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ARTICLE

Head birefringence properties are associated with acrosome reaction, sperm motility and morphology

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Abstract Birefringence in sperm heads reflects an organized and very compacted texture, indicating nuclear and acrosomal structural normality. This study performed a direct analysis of the acrosome integrity in single spermatozoa to verify whether a pattern of total or partial head birefringence reflected the acrosome status. The morphology in fresh samples was assessed according to World Health Organization criteria while the characteristics of birefringence were evaluated by polarized light. Acrosome integrity was evaluated by fluorescein isothiocyanate *Pisum sativum* agglutinin that binds selectively to the acrosome content. According to the results, a reacted acrosome was present in 96% of spermatozoa with partial birefringence and only in 35% of those with totally birefringent heads. A great proportion of sperm cells with normal morphology showed total birefringence both in the presence (59%) or in the absence of motility (45%; $P < 0.01$), while in morphologically abnormal spermatozoa the frequency of total birefringence was comparable to that of partial birefringence irrespective of motility (26% and 27%, respectively, in motile spermatozoa; 22% and 19%, respectively, in immotile spermatozoa). These data support a strong association between partial birefringence and reacted acrosome and show that the patterns of birefringence vary depending on sperm motility and morphology.

KEYWORDS: acrosome reaction, sperm birefringence, sperm motility, sperm morphology, sperm selection

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Introduction

The introduction of intracytoplasmic sperm injection (ICSI) in assisted reproduction treatment has drastically decreased the requirements in terms of concentration, motility and morphology for spermatozoa to be used in assisted conception cycles. Nevertheless, the capacity of an apparently normal, motile spermatozoon to generate a viable embryo remains related to the incidence of abnormalities in the sperm inner structures, including DNA integrity (Sakkas and Alvarez, 2010; Speyer et al., 2010), chromatin condensation (Angelopoulou et al., 2007; Hammoud et al., 2009) and the predisposition to chromosomal errors (Bernardini et al., 2000; Calogero et al., 2003; Gianaroli et al., 2005) as well as the capacity of inducing oocyte activation (Heytens et al., 2009). Most of these structural and functional anomalies increase proportionally to the severity of the male factor condition and reach the highest frequency in severely pathological sperm samples, which actually represent the most likely candidates to undergo ICSI (Gianaroli et al., 2005; Heytens et al., 2009; Magli et al., 2009; Speyer et al., 2010).

These considerations have motivated an increasing interest in defining suitable criteria that might reliably support the selection of the spermatozoon to be injected. In this respect, innovative techniques of sperm selection have been proposed for use during ICSI, taking into consideration that priority is generally given to morphologically normal, motile spermatozoa. Due to the small dimensions of the sperm cell, a meticulous analysis of its morphology requires the use of a high-magnification view that should be preferably compatible with a comfortable handling of the cells to be injected. Based on this consideration, two approaches have been described: (i) intracytoplasmic morphologically selected sperm injection, based on the use of high-resolution digital imaging to achieve a magnification of up to ×6300 (Bartoov et al., 2002); and (ii) a technique that expands the use of high magnification (up to ×5500) by including the evaluation of the birefringence properties that human sperm cells naturally possess due to the presence of longitudinally oriented protein filaments (Gianaroli et al., 2008). According to studies at transmission electron microscopy (TEM), the presence of sperm head birefringence reflects the normality of the structural organization in the nuclear and acrosomal regions (Baccetti, 2004).

An improved clinical outcome has been documented following the application of either both intracytoplasmic morphologically selected sperm injection (Antinori et al., 2008; Cassuto et al., 2009; Souza-Setti et al., 2010; Vanderzwalmen et al., 2008) or birefringence (Chattopadhyay et al., 2009; Gianaroli et al., 2008, 2010), implying that the selection of morphologically normal spermatozoa could have a positive impact on embryo viability. For both techniques, recent prospective randomized studies reported a significant advantage for severe cases of male infertility (Balaban et al., 2011; Gianaroli et al., 2008).

From a practical point of view, sperm assessment by polarization microscopy does not require software for the digital imaging processing, for which sperm viewing can be performed in real time. The detection of birefringence is very clear with the ×40 objective and Hoffman optics. The ×63 objective permits a detailed analysis of sperm morphology without the need of immersion oil for which ICSI can be performed concomitantly to the selection of the sperm cell in the same dish. In this way, an additional criterion for sperm selection is provided that goes beyond the characteristics of morphology and motility.

A previous study has documented the presence of different patterns of sperm head birefringence, of which two, either total or partial (i.e. localized in the post-acrosomal region), were reported to correlate on the basis of TEM observations with acrosome integrity (Gianaroli et al., 2010). More specifically, total birefringence was associated with an intact acrosome, whilst partial birefringence was proposed to indicate a sperm cell that had already undergone the acrosome reaction. Although no direct demonstration of this correlation was provided, the clinical performances of these two forms were found to be significantly different, those with partial birefringence being associated with the highest viability of the resulting embryos (Gianaroli et al., 2010). These results were also confirmed in another study (Chattopadhyay et al., 2009).

Recent studies have analysed the status of DNA integrity in relation to the patterns of birefringence. A significantly higher incidence of DNA fragmentation was reported in spermatozoa without birefringence compared with those with birefringent heads (Crippa et al., 2009). In addition, DNA fragmentation was found to be more frequent in cells with total birefringent heads compared with those with partial birefringence (Petersen et al., 2011).

Although these findings are not in contradiction as they both support the highest viability of birefringent spermatozoa and, particularly, those with partial birefringent heads, the question arises as to whether this characteristic is due to the acrosome status or to DNA integrity. As mentioned above, the relationship between the pattern of birefringence and the acrosome status was based on TEM results by assuming that when the acrosome reaction takes place the local protein organization disaggregates and the corresponding birefringence effect in the acrosomal region is lost. To confirm this hypothesis, the aim of this study was to perform a direct analysis of the acrosome integrity in single spermatozoa having different patterns of birefringence. Once the correlation between birefringence and acrosome status was validated, the distribution of the different patterns of birefringence was investigated in relation to sperm motility and morphology and finally it was verified whether the acrosome integrity could vary depending on these parameters.

Materials and methods

Patients

A spermiogram was performed on 50 samples from 50 infertile patients according to WHO criteria (World Health Organization, 1999). Of the 50 samples, 26 were normozoospermic, 12 were moderate oligoasthenoteratozoospermic (OAT) and 12 were severe OAT. Spermatozoa from these samples were then analysed for birefringence at the polarization microscope and the corresponding patterns were related to motility and morphology of each single cell. The study was discussed and approved by the Institutional Review Board (ref. No. 281008).

Analysis of birefringence

After liquefaction, a 10-μl aliquot of each sample was observed at an inverted microscope (Leica DMIRB; Leica Microsystem, Wetzlar, Germany) equipped with polarizing lenses and Hoffman Modulation Contrast (Gianaroli et al., 2008). Birefringence was already evident with the ×40 objective, but for a more detailed morphological analysis spermatozoa were evaluated through a PL Fluotar L63X objective and the images were enhanced by video coupler magnification (Leica Microsystem). The use of the zoom connected to the camera provided a total magnification on the monitor screen between ×2500 and ×5500.

The specific characteristics of birefringence were assessed in single spermatozoa by observing the corresponding images in real time on the computer screen and using specific tools for the concomitant measurement of dimensions and areas. In this way, sperm dimensions and vacuolar-like areas were recorded, providing an accurate classification of sperm morphology in fresh samples assisted by enhanced magnification.

For the evaluation of the sperm head birefringence, glass bottom dishes (Willco Wells, Amsterdam, The Netherlands) were used and prepared with 5 μl PVP (SAGE) and 1 μl spermatozoa. These dishes were preferred to plastic as a thin layer of glass minimizes the birefringence effect that is naturally related to the passage of light through the plate itself (Montag, 2008). The presence of PVP facilitated the analysis of motile spermatozoa that in most cases were also gently immobilized with the ICSI pipette. In the sperm head, three types of birefringent patterns were basically distinguished: (i) spermatozoa with a totally birefringent head (**Figure 1A**); (ii) spermatozoa with a partially birefringent head localized in the post-acrosomal region, considering that the acrosome represents 40—70% of the sperm head (**Figure 1B**); and (iii) sperm heads with an abnormal pattern of birefringence due to absence of birefringence, the presence of vacuole-like structures or small areas of birefringence localized either in the nuclear or acrosomal area (**Figure 1C**,**D**).

WHO parameters were used to assess morphology in fresh samples with the following categories of defects: (i) head defects (shape, dimensions, vacuoles, nucleus and acrosome); (ii) tail defects (including neck and mid-piece defects; and (iii) combined defects in the different sperm compartments (both head and tail). According to WHO criteria, normal sperm heads had length and width of 4.0—5.0 μm and 2.5—3.5 μm, respectively, vacuoles occupying an area not greater than 20% and the acrosome representing 40—70% of the head area (World Health Organization, 1999).

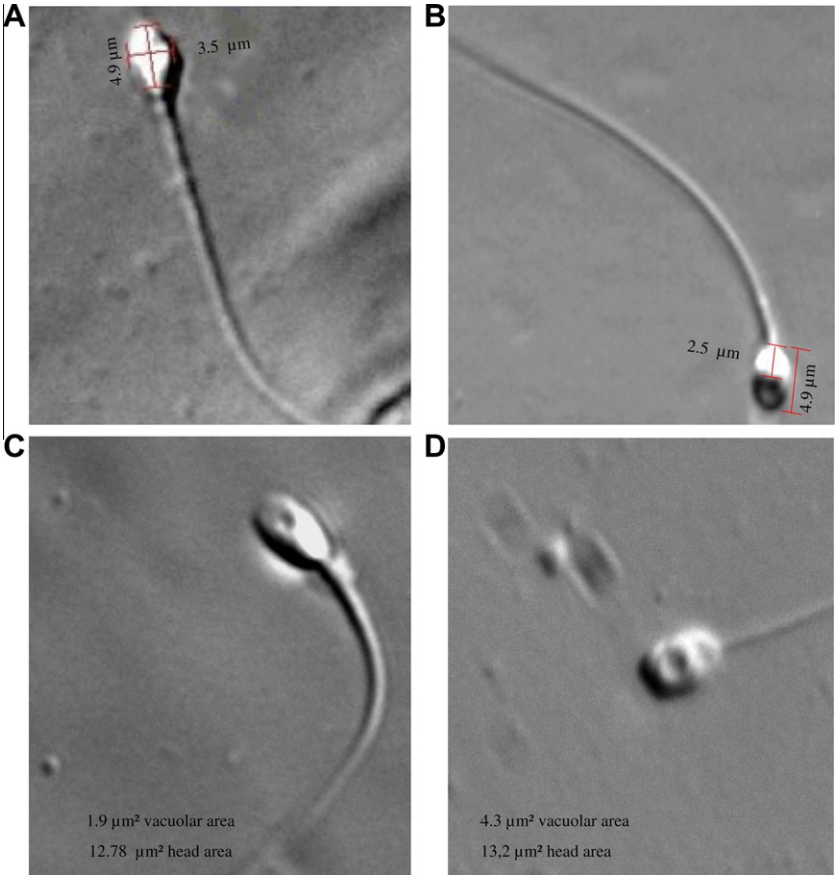

Figure 1 Observation and measurement of sperm heads under polarized light (×63 objective; magnification ×630). (**A**) Spermatozoon with normal morphology having a total head birefringence. (**B**) Spermatozoon with normal morphology having partial birefringence localized in the post-acrosomal region. (**C**) Presence of a small vacuole-like structure in a partially birefringent spermatozoon with normal morphology; the vacuole occupies 15% of the sperm head volume. (**D**) A large vacuole-like structure covering 33% of a non-birefringent head.

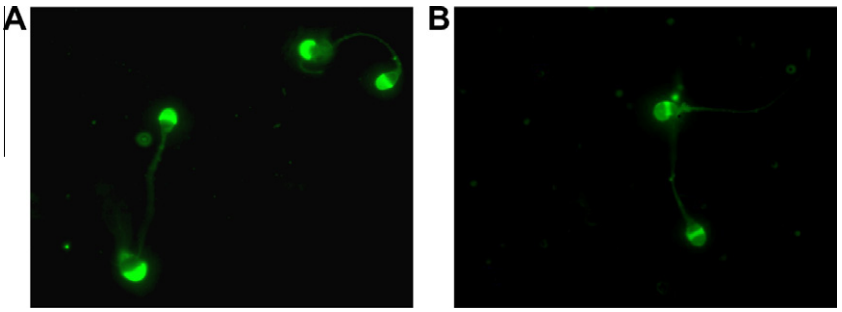

Figure 2 Scoring of sperm cells after treatment with fluorescein isothiocyanate *Pisum sativum* agglutinin by fluorescent illumination using a 520—540 nm filter. They were classified as acrosome intact when exhibiting uniform green fluorescence in the acrosomal region (**A**) or acrosome reacted in case of no fluorescence or when fluorescence was localized in an equatorial green band (**B**).

Examples of the observed sperm cells under the polarizing light with the corresponding measurements are presented in **Figure 1**. All analyses were performed by two independent operators and results were compared. In case of discrepancies, results were discussed for final agreement.

Separation of spermatozoa according to the pattern of head birefringence

After fluidification, 1 ml sperm samples were centrifuged at 300***g*** for 10 min and motile spermatozoa were recovered after 30 min of swim up. Immotile spermatozoa were not considered to exclude any possibly dead cells. Glass-bottomed culture dishes (Willco Wells) were prepared with 10 μl drop of pre-warmed buffered human tubal fluid (SAGE Cooper Surgical, Pasadena, CA, USA) supplemented with 5% human serum albumin (SAGE) overlaid with pre-equilibrated mineral oil (SAGE). An additional drop contained 5 μl polyvinylpyrrolidone (PVP; SAGE) and 1 μl sperm preparation. Based on the criteria described above, sperm cells with a totally birefringent head were separated by those having a partial birefringence localized in the post-acrosomal region and gently transferred with an ICSI pipette to two different drops of medium. In each group, a mean of 20 spermatozoa were isolated.

Evaluation of the acrosome reaction

The selected spermatozoa were all transferred with an ICSI pipette to 10-μl drops of distilled water in a slide with concave wells (12 wells, 5.2 mm; Thermo Scientific, Menzler-Glaser Diagnostika, Braunschweig, Germany). After complete evaporation, permeabilization in 95% ethanol in phosphate-buffered saline followed by incubation at 4°C for at least 30 min. When the ethanol was completely evaporated at room temperature, 20 μl of 100 μg/ml fluorescein isothiocyanate *Pisum sativum* agglutinin (PSA-FITC) were added and incubated in the dark in a humid chamber for 5 min. The slide was rinsed in distilled water followed by counterstaining with 4′6-diamidino-2-phenylindole. Sperm cells were scored under fluorescent illumination with a ×60 objective using a 520—540 nm filter and were classified as acrosome intact when exhibiting uniform green fluorescence or acrosome reacted in cases of no fluorescence or when fluorescence was localized in an equatorial green band (Bronson et al., 1999) (**Figure 2**).

Statistical analysis

Data were analysed by chi-squared analysis applying the Yates' correction with 2×2 contingency tables. Results were considered significant at $P < 0.05$.

The distribution of the four semen morphological types (normozoospermic, anomalies of the head, anomalies of mid-piece and tail and combined anomalies) in relation to the three birefringence patterns (total, partial or abnormal) was assessed with the Shannon distribution diversity index of categorical data (McDonald, 2003). The Shannon index is calculated as follows: $H' = \sum(p_i \ln p_i)$, for i to S and where n_i is the number of individuals in each defined species, S is the number of species (also called species richness) and p_i is the relative abundance of each species, calculated as the proportion of individuals of a given species to the total number of individuals in the community: n_i/N where N is the total number of all individuals. Typically the value of the index ranges from 1.5 (low species richness and evenness) to 3.5 (high species evenness and richness).

Results

Birefringence and acrosome reaction

To verify the correspondence between the patterns of total and partial birefringence with the acrosome status, a mean number of 20 motile sperm cells in each category (total birefringence and partial birefringence) were assessed accounting for a total of 1962 evaluated cells.

As represented in **Table 1**, 639 cells (65%) out of 988 spermatozoa with a pattern of total birefringence showed an intact acrosome at the analysis performed by PSA-FITC, while the acrosome was reacted in the remaining 349 (35%). In the presence of birefringence localized in the post-acrosomal region, 931 out of the 974 studied spermatozoa (96%) had undergone the acrosome reaction, whereas 43 (4%) still had an intact acrosome. This distribution was significantly different ($P < 0.001$).

Table 1 Pattern of sperm head birefringence in relation to acrosome status.

Acrosome status	*Total spermatozoa* (n = *1962*)	*Sperm head birefringence*	
		Total (n = *988*)	*Partial* (n = *974*)
Intact	682	639 (65)[a]	43 (4)[a]
Reacted	1280	349 (35)[b]	931 (96)[b]

Values are *n* (%). Partial birefringence = localized in the post-acrosomal region.
Values with same letters are significantly different: [a,b]$P < 0.001$.

Table 2 Sperm morphology in relation to sperm head birefringence: motile and immotile spermatozoa.

Birefringence	*Normal morphology* (n = *849*)		*Abnormal morphology* (n = *2097*)	
	Motile (n = *742*)	*Immotile* (n = *107*)	*Motile* (n = *1576*)	*Immotile* (n = *521*)
Total	437 (59)[a,b,c]	48 (45)[b,d]	409 (26)[a]	115 (22)[c,d]
Partial	218 (29)	25 (23)	422 (27)[e]	99 (19)[e]
Abnormal	87 (12)[f,g,h,i]	34 (32)[f,h,i]	745 (47)[f,g,j]	307 (59)[h,j]

Values are *n* (%). Partial birefringence = localized in the post-acrosomal region.
Values with same letters are significantly different: [a,c,d,f–j]$P < 0.001$.
[b]$P < 0.01$
[e]$P < 0.005$.

Distribution of birefringence patterns in relation to motility and morphology

To characterize the distribution of birefringence patterns in relation to motility and morphology, a total of 2946 spermatozoa (2318 motile and 628 immotile) from the 50 patients were assessed using the inverted microscope described above. For all the following experiments, morphology evaluation was performed in the fresh sample as previously described.

Table 2 shows that the distribution of birefringence properties was significantly different in relation to sperm morphology both in motile and in immotile spermatozoa ($P < 0.01$–0.001). The lowest level of abnormal birefringence was found in motile, morphologically normal spermatozoa (12%) and this figure increased proportionally to the loss of motility (32%) and to the presence of abnormal morphology (47% and 59% in motile and immotile sperm cells, respectively).

A great proportion of sperm cells with normal morphology showed a total head birefringence both in the presence (59%) or in the absence of motility (45%; $P < 0.01$), while in morphologically abnormal spermatozoa the frequency of total head birefringence was comparable to that of partial head birefringence irrespective of motility (26% and 27%, respectively, in motile spermatozoa; 22% and 19%, respectively, in immotile spermatozoa). More specifically, the ratio between total and partial birefringence was related to morphology and remained close to 2:1 in sperm cells with a normal morphology irrespective of their motility and decreased to approximately 1:1 in morphologically abnormal spermatozoa, both motile and immotile.

Figure 3 reports the data on the analysis of birefringence patterns in the different types of sperm morphological categories. To avoid any influence related to the presence or absence of motility, this analysis was only conducted in motile spermatozoa. When compared with spermatozoa with normal morphology, the proportion of spermatozoa with total birefringence was significantly lower in all the observed types of morphological abnormalities, with the lowest value (17%) in spermatozoa having combined defects ($P < 0.001$). The opposite was true for spermatozoa with abnormal head birefringence, whose proportion increased in all morphologically abnormal sperm categories with the highest value in sperm cells with combined defects. Partial birefringence was present at comparable frequency in all sperm types with the exception of those having combined defects where it reached the lowest value (16%, $P < 0.001$ versus all other groups). In agreement with the above reported observations, the ratio between total birefringence and partial birefringence was 1:1 in all sperm defect categories while it was 2:1 in spermatozoa with normal morphology.

When looking specifically at the presence of vacuole-like structures whose area exceeded the normal limit of 20%, it was found that they were equally distributed among the three patterns of birefringence.

Distribution of birefringence patterns in the different sperm categories

When analysing the different sperm categories, the proportion of spermatozoa with an abnormal pattern of birefringence was 26% in normospermic patients (415/1601

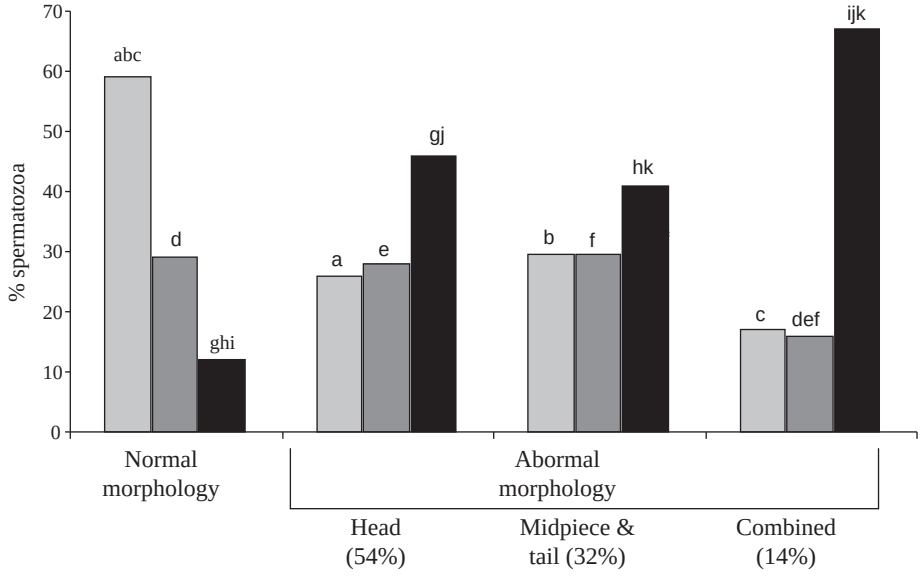

Figure 3 Sperm head birefringence in relation to morphology as assessed at the inverted microscope in fresh samples. The morphological abnormalities were grouped according to following types: head defects, tail defects (including neck and mid-piece) and presence of combined defects (both in head and tail). Three patterns of head birefringence were recognized: total (light grey bars), partial birefringence (dark grey bars) and abnormal birefringence (black bars). Values in groups with morphological defects were compared with spermatozoa with normal morphology. Bars with same letters are significantly different ($P < 0.001$).

spermatozoa), 38% in moderate OAT (259/677 spermatozoa) and 75% in severe OAT samples (499/668 spermatozoa). All these figures were significantly different ($P < 0.001$). In normospermic patients, total birefringence was present in 710 spermatozoa (44%) and partial birefringence was detected in 476 spermatozoa (30%). These figures were 222 (33%) and 196 (29%), respectively, in moderate OAT, and 77 (11%) and 92 (14%), respectively, in severe OAT.

Discussion

Birefringence in sperm heads reflects an organized and very compacted texture that indicates nuclear and acrosomal structural normality and presents different patterns in relation to the sperm acrosomal status. Until very recently, these observations were based on TEM results for which it was proposed that partial birefringence was due to an already occurred acrosome reaction. It is clear now, after direct analysis of the acrosome content, that a pattern of partial birefringence is a very strong predictor of a reacted acrosome (96% of cells showing a partial birefringence had a reacted acrosome; **Table 1**). On the other hand, this study observed that total birefringence denoted an intact acrosome in only 65% of spermatozoa. It can be postulated that this figure is conditioned by the dynamism of the acrosome reaction that represents the last event in a series of intracellular mechanisms triggered by the removal of the seminal plasma. In the experimental scheme of this work, motile spermatozoa were first selected on the basis of their birefringence pattern and then treated to test the acrosome status. It is possible indeed that during the time necessary to complete the sperm selection some cells in the human tubal fluid medium started or completed the acrosome exocytosis (Harper et al., 2006) and that for this reason they were negative at the PSA-FITC test.

The possibility of identifying the acrosome status has important clinical implications, as already pointed out by data reporting that reacted spermatozoa are associated with a higher development of ICSI embryos (Mansour et al., 2008). Accordingly, the injection of spermatozoa with partial head birefringence yielded an improved delivery rate when compared with spermatozoa with total birefringence (Gianaroli et al., 2010).

The recent studies documenting an association between sperm head birefringence and DNA integrity (Crippa et al., 2009; Petersen et al., 2011) contributed additional information about the sperm capacity to sustain fertilization and further development after ICSI. As far as is known, anomalies of sperm chromatin packaging and incomplete nuclear remodelling occurring during spermatogenesis could be associated with birefringence patterns, as expressed by the negative correlation between birefringence and DNA fragmentation (Crippa et al., 2009). In addition, DNA fragmentation is significantly more frequent in spermatozoa with total birefringence compared with those with partial birefringence (Petersen et al., 2011) that, according to this study centre's experience, are those having a reacted acrosome and a higher capacity of giving rise to implantation (Gianaroli et al., 2010). These findings altogether confirm what was already proposed by Ozmen et al. (2007); damage in DNA structure could negatively affect the sperm capacity to undergo the acrosome reaction and the consequent steps following its entry into the oocyte. This could explain the highest clinical outcome associated with the injection of reacted spermatozoa. Therefore, the evaluation of birefringence properties becomes important as it permits the identification with strong approximation of sperm cells having a reacted acrosome and the highest chances of DNA integrity, which are prerequisites for further development.

Not surprisingly, the data from the present study show that the great majority of morphologically normal, motile sperm cells also possess a normal head inner structure, as demonstrated by regular patterns of birefringence, either total (59%) or partial (29%), in 88% of sperm heads (**Table 2**). This proportion decreases significantly to 53% (26% total and 27% partial birefringence) in the category of morphologically abnormal, motile spermatozoa, suggesting that when these cells are selected for ICSI by the conventional method (inverted microscope at ×200–400 magnification and absence of birefringence evaluation), half of the oocytes would be injected with a sperm cell having structural abnormalities in its head.

Going to a more detailed analysis of spermatozoa with morphological abnormalities, it was evident that in these cells, irrespective of the type of defect, an abnormal pattern of birefringence occurred at higher incidence when compared with cells with a normal morphology (**Figure 3**). This was obviously true in the groups with sperm head abnormalities, standing either as single defect or in combination with others, but it also happened with tail defects. It is possible indeed that a morphological alteration could represent the tip of the iceberg of an abnormal condition, with profound defects being present in the protoplasmic compartment as well. A similar situation was reported for the presence of mid-piece defects that strongly correlated with DNA fragmentation (Speyer et al., 2010). Noteworthy, the ratio between total birefringence and partial birefringence in the considered morphological abnormalities underwent a dramatic change, with a significant decrease in the number of spermatozoa with total birefringence. Considering that totally birefringent spermatozoa are those with an acrosome that is still intact or, provided that DNA is not fragmented, in the process of initiating its reaction, the number of potentially 'useful' spermatozoa (light grey and dark grey bars in **Figure 3**) in teratozoospermic samples, especially in the presence of combined defects, becomes critically low.

As reported in Table 2, the worst situation is found – not surprisingly – in morphologically abnormal and immotile spermatozoa, where a disorganized inner structure is present in 59% of cells. This condition could contribute to the reduced viability of embryos generated by severely pathological sperm samples, including testicular spermatozoa that are mainly immotile, or very poorly motile, and often dimorphic. However, when considering the birefringence patterns of immotile spermatozoa with a normal morphology, it was found that even though they showed a proportion of birefringence abnormalities higher (32%) than the group with motility and normal morphology (12%), the distribution of their birefringence characteristics was closer to that of morphologically normal, motile spermatozoa (same ratio of total birefringence to partial birefringence, 2:1). The situation was completely different from that characterized in the group of motile spermatozoa with an abnormal morphology (47% abnormal birefringence, ratio of total birefringence to partial birefringence 1:1; **Table 2**) suggesting that sperm morphology has a stronger effect than motility on the organization of the inner sperm structures as expressed by birefringence characteristics. These considerations could be relevant when a decision has to be made to privilege motility or morphology for sperm selection for conventional ICSI.

In conclusion, a pattern of partial birefringence localized in the post-acrosomal region is strongly associated with a reacted acrosome as well as with DNA integrity (Petersen et al., 2011), representing a robust indicator for a 'healthy' sperm cell. In addition, the distribution of birefringence patterns is significantly different in relation to sperm morphology and motility, with morphology appearing to have a stronger effect than motility on birefringence characteristics. In view of these considerations, the study of sperm head birefringence seems to provide an extra tool that, along with the characteristics of motility and morphology, predisposes the best conditions for the selection of a competent spermatozoon.

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