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Review Article

Sperm function and assisted reproduction technology

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The evaluation of different functional sperm parameters has become a tool in andrological diagnosis. These assays determine the sperm's capability to fertilize an oocyte. It also appears that sperm functions and semen parameters are interrelated and interdependent. Therefore, the question arose whether a given laboratory test or a battery of tests can predict the outcome in *in vitro* fertilization (IVF).

One-hundred and sixty-one patients who underwent an IVF treatment were selected from a database of 4178 patients who had been examined for male infertility 3 months before or after IVF. Sperm concentration, motility, acrosin activity, acrosome reaction, sperm morphology, maternal age, number of transferred embryos, embryo score, fertilization rate and pregnancy rate were determined. In addition, logistic regression models to describe fertilization rate and pregnancy were developed. All the parameters in the models were dichotomized and intra- and interindividual variability of the parameters were assessed. Although the sperm parameters showed good correlations with IVF when correlated separately, the only essential parameter in the multivariate model was morphology. The enormous intra- and interindividual variability of the values was striking. In conclusion, our data indicate that the andrological status at the end of the respective treatment does not necessarily represent the status at the time of IVF. Despite a relatively low correlation coefficient in the logistic regression model, it appears that among the parameters tested, the most reliable parameter to predict fertilization is normal sperm morphology. (Reprod Med Biol 2005; 4: 7–30)

Key words: assisted reproduction, high intra- and interindividual variability, multivariate approach, prediction of outcome of IVF, sperm functions.

INTRODUCTION

MALE SUBFERTILITY IS the reason for an unfulfilled wish for children in approximately 50% of childless couples. In Germany alone, the number of andrologically caused childless partnerships amounts to more than 1 500 000. This high incidence of male factor infertility mandates a complete andrological consultation in all male partners of couples consulting for infertility. During recent years, apart from the light microscopical determination of sperm count and morphological malformations, evaluation of functional sperm parameters has become a powerful tool in andrological laboratories. Some of these assays determine biochemical parameters, such as α-glucosidase[1,2] or the polymorphonuclear granulocyte (PMN)-elastase[3,4] which have been found to be important for sperm function. Most of them, however, determine biological functions of spermatozoa, and consequently the capability to fertilize an oocyte (i.e. motility, membrane integrity, morphology, zona binding, acrosome reaction, acrosin activity, oolemma binding, chromatin condensation or DNA integrity) (Fig. 1). All these parameters repeatedly showed a moderate or strong relationship to both fertilization *in vitro* and pregnancy when they were examined in spermatozoa from the ejaculate, which was used for IVF treatment, at the same time. In this present review, the impact of these functional sperm parameters shall first be discussed separately and then in a multivariate approach.

In addition, the occurrence of leukocytes in ejaculates, which physiologically produce large amounts of highly detrimental substances, reactive oxygen species (ROS), is common, even in healthy men not regarded as leukocytospermic (leukocyte count $< 1 \times 10^6$/mL)[3] needs to be considered in order to assess the male fertility potential. There is: (i) still no common agreement on the accurate determination of active leukocytes in

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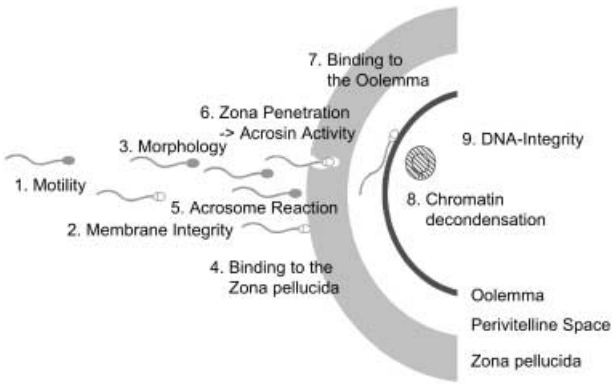

Figure 1 Schematic depiction of functional parameters of spermatozoa. Note that the function of capacitation goes together with acrosome reaction and sperm hyperactivation. In addition, chromatin decondensation goes together with the condensation of the sperm DNA material during spermatogenesis and subsequent sperm maturation in the epididymis.

ejaculates, the effect of (ii) ROS; and (iii) leukocytes on human sperm function and male fertility. Currently, no simple solution for these problems is available, especially in view of the high variability of these biological parameters.

Motility

Motility, the most obvious sperm function, is an essential prerequisite for fertilization and conventional methods of assisted reproduction. Under *in vivo* conditions, potentially fertile spermatozoa separate from immotile spermatozoa, debris and seminal plasma in the female genital tract by active migration through the cervical mucus.[5] During this process, not only progressively motile spermatozoa are selected, but male germ cells also undergo physiological changes called 'capacitation', which are fundamental prerequisites for the sperm's functional competence.[6] With regard to *in vitro* fertilization (IVF), Acosta *et al.*[7] reported that even low percentages of motile spermatozoa in the ejaculate did not have a significant negative influence on fertilization *in vitro* and pregnancy rates. However, it may be possible that motility values less than 10% may represent a problem in IVF. Sukcharoen and Keith[8] concluded that even detailed motility grading and sperm motility after 24 h does not have a practical value in predicting the fertilization outcome in an IVF program. However, Shulman *et al.*[9] emphasized that none of the standard semen characteristics, such as volume, sperm count or motility, has prognostic value for the outcome after intrauterine insemination. The only parameter that could predict treatment outcome was the percentage of motile spermatozoa after appropriate sperm separation. On principle, Kasai *et al.*[10] recently confirmed these results. These authors also concluded that there is a close positive relationship between mitochondrial membrane potential and sperm motility. Therefore, these parameters are indicative of the male fertility potential.

For assisted reproduction, motile spermatozoa are normally selected by different methods of sperm separation (i.e. swim-up, glass wool filtration, glass bead column separation, migration-sedimentation, density gradient centrifugation) (for review see Henkel & Schill[11]). Some of these methods can also be employed in cases in which epididymal or testicular spermatozoa were aspirated to be used in IVF or intracytoplasmic sperm injection (ICSI). Since the spermatozoon's ability to self-propelled movement is closely correlated with other parameters, such as morphology, this results in an increased percentage of morphologically normal sperm after sperm separation.[12–14] Therefore, motility is an important sperm parameter that is essential for successful fertilization in an assisted reproduction program. In addition, it is a sign of vitality, and scientists in the IVF laboratory make use of this feature to identify viable spermatozoa for ICSI. However, one must approach each male patient as an individual and assisted reproduction laboratories must have different separation techniques available in order to obtain the best result.

Morphology

Sperm morphology, as evaluated by strict criteria,[15] is one of the most important parameters of the standard semen analysis and has repeatedly been proven a good predictor for fertilization *in vivo*[16] and assisted reproduction.[17–19] In contrast to the evaluation of the other functional parameters of spermatozoa, morphology is a simple and cost-effective method that can be performed in every andrological and IVF laboratory after thorough training.[20] In this context, it is also important to mention that sperm morphology also correlates significantly with sperm motility[21] and its ability to bind to the zona pellucida (ZP).[22,23] In addition, Liu and Baker,[24] and Menkveld *et al.*[25] demonstrated that normal sperm acrosomal morphology correlated significantly with sperm binding to the ZP, while Franken *et al.*[26] and Menkveld *et al.*[27] showed a strong relationship between normal sperm morphology and the inducibility of the acrosome reaction.

It also appears that there is a correlation between poor sperm morphology, especially the presence of a residual cytoplasmic droplet, and the sperm cell's own excessive production of reactive oxygen species,[28,29] which significantly affects sperm fertilizing potential.[30] Spermatozoa that have cytoplasmic residues, have a higher content of cytoplasmic enzymes, such as creatine kinase or glucose-6-phosphate dehydrogenase,[31,32] which are thought to stimulate the generation of ROS in the spermatozoa themselves.[32,33] The clinical importance of this connection, between sperm morphology and the sperm cell's own ROS production, is underlined by considerably stronger correlations of the percentage of ROS-producing spermatozoa with the different parameters.[34]

The fact that morphological disturbances affect the sperm cell's functional competence to fertilize an oocyte, in many respects, is most probably the reason why this parameter has consistently been reported to have a high predictive power for the outcome of assisted reproduction (for review see Coetzee *et al*.[19]). Consequently, these manifold correlations, between a specific biological sperm function and its morphologically related structures, also reflect the importance of normal sperm morphology and its central role, which the evaluation of sperm morphology currently plays in many IVF centres. This is also an indication of the interdependent and interrelated nature of mammalian sperm functions and normal morphology. Nevertheless, the knowledge of specific disturbances of biological functions of spermatozoa is not less important, as this gives an insight in the pathophysiology of spermatozoa and their functions. These parameters are, therefore, discussed separately below.

Reactive oxygen species, membrane integrity and DNA integrity

Closely correlated with motility and sperm function is membrane integrity,[35,36] which is reportedly affected by ROS, such as hydrogen peroxide (H_2O_2), superoxide anion (O_2^-) and/or hydroxyl radical ($\cdot OH$).[37–40] These highly reactive substances, which exhibit half-life times in the nano-second ($\cdot OH$) to the milli-second range (O_2^-), are very strong oxidants and are physiologically produced in any living cell during respiration. Compared to somatic cells, sperm contain an unusually high percentage of polyunsaturated fatty acids in their membranes.[29] However, this feature is an essential prerequisite for normal sperm membrane function, but makes sperm in particular susceptible for oxidation by ROS, which causes lipid peroxidation. [28]

Since the first report by McLeod,[41] on the influence of ROS on human spermatozoa, it is now believed that oxidative stress is associated with male infertility.[29,37] In extreme cases this might result in a dramatic loss of normal sperm function (e.g. markedly reduced motility[36] and penetration in the zona-free hamster ovum penetration test,[42] or impaired membrane integrity[43]), therefore indicating decreased fertilizing capability of spermatozoa. In addition, oxidative damage to spermatozoa is closely correlated with inflammatory processes in the genital tract and occurrence of leukocytes, particularly granulocytes, that generate about 1000-times more ROS than spermatozoa themselves.[44] In addition, a highly positive correlation between ROS, PMN-elastase – a specific parameter of inflammation, sperm concentration and motility has been found.[45]

Several authors have revealed that 30–40% of ejaculates from infertile men generate excessive levels of ROS.[46,47] Oligozoospermic patients tend to have high ROS production of spermatozoa.[45] From a clinical view, it is therefore important to determine semen samples that produce excessive amounts of ROS, and to separate leukocytes and damaged spermatozoa from those sperm cells which still do not show signs of lipid peroxidation. Because of the sensitivity of spermatozoa to oxidative damage, sperm separation should be performed very carefully, preferably by means of density gradient centrifugation or glass wool filtration (for review see Henkel & Schill[11]). Although it is difficult to remove leukocytes completely from semen,[42] even after Percoll gradient centrifugation, leukocytes play a major role in the production of ROS.[48] Using the glass wool filtration technique, Sánchez *et al*.[49] were able to reduce leukocyte contamination in human ejaculates to an extent higher than 90%. Moreover, with this technique, it was possible to distinguish between ejaculates showing ROS production by spermatozoa or by leukocytes.[47] In addition, both density gradient centrifugation and glass wool filtration have been shown to maintain normal sperm function with regard to motility and penetration into zona-free hamster oocytes.[36,37]

At present, research is focused on scavenging free oxygen radicals, produced by either active leukocytes or the sperm cells themselves. This includes approaches to separate excessively high ROS-producing cells from those producing only very little amounts of free radicals by means of Percoll-centrifugation or glass wool filtration,[47] or by adding scavengers for ROS to the semen or sperm separation medium. For *in vitro* treatment of spermatozoa with glutathione during sperm separation, contradictory results have been published. Following

swim-up preparation of human spermatozoa in the presence of glutathione, Griveau and Le Lannou[50] found an improved acrosome reaction and 24 h-motility on the same level as for Percoll gradient centrifugation, and suggest that glutathione has a therapeutic potential. In contrast, Donnelly *et al*.[51] provided data indicating that this drug has no significant effect on progressive motility, neither by itself, nor in combination with hypotaurine. However, the treatment still afforded a significant protection against ROS-induced DNA damage. Another approach to treat oxidative stress-related male infertility was performed by Oeda *et al*.[52] These authors used N-acetyl-L-cysteine (ACC) and succeeded in a dose- and time-dependent significant reduction of the ROS production, and significantly improved motility. However, Hughes *et al*.[53] demonstrated that the addition of ACC to a sperm separation medium, induced sperm DNA damage. Moreover, *in vivo* attempts of scavenging ROS by antioxidants, such as vitamin E and glutathion, have been performed.[54–56]

However, ROS do not only oxidize the sperm plasma membrane, but also the DNA causing DNA fragmentation,[57] which is also closely related to fertilization.[34,58–60] It seems that patients treated with assisted reproductive technologies, especially with ICSI, have a significantly higher risk that sperm with fragmented DNA fertilize oocytes, which may lead to embryo death.[61] Since the ROS produced by leukocytes and pre-damaged spermatozoa affect sperm functions at a late stage, there is also evidence that ROS may be a cause of testicular damage.[62] This might be due to a production of ROS because of their regulatory role in programmed cell death, apoptosis.[63] In this regard, one can speculate whether increased levels of ROS in the testis are the reason for sperm damage or its consequence. The former is supported by the observation by Erkkilä *et al*.[64] that the antioxidant ACC significantly inhibits apoptosis in human male germ cells *in vitro*. This would not be possible if ROS production was a consequence of apoptotic events.

Causes of DNA fragmentation could be internal influences, such as apoptosis or ROS production of the spermatozoa or external inducers, such as leukocytes. ROS production in the ejaculate by leukocytes seems to have a low level of influence on sperm DNA fragmentation. However, as even low amounts of ROS are harmful to sperm DNA integrity,[65] a causality between leukocytes in the ejaculate and DNA fragmentation should not be neglected.[34] These cells play an important role in the immunosurveillance in the ejaculate and produce high amounts of oxidants, including hydrogen peroxide.[66] In addition, it has been shown that this oxygen metabolite accounts for most human sperm damage.[46] Also, because it is not charged, hydrogen peroxide can easily penetrate plasma membranes, enter the spermatozoa and damage DNA integrity. Henkel *et al*.[67] could corroborate this concept, and it even appeared that the cut-off value for leukocytospermia (1×10^6 leukocytes/mL ejaculate) set by the World Health Organization (WHO)[3] may be too high.

Finally, it is important to mention the consequences of fertilization of oocytes with sperm derived from an ejaculate containing a high incidence of DNA fragmentation in IVF and especially ICSI patients. According to present knowledge, sperm DNA fragmentation may not only cause impaired embryonic development and early embryonic death,[68–70] but also an increased risk of childhood cancer in the offspring.[71,72] The latter is due to the vulnerability of human sperm DNA during late stages of spermatogenesis and epididymal maturation. At this stage, DNA repair mechanisms have been switched off, resulting in a genetic instability of the male germ cells,[73] especially on the Y-chromosome, resulting in male-specific cancers.[74] Therefore, the pathophysiology of ROS and the impact of leukocytes on spermatozoa and DNA integrity should be better understood.

Zona pellucida binding

Direct interaction between mammalian spermatozoa and the oocyte is an essential step of fertilization taking place at two different physiological barriers. The first barrier for sperm entry into the oocyte is the ZP, and the second is the oolemma. The ZP is a non-cellular coat of the female gamete, which is synthesized by the oocyte and the surrounding follicle cells.[75] At its peak, the messenger ribonucleic acid (mRNA) content for zona proteins in oocytes amounts to approximately 1.5% of the total.[76] In the human, the ZP has an average thickness of about 22 µm. Early studies have shown the ZP to be composed of different layers with varying thickness among species and three to four glycoproteins.[77] Structural studies indicate that the ZP appears like a sponge,[78,79] and consists of interconnected microfilaments, each filament being formed by alternating molecules of ZP_2 and ZP_3. These filaments are long (2–3 µm) and of uniform width (7–18 nm), with the structure repeated every 14–15 nm, reflecting the periodic arrangement of several heterodimers ZP_2–ZP_3. They are bridged by the glycoprotein ZP_1, which itself is composed of two peptide chains connected by disulfide bridges.[80]

The ZP has several important features. Apart from mediation of sperm binding to the oocyte,[81] species-

specific recognition of spermatozoa,[82] and prevention of polyspermy,[83] the ZP is a physiological inducer of the acrosome reaction.[84,85] Sperm binding is mediated by means of O-linked carbohydrate side chains of the glycoproteins ZP_1/ZPB, ZP_2/ZPA and ZP_3/ZPC, composing the zona of many species.[81,86] In the pig, an additional low molecular weight (21 kDa) glycoprotein (ZP_4) has been identified.[80] Results obtained by Hasegawa *et al.*[87] have provided evidence that porcine ZP_4 and ZP_2 are derived from a common parent polypeptide by proteolytic cleavage. Porcine ZPC seems to be the primary receptor and ZPB the secondary receptor. Interestingly, only the ZPB-ZPC heterocomplex possesses zona-binding abilities in the pig, but not the free subunits.[80] The carbohydrate structures that are responsible for sperm binding in the pig have been clarified.[88,89] In acrosome-intact porcine spermatozoa, the binding site for zona proteins is located on the anterior portion of the sperm head, forming a band over the acrosomal ridge.[90]

ZP_3 is particularly involved in sperm-zona binding and induction of the acrosome reaction.[91] This protein serves as a primary receptor for spermatozoa and induces the acrosome reaction.[92] While ZP_2 is the secondary sperm receptor,[93] ZP_1 forms the matrix of the ZP.[94] In mice, Rankin *et al.*[95] showed that zonae without ZP_1 are structurally defective, resulting in decreased fecundity due to early embryonic loss. However, if ZP_3 is missing, no 2-cell embryos are formed and the respective females are infertile.[96] Meanwhile, full-length ZP cDNA from a series of species have been cloned, implying that most mammalian species express the ZPA, ZPB and ZPC proteins.[87] Recently, recombinant human ZP proteins were coexpressed in the human embryonic kidney cell line, 293T.[97] However, despite the presence of all three zona proteins, the biological activity to induce acrosome reaction was not observed.

Zona maturity[78,98] and proper sperm-zona binding ability have repeatedly been shown to be predictive of successful fertilization *in vitro*.[99,100] In order to test sperm-zona binding prior to IVF treatment, few zona binding assays have been developed in the past. In a competitive zona-binding assay, described by Liu *et al.*, spermatozoa from patients and donors were marked with different fluorescent dyes and the ratio of the differently marked spermatozoa bound to the zona was calculated.[101] In this assay, at least 20 oocytes are necessary to obtain valid results. The hemizona assay (HZA) gained practical importance in the diagnosis of male factor infertility and has been evaluated in an IVF program.[102] In this assay, only 2–4 devitalized human oocytes were microbisected into two hemispheres and incubated with the patient's or donor's sperm. A threshold of 30% for the hemizona assay index (HZI) was established, with better prognosis in IVF for those sperm samples with an index of >30%.[100] It is noteworthy that most of the spermatozoa bound to the hemizonae were morphologically normal[22] and 80% acrosome-reacted.[103] However, due to species specificity, human spermatozoa will bind firmly only to human ZP. In addition, availability of human ZP material is limited. Therefore, zona-binding assays using human material can only be performed in a selected group of patients. However, the test is complicated, time-consuming, requires highly skilled staff and an inverted microscope, including micromanipulation equipment.

Acrosome reaction

The acrosome reaction (AR) is another essential prerequisite for successful mammalian fertilization. The AR is a modified exocytotic event in which the outer acrosomal membrane fuses with the plasma membrane of the spermatozoon at discrete points,[104] resulting in hybrid membrane vesicles. These vesicles then detach from the spermatozoa and finally lead to the complete loss of the acrosome with the release of the acrosomal enzymes, which are thought to play a role in the penetration of spermatozoa through the outer oocyte vestments.[105] The AR can be induced after the spermatozoa have spent a period of time in the female genital tract or *in vitro* by incubating the spermatozoa in specific culture media. During this time, a series of poorly understood cellular and molecular changes, collectively known as capacitation, takes place.[104,106] The loss of cholesterol is an essential step in capacitation of human sperm, which is thought to increase membrane fluidity.[107] However, preventing the loss of sterols inhibited capacitation.[108] While capacitation is a reversible process, the execution of AR is irreversible. In addition, with the execution of the AR, spermatozoa not only render morphological changes, but also a functional change in terms of the loss of the ability to bind to the zona, and the acquisition of the ability to bind to the oolemma takes place.

Components of the natural environment of the spermatozoa along their way to the oocyte are of particular interest. In addition to the ZP,[85] the cumulus oophorus,[109] secretion products of the fallopian tube epithelium,[110] as well as follicular fluid have been discussed as possible inducers of the AR *in vitro*.[111] Recent studies with human follicular fluid have concentrated primarily on a 50 kDa protein[112–114] or progesterone[115] as the

inducer of which the corticosteroid binding globuline (CBG)-like protein-progesterone complex is thought to modulate AR *in vivo*.[110] Blackmore and Lattanzio,[116] Tesarik *et al*.[117] and Baldi *et al*.[118] found a novel non-genomic progesterone receptor on the plasma membrane, which, in contrast to the classical mechanism of steroid action, explains the velocity of the progesterone effects.

Apart from the physiological inducers, such as ZP, follicular fluid, progesterone or the cortico-steroid binding globulin progesterone complex, which have been shown to be predictive for fertilization *in vitro*, non-physiological inducers, such as calcium ionophore A 23187 or low temperature[119] can be used. Whereas a close correlation between the induction by means of low temperature and follicular fluid was observed,[120] no significant correlation between the ionophore induction and a physiological inducer could be found.[121] However, both methods are frequently used in andrological diagnosis and were shown to be predictive for fertilization *in vitro*.[122,123] In cases where the spermatozoa do not respond to the stimulus of the ZP to AR (disordered ZP-induced AR), the men are also infertile.[124,125]

Since only acrosome-reacted spermatozoa can penetrate the ZP, patients showing aberrations of the acrosome or an impaired AR are subfertile or infertile. Data obtained by Henkel *et al*.,[123] supports the hypothesis by Tesarik,[126] that higher levels of acrosome-reacted spermatozoa are required for fertilization, which will occur under physiologic induction of the AR. This means that the spontaneous AR of capacitated spermatozoa is not sufficient for fertilization of oocytes. Apart from a certain minimum of acrosome-reacted sperm in a sample, the inducibility of AR, that is, the difference between spontaneous AR and the percentage of acrosome-reacted sperm after induction of AR, is the most important parameter.[123] By means of receiver operating curve (ROC) analysis, Henkel *et al*.[123] calculated cut-off values for the induced AR and the inducibility of 13% and 7.5%, respectively. In patients whose sperm AR is above these cut-off values but showed poor fertilization, the cause for IVF failure can most obviously attributed to another sperm parameter, such as decreased acrosin activity.

Acrosin activity

Determination of acrosin, which is one of the best characterized sperm-specific enzymes, is a suitable approach to evaluate the fertilizing capacity of human spermatozoa. Acrosin is a trypsin-like serine proteinase that is exclusively located within the mammalian sperm acrosome.[127,128] It is considered the major penetration enzyme required for zona penetration through limited proteolysis of zona proteins. Another important function is its ability to bind to the ZP.[129] Acrosin is apparently also involved in capacitation and AR.[130,131] Although contradictory results on the contribution of acrosin to the fertilization process have been published,[132–135] its importance for fertilization and its determination for diagnostic purposes has repeatedly been emphasized.[136,138] In addition, it may act as a sperm-stimulating agent during intrauterine sperm migration when it is released from the acrosome of dead spermatozoa, since it is able to liberate kinins from kininogen. Kinins were demonstrated to enhance sperm metabolism and sperm motility *in vitro*.[139]

Several methods have been described to assess the acrosin activity in human spermatozoa.[139] A very simple method is the determination of the proteolytic potential of spermatozoa on gelatine plates.[138] Acrosin is released by hyperosmolaric rupture of the acrosome, and leads to halo formation during incubation in a humid chamber at 37°C. Halo formation is predominantly brought about by living spermatozoa, which is supported by correlation with the eosin test ($r = 0.619$). The more dead spermatozoa are identified, the lower is the halo formation rate. Normal acrosin activity indices are observed in men with high fertilization rates, whereas the halo diameters and halo formation rates are smaller in most cases of poor fertilization (<50%).[138] Therefore, the method may give information about the fertilizing potential of a sperm population. Patients showing normal acrosin activity index but low fertilization, probably have defects other than impaired acrosin activity (e.g. impaired AR, impaired sperm–oolemma interaction, or disturbance of chromatin decondensation). This is also a reason why statistical calculations show a low sensitivity (26%), whereas high specificity (98%), and a high predictive value (positive predictive value 90%, negative predictive value 74%) exist for human IVF outcome,[138] thus supporting the concept that acrosin determination is a useful parameter to predict the fertilizing potential of spermatozoa.[139] The rate of false negative results of this assay is 3.5%. No acrosin is available in case of globozoospermia.[141] The method of gelatinolysis is advantageous in that its equipment is simple and acrosin activity can be determined in individual spermatozoa. It shows good correlation with the biochemical assay.[142]

Compared to patients with normozoospermia, significantly lower acrosin activity is observed in patients with severe teratozoospermia and polyzoospermia, the

latter with an average of <60%.[139] By immunological methods, it was shown that the acrosomal membrane integrity is severely disturbed in most spermatozoa from polyzoospermic men. Therefore, polyzoospermic patients equal men with severe oligozoospermia, showing reduced fertility compared to normozoospermic controls.

Oolemma binding

Successful fertilization is the result of a variety of different interactive functional parameters of both the oocyte and the spermatozoon. Spermatozoa have to surmount two biological barriers before entry into the oocyte, the ZP and the oolemma. Therefore, direct interactions of spermatozoa with the oocyte can be divided into two phases, early and late. Following binding to the ZP, spermatozoa undergo AR, penetrate the zona and acquire their ability to bind to the oolemma. Therefore, only acrosome-reacted spermatozoa can penetrate the zona[143] and then get in contact with the oolemma.[144]

The morphological and functional changes in spermatozoa taking place during AR also reflect in the kind of interaction. While the interaction at the ZP is mediated by carbohydrate binding, adhesion molecules (integrins β1, β3, β4) and matrix proteins (fibronectin, laminin) mediate sperm–oolemma binding.[145,146] The molecular mechanism is thought to be analogous to the cell–cell interactions between somatic cells. Although the arginine-glycine-aspartic acid (RGD) sequence[147] is known to inhibit sperm–oolemma binding[148] and indicates the involvement of integrins, the actual role of integrins in sperm–egg interaction remains to be clarified. As the sperm–oolemma interaction also plays a critical role in the process of fertilization and can be regarded as an independent sperm function,[149] the determination of the sperm–oolemma binding has been suggested for andrological diagnosis by the WHO.[3]

A measure for the sperm–oolemma binding, is the sperm penetration assay (SPA) using zona-free hamster oocytes. This heterologous bioassay evaluates the ability of acrosome-reacted sperm to bind to the oolemma, to fuse with the oocyte, and to decondense within hamster eggs. Several authors demonstrated higher penetration rates in the sperm penetration assay after induction of AR.[150,151] Despite SPA being often used as a prognostic test to assess male fertility in many centres, no consensus of a correlation between SPA and conventional semen parameters has been attained. This is because of the varied experimental conditions and assessment criteria used by different laboratories. Moreover, the percentage of acrosome-reacted sperm in a certain sample has not been taken into account. Therefore, one does not know whether low binding and/or penetration results from a poorly induced AR or from an impaired binding of sperm to the oolemma. However, Henkel *et al.*[149] revealed that sperm binding to the oolemma has to be considered as a late interaction between spermatozoa and oocyte representing a discrete parameter of sperm function. Therefore, it seems obvious that spermatozoa showing insufficient penetration, express significantly less fibronectin, which might be one reason for poor results in the sperm penetration assay and failed fertilization in IVF. Miranda and Tezon[152] observed that human spermatozoa express fibronectin during epididymal maturation. Therefore, expression of fibronectin might be of particular importance for sperm–oolemma interaction, that is, binding of spermatozoa. Furthermore, expression of β1 integrins and fibronectin could be demonstrated on spermatogenic cells in human testis.[153] Recently, Ford *et al*. and Freeman *et al*. confirmed the diagnostic advantage of the SPA.[154,155]

Chromatin condensation

Another parameter of spermatozoal function that has been shown to be predictive of fertilization *in vitro* is chromatin condensation. During spermiogenesis, lysine-rich histones are normally replaced by protamines. This process is a prerequisite for the decondensation of the sperm head in the oocyte to form a male pronucleus. Recently, Steger *et al*. showed that the protamine 1-mRNA to protamine 2-mRNA ratio in round spermatids may serve as a predictive factor for the outcome of ICSI.[156] In case of disturbed chromatin condensation, histones persist and can be identified by staining with acidic aniline blue.[157] Therefore, the ratio of replacement is a measure to determine quality of chromatin condensation. Since nuclear proteins play a significant role in chromatin condensation, this method is an attempt to discriminate between fertile men and those suspected of being infertile,[158,159] using nuclear maturity as a parameter; disturbed chromatin condensation is often observed in combination with an increased number of acrosomal defects.[160]

According to studies by Dadoune *et al.*[161] and Hofmann *et al.*,[160] a normal ejaculate should contain at least 75% aniline blue-negative spermatozoa, which indicates normal chromatin condensation. These data were confirmed by Haidl and Schill[162] and Hammadeh *et al.*,[159] and

therefore show that normal chromatin condensation is mandatory to induce fertilization. The aniline blue stain is highly predictive and may be used as an easy performable laboratory test that should precede all methods of assisted reproduction. However, its value is apparently restricted to conventional IVF procedures, since recent studies assessing chromatin condensation in spermatozoa, used for intracytoplasmic sperm injection, failed to predict the outcome of fertilization by ICSI.[163,164] In this connection, it should be mentioned that Henkel *et al.* demonstrated that glass wool filtration has a selective capacity to enrich the number of normal chromatin condensed spermatozoa,[165] suggesting its beneficial effect for the various procedures of assisted reproduction. In addition, the same working group revealed that the chromatin condensation of human spermatozoa is clearly subject to seasonal changes which show a shift of 6 months on the southern hemisphere.[166] This might have a clinical impact on the results in IVF. Should a patient be examined in winter when the quality of sperm chromatin condensation is high, and referred to IVF in summer when the percentage of normally chromatin-condensed spermatozoa is significantly lower, IVF for this patient might fail. Thus, for these patients, a sperm separation by means of glass wool filtration (PureSperm; Hunter Scientific, Saffron Walden, UK) or migration-sedimentation might be beneficial.

Fertilization as a multifactorial process

It is postulated that if an abnormality in a specific step of the binding-fertilization chain could be identified, the information gained could then be used in selecting optimal therapy for a specific patient. Therefore, the fertilizing ability should be evaluated in a sequential analysis. Oehninger *et al.*[99] proposed that by combining the two bioassays, the HZA, a zona penetration assay, and the heterologous SPA, using zona-free hamster oocytes, it might be possible to evaluate tight sperm-oocyte binding, zona penetration, sperm oocyte fusion and sperm head decondensation in sequence. This is an interesting concept that may refine our ability to diagnose male factor infertility.

Since Amann already pointed out that fertilization is a multifactorial process,[167] prediction of the outcome of assisted reproduction technology (ART) will be more accurate the more parameters are tested.[168] This multifactorial nature of fertilization has to be considered even more as the female gamete, the oocyte, plays an essential role in the process as a whole as well. The female organism is coordinating and modulating events of the male gametes, such as capacitation, AR, adherence to the epithelial surface of the female reproductive tract or in sustaining normal sperm function.[169] All these complex, dynamic interactions eventually lead to the selection of functionally competent spermatozoa *in vivo*. Since the oocyte cannot be tested for diagnostic purposes in human ART, the question raises which of the functions of the male gamete, the spermatozoon, is most predictive.

However, clinicians and scientists are still confronted with the question of whether a given laboratory test or a battery of tests can predict the outcome in assisted reproduction. In addition, it is also not clear whether these tests should be performed in advance of an ART treatment, as is presently the rule in the clinical procedure of counseling patients and which actually makes sense, if the testing should be carried out shortly before IVF/ICSI, or if such tests are not useful because semen samples differ from each other. In 1987, Aitken *et al.* remarked that the prediction of the IVF outcome with sperm functional parameters was excellent when the same semen sample was used on which the IVF was performed, but markedly worse if the functional testing was performed on a previous sample.[170] Later, Sukcharoen *et al.* concluded in a study using two semen samples from IVF and from the same patient some weeks before IVF, that this time delay between testing of sperm fertilizing capacity and performing IVF did not affect predictive accuracy.[171] Considering that these questions are of paramount importance for clinical routine in andrology and assisted reproduction, we aimed at investigating the relationship of different functional parameters of spermatozoa on the outcome of IVF, if tested significantly, that is, up to 3 months before or after the IVF treatment.

MULTIVARIATE APPROACH

Materials and methods

FROM A DATABASE of 4178 patients who attended the Andrological Outpatient Clinic at the Center for Dermatology and Andrology, Justus Liebig University (JLU), Giessen, Germany, a total number of 161 patients who underwent an IVF treatment at the Institute of Reproductive Medicine, JLU, and had been examined for male infertility 3 months before or after the IVF treatment were selected. Andrological diagnosis and IVF techniques were performed according to standard procedures. On the andrological side, sperm

concentration, vitality, motility, acrosin activity, AR and normal sperm morphology were taken into consideration. On the female/IVF side, age of the female, number of transferred embryos, embryo score, fertilization rate and pregnancy rate were taken into consideration. Patients who received an ICSI treatment were not included in the study.

Considering that fertilization and pregnancy are processes that are influenced by a multitude of sequential parameters of both the male and the female partner, we tried to develop a logistic regression model to describe fertilization rate and pregnancy, and investigated the multivariate relationship of andrological parameters and the IVF success. At first, we examined the relationship of these parameters with embryo score, fertilization rate and pregnancy rate by means of the Spearman rank correlation and, due to the statistical characteristics of these parameters, the Median or Wilcoxon test. In addition, the correlation between the andrological parameters was investigated. From the clinical point of view and under consideration of the results of the above mentioned analyses, three sets of variables were selected as possible predictors for IVF success. Thus, the coefficients for the following models were calculated:

(i) Fertilization rate = $f_{(\text{motility, sperm concentration, morphology, AR})}$

(ii) Embryo score = $f_{(\text{motility, sperm concentration, morphology, female age})}$

(iii) Pregnancy = $f_{(\text{motility, morphology, female age, number of embryos})}$

To assess the inter- and intraindividual variability of sperm count, total motility, progressive motility and normal sperm morphology, 26 patients with six successive examinations were randomly chosen from the initial group of patients (n = 4178) who showed at least six repetitive measurements. To exclude the effects of therapy, seven patients with four successive measurements who had no therapy were selected from this subgroup. In order to obtain an unbiased estimation for the inter- and intraindividual variability, a variance component estimation was performed. This mathematical model is hierarchical and includes the factors 'the patient' and repeated measurements as random factors. Since normal distribution of the values was not given, a logarithmic transformation of the data was performed. Therefore, the variability between patients and within a patient can only be described by an interval around the geometric mean of all observed values.

Furthermore, to investigate the influence of leukocytes and ROS, we aimed at investigating the impact of extrinsic ROS produced by leukocytes, and intrinsic ROS produced by the spermatozoa themselves on motility and DNA fragmentation in 63 non-leukocytospermic (leukocyte count less than 1×10^6/mL semen) patients in a separate set of experiments. Ejaculates were randomly selected and analyzed for morphology, DNA fragmentation (TdT-mediated dUTP nick-end-labelling TUNEL assay), sperm count, and motility before and after sperm separation by swim-up. In addition, ROS production by leukocytes (extrinsic) and by the spermatozoa themselves (intrinsic) was evaluated by chemiluminescence and a fluorescence technique, respectively. The techniques applied for the TUNEL assay, the chemiluminescent and fluorescent detection of ROS are described elsewhere.[34]

RESULTS

IN THE FIRST data set, a relevant relation of the different andrological parameters with fertilization and pregnancy could be detected for progressive motility, sperm concentration and normal morphology (Table 1). For the other parameters (sperm vitality, AR and acrosin activity), no relevant association could be found.

Table 1 Results of the analysis of relation between different sperm parameters, fertilization and pregnancy. Only the sperm count and normal sperm morphology appeared to be significantly correlated with fertilization

	Fertilization rate			Pregnancy		
Sperm parameters	*n*	Statistics†	*P*-value	*n*	Statistics‡	*P*-value
Total motility	161	0.13	0.10	133	1.77	0.08
Progressive motility	161	0.14	0.08	133	1.49	0.14
Sperm concentration	161	0.21	0.01	133	0.34	0.73
Normal sperm morphology	156	0.16	0.04	128	1.04	0.30
Maximal induced acrosome reaction	69	−0.17	0.16	55	0.11	0.91
Inducibility of acrosome reaction	69	−0.07	0.58	55	−0.62	0.53

†Spearman rank correlation coefficient; ‡Wilcoxon test, z-approximation.

In order to assess the influence of andrological and gynecologic parameters on fertilization rate, embryo score and pregnancy, logistic regression model with the dependent variable dichotomised fertilization rate and the independent parameters sperm motility, sperm concentration, normal sperm morphology and AR were determined. Although the different sperm parameters showed moderate to good correlations/relations with fertilization *in vitro* in the univariate analysis and the model was successful ($P_{model} = 0.049$), the only essential parameter in this model was morphology ($P = 0.028$) (Table 2a). The attempt to apply a model for the embryo score with the explained independent parameters motility, sperm count, normal sperm morphology and female age was also successful ($P_{model} = 0.031$). However, the only essential parameter was female age ($P = 0.026$) (Table 2b). Finally, the attempt to develop a model for pregnancy with the independent parameters motility, normal sperm morphology, maternal age and number of transferred embryos failed ($P_{model} = 0.173$) (Table 2c). Not even one of the parameters included in the model showed a significant relationship to pregnancy. Due to weak (not relevant) correlations/relations with fertilization rate, pregnancy or embryo score, other parameters, such as acrosin activity, were not included in the models.

In all three models, only one or none the four dependent parameters were essential. Looking for explanations for this result, the intra- and interindividual variability were examined. The variability between the 26 patients selected for this analysis was determined for the first observation of each patient. As these parameters were not normally distributed, the geometric mean and the corresponding 2 s-intervals were computed: sperm concentration 26, 21.07 million/mL, (2.12 million/mL; 209.13 million/mL); motility 26, 32.20%, (7.36%; 140.78%); progressive motility: 25, 29.23%, (9.57%; 89.29%); normal sperm morphology: 24, 14.70%, (1.86%, 115.99%) (sample size, geometric mean, 2 s interval).

To obtain estimators for the intra- and interindividual variability for sperm count, total motility, progressive motility and normal sperm morphology, all patients ($n = 7$) who had no therapy during four successive measurements were selected. Using the method of variance component analysis under the assumption of log-normal distribution, estimations for the intra- and interindividual variability were computed. As the parameters were log-normal distributed, the results are described by means of geometric mean and the corresponding '2 s-interval'. For total motility and normal sperm morphology, we determined the following intervals: intra-individual variability (26.4%; 63.8%), (5.4%; 33.9%), and interindividual variability (18.3%; 92.1%), (2.1%; 86.5%). For the other parameters, these values are shown in Table 3. Very high values for intra- and interindividual variability are obvious and are depicted in Figure 2.

Table 2a Fertilization rate described as function of sperm motility, sperm concentration, normal sperm morphology and acrosome reaction, that is, model: fertilization rate = $f_{(motility,\ sperm\ concentration,\ morphology,\ scrosome\ teaction)}$, $n = 67$

Testing global null hypothesis (likelihood ratio)		
Chi-square	Degrees of freedom	*P*-value
9.550	4	0.049
Analysis of maximum likelihood estimates		
Parameter	Odds ratio and 95% CI	*P*-value
Motility	0.719 (0.250; 2.065)	0.540
Sperm concentration	2.238 (0.740; 6.767)	0.153
Morphology	3.348 (1.139; 9.841)	0.028
Acrosome reaction	0.625 (0.206; 1.898)	0.407

Table 2b Embryo score described as function of sperm motility, sperm concentration, normal sperm morphology and female age, that is, Embryo score = $f_{(motility,\ sperm\ concentration,\ morphology,\ female\ age)}$, $n = 156$

Testing global null hypothesis (likelihood ratio)		
Chi-squared	Degrees of freedom	*P*-value
10.657	4	0.031
Analysis of maximum likelihood estimates		
Parameter	Odds ratio and 95% CI	*P*-value
Motility	1.549 (0.793; 3.027)	0.200
Sperm concentration	0.887 (0.460; 1.711)	0.720
Morphology	1.602 (0.817; 3.140)	0.170
Female age	0.476 (0.247; 0.917)	0.026

Table 2c Pregnancy described as function of sperm motility, normal sperm morphology, female age and number of embryos transferred, that is, Pregnancy = $f_{(motility,\ morphology,\ female\ age,\ no.\ embryos)}$, $n = 128$

Testing global null hypothesis (likelihood ratio)		
Chi-squared	Degrees of freedom	*P*-value
6.380	4	0.173

Since fertilization and pregnancy are multifactorial processes influenced by sequential parameters of both the male and the female partner, a logistic regression model was applied to describe fertilization rate and pregnancy (no. patients: $n = 161$). Different sets of parameters were used as independent variables. All the parameters in the models were dichotomized.

Table 3 Variance component estimation for sperm parameters sperm count, total motility, progressive motility and morphology of seven patients who did not receive a treatment with four repeated measurements in order to obtain unbiased estimates. The high variability is obvious

Parameter	*n*	Geometric mean	Inter-individual variability† s-interval		Intra-individual variability† 2 s-interval	
			Lower border	Upper border	Lower border	Upper border
Sperm concentration (million/mL)	7	20.44	5.33	78.46	4.91	85.13
Total motility (%)	7	41.06	26.42	63.81	18.30	92.11
Progressive motility (%)	7	33.35	20.34	54.67	11.20	99.27
Morphology (%)	7	13.57	5.42	33.98	2.13	86.53

†Computation of the variance components is based on the assumption of log-normal distribution of the parameters of interest. The variability is characterized by the 2 s-interval calculated around the total geometric mean computed for all 28 observed values.

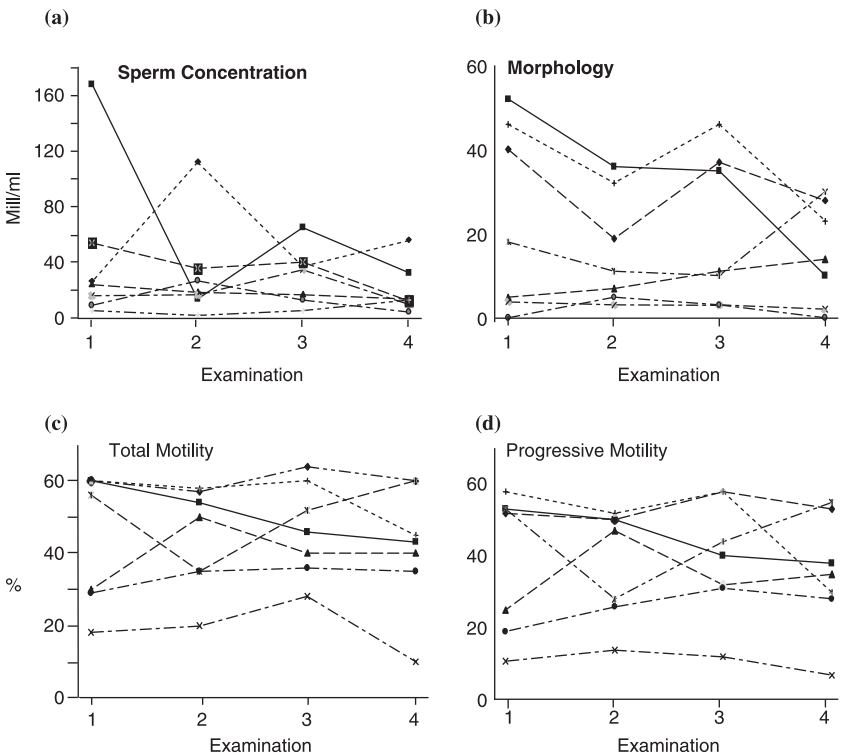

Figure 2 Intra- and interindividual variation of (a) sperm concentration, (b) morphology, (c) total motility, and (d) progressive motility of four successive examinations of seven different patients who had no therapy. The high variation of these sperm functions is obvious.

Seminal leukocytes correlated significantly with extrinsic ROS production ($r = 0.576$; $P < 0.0001$), but markedly less with intrinsic ROS production ($r = 0.296$; $P = 0.0218$). Sperm count, morphology and motility in the ejaculate were markedly more affected by extrinsic than by intrinsic ROS. However, DNA fragmentation was strongly positively correlated with intrinsic ROS production ($r = 0.504$; $P = 0.0001$), while this correlation was weaker for extrinsic ROS production ($r = 0.400$; $P = 0.0019$). No correlation was found between DNA fragmentation and the number of leukocytes ($r = 0.237$; $P = 0.0716$), while the correlations with motility in the ejaculate ($r = -0.523$; $P < 0.0001$) and the motile sperm count were highly significant ($r = -0.598$; $P < 0.0001$). Moreover, significant differences were observed for extrinsic ($P = 0.0003$) and intrinsic ROS production ($P = 0.0047$), sperm DNA fragmentation ($P = 0.0125$) and sperm motility in the ejaculate ($P = 0.0013$) between

groups of patients having a high (≥0.1 million/mL) and a low number (<0.1 million/mL) of leukocytes in the ejaculate.

DISCUSSION

Influence of different sperm parameters on the fertility status

THE QUESTIONS WHETHER a male is fertile and how fertility can be predicted have repeatedly been approached in domestic animals and human.[168,172–175] In stockbreeding, the aspects for a correct fertility evaluation and prediction are clearly oriented towards the selection of the most fertile animals at the lowest possible cost. In human reproduction, scientists and clinicians are dealing with men who are sub- or infertile due to various reasons and who want to father their own child. The high financial cost of ART can be restrictive for some patients. In human reproduction, health and ethical aspects weigh higher than in animals. However, the basic questions whether or not the outcome of ART can be predicted, and how accurate this prediction would be are the same. In addition, questions are raised as to which fertility tests should be applied and can fertility be predicted from a given semen sample.[176]

As fertilization is a multifactorial process that is not only influenced by the distinct sperm functions, as pointed out in Figure 1, but also by other sperm parameters, such as the ability of the spermatozoa to undergo capacitation or the mitochondrial membrane potential, an answer to the above questions is not easy. Also, the weight of each parameter contributing to the process as a whole is still unknown. However, in a meta-analysis, using data from studies that investigated sperm parameters in the same ejaculate that was also used for insemination, Oehninger *et al.*[175] reported a high predictive power of sperm-ZP binding and the inducibility of AR. Moreover, as the female organism is selecting functionally competent spermatozoa, modulating sperm functions and coordinating sperm and female genital tract functions, thus sustaining normal sperm functions, female parameters influencing the fertilization process must not be neglected. Therefore, factors that have an effect on the fertilization process and the onset of pregnancy are, to the present knowledge, summarized in Table 4, however, this Table may be still incomplete.

When focusing on andrology and the contribution of spermatozoa to the fertilization process in the past, the vast majority of papers published on the prediction of the outcome of ART investigated sperm functions when the tests were performed with spermatozoa from the same ejaculate that was also used for insemination. In these studies, significant correlations between the respective sperm functions and fertilization *in vitro* were observed repeatedly.[15,34,60,100,123,138] They reflect the importance of sperm functionality for the fertilization process, and significantly contributed to our understanding of the fertilization process itself. In addition, in most of the studies, only a single parameter was tested and correlated with the fertilization result and

Table 4 Factors influencing the (correlating with) fertilization process and the onset of pregnancy

Male/sperm factors	Female/oocyte factors
Sperm count	Maturity of oocyte
Motility	Maturity of spindle
Progressive motility	Maturity of zona pellucida
Morphology	Ability of the zona to induce acrosome reaction
Ability of spermatozoa to undergo capacitation	Intact cumulus-oocyte complex
Ability to induce hyperactivation	Ability to modulate sperm functions
Ability to bind to the zona pellucida	DNA fragmentation
Ability to undergo acrosome reaction	Sufficient selection of functionally competent spermatozoa
Energy metabolism	Sufficient transport of functionally competent spermatozoa
Mitochondrial membrane potential	Sufficient supply of energy resources for spermatozoa
Membrane integrity	Ability to decondense the sperm head
Normal chromatin condensation	Ability to restore limited DNA damages
Ability to: bind to and fuse with the oolemma	Endometriosis
DNA fragmentation	NA
Influence of leukocytes	NA
Sufficient contribution of accessory sex glands	NA
Autoantibodies against spermatozoa	NA
Sperm maturity as tested by creatine kinase or HspA2 chaperone levels	NA
Sufficient elimination of the element zinc from the spermatozoa during epididymal maturation	NA

pregnancy outcome. Most of these sperm function tests showed a more or less high predictive power as well as sensitivities and specificities of the test system. However, by all qualities of these test systems, they are used in andrological diagnosis under the premise that the conditions in an ejaculate obtained months before the actual fertility treatment takes place are the same in the ejaculate that will also be used for the insemination. In addition, as to the multifactorial nature of fertilization, most fertility centres perform only a selection of the apparently most relevant and most cost-effective tests, with the consequence that not all relevant male and sperm parameters are tested. This can lead to a lack of information and 'surprise' if the expected result, fertilization and pregnancy does not materialize. In turn, patients, especially the women, will be disappointed and may suffer from depression.

For this reason and in order to improve andrological diagnosis, Oehninger *et al.*[99] suggested investigating sperm-zona binding and sperm-oolemma binding as key sperm functions in a sequential manner. While Parinaud *et al.*[172,177] proposed a scoring method that included sperm functions, such as normal morphology, sperm viability, rapid motility, linearity of motility, spontaneous AR and the acrosomal response, Duran *et al.*[178] suggested a logistic regression model composed of the evaluation of sperm morphology and DNA strand breakages by means of the acridine orange stain. However, consideration was even given to describe the success of ART by means of complex mathematical formulas based on the ultrastructural evaluation of sperm morphology.[179,180] Since the attempt to predict male fertility is very difficult, in search of the most practical and most predictive, even different mathematical approaches have been discussed.[181] However, as mammalian reproduction is not simply an event of one single individual, but a result of the interaction of the gametes of both sexes, the female contribution has not yet been taken into consideration.

Mainly gynecological-oriented working groups have addressed this problem. In a multivariate analysis of factors predicting the success of live births after IVF, Minaretzis *et al.*[182] reported that previous pregnancies and the number of embryos transferred, correlated positively, while maternal age was negatively correlated with live birth. These conclusions could basically be confirmed by Hunault *et al.*[183] and Chuang *et al.*[184] In addition, there is evidence for a predictive value of the number of retrieved oocytes and an embryo score of the transferred embryos for ongoing pregnancy.[183,185] Our observation that maternal age is most important for the prediction of the embryo quality corresponds with these ideas. As a possible cause for the lower quality of oocytes from older women, Catt and Henman[186] discussed inherent age-related defects in oocytes and embryos, because oocytes of older women may suffer oxidative stress due to the higher production of reactive oxygen species by their mitochondria.[187]

However, in an attempt to address parameters of both sexes, Ashkenazi *et al.*[188] applied logistic regression analysis to sperm concentration, sperm motility index, hypoosmotic swelling and woman's age, and found that these parameters would be sufficient to predict sperm fertilizing capacity in IVF. In a study performed on 522 intrauterine insemination (IUI) cycles, the Tygerberg group[189] revealed that on the gynecologic side, the number of follicles and the woman's age were significantly correlated with pregnancy. On the andrological side, sperm motility and normal morphology have been identified as male factors that could significantly and independently predict the outcome of the fertility treatment. However, all these approaches to predict IVF outcome were still based on the same ejaculate, which was also used for the insemination in IUI and IVF.

In the present study, we analyzed data from IVF patients, where the male partner underwent andrological diagnostics 3 months (cycle of spermatogenesis) before or after the IVF treatment. The results of this study, and in particular the high variability of the sperm parameters analyzed, point out the problems of the prediction of fertilization and pregnancy in IVF. Since experienced and quality-controlled technologists performed all andrological laboratory diagnostics, these results indicate that the andrological status at the end of a respective andrological treatment seems not necessarily represent the status at the time of IVF. This assumption is supported by the variance component estimation. Despite a relatively low correlation coefficient in the logistic regression model, it appears that among the parameters tested, the most reliable parameter to predict fertilization *in vitro* is normal sperm morphology, even from ejaculates analyzed up to 3 months before or after the IVF treatment, which is the case in normal clinical routine. Therefore, our data confirmed the paramount contribution of normal sperm morphology to successful fertilization and the importance of an accurate evaluation of the percentage of morphologically normal spermatozoa, as well as its value for andrological diagnosis.[14,19,190–192]

Obviously, this good predictive power of normal sperm morphology is based on the fact that sperm

morphology appears to be closely correlated with other sperm functions, as it could be shown for sperm zona binding ability,[22,193] acrosomal functionality,[26,27,194] motility[21,58] or the sperm cell's own ROS production.[34] However, it is a good reason to believe that sperm morphology is a stable or even the most stable parameter, as the morphological phenotype of spermatozoa is genetically determined and stays relatively constant with respect to the type of abnormalities and the percentage of normal forms.[195–198] However, sperm morphology is also a very sensitive parameter with regard to physical conditions or environmental factors.[199,200] Nevertheless, the concept of the relative stability of sperm morphology could not be confirmed in this study, as there is a high intra-individual variability, which in turn would be in accordance with findings of Tielemans *et al.*[201] However, compared with the other functional parameters investigated, intra-individual variability of sperm morphology seems to be less, thus resulting in a parameter that is reliable and useful for the prediction of the outcome of assisted reproduction.[14,202] The only prerequisites for reproducible and reliable results are thorough training, continuous quality control of the laboratory personnel and a standardized methodology.[20,203]

According to Tielemans *et al.*,[201] sperm concentration had the largest reliability coefficient for conception, followed by motility and morphology. Although sperm concentration showed the highest level of significance with regard to fertilization in our correlation model, a significant relationship between these two parameters could no longer be observed after logistic regression, including motility, morphology and AR. Instead, normal sperm morphology was the only parameter that could predict fertilization. The fact that seminal sperm concentration does not count for successful fertilization or pregnancy, may be associated with the adjustment of sperm concentration for the *in vitro* insemination, together with the selection of functionally competent spermatozoa by means of sperm separation methods, even in patients with low seminal sperm concentration. In addition, Tielemans *et al.*[201] performed the study at a time where ICSI had just started and was therefore not available in many centers. However, since by ICSI, all physiological barriers are bypassed and therefore the sperm–oocyte interactions are reduced to a minimum, causing an anomalous fertilization process; ICSI was expressly excluded in our study. However, in a randomized controlled trial and a meta-analysis, Tournaye *et al.*[204] showed that sperm concentration has an influence on fertilization if patients were treated with normal IVF, although they should have been treated with ICSI because of their poor semen analysis. In these cases, the overall fertilization rate was significantly lower after IVF. Nevertheless, if the IVF insemination was performed with a five times higher sperm concentration, results in terms of fertilization were comparable with ICSI.

Our attempt to predict pregnancy by including those parameters from both sexes (sperm motility, normal sperm morphology, maternal age, number of transferred embryos) in our logistic regression model, that are reportedly most predictive for IVF success, failed, and not even one of the parameters included showed a significant relationship to pregnancy. The reasons for this failure are currently only speculative, but the accumulation of other parameters, such as oocyte or sperm DNA integrity, which has been shown to have a significant influence on the occurrence of pregnancy,[60,205] that were not included in the model, might be responsible. However, this impossibility to predict the outcome of ART also clearly reflects the enormous problems clinicians and scientists have in their endeavour to do so. The more parameters will be involved in the occurrence of a certain event, such as pregnancy, the more difficult is the prediction.

When focusing only on male fertility and on the relevant laboratory parameters, the first question that has to be addressed is about which of the different known sperm parameters should be tested. Obviously, this will also include a financial aspect, as some of the laboratory techniques are rather expensive, large-scaled and require highly trained personnel, and not every laboratory can afford these costs. Any seminal sample contains a heterogeneous population of spermatozoa of different functional competence, and fertilization is a multifunctional process that is also determined by the probability of the presence of a sufficient number of functionally competent spermatozoa in the close vicinity of a matured and functionally competent oocyte. Amann and Hammerstedt[168] described two prerequisites that must be met in order to predict the fertility status of a given male individual. Fertilizing competence must be given for: (i) each spermatozoon where all functional parameters of the spermatozoon must be normal at the right site and right time; and (ii) for the population of spermatozoa as a whole, where this population contains a certain percentage of functionally competent spermatozoa. Considering these two prerequisites, an individual spermatozoon would have to be considered *infertile* if only one of its functional parameter was low-ranged. However, the fertility status would

be *fertile*, if all of its functional parameters were normal. For a whole population of spermatozoa, either in the native semen or in a separated sperm fraction, this sperm population would have to be considered *infertile* if only one of the parameters tested was abnormal. However, if the tested parameters are normal, one cannot regard this sperm population as fertile yet, because another functional parameter that was not tested can be abnormal, therefore the fertility status of this sperm population has to be regarded as *unknown*.

This concept is depicted in Figure 3 for the percentage of acrosome-reacted spermatozoa (Fig. 3a) and for the acrosin activity index (Fig. 3b). The 'blank' bars represent patients who fertilized oocytes in IVF, the 'black' bars such patients who showed poor IVF results (fertilization rate < 50%). The cut-off value for the percentage of acrosome-reacted spermatozoa and the acrosin activity index are indicated at 13% and 6, respectively. A patient whose AR or acrosin activity was low-ranged, that is, had less than 13% acrosome-reacted spermatozoa after induction of AR in the ejaculate or had and acrosin activity index less than 6, showed poor fertilization results. However, there are also patients who had poor fertilization results in IVF, but whose test parameters were normal. Apparently, in these patients, a parameter other than the one tested was low-ranged, causing the poor fertilization. In this regard, functional parameters of the oocyte should of course not be forgotten. Therefore, if one does not count a diagnostic IVF, which is actually prohibited in countries such as Germany, the wish of some clinicians for the ultimate fertility assay is and will be in future utopia. Moreover, this is even more the case as other external parameters, such as leukocytes, do influence the fertilizing potential of individual spermatozoa as well as of whole sperm population.[206,207]

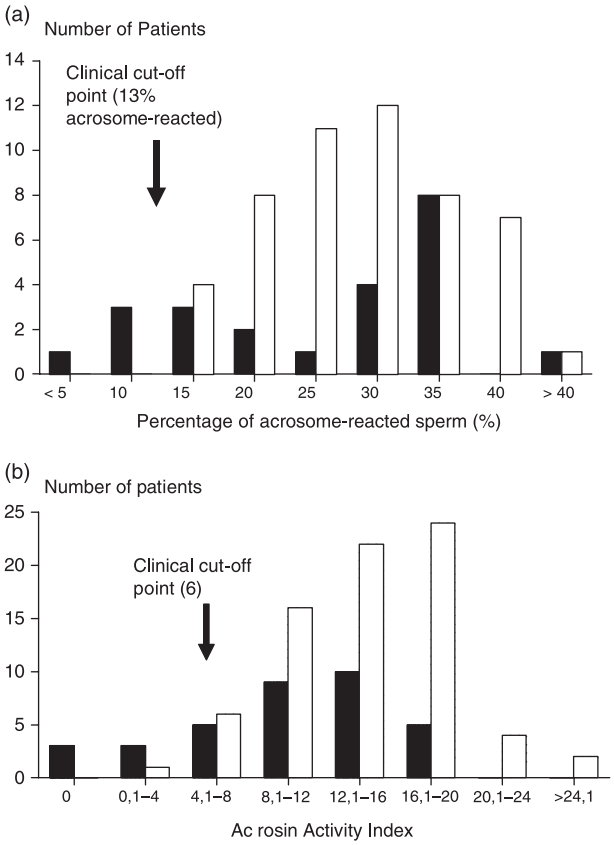

Figure 3 Cumulative number of patients for the percentage of acrosome-reacted spermatozoa after induction by means of the low-temperature method of 76 patients (a), and of the acrosin activity index measured by means of the gelatinolysis technique (b) of 110 patients. Patients with a fertilization rate higher than 50% show a normal distribution while those who show poor fertilization (fertilization rate < 50%) are left-shifted. Arrows indicate the cut-off points of the tests. Patients with a normal acrosome reaction and normal acrosin activity index, but low fertilization probably have fertilization disorders other than decreased acrosome reaction or acrosin activity. Therefore, only in those cases where the specific test, acrosome reaction or acrosin activity, is abnormally low, male infertility can be attributed to that particular sperm function. For patients where these functional tests were normal, the fertility status has therefore to be regarded as *unknown*. (■) Fertilization rate < 50%, (□) fertilization rate > 50%.

Influence of the oxidative status and leukocytes on sperm function

Previous findings revealed that leukocyte-derived extrinsic ROS production is positively correlated with the sperm cell's own intrinsic ROS production,[34,208] and could clearly be confirmed in this study. The noticeably lower correlation coefficient and level of significance for the correlation between the number of leukocytes in the ejaculate and intrinsic ROS production, as compared to the correlation between the number of leukocytes in the ejaculate and extrinsic ROS production, is indicative for a different action of ROS from a different origin. Therefore, the site of ROS production, either inside the spermatozoa themselves or outside by leukocytes, appears to play a role for sperm function. This effect can be explained by the fact that among the oxidants produced by leukocytes, H_2O_2 is persistent and can even penetrate plasma membranes, while other ROS-like superoxide or the ·OH are non-membrane permeable. Consequently, externally produced non-membrane penetrating ROS will oxidize the phospholipids in

the sperm plasma membrane and cause lipid peroxidation, which would affect sperm functions, such as motility.[36] H_2O_2, however, can penetrate the plasma membrane and therefore damage DNA integrity.[209] Considering the membrane permeability of H_2O_2, it is obvious that the external production of ROS must have a negative effect on sperm motility and DNA integrity. Here, and in a previous report,[34] we showed that extrinsic ROS significantly affects motility and DNA integrity, while intrinsic ROS affects DNA fragmentation.

This finding is consistent with those of Alvarez *et al*.[210] and Erenpreiss *et al*.,[211] who showed that leukocytospermia negatively affects sperm DNA integrity. Even in non-leukocytospermic patients, seminal leukocytes significantly impair sperm DNA integrity and motility. Therefore, we can confirm the observation of Sharma *et al*.[207] that oxidative stress, which reduces sperm fertilizing capacity, can even occur in patients with seminal leukocyte counts much less than 1×10^6/mL. Since Aitken *et al*.[212] demonstrated that leukocyte contaminations of more than 2×10^4 leukocytes/mL have detrimental effects on separated spermatozoa, the question raises whether the WHO threshold for leukocytospermia (1×10^6/mL) is still justified, because even low amounts of ROS are harmful to sperm DNA integrity.[65] Our results on ROS show that the origin of ROS deriving from both leukocytes and the sperm cells seems to have an influence on the site of the damage. In addition, since leukocyte counts much less than the normal value, as defined by the WHO, contributed to a significant decrease of motility and DNA integrity, this definition given by the WHO should be re-evaluated. Moreover, a significant effect of leukocyte action in the semen on fertilization and pregnancy might be possible in cases with elevated leukocyte concentrations and/or elevated levels of ROS accompanied by low levels of antioxidant capacity. Pasqualotto *et al*.[213] demonstrated that infertile patients did not only have elevated ROS levels, but also reduced levels of antioxidant capacity. This observation supports the concept that the balance between ROS generation and antioxidant capacity in the semen plays a critical role in the pathophysiology of genital tract inflammations and their impact on sperm functions and fertilization/pregnancy.[214]

Do sperm function tests contribute to fertility diagnosis?

From the above data and facts, it is obvious that fertilization is a multifactorial process and answering this question is not easy. If this question should be answered from a clinical point of view, the prediction of the male fertility status is certainly not accurate and the factor time must be taken into consideration, as the intra- and interindividual variation of the parameters is enormous. In addition, other parameters, such as the presence of leukocytes in the ejaculate, motility, chromatin condensation and seasonal variations of sperm concentration, are frequently found playing a paramount role and can therefore affect the outcome of assisted reproduction. Therefore, this study points out that the andrological status of a patient at the time of examination does not necessarily represent the status at the time of IVF.

Should the above question refer to the importance of functional sperm parameters influencing the fertilization process, it needs to be answered with a clear *yes*, if the correlation between these parameters and fertilization is investigated in the same ejaculate, which is also used for insemination. However, in a routine clinical setup, where patients are examined months before the treatment takes place, the high intra- and interindividual variability clearly reduces the clinical value of these parameters. Therefore, scientists and clinicians are urged to search for parameters with lower variability to improve and to standardize the laboratory methods.

Finally, as only a selection of diagnostic methods will be employed in the clinical routine, the examination of the male fertility status will always be fragmentary. However, in order to give the best counseling and most efficient treatment to a couple, an andrological examination should include as many functional parameters as possible, because only by doing so, the risk of a failed treatment in assisted reproduction with all its complications can be minimized. An example for this necessity of determining functional sperm parameters is the detrimental influence of smoking and varicocele on human sperm acrosin activity and AR.[214] It can be noted that not all these patients are prone to this negative influence and smoking appears not to affect sperm motility or sperm viability; a potential risk of fertilization failure in IVF would not be identified if these functional sperm parameters had been tested during andrological examination. In addition, the detrimental effect of leukocytes present in the ejaculate and/or an imbalanced antioxidative protection system in the seminal fluid on functional parameters of spermatozoa should not be neglected.

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