Birefringence characteristics in sperm heads allow for the selection of reacted spermatozoa for intracytoplasmic sperm injection

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Objective: To verify clinical outcome after injection of spermatozoa that have undergone the acrosome reaction (reacted spermatozoa) vs. those still having an intact acrosome (nonreacted spermatozoa).
Design: Prospective, randomized study.
Setting: Reproductive Medicine Unit, Italian Society for the Study of Reproductive Medicine, Bologna, Italy.
Patient(s): According to a prospective randomization including 71 couples with severe male factor infertility, intracytoplasmic sperm injection (ICSI) was performed under polarized light that permitted analysis of the pattern of birefringence in the sperm head. Twenty-three patients had their oocytes injected with reacted spermatozoa, 26 patient's oocytes were injected with nonreacted spermatozoa, and in 22 patients both reacted and nonreacted spermatozoa were injected.
Intervention(s): Intracytoplasmic sperm injection was performed under polarized light to selectively inject acrosome-reacted and acrosome-nonreacted spermatozoa.
Main Outcome Measure(s): Rates of fertilization, cleavage, pregnancy, implantation, and ongoing implantation.
Result(s): There was no effect on the fertilizing capacity and embryo development of either type of sperm, whereas the implantation rate was higher in oocytes injected with reacted spermatozoa (39.0%) vs. those injected with nonreacted spermatozoa (8.6%). The implantation rate was 24.4% in the group injected with both reacted and nonreacted spermatozoa. The delivery rate per cycle followed the same trend.
Conclusion(s): Spermatozoa that have undergone the acrosome reaction seem to be more prone to supporting the development of viable ICSI embryos. (Fertil Steril® 2010;93:807–13. ©2010 by American Society for Reproductive Medicine.)

Key Words: Acrosome reaction, birefringence, embryo implantation, microscopy, oligoasthenoteratospermia, sperm morphology, sperm selection, testicular sperm

The introduction of the intracytoplasmic sperm injection (ICSI) technique in assisted conception cycles has substantially reduced the threshold of requirements in terms of concentration, motility, and morphology of spermatozoa that are necessary for a patient to be treated. This has led to the extreme assumption that the minimum need for an ICSI cycle is a number of spermatozoa corresponding to that of the partner's oocytes to be inseminated, irrespective of motility and morphology characteristics. Nevertheless, several studies have reported that the quality of sperm samples is strictly related to the predisposition to chromosomal errors in sperm cells, as well as to the incidence of abnormalities in the sperm protoplasmic compartment (1–6). The frequency of these defects increases proportionally with the severity of the male factor condition, implying the necessity of having, for pathologic sperm samples, effective techniques that might reliably support the selection of fertilizing spermatozoa without compromising their vitality. In this respect, the analysis of birefringence in sperm cells was reported to be an indicator of structural normality (7).

As confirmed by transmission electron microscopy (TEM), the presence of birefringence is the expression of an organized and very compact texture that characterizes normal sperm nuclei, acrosomes, and motile tails (7). When light enters an anisotropic structure, it is refracted into two polarized rays that travel at different velocities, whose difference in phase is termed *retardance* (Fig. 1). On the basis of these considerations, the application of polarization microscopy to the ICSI technique has been proposed as a novel tool for sperm selection, based on the properties of birefringence that human spermatozoa naturally possess. According to a recent randomized, prospective study, the results derived from this approach are especially advantageous for the treatment of severely oligoasthenoteratospermic (OAT) samples, particularly those devoid of progressive motility, including testicular spermatozoa obtained by testicular sperm extraction (TESE) (6).

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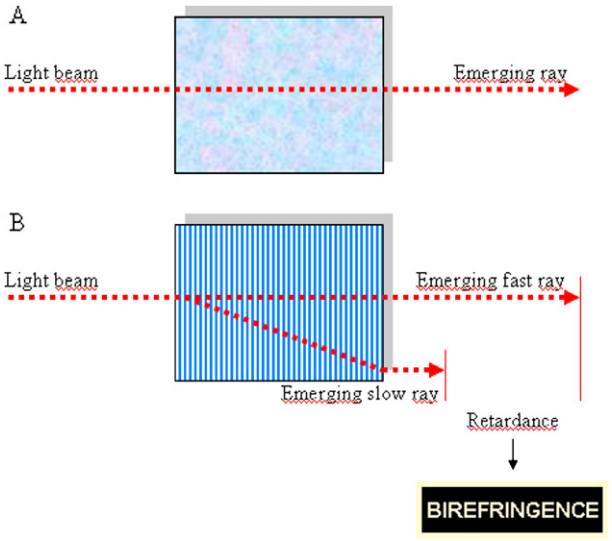
FIGURE 1

An incident light beam passing through an object. In the case of an isotropic structure (**A**), no birefringence effect is observed because the light propagates at a single velocity without being polarized. When crossing an anisotropic structure (**B**), the incident light beam is refracted into two rays traveling at different velocities. The retardance of the slow ray relative to the fast ray generates the birefringence effect.



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Careful analysis of the type of birefringence in the sperm head reveals specific details of the inner protoplasmic structure organization, providing a type of information much closer to that obtained by TEM than that routinely detected by phase contrast observation (7). One of the most interesting discoveries associated with this system is the possibility, confirmed by TEM, of distinguishing between spermatozoa that have undergone the acrosome reaction (reacted spermatozoa) and those in which the acrosome is still intact (nonreacted spermatozoa). Revisiting the specific literature on this subject, it is clear that the debate on the need for the acrosome reaction to occur in injected spermatozoa is still ongoing (8). Actually, most of the information regarding this critical subject derives from experimental or ultrastructural studies, which suggest the occurrence of the acrosome reaction being associated with the best performance in inducing oocyte activation and further development (9–11). These observations are in agreement with other studies performed in IVF settings, which report increased fertilization rates and accelerated cleavage rates after injection of reacted spermatozoa (8, 12). Unfortunately, no direct correlation with clinical outcome was attempted, but the above results suggest that the injection of reacted spermatozoa could be advantageous. For this reason, the availability of a technique allowing for the definition of the acrosomal status would possibly be of great benefit for ICSI patients.

The aim of this study was to verify whether the injection of reacted or nonreacted spermatozoa was associated with a different capacity for fertilizing the oocyte and inducing early cleavage divisions. The corresponding clinical outcome was also evaluated.

MATERIALS AND METHODS

Patients

A total of 71 ICSI cycles from 58 patients undergoing assisted conception cycles were included in the study. All patients had a normal karyotype. Mean (±SD) female age was 35.6 ± 4.5 years, and exclusion criteria were female obesity, diabetes, and polycystic ovary. The main indication for assisted reproductive technology was severe male factor infertility due to [1] severe OAT (45 samples) as defined by World Health Organization criteria (13), whereby no progressive motility was present, and [2] azoospermia (26 samples: 18 nonobstructive, 8 obstructive), in which case spermatozoa were retrieved by TESE.

During ICSI, the type of birefringence of the injected spermatozoa was analyzed to distinguish between reacted and nonreacted spermatozoa. Patients were divided into two groups according to an even–odd randomization to undergo ICSI with reacted or nonreacted spermatozoa. Allocation to either group was decided after oocyte retrieval. Oocytes from the same patient were injected with acrosome-reacted spermatozoa (reacted group, n = 23) and with acrosome-nonreacted spermatozoa (nonreacted group, n = 26). For some patients, not enough sperm cells of the same type were found within the usual timeframe requested to complete the procedure. These cases were gathered in a third group, in which both reacted and nonreacted spermatozoa were used (mixed group, n = 22).

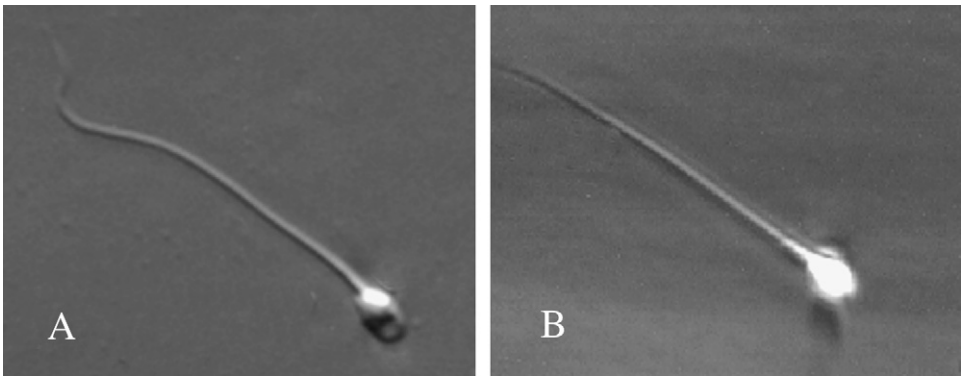
Assisted Reproductive Technology Procedure and Birefringence

Controlled ovarian stimulation was performed according to a long protocol, with pituitary down-regulation associated with the administration of recombinant human FSH (14). Ovulation was induced by hCG injection, followed by oocyte pick-up that was performed 34–36 hours later under ultrasound guidance. Oocytes were incubated in Quinn's advanced sequential media (Sage, CooperSurgical, Pasadena CA) supplemented with 5% human serum albumin (Sage), at 37.3°C in a 5.3% CO_2 humidified gas atmosphere. Insemination was performed by ICSI and, according to the local legislation on IVF prohibiting the formation of more than three embryos per patient, a maximum of three oocytes were inseminated per cycle (15).

Intracytoplasmic sperm injection was performed by selecting spermatozoa with a birefringent head by using an inverted microscope (Leica DMIRB; Leica Microsystem, Wetzlar, Germany) equipped with Leica modulation contrast, polarizing and analyzing lenses, and motorized micromanipulators (TransferMan NK; Eppendorf, McHenry, IL) (7). Birefringent

FIGURE 2

(**A**) The localization of birefringence in the postacrosomal region indicates that the acrosome reaction has already occurred. (**B**) The presence of birefringence in both compartments of the head, acrosome, and nucleus, identifies an intact acrosome in a nonreacted spermatozoon. The birefringence in the midpiece is clearly evident in **B**.



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spermatozoa were evaluated through a PL Fluotar L (Leica) at 63× objective. The analysis of the type of birefringence permitted distinguishing between reacted and nonreacted spermatozoa (Fig. 2). The localization of birefringence in the postacrosomal region was indicative of a spermatozoon that had undergone the acrosome reaction (Fig. 2A), whereas an intact acrosome conferred to the sperm head a uniform characteristic of birefringence (Fig. 2B).

Fertilization Assessment and Embryo Scoring

Oocytes were observed for the presence of pronuclei and polar bodies at 16 hours after insemination (16). Regularly fertilized oocytes were cultured individually and scored at 40 and 64 hours after insemination. Number and morphology of nuclei and blastomeres, as well as percentage and type of fragmentation, were recorded (17). According to the internal scoring system, embryos classified as grade 1 were those with regular blastomeres and no fragmentation. Top-quality embryos on day 2 were considered those with four cells and no fragments at 40 hours after insemination and, on day 3, those with eight cells and no fragments at 64 hours after insemination.

ET and Clinical Pregnancies

Embryo transfer was mainly performed on day 3. Clinical pregnancies were defined by the presence at ultrasonography of a gestational sac with fetal heartbeat. The implantation rate was expressed as the ratio between the number of gestational sacs with fetal heartbeat and the total number of embryos transferred. The ongoing pregnancy rate was calculated by considering clinical pregnancies proceeding normally beyond 12 weeks' gestation.

Statistical Analysis

Chi-square test or Fisher's exact test were used to compare categoric variables, whereas Student's *t*-test with unequal variances was applied to quantitative variables, one-way analysis of variance. The statistical package Paleontological Statistics Software (PAST; Hammer and Harper, http://paleo-electronica.org) was used for numeric calculations. Results were considered significant at $P<.05$ (18).

RESULTS

Table 1 summarizes the results derived from the treatment of the 71 cycles included in the study. Intracytoplasmic sperm injection was performed under polarized light on 214 oocytes, resulting in 150 regularly fertilized oocytes (70%). In all, 131 embryos resulted (87%), of which 118 were grade 1 (90%); 43% of the generated embryos had four cells with no fragments at the observation performed on day 2, and 43% of the 82 embryos that were cultured until day 3 had eight cells with no fragments. In 8 cycles (11%) no transfer was performed owing to failed fertilization or embryo development, whereas in the remaining 63 cycles a mean number of 1.9 ± 0.7 embryos were replaced. In all, 23 clinical pregnancies were generated (36% per transferred cycle and 32% per oocyte pick-up), with an implantation rate of 24.8%. Four pregnancies underwent spontaneous abortion, accounting for a delivery rate per oocyte pick-up of 27%.

Analysis of severe OAT and TESE samples revealed that the fertilization rate, embryo development, and clinical outcome were comparable, with a delivery rate per oocyte pick-up of 29% and 23%, respectively.

The results derived from the injection of reacted or nonreacted spermatozoa are shown in Table 2. As reported, ICSI was performed with reacted spermatozoa in 23 cycles (reacted group) and with nonreacted spermatozoa in 26 cycles (nonreacted group), whereas in the remaining 22 cycles

TABLE 1

Overall outcome in severe OAT and TESE cycles.

Variable	Severe OAT	TESE	TOTAL
Cycles	45	26	71
Age (y)	35.9 ± 4.2	35.1 ± 5.0	35.6 ± 4.5
Inseminated oocytes	128	86	214
Fertilized oocytes	93 (73)	57 (66)	150 (70)
Embryos	84 (90)	47 (82)	131 (87)
4 cells grade 1, day 2	41 (49)	15 (32)	56 (43)
8 cells grade 1, day 3[a]	25/51 (49)	10/31 (32)	35/82 (43)
Transferred cycles	40 (89)	23 (88)	63 (89)
Transferred embryos	1.9 ± 0.7	1.8 ± 0.7	1.9 ± 0.7
Clinical pregnancies	16	7	23
% per transferred cycle	40	30	36
% per pick-up	35	27	32
Implantation rate (%)	25.3	23.8	24.8
Spontaneous abortions	3 (18.7)	1 (14)	4 (17)
Delivery rate per pick-up	13 (29)	6 (23)	19 (27)

Note: Values are number (percentage) or mean ± SD, unless otherwise noted.
[a] Proportion of eight-cell embryos is calculated over the number of embryos that were cultured to day 3.

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both reacted and nonreacted spermatozoa were used (mixed group). The three groups were homogeneous in terms of maternal age, proportion of severe OAT vs. TESE samples, and proportion of TESE done for nonobstructive azoospermia (26%, 27%, and 23%, respectively). In the mixed group, ET was performed in 12 cases with embryos derived from the injection of both reacted and nonreacted spermatozoa, whereas in the remaining 8 cases only embryos derived from reacted (4 cases) or nonreacted (4 cases) spermatozoa were transferred.

The pregnancy rate per transferred cycle was significantly lower in the nonreacted group (14%) compared with the reacted group (54.5%, P=.006), whereas the difference from the mixed group showed borderline statistical significance (40%, P=.05). When calculated per pick-up, the pregnancy rate in the nonreacted group (11.5%) was decreased in relation not only to the reacted group (52%, P=.002) but also to the mixed group (36%, P=.037). The implantation rate showed the same trend (8.6% in the nonreacted group vs. 39.0% in the reacted group [P=.002] and 24.4% in the mixed group [P=.048]). The delivery rate per oocyte pick-up was significantly higher in the reacted group and mixed group (44% and 32%, respectively) than in the nonreacted group (8%, P=.004 and P=.033, respectively). In all, 24 healthy infants were born: 14 in the reacted group (4 sets of twins and 6 singletons), 2 in the nonreacted group (both singletons), and 8 in the mixed group (1 set of twins and 6 singletons).

DISCUSSION

As demonstrated by ultrastructural studies, human spermatozoa possess characteristics of birefringence that reflect the state of the inner protoplasmic structures (7). These observations supported the idea of selecting spermatozoa for ICSI under polarized light, with the aim of using birefringence as an additional noninvasive criterion (19–21) for the identification of the sperm cell to be injected (6). To realize this project it was necessary to set up a new optic system, because the commercially available PolScope (CRI, Woburn, MA) did not permit detection of the type and intensity of birefringence associated with sperm cells (7). This technical advance was proposed as an answer to the compelling need for a more accurate system guiding sperm selection, especially for cases of severely compromised sperm samples. Along this line of thought, the analysis of birefringence was also used to expand the criteria of sperm selection by taking advantage of its unique characteristics, which made it possible to distinguish between reacted and nonreacted sperm cells without affecting sperm vitality. The injection of either type of sperm was performed in this study according to a prospective randomization that permitted evaluation of the consequent outcome of the derived zygotes.

It is well known that the acrosome reaction includes a series of events that sperm cells need to undergo to naturally achieve fertilization of the oocyte. Because ICSI mechanically bypasses the zona pellucida and the oolemma, most injected spermatozoa possibly still contain intact acrosomes, implying that the interaction between the gamete membranes and the consequent trigger of the acrosome reaction are not necessary to induce activation of the oocyte, male nuclear decondensation, and pronuclear formation. Nevertheless, the question remains whether the acrosome reaction must occur within the injected oocyte to induce its fertilization; the main aim of this study was to address to this query.

TABLE 2

Injection of reacted and nonreacted spermatozoa according to the type of birefringence.

Variable	Reacted spermatozoa	Nonreacted spermatozoa	Mixed
Cycles	23	26	22
Age (y)	34.9 ± 4.0	36.3 ± 4.3	35.6 ± 5.3
TESE cycles	7 (30)	12 (46)	7 (32)
Inseminated oocytes	72	73	69
Fertilized oocytes	50 (69)	49 (67)	51 (74)
Embryos	45 (90)	42 (86)	44 (86)
4 cells grade 1, day 2 (%)	20 (44)	20 (48)	16 (36)
8 cells grade 1, day 3 (%)[a]	10/30 (33)	13/28 (46)	12/24 (50)
Transferred cycles	22 (96)	21 (81)	20 (91)
Transferred embryos	1.9 ± 0.8	1.7 ± 0.7	2.0 ± 0.8
Clinical pregnancies	12	3	8
% per transferred cycle	54.5[b]	14[b,c]	40[c]
% per pick-up	52[d]	11.5[d,e]	36[e]
Implantation rate (%)	39.0[f]	8.6[f,g]	24.4[g]
Spontaneous abortions	2 (17)	1 (33)	1 (12.5)
Delivery rate per pick-up	10 (44)[h]	2 (8)[h,i]	7 (32)[i]

Note: Values are number (percentage) or mean ± SD, unless otherwise noted.

[a] Proportion of eight-cell embryos is calculated over the number of embryos that were cultured to day 3.

[b] $P=.006$.

[c] $P=.05$ (not significant).

[d] $P=.002$.

[e] $P=.037$.

[f] $P=.002$.

[g] $P=.048$.

[h] $P=.004$.

[i] $P=.033$.

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Before commenting on the results derived from the injection of reacted vs. nonreacted spermatozoa, it is important to emphasize that the data from the present study confirm that the selection of birefringent spermatozoa supports a favorable clinical outcome in the treatment of the most severe male factor cases. The delivery rate per oocyte pick-up was 29% and 23% in the severe OAT and TESE groups, respectively (Table 1), which are especially valuable figures when considering that severe male factor patients are harshly penalized by the current national regulation on IVF that imposes a limit of insemination of no more than three oocytes per patient (15). Although the fertilization rate was slightly lower in the TESE group, the implantation rate did not differ between severe OAT and TESE, implying that the selection of sperm cells on the basis of their birefringence properties is indicative of their capability of giving rise to vital embryos. This happened with similar chances of success irrespective of the sperm source (Table 1).

In agreement with the conclusions derived from a previous study (6), these data suggest that there is still room for improvement for ICSI results if a more accurate selection of spermatozoa is performed, confirming that the sperm cell has a crucial role in determining embryo competence, especially in cases of severe male factor. A similar conclusion was reached by the application of intracytoplasmic morphologically selected sperm injection, which permits selection of sperm for ICSI with morphologically normal nuclei under a magnification up to ×6,300, yielding a higher pregnancy rate and a decreased incidence of abortion compared with a control group (22, 23). Regarding this approach, analysis

FIGURE 3

The pattern of birefringence is altered by the presence of a vacuole in the sperm head, whereas it is clearly visible in the midpiece.

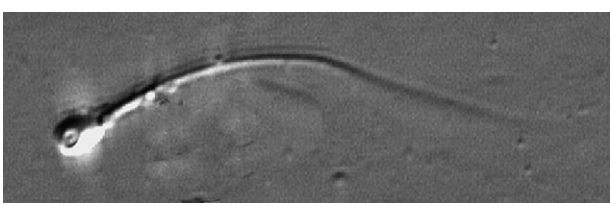

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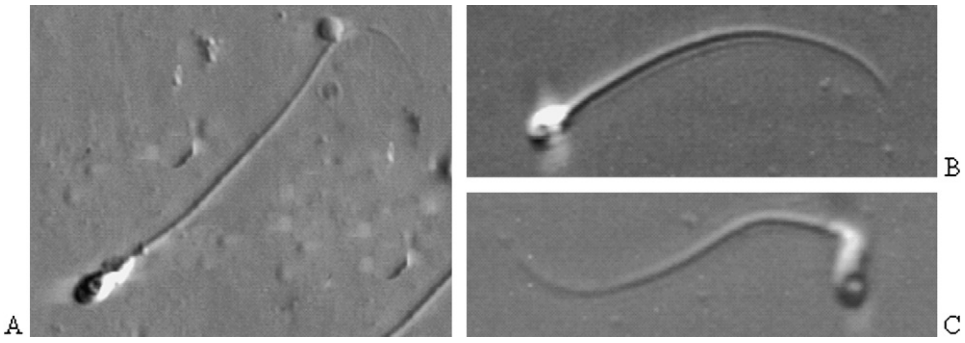
FIGURE 4

Under polarized light, vacuoles are evident in reacted (**A**), nonreacted (**B**) and non-birefringent (**C**) spermatozoa.



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of birefringence was intended to contribute information derived by evaluation of organellar organization in both compartments of the sperm head, nucleus and acrosome, and the tail. Actually, in addition to providing details on the protoplasmic structure of the sperm cell, the polarized light also permitted easily visualization of irregularities such as vacuoles in the sperm head (Fig. 3). Vacuoles could be detected in both reacted (Fig. 4A) and nonreacted (Fig. 4B) spermatozoa, as well as in non-birefringent sperm cells (Fig. 4C).

As a novel contribution to the knowledge of the process of fertilization occurring after ICSI, analysis of birefringence type was used to distinguish spermatozoa on the basis of their acrosome integrity, to evaluate whether some differences could derive from the injection of either reacted or nonreacted sperm cells. Apparently there was no effect on the fertilizing capacity of either type of sperm, nor on the initial cleavage divisions of the fertilized oocytes and consequent embryo quality. Nevertheless, the viability of the generated embryos was significantly different, as demonstrated by the higher implantation rate in the group of oocytes injected with reacted spermatozoa (39.0%) compared with those injected with nonreacted spermatozoa (8.6%, P=.002) (Table 2). Interestingly enough, in the group in which the type of injected spermatozoa was mixed, the implantation rate (24.4%) was still superior to that detected in the group of nonreacted spermatozoa (8.6%, P=.048). The delivery rate per oocyte pick-up followed the same trend, suggesting that spermatozoa that have undergone the acrosome reaction seem to be more prone to supporting the development of viable embryos.

The data from this study point to the injection of reacted spermatozoa as the best way to achieve pregnancy in patients with severe male factor infertility. This is in agreement with previous studies suggesting that the induction of the acrosome reaction in human spermatozoa is associated with an improved fertilization outcome and embryo development (8, 9, 12). Conversely, the absence of the acrosome in globozoospermia is known to be associated with extremely low or no fertilization (24). Accordingly, ultrastructural studies have reported that the acrosome reaction occurs in the ooplasm before sperm incorporation in the mature human oocyte and is preceded by acrosome swelling and followed by exposure of the inner membrane as observed on the surface of the zona pellucida during conventional IVF (10).

All these considerations and the results from this study indicate that the acrosome reaction could be a prerequisite for sperm incorporation to occur, not only after conventional IVF but also after ICSI. From a physiologic point of view, there is no doubt that the injection of acrosome-reacted sperm cells into the oocyte makes logical sense. In this manner, the fertilization process comes closer to the way it happens in nature, in a technique that tends to overcome any concept of natural progression.

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