Ultrastructure of gametes and intracytoplasmic sperm injection: the significance of sperm morphology

W.Küpker[1], W.Schulze and K.Diedrich

Department of Obstetrics/Gynecology, Medical University Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany

[1]To whom correspondence should be addressed

The aim of this study was to determine characteristic malformations of sperm ultrastructure in patients with severe subfertility undergoing intracytoplasmic sperm injection (ICSI). Although light microscopy (LM) can reveal major abnormalities of the three parts of the spermatozoon (head, mid-piece and flagellum), the various cell organelles of the spermatozoon and their fine structure remain unevaluated by LM. Insight into the submicroscopic organization of the spermatozoon and its complex organellar system may contribute to a better understanding of the preconditions for success or failure of fertilization. An in-depth evaluation of semen quality by transmission electron microscopy (TEM) can improve the diagnosis of male subfertility and can give substantial information about the fertilizing competence of spermatozoa. Thus, in this study 56 ejaculated sperm samples from patients with severe male subfertility or previous failed attempts at in-vitro fertilization were assessed by LM and TEM prior to ICSI to evaluate the most important sperm defects causing extreme subfertility. LM analysis was performed according to World Health Organization criteria. It could be confirmed that severe head defects are mostly involved in long-term infertility and fertilizing failure in classical IVF treatments. The most frequent head defects are disorders of the nuclear membranes and the acrosomal cap and disorganization of the chromatin structure. These defects of sperm fine structure seem to be associated with dysfunctional sperm–oocyte recognition, binding and fusion with the oolemma. Chromatin alterations and signs of decondensation or karyolysis are frequently associated with a deterioration of the nuclear membranes and may be due to impaired spermiogenesis. However, our results and the success of ICSI proved that severe sperm defects have no predictive value and do not impair the fertilization process, and also that the maturity of spermatozoa does not play an important role. Fine structure analysis revealed the pleiomorphology and heterogeneity of human spermatozoa.

Key words: ICSI/sperm morphology/sperm ultarstructure

Introduction

The fusion of the spermatozoon with the oocyte, the first step towards the assembly of a new individual, requires both gametes to be structurally normal, viable and functionally competent. The spermatozoon in particular must possess effective motility, as well as penetrating, fusiogenic and fertilizing capacities. The evaluation of the three most important semen parameters, i.e. sperm concentration, progressive motility and morphology, defines the capability of spermatozoa to effect oocyte fertilization. In particular, a link was established between sperm morphological characteristics and infertility. That sperm morphology plays a key role in the fertilization process was deduced by studies involving in-vitro fertilization (IVF). Light microscopy (LM) can reveal major abnormalities of the three parts of the spermatozoon, i.e. head, mid-piece and flagellum. Criteria for normozoospermia have been established by the World Health Organization (WHO, 1992), while sperm

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morphology criteria for IVF have resulted from recommendations by Kruger *et al.* (1986). However, the fine structure of the subcellular organelles within the spermatozoon cannot be evaluated using LM. Insight into the submicroscopic organization of the spermatozoon and its complex organellar system may contribute to better understanding of the preconditions for success or failure of fertilization. An in-depth evaluation of the quality of semen using transmission electron microscopy (TEM) can improve the diagnosis of male subfertility and provide substantial information about the fertilizing competence of spermatozoa. The introduction of intracytoplasmic sperm injection (ICSI) has shed new light on the fertilization process in the human and on the significance of the three major semen parameters in the treatment of severe male subfertility. However, while TEM analysis of the fine structure of spermatozoa provides a realistic prediction of their fertilizing capacity in IVF, its significance for the ICSI procedure remains questionable.

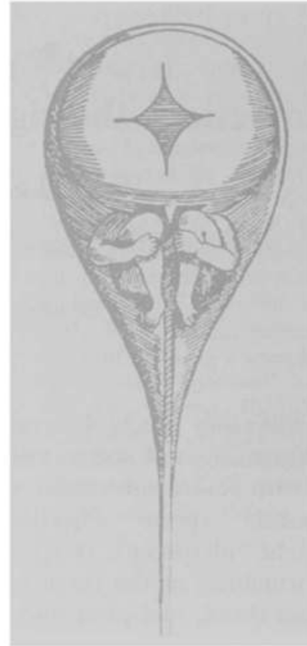

Figure 1. Homunculus (drawing by Hartsoeker, 1694).

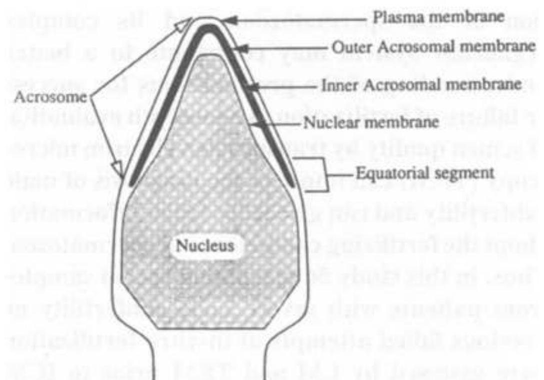

Figure 2. Fine structure of the sperm head.

Ultrastructure of abnormal spermatozoa

The characterization of normal-shaped spermatozoa and its plethora of deviations started with the introduction of the light microscope by Leeuwenhoek in the 18th century. The importance of spermatozoa for human reproduction was expressed in a drawing by Hartsoeker in 1694 (Figure 1) As early as 1902, Retzius stressed the pleiomorphology of human spermatozoa compared to those of other species. Ultimately, the technique of electron microscopy provided the realization and proof of historic ideas.

Pathology of the sperm head

The structural elements that confer upon spermatozoa the ability both to penetrate the oocyte's vestments and to fuse with the oolemma reside in the head. They are the acrosome and the sperm plasma membrane, which covers the equatorial segment of the acrosome and the post-acrosomal region of the head (Figure 2).

The acrosome is a Golgi-derived organelle covering more than two-thirds of the sperm nucleus. The acrosome results from envelopment of the spermatids by Sertoli cell cytoplasm in the late stages of spermiogenesis, and by contracting the nucleus directs its definitive shape (Holstein, 1976). Its membranes contain hydrolytic enzymes which play an essential role during the acrosome reaction and oocyte penetration. The physiological cascade, involving fusion of inner and outer membranes, vesiculation and fragmentation of the fused membranes and exocytosis of the enzyme-containing organelles, requires the integrity of the entire acrosome. In fact, pathology of the acrosome

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seems to be the most frequent cause of male subfertility.

The main structural acrosome defects are partial lack, complete lack, intra-organellar inclusions, degeneration and hypoplasia. Disorganization of the acrosomal membrane often leads to a change of the nuclear shape. Inclusions are characterized by the presence of pleiomorphic structures within the organelle, such as vesicles and granules of different size. These structures probably originated as remnants of Golgi elements and represent a failure of the late stages of spermiogenesis. These structural abnormalities are assumed to be associated with inability of the spermatozoon to undergo the acrosome reaction or to penetrate the zona pellucida. Complete lack of the acrosome changes the nuclear shape, resulting in the characteristic spherical (round) sperm head. This very rare condition, in which the male presents with ejaculates containing 100% round headed spermatozoa, was first described by Schirren *et al.* (1971) as globozoospermia. Complete lack of the acrosome is associated with failure or lack of the postacrosomal mitochondrial sheath, partial or total absence of the nuclear envelope and disorders of nuclear chromatin condensation. This monomorphous defect, which affects every single spermatozoon, is suspected to be inherited as a genetic defect (Flörke-Gerloff *et al.*, 1984). Only 20 cases had been reported worldwide up to 1988 (Lalonde *et al.*, 1988). Incomplete or type II globozoospermia occurs more frequently (Anton-Lambrecht *et al.*, 1976). This type of globozoospermia displays round headed spermatozoa with different features which can be differentiated exclusively by TEM semen analysis. Complete absence of the sperm head, partial lack of the acrosome, complete karyolysis or large cytoplasmic droplets surrounding the nucleus with disturbed mitochondria are responsible for the light microscopic aspect of round headed spermatozoa.

The ability of the spermatozoon to fertilize an oocyte and to contribute to the assembly of the genomic patrimony of the embryo resides within its nucleus. To achieve syngamy as the end point of the fertilization cascade, the nucleus of each of the two gametes must have a normal structural organization. With regard to the spermatozoon, a normal nuclear structure is essential for decondensation within the ooplasm, and for unravelling and hydration of its densely compacted chromatin. When analysed by TEM, the sperm nucleus appears as a homogeneous compact mass of chromatin displaying a high degree of electron density. During the late stages of spermiogenesis, elongation and progressive condensation of the chromatin takes place, which, with simultaneous acrosome attachment, results in the typical shape of the sperm head. Chromatin condensation is associated with biochemical changes such as elimination of RNA, replacement of somatic histones by protamines and the formation of chromatin-stabilizing disulphide bonds (Zamboni, 1987). The high degree of chromatin aggregation protects the mature spermatozoon against physical and chemical damage. It is only within the ooplasm of an activated oocyte that the sperm chromatin becomes decondensed as a result of the cleavage of disulphide bonds and the substitution of protamines by oocyte-derived histones (Longo, 1985). Structural abnormalities of the nucleus include incomplete or impaired chromatin condensation and nuclear vacuoles and inclusions. These defects often occur in association with alterations in the structure of the acrosome. Karyolytic changes or the presence of large intranuclear lacunae or vacuoles are the morphological manifestations of underlying biochemical alterations. Spermatozoa with incomplete chromatin condensation apparently more often display single-stranded rather than double-stranded DNA (Pedersen, 1987) or possess chromosomal abnormalities (Abramsson *et al.*, 1982).

Pathology of the flagellum

The structural elements responsible for progressive motility reside in the flagellum. This is divided into four segments: the neck, the mid-piece, the principal piece and the functionally unimportant terminal piece. The major components of the neck are the connecting piece just behind the nucleus and the proximal centriole, which consists of a circular system of nine triplet microtubules. Its long axis is oriented perpendicularly to that of the flagellum. The centriole is, after the nucleus, the most important sperm organelle for initiation of the intra-ooplasmic fertilization process, being

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responsible for the formation of the sperm aster (Van Blerkom and Davis, 1995). Extending from the proximal centriole to the end of the flagellum are the microtubules of the axonemal complex, organized in a characteristic pattern of 9+2, i.e. nine sets of double microtubules at the periphery surrounding two single microtubules in the centre. The axonemal complex is encircled by nine outer dense fibres. Cranially, these fibres fuse with the connecting piece. The fibres facilitate sperm movement, mediated by protein phosphorylation (Tash and Means, 1983), and serve as a protector against damage during sperm transit through the male and female reproductive tracts. The movement of the flagellum is mediated through action of the dynein arms, resulting in sliding of the axonemal microtubules alongside one another. Flagellar motility requires ATP, which originates from the mid-piece mitochondria and is hydrolysed by ATPase of the dynein arms in the presence of magnesium. In the mid-piece, axonema and outer dense fibres are ensheathed by mitochondria which are helically organized in 11–13 gyres with two mitochondria per gyre (Zamboni *et al.*, 1971). A serious defect is the separation of the flagellum from the head. This 'pin-head' defect (Zanefeld and Polakowski, 1977) is assumed to be genetically inherited. Cytoplasmic droplets surrounding the proximal segments of the decapitated flagellum appear as small 'pin-heads' under LM conditions. Other structural defects of the flagellum involve changes and alterations in the composition and numbers of the axonemal microtubules, particularly impairment of the dynein arms. Absence of dynein arms seems to be one of the most frequent causes of sperm immotility. Furthermore, disorganization of the mitochondrial sheath or its complete absence may impair motility due to the lack of ATP synthesis. Lack of ATP can also cause sperm degeneration and death, for mitochondria are essentially involved in cell metabolism.

Ultrastructure of spermatozoa and IVF

Before ICSI shed new light on the treatment of severe male subfertility, ultrastructural assessment of human spermatozoa aimed to relate fine morphological features with fertilizing potential. In contrast to the light microscopical criteria that were clinically practical appropriate for IVF, TEM analysis assesses with a high degree of sophistication the pleiomorphology and heterogeneity of human spermatozoa that is present even in ejaculates from proved fertile males. Scoring systems for >30 distinctive defects in the fine structure of spermatozoa have been established to determine fertilizing capacity (Bartoov *et al.*, 1980). Albeit, Mashiach *et al.* (1992) demonstrated that the ultrastructural abnormalities of the sperm head components are a key parameter for assessing sperm fertilizing competence *in vitro*. These authors claimed that sperm tail alterations presumably do not contribute to the fertilizing capacity of the spermatozoon *in vitro*, since spermatozoa are placed in culture in the immediate vicinity of the oocyte. This confirmed the finding of no correlation between sperm motility and IVF outcome (Kruger *et al.*, 1988). Mashiach *et al.* (1992) performed a computer-weighted quantitative analysis of the presence of ultramorphological head defects, consisting of hyperelongated head, acrosome deficiency and acrosome damage. A score was defined which was able to predict 90 and 76% of the cases with and without fertilizing potential respectively. Furthermore, it could be demonstrated that decondensation of nuclear chromatin was one of the most important causes of IVF failure, regardless of acrosomal defects (Chitale and Rathaur, 1995). Although it is difficult to give a clear answer to the question of which malformations are compatible with male fertility and what percentage of ultrastructurally defective spermatozoa results in infertility, after having evaluated more than 2000 ejaculates by TEM, W.Schulze (personal communication) suggested a minimum requirement for fertility of 12–18 % ultrastructurally intact normal spermatozoa.

Ultrastructure of spermatozoa and ICSI

Since the introduction of ICSI into the repertoire of techniques used in reproductive medicine (Palermo *et al.*, 1992), the importance of sperm morphology has greatly increased – fertilization and pregnancy rates are high even in the most severe cases of 0% normal sperm morphology, as determined by LM. Moreover, ICSI has permitted the use of epididymal and testicular spermatozoa for fertilization in cases

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of azoospermia or cryptozoospermia. Immature spermatids can replace fully mature spermatozoa for injection.

To evaluate the significance of sperm ultrastructure for the success of the ICSI procedure, we performed a study of the ultrastructural characteristics of semen samples of males referred to our ICSI programme.

Selection of patients

The study group comprised 56 couples with a history of long-term infertility (mean duration 5.6 years) who were referred to the Lübeck Center of Reproductive Medicine for ICSI treatment. Of these patients, 48% had a history of failed classical IVF. The males presented with severe oligoasthenoteratozoospermia according to WHO criteria.

Methods

Semen samples were obtained for LM evaluation of morphology and TEM analysis. For TEM analysis, 1–2 ml of the liquefied ejaculate was dripped into 5.5% glutaraldehyde. After centrifugation the supernatant was discarded and the sediment diluted with 1.5–2.0 ml of 1% OsO_4, followed by fixation for 1 h at 4°C and another centrifugation. The sediment was then dehydrated. Next, the sediment was subjected to an ascending alcohol series. Finally, the specimen was covered with propylene oxide–Epon (EPON 812).

Results

LM analysis was performed according to WHO (1992) criteria. The average percentage of normal sperm morphology (Figure 3) was 7.8% (range 0–30%). Severe teratozoospermia (Figure 4) was confirmed by TEM analysis, giving a mean percentage of normal shaped spermatozoa of 2.9% (range 0–20%). Statistical analysis showed a significant positive correlation between the results of light and electron microscopy ($r = 0.8122$, $P < 0.01$).

In most of the cases, severe head defects could be detected (Figures 5–9). In 77.6% of all semen samples, alterations of the acrosome could be seen such as hypoplasia, partial lack or detachment; 67.3% of the patients displayed a failure in their sperm chromatin condensation. Less frequent defects were fragmented nuclear plasmalemma and sperm head alterations, including disturbed karyoplasma or binucleated heads. Round headed and acrosomeless spermatozoa were detected in 16.3 and 4.1% of samples respectively. Neck and tail defects (defective axonema or defects of the basal plate and the distal part of the flagella) were also less frequent.. More than one third of the ejaculates presented with deteriorations of the mitochondrial sheath and the surrounding axonema (Table I).

Table I. Ultrastructural disorders of spermatozoa (56 ejaculates, analysed by transmission electron microscopy)

Defects	Samples with defects (%)
Head defects	
Acrosomal alterations (partial lack, detachment, hypoplasia)	77.6
Chromatin condensation failure	67.3
Fragmented nuclear plasmalemma	38.8
Disturbed karyoplasma	34.7
Binucleated head	22.4
Round headed	16.3
Lack of acrosome	4.1
Neck defects	
Defective axonema	6.1
Defective centriole	2.0
Mid-piece defects	
Mitochondrial sheath	34.7
Axonema and tubule system	22.5
Cytoplasmic droplets	2.0
Tail defects	2.0

Severe chromatin and acrosome disorders were detected in 70.8% of patients who had a history of failed classical IVF; 29% of patients with failed IVF had no severe sperm head disorders.

Of the 56 patients 28 were treated by ICSI, resulting in 12 pregnancies and a pregnancy rate of 25.5% per cycle. These results are comparable to our overall results using ICSI in cases of severe oligoasthenoteratozoospermia (Table II). In five cases, ICSI treatment was not successful (8.9%). TEM sperm analysis of those males revealed no differences compared to other patients. Moreover, acrosome disorders and chromatin condensation failure were less frequent in this group (16.3 and 17.6% respectively), suggesting the presence of different causes of fertilization failure after ICSI.

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Table II. Results of intracytoplasmic sperm injection in cases of severe oligoasthenoteratozoospermia (Lübeck)

	All cycles (3/94–6/96)	No. (%) of TEM analysis cycles
No. of cycles	1022	47
No. of MII oocytes injected	8923	396
No. (%) fertilized oocytes	5935 (67)	201 (51)
No. (%) with polyploidy	276 (3)	7 (2)
No. (% of cycles) pregnancies	280 (27.4)	12 (25.5)

MII=metaphase II.

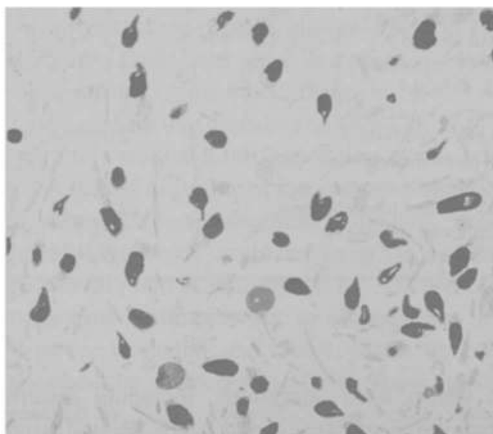

Figure 3. Normozoospermia (transmission electron microscopy: magnification ×3000).

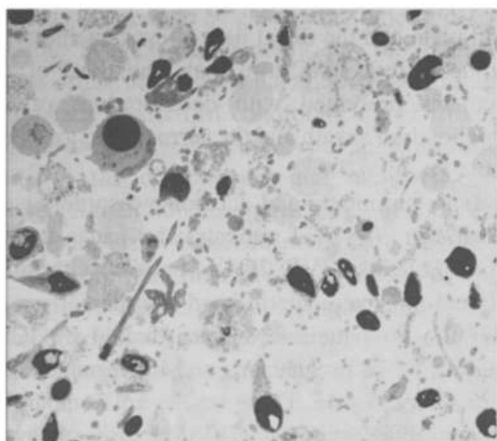

Figure 4. Teratozoospermia (transmission electron microscopy: magnification ×3000).

Conclusion

Fine structure analysis reveals the pleiomorphology and heterogeneity of human spermatozoa, and the fine structure of spermatozoa in cases of severe male subfertility and failed IVF has been thoroughly characterized. Data from our study confirm other findings suggesting that severe head defects are mainly associated with long-term infertility and failure of fertilization following classical IVF treatments. The most frequent head defects observed are disorders of the nuclear membranes and the acrosomal cap and disorganization of the chromatin structure. These defects in sperm fine structure seem to be associated with a functional failure of sperm–oocyte recognition, binding and fusion with the oolemma. Chromatin alterations and signs of decondensation or karyolysis are frequently associated with deterioration of the

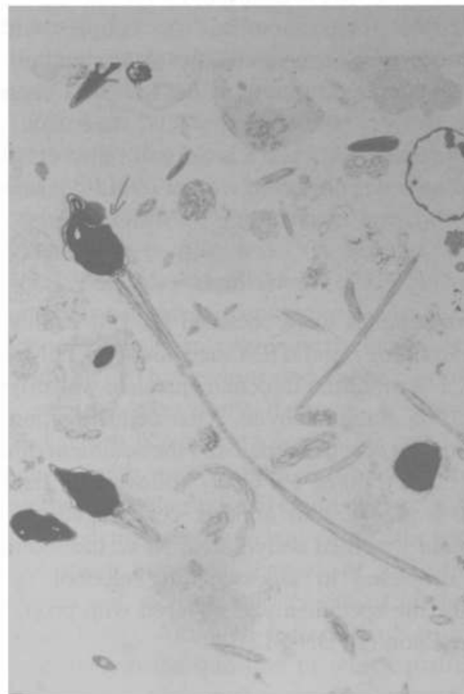

Figure 5. Severe acrosomal defect showing invagination (indicated by arrow), hypoplasia and vesicle (magnification ×8000).

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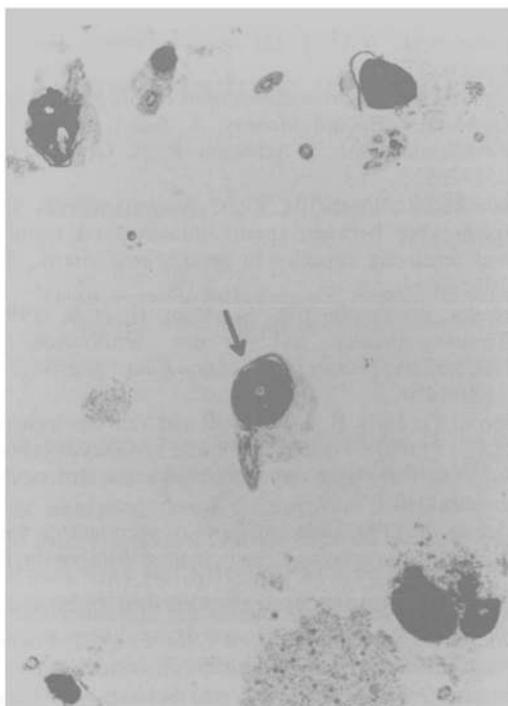

Figure 6. Round-headed spermatozoon showing intranuclear vacuole, cytoplasmic droplet (indicated by arrow) and partial lack of the acrosome (magnification ×8000).

Figure 8. Binucleated sperm head showing disorder of the mitochondrial sheath and a double tail (magnification ×8000).

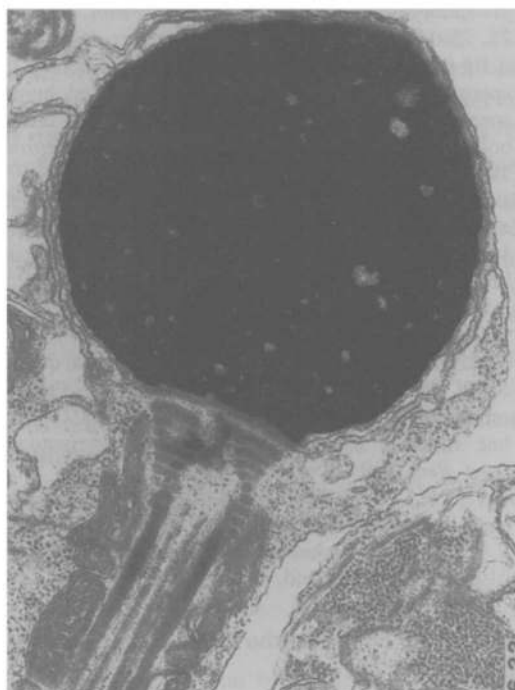

Figure 7. Globozoospermia (type I Schirren) (magnification ×40 000).

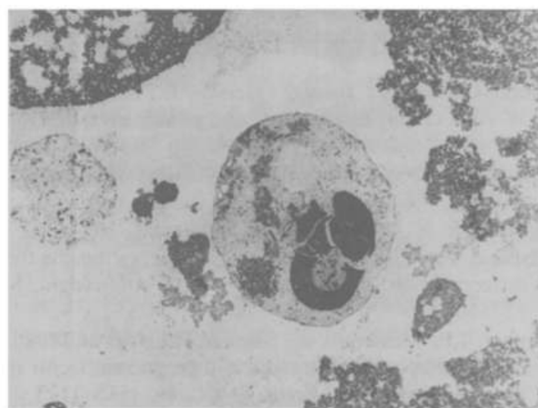

Figure 9. Macrophage showing phagocytosis of a sperm head (magnification ×8000).

nuclear membranes and may be due to impaired spermiogenesis. However, the severity of sperm defects appears to have no predictive value with regard to the success of ICSI. The maturity of spermatozoa does not play an important role in the fertilization process, and the role of the acrosome in ICSI remains to be clarified. Fertilization failure

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seems rather to be caused by impaired molecular events occurring in the ooplasma, at the point at which sperm–oocyte dialogue should be activated by the commencement of nuclear decondensation of the sperm head.

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