wright et al., 2014) and a huge amount of studies have investigated the relationship between ART outcomes and high DNA damage in sperm. Thus, outstanding reviews have described in details these studies, drawing the conclusion that contradictory evidence is associated with the importance of male gamete DNA fragmentation in predicting fertilization, embryo development, implantation, birth defects in the offspring and early pregnancy loss (Gandini et al., 2004; Huang et al., 2005; Borini et al., 2006; Bungum et al., 2007; Simon et al., 2011; Sakkas, 2013; Palermo et al., 2014) and we do not further discuss this topic.

Notwithstanding this, the strategy to identify those spermatozoa that are abnormal has gained popularity. DNA damage assays are just starting to be evaluated in a critical manner and the in-depth discussion about their clinical relevance in predicting clinical outcomes can be found elsewhere (Harper et al., 2012; Palermo et al., 2014).

Interventions

To date, there is no reliable approach to completely filter out spermatozoa with DNA strand breaks from an ejaculate (Zini et al., 2000; Gandini et al., 2004; Stevanato et al., 2008; Ebner et al., 2011). In daily laboratory routine, two major categories of selection method are currently used: those aiming to enhance the number of spermatozoa with intact DNA in the sperm population used for ICSI and those aiming to isolate the single spermatozoon with the lowest chance of having fragmented DNA for the injection.

Improvement of the sperm sample before ICSI procedure: annexin V–magnetic activated cell sorting. As mentioned, the main pathway leading to sperm DNA breaks is a process of apoptosis (Gorczyca et al., 1993; Muratori et al., 2015). During the early events of apoptosis, the phospholipid phosphatidylserine (PS), which is usually located in the cytosolic leaf of the plasma membrane lipid bilayer, shows a redistribution from the inner to the outer leaf (Shen et al., 2002). In necrosis, PS becomes accessible due to the disruption of membrane integrity. Annexin V is a major cell membrane component of macrophages that binds selectively PS in a Ca^{2+}-dependent manner. The approach combining density gradient centrifugation with annexin V–magnetic activated cell sorting (MACS) seems to be able to remove spermatozoa with PS externalization and to reduce the proportion of sperms having apoptotic features, including DNA fragmentation (Said et al., 2005, 2006; Grunewald et al., 2006; Aziz et al., 2007; Lee et al., 2010; Rawe et al., 2010; Degheidy et al., 2014; Nadalini et al., 2014; Vendrell et al., 2014; Bucar et al., 2015). By this technique, the apoptotic cells are magnetically labeled with annexin V-conjugated microbeads and pass through a MACS column placed in the magnetic field of a MACS separator. The PS-exposing cells, conjugated with the magnetic microbeads, are retained on the column, while the unlabeled, non-apoptotic spermatozoa can pass through (Miltenyi et al., 1990; Paasch et al., 2003). Two prospective randomized studies have evaluated the effectiveness of annexin V–MACS sperm sorting associated with density gradient centrifugation compared with density gradient centrifugation alone in terms of ICSI outcomes, with controversial results (Table VII). Dirican et al. (2008b) reported increased cleavage and chemical pregnancy rates in the study group compared with controls while implantation rate was of borderline statistical significance. Others authors have demonstrated an improved fertilization rate and embryo quality but similar pregnancy and live birth rates. Fifteen healthy babies born were reported (Sheikhi et al., 2013).

Interestingly, Dirican et al. (2008b) performed a gradient centrifugation only after the MACS technique in order to avoid the removal of capacitated normal spermatozoa. Indeed, albumin can stimulate capacitation and some studies showed that PS externalization would be not only a sign of early apoptosis but also may occur following capacitation (Kotwicka et al., 2002; Grunewald et al., 2006; Salicioni et al., 2007). However, it has to be underlined that both studies did not investigate apoptosis in spermatozoa and no comparison was made before and after the magnetic cell separation. Three case reports have described pregnancy achievement and three healthy babies born after sperm sorting with annexin V–MACS (Polak de Fried and Denady, 2010; Rawe et al., 2010; Herrero et al., 2013).

Some concern about the safety of this technique was raised regarding the possibility for microbeads to remain on the surface of the non-apoptotic spermatozoa used for ICSI. Based on experiments with transmission electron microscopy, this event would be unlikely (Paasch et al., 2003), although prospective studies are needed to establish the safety of this strategy.

Improvement of the sperm sample before ICSI procedure: zeta potential selection. An alternative method to select sperm prior to ICSI is the zeta potential procedure (Table VII).

Since it has been demonstrated that a mature sperm possesses a static surface electrical charge of −16 to −20 mV (Focarelli et al., 2009), named zeta potential, it has been proposed to exploit this characteristic for sperm selection. This method is simple and inexpensive and it was published for the first time by Chan et al. (2006): the electrostatic surface to immobilize the charged sperm is generated by simply placing a glass centrifuge tube inside a latex glove and rotating the tube two or three times. An incubation of 1 min at room temperature allows the adherence of the charged sperm to the tube wall and a short centrifugation is used to wash away sperm with less charge. Adhering spermatozoa can be detached from the tube surface by pipetting a serum-supplemented medium in order to neutralize the tube charge (Chan et al., 2006). Sperm cells isolated with this procedure have high DNA integrity and low histone content (Chan et al., 2006), making the zeta potential method an effective procedure for sperm selection. Moreover, several researchers highlighted that the zeta method was able to select a higher percentage of spermatozoa with intact DNA, in comparison with the hyaluronic acid binding (Razavi et al., 2010). However, only one prospective randomized study analyzed ICSI outcomes when zeta sperm selection was applied (Kheirollahi-Kouhestani et al., 2009): in this study, 30 couples agreed to have half of their oocytes inseminated by sperms processed by the combined density gradient centrifugation/zeta procedure, and half with density gradient centrifugation only. Fertilization rate was improved following the combined density gradient centrifugation/zeta procedure and it was shown that the selected spermatozoa had less DNA fragmentation.

Improvement of the sperm sample before ICSI procedure: electrophoretic separation. Electrophoretic systems for the rapid isolation of sperm exhibiting high levels of DNA integrity have been recently described. This

Table VII Summary of 10 papers identified in a systematic review of the literature in order to investigate the effects of different selection methods to manage sperm DNA fragmentation before ICSI on embryo development and clinical outcomes.

			Sperm sample	Notes	FR	Good EQ rate	Cleavage rate	BL rate	CPR	IR	AbR	LBR	P/PR/R/CR/CS study
Before ICSI procedure	MACS	Dirican *et al.* (2008a, b)	Ejaculated	MACS versus DGC	Unchanged	Unchanged	Improved	—	Improved	Unchanged	—	—	PR
		Rawe *et al.* (2010)	Ejaculated	MACS selection	—	—	—	—	—	—		One live birth	CR
		Polak de Fried and Denaday (2010)	Ejaculated	MACS selection	—	—	—	—	Twin ongoing pregnancies	—	—		CS
		Sheikhi *et al.* (2013)	Ejaculated	MACS versus DGC	Improved	Improved	Unchanged	—	Unchanged	Unchanged	—	Unchanged	PR
		Herrero *et al.* (2013)	Ejaculated	MACS selection	—	—	—	—	—	—	—	Twin live births	CR
	Zeta potential method	Kheirollahi-Kouhestani *et al.* (2009)	Ejaculated	DGC versus DGC-Zeta	Improved	Unchanged	Unchanged		Unchanged	Unchanged	—		PR
	Electrophoretic system	Ainsworth *et al.* (2007)	Ejaculated		—	—	—	—	—	—	—	One live birth	CR
		Fleming *et al.* (2008)	Ejaculated	DGC versus CS-10	Unchanged	Unchanged	Unchanged	—	—	—	—		PR
During ICSI procedure	HAB	Nasr-Esfahani *et al.* (2008a, b)	Ejaculated	Routine sperm selection versus HAB selection	Improved	Unchanged	Unchanged	—	Unchanged	Unchanged	—		PR
		Parmegiani *et al.* (2010)	Ejaculated	HAB selection versus PVP selection	Unchanged	Improved	—	—	Unchanged	Unchanged	Unchanged	Unchanged	PR

Improved or decreased results are indicated only when statistically significant.
MACS, magnetic cell sorting; DGC, density gradient centrifugation; CS-10, Cell Sorter-10; HAB, hyaluronic acid binding assay; PVP, polyvinylpyrrolidone.

method is based on the principles that the highest quality spermatozoa within the ejaculate carry the greatest net negative charge (Giuliani *et al.*, 2004; Ainsworth *et al.*, 2005) and that they can be separated from other electronegative cells, such as leucocytes and immature germ cells, because of their smaller size. Hence, the separation is obtained by the application of an electric field that forces the spermatozoa to pass through a polycarbonated filter containing 5 μm pores (Ainsworth *et al.*, 2005). A case report has reported the first human pregnancy obtained by using this procedure in a couple suffering from long-term infertility associated with extensive sperm DNA damage (Ainsworth *et al.*, 2007). However, the only published prospective randomized controlled clinical trial designed to compare spermatozoa prepared using this technique and spermatozoa prepared by density gradient centrifugation has demonstrated comparable rates of fertilization, cleavage and high-quality embryos (Fleming *et al.*, 2008). Fleming *et al.* reported also three normal deliveries from embryos derived from electrophoretically selected spermatozoa. Of course, further proof of safety is necessary to validate this method. One of the exciting prospects of this method is its potential application for the isolation of spermatozoa exhibiting low levels of DNA damage from complex cellular mixtures such as testicular or epididymal biopsies.

Selection of sperm during ICSI procedure: hyaluronic acid binding. Hyaluronic acid is present in the cumulus oophorus surrounding ovulated eggs. Current models suggest that spermatozoa pave their way through the cumulus by binding hyaluronic acid to a specific protein located in the sperm head, PH20. On this basis, a hyaluronan-binding assay has been developed, representing a suitable and reproducible laboratory test for selecting good-quality spermatozoa able to bind hyaluronan through specific receptors (Cayli *et al.*, 2003; Huszar *et al.*, 2003, 2007; Parmegiani *et al.*, 2010). Moreover, selection of hyaluronan-bound spermatozoa has been reported to significantly decrease the percentage of sperm showing apoptotic marker proteins (Sakkas, 2013). An accurate analysis of all the contributions reporting on ART outcomes derived from sperm selected by hyaluronic acid binding can be found elsewhere (McDowell *et al.*, 2014). We have included herein only the studies that have addressed the efficacy of spermatozoa selection by hyaluronan-binding in reducing DNA fragmentation. Basically, two protocols are used for the sperm selection: in the first protocol, a drop of diluted hyaluronic acid is allowed to dry on the bottom of an ICSI dish. Sperm cells are selected according to their ability to bind the solid hyaluronic acid attached to the bottom of the dish (Nasr-Esfahani *et al.*, 2008b). Ready-to-use dishes are also available (Parmegiani *et al.*, 2012). In the second protocol, a ready-to-use viscous medium containing hyaluronic acid is used. A drop of sperm suspension is mixed with a drop of medium containing the hyaluronic acid on the base of the ICSI dish, spermatozoa bound to hyaluronic acid are slowed in the junction zone of the droplets and are selected for ICSI (Parmegiani *et al.*, 2012). In both protocols, spermatozoa have to be prepared by means of swim-up or density gradient before sperm selection by hyaluronic acid can be applied. The effectiveness of this procedure on ICSI outcomes has been investigated, according to our searching criteria, by two prospective studies (Table VII). Nasr-Esfahani *et al.* (2008b) observed a significant inverse correlation between percentage of hyaluronic acid binding and DNA fragmentation. Furthermore, when a hyaluronan-binding procedure was applied for sperm selection prior to ICSI, a significantly higher fertilization rate was observed, while pregnancy and implantation rates remain unchanged. In a prospective randomized study, Parmegiani *et al.* (2010) reported that hyaluronan-selected sperm cells show a lower DNA fragmentation level and less nuclear abnormality in comparison with PVP selection. Significantly, improved embryo quality and development after ICSI were also observed.

Overall, the evidence is insufficient to recommend one specific method of sperm selection in the case of absolute asthenozoospermia or high sperm DNA fragmentation.

Indeed, a considerable number of studies reported in the section 'ICSI and male gametes' were classified as case series or case reports. Few studies were prospective and randomized, and in most of them, the study group was not compared with standard ICSI as a control group but with a different advanced sperm selection method. Results are discordant and important clinical outcomes, such as miscarriage and fetal abnormalities, are lacking. No clear data on possible adverse effects of these techniques have been reported.

Discussion

After decades of practice using IVF, the current strategy is represented by the personalization of treatment. This strategy should lead to an improvement in patient compliance and better clinical practice. Individualization of treatment is not new to the field of medicine, although this concept is relatively fresh in reproductive medicine. In clinics, the availability of new markers of ovarian reserve, the improvement in methodology for their measurement and the huge amount of clinical data have supported the view that individualization in IVF is the way forward (La Marca and Sumkara, 2014). In laboratory practice, this view is only emerging, although, especially in centers performing many cycles, it is always difficult to abandon well-established protocols in order to solve specific problems. This review has been prepared mostly with the idea to support embryologists in trying to offer to the couples the best treatment tailored to their unique characteristics. We have detailed the current knowledge on procedures to improve the use of oocyte without the zona pellucida, to solve difficult issues related to fertilization failure or oocyte degeneration, to use round-headed spermatozoa and to improve selection of viable sperm in the case of absolute asthenozoospermia or high fragmentation rate (Fig. 5). In this context, this analysis might represent a platform to favor a rethinking of established procedure modalities in the IVF laboratory and to support the individualization of treatment.

On the other hand, two important issues may be deduced from this analysis. The first is represented by the safety concern. The list of techniques and procedures that have been brought into the IVF clinic over the last years is long and it continues to grow. The ideal paradigm for the introduction of new procedures or technologies implies initially their development in animals (Harper *et al.*, 2012). Whether animal studies are appropriate or not, research should then be performed on human gametes or embryos donated for research and with an appropriate risk assessment. Moreover, sufficiently powered RCTs need to show that there is a clinical benefit and that the technique is safe, with neonatal follow-ups if appropriate. For certain procedures, such as PGD, RCTs are not appropriate because it would be impossible to have a control group but for procedures that claim to increase delivery rates, RCTs are essential (Harper *et al.*, 2012). All deliveries need to be followed up to ensure that any risks are within an acceptable range. Considering the number of different procedures and treatments described in this review, the number of published RCTs is very few, thus supporting the idea that the necessary research for the introduction of a novel

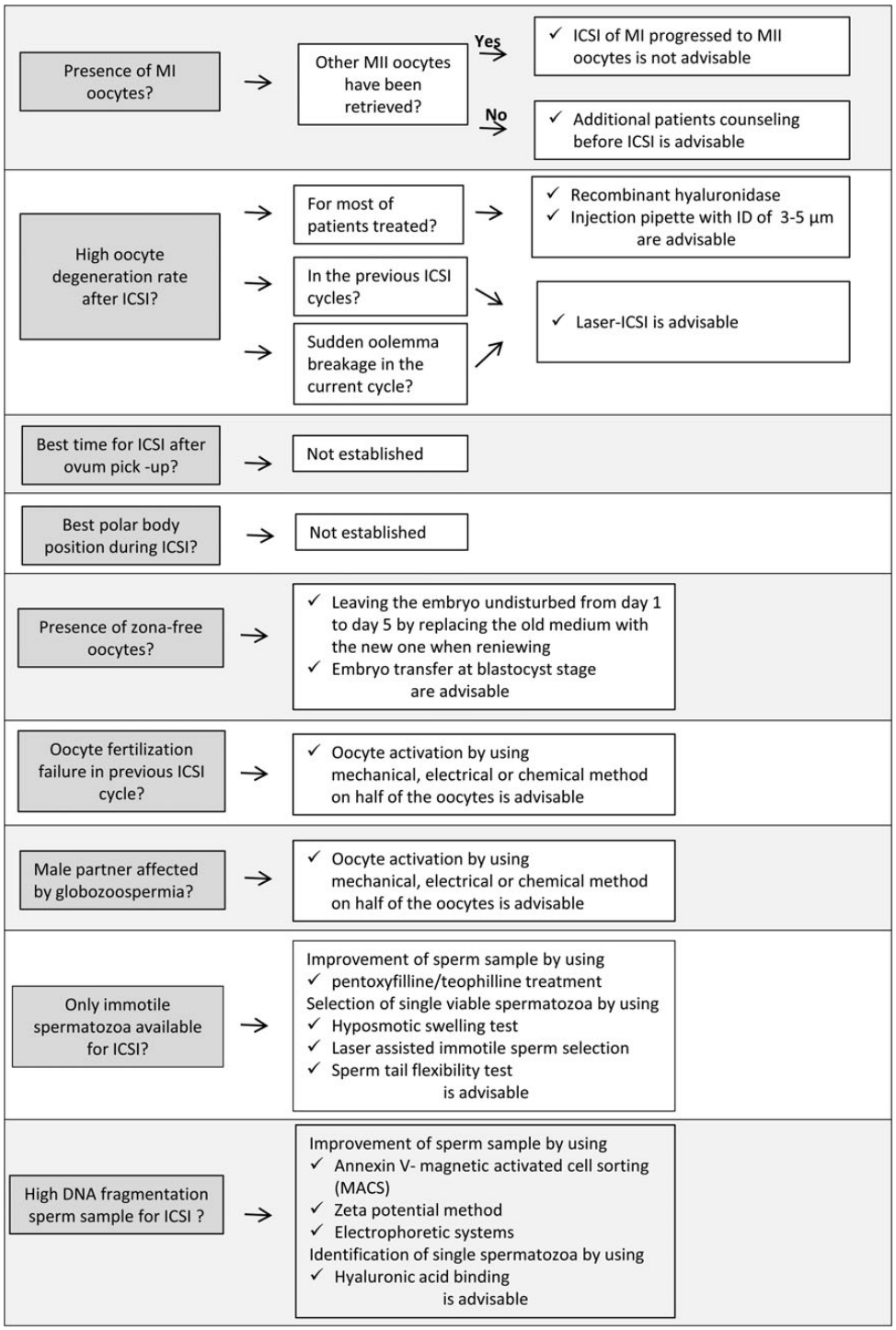

Figure 5 Summary of the practices advisable for specific situations during ICSI. Limitations of every single procedure are discussed in the text. MI/MII: metaphase I/II.

procedure is often not conducted properly. In this regard, some concerns have been raised recently for the introduction in the market of a commercially available medium containing Calmicyn to largely eliminate the possibility of fertilization failure (van Blerkom *et al.*, 2015). The risk here is that the strategy will be used not only for selected patients, without obtaining the necessary information to establish its safety. This deficiency emerges dramatically for several procedures evaluated in this analysis and not only for oocytes defects. Impressively, we were unable to find a single study evaluating short- and long-term follow-up of children born after using pentoxifylline-selected sperm for ICSI, notwithstanding that several IVF centers worldwide are known to have used or use the compound in daily practice.

This brings us to the second important point derived from this analysis. Embryologists should be encouraged to set up well-conducted studies, to validate the techniques used and to report their findings. One may argue that since it takes years to do the necessary research and development and obtain the results of RCTs, such an approach will dampen the development of the field but previous examples have shown that it is possible to introduce technology bringing no clinical benefit or even adverse effects (Gleicher *et al.*, 2014). On the other hand, huge amounts of potentially valuable data deriving from the performance of thousands of cycle all over the world using various laboratory approaches are probably never published. While ICSI introduction has probably not been achieved by following the classical steps of looking for new technologies to be applied to humans (Harper *et al.*, 2012), it implies so many critical phases potentially affecting epigenetic, genetic or chromosomal errors that follow-up studies in the human embryo and pediatric follow-ups are needed for each of them. Thus, embryologists should be also encouraged to share their follow-up data deriving from the various technical approaches used.

Finally, for a complete understanding of the potential consequences of the ICSI technique, the biological basis of each step of the ICSI procedure needs to be provided to embryologists together with their training on the mechanical aspects of the injection. This is particularly important considering that some steps of the procedure, such as timing of the injection after oocyte retrieval, have reached a high level of standardization without a strong scientific literature supporting it.

We are aware not to have included in this work important issues such as the sperm selection using ultra-high magnification (IMSI). However, IMSI has already been the focus of important analyses and discussions (Teixeira *et al.*, 2013; Setti *et al.*, 2014). Our aim was to focus on aspects critical to embryologists but for which review contributions were absent or limited.

Conclusions

This review provides the current knowledge on some controversial technical aspects of the ICSI procedure in order to improve its efficacy in specific contexts. The introduction of ICSI was a revolution in reproductive medicine, but the technique was introduced into clinical practice with minimal proof of safety. The refinements of the technique based on evidence-based medicine represent a priority for the future. Even many years after its introduction, the improvements and technical developments in ICSI still lack sufficient well-designed studies.

Authors' roles

P.R., A.L., P.V. and P.P. provided a substantial contribution to the review conception and drafted the article. All authors approved the final version of the article.

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Conflict of interest

None declared.

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