Effects of advanced selection methods on sperm quality and ART outcome: a systematic review

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BACKGROUND: Current routine semen preparation techniques do not inclusively target all intrinsic sperm characteristics that may impact the fertilization potential. In order to address these characteristics, several methods have been recently developed and applied to sperm selection. The objective of this study was to systematically review the literature describing these advanced sperm selection methods focusing on their anticipated benefits on sperm quality and assisted reproductive technique (ART) outcome.

METHODS: Systematic literature review was conducted by means of a Medline literature search. Sperm quality parameters assessed included: motility, morphology, viability, DNA integrity, apoptosis and maturity. ART outcomes assessed included: fertilization, embryo quality, pregnancy, abortion and live birth rates.

RESULTS: A total of 44 studies were identified describing four advanced sperm selection methods based on: (i) surface charge (electrophoresis and zeta potential), (ii) apoptosis (magnetic cell sorting and glass wool), (iii) membrane maturity (hyaluronic acid binding) and (iv) ultramorphology (high magnification). Selection of high-quality sperm including improvements in DNA integrity, resulted from the application of these methods. Fertilization and pregnancy rates showed improvement following some of the advanced sperm selection techniques.

CONCLUSIONS: While some of the advanced sperm selection methods are of value in specific clinical ART settings, others are in need of further evaluation. More clinical studies on safety and efficacy are needed before the implementation of advanced sperm selection methods could be universally recommended in ART.

Key words: assisted reproductive techniques / hyaluronic acid binding / IMSI / magnetic cell sorting / sperm selection

Introduction

Despite the widespread use of assisted reproductive techniques (ARTs) for many years in the treatment of infertility, live birth rates remain relatively low and could be improved (Wright et al., 2008). The possible contribution of sperm selection to this improvement has yet to be established. Previously, the role of spermatozoa in fertilization and embryo development was minimized to being a carrier that transports DNA to the oocyte. It is now proved that human spermatozoa play an extensive role that extends even beyond the early...
stages of fertilization to include abnormal embryogenesis leading to implantation failure (Barroso et al., 2009).

Routine sperm preparation techniques such as density gradient centrifugation (DGC) and swim-up are currently used as main components of ART procedures. They depend on sedimentation or migration approaches to separate spermatozoa (Akerlof et al., 1987). These routine techniques appear to be equally effective in selecting motile, morphologically normal sperm (Le Lannou and Blanchard, 1988). However, other sperm characteristics such as apoptosis and apoptosis-like manifestations, DNA integrity, membrane maturation and ultrastructure are not directly targeted by routine sperm preparation techniques. These characteristics could be influenced by sperm selection and concomitantly be important determinants of fertility. In support, testicular sperm retrieval was proved to result in the retrieval of sperm with significantly lower DNA fragmentation compared with ejaculated sperm in the same patient (Moskovtsev et al., 2010). The same concept is validated in a study documenting higher ongoing clinical pregnancy rates following ICSI with testicular sperm compared with ICSI using ejaculated sperm in patients with high DNA fragmentation (Greco et al., 2005). Therefore, the need for advanced sperm selection methods is clearly evident.

Several advanced sperm selection methods have been developed with the objective of improving sperm preparation protocols used during ART. These methods aim at isolating mature, structurally intact and non-apoptotic spermatozoa with high DNA integrity. Similar advanced sperm selection methods are highly needed due to the extensive use of ICSI in the management of infertile couples. While ICSI has revolutionized ART and offered an effective treatment option for severe male factor infertility, its application, if using spermatozoa with defective DNA, may result in serious consequences for the offspring (Ji et al., 1997; Aitken et al., 2003; Aitken and De Iuliis, 2007).

The objective of this review is to summarize the relevant literature on advanced sperm selection methods used in routine ART treatment and to make recommendations on their clinical application. We will describe their feasibility, safety and effects on sperm quality and ART outcomes. The limitations of studies describing the effects of using advanced sperm selection methods on ART outcomes, including small sample size will be highlighted. Controversies will be discussed and suggestions for further research provided.

Methods

A MEDLINE literature search was conducted using the key words: semen preparation or sperm preparation or sperm selection in combination with any of the following: ART outcome, ICSI, IUI, IVF, sperm parameter, sperm quality, apoptosis, DNA, motility, morphology and viability. The search was restricted to human studies published from January 1990 to October 2010. No language restrictions were applied. Only original articles that assessed the effects of advanced sperm selection methods on sperm characteristics and/or ART outcome were included in this systematic review. Cross-referencing was also conducted from the citations in the relevant articles obtained from the MEDLINE search. Meeting abstracts were not included in this study.

Results

Our initial literature search resulted in 943 articles. Articles not reporting on sperm quality or ART outcome and those describing routine sperm preparation methods such as density gradient and swim-up were excluded on the basis of title and abstract content (n = 912). Cross-referencing of relevant articles resulted in the inclusion of an additional 13 articles. Based on the 44 studies included, we identified four advanced sperm selection methods: (i) selection based on sperm surface charge (6 studies), (ii) non-apoptotic sperm selection (12 studies), (iii) selection based on sperm membrane maturity (12 studies) and (iv) selection based on sperm ultramorphology (14 studies). For each method, the principles, safety and feasibility will be described first, and this will be partly based on literature that was not included in the systematic search. Table I summarizes the studies identified in the systematic literature search, describing the effects of advanced sperm selection methods on sperm quality, while Table II shows a summary of studies describing effects of advanced sperm selection methods on ART outcomes, including fertilization rates and clinical pregnancy rates. No articles on the use of advanced sperm selection methods prior to IUI were identified.

Selection based on sperm surface charge

Principles, safety and feasibility

An electrophoresis-based technology (Microflow® CS-10, Nusep Ltd., Frenchs Forest, Australia) has been developed to separate spermatozoa based on size and electronegative charge. The sperm sample solution is loaded into the apparatus’ reservoirs and allowed to equilibrate with special buffer for only 5 min prior to application of an electric field in the form of constant applied current of 75 mA and a variable voltage of 18–21 V. Sorted sperm can then be retrieved from the collection chamber (Fig. 1) (Ainsworth et al., 2005). The size criterion ensures that only spermatozoa are included, while leukocytes and immature germ cells are excluded. An electronegative surface charge indicates that the sperm is normally differentiated and has CD52 on its surface (Schoeter et al., 1999). This could be the reason for the higher quality of spermatozoa selected by electrophoresis since CD52 expression was found to be correlated with normal sperm morphology and capacitation (Giuliani et al., 2004). The use of electrophoresis in sperm selection may prompt some safety concerns due to the reported negative effects on sperm motility (Engelmann et al., 1988; Ainsworth et al., 2005).

Electrophoretic sperm selection is relatively fast as it requires only 5 min of current application in addition to time for loading, removal and dilution of samples (Ainsworth et al., 2005). It does not entail any centrifugation steps; thus, it avoids the generation of reactive oxygen species (ROS) that usually occur with centrifugation (Aitken and Clarkson, 1988). Consequently, the technique may be of great value in minimizing oxidative stress to spermatozoa. Also, the technique has the ability to exclude major sources of ROS such as leukocytes and immature germ cells. The efficiency of this technique has been proved when applied to oligozoospermic samples, testicular sperm and frozen spermatozoa. Despite the undisputed benefits of this technique, the complexity of the separation apparatus used may be a limiting factor against its daily routine use in Andrology Laboratories, specifically those with limited resources.

Another method has been developed based on the sperm zeta potential (electrokinetic potential) (Chan et al., 2006), which is the electric potential between the sperm membrane and its surroundings measuring −16 to −20 mV in mature sperm (Ishijima et al., 1991).
Table I Summary of studies describing the effects of advanced sperm selection methods on sperm quality, comparing outcomes for selected and unselected sperm in the study populations.

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<tr>
<th>Sperm selection approach</th>
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<th>Sperm selection method</th>
<th>Study design</th>
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<td>Electrophoretic separation (Microflow®, CS-10)</td>
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<td>Healthy donors (n = 31)</td>
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<td>Chan et al. (2006)</td>
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<td>Kam et al. (2007)</td>
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<td>Fleming et al. (2008)</td>
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<td>Razavi et al. (2010)</td>
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<td>Healthy donors (n = 15)</td>
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<td>Said et al. (2006a, b)</td>
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<td>Grunewald et al. (2006)</td>
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<td>Apoptosis marker (MMP) following cryopreservation (S)</td>
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<td>(i) Sperm selection</td>
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<td>Grunewald et al. (2007)</td>
<td>Molecular glass wool MACS</td>
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<td>Grunewald et al. (2008)</td>
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<td>de Vantery Arrighi et al. (2009)</td>
<td>DGC + MACS</td>
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<td>Apoptosis markers (EPS, MMP) (S)</td>
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<td>Lee et al. (2010)</td>
<td>DGC + MACS</td>
<td>Prospective, controlled</td>
<td>Men from couple with unexplained infertility and two failed IUI (n = 60)</td>
<td>Apoptosis markers (EPS, MMP) (S)</td>
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<td>Huszar et al. (2003)</td>
<td>HA binding</td>
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<td>Men undergoing fertility evaluation: Maturity markers (n = 30), acrosomal integrity (n = 5)</td>
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<td>Cayli et al. (2004)</td>
<td>HA binding</td>
<td>Prospective, controlled</td>
<td>Men undergoing fertility evaluation (n = 10)</td>
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<td>Jakab et al. (2005)</td>
<td>HA binding</td>
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<td>Ye et al. (2006)</td>
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<td>Prinosilova et al. (2009)</td>
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<td>Tarozzi et al. (2009)</td>
<td>HA binding</td>
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<td>Parmegiani et al. (2010a, b)</td>
<td>HA binding</td>
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<td>Males in ICSI program: DNA fragmentation (n = 20), MSOME (n = 15)</td>
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<td>(iv) Selection based on ultramorphology</td>
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<td>Garolla et al. (2008)</td>
<td>High magnification (× 13 000)</td>
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<td>Franco et al. (2008)</td>
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<td>Men undergoing fertility evaluation (n = 30)</td>
<td>DNA fragmentation (S)</td>
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</tbody>
</table>

ART, assisted reproductive techniques; Bcl, B cell lymphoma protein; CK, creatine kinase; CP-3, caspase-3; DGC, density gradient centrifugation; EPS, externalized phosphatidylserine; HA, hyaluronic acid; HICSI, hamster oocyte ICSI; HspA2, heat shock protein; IAR, induced acrosome reaction; ICSI, intracytoplasmic sperm injection; IUI, intrauterine insemination; IVF, in vitro fertilization; MACS, magnetic-activated cell sorting; MSOME, motile sperm organelle morphology examination; MMP, mitochondrial membrane potential; NS, no statistically significant difference; S, statistically significant difference; SDI, sperm deformity index.
The zeta potential further decreases with capacitation (Della Giovampaola et al., 2001). The method entails pipetting washed sperm into a positively charged centrifuge tubes, which can be achieved by simply rotating a tube two or three times in a latex glove. After 1 min, the tube is centrifuged and inverted to remove all the non-adhering sperm and other contaminants. Thereafter, adhering (negatively charged, mature) sperm can be retrieved by rinsing the tube with serum-supplemented media (Fig. 2).

The zeta method appears to be easy to perform and is inexpensive since no electrophoresis equipment is required. Zeta processing was successfully applied on cryopreserved-thawed sperm (Kam et al., 2007). In terms of safety, the method does not include the use of high-voltage electricity; however, there are certain limitations that should be noted. Low sperm recovery was noted following the application of zeta method, which limits its use for oligozoospermic samples. In addition, the zeta method was not tested on testicular/epididymal sperm or in a humid environment that is known to neutralize electrical surface charges (Chan et al., 2006).

Effects on sperm quality (Table I)
Data evaluating electrophoretic sperm selection using Microflow® showed that the apparatus yields adequate numbers of spermatozoa compared with DGC and results in the isolation of a sperm population with significantly improved morphology and lower leukocyte contamination compared with DGC and ×3 centrifugation methods (Ainsworth et al., 2005). Sperm DNA analysis revealed that only samples processed by Microflow® had significantly lower damage than that in the raw ejaculate in contrast to samples processed by DGC. There was a decrease in sperm motility consistent with other reports about the negative effects of electrophoresis on sperm motility (Engelmann et al., 1988); nevertheless, sperm motion kinetics were comparable with samples processed by DGC and ×3 centrifugation methods (Ainsworth et al., 2005). Similarly, the zeta potential selection method showed an ability to yield spermatozoa with significantly higher morphology, hyperactivation, DNA integrity and maturity, but not motility, compared with control samples processed by DGC (Chan et al., 2006; Kam et al., 2007; Kheirollahi-Kouhestani et al., 2009; Razavi et al., 2010). The lack of improvement in motility does not indicate a negative effect, unlike the Microflow® electrophoretic separation, which showed a decrease in sperm motility following its application (Ainsworth et al., 2005).

Effects on ART outcome (Table II)
The first human live birth following electrophoretic sperm selection (Microflow®, CS-10) and ICSI was reported in a couple that had seven IVF/ICSI cycles characterized by good fertilization but poor embryo cleavage rates, associated with extensive sperm DNA damage (Ainsworth et al., 2007). Sperm selection by surface charge resulted in a decrease in DNA fragmentation and a pregnancy. Although this report described the benefit of using Microflow® in a single case characterized by high sperm DNA fragmentation, a prospective study revealed contradicting results. In this case-series, each semen sample was split between preparation with the CS-10 and preparation by standard DGC, and each cohort of oocytes was split for insemination (IVF) or injection (ICSI) using either CS-10 or DGC-prepared spermatozoa. Results showed no significant difference between the ability of CS-10 and DGC-prepared spermatozoa to produce fertilization, embryo cleavage and high-quality embryos. The only advantage noted by the authors was that CS-10 was less time-consuming than DGC (Fleming et al., 2008).

In order to evaluate a combined DGC/zeta potential sperm selection method, a trial was conducted in couples undergoing ICSI due to male factor infertility. Half of oocytes retrieved were inseminated with sperm prepared using the combined protocol, while the other half was inseminated with sperm prepared using DGC only to serve as controls. The fertilization rates were significantly higher in couples receiving at least one embryo from the zeta group. No differences were seen in embryo cleavage or quality (Kheirollahi-Kouhestani et al., 2009). However, actual pregnancy rates could not be evaluated since embryo transfers were done with embryos from both treatment and control group in the same patient.

Non-apoptotic sperm selection
Principles, safety and feasibility
The externalization of phosphatidylserine (PS) to the outer surface of the sperm membrane, a feature of early apoptosis, has been used as a basis for selection of non-apoptotic spermatozoa. The externalization of PS allows for its binding with Annexin-V-conjugated paramagnetic microbeads, which could be used to label and separate apoptotic spermatozoa using a magnetic-activated cell sorting system (MACS, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) (Grunewald et al., 2001). Initially, a heterogeneous sperm cell suspension is incubated with Annexin-V-conjugated microbeads, which bind to only apoptotic sperm with externalized PS. Thereafter, the bead/sperm mixture is allowed to run through the MACS column, which is placed inside a magnet. The magnetic force will cause the retention of the cells labeled with microbeads inside the column, while the non-labeled cells will freely flow (Fig. 3) (Manz et al., 1995). Since MACS does not have the ability to remove leukocytes or immature germ cells, the technique was used in conjunction with DGC to exclude the seminal plasma and other contaminants (Said et al., 2005a, b).

Annexin V magnetic cell separation of non-apoptotic spermatozoa is simple, fast, inexpensive and highly specific (Said et al., 2008). However, the technique still requires special laboratory equipment, which may not be feasible or available in all settings. In addition, the combination of DGC and MACS will involve repeated steps of centrifugation and re-suspension, which might be detrimental when applied to semen samples characterized by limited sperm counts, as low sperm recovery may be expected (Said et al., 2008).

Some safety aspects should be considered before using MACS for sperm selection. MACS microbeads are biodegradable and do not affect cell viability (Miltenyi et al., 1990); however, research is still needed to ensure the complete absence of freely floating microbeads in the non-apoptotic sperm fraction.

In order to avoid the problems associated with freely floating microbeads, another system for selection of non-apoptotic spermatozoa has been recently described. The system is based on modifying commercially available glass wool separation columns (SpermFertil, TranMIT, GmbH, Giessen, Germany) to add the ability of retaining apoptotic sperm. This was achieved by coating the glass wool with Annexin V that binds to apoptotic sperm. The technique referred to as Annexin V glass wool (annexin V-GW) or molecular glass wool is easy to use on both fresh and cryopreserved semen samples;
Table II  Summary of studies describing the effects of advanced sperm selection methods on ART outcomes including fertilization rates and clinical pregnancy rates.

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<tr>
<th>Sperm selection approach</th>
<th>Author (year)</th>
<th>Sperm selection method</th>
<th>Study design</th>
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<td>One infertile couple</td>
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<td>Fleming et al. (2008)</td>
<td>Electrophoretic separation (Microflow®, CS-10)</td>
<td>Prospective, controlled</td>
<td>Couples undergoing IVF (n = 17) or ICSI (n = 11), split oocytes inseminated with sperm prepared by CS-10 (n = 197) versus DGC (n = 195)</td>
<td>FR (IVF) 62 versus 69% (NS) FR (ICSI) 64 versus 52% (NS) PR NA</td>
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<td>Kheirollahi-Kouhestani et al. (2009)</td>
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<td>(iii) Mature sperm selection</td>
<td>Ye et al. (2006)</td>
<td>HA binding</td>
<td>Prospective</td>
<td>Couples undergoing IVF (n = 175)</td>
<td>PR 48% versus 37% (S) HBA score when FR &gt;50 versus HBA score when FR ≤50: 75% versus 69% (marginal S)</td>
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<td>Nasr-Esfahani et al. (2008)</td>
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<td>Tarozzi et al. (2009)</td>
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<td>PR 46% versus 40% (NS) FR when HBA score ≥80% versus FR when HBA score &lt;80%: 86% versus 87% (NS) PR when HBA score ≥80% versus PR when HBA score &lt;80%: 36% versus 32% (NS)</td>
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<td></td>
<td>Van Den Bergh et al. (2009)</td>
<td>HA binding</td>
<td>Prospective, randomized</td>
<td>Couples undergoing ICSI (n = 44), oocytes injected with HA bound (n = 204) versus non-bound sperm (n = 203)</td>
<td>FR 76% versus 70% (NS)</td>
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<td>Parmegiani et al. (2010a, b)</td>
<td>HA binding</td>
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Results are presented as comparison between infertile couples in whom advanced sperm selection methods were performed (study group) versus infertile couples in whom no specific sperm selection was done (controls), unless otherwise noted.

ART, assisted reproductive techniques; DGC, density gradient centrifugation; FR, fertilization rate; HA, hyaluronic acid; HBA, hyaluronic acid binding assay; ICSI, intracytoplasmic sperm injection; IMSI, intracytoplasmic morphologically selected sperm injection; IVF, in vitro fertilization; MACS, magnetic-activated cell sorting; MSOME, motile sperm organelle morphology examination; NA, not available; NS, non-significant; OAT, oligoasthenoteratozoospermia; S, statistically significant.
nevertheless, more studies are still needed to validate its efficiency and standardization (Grunewald et al., 2007).

**Effects on sperm quality (Table I)**

In fresh and cryopreserved-thawed samples of healthy donors, MACS used in combination with DGC selected spermatozoa with higher motility and mitochondrial membrane potential but lower active caspase-3 and PS externalization compared with sperm selected by DGC only (Said et al., 2005a, b; Grunewald et al., 2006, 2008). Results from a study on samples from healthy donors showed that the average number of spermatozoa lost during the combined approach was an additional 15% compared with samples prepared by DGC only (Said et al., 2006a, b). Similar to MACS, annexin V-GW showed an ability to select spermatozoa with lower caspase activation and higher mitochondrial membrane potential (Grunewald et al., 2007). Significantly higher motility, mitochondrial membrane potential, survival following 24 h of incubation and normal sperm morphology as assessed by the strict criteria and the sperm deformity index were seen in fractions prepared by DGC + MACS compared with fractions prepared by DGC only (Aziz et al., 2007; de Vantery Arrighi et al., 2009).

In healthy donors and men with unexplained infertility, spermatozoa prepared by MACS after DGC had 30% lower rate of DNA damage compared with those prepared by only DGC (Said et al., 2006a, b; Lee et al., 2010). Since DGC by itself was reported to select spermatozoa with 50% lower DNA damage (Donnelly et al., 2000), the results evaluating MACS indicate that a further decrease in percentage of DNA-damaged sperm is possible, which can be of benefit in some patients that present with a high percentage of DNA-fragmented sperm. The combination of DGC and MACS also appears to be of benefit in enhancing sperm cryosurvival rates. Non-apoptotic spermatozoa selected by DGC and MACS displayed significantly higher cryosurvival rates following cryopreservation-thawing compared with samples prepared by DGC only (Said et al., 2005a, b).

Hamster oocytes were used to evaluate oocyte penetration potential and chromatin decondensation of non-apoptotic sperm separated by DGC and MACS. When selected from healthy donors, non-apoptotic sperm exhibited significantly higher oocyte penetration potential than controls prepared by DGC (Said et al., 2006a, b). Sperm chromatin decondensation up to 18 h following hamster oocyte ICSI in fractions selected by DGC and MACS from samples of healthy donors was comparable with controls prepared by DGC only (Said et al., 2006a, b). However, when using samples from infertile men with abnormal sperm parameters, sperm chromatin...
decondensation up to 18 h following hamster oocyte ICSI was significantly higher than that of controls prepared by DGC only (Grunewald et al., 2009). Samples from infertile patients with abnormal sperm parameters show higher levels of apoptosis markers. This may be the reason why the beneficial effects of MACS on sperm chromatin decondensation were noted only in samples from infertile patients and not in samples from healthy donors.

Effects on ART outcome (Table II)
In order to assess the value of using a combination of DGC and MACS in a clinical ART setting, a study was conducted by subjecting semen samples from men with abnormal sperm parameters to preparation by DGC and MACS before undergoing ICSI (Dirican et al., 2008). Outcomes were compared with controls whose semen samples were prepared by DGC only. There were no differences in fertilization rates between the study group and the control group. On the other hand, higher embryo cleavage rates and clinical pregnancy rates were seen in the study groups compared with controls (Dirican et al., 2008). Recently, case reports described the use of MACS prior to an ICSI cycle that resulted in the birth of healthy children (Polak de Fried and Denaday, 2010; Rawe et al., 2010). The cases reported had a history of failure of fertilization and poor embryo development following routine ICSI, and the male partners had a significant level of sperm DNA fragmentation and activated caspase-3. The findings of these cases support the benefit of integrating MACS into the ART protocol in cases with a high incidence of apoptotic, DNA-fragmented sperm.

Selection based on sperm membrane maturity
Principles, safety and feasibility
The formation of hyaluronic-acid (HA)-binding sites on the sperm plasma membrane is one of the signs of sperm maturity that has been used as a basis for sperm selection (Huszar et al., 1997). A device called a PICSI dish (MidAtlantic Diagnostics Inc., Mt Laurel, NJ, USA) has been developed by adding four marked spots of

**Figure 3** Schematic diagram of MiniMACSTM magnetic cell separation column. (A) The column that contains steel spheres is placed inside an external magnet. (B) The non-labeled cells flow through the column to be collected. (C) The immuno-magnetically labeled cells remain attached to the magnetized spheres and are retained inside the column.

**Figure 4** Sperm selection using PICSI dishes. (A) A sperm drop is placed at the periphery of a HA drop, mature sperm binds to the HA-spot, while immature sperm moves freely. (B) Bound sperm could be picked up with the ICSI pipette. (Jakab et al., 2005, with permission from Elsevier.)
immobilized HA in a Falcon Petri dish. One drop of washed spermatozoa is placed at the edge of the HA spot and the HA-bound spermatozoa are collected after 15 min in an ICSI pipette and used for injection (Fig. 4) (Jakab et al., 2005). This method of sperm selection is highly specific and has minimal safety concerns (Huszar et al., 2003). While it is possible that HA molecules will be carried with the selected sperm within the ICSI pipette, HA is a normal component in cervical mucus, cumulus cells and follicular fluid (Cayli et al., 2003). To date, no adverse reactions on fertilization or embryo development have been reported following the use of HA-selected spermatozoa in clinical IVF settings (Huszar et al., 2007). However, it is important to note that this added step will eventually require embryologists to invest more time into preparatory steps since sperm binding by itself may take up to 30 min, which could be a challenge when injecting a large number of oocytes.

Effects on sperm quality (Table I)
Spermatozoa selected via HA binding display manifestations of maturity as defined by creatine kinase, heat shock-related protein 2 (HspA2) and aniline blue staining. HA-selected spermatozoa were also identified as viable, with non-reacted acrosomes, lower caspase-3 activation and positive correlation with motility (Huszar et al., 2003; Cayli et al., 2004; Ye et al., 2006). HA-bound spermatozoa have also displayed significantly less DNA fragmentation compared with spermatozoa prepared by DGC and spermatozoa in unprocessed semen (Tarozzi et al., 2009). The likelihood of improving a sperm population to the level where it includes >14% morphologically normal sperm was estimated to increase 3-fold following selection by HA binding (Prinosilova et al., 2009).

A correlation exists between sperm maturity and the frequency of chromosomal aneuploidies as shown by the decreased expression of HspA2 as a common factor underlying both sperm immaturity and aneuploidy (Kovanci et al., 2001). Thus, it has been suggested that HA-binding could be used to select mature sperm with low frequency of chromosomal abnormalities to reduce the risks for genetic complications following ICSI associated with the use of abnormal spermatozoa. In semen samples from men undergoing fertility assessment, HA-bound spermatozoa had a significantly lower frequency of autosomal disomy, diploidy and sex chromosome disomy compared with spermatozoa not selected by HA binding (Jakab et al., 2005).

Effects on ART outcome (Table II)
The proportion of sperm capable of binding to HA had no correlation with fertilization, cleavage, good-quality embryos, miscarriage and pregnancy rates in couples undergoing IVF (Tarozzi et al., 2009). Consistently, patients with clinical pregnancies had a percentage of HA-bound sperm that was comparable to those without pregnancy (Tarozzi et al., 2009). Only a weak correlation between the proportion of HA-bound sperm and fertilization rates following IVF was once demonstrated (Ye et al., 2006). Currently there are no threshold values for sperm HA binding that could predict IVF outcome, which limits the value of estimating the proportion of HA-bound sperm in predicting IVF outcome (Nijs et al., 2010).

Few studies have documented the use of HA-selected sperm in clinical ART settings. In comparison with routine sperm preparation techniques, spermatozoa selected by HA binding resulted in significantly higher fertilization rates following ICSI, while pregnancy rates were only slightly increased (Nasr-Esfahani et al., 2008). The positive effects of HA-bound sperm were associated with lower DNA fragmentation, which suggests that these effects may be attributed at least in part to selecting spermatozoa with higher DNA integrity (Nasr-Esfahani et al., 2008). In different studies, HA-bound spermatozoa used for ICSI resulted in significantly higher embryo quality and cleavage rates but not fertilization or pregnancy rates compared with spermatozoa conventionally selected (Parmegiani et al., 2010a, b).

In a randomized study, the effects of HA-selected sperm on embryo development as measured by the zygote score (Z-score) were assessed. Sibling mature oocytes were injected in a randomized manner, with either HA-bound or HA-non-bound spermatozoa; no differences were found in fertilization rates or Z-scores between both groups (Van Den Bergh et al., 2009). However, it has been argued that the use of non-HA-bound sperm in ICSI may result in risks for the conceptus since such sperm is documented to have a higher incidence of aneuploidies and DNA fragmentation (Parmegiani et al., 2009).

Selection based on sperm ultramorphology
Principles, safety and feasibility
Sperm morphology has been described as one of the major determinants of male in vivo and in vitro fertility (Kruger and Coetzee, 1999; Van Waar et al., 2001; van der Merwe et al., 2005). However, it has been debated that morphology evaluated on random stained cells from the ejaculate at ×1000 magnification is of limited value during ICSI where sperm is selected unstained at ×400 magnification (Bartoov et al., 2002). Alternatively, a new sperm selection method has been developed based on the inclusion of only normal sperm assessed using real-time motile sperm organelle morphology examination (MSOME) at a magnification of ×6300 (Bartoov et al., 2002). During MSOME, a micro-droplet of motile sperm suspension prepared by a routine sperm preparation technique is examined under oil immersion, with an inverted light microscope fitted with high-power Nomarski optics with digital enhancement.

MSOME assesses five sperm organelles (acrosome, postacrosomal lamina, neck, tail and mitochondria) that can be classified as either normal or abnormal. The sixth organelle (the nucleus) is evaluated for both shape and chromat in content (vacuolar area) (Fig. 5). Among the six organelles, the sperm nucleus appears to be the most important in influencing ART outcome (Bartoov et al., 2002). Subsequently, a modification of ICSI termed intracytoplasmic morphologically selected sperm injection (IMSI) has been developed (Bartoov et al., 2003). This approach is of particular benefit when used in situations where identification of specific sperm organelles is required, such as the acrosomal components in cases of globozoospermia (Check et al., 2007).

MSOME followed by IMSI is an elaborate procedure that involves prolonged sperm manipulation, adding significantly to the routine ICSI processing times. It was reported that it could take up to 5 h to perform (Berkovitz et al., 2005). It also requires special instrumentation with considerable expense. The subjectivity of the sperm ultramorphology assessment may be another limiting factor that prevents its widespread use. Classification of normal sperm ultramorphology will depend on the technician’s training and experience. While intra-observer variability was limited during MSOME (Bartoov et al.,
2002), the technique will always require a high level of technical expertise and inter-observer reproducibility.

A different optical system has been developed to identify the sperm birefringence, which occurs in mature forms due to the presence of subacrosomal protein filaments that are longitudinally oriented (Baccetti, 2004). Sperm birefringence can be evaluated using an inverted microscope equipped with polarizing and analyzing lenses, which allows the selection of birefringent, acrosome-reacted spermatozoa during ICSI without negatively impacting sperm motility or viability (Gianaroli et al., 2008). Birefringent spermatozoa can be selected for micromanipulation and these are thought to present with higher quality as the proportion of birefringent sperm has a significant positive correlation with other sperm parameters such as concentration, motility and viability (Gianaroli et al., 2008). As for MSOME and IMSI, the selection of spermatozoa using polarizing microscopy will require additional instrumentation, time and technical expertise.

Confocal light absorption and scattering spectroscopic (CLASS) microscopy is an optical system capable of evaluating single subcellular organelles without resulting in cell destruction (Itzkan et al., 2007). When applied to human sperm, CLASS can be used to visualize sperm organelles including the chromatin. Subsequently, this approach may be used in the future to select spermatozoa with intact chromatin prior to ICSI. The extremely limited time of sperm exposure to light, estimated as 1 second, may favor the occurrence of fewer side effects (Sakkas and Alvarez, 2010). No published reports regarding the impact of using CLASS on sperm quality and ART outcome are currently available.

**Effects on sperm quality (Table I)**

Immotile spermatozoa identified as normal with no nuclear vacuoles using high magnification microscopy ($\times13000$) have significantly higher mitochondrial membrane potential and lower DNA fragmentation and aneuploidy compared with unselected sperm (Garolla et al., 2008). Consistently, the presence of large nuclear vacuoles ($\geq50\%$ of sperm nuclear area) is associated with significantly higher DNA fragmentation and denaturation compared with spermatozoa with a normal nucleus (Franco et al., 2008).

**Effects on ART outcome (Table II)**

In a feasibility study, assessment by MSOME of spermatozoa remaining from a pool used in routine ICSI revealed that the incidence of morphologically normal sperm has a significant correlation with fertilization rates (Bartoov et al., 2002). Additionally, a threshold of 2.5% normal morphology as assessed by MSOME was highly predictive of fertilization rates following ICSI (sensitivity 81%, specificity 78%) (Bartoov et al., 2002). Clinical outcomes such as pregnancy and live birth rates were significantly higher following IMSI than routine ICSI, but no differences were noted as regards to fertilization and cleavage rates and embryo morphology (Hazout et al., 2006; Antinori et al., 2008; Mauri et al., 2010). This may suggest that IMSI is not of benefit in improving the paternal component in early steps of fertilization (Mauri et al., 2010). However, later effects on implantation should not be dismissed since higher pregnancy rates and diminished abortion rates were reported in couples that underwent IMSI when compared with couples that underwent routine ICSI (Bartoov et al., 2003; Berkovitz et al., 2006a, b). In support, a recent meta-analysis was conducted by comparing results from 357 IMSI cycles versus 349 routine ICSI cycles. Results showed significant improvement with IMSI in pregnancy and abortion rates but not in fertilization rates (Souza Setti et al., 2010).

Embryos resulting from the microinjection of birefringent spermatozoa only of men with severe male factor infertility had significantly higher pregnancy rates compared with rates observed after routine ICSI (Gianaroli et al., 2008), and lower abortion rates. On the other hand, fertilization and cleavage rates were comparable with rates resulting from routine ICSI (Gianaroli et al., 2008). The use of polarizing microscopy allows the selection of acrosome-reacted sperm to be used in routine ICSI, which resulted in significantly higher pregnancy rates compared with spermatozoa with non-reacted acrosomes (Gianaroli et al., 2010).

**Conclusions**

The evidence supporting the need for specific advanced sperm selection methods to be implemented in ART is considerable. Data indicate that even if the best quality spermatozoa are used in ICSI, no
more than 55% of the selected sperm have normal DNA (Ramos et al., 2004). This further supports the hypothesis that sperm selection methods currently used prior to ART are inadequate and that other methods need to be considered to ensure that only spermatozoa with optimum quality are included. Several advanced sperm selection methods have been described based on different approaches for targeting functionally competent and intact spermatozoa. This review focused on advanced sperm selection methods that could play a role routinely in the clinical ART practice. Other methods that could be used in unique situations to identify live sperm in cases of specific defects such as total lack of motility (El-Nour et al., 2001; Gerber et al., 2008) were beyond the scope of this article.

Selection of spermatozoa based on their electronegative surface charge, apoptosis markers, membrane maturity (HA binding) and ultramorphology has been used to develop technical protocols for sperm isolation. These different approaches aim at including only mature, non-apoptotic, DNA intact, morphologically normal spermatozoa during IVF or ICSI. Published data document the ability of these advanced sperm selection methods to isolate spermatozoa with higher quality in terms of motility, morphology, viability, DNA integrity, apoptosis and maturity markers.

There are contradicting reports regarding whether ART outcomes may be improved by using advanced sperm selection methods. Electrophoretic preparation using MicroFlow® may be considered for samples with high DNA damage since it is capable of selecting sperm with intact DNA (Ainsworth et al., 2007). However, the technique did not lead to any improvement in fertilization rates or embryo quality following ICSI (Fleming et al., 2008). Conversely, the zeta potential method was reported in only one study to increase fertilization, implantation and pregnancy rates (Kheirollahi-Kouhestani et al., 2009). MACS for the selection of non-apoptotic sperm was proved to result in higher embryo cleavage and pregnancy rates but not in fertilization or implantation rates (Dirican et al., 2008). The technique appears to be of most benefit in samples having significant levels of DNA damage and apoptosis (Polak de Fried and Denaday, 2010; Rawe et al., 2010).

As regards HA-binding for sperm selection, some reports show increased fertilization rates with no effects on pregnancy rates, while others report positive effects on embryo development and pregnancy rates and none on fertilization rates (Ye et al., 2006; Nasr-Esfahani et al., 2008; Tarozzi et al., 2009; Parmegiani et al., 2010a, b). These discrepancies may be due to subtle differences in study designs and data analysis. A relatively recent randomized, double-blinded, multisite study reported significantly higher clinical pregnancy rates following PICSI compared with ICSI. It has also been reported that patients with lower HBA scores stand to benefit the most from the application of PICSI (Worrolow et al., 2009). As regards IMSI, ample evidence documents the benefits of the technique on late ART outcomes such as higher pregnancy and live birth rates, and lower abortion rates but not on earlier outcomes such as fertilization, embryo cleavage rates and embryo morphology (Bartoov et al., 2003; Berkovitz et al., 2006a, b; Hazout et al., 2006; Antinori et al., 2008; Mauri et al., 2010). Finally, it is important to note that healthy live births were reported following the use of spermatozoa selected using electrophoretic separation, MACS, HA binding and IMSI. Nevertheless, it is not possible, based on the current published data, to identify which sperm selection method will result in the highest birth rate of healthy offspring.

Most of the evidence provided regarding the advantages of using advanced sperm selection techniques remains to date preliminary in nature. Recent presented studies on MACS showed an improvement in pregnancy rates following IUI with Annexin-V-sorted sperm although the recovery of motile sperm was considerably diminished (Romany et al., 2010). Also, MACS application did not result in any significant benefit in cases with high DNA fragmentation or apoptosis (Alvarez Sodo et al., 2010). Similarly, doubling of ongoing pregnancy rate and halving of abortion rate was achieved following IMSI compared with ICSI but yet the numbers reported did not reach statistical significance (Oliveira et al., 2010).

The suggested new methodologies vary in terms of instrumentation and time required. Selection using zeta potential and MACS are fairly quick and inexpensive. Electrophoretic separation, HA binding and IMSI do require relatively expensive instrumentation and/or considerable time to perform. All advanced sperm selection methods involve sperm manipulations that are more elaborate and time-consuming than routine sperm preparation techniques currently used. Furthermore, some proposed sperm selection protocols use a combination of both advanced methods and the routinely used ones. The added technical steps and processing times warrant careful assessment of some safety aspects since prolonged sperm exposure to non-physiologic conditions may induce iatrogenic damage (Agarwal et al., 1994). This may result in sperm DNA damage (Twigg et al., 1998) leading to aberrant embryo development and most importantly abnormalities in the offspring presenting as birth defects or genetic disorders (Marchetti and Wyrobek, 2005; Verhofstad et al., 2008). Most recently, higher frequency of congenital abnormalities and lower birthweight was reported following IMSI compared with ICSI (Junca et al., 2010), although the differences were not statistically significant. Further assessment of embryo development and long-term follow up for anomalies in the offspring, which are currently lacking, should be conducted to provide assurance regarding the use of advanced sperm selection methods in ART. Additionally, other unwanted effects such as influencing the sex ratio of the offspring should also be evaluated.

In conclusion, the first results following IVF and mostly ICSI after the use of electrophoretic separation, MACS, HA binding and IMSI are encouraging concerning fertilization and pregnancy rates. Despite these encouraging results, it should be noted that the numbers of patients assessed are limited, and most studies are underpowered to conclude on differences in pregnancy rates and live births. More research is needed to identify which infertility cases, if not all, will benefit from the application of these selection methods. Care should be taken to investigate safety and efficacy aspects of advanced sperm selection methods before their widespread implementation in ART. Animal models could be used initially and when proved safe, there will still be a need for adequately powered randomized trials in the human setting on efficacy with long-term follow-up of children.

Authors’ roles

T.M.S. took part in acquisition of data, data analysis and interpretation, drafting of article and final approval of the version to be published. J.L.
played a role in drafting of article, critical revision of article, and final approval of the version to be published.

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**References**


