Sperm head's birefringence: a new criterion for sperm selection

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Objective: To investigate the characteristics of birefringence in human sperm heads and apply polarization microscopy for sperm selection at intracytoplasmic sperm injection (ICSI).
Design: Prospective randomized study.
Setting: Reproductive Medicine Unit, Società Italiana Studi Medicina della Riproduzione, Bologna, Italy.
Patient(s): A total of 112 male patients had birefringent sperm selected for ICSI (study group). The clinical outcome was compared with that obtained in 119 couples who underwent a conventional ICSI cycle (control group).
Intervention(s): The proportion of birefringent spermatozoa was evaluated before and after treatment in relation to the sperm sample quality. Embryo development and clinical outcome in the study group were compared with those in the controls.
Main Outcome Measure(s): Proportion of birefringent sperm heads, rates of fertilization, cleavage, pregnancy, implantation, and ongoing implantation.
Result(s): The proportion of birefringent spermatozoa was significantly higher in normospermic samples when compared with oligoasthenoteratospermic samples with no progressive motility and testicular sperm extraction samples. Although fertilization and cleavage rates did not differ between the study and control groups, in the most severe male factor condition (oligoasthenoteratospermic with no progressive motility and testicular sperm extraction), the rates of clinical pregnancy, ongoing pregnancy, and implantation were significantly higher in the study group versus the controls.
Conclusion(s): The analysis of birefringence in the sperm head could represent both a diagnostic tool and a novel method for sperm selection. (Fertil Steril® 2008;90:104–12. ©2008 by American Society for Reproductive Medicine.)

Key Words: Birefringence, embryo implantation, microscopy, oligoasthenoteratospermia, sperm morphology, sperm selection, testicular sperm

The never-ending search for a method providing the possibility of selecting the most viable embryos gave impulse to deepen the investigation of the quality of gametes. This resulted in a meticulous inspection of oocyte quality, for which the morphologic evaluation at the time of intracytoplasmic sperm injection (ICSI) can be combined with the assessment of the oocytes' chromosomal status (1). Similarly, the methods of sperm analysis and selection have been improved, and a closer correlation between sperm analyses and male factor infertility has been achieved (2–4). Recently, a new method of intracytoplasmic morphologically selected sperm injection has been proposed, based on motile sperm organellar examination, that results in a higher pregnancy rate and a decreased incidence of abortion (5–7). According to this strategy, sperm examination is performed during ICSI with use of an inverted light microscope equipped with high-power Normansky optics enhanced by digital imaging to achieve a magnification up to ×6,300 and permitting insemination using spermatozoa carrying morphologically normal nuclei.

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Based on a similar approach, but with the idea of evaluating the organellar organization in both compartments of the sperm head, nucleus and acrosome, and in the tail, the application of polarization microscopy to the ICSI technique has been proposed as a novel tool for sperm selection (8). This approach was based on the birefringence characteristics of the spermatozoa due to anisotropic properties of their protoplasmic texture. In the mature sperm nucleus, there is a strong intrinsic birefringence associated with nucleoprotein filaments that are ordered in rods and longitudinally oriented. The presence of subacrosomal protein filaments that are longitudinally oriented gives to the mature acrosomal complex a similar type of birefringence. The same is true for large portions of the tail texture, in which the microtubular organization of the axoneme and the chondriome in the midpiece are birefringent (8). These patterns have been confirmed by the analysis performed by transmission electron microscopy (TEM).

The major advantage related to the introduction of polarizing and analyzing lenses in the inverted microscope, equipped with a micromanipulation system, is due to the possibility of selecting birefringent sperm cells for ICSI without affecting their vitality or motility. In this way, the birefringence of sperm cells can be evaluated, providing information on the inner

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protoplasmic structures that is closer to the information provided by TEM than that obtained by phase-contrast observation.

The purpose of this study was to evaluate the proportion of birefringent spermatozoa in samples obtained in patients undergoing an assisted conception cycle aiming [1] to assess a possible correlation between morphologic parameters of the sperm samples and their protoplasmic structures and [2] to apply polarization microscopy to the ICSI technique to attempt a structural evaluation of the human spermatozoon selected for insemination. Embryo development and implantation in this group were evaluated and compared with those from a control group subjected to routine ICSI procedure to verify whether any possible advantage could be obtained by selecting the sperm for injection on the basis of its birefringence properties.

MATERIALS AND METHODS

Study Design

A total of 112 sperm samples from patients undergoing assisted conception cycles (85 with fresh oocytes and 27 with thawed oocytes) were evaluated with use of an inverted microscope with polarizing and analyzing lenses (study group) (Fig. 1). Nine samples were normospermic, 89 were oligoasthenoteratospermic (46 were oligoasthenoteratospermic with progressive motility and 43 oligoasthenoteratospermic without progressive motility), and 14 were testicular sperm retrieved by testicular sperm extraction (TESE). In the ejaculated samples, the presence of birefringence in the sperm head was evaluated before and after sperm preparation. Intracytoplasmic sperm injection was performed under the polarizing microscope by selecting for injection birefringent spermatozoa (Fig. 2). The clinical outcome was compared with that derived from 119 cycles, 90 fresh cycles and 29 with thawed oocytes, with similar characteristics, which were treated by routine ICSI procedure in the same period. Table 1 shows the number of cycles in each semen sample category and the corresponding mean maternal age. Exclusion criteria were female obesity, diabetes, and polycystic ovary.

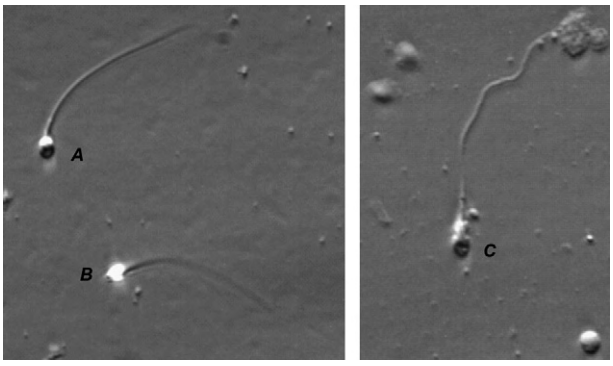
Allocation to the study or the control group was done according to an even-odd randomization and was mutually exclusive. The study was discussed and approved by our Institutional Review Board and was performed between September 2005 and July 2006.

Assisted Reproductive Technology Procedure

Controlled ovarian stimulation was performed following a long protocol with a pituitary down-regulation, associated with the administration of recombinant human FSH (9). Ovulation was triggered by hCG injection, and, 36 hours later, oocyte retrieval was performed transvaginally via ultrasound guidance. Oocytes were cultured in Quinn's advanced sequential media (Sage, CooperSurgical Inc., Pasadena, CA) supplemented with 5% human serum albumin (Sage), in a 5.3% CO_2 humidified gas atmosphere at 37.3°C. Insemination was performed by ICSI, and, according to the current national legislation on IVF stating that no more than three embryos can be generated, a maximum of three oocytes were inseminated per cycle (10).

FIGURE 1

The acrosome is reacted in spermatozoon (*A*) and not reacted in sperm (*B*). Sperm *C* is devoid of birefringence because of the absence of a conventional sperm texture, with a vacuole in its head. Only nonpyknotic nuclei can be birefringent, because of the organization of nucleoprotein filaments, strictly packed in a very compact texture.



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Fertilization Assessment and Embryo Scoring

Oocytes were checked for the presence of pronuclei and polar bodies at 16 hours after insemination (11). Regularly fertilized oocytes were cultured individually and scored at 40 and 64 hours after insemination. Number and morphology of nuclei and blastomeres and the percentage and type of fragmentation were recorded (12). According to the internal embryo scoring system, embryos classified as grade 1 were those having regular blastomeres and no fragmentation. Excellent embryos were defined as those having four cells, no fragments, at 40 hours after insemination and eight cells, no fragments, at 64 hours after insemination.

Pregnancies were defined as clinical 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 heartbeats and the total number of embryos transferred.

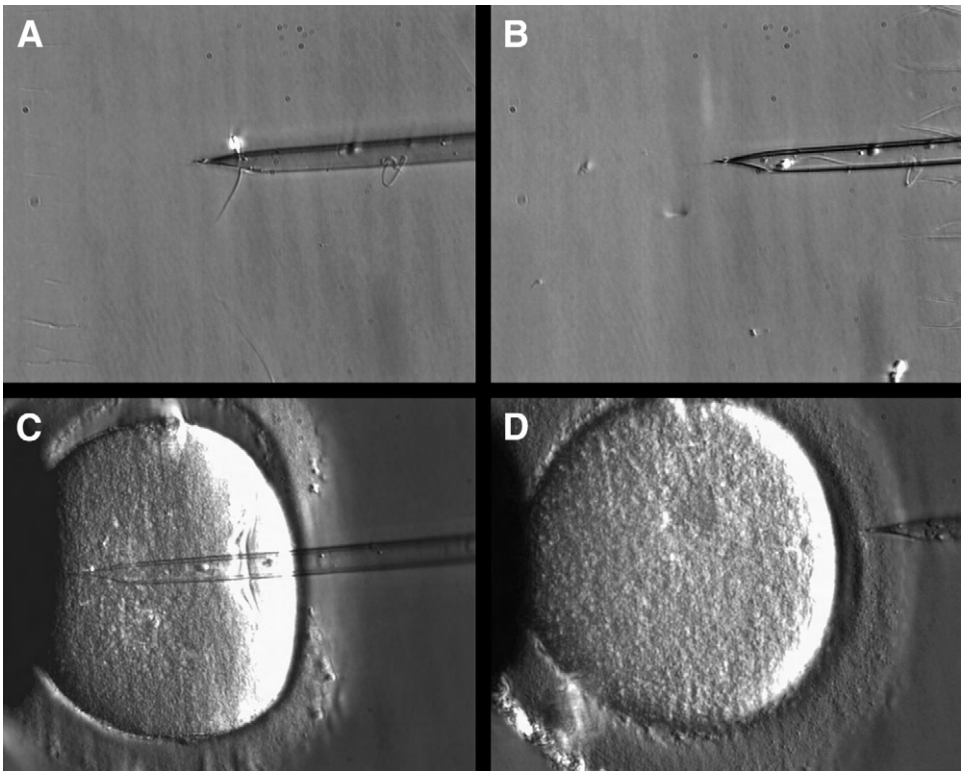
Sperm Analysis

The morphologic parameters of the sperm samples included in the study were analyzed according to the criteria established by the World Health Organization (13) and, for the morphology, by Krüger et al. (14).

Sperm preparation was performed either by swim-up or by discontinuous gradient separation (15). One hundred sperm

FIGURE 2

The ICSI procedure is performed under the polarizing light. A birefringent spermatozoon is selected and immobilized (**A**), aspirated (**B**), injected (**C**), and released in the ooplasm (**D**). The birefringence of the head is evident at every step of ICSI.



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cells were evaluated for the presence of birefringence before and after sperm preparation.

Sperm birefringence was assessed by using an inverted microscope (Leica DM IRB, Leica Microsystems, Wetzlar, Germany)* equipped with Hoffman contrast and polarizing and analyzing lenses (8).

The objective through which the source of light went after crossing the polarizing lens, the Hoffman lens, the condenser, and the specimen was a PL Fluotar L63X (Leica Microsystems, Wetzlar, Germany). After crossing the objective, the beam of polarized light went through a compensator and an analyzer, entering the first optical unit that was made by a lens forming the image and a transmission prism. The resulting ray hit a mirror and reflected along a second optic pathway through which the polarized image of the specimen was formed and entered the ocular. The images were transmitted to a camera connected to a monitor and captured in a computer. The microscope has also been equipped with motorized micromanipulators (Eppendorf TransferMan NK, McHenry, IL) (Fig. 3).

The criterion regulating sperm selection for ICSI was the presence of birefringence in the sperm head, whereas the tail was not evaluated to simplify the axonemal analysis using the available optic system. Both types of birefringent sperm heads were injected: those with a nonreacted acrosome (Fig. 1[*A*]) and those with a reacted acrosome (Fig. 1[*B*]). The quality of the image also permitted identifying sperm cells with vacuoles because the corresponding area was devoid of birefringence (Fig. 1[*C*]).

Statistical Analysis

The linear correlation coefficient (r) was used to evaluate the strength and the direction of a linear relationship between

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Date of filing: 25.02.05
Priority: IT/27.02.04/ITA RM20040102
Title: Improved inverted light optical microscope
Designed states: AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IS IT LI LT LU MC NL PL PT RO SE SI SK TR
Receiving section: European Patent Office

TABLE 1

Number of cycles and maternal age in the four sperm categories.

	Normospermic		OAT with progressive motility		OAT without progressive motility		TESE	
	Study group	Control group	Study group	Control group	Study group	Control group	Study group	Control group
No. cycles	9	9	46	53	43	44	14	13
Maternal age (y)	37.0 ± 5.0	35.1 ± 3.5	35.2 ± 4.5	35.8 ± 4.5	34.2 ± 4.2	34.9 ± 4.1	33.9 ± 4.7	33.6 ± 5.9

Note: Values are expressed as mean ± SD. OAT = oligoasthenoteratospermia.

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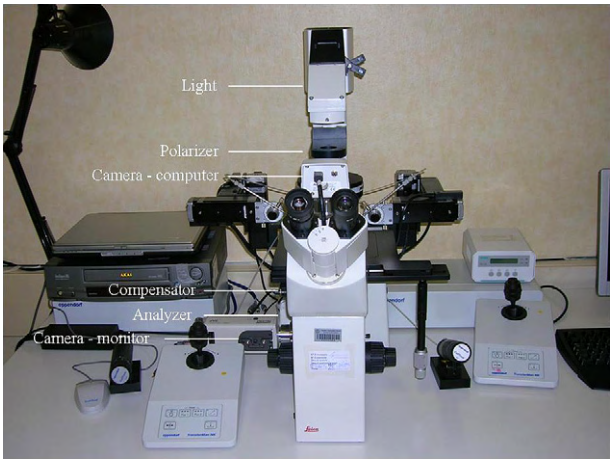
two variables. The r value is a number between −1 and 1 that measures how close to a straight line a set of points falls. The closer to zero the correlation coefficient is, the less the points fall on a straight line. When r is >0.5, the variables are considered to be significantly correlated.

The χ^2 test or Fisher's exact test was used to compare categorical variables, and Student's t-test with unequal variances was applied to quantitative variables, one-way analysis of variance (ANOVA). The statistical package PAST (2006; available at http://www.nhm.uio.no/-ohammer/past) was used for the numerical calculations. The results were considered statistically significant if $P<.05$ (16).

FIGURE 3

The polarization inverted microscope equipped with the micromanipulators seen in frontal view. The light source, the polarizer, the compensator, and the analyzer are indicated. There are two cameras, one transmitting the image to the computer and the other to the monitor.



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RESULTS

The proportion of birefringent spermatozoa in the sperm samples before preparation was positively correlated with concentration ($r = 0.67$), progressive motility ($r = 0.64$), and vitality ($r = 0.63$).

After sperm preparation (Table 2), the proportion of birefringent spermatozoa was significantly higher in normospermic samples (87.0% ± 11.1%) compared with oligoasthenoteratospermic samples with progressive motility (55.3 ± 27.6), oligoasthenoteratospermic samples without progressive motility (22.1% ± 17.0%), and TESE samples (13.8% ± 9.1%). Although the proportion of birefringent spermatozoa between oligoasthenoteratospermic with progressive motility and TESE was significantly different ($P=8.43384^{-07}$), this figure was similar when comparing oligoasthenoteratospermic with no progressive motility and TESE samples ($P=.0866$).

Table 3 summarizes the results of the laboratory and clinical performance in the study group versus the controls. Apparently, no differences derived from the injection of spermatozoa carrying reacted or nonreacted acrosome, as indicated by the type of birefringence. For this reason, the results were not broken down for this factor.

The fertilization and cleavage rates did not differ between the two groups, with the exception of excellent embryos on day 3 (eight-cell embryos, grade 1) that were more frequent in the study group (33%) than in the controls (20%, $P<.025$). Accordingly, the implantation potential of the embryos generated after the selection of birefringent spermatozoa (19%) was superior to that observed in the conventional treatment group (11.2%, $P<.05$).

Regarding the clinical outcome, pregnancies occurred at similar rates: 31% in the study group and 21% in the control group. However, because of a higher incidence of abortions in the controls (41% vs. 16% in the study groups, $P=.035$), the ongoing pregnancy rate was superior in the study group (23%) when compared with the controls (11%; $P<.025$).

The results were then evaluated in relation to the category of the sperm sample. As for the overall data, no differences

TABLE 2

Proportion of birefringent spermatozoa after sperm preparation.

No. samples	Type	Birefringence	
		Mean ± SD	Range
9	Normospermic	87.0% ± 11.1%[a,b,c]	65%–97%
46	OAT with progressive motility	55.3% ± 27.6%[a,d,e]	8%–98%
43	OAT without progressive motility	22.1% ± 17.0%[b,d]	2%–73%
14	TESE	13.8% ± 9.1%[c,e]	4%–32%

Note: OAT = oligoasthenoteratospermia.
[a] $P=.0008$.
[b] $P=6.843154^{-15}$.
[c] $P=6.926984^{-14}$.
[d] $P=1.749424^{-08}$.
[e] $P=8.43384^{-07}$.

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were detected in the fertilization and cleavage rates in the examined categories. As represented in Figure 4, the pregnancy rate per ET was analyzed in the four categories. Similar rates between study and control groups were observed (Fig. 4A), although for TESE couples the corresponding figures almost reached a statistical significance (58% in the study group vs. 18% in the control group, $P=.053$). However, if the performance of the couples with the most severe male factor was combined (oligoasthenoteratospermic with no progressive motility and TESE), the pregnancy rate was significantly higher in the study group (35%) compared with the controls (16%, $P=.018$). Similarly, when considering the ongoing pregnancy rate per ET (Fig. 4B), the differences were statistically significant for oligoasthenoteratospermic with no progressive motility (23% in the study group vs. 8% in the controls, $P=.049$) and for TESE (58% in the study group vs. 9% in the controls, $P=.018$). If these two groups were combined, the differences had a stronger significant difference (31% vs. 8%, respectively, $P=.003$).

The same performance was observed when evaluating the ongoing pregnancy rate per cycle (Fig. 5A) and the implantation rate (Fig. 5B). In patients who had TESE, the implantation rate was 42.1% in the study group and 12.5% in the controls, $P=.049$. In the other categories the differences were not statistically significant, but when combining oligoasthenoteratospermic with no progressive motility and TESE the figures were highly different (22% in the study group vs. 9% in the control group, $P<.025$). When the analysis of the results was conducted separately in cycles with fresh and frozen oocytes, the results did not differ between the two groups.

TABLE 3

Laboratory and clinical results in the study and control groups.

	Study group	Control group	*P*
No. cycles	112	119	
Age (y, mean ± SD)	34.8 ± 4.4	35.2 ± 4.5	
No. fertilized oocytes (%)	235/317 (74)	248/342 (72)	
No. embryos (%)	204 (87)	210 (85)	
Grade 1—day 2 (%)	178 (87)	177 (84)	
Four cells, grade 1—day 2 (%)	66 (32)	57 (27)	
Grade 1—day 3 (%)	144/164 (88)	117/135 (87)	
Eight cells, grade 1—day 3 (%)	54 (33)	27 (20)	<.025
No. transferred cycles	101	104	
No. transferred embryos (mean ± SD)	184 (1.8 ± 0.7)	196 (1.9 ± 0.7)	
No. clinical pregnancies (%)	31 (31)	22 (21)	
Implantation rate (%)	35/184 (19.0)	22/196 (11.2)	<.05
Abortions (%)	5 (16)	9 (41)	.035
Ongoing pregnancy rate (%)	26/112 (23)	13/119 (11)	<.025

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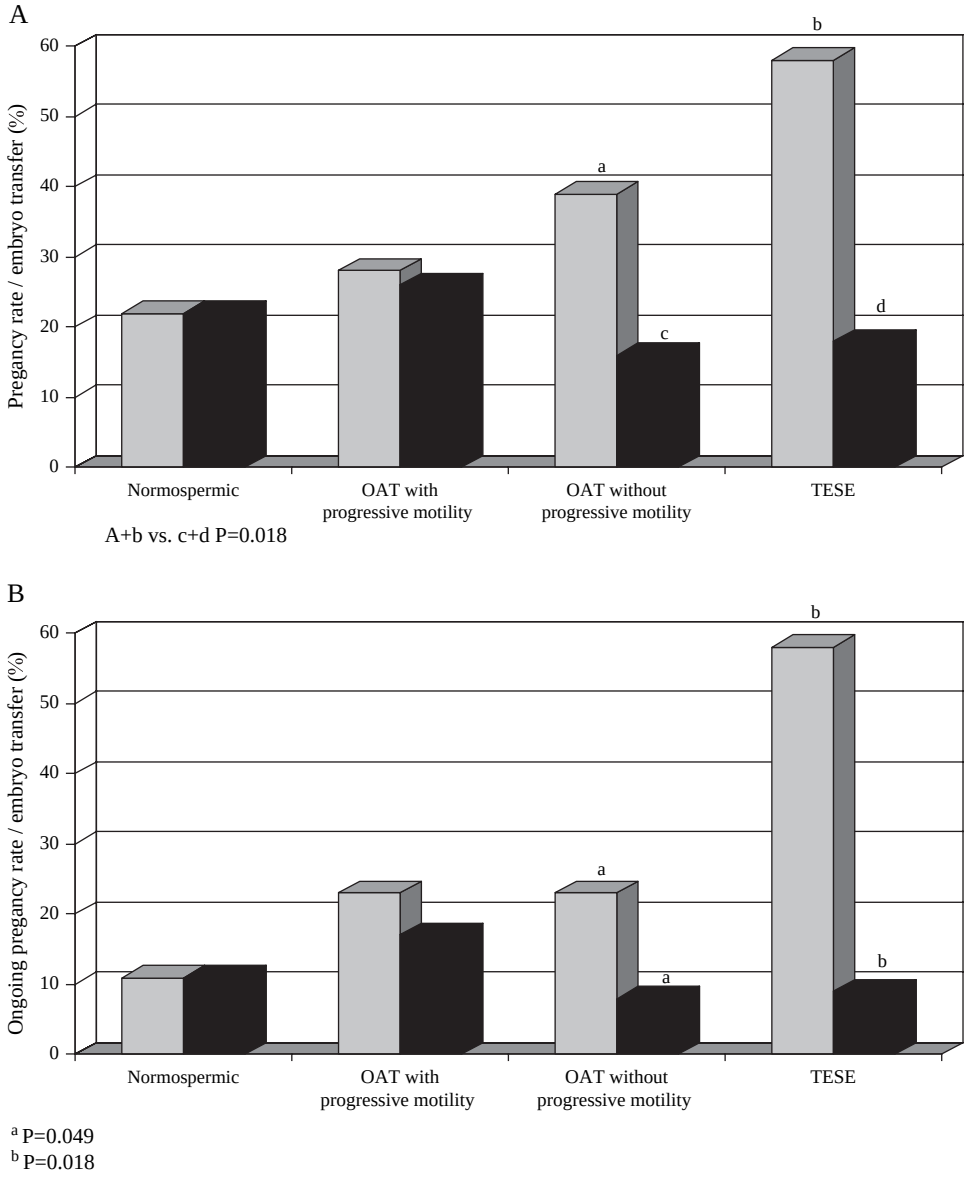
FIGURE 4

Pregnancy rate per ET (**A**) and ongoing pregnancy rate (**B**) in relation to the quality of the sperm sample. *Bars* with same letters are statistically significantly different. OAT = oligoasthenoteratospermic.



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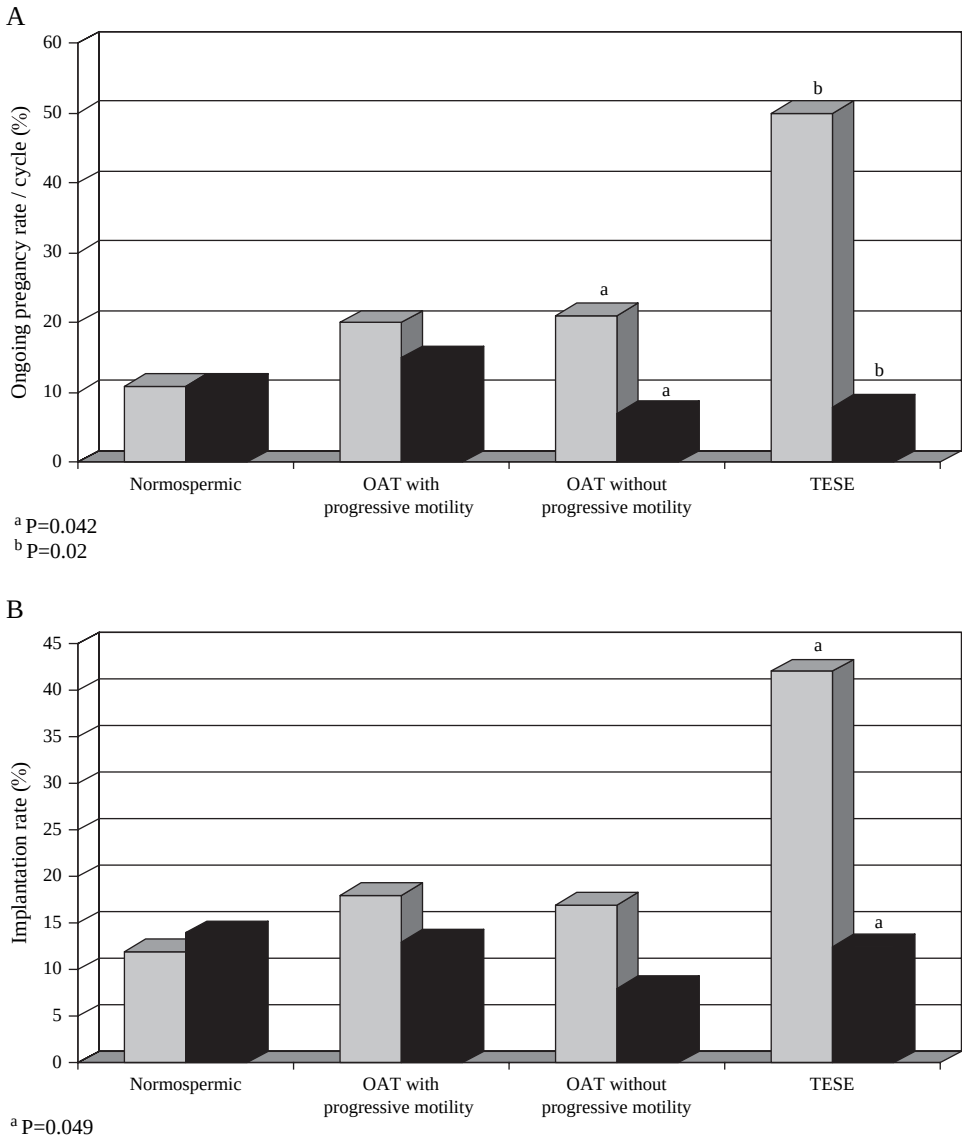
DISCUSSION

The association between semen quality and male factor infertility has been known for more than 40 years. Accordingly, the possibility of estimating precisely a man's fertility potential has long represented a major goal for practitioners and researchers. In this context, the term "male factor infertility" does not represent a defined clinical syndrome but rather an assortment of different conditions having varying etiologies and prognoses (17). For this reason, having tests that will indicate with absolute certainty that a man is fertile or infertile is unattainable, and the debate is still ongoing on which criteria should be adopted to define normal spermatozoa and which classification of abnormal forms is most correct to predict the fertilizing capacity of male gametes.

In assisted conception cycles, the introduction of the ICSI technique has substantially decreased the threshold of requirements in terms of concentration, motility, and morphology of spermatozoa that can be used to inseminate the partner's oocytes with reasonable hope of success. Nevertheless, the quality of sperm samples has an effect not only on the ICSI outcome but also on the incidence of abnormalities in their protoplasmic compartment and in the predisposition to chromosomal errors, which increase proportionally to the

FIGURE 5

Ongoing pregnancy rate per cycle (**A**) and implantation rate (**B**) in relation to the quality of the sperm sample. *Bars* with same letters are statistically significantly different. OAT = oligoasthenoteratospermic.



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severity of the male factor condition (3, 5, 18–21). Unfortunately, most of the techniques used to investigate sperm cells are highly invasive and cannot be adopted to select the fertilizing spermatozoon. An exception is represented by intracytoplasmic morphologically selected sperm injection, which permits selecting sperm for ICSI with morphologically normal nuclei under a magnification up to ×6,300 (5).

These observations gave impulse to find new strategies that could contribute valuable information to the definition of "normal spermatozoa" without compromising their motility or vitality, meaning by normal spermatozoa those that can generate a viable, healthy pregnancy. In this context, the analysis of birefringence in sperm cells was proposed as an indicator of normality, being the expression of an organized and a very compact texture that characterizes nonpyknotic sperm nuclei, normal acrosomes, and motile tails. As previously reported, there is a strong negative intrinsic birefringence in the mature nucleus of sperm heads to which a weak form of birefringence is superimposed (8). This is due to the presence of nucleoprotein filaments arranged in rods and oriented longitudinally. The mature acrosomes and sperm tails also show a similar pattern of birefringence, suggesting the presence of longitudinally oriented filamentous texture that in the tail is due to the microtubular organization of the axoneme and the chondriome in the midpiece. These observations have been confirmed by TEM.

The data from this study confirm that the presence of birefringence in the sperm head might reflect the good health of a sperm cell, because the proportion of birefringent spermatozoa varied significantly in relation to the sample concentration, vitality, and motility (Table 2). This correlation was directly proportional to the quality of the sperm sample, suggesting that the birefringence in human spermatozoa appears to be disturbed in pathologic sperm samples, in which the inner protoplasmic structures are also affected. However, with the correlation coefficients being statistically significant, but far from 1 (either −1 or +1), it was postulated that a benefit could derive in terms of oocyte fertilization, development, and implantation when performing ICSI with use of an inverted microscope equipped with polarizing and analyzing lenses.

The comparison with the data obtained from the control group demonstrated no effect on the fertilization rate, giving an additional confirmation of the potentiality of ICSI to achieve high fertilization rates irrespective of sperm quality. Conversely, the proportion of excellent embryos on day 3 and their capacity to implant and to progress at least beyond 16 weeks of gestation were superior in the study group versus the controls (Table 3). These data imply that the presence of birefringence in the sperm head identifies a normal sperm structure, as previously demonstrated by TEM, and that this is typical of a vital cell with the highest potential of development.

The relevance of these results was especially evident when the data were analyzed according to the quality of the sperm sample. It has to be acknowledged that the type of randomization performed in this study possibly included potential biases because of female factors and the different techniques used for sperm preparation (swim-up or discontinuous gradient separation) and oocyte handling (fresh and frozen). Although the variability of female factors was partially controlled by excluding some categories predisposing to ovulatory dysfunctions, the optimal situation would be to eliminate all the sources of dyshomogeneity among the studied groups and subgroups.

Nevertheless, the results suggested that the differences in the clinical outcome between the study groups and the controls were probably dependent on the type of sperm samples (Figs. 4 and 5). Patients with normospermia and patients with oligoasthenoteratospermia with progressive motility yielded similar rates of pregnancy, ongoing pregnancy, and implantation irrespective of the selection of birefringent spermatozoa under the polarizing light during ICSI. However, in the categories with the most severe male factor condition, oligoasthenoteratospermic without progressive motility and TESE, the clinical outcome was superior in the study group compared with the controls. This was especially notable when considering the ongoing implantation rate that was significantly higher when calculated either per ET or per cycle. Even with the above-mentioned limitations because of female factors, besides reaffirming the importance of the quality of the male gamete to achieve a regularly developing pregnancy, these data suggest that the selection of spermatozoa for ICSI based on their birefringence properties might find its major application in those cases where the proportion of non-birefringent spermatozoa is more frequent.

The information provided by this system permits selection of not only spermatozoa with a normal nuclear morphology, as intracytoplasmic morphologically selected sperm injection does, but also of those with a regular protoplasmic structure in the acrosome and the tail.

In conclusion, according to the data reported in this study, the use of the proposed instrumentation for ICSI could represent not only a diagnostic tool but also an accurate and novel method for sperm selection. Future perspectives include [1] to increase the number of cycles to confirm these preliminary data by following a strict experimental design in which the homogeneity of groups is strictly controlled, [2] to verify the clinical outcome derived by the injection of reacted and non-reacted spermatozoa as identified by the two patterns of birefringence (Fig. 1), and [3] to study the effects related to the presence or absence of birefringence in the alive sperm tail.

The answer to these points could provide not only a strategy guiding the criteria for sperm selection but also a tool to better understand about sperm physiology. In other words, the evaluation of birefringence in sperm cells could dramatically change the definition of infertility by contributing additional information on sperm physiologic condition and potential in the routine evaluation of the male factor, as well as modifying the technique of sperm preparation.

This study was realized in the belief that every approach aimed at increasing the efficiency of IVF techniques deserves to be investigated. It is concluded that improving the selection criteria that help identify normal spermatozoa in vivo might substantially contribute to the generation of embryos with high chances of implantation.

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