Polish Journal of Veterinary Sciences Vol. 16, No. 4 (2013), 773–785

DOI 10.2478/pjvs-2013-0112

Review

Evaluation of morphological criteria of sperm quality before *in vitro* fertilization and intracytoplasmic sperm injection

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Abstract

The quality of sperm has a direct influence on the fertilization and developmental competence of embryos. In the literature we did not find defined criteria for evaluation of normal sperm parameters in various species of domestic mammals. Therefore we attempted to review evaluation of criteria of morphologically normal human sperm and their abnormalities. All sperm cells observed in the stained sample are classified as normal or abnormal. Any abnormalities in morphology of sperm have a negative effect on the outcome in *in vitro* fertilization and intracytoplasmic sperm injection. Abnormal sperm are categorized into subgroups according to the observed defects (concerning the head and/or midpiece and/or tail). Most morphologically abnormal sperm have multiple defects. This article can be considered as guideline for the manual of sperm quality evaluation in different species of domestic mammals.

Key words: sperm, quality, morphological criteria

Introduction

The development of *in vitro* produced domestic animals and human embryos is directly dependent on the quality of the oocytes and sperm which are used for *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI). The examination of oocyte (Lasiene et al. 2009, 2011) and sperm morphology is currently considered to be a tool devoted to the fertility prognosis. Sperm morphology has been recognized as the best predictor of outcome for natural fertilization, intra-uterine insemination and IVF. The sperm morphology also plays a significant role in ICSI outcome (Berkovitz et al. 2005). Morphological criteria

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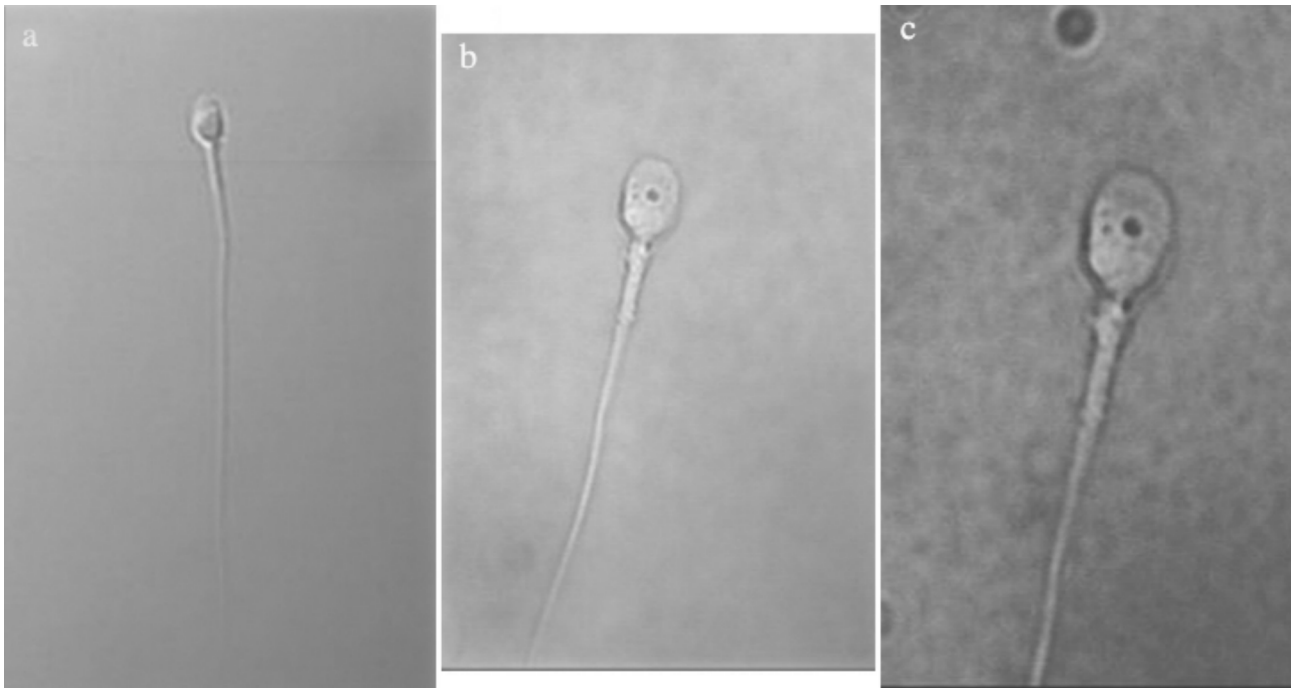

Fig. 1. A human sperm (Antinori et al. 2008): (a) ×400, apparently suitable for intracytoplasmic sperm injection procedure; (b) ×3670, the same spermatozoon showing head (vacuoles, vesicles, irregular post-acrosomal lamina) and neck malformations; (c) ×5880, a close-up of the previous malformations.

of sperm can show differences in sperm quality of men in different regions and countries (Jørgensen et al. 2001, 2002, Punab et al. 2002, Haugen et al. 2006, López-Teijón et al. 2008, Fernandez et al. 2012).

We did not find the guidelines for evaluation of normal sperm parameters in domestic mammals. Many authors using different staining methods describe various morphologic abnormalities of sperm in bulls (Revay et al. 2009, Freneau et al. 2010, Mandal et al. 2010, Enciso et al. 2011), stallions (Kavak et al. 2004, Morrell et al. 2008, Einarsson et al. 2009, Brito et al. 2011, Love 2011), rams (Lambrechts et al. 2000, Janett et al. 2001, Sweeney et al. 2007, Lopez Armengol et al. 2012) and dogs (Kawakami et al. 2005, Nxñez-Martinez et al. 2005, Peña et al. 2007, Lange-Consiglio et al. 2010). But we did not find concrete references what is the morphologically normal sperm cell in domestic animals. Only World Health Organization (WHO) published laboratory manuals which determined normal values criteria for the analysis of human sperm and seminal fluid on the basis of laboratory tests.

Therefore using two last WHO manuals (1999, 2010) and many researchers' references we attempted to review criteria of normal human sperm cells and their abnormalities.

The fourth edition of the WHO manual lists categories of sperm defects that should be considered; however, precise definitions of each category are not given (WHO 1999). WHO revised criteria substantially and provided the 5th edition of manual for the human semen analysis (WHO 2010) that addresses a lot of criticisms (Cooper et al. 2009, Brazil 2010, Ford 2010, Menkveld 2010, Skakkebaek 2010, Menkveld et al. 2011). We do not want to get involved in scientific discussions; we just want to review the morphological criteria of the normal sperm and various abnormalities of sperm.

Evaluation of sperm morphology

The sample should be collected after a minimum of 2 days and a maximum of 7 days of sexual abstinence. If additional samples are required, the number of days of sexual abstinence should be as constant as possible at each visit. It is impossible to characterize a man's semen quality based on evaluation of a single semen sample, because intra-individual variation is very significant. The first ejaculate gives a correct conclusion in at least 85% of cases. It is helpful to examine two or three samples to obtain more precise data (Rylander et al. 2009, WHO 2010).

There are two methods of examination of sperm morphology based on the microscopic analysis of stained samples, either through visual observations (manual methods) or by using computer vision-derived methods. For either of these methods, the drop-

let of the semen sample was smeared on a glass-slide, air-dried and fixed. Sperm samples must be optimally stained for the providing of sharp contrast for defining the sperm outline and cell details.

There are a lot of methods for the staining of sperm. World Health Organization recommends three staining methods for the evaluation of morphology of human sperm: Papanicolaou, Shorr or Diff-Quik (WHO 1999, 2010).

The stained samples are analyzed by the final magnification ×1000 with oil immersion. Using a standard microscopic approach, the laboratory technician examines 100 spermatozoa and categorizes each sperm cell as normal or abnormal. Subsequently the anomalies are classified using strictly defined criteria. By contrast, computer-assisted technology measures different morphological features (mostly head parameters) for each selected sperm cell (van der Horst et al. 2009, Auger 2010, Bellastella et al. 2010, Blanchard et al. 2011, Butts et al. 2011). Therefore, the parameters of normal sperm can differ according to staining method of sperm sample (Maree et al. 2010).

When a lot of abnormal sperm (teratozoospermia), low concentration of sperm (oligozoospermia) or reduced sperm motility (asthenoteratozoospermia) were found in the semen, some laboratories have started use to methods for analysis of chromatin and chromosomal abnormalities, apoptosis, mitochondrial membrane potential and ultramorphology of sperm.

The ultrastructure of sperm head, neck and tail can be analyzed and quantified by scanning or transmission electron microscopy (Moretti et al. 2007, Collodel et al. 2009a,b, Skowronek et al. 2010, Visco et al 2010, Skowronek et al. 2012, Collodel et al. 2013, Gatimel et al. 2013).

Fuorescence microscopy of samples stained with acridine orange, aniline blue and toluidine blue, acridine orange-propidium iodide is used for identification of sperm nuclei with the abnormal chromatin condensation and the ploidy (Erenpreiss et al. 2001, Erenpreisa et al. 2002, Bungum et al. 2007, Mahfouz et al. 2009, Moskovtsev et al. 2009, Lazaros et al. 2011, Montjean et al. 2012).

Single fluorescence Hoechst 33258 dye can be used to determine the live and dead sperm, which could have presented a loss in acrosomal content. Living X- and Y-chromosome bearing sperm can be sorted using Hoechst 33342 (Vigil et al. 2008, Garner 2009, Karabinus 2009, Alçada-Morais et al. 2013). Apoptotic sperm are differentiated from necrotic using double staining with fluorescein isothiocyanate (FITC)-labeled annexin V and Hoechst 33258 (Collodel et al. 2009b, de Vantery Arrighiet al. 2009). The number of viable acrosome-reacted sperm can be evaluated by staining with Hoechst 33258 and fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin lectin (Vigil et al. 2008, Tapia et al. 2011, Montjean et al. 2012, Vigil et al. 2012).

The labeling of DNA strand breaks (the terminal transferased UTP nick-end labeling (TUNEL) assay), Annexin V- propidium iodide assay and Sperm Chromatin Structure Assay are used for the detection and quantification of apoptosis in sperm (DNA fragmentation) (Erenpreisa et al. 2002, Ricci et al. 2002, Oosterhuis and Vermes 2004, Mahfouz 2009, Rylander et al. 2009, Gatimel et al. 2013, Gomez-Lopez et al. 2013, Ruvolo et al. 2013).

Meiotic chromosome segregation in sperm nuclei (disomy and diploidy) is investigated by fiuorescence in situ hybridisation (FISH) method using probes for chromosomes 13, 17, 18, 21, X and Y (Bielanska et al. 2000, Moretti et al. 2007, Collodel et al. 2009b, Zhou et al. 2011, Mokánszki et al. 2012, Perdrix et al. 2013).

Acrosomal content before acrosome reaction is labelled with fiuorescein-iso-thiocyanate (FITC)-conjugated peanut agglutinin. Inner acrosomal membrane is labelled before membrane permeabilization with a mouse anti-CD46 monoclonal antibody and TexasRed-conjugated anti-IgG secondary antibody (Gatimel et al. 2013, Zou et al. 2013).

The integrity of the mitochondrial membrane potential in sperm can be detected by a cell-permeant, cationic, fluorescent dye Rhodamine 123 that is readily sequestered by active mitochondria without inducing cytotoxic effects (O'Connell et al. 2002, de Vantery Arrighi et al. 2009).

Prior to the ICSI procedure it is necessary to evaluate live sperm morphology. A new method for observing spermatozoa prior ICSI is called "motile sperm organelle morphology examination" (MSOME), which enables the evaluation of the fine nuclear morphology of motile spermatozoa in real time at high magnification (×6000 and more). As a consequence, a new microinjection procedure is called "intracytoplasmic morphologically selected sperm injection" (IMSI). Six sperm cellular elements (neck, tail, midpiece, mitochondria, acrosome and post-acrosomal lamina) and the sperm nucleus (shape, chromatin content) are morphologically examined at high magnification by the inverted computerized microscope. Using this technique, it is possible to observe morphological abnormalities in sperm cells, which are not visible with magnification ×400 (Fig. 1) (Antinori et al. 2008, Nadalini et al. 2009, Sermondade et al. 2011, Sermondade and Sifer 2011, Montjean et al. 2012, Silva et al. 2012, Gatimel et al. 2013).

A new method for analysis of sperm quality is the estimation of sperm head birefringence by polarized light. The birefringence of sperm head can be studied

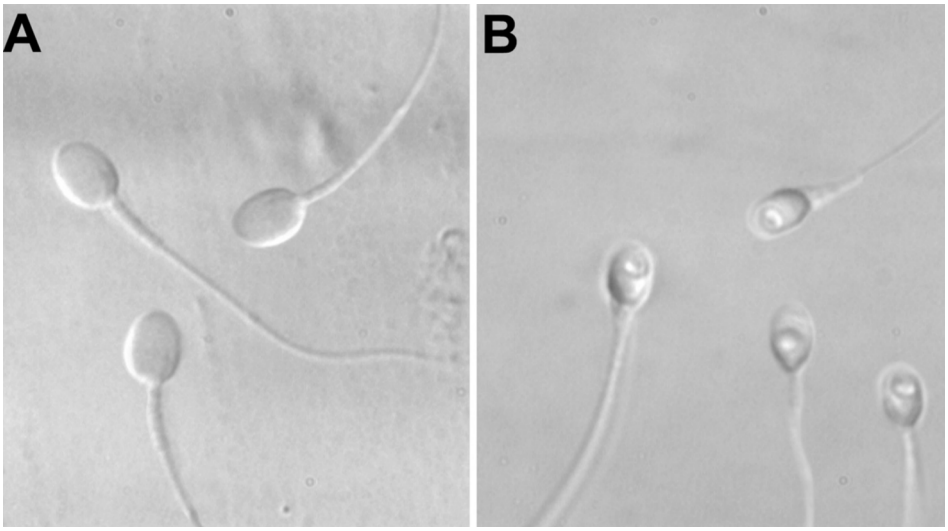

Fig. 2. A. Normal sperm. B. Sperm with large nuclear vacuoles (magnification ×8400) (Oliveira et al. 2009).

using polarized microscope. Sperm cells are birefringent (optically anisotropic). When the microscope light enters an anisotropic structure, it is refracted into two different refractive rays indicating this double refraction (Gianaroli et al. 2008, 2010, Petersen et al. 2011, Magli et al. 2012, Collodel et al. 2013). Microinjection into retrieved oocytes of individually selected spermatozoa with a strictly defined morphologically normal nuclear shape and content resulted in significantly higher pregnancy rates compared to the conventional ICSI (Bartoov et al. 2003, Berkovitz et al. 2005, Antinori et al. 2008, Cassuto et al. 2009).

Morphologically normal sperm

The analysis of sperm morphology includes the assessment of sperm head, neck, midpiece and tail.

The analysis of sperm head includes the assessment of head size, shape, covering acrosomal area (acrosomal index) and acrosomal vacuoles. The acrosomal index of the sample is defined as the percentage of sperm (out of 100) that exhibited a normal-sized acrosome. The presence of the acrosomal vacuoles is also confirmed by visual examination (Menkveld et al. 1990, Lee 1996, El-Ghobashy and West 2003).

All sperm cells observed in the stained sample are classified as normal (Fig. 2) or abnormal. The criteria for a normal sperm head are as follows: head length is 4 to 5 µm, width is 2.5 to 3.5 µm and length-width ratio is 1.5 to 1.75 (WHO 1999).

The normal shape of the nucleus is smooth, symmetric and oval. The normal nucleus should have an average length of 4.75 ± 0.28 µm and an average width of 3.28 ± 0.20 µm with no regional nuclear disorders and with no more than one vacuole that occupies less than 4% of the nuclear area (Bartoov et al. 2002, Berkovitz et al. 2005, Ubaldi and Rienzi 2008).

The normal acrosomal size is 40% to 70% of total sperm head area (WHO 1999). The presence of acrosomal vacuoles is considered as a significant marker of successful fertilization of oocytes *in vitro*. Men whose semen had 72% of sperm with acrosomal vacuoles showed better fertilization rate than men with 61.7% of sperm without acrosomal vacuoles. These vacuoles were concentrated between the inner and outer acrosomal membranes. The scientists suppose that the acrosomal vacuoles can present the migration of limited amounts of acrosin to the sperm surface. It may be the earliest event characterizing the beginning of the acrosome reaction (Tesarik et al. 1988, El-Ghobashy and West 2003).

Sperm head birefringence evaluation was used first by Gianaroli with co-authors (2008) for selection of sperm for ICSI. The presence of birefringence in the sperm head indicates the good morphology of a sperm cell. Normal sperm that has non-pyknotic nucleus and normal acrosome shows a strong negative birefringence. This birefringence is associated with longitudinally oriented subacrosomal protein filaments and nucleoprotein filaments which form longitudinally oriented strands. Gianaroli et al. (2008) demonstrated that significantly higher number of excellent quality embryos and a higher implantation rate were after injection of sperm with head birefringence when compared with sperm without head birefringence. These scientists carefully evaluated two types of sperm head birefringence on the basis of their acrosome integrity (partial head birefringence/acrosome-reacted and total head birefringence/acrosome-non-reacted sperm) and demonstrated that using of acrosome-reacted sperm for ICSI showed better

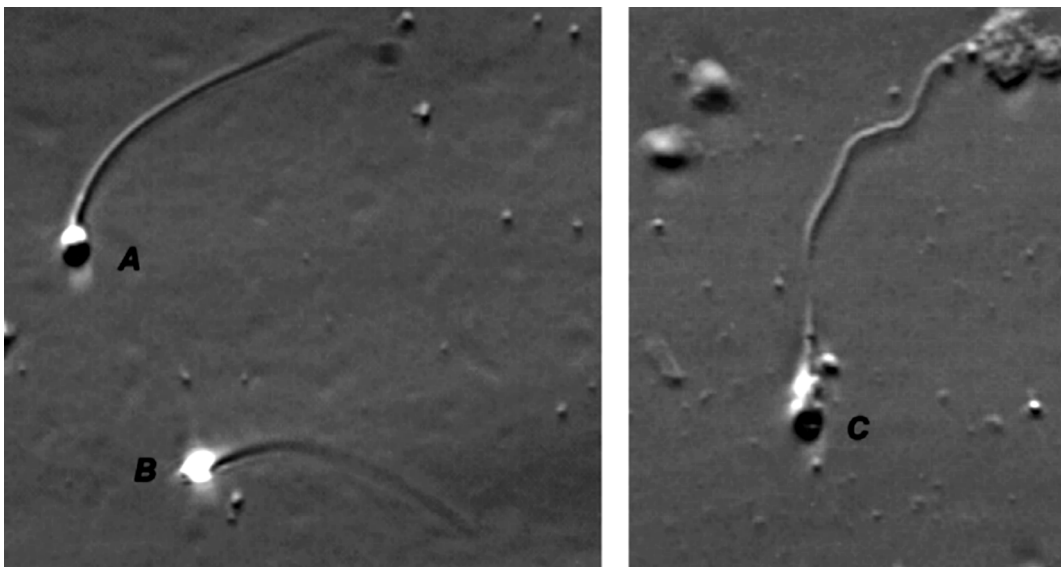

Fig. 3. The birefringence of human sperm head (Gianaroli et al. 2008). The birefringence in the postacrosomal region of sperm head indicates that the acrosome reaction has already occurred (A). The presence of birefringence in both compartments of the head, acrosome and nucleus, shows an intact acrosome in a nonreacted sperm (B). Sperm C is devoid of birefringence in the head because of the absence of a conventional sperm texture, with a vacuole in its head.

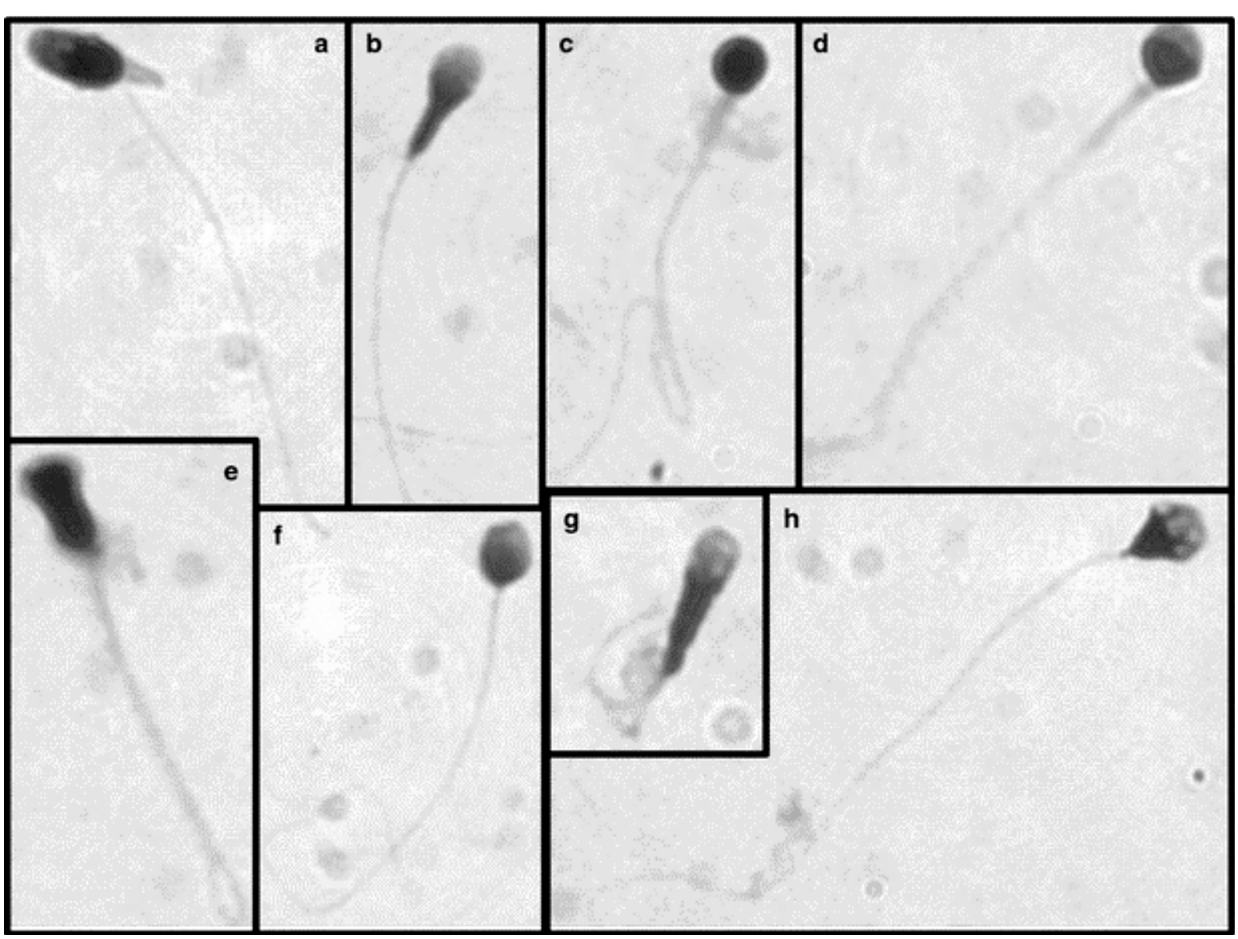

Fig. 4. Sperm with abnormal head morphology (Tang et al. 2010): (a) slightly tapered sperm head with bent neck and small cytoplasmic droplet; (b) pyriform sperm head; (c) small, round sperm head with no acrosome and thick midpiece; (d) round sperm head with normal acrosome area; (e) amorphous sperm head with no acrosome; (f) amorphous sperm head (small posterior indentation); (g) amorphous sperm head with coiled tail; (h) amorphous sperm head with abnormally large amount of vacuoles. Analysis of sperm morphology was performed on a phase-contrast microscope (Nikon Eclipse E600) equipped with a 100× oil-immersion objective lens and an eye piece micrometer calibrated (with a stage micrometer) to allow differentiation of 1 μm.

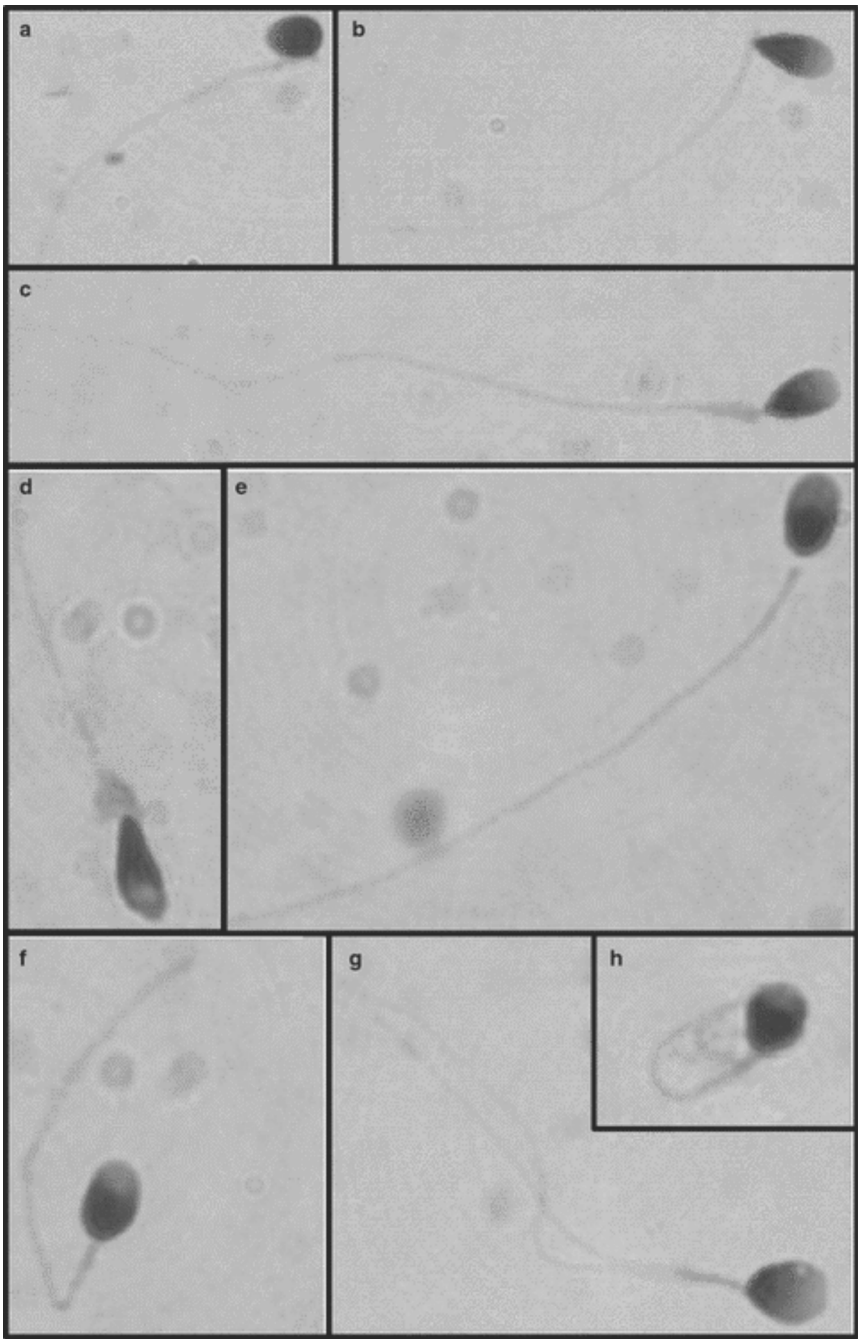

Fig. 5. Sperm with abnormal neck, midpiece and tail morphology (Tang et al. 2010): (a) asymmetric neck insertion with small acrosome area; (b) bent neck; (c) thick midpiece; (d) cytoplasmic droplet with amorphous sperm head; (e) thin midpiece; (f) bent tail; (g) two-tailed sperm; (h) coiled sperm tail. Analysis of sperm morphology was performed on a phase-contrast microscope (Nikon Eclipse E600) equipped with a 100× oil-immersion objective lens and an eye piece micrometer calibrated (with a stage micrometer) to allow differentiation of 1 µm.

results (Fig. 3) (Gianaroli et al. 2008, 2010, 2011, Magli et al. 2012). Acrosome-reacted sperm selected for ICSI presented lower ratio of DNA fragmentation and higher ratio of normal nucleus (Petersen et al. 2011).

The midpiece should be slender, 7-8 µm long, less than 1 µm in width and attached axially to the head. It must not have cytoplasmic droplets and/or disorders. The mitochondria must not be fragmented or damaged (Bartoov et al. 2002, Berkovitz et al. 2005, Ubaldi and Rienzi 2008, Oliveira et al. 2009).

The tail should be straight, uniform, and thinner than the midpiece, uncoiled and approximately 45-50 µm long. The normal tail/head length ratio is 10.3 ± 0.2 (Gergely et al. 1999, Celik-Ozenci et al. 2003, Oliveira et al. 2009).

Table 1. Morphological abnormalities and sperm pathology (Auger 2010).

Sperm defect (Light microscopy, ×1000)	Possible related TEM observations	Possible associated functional anomaly	Reference
1	2	3	4
Elongated head Major axis ↑ Minor axis =	Abnormally shaped head and abnormally condensed chromatin	Immature chromatin /fragmented DNA (possible disadvantage for embryo development) /increased aneuploidy	Dadoune et al. 1988 Gandini et al. 2000 Prisant et al. 2007
Thin head Major axis = Minor axis ↑	Abnormally shaped head and abnormally condensed chromatin	Immature chromatin/ fragmented DNA (possible disadvantage for embryo development)	Dadoune et al. 1988 Gandini et al. 2000
Microcephalous head Major axis ↓ Minor axis ↓	Excessive shrinking of the nucleus and abnormally condensed chromatin	Immature chromatin /fragmented DNA	Dadoune et al. 1988 Gandini et al. 2000
Macrocephalous head Major axis ↑ Minor axis ↑	Insufficient shrinking of the nucleus and abnormally condensed chromatin	Increased aneuploidy	Benzacken et al. 2001
Multiple heads More than one head	Two or more closed or dissociated heads with or without a common acrosome or midpiece	Handicaps migration through mucus and oocyte vestments /fragmented DNA	Gandini et al. 2000
Abnormal postacrosome region All outline and texture anomalies of the region	Abnormally shaped region post--acrosomal component and disorganization of the cape structures; abnormal DNA condensation	Possible disadvantage for gamete interaction	Gandini et al. 2000 Courtot et al. 1987 Longo et al. 1987 Escalier 1990
Abnormal acrosome region All outline, size and texture anomalies of region	Absent or abnormally shaped or sized acrosome, incomplete acrosome and/or abnormal appearance of the underlying nucleus	Abnormal-impossible acrosome reaction /fragmented DNA (possible disadvantage for gamete interaction/ embryo development)	Albert et al. 1992 Gandini et al. 2000 Holstein et al. 1973
Abnormal residual cytoplasm Residual cytoplasm >30% of head size	Abnormally wide cytoplasmic remnant containing subcellular components	Handicaps migration through cervical mucus and oocyte vestments, a possible disadvantage for gamete interaction	Courtot et al. 1987 Gomez et al. 1996
Thin midpiece Diameter of midpiece < diameter of the proximal principal piece	Partial or absent mitochondrial sheath	No or reduced ATP available for cell propelling	Zamboni 1992
Bent tail Not aligned with midpiece and head or sharply bent midpiece/tail	Misaligned midpiece and head or sharply bent midpiece/tail	Impairment of syngamy and cleavage; abnormal cell propelling: handicaps migration handicaps migration	Chemes et al. 1999 Sadas-Magnan 1999
Absent tail Isolated head, no tail observed	Various anomalies of the neck region	Fragility of the neck structures Sperm propulsion not possible	–
Short tail Tail length <5 head lengths	Abnormally shaped periaxonemal and sometimes axonemal structures/ dysplasia of the fibrous sheath	Immotility or severe dyskinesia	Chemes et al. 1998

cont. Table 1

1	2	3	4
Irregularly shaped tail Irregular/ changing calibre along the tail	Abnormally shaped periaxonemal and sometimes axonemal structures	Abnormal motion (sliding spermatozoa, for example) (possible disadvantage for gamete interaction)	Feneux et al. 1985
Coiled tail Completely or partially coiled tail	Completely or partially coiled tail often within a huge cytoplasmic remnant	Sperm propulsion not possible	–
Multiple tails More than one tail	Partially dissociated tails connected to a single or to multiple heads or tails knitted together over a variable length	Abnormal motion: handicaps migration through mucus, oocyte vestments (possible disadvantage for gamete interaction)	–

Table 2. Specific morphological malformations of the sperm cell organelles (Bartoov et al. 2002).

Acrosome	Postacrosomal lamina	Nucleus		Neck	Mitochondria
		Shape	Chromatin content		
Lack	Lack	Small oval	Vacuolar area >4% of the whole nuclear area	Abaxial	Lack
Partial	Vesiculated	Large oval		Disorder	Partial
Vesiculated		Narrow (<2.9 μm in width)		Cytoplasmic droplet	Disorganization
		Wide >3.7 μm in width)			
		Short (<4.2 μm in length)			
		Regional disorder			
		Large vacuoles + normal shape/size			
		Narrow forms + large vacuoles			

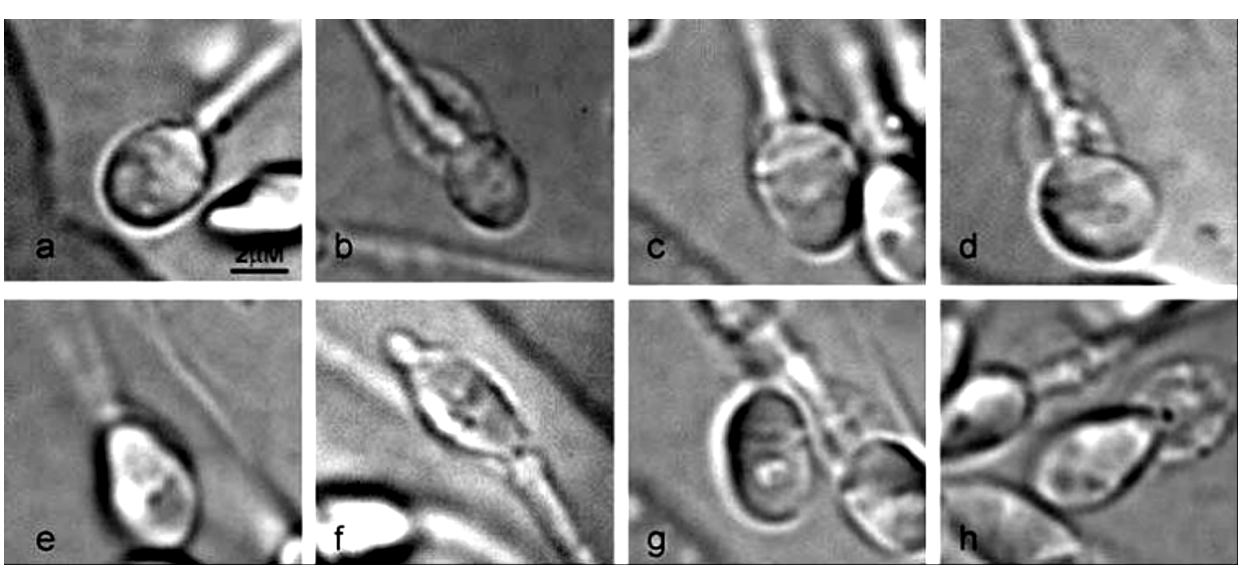

Fig. 6. Micrographs of sperm with nuclear abnormalities (Berkovitz et al. 2005): a – sperm cell with a morphologically normal nucleus; b – small oval nuclear form; c – large oval nuclear form; d – wide nuclear form; e – narrow nuclear form; f – regional (acrosomal) nuclear shape disorder; g – oval nuclear shape + large nuclear vacuoles; h – abnormal (narrow) nuclear shape + large nuclear vacuoles. Bar = 2 μm.

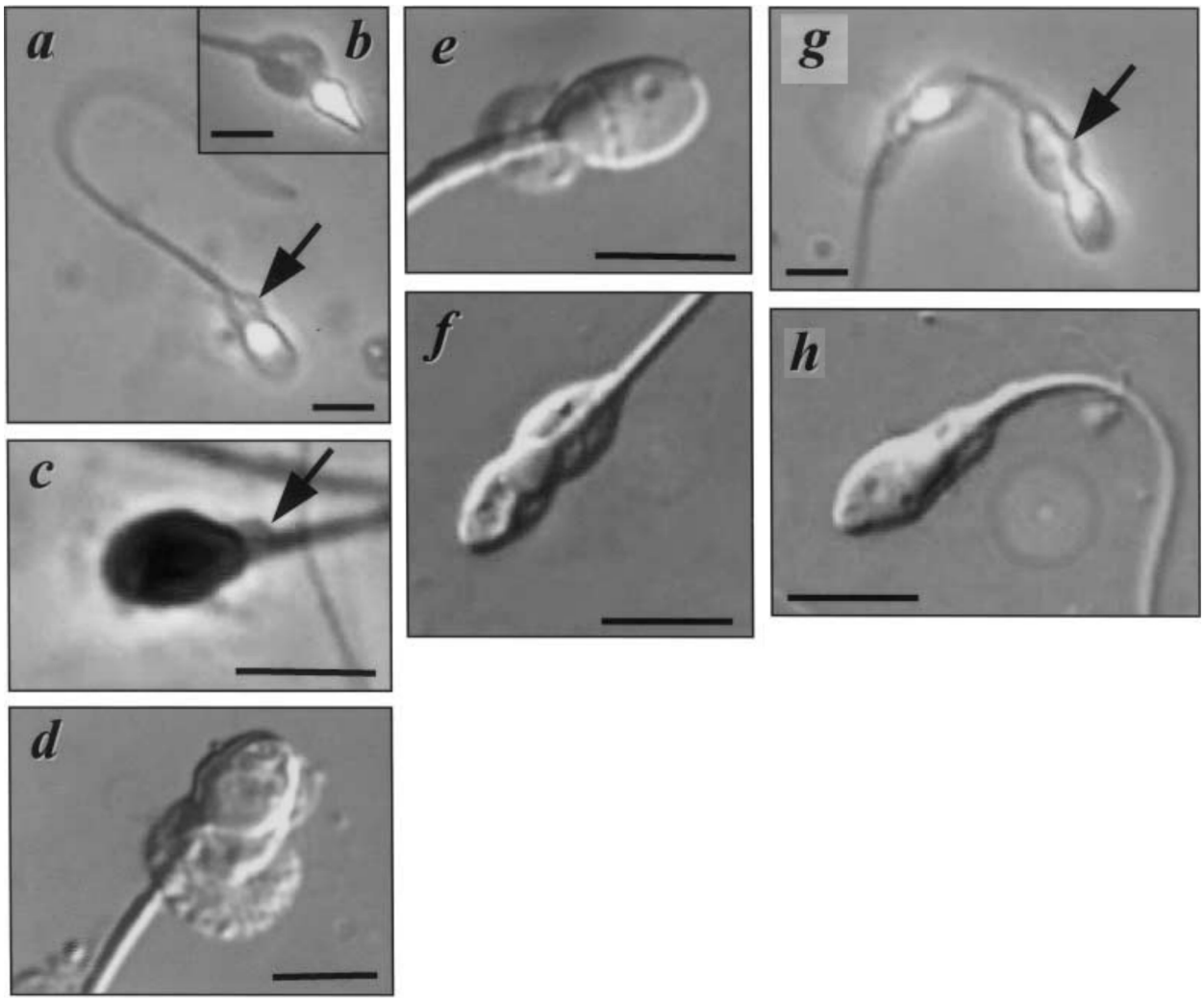

Fig. 7. Human sperm with cytoplasmic droplets and cytoplasmic residues (Cooper et al. 2004). Examples of true cytoplasmic droplets (a, b, c, e, f), abnormal cytoplasmic residues (d, g, h). Bars = 5 µm.

The X and Y bearing sperm differ in size. The X bearing sperm have greater head length, neck and tail length, head perimeter and head area (Cui and Matthews 1993, Cui 1997).

Morphological abnormalities of sperm

Any abnormalities in morphology of sperm have a negative effect on the outcome of IVF and ICSI. Abnormal sperm cells are categorized into subgroups according to the defects observed (concerning the head and/or midpiece and/or tail). Defects in these subgroups are related to analysis of sperm in electronic microscopy and functional anomalies (Tab. 1, Fig. 4, 5) (Auger 2010, Tang et al. 2010, Chemes and Alvarez Sedo 2012).

In the other hand, the sperm cell can have specific morphological malformations in cellular organelles (Tab. 2). The small oval, large oval, wide and narrow nucleus, regional shape disorders and multiple disorders can be observed in the head of human sperm (Fig. 6). Large vacuoles in sperm nucleus show aneuploidy and chromatin condensation defects in the cell (Berkovitz et al. 2006, Perdrix et al. 2011, Franco et al. 2012, Silva et al. 2012).

The sperm cells often have cytoplasmic droplets (small, regular distensions at the neck or midpiece) and cytoplasmic residues (large, irregular material along the midpiece) (Fig. 7) (Bartoov et al. 2002, Cooper et al. 2004, Berkovitz et al. 2005).

Most morphologically abnormal sperm have multiple defects. Three indices are proposed (Auger 2010): i) the multiple anomalies index (MAI), ii) the teratozoospermia index (TZI), and iii) the sperm deformity index (SDI). The MAI is calculated as the mean number of anomalies per abnormal sperm. All head, midpiece and tail anomalies are included

in the calculation. The TZI is similar, but as only one abnormality per sperm compartment is counted, it only accounts for at most one head, one midpiece and one tail anomaly for each abnormal spermatozoon, independent of the real number of total anomalies. The SDI is the number of defects divided by the total number of spermatozoa (including abnormal and normal spermatozoa); it has several categories of head anomaly, but only one for each midpiece and tail defect (Jouannet et al. 1988, Aziz et al. 1996, Menkveld et al. 2001, Berkovitz et al. 2006).

Conclusion

Before IVF and ICSI the quality of sperm must be estimated exactly, because this has the high influence on embryo development. The suitability of sperm for IVF and ICSI must be estimated most precisely using complex evaluation of peculiarities of head, neck, midpiece and tail at the same time.

Recent data in the literature indicate that the morphological classification of sperm can be used as a determinant of fertilizing capacity in standard IVF treatment The improved assessment of sperm morphology can be used to discriminate three categories in relation to the predicted outcome, of standard IVF treatment: excellent prognosis (>14% morphologically normal spermatozoa), good prognosis (4-14%) and poor prognosis (<4%) (Kruger et al. 1986, Kruger et al. 1988, Menkveld et al. 1990, Grow et al. 1994, Sukcharoen et al. 1995).

Therefore in the future the analysis of the morphological parameters of one sperm cell using the light microscopy, in combination with precise sperm head birefringence detection using the polarized microscopy, could give the best fertilisation rate and embryo quality after ICSI.

We hope that this article will be considered as the background for manual of criteria of normal sperm cells and their abnormalities in different species of domestic mammals.

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