The effect of solubilized homologous zona pellucida on the human acrosome reaction, sperm-zona binding and motion characteristics of capacitated human spermatozoa

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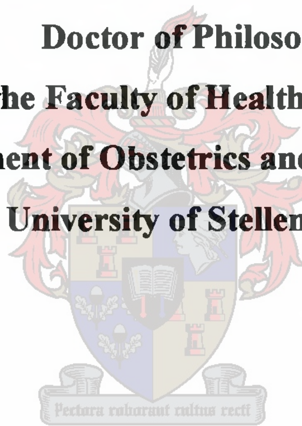

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DECLARATION

I, the undersigned, hereby declare that the work in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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ABSTRACT

CHAPTER 1 provides literature based background information to emphasize the use of a sequential, multistep diagnostic schedule for couples in an assisted reproductive program as well as the clinical importance of sperm morphology as recorded by strict criteria during the diagnostic approach of the infertile couple. Furthermore, the chapter includes evidence underlining the growing need for the implementation of the physiologically induced acrosome reaction as an important contribution to the assisted reproductive program. The zona pellucida-induced acrosome reaction (ZIAR), sperm-zona interaction as well as computer-assisted semen analyses were investigated. **CHAPTER 2** provides detailed experimental protocols of the materials and methods used in the study. **CHAPTERS 3-6** each represent a separate study that was prepared as a scientific paper and encompass the experimental research undertaken in the reproductive biology research laboratory at Tygerberg Hospital to address important aspects of human acrosome processes. In the **first study**, we aimed to evaluate the regulatory role of the G_i-like protein during the AR of normal sperm donors and the role of intact acrosomes during sperm-zona binding. It seems that pertussis toxin-sensitive G_i-like protein in human spermatozoa plays an important regulatory role in the ZIAR and this underlines the importance of intact acrosomes during sperm-zona binding. In the **second study**, we aimed to evaluate the relationship between the ZIAR and the percentage normal spermatozoa as well as the sperm-ZP binding potential among men referred for a routine semen analysis. ZIAR testing should become part of the second level of male fertility investigations, *i.e.*, sperm functional testing, since 15% of the andrology referrals revealed an impaired AR response to solubilized ZP. In the **third study**, we aimed to evaluate the possible relationships between the sperm morphology, the acrosome responsiveness to solubulized human ZP and the sperm-zona binding potential among consecutive andrology referrals and randomly selected IVF cases. ZIAR results provide further information regarding dysfunctional sperm and can be used as an additional diagnostic test since the results predicted fertilization failure during IVF treatment. In the **fourth study**, we aimed to evaluate changes in the sperm motion characteristics and the occurrence of hyperactivated motility after exposure to ZP among andrology referrals. Solubilized human ZP induces hyperactivated motility among sperm populations that have been capacitated under laboratory conditions. Capacitated spermatozoa have an elevated percentage hyperactivated cells that correlate with the percentage normal spermatozoa in the ejaculate. **CHAPTER 7**, the general discussion, is brief and concise to avoid unnecessary repetition, underlines the validity of a sequential, multistep diagnostic approach and concludes with the recommendation that the ZIAR should form part of the diagnostic tools in the assisted reproductive program.

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OPSOMMING

HOOFSTUK 1 bestaan uit 'n omvattende agtergrondstudie wat bestaan uit die ontwikkeling van diagnostiese toetse, die hantering van die egpaar in die reproduktiewe ondersteunings-program asook op die kliniese belang van spermmorfologie. Die toenemende behoefte aan die implementering van die fisiologies-geïnduseerde akrosoomreaksie, as 'n belangrike bydrae tot die reproduktiewe ondersteuningsprogram, word ook beklemtoon. Die zona pellucida geïnduseerde akrosoomreaksie (ZIAR), sperm-zonabinding asook rekenaar-bemiddelde semenanalises is ondersoek. **HOOFSTUK 2** dek gedetailleerde eksperimentele protokolle van die materiale en metodes wat in die studie gebruik is. **HOOFSTUKKE 3-6** behandel die eksperimentele navorsing wat in die laboratorium van die reproduktiewe biologie-eenheid te Tygerberg hospitaal uitgevoer is en wat as ses afsonderlike wetenskaplike publikasies aangebied word. Die doel van die **eerste studie** was om die regulerende rol van G_i-proteïene tydens die AR van normale spermdonors asook die rol van intakte akrosome tydens sperm-zonabinding te evalueer. Dit kom voor asof G_i-proteïene in spermatozoa 'n belangrike regulerende rol in die ZIAR speel. Dit beklemtoon die belangrikheid van intakte akrosome tydens sperm-zonabinding. Die doel van die **tweede studie** was om die verhouding tussen die ZIAR en die persentasie normale spermatozoa asook die sperm-zonabindingspotensiaal tussen mans wat vir 'n roetine semenanalise verwys is te evalueer. ZIAR-toetsing moet deel uitmaak van die tweede vlak van manlike fertiliteitsondersoeke, d.w.s. funksionele toetsing, aangesien 15% van die andrologie pasiënte 'n verswakte AR respons tot opgeloste ZP openbaar. In die **derde studie** was die doel om die moontlike verhoudinge tussen sperm-morfologie, die ZIAR en die sperm-zonabindingspotensiaal onder opeenvolgende andrologie-pasiënte asook lukraak geselekteerde IVB-pasiënte te evalueer. Die ZIAR-resultate bied verdere informasie aangaande disfunksionele spermatozoa en kan gebruik word as 'n addisionele diagnostiese toets aangesien hierdie resultate mislukte bevrugting tydens IVB behandeling voorspel. Die **vierde studie** het ten doel gehad om veranderinge in sperm-motiliteitseienskappe asook hiperaktiwiteit na die blootstelling aan opgeloste zona onder andrologie-pasiënte te evalueer. Daar is afgelei dat opgeloste menslike zona hiperaktiwiteit induseer in spermpopulasies wat onder gunstige laboratoriumkondisies gekapasiteer is. Die gekapasiteerde spermatozoa het 'n verhoogde persentasie gehiperaktiveerde spermatozoa getoon wat met die persentasie normale spermatozoa in die ejakulaat korreleer. In **HOOFSTUK 7** word aangetoon dat dit noodsaaklik is om die diagnostiese skedule by die hantering van die onvrugbare egpaar te gebruik asook dat die ontwikkeling van die funksionele toestand belangrik is vir die bepaling van ZIAR.

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Dedicated to

Rosemund, Maud & Muriel

for your unconditional love and support

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CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Considerable progress has been made towards the understanding of sperm physiology and the biology of gamete interaction, but still more work is needed to achieve objectivity and standardization of some of the andrological diagnostic methods used in the clinical setting. More information is needed to definitively establish which tests are more accurate predictors of sperm performance and how they correlate with pregnancy potential following in vivo and in vitro interventions.

Infertile men can in some cases be successfully treated with defined urological and medical therapies or with assisted reproductive technologies. Among the latter, in vitro fertilization (IVF) and embryo transfer, augmented with intracytoplasmic sperm injection (ICSI) in moderate and severe cases, constitute validated and successful ways to assist fertilization and conception (Oehninger *et al.*, 2000). Nevertheless, it is expected that simplified and more cost-efficient therapeutic modalities will be developed as additional basic (cellular-molecular) and clinical knowledge is gained.

The cornerstone of the andrological evaluation in all cases is an exhaustive history and a physical examination followed by repeated semen analyses. A urological, endocrine and/or imaging workup should be implemented as appropriate. A comprehensive analysis following the World Health Organization guidelines (WHO, 1999) is fundamental at the primary care level to make a rationale initial diagnosis and to select the appropriate clinical management. The collection and analysis of the semen must be undertaken by properly standardized procedures in appropriately qualified and accredited laboratories (De Jonge, 2000).

The basic semen evaluation should include: (i) assessment of physical semen characteristics (volume, liquefaction, appearance, consistency, pH and agglutination) (WHO, 1999); (ii) evaluation of sperm concentration, grading of motility and analysis of morphological characteristics (using strict criteria) (Kruger *et al.*, 1986; Menkveld *et al.*, 1990; Kruger & Coetzee, 1999); (iii) determination of sperm vitality (viability), testing for sperm auto-antibodies (using the mixed antiglobulin reaction and/or the direct immunobead tests), presence of leukocytospermia and immature sperm cells (WHO, 1999); and (iv) bacteriological studies (WHO, 1999). The identification and separation of the motile sperm fraction is also regarded as an important factor as far as semen quality is concerned (Oehninger, 1995; Mortimer, 2000).

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Other categories of assays that are usually considered include: (i) tests that examine defective sperm functions indirectly through the use of biochemical tests (*i.e.*, measurement of the generation of reactive oxygen species or evidence of peroxidative damage, measurement of enzyme activities such as creatine phosphokinase and others) (Aitken *et al.*, 1991; Huszar & Vigue, 1994); (ii) bioassays of gamete interaction (*i.e.*, the heterologous zona-free hamster oocyte test and the homologous sperm-zona pellucida binding assays) (Burkman *et al.*, 1988; Liu *et al.*, 1988; Franken, 1998; Oehninger *et al.*, 2000) and induced acrosome reaction evaluation (ESHRE, 1996); and (iii) computer-assisted sperm motion analysis (CASA) for the evaluation of sperm motion characteristics (WHO, 1999).

1.2 The basic semen analysis

The basic semen analysis is the cornerstone of laboratory evaluation of the male as far as routine evaluation of male infertility is concerned. The physical examination of semen (e.g. coagulation, liquefaction and viscosity) is important and gross abnormalities, when present, should be detected and carefully reported by the andrology laboratory regardless of the quality of other semen parameters. Semen processing using different separation techniques is often hampered by physical abnormalities (Menkveld & Kruger, 1990).

Normal semen is a mixture of spermatozoa suspended in secretions from the testis and epididymis which, at the time of ejaculation, are combined with secretions from the prostate, seminal vesicles and bulbourethral glands (Yanagimachi, 1994). The final composition is a viscous fluid that comprises the ejaculate.

Measurements made on the whole population of ejaculated spermatozoa cannot define the fertilizing capacity of the few that reach the site of fertilization. Nevertheless, semen analysis provides essential information on the clinical status of the individual. Clearly, the collection and analysis of semen must be undertaken by properly standardized procedures if the results are to provide valid information.

The semen sample should be collected after a minimum of 48 hours but not longer than seven days of sexual abstinence (WHO, 1999). To reduce the variability of semen analysis results, the number of days of abstinence should be as constant as possible. The sample should be obtained by masturbation and ejaculated into a clean, wide-mouthed container made of glass or plastic. It is important to emphasize to the patient that the semen sample should be complete.

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1.2.1 Macroscopic examination

1.2.1.1 Liquefaction

A normal semen sample liquefies within 60 minutes at 37°C, although usually this occurs within 15 minutes (WHO, 1999). In some cases, complete liquefaction does not occur within 60 minutes and this should be recorded.

The sample must be well mixed in the original container and must not be shaken vigorously. During liquefaction, continuous gentle mixing or rotation of the sample container may reduce errors in determining sperm concentration (de Ziegler *et al.*, 1987). If sperm motility is to be assessed at 37°C, the sample should be equilibrated to this temperature during liquefaction and mixing.

1.2.1.2 Appearance

The semen sample should be examined immediately after liquefaction or within one hour of ejaculation, first by simple inspection at room temperature (WHO, 1999). A normal sample has a homogenous, grey-opalescent appearance. It may appear less opaque if the sperm concentration is very low, red-brown when red blood cells are present or yellow in a patient with jaundice or taking some vitamins.

1.2.1.3 Volume

The volume (2-6 ml) of an ejaculate may be measured using a graduated cylinder with a conical base or by weighing standard containers with and without semen (WHO, 1999). Plastic syringes should not be used because they may affect sperm motility and hypodermic needles are unsafe.

1.2.1.4 Viscosity

The viscosity (sometimes referred to as “consistency”) of the liquefied sample can be measured after semen has been evenly mixed and the length of a thread produced by a drop from a glass pipette when it is slowly released is estimated (WHO, 1999). If the viscosity is normal, the drop of semen, stretched like a thread of about 1cm in length, will hang for 1-1.5 seconds. In cases of abnormal viscosity, the drop will form a thread more than 2cm long.

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High viscosity can interfere with determination of sperm motility, concentration and antibody coating of spermatozoa (WHO, 1999). To reduce viscosity, additional treatment, mechanical mixing or enzyme digestion (e.g. bromelain) may be necessary.

1.2.1.5 pH

The pH of a normal ejaculate may vary between 7.2 and 7.8, and is measured after liquefaction (WHO, 1999). Above or below this range may be an indication of inflammation of the male accessory sex organs or chronic disease of the prostate glands and/or seminal vesicles. If the pH is less than 7.0 in a sample with azoospermia, there may be obstruction of the ejaculatory ducts or bilateral congenital absence of the vasa.

1.2.2 Microscopic examination

During the initial microscopic investigation of the sample, estimates are made of the concentration, motility and agglutination of spermatozoa. A phase-contrast microscope is recommended for all examinations of unstained preparations of fresh semen or washed spermatozoa.

1.2.2.1 Sperm concentration

Accurate determination of the sperm concentration (millions of spermatozoa per milliliter of semen) and the total sperm count (millions of spermatozoa per ejaculate) are important parameters for evaluating the quantitative aspects of spermatogenesis. The number of spermatozoa is evaluated in terms of both sperm density and recoverable spermatozoa. If the sperm density is $<20x10^6$/ml, the specimen is considered abnormal (WHO, 1999). The most accurate method determining sperm concentration applicable in the routine laboratory situation is volumetric dilution and hemocytometry.

If the number of spermatozoa per visual field varies considerably, it indicates that the sample is not homogenous and should be mixed again. Lack of homogeneity may result from abnormal consistency, abnormal liquefaction, aggregation of spermatozoa in mucous threads or from sperm agglutination (WHO, 1999). All samples in which no spermatozoa are detected by microscopy should be centrifuged to detect the presence of spermatozoa in the sediment. When no spermatozoa are found after a complete and systematic search of all the resuspended precipitate, should samples be classified as azoospermia.

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1.2.2.2 Sperm motility

Sperm motility is essential for transport through the female reproductive tract and for fertilization. Vigorous motility, also called hyperactivated motility, is necessary in certain sites and at certain times to ensure successful sperm migration to the site of fertilization, and transit through the granulosa cell vestments and the zona pellucida of the oocytes (Yanagimachi, 1994). Increased motile vigour and directionality of motion are also associated with semen of increased quality.

A simple grading system is recommended which provides an assessment of sperm motility without the need for complex equipment. According to the WHO (1999), the motility of each spermatozoon is graded “a”, “b”, “c” or “d”, according to whether it shows:

a. rapid progressive motility (*i.e.*, =25µm/s at 37°C and =20µm/s at 20°C; note that 25µm is approximately equal to five head lengths or half a tail length);
b. slow or sluggish progressive motility;
c. nonprogressive motility (<5µm/s);
d. immotility.

The number of spermatozoa in each category can be tallied with the aid of a laboratory counter. All spermatozoa with grade a and b motility are counted first. Subsequently, spermatozoa with nonprogressive motility (grade c) and immotile spermatozoa (grade d) are counted in the same area.

In our laboratory, qualitative motility is evaluated on a scale of 0 to 4 (Menkveld & Kruger, 1996):

0 no movement.
1 movement – none forward.
1+ movement – a few now and then.
2 movement – slowly and undirected.
2+ movement – slowly and directly forward.
3- movement – fast and undirected.
3 movement – fast and directly forward.
3+ movement – very fast and directly forward.
4 movement – extremely fast and directly forward.

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1.2.2.3 Sperm agglutination

Agglutination of spermatozoa means that motile spermatozoa stick to each other head-to-head, tail-to-tail or in a mixed way, e.g. head-to-tail (WHO, 1999). The adherence either of immotile spermatozoa to each other or of motile spermatozoa to mucous threads, cells other than spermatozoa, or debris is considered to be nonspecific aggregation rather than agglutination and should be recorded as such (WHO, 1999).

The presence of agglutination is suggestive of, but not sufficient evidence for, an immunological cause of infertility. Agglutination is assessed at the time of determining sperm motility. The type of agglutination should be recorded, e.g. head-to-head, tail-to-tail or mixed. A semiquantitative grading from – (no agglutination) to +++ (severe clumping in which all the motile spermatozoa are agglutinated), can be used (WHO, 1999).

1.2.2.4 Sperm morphology

The morphologic characteristics of spermatozoa appear to be the best predictor for fertilization. Based on previous publications, strict criteria should be employed during the morphological examination of spermatozoa since it gives the best predictive ability (Kruger *et al.*, 1986, 1988, 1993; Menkveld *et al.*, 1990; Van der Merwe *et al.*, 1992). The morphology threshold for assisted reproduction, according to the Tygerberg (Kruger *et al.*, 1986) and Norfolk groups (Oehninger *et al.*, 1988), is at the level of 14% normal forms if strict criteria are used.

The normal spermatozoon (Fig. 1) should have a single head with a well-defined acrosome comprising between 40% to 70% of the sperm head area and a perfectly smooth oval head or one that tapered slightly at the level of the postacrosomal region. The head length should be 4µm to 5µm and the width 2.5µm to 3µm when Papanicolaou staining is utilized. When Diff-Quik stain is used, the length should be 5µm to 6µm and the width 2.5µm to 3.5µm (Kruger *et al.*, 1987). No abnormalities in the neck, midpiece or tail should be present. The midpiece should be slender, axially attached, and less than 1µm wide and its length should be approximately 1.5 times the head length. No cytoplasmic droplet greater than half the area of the sperm head should be present. The tail should be uniform, slightly thinner than the midpiece, uncoiled and approximately 45µm long. Trival variations in head morphology should be considered normal, but borderline normal heads are classified in a subgroup called slightly abnormal. Spermatozoa are classified into seven groups according to their head morphology, namely, normal spermatozoa, large heads, small

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heads, elongated or tapered heads, duplicated heads, amorphous heads, and normal sperm heads with neck and/or midpiece and/or tail and cytoplasmic droplet defects (strict criteria) (Menkveld *et al*., 1990).

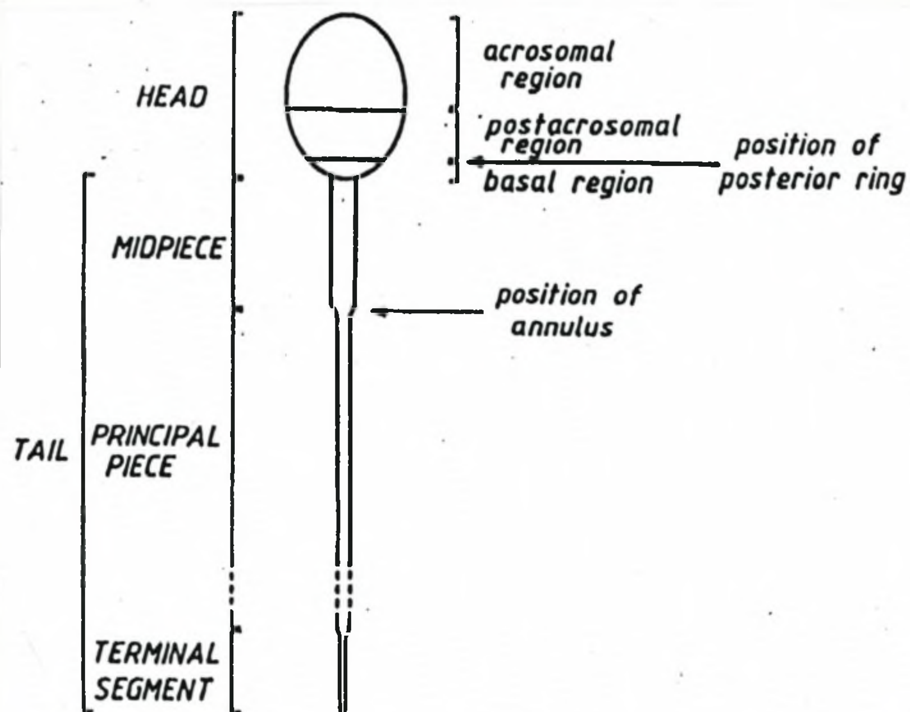

FIGURE 1: The mature spermatozoon.

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When strict criteria for normality are utilized in patients undergoing IVF, patients with <14% normal morphologic forms were found to have a decreased fertilization rate (Kruger *et al.*, 1988). Furthermore, in this same group of patients, two subgroups could be identified. The first appeared to have a good prognosis in terms of fertilizing ability, although the percentage of eggs that fertilized was lower than normal. This group was said to demonstrate the good prognostic pattern (G-pattern; 4-14% normal forms). The second group, in which fertilization was observed, exhibited a poor prognostic pattern (P-pattern; <4% normal forms). These morphological forms have been found to be consistent and interpretation of the slides was reproducible among technicians. In patients with <4% normal sperm morphology, fertilization rates per oocytes were found to be 7.6%, compared to the fertilization rate of 63.9% in the g-pattern group ($P<0.0001$) (Kruger *et al.*, 1988). Independent researchers from various international centres looking at the role of sperm morphology in assisted reproduction confirmed these results (Oehninger *et al.*, 1988; Enginsu *et al.*, 1991, 1993).

Of all the semen parameters, sperm morphology has consistently been the best indicator of male in vitro fertility. This predictive potential has also been shown to be independent of other semen variables as well as being one of the most stable parameters (Coetzee *et al.*, 1998). Many authors have gone as far as to argue that sperm morphology is a reflection of sperm functional competence (Coetzee *et al.*, 1998). The evaluation of sperm morphology is a relatively simple procedure and can be performed in the majority of laboratories, but technical problems and lack of standardization in its evaluation has precluded worldwide consensus on its true value. The lack of objectivity in evaluating sperm morphology, the difficulty of standardization, implementing and controlling manual methods, and the high degree of variation within and between laboratories and technicians have provided the incentives for the development of automated sperm morphology analysis (ASMA) instruments (Davis *et al.*, 1992).

Wang *et al.* (1991) was one of the first studies to assess the usefulness of computerized sperm morphology evaluation to predict the outcome of sperm fertilizing capacity. Kruger *et al.* (1993) evaluated the prognostic value of computerized evaluation in the human system by evaluating 21 slides from Tygerberg Hospital's GIFT program and 21 slides from Norfolk's IVF program. Both sets of slides were evaluated both manually and by computer. The fertilization rates for the groups with <14% and >14% sperm normal forms, manually evaluated, were 33.3% and 76.7%, respectively. The fertilization rates were 46.8% and 75.6%, respectively, for the computerized evaluation. The fertilization rates within these two groups (Norfolk slides) were 27.4% and 90.0%, and 33.9% and 88.4% for the manual and automatic systems,

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respectively. ASMA systems have been shown to have the potential to eliminate the biases and subjectivity plaguing the manual evaluation of sperm morphology.

The replacement of the light microscopic evaluation of sperm morphology with computer-assisted evaluation, as a means of eliminating some of the technical variation and facilitating standardization, however, first requires unequivocal proof of the predictive (in vitro) value of the normal sperm morphology outcomes of the computerized systems. This precondition was confirmed in a study performed by Kruger *et al.* (1995) who found unique differences between manual and computer-assisted sperm morphology. The differences can probably be ascribed to two factors: (i) the shift to a greater reliance on quantitative input (morphometric) and (ii) the greater influence of sample preparation and staining on the correct digitization (interpretation) sperm images. Computer-assisted sperm morphology analyzers do, however, possess the capacity, due to their greater objectivity, to eliminate most of the variation producing factors (*i.e.*, classification systems, interpretation and technician experience) so inherent to the manual evaluation of sperm morphology.

Progress in the evaluation of semen ejaculates demands the development of accurate and reliable automated semen analyzers, not only to eliminate the inherent subjectivity and variability associated with performance of a standard semen analysis, but also to increase its diagnostic value through quantification and pertinent statistical evaluation. The increased diagnostic resolution of automated semen analyzers could in future help to identify more specific male fertility groups – biologically (functionally) different groups (Davis & Katz, 1993); thereby allowing for employing more optimal treatment regiments for individual patients. The greatest impediments to automated systems achieving this goal have been the technical limitations of such systems – the establishment of algorithms for the accurate identification of seminal debris and deficiencies in image digitization (Garrett & Baker, 1995). The Hamilton-Research semen analyzer has produced promising results in the quest to achieve the stated objectives (Kruger & Coetzee, 1999).

1.2.3 Testing for antibody coating of spermatozoa

Sperm antibodies in semen belong almost exclusively to two immunoglobulin classes, namely, IgA and IgG. IgA antibodies might have greater clinical importance than do IgG antibodies (Kremer & Jager, 1980). IgM antibodies, because of their large molecular size, are rarely found in semen.

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The screening test for antibodies is performed on the fresh semen sample and makes use of either the immunobead test (IBT) (Clarke *et al.*, 1982) or the mixed antiglobulin reaction (MAR) test (Jager *et al.*, 1978). The results from these tests do not always agree (Hellstrom *et al.*, 1989). The IBT correlates well with immobilization tests carried out on serum (Clarke *et al.*, 1985). When these tests are positive, additional tests such as sperm-cervical mucus contact tests or sperm-cervical mucus capillary tube tests, should be done. These additional tests provide information regarding the influence of the specific antibody on sperm-mucus penetration and migration.

1.2.3.1 Immunobead test

Antibodies on the sperm surface can be detected by the direct IBT (Clarke *et al.*, 1985). The presence of IgA and IgG antibodies can be assessed independently with this test. Immunobeads adhere to the motile spermatozoa that have surface-bound antibodies. The percentage of motile spermatozoa with surface antibodies is determined, the pattern of binding is noted and the class (IgA or IgG) of these antibodies can be identified using different sets of immunobeads.

Sperm penetration into the cervical mucus and in vivo fertilization tend not to be significantly impaired unless 50% or more of the motile spermatozoa have antibody bound to them (Jager *et al.*, 1978). On this basis, at least 50% of the motile spermatozoa must be coated with immunobeads before the test is considered to be clinically significant. Furthermore, immunobead binding restricted to the tail tip is not associated with impaired fertility and can be present in fertilization.

1.2.3.2 Mixed antiglobulin reaction test

The MAR test using antibody coated red blood cells was originally described by Jager *et al.* (1978) but due to problems encountered with the availability of fresh red blood cells the test was devised by Vermeulen and Comhaire (1983) using antibody coated latex particles instead. The IgA and IgG MAR tests are performed by mixing fresh, untreated semen with latex particles or red blood cells coated with human IgA or IgG. To this mixture is added a monospecific anti-human-IgG antiserum. The formation of mixed agglutinates between particles and motile spermatozoa indicates the presence of IgA or IgG antibodies on the spermatozoa. The diagnosis of immunological infertility is possible when 50% or more of the motile spermatozoa have adherent particles (Barratt *et al.*, 1992), but the diagnosis must be confirmed by sperm-mucus interaction tests.

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1.3 Sperm preparation techniques

The separation of spermatozoa from seminal plasma to achieve a final preparation with a high percentage of motile spermatozoa, free from debris and dead spermatozoa, is important for several therapeutic and diagnostic techniques in clinical andrology. Many procedures may be used but there are two main methods of separation (Mortimer, 1994a).

Firstly, spermatozoa may be selected on their ability to swim, known as the swim up technique. Centrifugation of spermatozoa (including cell debris and leukocytes) prior to swim up should be avoided because it can result in damage of the sperm membranes, probably by the production of reactive oxygen species. A direct swim up from semen is the preferred method for the separation of motile spermatozoa. This technique is performed by layering culture medium over the liquefied semen so that motile spermatozoa then swim into the medium.

The second method of selecting spermatozoa is by the use of density gradients. A simple two-step preparation is the most extensively used of such methods. In general, the direct swim up technique is used when the semen samples are considered to be normal. For semen with suboptimal characteristics, alternative preparations are generally preferred.

It may be necessary to select the sperm preparation technique according to the individual semen samples (Canale *et al.*, 1994). The efficiency of the various techniques is usually expressed as the absolute number or the relative yield of morphologically normal motile spermatozoa. In some cases, the functional capacity of the prepared spermatozoa may be determined, for example, in the zona-free hamster oocyte test, to identify the most suitable method of preparation (Aitken *et al.*, 1983).

1.4 Biochemical markers

1.4.1 Reactive oxygen species

Reactive oxygen species (ROS) are metabolites of oxygen and include superoxide anion, hydrogen peroxide, hydroxyl radical, hydroperoxyl radical and nitric oxide (Aitken & Clarkson, 1987). When present in excess, such ROS can initiate pathological damage by inducing oxidative damage to cellular lipids, proteins and DNA (Griveau & Le Lannou, 1997). Most cells are equipped with either enzymatic

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antioxidant systems (superoxide dismutase, gluthatione peroxidase and catalase) or nonenzymatic antioxidant systems (uric acid, vitamin C and vitamin E). When these defences are overwhelmed, cell function is affected (Smith *et al.*, 1996).

In the human ejaculate, ROS are produced by both spermatozoa (Aitken & Clarkson, 1987; Iwasaki & Gagnon, 1992) and leukocytes (Aitken & West, 1990). Seminal plasma possesses antioxidant scavengers and enzymes which may be deficient in some patients (Smith *et al.*, 1996). The removal of seminal plasma during the preparation of spermatozoa for assisted conception may render these cells vulnerable to oxidative attack. About 40% of men attending infertility clinics exhibit detectable levels of ROS generation in their semen (Iwasaki & Gagnon, 1992). High ROS production may cause peroxidative damage and loss of sperm function.

1.4.2 Creatine kinase

The excessive generation of ROS and the presence of high concentrations of cytoplasmic enzymes such as creatine kinase (CK) may both reflect abnormal or immature spermatozoa with excessively retained cytoplasm in the midpiece (Gomez *et al.*, 1996). CK is a key enzyme in the synthesis and transport of energy (Huszar & Vigue, 1994). ATP, generated in mitochondria, is used for the phosphorylation of creatine to creatine phosphate, which migrates to the contractile elements of the sperm tail where CK uses it for the phosphorylation of ADP to ATP. Dephosphorylated creatine moves back to the mitochondria where the cycle starts over again.

Sperm CK levels show a highly significant inverse correlation with ejaculate sperm concentration (Huszar *et al.*, 1988). Spermatozoa from normozoospermic ejaculates possess much higher CK levels than spermatozoa from oligozoospermic specimen. Selection of spermatozoa by a swim up procedure results in a highly motile sperm population with low CK levels. No correlation exists between sperm motility, sperm morphology or seminal plasma CK and sperm CK (Huszar *et al.*, 1988). The levels of CK in sperm populations have been shown to be a good predictor of sperm fertilizing potential for oligozoospermic men during IUI (Huszar *et al.*, 1990). Whether this marker will be more generally applicable, e.g. for idiopathic infertility, remains unknown, but it does show great promise as an additional biochemical test that might be related to sperm functional ability. Basically, it seems that the spermatozoa containing high levels of CK activity do so because of a relatively large volume of retained cytoplasm. This morphological feature is

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abnormal, immature or both and apparently is closely related to these spermatozoa's ability to produce ROS (Gomez *et al.*, 1996).

1.5 Sperm function tests

1.5.1 Sperm-zona pellucida binding

Sperm-zona binding has been illustrated to be an essential requisite during fertilization and can be measured under hemizona assay (HZA) (Burkman *et al.*, 1988) as well as intact zona pellucida (ZP) conditions (Liu *et al.*, 1988). Both bioassays have the advantage of providing a functional homologous test for sperm binding to the ZP, comparing populations of fertile and infertile spermatozoa in the same assay. The sensitivity and specificity of sperm-zona binding results indicated the assay to be positively and significantly correlated with IVF outcome. The scarcity of human oocytes stimulated the development of the HZA concept of using identical, matching halves from the same ZP. The availability of oocytes still remains an important limiting factor for laboratories to embark on the methodology of the assay.

The HZA measures a sperm function based on the relative binding of patient versus control spermatozoa to the matching halves of a bisected human oocyte (Burkman *et al.*, 1988; Franken *et al.*, 1993). This assay assesses tight binding of sperm to the outer surface of the ZP hemisphere. Clear advantages of the HZA include: (i) the two halves (hemizonae) are functionally (qualitatively) equal zonae surfaces, allowing a controlled comparison of sperm-zona binding; (ii) the very limited number of recovered human oocytes is amplified, since an internally controlled test can be carried out on a single oocyte; and (iii) ethical objections to possible inadvertent fertilization of a viable oocyte are culminated by first cutting the egg into halves.

The competitive intact ZP binding test is one using oocytes that failed to fertilize in vitro to determine a sperm-ZP binding ratio between control and test spermatozoa (Liu *et al.*, 1988). Oocytes that show no evidence of either pronuclei or cleavage 48 to 60 hours after insemination are placed in 1*M* ammonium sulphate solution and stored at 4°C. The test is based on competitive binding of two sperm populations (test patient and fertile control sperm donor) to several oocytes. Test and control sperm are labeled with different fluorochrome suspensions, namely, fluorescenated *Pisum Sativum* agglutinin (green) and tetramethyl rhodamine isothiocyanate (red). Following labelling, sperm populations, *i.e.*, control and tests

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are simultaneously coincubated with several oocytes, after which zona binding is assessed by counting zona-bound sperm under fluorescence microscopy.

The identification of specific gamete dysfunctions remains one of andrology's most formidable tasks (ESHRE, 1996). Fertilization disorders, due to a defective sperm-ZP interaction, are relatively common in clinical practice, thereby underscoring the importance of sperm-zona binding tests as diagnostic/predictive tests. Independent publications from Norfolk (USA), Melbourne (Australia) and Israel of highly comparable results, confirmed that sperm-zona binding tests are good predictors for fertilization (Liu & Baker, 1993; Oehninger, 1995; ESHRE, 1996). In these centers the use and development of the indirect zona binding test is advised in cases of total IVF failure and all oocytes should be examined for the presence of spermatozoa bound to the ZP. While this is not as reliable as a direct sperm-zona binding test, being a qualitative rather than a quantitative evaluation, it may be the only source of such information if the direct method is not available (Franken *et al.*, 1989a).

The Tygerberg and Norfolk laboratories jointly evaluated: (i) the relationship between different sperm characteristics (from original semen samples as well as from retrieved motile fractions) and sperm-zona binding potential; and (ii) the role of the HZA as a diagnostic tool for predicting fertilization in vitro. A large number of infertile patients were studied before IVF therapy and the main outcome measured included CASA, sperm morphology and HZA data correlated with fertilization rates achieved of the patients under investigation. Multiple regression analyses have demonstrated that sperm morphology is the most significant predictor of sperm-zona binding in the HZA, when compared to other sperm variables from the original semen sample ($r=0.83$, $P=0.0001$). On the other hand, curvilinear velocity and hyperactivated motility were the most significant predictors of successful zona binding, after separation of the motile sperm fraction ($r=0.47$ and $r=0.46$, respectively, $P=0.001$) (Oehninger *et al.*, 1997b).

Sperm morphology remains, not only one of the best predictors of the ability of sperm to bind to the ZP, but also it correlates well with fertilization outcome under IVF conditions. This might be due to the presence of a specific membrane/receptor deficiency among teratozoospermic men. When the HZA results are removed from a logistic regression analysis in order to identify the predictive value of other sperm parameters (sperm concentration, morphology and motion characteristics), the percentage progressive motile cells was the best predictor of fertilization outcome (Oehninger, 1992; Franken *et al.*, 1996b). Logistic regression analysis provided a robust hemizona index range predictive of the oocyte's potential to be fertilized. This cut-off value was determined to be 35%. The discriminating power of the HZA is

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constant for different sperm samples from the same patient in different (consecutive) IVF cycles (ESHRE, 1996).

The basic underlying gamete defects responsible for failed fertilization is poorly understood in the majority of cases. The development of zona binding technology enhanced the awareness of sperm dysfunction so far as zona binding is concerned. On the other hand, it is accepted that oocyte abnormalities may also be responsible for fertilization failure, excluding "descriptive" oocyte morphology abnormalities. The role of the ZP, the primary structure responsible for gamete recognition, has been evaluated in the context of the IVF setting using oocytes from failed fertilization attempts (Hinsch *et al.*, 1994). These immunocytochemical studies showed a statistical significant incidence of ZP abnormalities in patients with both morphologically normal and abnormal oocytes, and in the absence of sperm defects (Oehninger, 1995).

1.5.2 The acrosome reaction

Spermatozoa are not able to fertilize an oocyte upon ejaculation. Before gaining fertilizing ability, spermatozoa should first undergo capacitation, which is considered to be completed when the spermatozoa are able to undergo the acrosome reaction (AR). The process of capacitation, first identified by Austin (1951) and Chang (1951), consists of a series of functional, biochemical and biophysical modifications that render the ejaculated spermatozoa competent for fertilization of the oocyte. These fundamental processes normally take place in the female genital tract during the migration of spermatozoa to the site of fertilization (Yanagimachi, 1994). However, under appropriate conditions, capacitation can also be induced in vitro (Fraser, 1995). One of the functional consequences of capacitation is the development of a distinct motility pattern called hyperactivation, which is characterized by pronounced flagellar movements, marked lateral excursion of the sperm head and a non-linear trajectory. An additional manifestation of sperm capacitation is the acquisition of the ability to undergo the AR in response to physiological stimuli such as ZP3 and progesterone. The responsiveness of spermatozoa to ZP3 (Wassarman, 1994; Saling, 1995) and progesterone (Mendoza & Tesarik, 1993) increases during capacitation, assuring maximal responsiveness at the site of fertilization. Capacitation is associated with modifications in sperm surface protein distribution, alterations in plasma membrane characteristics, changes in enzymatic activities and modulation of expression of intracellular constituents (Yanagimachi, 1994).

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The AR is the exocytosis of the sperm acrosome, a secretory granule localized at the anterior part of the sperm head, with the outer acrosomal membrane and overlying plasma membrane fusing, the fused membranes undergoing fenestration and vesiculation, and the acrosomal contents, mainly the enzymes hyaluronidase and acrosin, are released (Fig. 2) (Mortimer, 1994b). The AR has become one of the best studies in the process of fertilization, a very complex phenomenon involving sequential interactions between the fertilizing spermatozoon, and cumulus oophorus, ZP and oolemma (Yanagimachi, 1994).

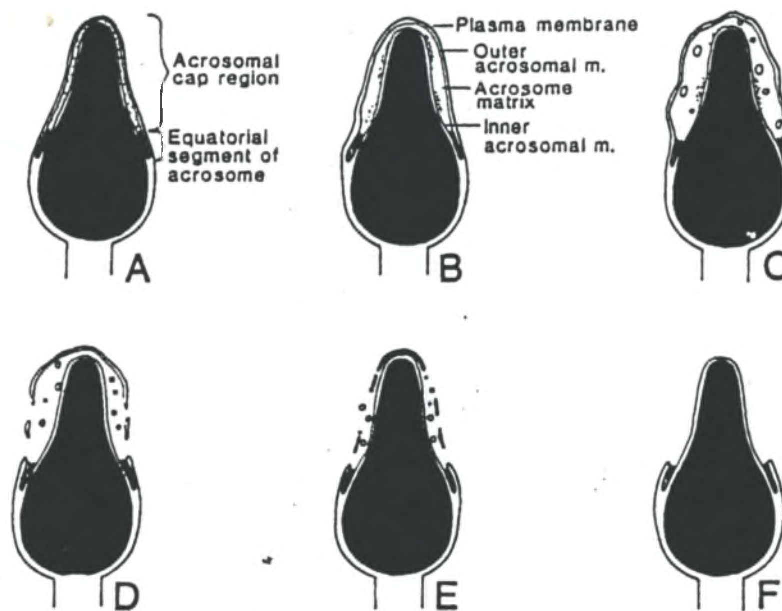

FIGURE 2: Probable sequence of the morphological events in the human sperm acrosome reaction (*Mortimer, 1994b*).

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The fertilizing spermatozoon first penetrates the cumulus oophorus (Fig. 3; step a), consisting of cumulus cells (somatic cells from the ovarian follicle) embedded in an extracellular matrix (ECM). The spermatozoon then contacts the ZP (Fig. 3; step b), where the AR is triggered by ZP3. Acrosome-reacted spermatozoa penetrate the ZP, enter the perivitelline space, and then adhere to (Fig. 3; step c) and fuse with (Fig. 3; step d) the plasma membrane of the egg. The egg has extruded the first polar body (PB1) and progressed to metaphase II. This model (Fig. 3) is based on in vitro studies of gamete interactions and is consistent with in vivo fertilization, which occurs in the oviduct (Yanagimachi, 1994).

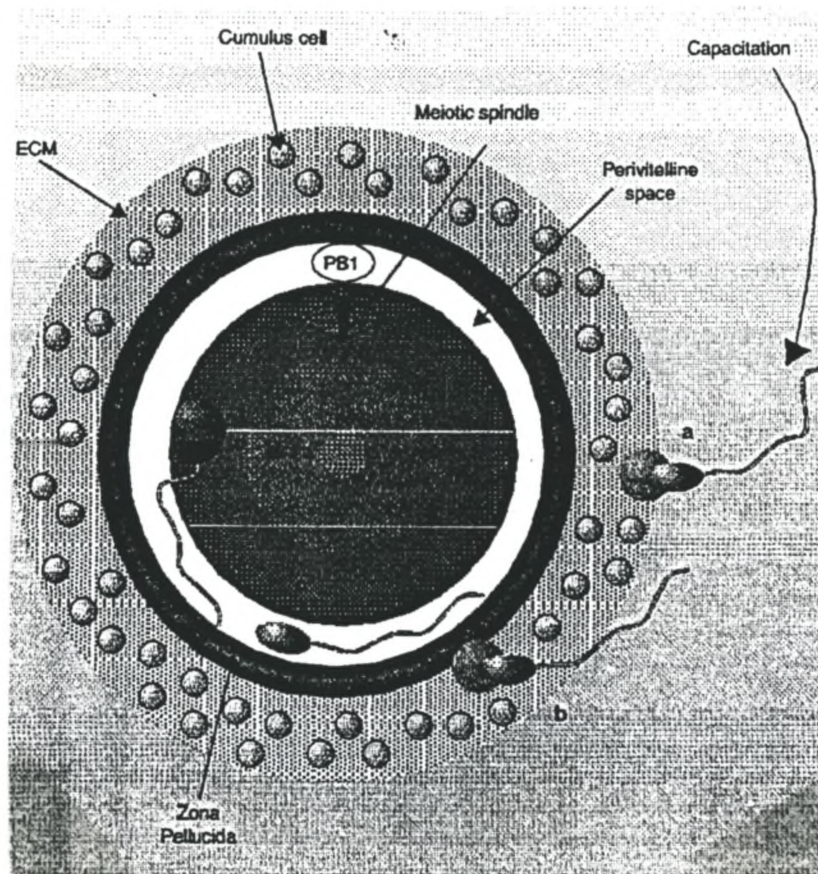

FIGURE 3: The sequence of early events in fertilization (*Evans & Florman, 2002*).

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The importance of the acrosome for fertilization in the human is demonstrated by a rare congenital condition in which each sperm has a small spherical head without an acrosome called globozoospermia. Men with this condition are sterile and unable to produce pregnancies either by natural insemination or by standard IVF procedures. These acrosomeless sperm are unable to bind to or penetrate the ZP. This condition can be treated by ICSI with reasonable fertilization rates and pregnancies in some patients (Bourne *et al.*, 1995). However, in others the oocytes fail to fertilize despite ICSI, usually because the sperm heads do not decondense normally in the ooplasm (Nagy *et al.*, 1995).

Although the acrosomal region can be assessed by light microscopy and computer-assisted morphometry, the technique cannot be used to distinguish between acrosome-intact and acrosome-reacted sperm cells. However, labelled lectins or antibodies that bind to acrosomal components can be used to determine acrosome status. Liu & Baker (1992) have used either *Pisum Sativum* agglutinin or a monoclonal antibody to a form of clusterin (E5) that is present in normal intact acrosomes. On a number of occasions they have found some relationship between the proportion of sperm with intact acrosomes in the insemination medium and the fertilization rates in vitro. Similarly, there is a relationship between the proportion of acrosome-intact sperm in the medium and the numbers of sperm bound to the ZP (Liu & Baker, 1988, 1990a, 1994a, 1997). These findings are consistent with the proposition that human sperm normally bind to the surface of the ZP with the acrosome intact.

To test this proposition, Liu & Baker (1994a) artificially induced the AR with calcium ionophore (A23187). This agent is a powerful inducer of the AR but also reduces sperm motility and therefore experiments had to be carefully performed to adjust the concentrations of acrosome-intact and acrosome-reacted motile sperm, which were exposed to the oocytes. Using this technique, Liu & Baker (1990b; 1994a) showed that the numbers of sperm bound to the oolemma of zona-free oocytes increased as expected with increasing proportions of acrosome-reacted sperm in the insemination medium, but the numbers of sperm bound to zona-intact oocytes declined. This indicates that induction of the AR in the insemination medium reduces or prevents sperm from binding to the ZP. Although experimental models have been developed to show that acrosome reacting sperm are still capable of binding to the ZP (Morales *et al.*, 1989), the physiological fertilization process involves binding of acrosome-intact sperm to the surface of the ZP and the binding process stimulates the AR to occur on the surface of the zona. Patients with poor zona-induced acrosome reaction (ZIAR) may have normal A23187-induced AR and vice versa (Liu & Baker, 1996). Thus, it is important to use the physiological inducer, namely the ZP, when studying the AR. In future, rhuZP3 may become available for this purpose.

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Esterhuizen *et al.* (2001) described cases where the ZIAR was significantly correlated with IVF when the ZIAR was >15%. Impaired ZIAR results were identified where ZIAR was <15% and typically associated with fertilization failure in a group of patients with normal conventional semen analyses. These results are in close agreement with other reports (Liu & Baker, 1994a) during which ten couples with long-standing infertility revealed a reduced frequency of acrosome-reacted spermatozoa bound to the ZP. That study suggested the existence of a zona-bound sperm population with a disordered AR, thus causing impaired fertility (Liu & Baker, 1994a). Patients with a long history of idiopathic infertility with poor fertilization or complete fertilization failure should be tested for this defect using sperm-ZP interaction tests. Reduced ZIAR patients should be directed to ICSI rather than standard IVF (Esterhuizen *et al.*, 2001; Liu *et al.*, 2001).

Equally important is the finding that the AR must be precisely timed with respect to sperm-ZP interaction to ensure subsequent events, *i.e.*, zona penetration and oolemma fusion. Therefore, precise timing of acrosomal response was the rationale for the development of the acrosome reaction ionophore challenge test (ARIC-test) (Cummins *et al.*, 1991; Tesarik, 1996). The ARIC-test as well as the concept of acrosomal inducibility (Henkel *et al.*, 1993) is a reliable predictive tool of sperm fertilizing ability as compared with tests that simply measure the frequency of spontaneous ARs. The inducibility of the AR, *i.e.*, the difference between spontaneous and percentage acrosome-reacted spermatozoa after induction, correlates significantly with fertilization rates (Henkel *et al.*, 1993). The acrosome inducing activity of the ZP, in both the intact and solubilized state, has been illustrated to be powerful (Cross *et* al., 1988; Mahony *et. al.*, 1991; Bielfeld *et al.*, 1994a; Liu & Baker, 1994a; Franken *et al.*, 1996a, 1997). The implementation of assays using small volumes of human solubilized ZP, biologically active recombinant human ZP3 (rhuZP3) or active, synthetic ZP3 peptides (or analogs) will probably allow for the design of improved, physiologically oriented AR assays (Van Duin *et al.*, 1994; Chapman & Barratt, 1996; Franken *et al.*, 1996a, 1997, 2000; Dong *et al.*, 2001; Bastiaan *et al.*, 2002, 2003).

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1.5.3 Introduction of the acrosome reaction:

Preliminary investigations performed as introduction to this study

1.5.3.1 Zona pellucida mediated acrosome reaction and sperm morphology

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(Andrologia, 1997, Vol. 29, 311-317)

Summary

Sperm samples from 29 men randomly selected from the andrology laboratory were used to evaluate the AR response to solubilized human ZP. Capacitated sperm samples were exposed to a solution containing 2.5ZP/µl for 60 minutes, after which acrosomal status were recorded using a PSA-FITC technique. Controls included samples supplied by fertile sperm donors. After completion of the AR studies, patient samples were divided according to the percentage of morphologically normal spermatozoa. Three basic groups were identified, namely, fertile donors (normal sperm morphology >14%; n=3), teratozoospermic (normal sperm morphology 4-14%; n=25) and severely teratozoospermic (normal sperm morphology <4%; n=4) groups. The mean percentage normal spermatozoa were (15.8±0.9)%, (10.4±0.7)% and (2.7±0.7)%, respectively, for normozoospermic donors, teratozoospermic and severely teratozoospermic men. The mean percentage (±SE) ZP-mediated acrosome-reacted spermatozoa among teratozoospermic and severely teratozoospermic cases were (25.8±0.9)% and (19.0±0.9)% (P=0.001), compared to (36.8±0.9)% for the donor controls. Results were analyzed and expressed as correlations between sperm morphology and acrosomal response to human solubilized ZP, spontaneous and A23187-induced ARs. Predictive values for acrosome responsiveness were depicted with receiver operating characteristics (ROC) curve analyses. Sperm morphology evaluated by strict criteria correlated positively and highly significantly with the responsiveness of the AR (r=0.91, P=0.0001). At a morphology cut-off value of 4%, the ROC curve analysis showed sperm morphology to be highly predictive of ZP-induced acrosome responsiveness with a sensitivity of 100% and negative predictive value of 100%. Spontaneous and A23187-induced ARs revealed no correlation with sperm morphology. It was concluded that (i) morphological features of human spermatozoa are indicative of specific functional characteristics and (ii) ZP induction of the AR is superior, as a predictor of sperm morphology, compared to A23187-induced and spontaneous ARs.

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Introduction

With the onset of assisted reproductive technologies, refined sperm functional evaluation in the 1990s has become a mandatory part of the andrological workup schedule. The astounding success rates achieved by ICSI (Van Steirteghem *et al.*, 1993), in cases of profound male factors, now have enhanced the need to fully understand human fertilization and all subsequent related processes leading to the formation of the normal embryo. Contemporary andrology laboratories should be able to select the most appropriate form of treatment for each couple, especially those diagnosed as male factor infertility (Oehninger *et al.*, 1991; Oehninger, 1992). It remains imperative to understand the role and diagnostic power of a sequential analytical approach to properly interpret the existing laboratory methods for evaluation of male gamete fertilizing ability.

In contrast to many animal species, human semen is virtually unique because of its heterogeneity in sperm morphology (pleomorphism); both fertile and infertile men produce high numbers of morphologically abnormal spermatozoa (Katz *et al.*, 1982; Kruger *et al.*, 1986). This phenomenon is of paramount clinical importance as sperm morphology is regarded as a significant prognostic factor for fertilization and pregnancy outcome in the IVF/GIFT/IUI settings (Kruger *et al.*, 1986; Grow *et al.*, 1994; Toner *et al.*, 1995). Multiple regression analyses have demonstrated sperm morphology to be the most significant predictor of sperm-zona binding in the HZA (Franken *et al.*, 1989b, 1991a, 1993) when compared to other sperm variables from the original semen sample (r=0.83, P=0.0001). On the other hand, VCL and hyperactivation were the most significant predictors of successful zona binding after separation of the motile sperm fraction (r=0.47 and r=0.46, respectively, P=0.001).

Equally important is the finding that the AR must be precisely timed with respect to sperm-ZP interaction to ensure subsequent events, *i.e.*, zona penetration and oolemma fusion. Therefore, precise timing of acrosomal response was the rationale for the development of the acrosome reaction ionophore challenge test (ARIC-test) (Cummins *et al.*, 1991; Tesarik, 1996). The ARIC-test as well as the concept of acrosomal inducibility (Henkel *et al.*, 1993) is a reliable predictive tool of sperm fertilizing ability as compared with tests that simply measure the frequency of spontaneous ARs. The inducibility of the AR, *i.e.*, the difference between spontaneous and percentage acrosome-reacted spermatozoa after induction, correlates significantly with fertilization rates (Henkel *et al.*, 1993). The acrosome inducing activity of the ZP, in both the intact and solubilized state, has been illustrated to be powerful (Bleil & Wassarman, 1980; Cross *et al.*, 1988; Bielfeld *et al.*, 1994a, b; Liu & Baker, 1994b, 1996). The ZP, and specifically

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glycoprotein 3 (ZP3), is thought to be the primary zona protein involved in the initial sperm-egg recognition and mediation of the AR. The present study aimed to evaluate the acrosome responsiveness of spermatozoa from different men to solubilized ZP.

Materials and Methods

Sperm samples and preparation

Semen samples were obtained by masturbation after 2-3 days of sexual abstinence from normozoospermic fertile donors and men visiting the andrology laboratory for routine analysis. Semen samples were analyzed according to the World Health Organization criteria (WHO, 1992) together with strict sperm morphology assessment (Kruger *et al.*, 1986). Motile sperm fractions were collected from samples using a slightly modified double-wash swim up technique (Franken *et al.*, 1996a, b). From each sample, 1ml semen was placed in a plastic centrifuge tube (10mm diameter). During the first and second washes, 2ml of synthetic HTF (Quinn *et al.*, 1985) was added to the semen and thoroughly mixed, after which the suspension was centrifuged for 5 minutes at 300 x*g*. After removal of the supernatant, the sperm pellet was gently overlaid with 0.5ml HTF supplemented with 3% BSA. The tube was then incubated at an angle of 45° for 1 hour in 5% CO_2 in air at 37°C. This procedure resulted in post-swim up sperm concentrations of >$25x10^6$ cells/ml. Retrieved sperm samples were resuspended in HTF supplemented with 3% BSA to a sperm concentration of $25x10^6$ cells/ml. Before the onset of AR studies, sperm samples were allowed to capacitate at 37°C in 5% CO_2 in air for 3 hours in HTF supplemented with 3% BSA.

Preparation of solubilized zona pellucida

Oocytes were retrieved from post mortem derived ovarian material. Great care was taken to ensure that all legal, ethical and moral guidelines were adhered to at all times during oocyte collection. After retrieval, oocytes were stored at 4°C under mineral oil in concentrated salt solutions, pH 7.4, containing 1.5*M* $MgCl_2$, 0.01% PVP and 40 m*M* of Hepes buffer (Franken *et al.*, 1991b). Twenty-four hours prior to each test, oocytes were removed from storage and desalted using 5 changes in fresh HTF containing 3% BSA. Prior to solubilization, the oocytes were denuded and cleaned using small bore glass micropipettes. On the day of the experiment, 40 oocytes were removed from the HTF droplet containing desalted oocytes and placed in a plastic (1ml) Eppendorf tube. The Eppendorf tubes containing oocytes were centrifuged for 15 minutes at 1800 x*g*, after which the HTF was removed under microscopic vision, leaving only the 40

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oocytes at the bottom of the tube. A total volume of 4µl of 10m*M* HCl was then added to the oocytes in the tube; solubilization of the ZP was microscopically observed and controlled. Ooplasma of all oocytes were left at the bottom of the tube. Pilot studies performed in our laboratory and that of others (Liu & Baker, 1990b) showed no interference with the AR using ZP-free oocytes in control or test samples. All control and test samples included ZP-free oocytes. Following solubilization, 4µl of 10m*M* NaOH was added to the solubilized ZP to render a final zona volume of 8µl, containing 5ZP/µl. The zona solution was aliquoted in 4µl portions in plastic tubes to which 4µl of HTF supplemented with 3% BSA was added, resulting in zona concentrations of 2.5ZP/µl. The final concentrations (after addition of spermatozoa) of the ZP solutions were 2.5ZP/µl.

Acrosome reaction studies

Acrosomal status was determined following procedures previously described (Cross *et al.*, 1988; Morales *et al.*, 1989). The fixed spermatozoa were air-dried, after which the acrosomal status of spermatozoa capable of excluding the Hoechst dye was determined, using FITC-PSA (125µg/ml). ARs were determined among 3 hour capacitated spermatozoa incubated for 60 minutes with the following: (i) HTF (control); (ii) 20µ*M* A23187 (control); (iii) 2.5ZP/µl (test). From the results of the pilot study, a ZP concentration of 2.5ZP/µl was chosen for the acrosomal studies on andrology patients.

Semen samples from 29 men visiting the andrology laboratory were evaluated for AR after spermatozoa were allowed to capacitate for 3 hours. Spermatozoa were incubated with the following: (i) 2.5ZP/µl (test); (ii) spontaneous (control); and (iii) 20µ*M* A23187 (control). During all acrosomal studies, 4µl of the prepared zona solution, HTF or A23187 was carefully placed in a 1ml plastic tube, after which 1µl of the motile sperm fraction containing 25×10^6 cells/ml was added; the final zona concentration being 2.5ZP/µl. Tubes were sealed and placed at 37°C for 60 minutes. Control samples were incubated with neutralized acidic buffer without ZP for 60 minutes. Ten minutes prior to the completion of each experiment, supravital stain Hoechst 33258 (1µg/ml) was added to each sperm/zona droplet to determine live/dead spermatozoa ratio during the AR evaluation. The AR results represent only those observed among the dead/live sperm population.

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Statistical analysis

Comparisons between normal sperm morphology and percentage acrosome-reacted spermatozoa under different laboratory conditions were done with Wilcoxon *t*-test. The association between percentage normal spermatozoa and percentage acrosome-reacted spermatozoa under various experimental conditions was reported, using multiple regression analysis as well as correlation analysis. The discriminating power of sperm morphology as a screening test for the identification of AR responsiveness was illustrated with ROC curve analysis.

Results

The mean (±SE) values for sperm parameters recorded during semen analysis of donors (n=3) and men referred to the andrology laboratory (n=29) are depicted in Table 1. All semen samples (apart from the donors) were randomly selected from the andrology laboratory and acrosomal induction response was determined in a blind fashion. Sperm morphology was only known after the acrosomal studies were recorded. The mean percentage AR after exposure to 2.5ZP/µl, spontaneous and A23187-induced AR results of different morphological groups are depicted in Table 2. Retrospective classification of semen samples after acrosome determination showed significant differences between acrosomal response of the normozoospermic donors, teratozoospermic and severely teratozoospermic samples, using 2.5ZP/µl and spontaneous AR.

Zona pellucida mediated acrosome reaction

Sperm morphology values, reported according to strict criteria of each sample, were correlated with acrosomal activity following exposure to 2.5ZP/µl human ZP. The ZP-induced acrosome-reacted spermatozoa showed a significant and positive correlation with the percentage normal spermatozoa in the populations (r=0.91, P=0.0001). At a morphology cut-off value of 4% (threshold value for P-patterns), the ROC curve analysis showed sperm morphology to be highly predictive of the zona-induced acrosome responsiveness. The sensitivity and negative predictive value was calculated at 100% in both cases. The calculated cut-off value for percentage acrosome-reacted spermatozoa was 18% under these conditions.

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Inducibility of the acrosome reaction

Inducibility of the AR was reported as the difference between AR values after induction with 2.5ZP/µl and the percentage spontaneous acrosome-reacted spermatozoa. A highly positive and significant correlation existed between normal sperm morphology and the inducibility of the AR ($r=0.88$, $P=0.0001$). ROC curve analysis showed a sensitivity and negative predictive value of 100% in both cases for predicting a given sperm sample acrosome responsiveness within the morphology groups >4% or <4%. The cut-off value between induced and spontaneous acrosome-reacted sperm populations (*i.e.*, acrosome inducibility) within the two morphology groups was calculated by ROC curve analysis as 7% (Fig. 4).

Spontaneous acrosome reaction

Spontaneous ARs were recorded among control sperm samples that were allowed to capacitate for 3 hours and exposed to HTF only for 60 minutes. No correlation was detected between the AR and normal sperm morphology, using 4% as cut-off value ($r=0.16$, $P=0.4$). ROC curve analysis indicated that with a cut-off value of 4% normal sperm morphology, the percentage acrosome-reacted spermatozoa was 12% with a sensitivity and negative predictive value of 80% and 29%, respectively (Fig. 4).

Calcium ionophore-induced acrosome reaction

A23187 (20µ*M*)-induced AR data showed no correlation with the percentage normal spermatozoa ($r=0.012$, $P=0.9$). Likewise, ROC curve analysis revealed a sensitivity of 44% and a negative predictive value of 12% at a morphology cut-off of 4% and AR value of 52% (Fig. 4).

Multiple regression analyses have demonstrated ZP-mediated AR ($r=0.91$, $P=0.0001$) and the concept of acrosome inducibility ($r=0.88$, $P=0.0001$) (*i.e.*, percent following induction minus percentage spontaneous-reacted) to be the most significant predictors of sperm morphology.

Discussion

The semen analysis still remains a cornerstone of the diagnostic management of men in an assisted reproductive program. We have been supporting the concept of a sequential, multistep diagnostic approach for the evaluation of the various structural, dynamic and functional sperm characteristics (Oehninger *et al.*,

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1991). This approach has been the result of combined information derived from the basic and clinical areas of the andrology and reproductive endocrinology disciplines. It is our opinion that this diagnostic scheme should include: (i) assessment of the basic semen analysis and (ii) extended functional testing of spermatozoa. Sperm function assessments currently used with satisfying results are: (i) zona binding (Franken *et al.*, 1996a) and zona penetration potential (Lanzendorf *et al.*, 1994), (ii) evaluation of sperm motion characteristics (Barratt, 1996) and (iii) inducibility of the AR (Henkel *et al.*, 1993; Tesarik, 1996).

Clinicians are sometimes faced with unexpected pregnancies among severely teratozoospermic patients, who were diagnosed as having a poor chance of achieving fertilization and/or pregnancy. This phenomenon may be caused by the presence of morphologically abnormal spermatozoa with normal function, *i.e.*, adequate zona binding and AR potential. The present results are the first steps towards the development of refined functional diagnostics, such as an ideal acrosome test, *i.e.*, acrosome responsiveness to ZP. Results indicate the clinical importance of using physiological inducers of the AR, since we obtained excellent correlations between sperm morphology and the ability of spermatozoa to undergo ARs when exposed to solubilized ZP. Normal sperm morphology and ZP-induced AR results correlated well ($r=0.91$, $P=0.0001$), similarly high was the correlation ($r=0.88$, $P=0.0001$) when we employed the concept of acrosome inducibility previously described (Henkel *et al.*, 1993). Acrosome inducibility is defined by the difference of stimulated/induced and spontaneous AR values. Future studies will focus on patients in the IVF setting, evaluating during a multistep analysis, acrosomal response to solubilized ZP and IVF rates. The inclusion of clinically controlled ZP-mediated AR studies in the assisted reproductive areas will be a valuable additional indication for selected criteria's, eventually set for IVF/ICSI/GIFT/IUI patients.

Although it is generally accepted that spermatozoa must be acrosome-reacted to complete penetration of the zonae (Franken *et al.*, 1991b), the exact site of the AR has not been defined and appears to differ between species. However, only acrosome-reacted spermatozoa were found in the inner ZP after 4 hours of coincubation with hemizonae, thus indicating the ZP as a prime candidate for mediating the AR (Franken *et al.*, 1991b). Despite the uncertainties (Kopf & Gerton, 1991; Bielfeld *et al.*, 1994a; Brucker & Lipford, 1995), the acrosome seems to play an imperative role during ZP binding, since acrosomal loss was associated with decreased zona binding capacity (Franken *et al.*, 1991b, 1996b). On the other hand, there is some debate as to whether human spermatozoa that have undergone acrosomal exocytosis on the ZP represent the fertilizing spermatozoa, since both acrosome-intact and acrosome-reacted spermatozoa can be involved during the initial stages of zona binding to the human ZP (Morales *et al.*, 1989). Binding of spermatozoa to the ZP can be divided into two categories, namely, first, a situation involving spermatozoa

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that have initiated in zona binding, but where the binding as such is restricted to a temporary attachment to the ZP which presumably takes place immediately (<1 minute) after gamete contact (Morales *et al*., 1989). Second, zona binding can result in a permanently irreversible phenomenon, which occurs after extended coincubation periods (>2 hours) of the gametes and possibly reflects a semi-penetrated status of the spermatozoa. In general, we can speculate that acrosome-reacted spermatozoa have the capacity to loosely associate with the zonae by a reversible attachment to the outer zonae surfaces, which will partly be dislodged during the washing procedure of the ZP. Acrosome-intact spermatozoa, however, will achieve irreversible binding with the zonae, using the outer acrosomal membrane, and prior to penetrating undergo the AR on the surface of the zonae.

It is important to remember that the spontaneous AR merely reflects sperm dysfunction and should not be regarded as a functional sperm assay, *i.e*., men whose spermatozoa reveal a high level of spontaneous AR within a short time after separation from seminal plasma are likely to have an acrosome instability problem. Our results indicated a very poor (r=0.16) correlation between the percentage of normal spermatozoa and the percentage of spermatozoa that underwent spontaneous ARs. Simultaneously conducted A23187-induced acrosome studies showed very poor correlations and low positive and negative predictive values for normal sperm morphology (Fig. 4). Both spontaneous and A23187-induced ARs are therefore not suitable for use as screening tests for sperm function, since they demonstrated decreased negative predictive values, *i.e*., 28% and 12%, respectively. The normal situation for spermatozoa is not to show increased levels of spontaneous acrosomal loss during in vitro capacitation conditions. Rather, at least in vivo, capacitated spermatozoa attach to and ultimately bind to the ZP3 in the ZP, after which the AR occurs (Franken *et al*., 1991b, 1996b; Tesarik, 1996).

In conclusion, it is well recognized that the lack of information regarding the zona-induced AR in human spermatozoa is largely due to the paucity of human zonae available for diagnostic laboratory testing and research. Following the original elucidation of the cDNA sequence of the human ZP3 gene (Chamberlain & Dean, 1990), biologically active rhuZP3 has been recently expressed in Chinese hamster ovary cells (Van Duin *et al*., 1994; Barratt & Hornby, 1995; Brewis *et al*., 1996). The rhuZP3 will be the ultimate agonist for the human AR and will form the basis of the ideal diagnostic test in future programmes (Tesarik, 1996). The acrosome stimulation test using rhuZP3 as stimulant will add valuable information to the sequential approach for identifying the causative pathophysiology.

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TABLE 1

The mean (±SE) values for sperm parameters recorded for fertile sperm donors and andrology patients.

Semen source	Sperm concentration (10^6/ml)	% Motility	% Normal morphology
Donors (*n*=3)*	199.4±7.8	56.5±0.8	15.8±0.9
Teratozoospermic men (*n*=25)	153.1±14.7	52.6±1.5	10.0±0.7
Severely teratozoo-spermic men (*n*=4)	179.0±51.4	43.7±2.3	2.7±0.8

*mean of 29 semen samples

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TABLE 2

The mean percentage acrosome-reacted spermatozoa and morphologically normal spermatozoa from teratozoospermic men following solubilized ZP, A23187 and spontaneous induction.

		% Acrosome-reacted after 3 h capacitation		
Semen source	**% Morphologically normal spermatozoa**	**Solubilized ZP-induced (2ZP/µl)**	**Spontaneous**	**A23187-induced**
Sperm donors (*n*=3) (29 samples)	15.8±0.9[a]	36.8±0.9[d]	17.0±0.2[g]	52.7±0.3
Terato-zoospermic men (*n*=25)	10.4±0.7[b]	25.8±0.9[e]	9.1±0.2	51.3±0.7
Severely terato-zoospermic men (*n*=4)	2.7±0.7[c]	19.0±0.9[f]	10.5±1.1[h]	51.5±1.6

a vs. b, P=0.001; a vs. c, P=0.0001; b vs. c, P=0.0001; d vs. e, P=0.01; d vs. f, P=0.001; e vs. f, P=0.001; g vs. h, P=0.01.

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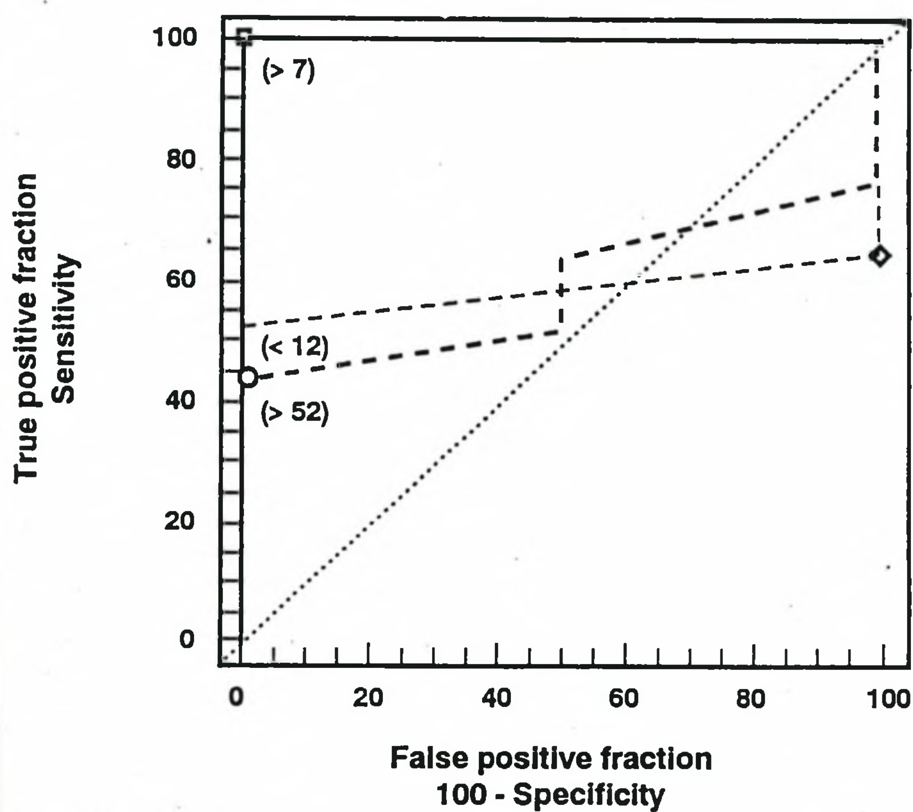

FIGURE 4: **ROC curve analysis for sperm morphology and ARs mediated by solubilized ZP.**

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Subscript to Figure 4:

__________A highly positive and significant correlation existed between normal sperm morphology and the inducibility of the AR (r=0.88, *P*=0.0001). ROC curve analysis showed a sensitivity and negative predictive value of 100% in both cases for predicting a given sperm sample acrosome responsiveness within the morphology groups >4% or <4% and an AR cut-off value of >7%.

..................No correlation was detected between the AR and normal sperm morphology using 4% as cut-off value (r=0.16, *P*=0.4). ROC curve analysis indicated with a cut-off value of 4% normal sperm morphology, the percentage acrosome-reacted spermatozoa was 12% with a sensitivity and negative predictive value of 80% and 29%, respectively.

\- - - - - - - A23187-induced AR data showed no correlation with the percentage normal spermatozoa (r=0.012, *P*=0.9). Likewise, ROC curve analysis revealed a sensitivity of 44% and a negative predictive value of 12% at a morphology cut-off of 4% and AR value of 52%.

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1.5.3.2 Physiological induction of the acrosome reaction in human sperm: validation of a microassay using minimal volumes of solubilized, homologous zona pellucida

Franken, D.R., Bastiaan, H.S. and Oehninger, S.C.

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Abstract

Purpose: The objective was to develop a method that could accommodate microvolumes of solubilized human ZP and sperm for assessing the induction of the AR.

Methods: A microassay using 1µl of 2.5, 1.25, 0.6, 0.3 and 0.125ZP/µl incubated with 1µl of a highly motile sperm suspension for 60 minutes. As a control and parallel to the microassay, a standard AR technique was performed.

Results: No significant differences were observed between the percentage acrosome-reacted sperm reported by the two assays under basal conditions (spontaneous) or after induction with A23187 or solubilized ZP. At a ZP concentration of 0.6ZP/µl, the percentages of acrosome-reacted spermatozoa in both techniques were significantly higher compared to the spontaneous AR results, namely, 18% and 17%, compared to 10% and 10%, respectively. Approximately a 30% level of acrosomal exocytosis was induced with 2.5ZP/µl in both methods.

Conclusions: This newly devised microtechnique is easy and rapid to perform, is repeatable and facilitates the use of minimal volumes of solubilized human ZP (even a single ZP) for assessment of the inducibility of the AR of a homologous sperm population.

Introduction

The andrologic evaluation of the male partner relies on a thorough history and physical examination, followed by a urologic and endocrinologic workup as indicated. Still, the semen analysis remains the cornerstone of diagnostic management. We, and others, have been promoters of a sequential, multistep diagnostic approach for the evaluation of the various structural, dynamic and functional sperm characteristics (Oehninger *et al.*, 1991; Amman & Hammerstedt, 1993; Oehninger *et al.*, 1997a). The proposed diagnostic scheme should include (i) assessment of the "basic" semen analysis and (ii) functional testing of spermatozoa (WHO, 1992; Oehninger, 1995).

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Different laboratories have highlighted the diagnostic power of a variety of tests that examine the functional competence of the male gamete. The World Health Organization has incorporated some of them under the category of functional tests (WHO, 1992). At a recent consensus workshop on advanced diagnostic andrology techniques (ESHRE, 1996), it was concluded that because of their validation and unquestioned clinical value, the homologous sperm-ZP binding tests should be incorporated in the advanced stages of the workup. However, it also was agreed that better standardization of the currently used AR techniques should be implemented prior to their introduction as a routine clinical tool. At the present time, there seems to be general agreement that more clinical information can be gained by the analysis of a stimulant-induced acrosomal exocytosis compared to the assessment of the spontaneous frequency of ARs (basal rate). The most widely utilized method is the challenge with a calcium ionophore agent where the AR is identified with defined lectins in combination with indirect immunofluorescence (ESHRE, 1996).

The acrosomal response of a given sperm sample has been illustrated to be a crucial event leading to fertilization and many reports have aimed to correlate AR response with IVF rates. Moreover, the precise timing of the AR formed the rationale for the development of the acrosome reaction ionophore challenge test (ARIC-test) (Cummins *et al.*, 1991; Tesarik, 1996). The concept of acrosomal inducibility (Henkel *et al.*, 1993, 1998) and the ARIC-test have gained more recognition, and there seems to be agreement that this method of evaluation is a more reliable predictor of sperm fertilizing ability than those tests that simply measure the frequency of spontaneous ARs. The inducibility of the AR, *i.e.*, the difference between spontaneous and percentage acrosome-reacted sperm after induction, correlates significantly with IVF outcome (Henkel *et al.*, 1993).

The ZP in both the intact and solubilized state has been demonstrated to be a powerful and physiological inducer of the AR (Cross *et* al., 1988; Mahony *et. al.*, 1991; Bielfeld *et al.*, 1994a; Liu & Baker, 1994b; Franken *et al.*, 1996a, 1997). During fertilization, AR failure can be caused by multiple factors, such as (i) inadequate sperm capacitation; (ii) an inability of the sperm membrane to undergo specific structural-functional changes after binding to the ZP; or (iii) an impaired capacity of the ZP of a specific oocyte to induce the acrosomal cascade.

In this investigation, we aimed to develop and validate a simple and rapid microassay for the accurate determination of the human sperm AR mediated by minimal volumes of solubilized (or even a single ZP) homologous ZP.

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Materials and methods

Preparation of sperm samples

Ejaculates from fertile men (donors) were used in these studies after approval by the local ethics committee. The sperm parameters of samples used were as follows (mean±SD): concentration, $117.4\pm16x10^6$/ml; sperm motility, (60±5)%; and normal morphology (strict criteria), (17±2)%. Motile sperm fractions ($10x10^6$ cells/ml, >90% motility) were retrieved using a double-wash swim up technique (Franken *et al.*, 1996a, 1997). Before the onset of AR studies, sperm were allowed to capacitate at 37°C in 5% CO_2 in air for 3 hours in synthetic HTF supplemented with 3% BSA (Quinn *et al.*, 1985).

Preparation of solubilized zona pellucida

Human oocytes were retrieved from post-mortem derived human ovarian tissue following approval by the local ethics committee. Oocytes were stored in DMSO/sucrose at -196°C in liquid nitrogen (Hammit *et* al., 1991). Twelve hours prior to each experiment, oocytes were removed from storage and thawed at 37°C. Oocytes were placed in 0.25*M* sucrose and 3% BSA in HTF for 20 minutes at room temperature, after which the oocytes were placed under mineral oil until used.

On the day of each experiment, 50 oocytes were vigorously pipetted with a small-bore glass pipette (inner diameter 80 µm) to separate the ZP from the ooplasm. The separated ZP were then placed in a plastic Eppendorf tube containing HTF supplemented with 3% BSA. The tubes were centrifuged for 15 minutes at 1800 x*g*, after which the HTF was carefully removed under microscopic vision, leaving only 50 ZP at the bottom of the tube. A total volume of 5µl 10m*M* HCl was added to the ZP. Solubilization of the ZP was microscopically observed and controlled after which 5 µl of 10m*M* NaOH was added to the ZP to render a final zona volume of 10µl, containing 5ZP/µl. The final pH of the zona solution was 7.4.

Acrosome reaction studies

Two sets of experiments, each using different volumes of solubilized ZP, were performed in a parallel fashion, namely, a microassay and a standard AR assay. Following solubilization, ZP was kept at 4°C for 7 days during which all experiments were performed. Ongoing studies in our laboratory currently evaluate the AR inducibility of solubilized ZP recorded over an extended time period.

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For the microassay, 1µl of ZP stock solution (5ZP/µl) was aspirated into a Teflon pipette tip (Hamilton Pipette-tip; Cat. No. 84254; Separations, Cape Town, South Africa), fitted to a microsyringe (Hamilton 702; Separations, Cape Town, South Africa) with 1µl of sperm ($10x10^6$ sperm/ml, >90% motility), to render a final ZP concentration of 2.5ZP/µl. Serial dilutions were made from the 5ZP/µl solutions (1:1, vol:vol) using HTF to equal final zona concentrations of 2.5, 1.25, 0.6, and 0.3ZP/µl. One microliter of each dilution was separately added to 1µl of sperm to equal a final ZP concentration of 1.25, 0.6, 0.3 and 0.15ZP/µl. Prior to aspiration into Teflon tips, all sperm-ZP suspensions were gently mixed in a well of a microtiter plate (Microtest plate; P-43; Laboratory and Scientific, Cape Town, South Africa). To prevent evaporation from the Teflon tips, aspirating HTF droplets into both sides of the Teflon tip sealed off sperm-ZP suspensions. Each sperm-ZP suspension was separated from the HTF droplets by air bubbles on both sides. Due to the small volumes involved, progressive motility and percentage live cells (WHO, 1992) for both AR techniques were manually performed on spotted slides (MAGV, Germany, XER 201B). Control and treated sperm samples were carefully removed from the Teflon tips and placed on separate spots on the spotted slide and immediately evaluated for percentage live sperm under inverted phase-contrast microscopy (Nikon TMS-F, Research Instruments, Johannesburg, South Africa). During both techniques the percentage live acrosome-reacted cells were recorded by aspirating/adding 1µl supravital stain Hoechst 33258 (1µg/ml) 5 minutes before termination of incubation of sperm and solubilized zona. During the evaluation of the acrosome status of each experiment, only live acrosome-reacted spermatozoa were recorded. In each study, negative and positive control experiments consisted of 1µl of sperm suspension plus 1µl HTF or 1µl 10µ*M* A23187 incubated as the test conditions at 37°C, 5% CO_2, 95% humidity for 1 hour. For the standard AR assay, larger volumes [*i.e.*, 5µl of the sperm suspension plus 5µl of the above-mentioned zona solutions (*i.e.*, 2.5, 1.2, 0.6, 0.3, and 0.15ZP/µl)] were incubated in 0.4 ml Eppendorf tubes, under similar laboratory conditions for 1 hour. Prior to the onset of the study control experiments (*i.e.*, exposure of sperm to HTF and A23187) were also included in the standard acrosome assay as described above.

Following the motility assessments, sperm droplets were allowed to air-dry, after which the sperm were fixed in 70% ethanol for 20 minutes and evaluated for percentage live cells. Acrosomal status for both assays (and respective control conditions) was evaluated using FITC-PSA staining with epifluorescence microscopy (Cross *et* al., 1988; Morales & Cross, 1989; Morales *et al.*, 1989; Mahony *et al.*, 1991). Two hundred cells were counted in a blinded fashion in each well of the spotted slide and results were expressed as percentage acrosome-reacted sperm. The following staining patterns were evaluated as acrosome-

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reacted spermatozoa: (i) patchy staining on acrosomal region; (ii) distinct staining in the equatorial region occurring as an equatorial bar; and (iii) no staining observed over entire sperm surface.

Intact zona pellucida-induced acrosome reaction

Additional AR studies were performed on intact ZP. Using the same sperm samples, parallel experiments to the microassays and standard acrosome assays were performed where the acrosomal status of ZP-bound sperm was examined after coincubation of the male gametes with intact, previously salt-stored human oocytes. It has been demonstrated that oocytes stored under these conditions retain biophysical, biochemical and functional properties (Yanagimachi *et al.*, 1979; Burkman *et al.*, 1988; Franken *et al.*, 1991b). At the time of the experiments, the oocytes were desalted, washed in culture medium and microbisected into matching hemizonae as previously described (Burkman *et al.*, 1988). A total of 60 hemizonae (matching pairs from 30 oocytes) were individually incubated under oil in 50 µl droplets containing $25x10^6$/ml motile sperm (post-swim up) for 60 minutes at 37°C in 5% CO_2 in air. The hemizonae were then removed from the suspension and after pipetting using a fine glass pipette to remove loosely attached sperm, the number of sperm tightly bound to each hemizonae were counted under phase-contrast microscopy (Burkman *et al.*, 1988; Franken *et al.*, 1991b). Thereafter, the sperm tightly bound to the zonae were removed (stripped) by a shearing action, using a small-bore glass (60 µm inner diameter) pipette. Individual sperm were then placed on a spot glass slide, allowed to air-dry, after which the AR was determined as described above. The number of sperm tightly bound to each hemizona under these conditions were always >300 cells. Most of the zona-bound sperm could be stripped during the experiments for all hemizonae evaluated and at least 200 spermatozoa (per hemizona) could be assessed for acrosomal status.

Statistical Analysis

Comparisons of the percentage AR for both methods (microassays and standard assays) under different experimental conditions (*i.e.*, spontaneous, A23187 and ZP-induced conditions) were performed with Fisher's exact paired *t*-test. The overall dose-dependency effect of varying solubilized ZP concentrations on acrosomal exocytosis was assessed by analysis of variance (ANOVA).

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Results

The percentage AR recorded for spontaneous (in HTF) and A23187-induced did not differ between the microassays and standard AR assays (Table 3). Table 4 shows AR results for the various solubilized ZP concentrations used. Again, there were no differences in the induction of acrosomal exocytosis for the two methods. The percentage of live acrosome-reacted sperm in both techniques were >80%. In addition, an obvious dose-dependent effect of solubilized ZP on the AR was observed reaching an approximate 30% induction using a maximun dose of 2.5ZP/µl for both the microassays and standard assays. Following a 1-hour incubation with intact hemizonae (intact ZP), on the other hand, the percentage acrosome-reacted zona-bound sperm was significantly higher than the levels obtained with solubilized ZP or the A23187 agent [(84±9)% vs. (32±2)% and (28±3)%, respectively, $P < 0.001$].

Discussion

The need for a microvolume assay to assess ARs has been identified previously (Morales & Cross, 1989). This is due to the fact that diagnostic andrology laboratories often lack sufficient biological material (*i.e.*, human ZP) to perform a defined and specific test such as the examination of the physiological AR. This is true for the natural ZP protein(s), but also will be relevant when recombinant human ZP proteins are to be tested for corroboration of their biological activity (Brewis *et al.*, 1996; Chapman & Barratt, 1996).

The newly described assay is simple, can be performed quickly, and the results are reliable and repeatable. Therefore, because of the small volumes employed, it is an ideal technique for testing native and recombinant ZP (highly precious or scant material). The results of the present study indicated the use of a single ZP to be adequate for mediating the AR of a sperm population in suspension. At a ZP concentration of 0.6ZP/µl, the percentage acrosome-reacted sperm, as determined by both the standard assays and microassays, was significantly higher than the spontaneous reaction, *i.e.*, 18% (standard assay) and 17% (microassay) compared to the spontaneous reaction, namely, 10% (standard assay) and 10% (microassay). The maximum levels of acrosomal exocytosis (28%) induced with the highest ZP concentration (2.5ZP/µl final concentration) in the microassay were similar to the ones observed with the standard assay in our laboratory and in those of others (Mahony *et al.*, 1991; Liu & Baker, 1994b; Franken *et al.*, 1996a). On the other hand, AR induction by intact ZP as detected during sperm-zona binding control assays as expected was higher than with the use of solubilized ZP. The incidence of zona-bound acrosome-reacted spermatozoa found here is similar to the one we reported using the HZA model combined with

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transmission electron microscopy and the monoclonal T-6 antibody (Coddington *et al.*, 1990; Franken *et al.*, 1991b). A more adequate configuration of the ZP proteins in the intact zona matrix and a higher number of zona protein molecules in the hemizona (as compared to lower numbers present in the very small volumes of solubilized ZP used here) are a possible explanation for this finding.

The routine introduction of a simple and reliable assay for the evaluation of the physiologically induced AR as a component of the previously proposed sequential diagnostic workup program will assist in the identification of specific sperm defects and may allow the development of more directed therapies. Andrology testing remains, in our opinion as well as those of others (Mortimer, 1994c), an ever-growing component in the workup of the infertile couple. We enter the next millennium with many questions that remain to be answered by the hand of efficacious screening techniques and a new formidable therapy in ICSI (Oehninger *et al.*, 1997a). The analysis of the inducibility of the AR, a critical step during fertilization, aids the clinician in the management of male infertility. Finally, it is accepted that, once available, biologically active rhuZP3 will be the ultimate agonist or trigger substance for human sperm AR. Such a test will most certainly become the basis of the ideal AR test (Tesarik, 1996). Once available in sufficient amounts, rhuZP3 moieties will have to go through comparative evaluation studies, using natural (solubilized) ZP as controls in subsequent (microvolume) AR assays.

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TABLE 3

AR results recorded with a microassay and a standard technique using A23187-induced and spontaneous reactions.

Standard assay*		**Microassay***		**Intact ZP**
Spontaneous AR (%)	**% A23187-induced AR**	**Spontaneous AR (%)**	**% A23187-induced AR**	**(% AR of zona-bound spermatozoa)**
10±2[a]	51±2[b]	10±3[c]	47±14[d]	84±9%

*Mean % ± SD.

a vs. c, not significant; Fisher exact paired *t*-test.

b vs. d, not significant; Fisher exact paired *t*-test.

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TABLE 4

The mean (±SD) percentage acrosome-reacted spermatozoa recorded for varying solubilized ZP concentrations.

ZP concentration (ZP/µl)	Standard Assay Percentage AR (mean±SD)	n	Microassay Percentage AR (mean±SD)	n
2.50	32^{Φ}±2%	4	28±3%	6
1.25	26^{Φ}±2%	6	23±2%	5
0.60	18^{Φ}±3%	5	17±3%	6
0.30	14±2%	6	16±2%	4
0.15	14±3%	4	14±3%	6
Spontaneous	10^{Φ}±2%	6	10±3%	6

*Overall dose-dependency effect for both methods, $P<0.0001$ by analysis of variance.

$^{\Phi}P=0.05$ for 2.5, 1.25 and 0.6ZP/µl when compared to spontaneous AR results.

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1.6 Computer-assisted sperm motion characteristics

Despite many years spent developing and refining methods of testing sperm function, there is no consensus as to which laboratory tests can predict the ability of the spermatozoon to fertilize the oocyte, and it is generally agreed that descriptive semen analysis is of limited value in the assessment of male infertility (WHO, 1999). One parameter of descriptive semen analysis, the assessment of sperm morphology, if defined by strict criteria as proposed by the Tygerberg group (Kruger *et al.*, 1986), is said to have a high predictive power of fertilization success in assisted reproduction. However, researchers continue to seek physiological indicators of the functional competence of spermatozoa as this may allow a more rational treatment of sperm dysfunction. New measuring instruments, such as computer-assisted sperm analyzers, have opened new areas for investigation. One such area is the use of the motility pattern called hyperactivation as a biomarker of the ability of spermatozoa to undergo capacitation.

Hyperactivated motility, a functional change in the sperm movement pattern which usually occurs during the capacitation process (Burkman, 1984), was first assessed subjectively by visual observations of the flagellar beat using descriptive criteria such as "serpentine" (Yanagimachi, 1972), "figure-of-eight" (Fraser, 1977) and "whip-lash" (Cooper *et al.*, 1979). The fundamental movement change, which occurs during hyperactivation, is an increase in the amplitude of the flagellar beat caused by an increase in the bending of the proximal flagellum (Katz *et al.*, 1989).

Since the pattern and velocity of sperm head motion are consequences of flagellar beat, their measurement can be used in the assessment of motility, though the kinetic and mechanical information provided is inferior to that produced by analysis of flagellar beat. Manual measurement of head movements (Yanagimachi, 1970) has been gradually superseded by semi-automated (Mortimer *et al.*, 1988) and finally automated measuring methods exploiting video microscopy and computed image analysis (Davis *et al.*, 1992).

Several different computer-assisted systems for analyzing sperm movement are currently available, e.g. Hamilton-Thorne. An automated system is capable of acquiring more information about sperm motility and morphology, and an automated analysis offers the advantage of being able to determine sperm velocity and linearity of the sperm track, and also is capable of saving the moving sperm image onto an optical disk or some other medium for later retrieval and reanalysis.

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This type of equipment reconstructs sperm trajectories from the position of the sperm head in successive video frames for a minimum and maximum number of video frames defined by the user, within the limits of the equipment. Movement parameters are derived according to the following definitions (Mortimer, 1990): Curvilinear velocity (VCL, µm/s) is the total distance between each head point for a given cell during the acquisition period, divided by the time elapsed. Straight line velocity (VSL, µm/s) is the straight line distance between the first and last head point, divided by the acquisition time. Average path velocity (VAP, µm/s) is a smoothed path constructed by averaging several neighbouring positions on the track (five or nine points) and joining the averaged positions. Amplitude of lateral head displacement (ALH, µm) is intended to give a measure of sperm head oscillation. It is calculated from either the maximum sperm head departure from the average path (ALH_{max}) or the mean sperm head departure from the average path (ALH_{mean}). Since this represents departure from the path in only one direction, this figure is doubled to give full-width amplitude. Beat cross frequency (BCF, Hz) is determined by measuring the frequency with which the sperm track crosses the average path in either direction. Linearity (LIN, %) measures departure from linear progression. This parameter is defined as VSL/VCLx100, with 100% representing an absolutely straight track. Straightness (STR, %) gives a similar measure using the ratio VAP/VCLx100.

Sperm motility appears to be a sensitive parameter of sperm function (Hall, 1981). The potential use of hyperactivation as a biomarker of fertilizing ability has created much interest, and a correlation between hyperactivation and fertilization has been sought for human spermatozoa. Burkman (1995) showed that sperm samples from subfertile men exhibited significantly less hyperactivated motility when compared with samples from men of proven fertility during IVF. However, studies in which the incidence of spontaneous pregnancies is recorded are of limited prognostic value due to the inter-ejaculatory variability and by the possibility of coexisting female pathology. The study of semen used for IVF overcomes the problem of inter-ejaculatory variability, with the same semen sample being used for both sperm function and attempted fertilization, but the results may not be applicable to the achievement of spontaneous pregnancies. Sperm motility parameters have been analyzed by CASA to determine any correlation with the outcome of IVF. LIN, VAP and VSL have all been shown to correlate significantly with the fertilization rate in vitro (Liu *et al.*, 1991). Sperm populations with low fertilization success rates in vitro have been shown to possess a significantly lower proportion of hyperactivated cells (Burkman, 1991). In addition, hyperactivation was closely correlated with binding to the ZP (Coddington *et al.*, 1991).

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CHAPTER 2

MATERIALS AND METHODS

The materials and methods used for each study are outlined in the individual articles presented in Chapters 3-6. The methods differed slightly between these studies; however, the basic procedures followed are described in this chapter.

2.1 Semen collection and analysis

Modern andrological evaluation in all cases is an exhaustive history and physical examination followed by repeated semen analyses (Oehninger, 1995). The subsequent spermiogram, established from these analyses, may be used for an initial evaluation of fertility status. Using these criteria, a patient may be classified into a particular "fertility status group" and the clinician may evaluate the patient's potential or requirement for assisted reproductive technology.

Semen samples, used in the present studies, were obtained from normozoospermic fertile donors as well as from men visiting the andrology laboratory at Tygerberg Hospital. All samples were provided following masturbation after a period of 2-4 days of sexual abstinence. These samples were kept at 37°C until liquefaction had occurred and analyzed according to the World Health Organization criteria (WHO, 1992, 1999) together with strict sperm morphology assessment (Kruger *et al.*, 1986; Menkveld & Kruger, 1996; Menkveld *et al.*, 1990, 1996). The percentage of motile spermatozoa and the average grade of sperm velocity (on a scale of 0-4) were visually assessed under phase-contrast microscopy, and the sperm concentration was determined using a Neubauer haemocytometer (Menkveld & Kruger, 1996).

2.2 Computer-assisted semen analysis

Sperm motility/kinematics were determined with the HTM-IVOS analyzer (Hamilton-Thorne Research, Beverly, MA) with standard set-up parameters. The HTM-IVOS combines an internal optical system with an internal computer and image digitizing and analyzing systems. A computer-controlled stage moves the specimen slide between fields. The system can be used for movement analysis as well as static cell morphology determination.

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The HTM-IVOS operates as a computerized cell motion analyzer. Integrated hardware components include an internal phase-contrast optics system with stroboscopic illumination, an internal automated, heated specimen stage, an image digitizer and an 80486 computer. Specimens are viewed under a 10x objective and IVOS can either evaluate the sample automatically, selecting random fields (maximum 10 fields) or the user can manually select the fields to be examined.

The HTM-IVOS is capable of evaluating the following parameters: sperm concentration; motile and progressively motile concentrations; percentage motile and progressively motile; VAP; VSL; VCL; ALH; BCF; STR; LIN; hyperactivation; elongation; and area (Fig. 5).

The analyzer settings were as follows: 30 frames/60 Hz; minimum contrast, 80; minimum cell size, 2; minimum static contrast, 30; low VAP cut-off, 5 µm/s; low VSL cut-off, 11 µm/s; head size, non-motile, 3; head intensity, non-motile, 160; static head size, 1.01-2.91; static head intensity, 0.60-1.40; slow cells, non-motile; magnification, 2.01; and temperature, 37°C.

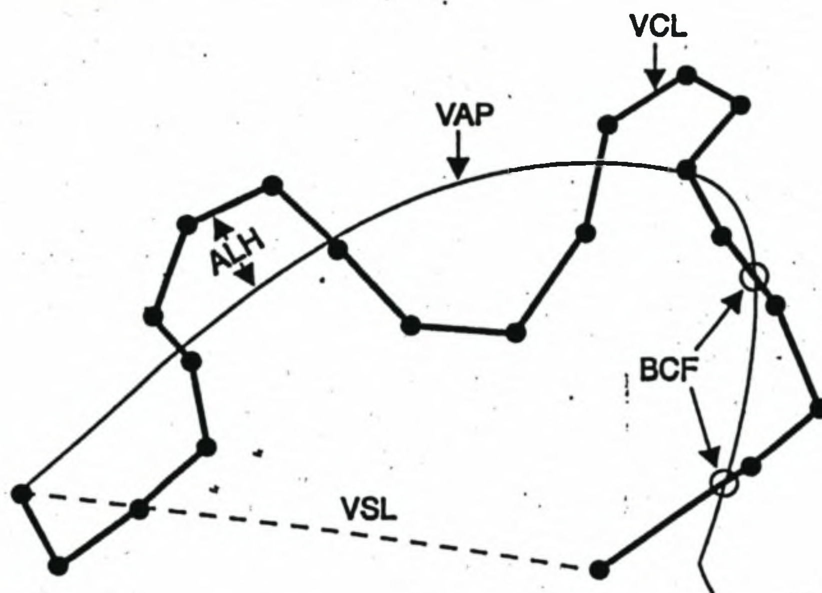

FIGURE 5: Sperm head motion parameters typically determined by CASA (*WHO, 1999*).

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2.3 Standard swim-up method

The double-wash swim up separation method is a sperm preparation technique used to isolate highly motile fractions from the human ejaculate (Purdy, 1982). The procedure is simple, rapid and effective, whereby highly progressively motile spermatozoa are allowed to swim up from a concentrated sperm pellet into a small volume of overlying culture medium. This may be regarded as a mimic to the in vivo transit of spermatozoa from the cervical mucus to the fallopian tubes for fertilization. The technique also serves to separate motile sperm cells from other cells, and debris that may occur in the ejaculate. The wash and centrifuge steps in this procedure separate sperm cells from seminal plasma. Such separation is necessary for capacitation since seminal plasma contains decapacitation factors. Isolation and recovery of motile sperm fractions enhance the quality of the insemination sample used in IVF procedures (among others) and thereby increase the possibility of fertilization.

Motile sperm fractions were collected from samples using a slightly modified double-wash swim up technique (Franken *et al.*, 1996, 1997). From each sample, 1ml semen was placed in a plastic centrifuge tube (10mm diameter). During the first and second washes, twice the volume of synthetic human tubal fluid medium (HTF; Quinn *et al.*, 1985) supplemented with 3% bovine serum albumin (BSA; Seravac, Cape Town, South Africa) was added to the semen and thoroughly mixed, after which the suspension was centrifuged for 5 minutes at 300 x*g*. After removal of the supernatant, the sperm pellet was gently overlaid with half the volume of HTF supplemented with 3% BSA. The tube was then incubated at an angle of 45° for 1 hour in 5% CO_2 in air at 37°C to allow the spermatozoa to "rise" from the pellet at the bottom of the centrifuge tube. Retrieved sperm samples were resuspended in HTF supplemented with 3% BSA to the desired sperm concentration. Before the onset of AR studies, these samples were allowed to capacitate at 37°C in 5% CO_2 in air for 3 hours in HTF supplemented with 3% BSA.

2.4 Oocyte collection and storage

All oocytes used in the studies were non-living, with no developmental potential. Initially, oocytes were obtained from ovarian tissue that was collected post mortem. Great care was taken to ensure that all legal, ethical and moral guidelines were adhered to at all times during oocyte collection. All ovarian tissue was excised within 24 hours of death and stored at 4°C in phosphate-buffered saline. Oocyte retrieval was accomplished following described protocols (Overstreet *et al.*, 1980).

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Zona-intact oocytes denuded of granulosa cells were recovered, aspirated into standard cryopreservation straws and stored in dimethylsulphoxide (DMSO)/sucrose solution at minus 196°C (Hammit *et al.*, 1991). Twenty-four hours prior to each test, the oocytes were removed from storage, thawed at 37°C, placed in 0.25*M* sucrose and HTF supplemented with 3% BSA for 7-10 minutes, and washed 5x in fresh HTF containing 3% BSA by pipetting with a finely drawn micropipette.

A salt storage method (Franken *et* al., 1991) was used as an alternative for the DMSO method. After retrieval, oocytes were stored at 4°C under mineral oil (M-3516; Sigma Chemical Co, St Louis, MO, USA) in concentrated salt solutions, containing 1.5*M* $MgCl_2$ (Mallinckrodt Chemical Work, St Louis, MO, USA), 0.01% polyvinylpyrrolidone (PVP) and 40m*M* of Hepes buffer, at pH 7.4. Twenty-four hours prior to each test, oocytes were removed from storage and desalted using 5 changes in fresh HTF supplemented with 3% BSA.

2.5 Induction of the acrosome reaction

The inducibility of the AR (*i.e.*, the difference between induced and spontaneous AR), which is of predictive value for the fertilizing capacity of spermatozoa (Henkel *et al.*, 1993), depends on the inducing agent used, and may be influenced by sperm malformations and environmental factors. The induction of the human sperm AR by various agents, namely, A23187 and acid-solubilized ZP, was studied among normozoospermic fertile donors as well as men visiting the andrology laboratory at Tygerberg Hospital.

2.5.1 Acrosome reaction induced by calcium ionophore

On the day of use, an aliquot of 5m*M* stock solution of A23187 (C-7522; Sigma Chemical Co, St Louis, MO, USA) in DMSO (Merck, Darmstadt, Germany) was diluted 5 times with serum-free HTF and 1µl was added to 50µl of the motile sperm suspension, making a final concentration of 20µ*M* A23187. To render a final concentration of 10µ*M* A23187, an aliquot of 5m*M* stock solution of A23187 in DMSO was diluted 10 times with serum-free HTF, after which 1µl was added to 50µl of the motile sperm suspension (Liu & Baker, 1996).

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2.5.2 Acrosome reaction induced by human follicular fluid

HFF was obtained from women attending the IVF program at Tygerberg Hospital. The patients were stimulated using 100mg clomiphene citrate daily for 5 days. 150 IU of hMG was administered intramuscularly on alternate days starting on day 2 of clomiphene citrate cycle. Follicular growth was monitored by ultrasonography and the serum estradiol was measured. hCG was administered as soon as the leading follicle reached 18mm in diameter, with two additional follicles of at least 16mm diameter. Follicle aspiration was performed 36 hours after hCG administration. Only mature oocytes (metaphase II) (Veeck, 1988) containing straw-coloured HFF was used, without heparin. All collected HFF samples were centrifuged immediately to remove contaminating granulosa cells and the pooled supernatants stored at -20°C.

2.5.3 Acrosome reaction induced by solubilized human zona pellucida

On the day of the experiment, oocytes were placed in a plastic Eppendorf tube (1ml) containing HTF supplemented with 3% BSA, centrifuged for 15 minutes at 1800 x*g*, after which the HTF was removed under microscopic vision (Olympus Stereoscope Model SZ40, Wirsam Scientific, Cape Town, South Africa), leaving only the oocytes at the bottom of the tube. A 10m*M* HCl was added to oocytes in the Eppendorf tube to solubilize the ZP; solubilization of the ZP was microscopically observed and controlled. Ooplasm of all oocytes were left at the bottom of the tube. Pilot studies performed in our laboratory and that of others (Liu & Baker, 1990) showed no interference with the AR using ZP-free oocytes in control or test samples. All control and test samples included ZP-free oocytes. Following solubilization, 10m*M* NaOH was added to the solubilized ZP to neutralize the acidic pH. The zona solution was aliquoted in portions to which HTF supplemented with 3% BSA was added to render the desired concentration(s) of the ZP solution(s) (Liu & Baker, 1996).

2.6 Inhibition of G_i proteins by pertussis toxin

G proteins appear to couple putative ZP3 receptors to intracellular signalling events required for induction of the AR (Lee *et al.*, 1992). These proteins play critical roles as signal-transducing elements in coupling many ligand-receptor interactions to intracellular second messenger cascades/ionic changes (Ross, 1989). Functional inactivation of sperm guanine nucleotide-binding regulatory (G_i) proteins by pertussis toxin (PT) treatment does not affect the ability of sperm to bind to the ZP, but inhibits specifically the AR

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induced by ZP3 (Lee *et al.*, 1992; Franken *et al.*, 1996). PT-treated sperm samples were evaluated for acrosomal status and ZP binding.

Prepared sperm samples were exposed to varying concentrations (1, 10 and 100ng/ml) of PT (P-9452; Sigma Chemical Co, St Louis, MO, USA). The acrosomal status were determined for PT-treated spermatozoa exposed for 15, 30 and 60 minutes at 37 °C in 5% CO_2 in air.

2.7 Assessment of the acrosomal status

The acrosomal cap of some mammalian spermatozoa is in general sufficiently large enough to be visualized by phase-contrast microscopy but in others, including humans; it is too small to be evaluated directly for acrosomal reacted state. Consequently, indirect methods using fluorescein-labelled lectins (Cross *et al.*, 1988; Morales *et al.*, 1989), monoclonal antibodies (Wolf *et al.*, 1985), and staining techniques (Yanagimachi, 1994) have been developed to visualize the presence or absence of the human acrosome. Although interpretation of investigations based on AR scoring have not yet attracted widespread clinical interest in infertility management, it is a research area of great current interest and an increasing number of laboratories (Cummins *et al.*, 1991; Tesarik, 1995; Barratt & Hornby, 1995; Liu & Baker, 1996; Brewis *et al.*, 1996; Esterhuizen *et al.*, 2001) are establishing methods for monitoring the acrosomal status of human sperm populations.

Before the onset of AR studies, sperm samples were allowed to capactitate at 37°C in 5% CO_2 in air for 3 hours in HTF supplemented with 3% BSA. ARs were determined among capacitated spermatozoa with the following: HTF (control); HCl/NaOH (control); A23187 (control); 30% (v/v) HFF (control); PT (control); and ZP (test). Ten minutes prior to each experiment, spermatozoa were stained with the supravital stain Hoechst 33258 (1µg/ml; B-2883; Sigma Chemical Co, St Louis, MO, USA) to determine vital status of spermatozoa at 37°C in 5% CO_2 in air for 10-15 minutes. Excess stain was removed by centrifuging spermatozoa at 900 x*g* for 5 minutes through a solution of 2% PVP (PVP-40; Sigma Chemical Co, St Louis, MO, USA) in phosphate-buffered saline (pH 7.4), after which spermatozoa were fixed in 95% ethanol (BDH Laboratory Supplies, England) for 30 minutes at 4°C and air-dried onto a microscope slide. The acrosomal status of spermatozoa capable of excluding the Hoechst dye was determined using fluorescenated *Pisum Sativum* agglutinin (FITC-PSA; 125µg/ml; L-0770; Sigma Chemical Co, St Louis, MO, USA) (Cross *et al.*, 1988; Morales *et al.*, 1989; Liu *et al.*, 2001). Hoechst 33258 labels dead (spermatozoa show bright blue-white fluorescence) but not living (spermatozoa show a pale blue

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fluorescence) spermatozoa (Mortimer, 1994). A total of 200 spermatozoa per slide were counted using a fluorescence microscope (Olympus System Attachment Model BX40).

Four classes of PSA labelling were distinguished (Fig. 6):

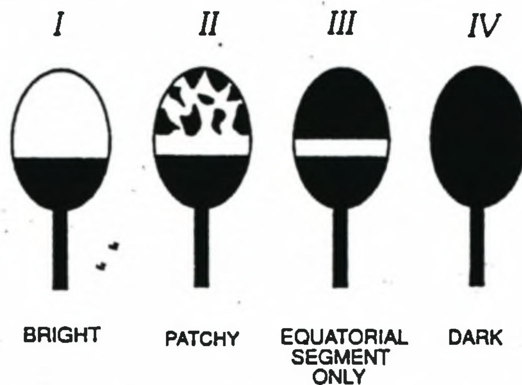

FIGURE 6: PSA lectin-labelling patterns of human spermatozoa.

I. *Whole acrosome labelling* denoting a reacted acrosomal membrane.

II. *Patchy acrosome labelling* suggestive of a transition stage where the outer acrosomal membrane is fenestrated, thus causing a "patchy" uneven stained acrosomal area.

III. *Equatorial segment only labelling* denoting an acrosome-reacted spermatozoon that has lost the outer acrosomal membrane over the anterior cap portion of the acrosome, but has retained the equatorial segment of the acrosome-intact.

IV. *No labelling* denoting a spermatozoon with no outer acrosomal membrane, either as a result of total acrosome loss or a morphological defect in which the spermatozoon had lacked an acrosome.

2.8 Assessment of sperm-zona binding

The current high incidence of human infertility caused by the male factor has promoted an intense search for reliable means to predict human sperm fertilizing potential in vivo and in vitro. The optimum human assay would ideally utilize living, fertilizable human oocytes. Ethical reasons, however, prevented scientists and physicians from performing such direct diagnostic functional assessments (Overstreet & Hembree, 1976). However, reliable and discriminating prognostic assays are needed to determine which infertile men are likely to achieve fertilization in vitro or in vivo. The HZA represents a direct biological test for the binding of a particular sperm sample (Burkman *et al.*, 1988; Franken *et al.*, 1989).

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For the HZA, fresh, excess pre-ovulatory oocytes (donated by couples attending the infertility clinic at Tygerberg Hospital) were microbisected into two identical hemizonae, using previously reported micromanipulation techniques (Burkman *et al.*, 1988; Franken *et al.*, 1989; Oehninger *et al.*, 1989). Two sperm populations are used to perform a single HZA (Fig. 7), namely, (i) control sperm population (sperm samples were obtained from proven fertile men who repeatedly showed normal zona binding capacity during previously testing); and (ii) test sperm population (sperm samples were obtained from men visiting the andrology laboratory at Tygerberg Hospital and who had indications for male factor infertility). Fifty microliter droplets (25 000 sperm total count) were used in the HZA, which was set up in 25mm plastic petri dishes. In each dish, 50µl control and 50µl test sperm droplets were separately placed. The hemizonae were removed from storage droplets, placed in separate sperm droplets, covered with mineral oil and incubated for 4 hours at 37°C in 5% CO_2 in air. After incubation, control and test hemizonae were removed and placed in 50µl droplets of culture medium. Loosely attached spermatozoa were then removed by vigorously pipetting in a micropipette and the number of tightly bound sperm cells on each hemizona was counted.

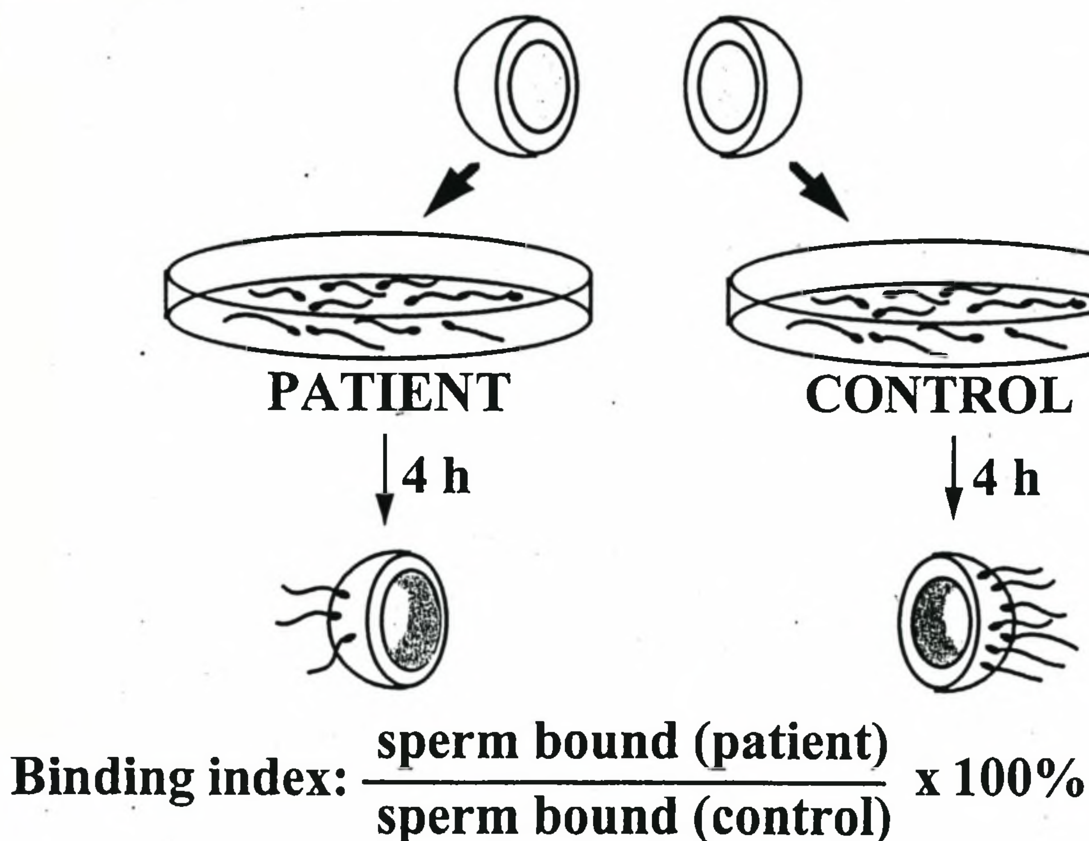

FIGURE 7: Flow diagram of the procedures for the HZA.

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CHAPTER 3

G-protein regulation of the solubilized human zona pellucida-mediated acrosome reaction and zona pellucida binding

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3.1 Abstract

Purpose: The study aimed to evaluate (i) the regulatory role of G_i-like protein during the acrosome reaction (AR) of normal sperm donors and (ii) the role of intact acrosomes during sperm-zona binding.

Methods: The acrosomal exocytosis of spermatozoa incubated with solubilized zona pellucida (ZP) at a final concentration of 1ZP/µl was compared with 10µ*M* A23187 and 30% (v/v) pooled HFF. Spermatozoa were incubated with 1, 10 and 100ng/ml pertussis toxin (PT) during capacitation to inactivate functionally the G_i-like protein. The sperm-zona binding potential of 100ng/ml PT-treated spermatozoa followed by exposure to 1ZP/µl, revealed significantly higher zona-bound spermatozoa compared to controls treated with 1ZP/µl only.

Results: PT treatment of spermatozoa did not affect sperm motility, however, inhibited the percentage AR induced by solubilized ZP. In contrast, the A23187 and HFF-induced ARs were not sensitive to PT treatment. PT inhibition of the ZP-induced AR occurred in a concentration-dependent manner with maximal effects observed at 100ng/ml PT.

Conclusions: In conclusion, it seems that PT-sensitive G_i-like protein in human spermatozoa plays an important regulatory role in the AR induced by the human ZP, and this underlines the importance of intact acrosomes during sperm-zona binding.

3.2 Introduction

The astounding success rates achieved by ICSI (Van Steirteghem *et al.*, 1993) emphasized the need to refine sperm functional evaluation. This is particularly true in cases of profound male factor infertility and contemporary andrology laboratories therefore should be able to select the most appropriate form of treatment for each couple, especially those diagnosed with male factor infertility (Oehninger *et al.*, 1997).

Precise timing of acrosomal response was the rationale for the development of the acrosome reaction ionophore challenge test (ARIC-test) (Cummins *et al.*, 1991; Tesarik, 1996). The ARIC-test as well as the concept of acrosomal inducibility (Henkel *et al.*, 1993) is a reliable predictive tool of sperm fertilizing

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ability as compared with tests that simply measure the frequency of spontaneous AR. The inducibility of the AR, *i.e.*, the difference between spontaneous and percentage acrosome-reacted spermatozoa after induction, correlates significantly with fertilization rates (Henkel *et al.*, 1993). The acrosome inducing activity of the ZP in both the intact and solubilized state has been illustrated to be powerful (Bielfeld *et al.*, 1994; Liu & Baker, 1994; Franken *et al.*, 1996).

The ZP, and specifically glycoprotein 3 (ZP3), is thought to be the primary zona protein involved in the initial sperm-egg recognition and mediation of the AR. A sperm-associated G protein of the G_i-type mediates the ZP3-induced AR in mouse spermatozoa (Endo *et al.*, 1988) and the ZP-induced AR in bovine spermatozoa (Florman *et al.*, 1989). This particular class of G proteins are substrates for PT-catalysed ADP-ribosylation and are functionally inactivated by such a covalent modification. G proteins play important intermediary roles as signal transducing elements in coupling many ligand-receptor interactions to intracellular second messenger cascades/ionic changes (Ross, 1989) and all mammalian spermatozoa studied thus far, including the human, contain G_i-like proteins (Kopf *et al.*, 1986).

Little is known about ZP-mediated sperm signal transduction in the human, due, for the most part, to an inability to obtain sufficient quantities of human ZP for experimental purposes. The human ZP has been shown to bind human spermatozoa and to induce the AR of spermatozoa (Cross *et al.*, 1988; Morales *et al.*, 1989). The present study aimed (i) to determine optimal exposure time of varying PT; (ii) to compare PT exposed spermatozoa's AR results, after induction with A23187, HFF and solubilized human ZP in parallel experiments; and (iii) to evaluate zona binding capacity of PT-treated sperm samples that were exposed to 1ZP/µl solubilized ZP.

3.3 Materials and methods

3.3.1 Preparation of sperm samples

Semen samples were obtained by masturbation after 2-3 days of sexual abstinence from normozoospermic fertile donors. Semen samples were analysed according to the World Health Organisation criteria (WHO, 1992) together with strict sperm morphology assessment (Kruger *et al.*, 1986). Motile sperm fractions were collected from samples using a slightly modified double-wash swim up technique (Franken *et al.*, 1996, 1997). Retrieved sperm samples were resuspended in HTF (Quinn *et al.*, 1985) supplemented with 3% BSA to a sperm concentration of 10×10^6 cells/ml. Before the onset of AR studies, sperm samples were

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allowed to capacitate at 37°C in 5% CO_2 for 3 hours in HTF-BSA. Prepared sperm samples were exposed to varying concentrations (1, 10 and 100ng/ml) of PT for 15, 30 and 60 minutes.

3.3.2 Preparation of solubilized zona pellucida

Oocytes were retrieved from post mortem derived ovarian material. Great care was taken to ensure that all legal, ethical and moral guidelines were adhered to at all time during oocyte collection. Oocytes were stored in a DMSO/sucrose solution at -196°C in liquid nitrogen (Hammit *et al.*, 1991). Twenty-four hours prior to each test, oocytes were removed from storage and thawed at 37°C. Retrieved oocytes were placed in 0.25*M* sucrose and 3% BSA in HTF. On the day of the experiment, 50 oocytes were placed in a plastic Eppendorf tube containing 3% BSA in HTF, centrifuged for 15 minutes at 1800 x*g*, after which the HTF was removed under microscopic vision, leaving only the 50 oocytes at the bottom of the tube. A total volume of 5µl of 10m*M* HCl was then added to the oocytes in the tube; solubilization of the ZP was microscopically observed and controlled. Ooplasma of all oocytes were left at the bottom of the Eppendorf tube; test samples included ZP-free oocytes. Following solubilization, 5µl of 10m*M* NaOH was added to the solubilized ZP to render a final zona volume of 10µl, containing 5ZP/µl. The final ZP concentration, after addition of spermatozoa, was 1ZP/µl.

3.3.3 Acrosome reaction studies

The procedure to determine the AR has been published in detail elsewhere (Cross *et al.*, 1988; Morales *et al.*, 1989). AR status were determined for PT-treated spermatozoa incubated in 5% CO_2 at 37°C for 15, 30 and 60 minutes, respectively, with the following: (i) 1ZP/µl (test); (ii) spontaneous (control); (iii) 10µ*M* A23187 (control); and (iv) pooled HFF (30%, v/v) (control). Blood-free follicular fluid was aspirated from mature follicles of females attending the assisted reproductive program. Spermatozoa from the different experiments described above were fixed and air-dried, after which the acrosomal status was determined using FITC-PSA (125µg/ml). A minimum of 200 spermatozoa was scored for each determination at the different time points.

3.3.4 Zona pellucida binding

Parallel with the acrosomal studies, spermatozoa (10×10^6 cells/ml; test) were pre-treated with 100ng/ml PT (60 minutes) before exposure to 1ZP/µl for 60 minutes. Control spermatozoa were simultaneously

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incubated in synthetic HTF prior to ZP exposure. Both test and control sperm droplets (50µL) were incubated under mineral oil for 30 minutes. Hemizonae were then added in a match-controlled fashion. HZAs were performed 10-fold and coincubation was for 4 hours. Following the coincubation period, hemizonae were removed and washed (5X) to strip the loosely attached spermatozoa from the hemizonae. Hemizonae were then evaluated while the number of spermatozoa tightly bound to the ZP was recorded for each test and matching control hemizona.

3.3.5 Statistical analysis

Sperm-zona binding results were expressed as the mean number of sperm bound to matching hemizonae that were used as tests and controls during the experiments. HZA results were compared using Student's paired *t*-test. The percentage acrosome-reacted spermatozoa were compared using Student's paired *t*-test for control and test samples.

3.4 Results

3.4.1 Pertussis toxin and the acrosome reaction

ARs induced either spontaneously (observed in the absence of ZP) or non-specifically (in the presence of A23187 and HFF), in contrast, were completely insensitive to PT treatment of the spermatozoa. Following exposure to 100ng/ml PT for 15, 30 and 60 minutes, the mean percentage of acrosome-reacted spermatozoa remains unchanged, namely, 47%, 51% and 50%, respectively. Similar results were observed in the presence of 30% (v/v) HFF which mediated AR among 30%, 29% and 27% of the spermatozoa following PT pre-treatment periods of 15, 30 and 60 minutes, respectively. The inhibitory effect of PT on the ZP-induced AR was dependent on the concentration and exposing time of PT. The maximum AR inhibition occurred after 60 minutes PT treatment, when only 14% spermatozoa were reported to be acrosome-reacted (Table 5).

3.4.2 Sperm-zona binding

Since the acrosome plays an important role during the binding and penetration of the ZP, zona-binding capacity of PT-treated sperm populations was recorded. PT-treated sperm populations exposed to 1ZP/µl for 60 minutes bound significantly more spermatozoa (mean±SD) to the ZP compared with the control

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spermatozoa that were incubated in a solution containing 1ZP/µl only, namely, 134.1±15 compared to 84.3±19 (P<0.001) (Table 6). The PT-treated sperm population therefore bound significantly more spermatozoa compared to the sperm population (control) that was exposed to 1ZP/µl only.

3.5 Discussion

Sperm-associated G protein has been shown to be involved during induced acrosomal exocytosis of different species (Lee *et al.*, 1992). The present results illustrate the possible regulatory effect of PT on the ZP-induced AR. Functional inactivation of G_i by PT inhibits downstream events leading to acrosomal exocytosis. Stimulation of spermatozoa with ZP depolarises sperm membrane potential. The ZP and specific ZP3 stimulation therefore activates a depolarization mechanism with the characteristics of a poorly selective cation channel. Pre-treatment of spermatozoa with PT prevents activation of the Ca^{2+}-selective channel by ZP3/ZP (Florman *et al.*, 1995). The results can be interpreted to support the idea that the ZP-induced AR is the physiologically relevant exocytotic event since it is the ZP-induced AR, and not the spontaneous or A23187 and HFF-induced AR, which appears to be mediated through a G protein-mediated signal transduction process.

Human spermatozoa were capacitated in the presence of PT in order to determine whether functional inactivation of sperm G_i affected the ability of the treated cells to undergo the AR. PT treatment of spermatozoa inhibited the ability of the cells to undergo acrosomal exocytosis in the presence of solubilized ZP. Fertile donor spermatozoa that were first exposed to 1, 10 and 100ng/ml PT concentrations for 15, 30 and 60 minutes; followed by a second incubation in 1ZP/ul for 60 minutes, showed increasing inhibition of the AR. Sperm-zona binding studies with PT-treated acrosome-intact spermatozoa, however, revealed significant higher numbers of spermatozoa firmly bound to the zona under controlled HZA conditions. It is interesting to note that a difference of 15% in the acrosome-reacted sperm population following treatment with solubilized ZP caused a significant decrease in the zona binding potential of the spermatozoa (Table 6). Despite the presence of 70% (350 000 sperm/hemizona) acrosome-intact sperm in the sample, significant less sperm were reported bound to the zona under HZA conditions. The inhibition of sperm-ZP binding by previous incubation with solubilized ZP may also be partly due to the occupation of ZP binding sites on still acrosome-intact spermatozoa. PT may moderate this effect not only by inhibiting the AR, but also by delaying sperm capacitation, leading to reduced availability of sperm plasma membrane binding sites for solubilized ZP. Comparing ZP binding ability of PT-treated and untreated spermatozoa with exposure to solubilized ZP during pilot studies tested the possibility. The mean number of PT-treated

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sperm versus untreated sperm that were zona-bound after coincubation was 118±12 and 124±17, respectively.

The results highlight the importance of intact acrosomes during tight zona binding and underline the possible regulatory effect of PT on the ZP-induced AR. Although it is generally accepted that the spermatozoa must be acrosome-reacted to complete penetration of the zona (Franken *et al.*, 1991), the exact site of the AR has not been defined and appears to differ between species. PT treatment of human spermatozoa does not affect the ability of spermatozoa to bind to structural intact human ZP. The results indicate the importance of intact acrosomes on the spermatozoa to ensure tight binding to the ZP, *i.e.*, those sperm populations with a decreased AR, namely, the PT-treated spermatozoa, bound significantly higher numbers of sperm during HZA conditions (Franken *et* al., 1996).

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TABLE 5

The influence of PT exposure on the ARs (mean±SD) mediated by ZP, HFF and A23187.

% Acrosome-reacted spermatozoa in triplicate experiments			
		Exposure time	
AR inducer	**15 minutes**	**30 minutes**	**60 minutes**
Culture medium	18±2	19±3	18[g]±3
1ZP/µl	18[h]±3	24±5	30[i]±3
		1ng/ml PT	
A23187	49±6	50±4	47±4
FF	31±7	30±7	28±9
ZP	30[a]±4	20±7	18[b]±3
		10ng/ml PT	
A23187	51±7	48±8	49±5
FF	29±7	32±9	30±4
ZP	30[c]±4	19±2	17[d]±1
		100ng/ml PT	
A23187	47±3	51±7	50±8
FF	30±4	29±3	27±7
ZP	19[e]±3	17±5	15[f]±3

g vs. i, P=0.001; g vs. h, P=0.001; g vs. b, g vs. d, g vs. f, not significant.

b vs. a, P=0.001; b vs. d, b vs. f, not significant.

c vs. d, P=0.001; d vs. f, not significant.

e vs. f, not significant.

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TABLE 6

Sperm-zona binding and AR results after PT treatment followed by exposure to solubilized ZP.

Tests: **Spermatozoa exposed to PT followed by treatment of 1ZP/µl solubilized zona**		**Control:** **Spermatozoa treated with 1ZP/µl solubilized zona only**	
% Acrosome-reacted sperm (*n*=3)	Mean (±SD) number zona-bound sperm (*n*=10)	% Acrosome-reacted sperm (*n*=3)	Mean (±SD) number zona-bound sperm (*n*=10)
15[a]±3	134.1[c]±15	30[b]±3	84.3[d]±19

a vs. b, P=0.0001; c vs. d, P=0.001.

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CHAPTER 4

Zona pellucida-induced acrosome reaction, sperm morphology and sperm-zona binding assessments among subfertile men

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4.1 Abstract

Purpose: The study aimed to evaluate the relationship between the zona pellucida-induced acrosome reaction (ZIAR) and (i) percentage normal spermatozoa as well as (ii) sperm-ZP binding potential among men referred for a routine semen analysis.

Methods: Semen samples of 164 consecutive men referred to the andrology laboratory for routine semen analysis were studied. Semen samples were analyzed using the new WHO standards (strict criteria). ZIAR was recorded with a lectin conjugated *Pisum Sativum* agglutinin microassay, while sperm-zona binding was evaluated with a standard HZA.

Results: Andrology patients were divided according to the percentage normal spermatozoa in the ejaculate, namely, <4% normal forms (*n*=71), 4-14% normal forms (*n*=73) and >14% normal forms (*n*=20). The mean (±SD) ZIAR data of the <4%, 4-14% and >14% groups were (9.6±0.6)%, (13.9±0.5)% and (15.0±1.1)%, respectively. The mean (±SD) ZIAR data of fertile control men was (26.6±1.4)% that differed significantly from the three andrology referral groups. Likewise, significant differences were recorded during the HZA, namely, 38.0% (<4% normal forms), 54.5% (4-14% normal forms) and 62.6% (>14% normal forms). Among the group with >14% normal forms, five cases had impaired ZIAR outcome (<15%). Three of these men had normal morphology and HZAs.

Conclusions: ZIAR testing should become part of the second level of male fertility investigations, *i.e.*, sperm functional testing, since 3 out of 20 (15%) of andrology referrals with normal semen parameters revealed an impaired AR response to solubilized ZP.

4.2 Introduction

Intracytoplasmic sperm injection (ICSI) has provided a unique technique to allow couples, diagnosed with severe male infertility (Van Steirteghem *et al.*, 1993), to achieve their reproductive goals (Oehninger, 1995). However, several questions obviously arise including (i) what are the diagnostic steps that we should use to direct infertile men to a specific therapeutic modality? and (ii) what are the current indications for ICSI? (Oehninger, 1995).

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Despite the questions surrounding the clinical importance of the semen analyses (McDonough, 1997), the andrologic investigation still relies on a thorough history and physical examination of the male partner (Oehninger, 1995). Additionally, a urological and endocrinological workup should be implemented as needed. The semen analysis therefore still remains the cornerstone of the diagnostic management (Oehninger *et al.*, 1991; Oehninger, 1995). A multistep diagnostic approach for the evaluation of the various structural, dynamic and functional sperm characteristics have been advocated by scientists and clinicians (Oehninger *et al.*, 1991). This approach has been the result of combined information derived from the basic and clinical areas of the andrology and reproductive endocrinology disciplines. It is our opinion that this diagnostic scheme should include a first level assessment of the "basic" semen analysis as outlined by the WHO (Oehninger *et al.*, 1992; Oehninger, 1995; WHO, 1999). The second level of approach should include functional testing of spermatozoa, *i.e.*, sperm-zona binding (Franken *et al.*, 1989; Oehninger *et al.*, 1992; Franken *et al.*, 1993), AR (Liu & Baker, 1994; Esterhuizen *et al.*, 2001) and chromatin packaging (Sakkas *et al.*, 1996).

Sperm morphology is regarded as possibly the most consistent sperm variable that appears to be related to IVF success (Kruger *et al.*, 1986; Liu & Baker, 1992; Ombelet *et al.*, 1995). This observation has therefore not only a very important clinical and diagnostic role to play in the structured management of infertile couples, but also serves as a reference point in many research projects that aim to establish the importance of a new diagnostic test (Franken *et al.*, 1989; Esterhuizen *et al.*, 2001).

The present study aimed to evaluate the relationship between the ZIAR and (i) percentage normal spermatozoa and (ii) sperm-zona binding potential among men referred for a routine semen analysis.

4.3 Materials & Methods

4.3.1 Spermatozoa

The semen samples of 164 consecutive men referred to the andrology laboratory at Tygerberg Hospital for routine semen analyses were additionally tested for sperm function. This included sperm-zona binding (HZA) and AR induced by solubilized human ZP (ZIAR). Samples were analyzed based on strict criteria suggested by the WHO's criteria (Menkveld *et al.*, 1990; WHO, 1992; Menkveld & Kruger, 1996; Menkveld *et al.*, 1996; WHO, 1999). Results of the semen analyses were kept blind to the diagnostic sperm laboratory personnel until AR and sperm-zona binding results were completed.

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4.3.2 Microassay for the evaluation of the acrosome reaction

During the initial stages of the work, semen samples from 11 fertile sperm donors were used to establish (i) the dose-response curve for ARs after stimulation with solubilized human ZP and (ii) the intertechnician and intratechnician and sample variation for sperm morphology and acrosome staining with PSA-FITC (see Chapter 2). Coefficient of variations for both intraassay and interassay and technician values were calculated by dividing the mean with standard deviation x100% for each observation. The interassay and intraassay as well as intertechnician and intratechnician coefficient of variation was <15% among the slides (Franken *et al.*, 2000b; Esterhuizen *et al.*, 2001).

Capacitated motile sperm fractions from the 164 men were incubated for 60 minutes with (i) HTF (spontaneous AR, control), and (ii) 0.6ZP/µl (ZIAR, test). Results were recorded as the difference between zona-induced acrosome-reacted and spontaneous acrosome-reacted sperm and expressed as percentage ZIAR.

For the microassay, 1µl of ZP solution (5ZP/µl) was aspirated into a Teflon pipette tip, fitted to a microsyringe with 1µl of sperm ($10x10^6$ sperm/ml, >90% motility), to render a final ZP concentration of 2.5ZP/µl (stock solution). The stock solution was stored at 4°C for a maximum period of 7 days. On each day of the experiment, 1µl volumes were removed from this solution to perform serial dilutions using HTF to equal a final zona concentration (after adding 1µl sperm) of 0.6ZP/µl.

Prior to aspiration into Teflon tips, all sperm-ZP suspensions were gently mixed in a well of a microtiter plate. To prevent evaporation from the Teflon tips, aspirating HTF droplets into both sides of the Teflon tip sealed off sperm-ZP suspensions. Each sperm-ZP suspension was separated from the HTF droplets by air bubbles on both sides. Monitoring of progressive motility for both AR techniques was manually performed on spotted slides. Sperm droplets were carefully placed on separate spots and immediately evaluated for percentage live sperm under inverted phase-contrast microscopy (Franken *et al.*, 2000a).

4.3.3 Sperm-zona binding

For the HZA, oocytes were microbisected into two identical hemizonae using previously reported micromanipulation techniques (Franken *et al.*, 1989). In each assay, matching hemizonae was separately incubated to a sperm concentration of $5x10^6$/ml from a fertile donor (control) and patient (test). After 4 hours of coincubation (at 37°C, in 5% CO_2 in air), hemizonae were rinsed in HTF by pipetting 5x with a

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finely drawn micropipette (100 µm inner diameter) to dislodge loosely attached sperm. HZA results were calculated as the absolute number of tightly bound sperm per hemizona and results were expressed as hemizonae indices (HZI, see Chapter 2).

4.3.4 Statistical analysis

Comparisons between normal sperm morphology, percentage acrosome-reacted sperm and sperm-zona binding data were done with Fisher's exact *t*-test and the Wilcoxon *t*-test. The association between percentage normal spermatozoa and percentage acrosome-reacted sperm were reported by using a correlation analysis. The discriminating power of sperm morphology and sperm-zona binding as a screening test for the identification of AR responsiveness was illustrated with the ROC curve analysis.

4.4 Results

The dose-response results recorded with varying dosages of solubilized human ZP and the AR data is represented in Table 7. Due to the scarcity of human material, a ZP concentration of 0.6ZP/µl was chosen for acrosome induction studies.

Sperm parameters and results from the functional assays, *i.e.*, AR data as well as the HZA data of the fertile control group and 164 andrology patients, are depicted in Table 8. A significant difference was recorded between the percentages of morphological normal spermatozoa among the fertile controls compared to that of the andrology patients (P=0.0001, Fisher's exact *t*-test). Furthermore, significant differences existed between ZIARs (P=0.001) and sperm-zona binding data (P=0.001) of the fertile and subfertile groups.

The andrology patients were further subdivided into three groups according to the percentage normal morphology present in the semen, *i.e.*, $\leq$4% (P-pattern, n=71), 4-14% (G-pattern, n=73), >14% (normal, n=20) (Table 9). Since we did not have fertilization rates to calculate cut-off values for the ZIAR results, the data was analyzed according to the distribution plots for the percentage ZIAR recorded among each morphological group (Fig. 8). Impairment ZIAR results were identified in cases where the values fell outside the lower 95% confidence interval (95% CI) of the group. For the morphological normal group (>14% normal forms) the lower 95% CI for the ZIAR was 12.7%, for G-patterns (4-14% normal forms) the lower 95% CI was 10% and for the P-patterns (<4% normal forms) the ZIAR was 6%. In the normal, G-pattern and P-pattern groups, 75% (15 out of 20), 76% (55 out of 73) and 59% (49 out of 71) men,

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respectively, had ZIAR results above the lower 95% CI of that group. Five cases (25%) among the normal group reported in Table 10 had impaired ZIAR results, *i.e.*, <12.7% ZIAR. The HZA results of three of the five cases were normal HZA (HZI>40%), while two men also had impaired sperm-zona binding (HZI<40%). The HZI is defined as a ratio between the number of control sperm bound to the zona. All 5 cases had >14% normal spermatozoa (Table 10).

4.4.1 Receiver Operator Characteristics curve analysis

In order to evaluate the relationship of the ZIAR results and percentage normal spermatozoa, the data were analyzed with the ROC curve analyses. ZIAR data were able to discriminate (sensitivity 60% and specificity 82%) between sperm populations with sperm morphology of >4% and <4% normal forms at a cut-off value for percentage ZIAR of 13%. The areas under the curve for ZIAR and HZI were 0.76 (95% CI, 0.67 to 0.82) and 0.80 (95% CI 0.72 to 0.86), respectively (Fig. 9). This implies that a randomly selected individual from the >4% normal spermatozoa group has a ZIAR value larger than that for a randomly chosen individual from a <4% morphology group in 76% of cases. Likewise, a randomly selected individual from the >4% normal spermatozoa group has a HZI value larger than that for a randomly chosen individual from a <4% morphology group in 80% of cases. The calculated cut-off values for ZIAR and HZI were 13% and 46%, respectively.

4.5 Discussion

Standard IVF requires good sperm function, particular sperm-zona binding and penetration that are essential for fertilization. With ICSI several sperm functions are not required for fertilization, especially those associated with sperm-ZP interaction. Couples with severe spermatozoa defects such as teratozoospermia can usually be identified by routine semen analysis (WHO, 1999) and ICSI is recommended for the first treatment. However, couples with unexplained infertility with normal semen analysis are usually treated with standard IVF. Studies have shown that between 10% (Esterhuizen *et al.*, 2001) to 25% (Liu *et al.*, 2001) of these couples may have a low ZIAR result and are at risk of zero or very low fertilization rates in standard IVF (Esterhuizen *et al.*, 2001). Although these couples can be treated with ICSI in the second cycle there is a high cost to the patients both financially and emotionally. Failed attempts can also decrease the confidence of the patient in the therapy and therefore reduce the chance of success during future attempts.

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Sperm morphology has been recognized as a clinical discriminator of male fertility potential (Kruger *et al.*, 1986). Likewise, in close correlation with the percentage of normal spermatozoa, sperm-ZP binding was described as an additional clinical important characteristic of spermatozoa (Franken *et al.*, 1989; Oehninger *et al.*, 1991).

Sperm functional information is important since it could assist in therapeutic choices, such as IVF or ICSI. The role of impaired ZIAR in a clinical set-up is crucial when reported among cases with apparently normal sperm characteristics. Five of the 20 cases (25%) with normozoospermic semen had a ZIAR value of <12% that is an indication of slight impairment of acrosomal response to the ZP. Esterhuizen *et al.* (2001) described among IVF couples two groups of patients, *i.e.*, ZIAR<15% and ZIAR>15% with mean fertilization rates of 49% and 79%, respectively. Although the present report does not include IVF results, we believe that the reported decrease in the percentage ZIAR, especially among the three men (cases 1, 2 and 3) with normal semen parameters and HZAs, should be regarded as a clinical warning.

In a selected patients population, ZIAR results can be used to indicate IVF failure in >90% of cases and patients could accordingly be referred to an ICSI program (Liu & Baker, 1997, 2000; Esterhuizen *et al.*, 2001). It is known that about 10% of patients repeatedly have zero or low fertilization with standard IVF. Although oocyte immaturity or abnormalities can contribute to fertilization failure, sperm defects are regarded as the most frequent contributors in cases where complete fertilization failure is reported (Liu & Baker, 1997; Liu *et al.*, 2001).

Present results and that reported by others (Esterhuizen *et al.*, 2001; Liu *et al.*, 2001), underline the importance of a multistep diagnostic approach to identify the specific cause of male factor infertility. When sperm morphology is used as a first-step clinical guideline of male factor infertility, investigations such as sperm-zona binding and acrosomal response to homologous ZP plays an important role during the diagnostic procedure (ESHRE, 1996). We suggest that ZIAR evaluation should not be part of the first level of clinical approach, but instead form part of the second level of the diagnostic scheme that includes testing of the functional capacity of spermatozoa. In conclusion, the implementation of acrosome assays using small volumes of human solubilized ZP (Liu & Baker, 1997), biologically active rhuZP3 (Barrat & Hornby, 1995) or active, synthetic ZP3 peptides (or analogs) (Hinsch *et al.*, 1994) will probably allow for the design of improved, physiologically oriented assays (Tesarik, 1996).

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TABLE 7

Dose-response results of percentage acrosome-reacted sperm mediated by varying concentrations of solubilized human ZP.

ZP concentration (ZP/µl)	% Acrosome-reacted sperm
Control	10.0±0.2[a]
0.30	14.8±0.3[b]
0.60	19.3±0.4[c]
1.25	25.1±0.4[d]
2.50	32.5±0.7[e]

Fisher's exact t-test: a vs. b, $P>0.5$; a vs. c, $P=0.001$; a vs. d, $P=0.001$; a vs. e, $P=0.001$.

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TABLE 8

Results of semen parameters from 164 andrology patients to determine ARs mediated by human ZP and sperm-zona binding capacity.

	Fertile controls (*n*=11)	**Andrology patients (*n*=164)**	***P* values (Fisher's exact *t*-test)**
Sperm concentration ($x10^6$/ml)	199.4±7.8	74.1±6.6	
Motile cells (%)	56.5±0.80	52.1±1.5	
Normal cells (%)	15.8±0.50[a]	5.5±0.1[e]	a vs. e, *P*=0.0001
Spontaneous AR (%)	10.3±0.55[b]	12.1±1.0[f]	b vs. f, *P*=0.0001
ZIAR (0.6ZP/µl) (%)	26.6±1.40[c]	11.9±0.5[g]	c vs. g, *P*=0.0001
HZA (%)	84.8±2.90[d]	48.3±1.9[h]	d vs. h, *P*=0.001

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TABLE 9

Results (mean±SEM) of ARs mediated by human ZP and sperm-zona binding capacity according to percentage normal spermatozoa.

	P-patterns (n=71)	**G-patterns (n=73)**	**Normal (n=20)**
Sperm concentration ($x10^6$/ml)	42.4±6.6	89.5±6.3	121.7±16.0
Normal cells (%)	2.2±0.1[a]	6.9±0.2[b]	14.5±0.1[c]
Motile cells (%)	46.0±1.9	55.0±1.2	58.5±2.2
Spontaneous acrosome (%)	12.2±0.3[d]	11.9±0.3[e]	11.9±0.6[f]
ZIAR (Mean±SEM) (%)	9.6±0.6[g]	13.9±0.5[h]	15.0±1.1[i]
ZIAR median (range) (%)	9.0 (2-23)[j]	14.0 (4-26)[k]	15.0 (6-26)[l]
HZI (%)	38.0±1.6[m]	54.5±2.2[n]	62.6±4.2[o]

Unpaired *t*-test:

a vs. b, $P \leq 0.0001$; a vs. c, $P \leq 0.0001$; b vs. c, $P < 0.0001$.

motile cells, $P > 0.05$.

d vs. e, $P \leq 0.003$; d vs. f, $P \leq 0.003$; e vs. f, $P \leq 0.002$.

g vs. h, $P \geq 0.05$; g vs. i, $P \geq 0.05$; h vs. i, $P \geq 0.05$.

j vs. k, $P < 0.0001$; j vs. l, $P \leq 0.001$; k vs. l, $P \geq 0.05$.

m vs. n, $P \leq 0.01$; m vs. o, $P \leq 0.001$; n vs. o, $P \geq 0.05$.

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TABLE 10

Results of HZAs and sperm morphology of five cases with impaired ZIAR.

Case	HZI (%)	ZIAR (%)	Morphology (% normal)
1	77	11	16
2	92	6	16
3	63	9	15
4	26	6	14
5	37	12	14

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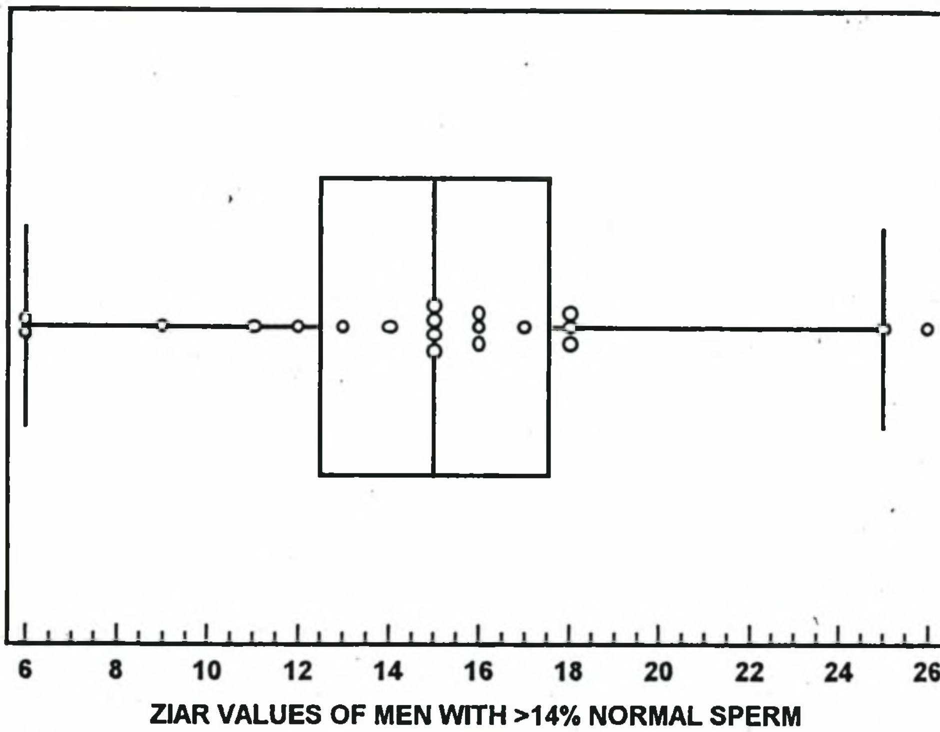

FIGURE 8: **Distribution of ZIAR data recorded for normozoospermic men.**

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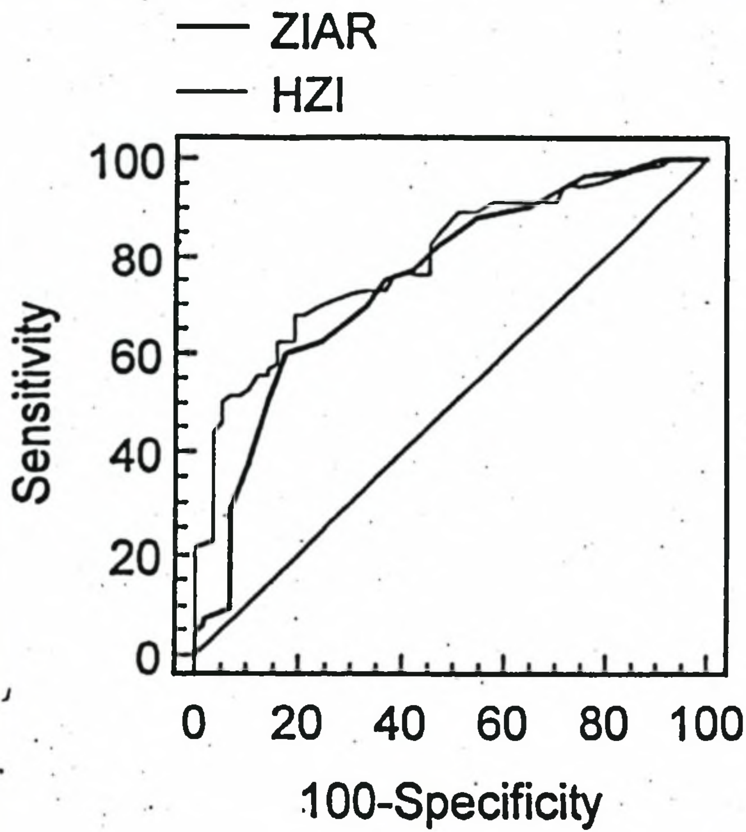

FIGURE 9: **ROC curve analysis for percentage ZIAR and HZI versus sperm morphology.**

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CHAPTER 5

Relationship between zona pellucida-induced acrosome reaction, sperm morphology, sperm-zona pellucida binding and in vitro fertilization

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5.1 Abstract

Objective: To evaluate the possible relationships between sperm morphology, acrosome responsiveness to solubilized human ZP and sperm-zona binding potential among (i) consecutive andrology referrals and (ii) randomly selected IVF cases.

Patients: Randomly selected couples consulting for infertility.

Interventions: AR response to solubilized human ZP was recorded.

Main outcome measures: We determined the difference in percentage of sperm that acrosome-reacted after exposure to solubilized ZP and spontaneous AR. The results were expressed as percentage ZIAR.

Results: Data were analyzed using correlation coefficients (r) and ROC curve analyses. The ROC curve analyses indicated ZIAR to be a sensitive indicator for fertilization failure during IVF therapy, with sensitivity and specificity of 81% and 75%, respectively. For andrology referrals, a positive and statistically significant correlation existed between ZIAR data and sperm morphology (r=0.65) and sperm-zona binding (r=0.57).

Conclusions: ZIAR results provide further information regarding dysfunctional sperm and can be used as an additional diagnostic test since results predicted fertilization failure during IVF treatment.

5.2 Introduction

The past 10-15 years have brought not only an explosion in the number of laboratory tests for human sperm functions, but certainly also the belief among many clinicians that sperm function testing is now irrelevant due to the advances in assisted reproduction and especially ICSI. Despite the advantages of ICSI therapy, the need for an accurate and inexpensive test that could be used to determine which men require ICSI and which do not will benefit both patient as well as clinician.

Sperm dysfunction is one of the most common single causes of infertility; yet remarkably, our knowledge of the cellular and biochemical basis for this condition is very limited (ESHRE, 1996; Oehninger *et al.*, 2000). Indeed, our understanding of the physiology of the normal human spermatozoon, let alone the dysfunctional spermatozoon, is incomplete. Contemporary andrology laboratories should provide two

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levels of andrologic investigations, namely, a standardized quality controlled semen analysis as a first level of approach, supported by the second level that consists of appropriate sperm functional assays. This will enable the clinician to select the most practical and cost-effective appropriate form of treatment for each couple, especially those diagnosed with male factor infertility (Oehninger *et al.*, 1992; Kruger & Coetzee, 1999).

The unique nature and composition of human semen is underlined by its heterogeneity in sperm morphology (Menkveld *et al.*, 1990; Menkveld & Kruger, 1996); this phenomenon is of paramount clinical importance as sperm morphology is regarded to be a significant prognostic factor for fertilization and pregnancy outcome in the IVF/GIFT/IUI settings (Katz *et al.*, 1982; Kruger *et al.*, 1986; Grow *et al.*, 1994; Toner *et al.*, 1995). Multiple regression analyses have demonstrated sperm morphology to be the most significant predictor of sperm-zona binding in the HZA when compared to other sperm variables from the original semen sample ($r=0.83$, $P=0.0001$). VCL and hyperactivation were the most significant predictors ($r=0.47$ and $r=0.46$, respectively; $P=0.001$) of successful zona binding after separation of the motile sperm fraction (Oehninger *et al.*, 1992).

If abnormalities are found during the basic investigation, the workup should progress to the examination of specific sperm functions. Four categories of diagnostic tests have been proposed as components of the second level of the diagnostic approach: (i) CASA, (ii) inducibility of the AR and (iii) bioassays that sequentially assess gamete interaction including sperm-ZP binding tests and (iv) sperm-hamster egg penetration assay (Oehninger *et al.*, 1992; ESHRE, 1996). Different laboratories have highlighted the diagnostic power of these tests and the WHO has incorporated them under the category of functional tests (WHO, 1999). Importantly, among the bioassays of sperm-egg interaction, it was recommended that because of the powerful evidence for prediction of both fertilization and its failure in the IVF setting, sperm-zona binding tests should be favored among the present available functional assays (ESHRE, 1996).

Important is the finding that in vitro the AR must be precisely timed, with respect to sperm-ZP interaction to ensure subsequent events, *i.e.*, zona penetration and oolemma fusion (Cummins *et al.*, 1991; Tesarik, 1996). During a previous study, Esterhuizen *et al.* (2001) described cases where the ZIAR was significantly correlated with IVF when the ZIAR was >15%. Impaired ZIAR results were identified where ZIAR was <15% and typically associated with fertilization failure in a group of patients with normal conventional semen analyses. These results are in close agreement with other reports (Liu & Baker, 1994) during which ten couples with long-standing infertility revealed a reduced frequency of acrosome-reacted

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spermatozoa bound to the ZP. That study suggested the existence of a zona-bound sperm population with a disordered AR, thus causing impaired fertility (Liu & Baker, 1994). Patients with a long history of idiopathic infertility with poor fertilization or complete fertilization failure should be tested for this defect using sperm-ZP interaction tests. Reduced ZIAR patients should be directed to ICSI rather than standard IVF (Liu & Baker, 1997; Esterhuizen *et al.*, 2001).

The present prospective study aimed to evaluate the possible relationships between sperm morphology, acrosome responsiveness to solubilized human ZP and sperm-zona binding potential among (i) andrology referrals with normozoospermic, teratozoospermic and severely teratozoospermic semen and (ii) IVF couples.

5.3 Materials & Methods

All patients signed informed consent forms after the study had received institutional review board approval. In this ongoing prospective study a total of 163 semen samples at Tygerberg Hospital were used to test acrosome responsiveness to solubilized human ZP (ZIAR) and sperm-zona binding capacity (HZA). The samples were collected from the following groups. During a pilot study, semen samples from 11 fertile sperm donors were used to determine the optimum solubilized ZP concentration to induce the AR. Also, samples were obtained from 122 consecutive men referred to the andrology laboratory for routine semen analysis. From couples undergoing IVF treatment, 30 sperm samples were obtained. Semen samples were analyzed according to the WHO guidelines together with strict sperm morphology assessment (Menkveld *et al.*, 1990; WHO, 1999). The diagnostic sperm laboratory was blinded to semen analyses results until the acrosome and sperm-zona binding results had been completed.

5.3.1 Assay for detection of the acrosome reaction

Capacitated motile sperm fractions were incubated for 60 minutes with HTF (Quinn *et al.*, 1985) and 1ZP/µl (induced AR, test). Results were recorded as the difference between zona-induced acrosome-reacted and spontaneous acrosome-reacted sperm and expressed as percentage ZIAR. From the results of the pilot study, a ZP concentration of 1ZP/µl was chosen as acrosome inducing concentration for the acrosomal studies on andrology patients.

During all acrosomal studies, 4µl of either prepared zona solution (induced AR) or HTF (negative control = spontaneous AR) or A23187 (positive control) was carefully placed in a 1ml plastic tube after which

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45µl of the motile sperm fraction containing $5x10^6$ cells/ml was added. The final zona concentration was 1ZP/µl. Tubes were sealed and placed at 37°C for 60 minutes. After incubation, sperm-zona suspensions were placed in a well of a spotted slide and evaluated for the percentage motile cells in each droplet. The droplets were then allowed to air-dry for 24 hours. Air-dried ethanol fixed sperm smears were stained with PSA-FITC stain after which 200 cells were evaluated for acrosome integrity (Liu *et al.*, 2001).

Before the study, technician and sample variations were recorded by establishing intraassay and interassay/technician coefficient of variations for PSA-FITC staining. Coefficients of variation for both intraassay and interassay and intratechnician and intertechnician values were calculated by dividing the mean with standard deviation x100% for each observation. The interassay and intraassay as well as intertechnician and intratechnician coefficient of variation was <15%. Results were discarded in cases where the coefficient of variation for assay and technician exceeded 15%. Acrosome responsiveness to different concentrations of solubilized human ZP was performed to determine the optimum zona concentration to mediate the AR (Esterhuizen *et al.*, 2001; Liu *et al.*, 2001).

5.3.2 Sperm-zona binding test

For the HZA, oocytes were microbisected into two identical hemizonae using previously reported micromanipulation techniques (Franken *et al.*, 1989). In each assay, matching hemizonae were separately incubated to a sperm concentration of $5x10^6$/ml from a fertile donor (control) and patient (test). After 4 hours of coincubation (at 37°C, in 5% CO_2 in air), hemizonae were rinsed in HTF by pipetting 5x with a finely drawn micropipette (100 µm inner diameter) to dislodge loosely attached sperm. The HZA results were calculated as the absolute number of tightly bound sperm per hemizona for test and control halves and results were expressed as the HZI, *i.e.*, test results divided by control results times 100.

5.3.3 Ovarian stimulation and fertilization outcome

Clomiphene citrate (Serophene, Serono, Johannesburg, South Africa) and hMG (Pergonal, Serono) were used for ovarian hyperstimulation while follicular growth monitoring was done by serial ultrasound measurements and serum luteinizing hormone determinations. hCG (10 000 IU, Profasi) was administered when the dominant follicle reached a diameter of >18mm and follicular aspiration performed 36 hours after hCG administration.

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In a previous study, we defined normal fertilization using normal gametes in the assisted reproduction program at Tygerberg Hospital (Franken *et al.*, 1993). During that study, the total fertilization rate (total number of preovulatory oocytes fertilized/total number of preovulatory oocytes inseminated) was (88.6±17)% (mean±SD) and the normal fertilization rate (total number of preovulatory oocytes with normal fertilization/total number of oocytes inseminated) was (81.3±22)% (mean±SD).

The minimum total fertilization rate (mean minus 2xSD) that can be considered normal in the Tygerberg program is 55% and the minimum normal fertilization rate is 37%. Due to the employment of corrective measurements during the present IVF cycles, the 55% fertilization rate was chosen as the cut-off value for normal and abnormal fertilization. During these measurements the sperm insemination concentration used per oocyte was determined by adjusting the sperm concentration according to the percentage normal spermatozoa present in ejaculates: 500 000 to $1x10^6$ cells (P-pattern, <4%) or 500 000 cells (G-pattern, 5-14%) or 100 000 cells (normal pattern, >14%).

5.3.4 Statistical analysis

Comparisons between normal sperm morphology, percentage ZIAR and sperm-zona binding data were done with Fisher's exact *t*-test and the Wilcoxon *t*-test. The associations between percentage normal spermatozoa, ZIAR and HZA results were investigated by correlation analysis. Sperm morphology and sperm-zona binding were used to determine cut-off values for AR responsiveness by means of ROC curve analysis with the semen samples obtained from the andrology laboratory. ROC curve analyses were employed to determine the role of ZIAR results on the fertilization rates reported during IVF treatment.

5.4 Results

The mean (±SEM) values of semen parameters for the fertile controls, andrology referrals and IVF cases are depicted in Table 11. Andrology referrals were subdivided into three groups according to the percentage normal morphology. The obtained data were analyzed using correlation coefficients and ROC curve analyses. Normal distributions of the ZIAR results were recorded and are depicted in Figures 10 and 11.

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5.4.1 Andrology referrals

5.4.1.1 Morphology

Among the 122 men tested, the mean percentage sperm with normal morphology (mean±SEM) was (2.5±0.1)%, (8.4±0.3)% and (15.5±0.8)%, for the samples <4% (*n*=50), 4-14% (*n*=63) and >14% (*n*=9), respectively, of sperm with normal morphology. A positive and significant correlation existed between the percentage sperm with normal morphology and (i) sperm-zona binding (r=0.68, *P*=0.0001) as well as (ii) ZIAR values (r=0.65, *P*=0.0001).

5.4.1.2 Sperm-zona binding

The HZI (mean±SEM) differed significantly among the three morphology groups, namely, (44.9±2.7)%, (59.6±2.5)% and (84.8±2.9)% for samples with <4%, 4-14% and >14% normal sperm, respectively. A positive and significant correlation (r=0.57, *P*=0.0001) existed between HZI and ZIAR values.

5.4.1.3 Acrosome reaction

The mean percentage acrosome-reacted sperm recorded after exposure to solubilized human ZP also showed significant differences: (13.4±0.6)%, (16.1±0.6)% and (22.8±0.9)% for morphology groups <4%, 4-14% and >14% sperm with normal morphology, respectively. The mean ZIAR value correlated with morphology (r=0.6, *P*=0.001) and HZI (r=68, *P*=0.0001). However, the mean percentage spontaneous acrosome-reacted sperm did not correlate with sperm morphology or HZI.

The severe teratozoospermic cases (Table 11) had not only impairment of sperm morphology [(2.5±0.1)% sperm with normal morphology], but also a decreased sperm count (34.6±4.9 x10^6 cells/ml) compared to the remaining two morphology groups (80.4±8 x10^6 cells/ml and 187.7±8.2 x10^6 cells/ml, respectively; Table 11) that revealed a relationship with the ZIAR values.

5.4.2 Receiver operator characteristics and ZIAR cut-off value

Using ROC curve analysis, the diagnostic accuracy and the ability for correct classification of subjects in teratozoospermic and normozoospermic subgroups were documented for the recorded ZIAR and HZI values. The men's acrosome responsiveness to solubilized human ZP and the sperm-zona binding

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capacity was analyzed in terms of diagnostic sensitivity, specificity, and positive and negative predictive value.

During the calculation of the percentage ZIAR cut-off value, we based our evaluation on the percentage normal sperm cells in a given ejaculate and a HZI of >37%, which both correlate positively and significantly with the IVF rates (Kruger *et al.*, 1986; Franken *et al.*, 1991). Combined ROC curve analyses were accordingly used to calculate at which point the percentage ZIAR discriminates significantly between HZI>37% and HZI<37%, and morphology values of >4% and <4%. In both cases, *i.e.*, normal morphology and HZI, the cut-off value for ZIAR was calculated at 16%.

For morphology, the sensitivity was 65% and specificity 86%, and for HZI the sensitivity was 70% and specificity 76%. The areas under the curve for ZIAR and HZI were 0.78 (95% CI 0.69-0.85) and 0.83 (95% CI 0.75-0.89), respectively. This implies that a randomly selected individual from the >4% normal spermatozoa group has a ZIAR value larger than that for a randomly chosen individual from a <4% morphology group in 78% of cases. Likewise, a randomly selected individual from the >4% normal spermatozoa group has a HZI value larger than that for a randomly chosen individual from a <4% morphology group in 83% of cases.

5.4.3 In vitro fertilization

The results of semen parameters, sperm-zona binding, AR and fertilization rates of the 30 couples undergoing IVF are presented in Table 11. The ROC curve analysis revealed the ZIAR to have a sensitivity and specificity of 100% and 60%, respectively, to predict the IVF fertilization rates of <50% and >50%. The cut-off for the ZIAR value was calculated at 19%. The area under the curve was 0.90, which implies that a randomly selected individual from the >50% fertilization group has a ZIAR value larger than that of a randomly chosen individual from the <50% fertilization group in 90% of cases. The interactive dot diagram separated for fertilization categories, *i.e.*, >50% (n=22) and <50% (n=8) with a cut-off value for ZIAR of 8%. Distribution of ZIAR results among the <50% and >50% fertilization groups are represented in Figures 12 and 13, respectively.

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5.5 Discussion

The prevalence of male infertility and the availability of new, highly successful therapeutic options make the testing of sperm functional competence mandatory. The identification of these tests followed the recommendations of the WHO (1999) and discussions of a consensus workshop on advanced diagnostic techniques organized by the ESHRE Andrology Special Interest Group (ESHRE, 1996). There is a real need for the optimization of predictive tests of sperm function. Although the components of the "basic" semen evaluation provide an approximate idea of the functional competence of the male gamete, a better prediction can be gained from validated sperm function assays. Severe teratozoospermic men showed an impaired ZIAR in the presence of solubilized ZP when compared to normozoospermic men [Table 11; (13.4±0.6)% versus (22.8±0.9)%, P=0.001].

In current clinical practice, treatment of patients by either standard IVF or ICSI in the first cycle is usually decided on semen analysis results. Standard IVF requires good sperm function, particular sperm-zona binding and penetration that are essential for fertilization. With ICSI, several sperm functions are not required for fertilization, especially those associated with sperm-zona interaction. Couples with severe spermatozoa defects such as teratozoospermia can be identified by routine semen analysis and ICSI is recommended for the first treatment. On the other hand, couples with unexplained infertility with normal semen analysis are usually treated with standard IVF. Previous studies have shown that between 10% and 25% (Esterhuizen *et al.*, 2001; Liu *et al.*, 2001) of these couples may have low ZIAR and are at risk of zero or very low fertilization rates in standard IVF. Although these cases can be treated with ICSI in the second cycle, there is a high cost to the patients both financially and emotionally. Failed attempts can also decrease the confidence of the patient in the therapy and therefore reduce the chances of success during future attempts.

Previous reports described the implementation and diagnostic value of the ZIAR among highly selected patient population (Esterhuizen *et al.*, 2001; Liu *et al.*, 2001). The results of that study indicated that the diagnostic value of sperm functional assays could only be appreciated if the specific assay has been implemented within a suitable patient population. The importance of a suitable sperm diagnostic test lies not only in its discriminative power to predict fertilization failure but also to assist clinicians in the choice of therapeutic approaches. In previous studies, ZIAR results could be used to indicate IVF failure in >90% of cases and patients could accordingly be referred to an ICSI program (Esterhuizen *et al.*, 2001).

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In close agreement with previous reports (Esterhuizen *et al.*, 2001; Liu *et al.*, 2001), the cut-off value for ZIAR seems to be close to 15%. Esterhuizen *et al.* (2001) calculated a cut-off value for ZIAR at 15% during a previous study of only men with non-male factor infertility with repeated poor performance, or no fertilization no known female factors. In the present study, the cut-off value was recorded at a ZIAR value of 16% to discriminate between normal and abnormal sperm-zona binding. A ZIAR value of 16% was also recorded to predict normal morphology classification and sperm-zona binding capacity. ROC curve analysis of the present IVF data revealed the ZIAR data to have powerful discriminating power as far as IVF success or failure is concerned (sensitivity = 100% and specificity = 60%).

The cases where >50% fertilization rates were reported (Table 11) had a mean (±SD) percentage ZIAR of (13.5±0.9)%; although this is lower than the cut-off value of 16%, it is still a significant difference from the cases where <50% fertilization was reported. The IVF population in this study was a randomly selected population that included men with <14% normal forms. The mean (±SD) percentage normal sperm in the >50% fertilization group was (6.1±0.6)% and this impaired morphology value can be used as an explanation why the >50% fertilization group had a percentage ZIAR below the cut-off value of 16%. Previous studies (Esterhuizen *et al.*, 2001) indicated that patients with poor IVF results (<30% mature oocytes fertilized) obtained better fertilization rates during an ICSI cycle. The IVF rates recorded for 16 couples were significantly lower compared to those obtained for the same group during rescued ICSI therapy.

The ZIAR is able to identify a sperm population that has normal sperm parameters and normal sperm-zona binding potential but with dysfunctional acrosome responsiveness to solubilized human ZP. Thus, ZIAR test results can be clinically used to distinguish between couples that will benefit from IVF treatment and which may require ICSI therapy.

The fundamental utility of the ZIAR test stems from its being a functional bioassay of sperm performance in relation to the human ZP. Together with tight binding to the ZP, the ZIAR potential of a sperm population can be seen as a requisite for IVF. The ZIAR results provide useful discrimination between men capable of achieving fertilization in vitro versus those who are unlikely to be successful. The results can become a valuable tool in the diagnostic scheme of the consulting clinician, because the outcome of the test points to IVF or ICSI as the most effective clinical option. In conclusion, the implementation of acrosome assays using small volumes of human solubilized ZP (Franken *et al.*, 2000), biologically active rhuZP3 (Barrat & Hornby, 1995; Dong *et al.*, 2001) or active, synthetic ZP3 peptides (or analogs) (Hinsch

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et al., 1994), will probably allow for the design of improved, readably available and physiologically oriented assays.

5.6 References

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TABLE 11

The mean (±SEM) results for sperm parameters, ZIAR and HZI values recorded for fertile sperm donors, andrology referrals and IVF cases.

		Andrology referrals			IVF cases	
	Sperm donors	<4% normal	4-14% normal	>14% normal	>50% fertilization	<50% fertilization
n	11	50	63	9	22	8
Sperm concentration ($x10^6$/ml)	199.4 ±7.8	34.6 ±4.9	80.4 ±8.0	187.7 ±8.2	69.9 ±6.9	69.1 ±19.8
Morphology (%)	15.8 ±0.9[a]	2.5 ±0.1[b]	8.4 ±0.3[c]	15.5 ±0.8[d]	6.1 ±0.6[e]	5.8 ±1.3[f]
HZI (%)	84.8 ±3.0[g]	44.9 ±2.7[h]	59.6 ±2.5[i]	84.8 ±2.9[j]	55.8 ±4.3[k]	52.0 ±9.9[l]
Spontaneous AR (%)	10.9 ±1.8	13.0 ±0.4	13.4 ±0.3	11.9 ±0.4	12.4 ±0.2	14.3 ±0.1
% ZIAR (mean±SEM)	26.6 ±2.0[m]	13.4 ±0.6[n]	16.1 ±0.6[o]	22.8 ±0.9[p]	13.5 ±0.9[q]	7.1 ±1.1[r]
% ZIAR median (range)	25.4 (9-33)	13.5 (6-23)	16.0 (8-26)	22.0 (6-34)	11.0 (9-23)	5.5 (4-12)
n (oocytes)					116/138[s] (84%)	12/33[t] (36%)

Fisher's exact *t*-test:

a vs. b, *P*=0.001; a vs. c, *P*=0.001; a vs. d, *P*=not significant; a vs. e, *P*=0.01; a vs. f, *P*=0.01; b vs. c, *P*=0.001; b vs. d, *P*=0.001; b vs. e, *P*=0.01; b vs. f, *P*=0.01; c vs. d, *P*=0.01; c vs. e, *P*=not significant; c vs. f, *P*=not significant; g vs. h, *P*=0.001; g vs. i, *P*=0.01; g vs. j, *P*=not significant; g vs. k, *P*=0.01; g vs. l, *P*=0.01; h vs. i, *P*=not significant; h vs. j, *P*=0.01; h vs. k, *P*=not significant; h vs. l, *P*=not significant; m vs. n, *P*=0.01; m vs. o, *P*=0.01; m vs. p, *P*=not significant; m vs. q, *P*=0.01; m vs. r, *P*=0.001; n vs. o, *P*=not significant; n vs. p, *P*=0.01; n vs. q, *P*=not significant; n vs. r, *P*=0.01; o vs. p, *P*=not significant; o vs. q, *P*=not significant; o vs. r, *P*= 0.01; s vs. t, *P*=0.001.

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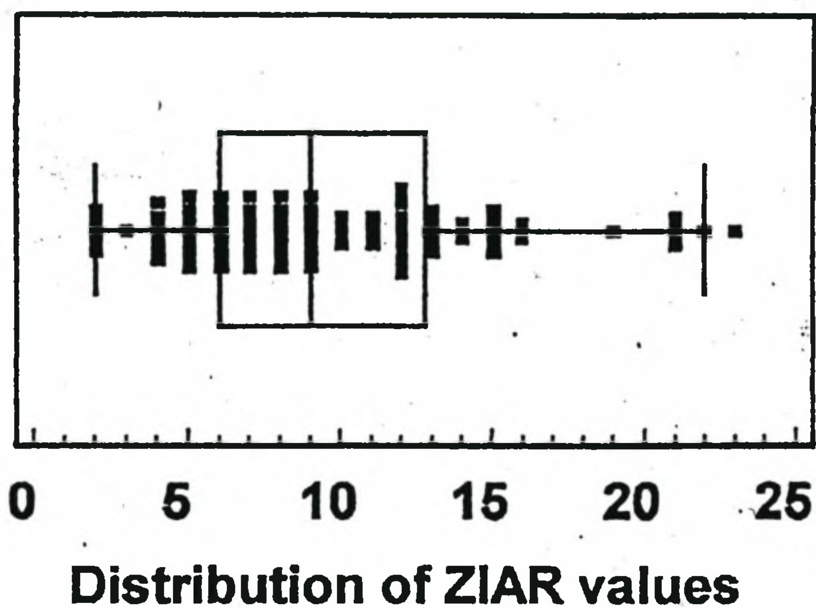

FIGURE 10: **Distribution recorded for ZIAR results among andrology referrals with <4% morphologically normal sperm in the ejaculate.**

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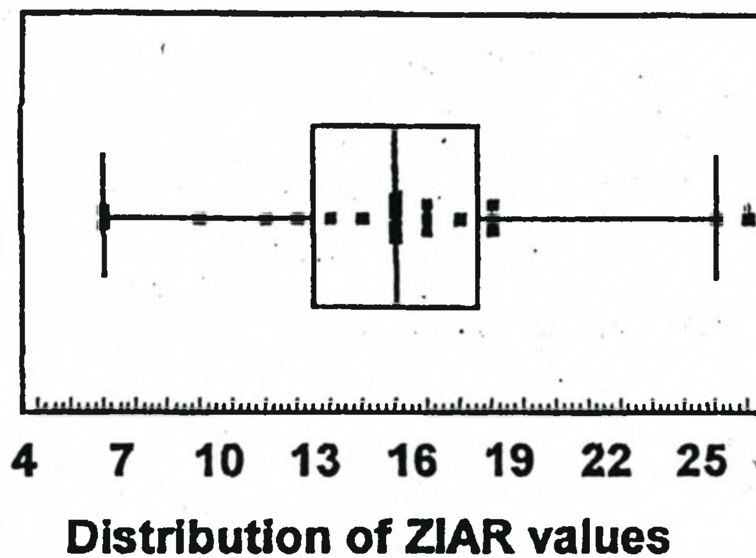

FIGURE 11: Distribution recorded for ZIAR results among andrology referrals with >14% morphologically normal sperm in the ejaculate.

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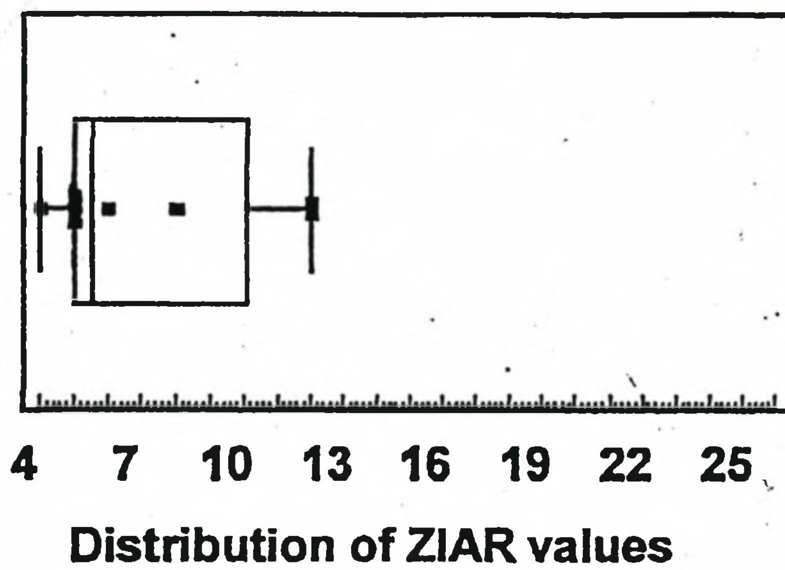

FIGURE 12: **Distribution of ZIAR results among IVF cases with <50% of oocytes fertilized.**

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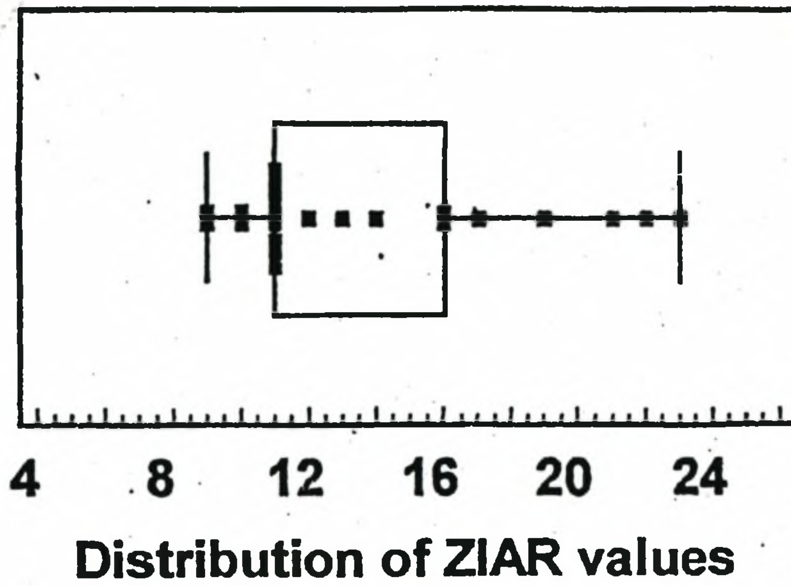

FIGURE 13: **Distribution of ZIAR results among IVF cases with >50% of oocytes fertilized.**

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CHAPTER 6

Hyperactivation of human spermatozoa following exposure to homogenous zona pellucida

6.1 Abstract

Purpose: The study aimed to evaluate changes in the sperm motion characteristics and the occurrence of hyperactivated motility after exposure to solubilized zona pellucida (ZP) among andrology referrals.

Methods: Semen samples of 64 consecutive men referred to the andrology laboratory for routine semen analysis were analyzed using the new WHO standards (strict criteria). Sperm motility/kinematics were determined with the HTM-IVOS analyzer.

Results: Andrology patients were divided according to the percentage normal spermatozoa in the ejaculate, namely, P-pattern, *i.e.*, <4% normal forms (n=27), G-pattern, *i.e.*, 4-14% normal forms (n=31) and normal group, *i.e.*, >14% normal forms (n=6). The mean percentage normal spermatozoa were (16.0±1.4)%, (8.0±1.7)% and (2.8±1.3)%, respectively, for normozoospermic, G and P-pattern men. Human zona-induced acrosome reaction (ZIAR) data of the normozoospermic, G and P-pattern groups were (25.0±2.3)%, (18.0±2.0)% and (14.1±0.6)%, respectively. Likewise, significant differences were recorded during the hemizona assay (HZA), namely, (41.1±4.6)% (P-patterns), (60.9±4.7)% (G-patterns) and (77.0±6.2)% (normal group). When divided into the three morphology groups, the number of spermatozoa with hyperactivated movement were significant higher in the normozoospermic group compared to men with G and P-patterns, namely, 7.5% versus 3.56% and 2.39% for G and P-patterns, respectively ($P<0.0001$).

Conclusions: Solubilized human ZP induces hyperactivated motility among sperm populations that have been capacitated under laboratory conditions. Normozoospermic semen samples have a higher hyperactivation capacity compared to teratozoospermic semen.

6.2 Introduction

Hyperactivation can be described as a pattern of sperm movement observed at the site of fertilization in mammals. It may be critical to fertilization success, because it increases the ability of spermatozoa to move through the female genital tract and, finally, to bind and penetrate the ZP of the oocyte. The movement of hyperactivated spermatozoa appears different under different physical conditions and in different species, but basically it involves an increase in flagellar bend amplitude and beat asymmetry (Ho & Suarez, 2001). The identification of human sperm hyperactivated motility has potential importance in

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sperm function tests, as well as in quality control assays and in reproductive toxicology investigations (Mortimer, 1997).

It is believed that there is a signal or signals to initiate hyperactivation at the appropriate time; and there is some evidence that the source of the signal might be follicular fluid, yet spermatozoa are known to hyperactivate before ovulation would release the fluid into the oviduct (Ho & Suarez, 2001; Peedicayil *et al.*, 1997). Although the signal transduction cascade regulating hyperactivation remains to be described completely, it is clear that calcium ions interact with the axoneme of the flagellum to switch on hyperactivation (Ho & Suarez, 2001; Suarez & Ho, 2003).

The process may also involve increases in intracellular cAMP, which at least is required to support motility in general. Although hyperactivation usually occurs during capacitation, the two events are regulated by different pathways. Bajpai *et al.* (2003) showed tyrosine phosphorylation can be associated with capacitation and can be suggested as a regulator of sperm movement, especially characterizing hyperactivation.

Improvement in sperm motility and motion characteristics after exposure to a wide variety of stimulants has been described in great length. Compounds such as caffeine, pentoxifylline, 2-deoxyadenosine, cyclic adenosine monophosphate (cAMP), relaxin, adenosine, kallikrein and calcium were compared for their ability to stimulate the motility of fresh and cryopreserved spermatozoa (Hammit *et al.*, 1989).

Furthermore, data provided evidence covering various studies on the use of the phosphodiesterase inhibitor, pentoxifylline (PF), in the sperm preparation for procedures in assisted reproduction (Yovich, 1993). Significant improvements have been shown in the fertilization rate of oocytes along with a reduced risk of failed fertilization cycles utilizing oligo-/asthenozoospermic semen samples. PF has proven effects on sperm motility, increasing the proportion of hyperactivated spermatozoa. It can also enhance the acrosome reaction and this may be the more relevant function for clinical prediction (Yovich, 1993).

There is a further action as a suppressor or scavenger of reactive oxygen species, although higher concentrations than that in current clinical use may be required to optimize this effect. PF should be washed out of the sample used for insemination to avoid inhibiting the completion of oocyte maturation (Yovich, 1993). Incubation with PF significantly enhanced sperm motility ($P<0.05$) and viability without affecting membrane integrity ($P<0.05$). Spermatozoa incubated with sildenafil and PF from both normal

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donors and infertile patients demonstrated no significant change in sperm penetration assays from respective controls (Yovich, 1993).

During previous studies, the role and influence of solubilized ZP on specific sperm functions, *i.e.*, sperm-zona binding (Franken *et al.*, 1996b) and the acrosome reaction (Esterhuizen *et al.*, 2001), were recorded. These studies aimed to characterize the effects of the physiological, homologous agonists of the acrosome reaction, *i.e.*, the ZP and progesterone/follicular fluid (Schuffner *et al.*, 2002), on human spermatozoa. Highly motile spermatozoa from fertile donors were exposed to the agonists in a microassay for the acrosomal status (Franken *et al.*, 2000). Progesterone and human follicular fluid exerted a priming, time-dependent effect on the ZIAR. These studies demonstrated that progesterone and follicular fluid exert a priming effect on the ZIAR (Schuffner *et al.*, 2002).

The present study aimed to record the influence of solubilized ZP on (i) the prevalence of hyperactivation and motion characteristics changes and (ii) sperm-zona binding and the ZIAR among sperm morphological groups, *i.e.*, normal, G and P-patterns (Kruger *et al.*, 1988; Menkveld *et al.*, 1990), among andrology referrals.

6.3 Materials and Methods

A total of 64 semen samples of consecutive men referred to the andrology laboratory at Tygerberg Hospital for routine semen analysis were analyzed according to the WHO guidelines (WHO, 1999) together with strict sperm morphology assessment (Menkveld *et al.*, 1990). Sperm motility/kinematics were determined with the HTM-IVOS analyzer (Hamilton-Thorne Research Inc., Beverley, MA, USA) with standard set-up parameters: 30 frames/60 Hz; minimum contrast, 80; minimum cell size, 2; minimum static contrast, 30; low VAP cut-off, 5 μm/s; low VSL cut-off, 11 μm/s; head size, non-motile, 3; head intensity, non-motile, 160; static head size, 1.01-2.91; static head intensity, 0.60-1.40; slow cells, non-motile; magnification, 2.01; and temperature, 37°C. The following parameters were evaluated: sperm concentration; motile and progressively motile concentrations; percentage motile and progressively motile; VAP; VSL; VCL; ALH; BCF; STR; LIN; and hyperactivation. Motion characteristics were recorded in all samples using 10 randomly selected microscopic fields.

The semen samples were also used to test acrosome responsiveness to solubilized human ZP (ZIAR) and sperm-zona binding capacity (HZA).

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6.3.1 Hyperactivation

The proportion of hyperactivated spermatozoa in each treatment was determined using the SORT function of the computer-assisted semen analysis (CASA) instrument. To be classified as hyperactivated, a trajectory had to meet all of the 60 Hz SORT criteria, *i.e.*, VCL≥150μm/s, LIN≤50% and ALH≥7μm (Mortimer *et al.*, 1998). In order to obtain standardization results from CASA to allow comparison between laboratories and quality control, it is obviously important that instruments are operated under standardized conditions.

6.3.2 Inducibility of the acrosome reaction

Capacitated motile sperm fractions were incubated for 30 minutes at 37°C in 5% CO_2 in air with human tubal fluid medium (HTF) and 1ZP/μl (ZIAR, test). Results were recorded as the difference between zona-induced acrosome-reacted and spontaneous acrosome-reacted spermatozoa and expressed as percentage ZIAR. During a pilot study, semen samples from fertile sperm donors were used to determine the optimum solubilized ZP concentration of 1ZP/μl as acrosome inducing concentration for the acrosomal studies on andrology patients. Acrosomal status was determined following procedures previously described (Cross *et al.*, 1988; Morales *et al.*, 1989). The fixed spermatozoa were air-dried, after which the acrosomal status of spermatozoa capable of excluding the Hoechst dye was determined, using fluorescenated *Pisum Sativum* agglutinin (125μg/ml). A minimum of 200 spermatozoa was scored for each determination at the different time points. The following staining patterns were evaluated as acrosome-reacted spermatozoa: (i) patchy staining on acrosomal region; (ii) distinct staining in the equatorial region occurring as an equatorial bar; and (iii) no staining observed over the entire sperm surface.

6.3.3 Sperm-zona binding test

For the HZA, oocytes were microbisected into two identical hemizonae using previously reported micromanipulation techniques (Franken *et al.*, 1989). In each assay, matching hemizonae were separately incubated to a sperm concentration of $5x10^6$/ml from a fertile donor (control) and patient (test). After 4 hours of coincubation (at 37°C, in 5% CO_2 in air), hemizonae were rinsed in HTF by pipetting 5x with a finely drawn micropipette (100 μm inner diameter) to dislodge loosely attached spermatozoa. The HZA results were calculated as the absolute number of tightly bound spermatozoa per hemizona for test and control halves and results were expressed as the HZI, *i.e.*, test results divided by control results times 100.

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6.3.4 Statistical analysis

Semen parameters, *i.e.*, sperm concentration, motility, morphology, sperm-zona binding and ZIAR were compared using the Student's *t*-test. The differences between the hyperactivated spermatozoa were calculated using comparisons between percentages *t*-test (*Medcalc Statistics in Medicine*, 1995). Calculations between hyperactivated sperm populations were performed using the comparisons between proportions *t* test.

6.4 Results

Semen samples were divided according to the percentage normal spermatozoa in the ejaculate, namely, <4% normal forms (*n*=27), 4-14% normal forms (*n*=31) and >14% normal forms (*n*=6). The mean (±SD) values for semen parameters, percentage ZIAR, % sperm-zona binding (HZI) and percentage hyperactivated spermatozoa (%HA) are depicted in Table 12. The mean (±SD) percentage normal spermatozoa were (2.8±1.3)%, (8.0±1.7)% and (16.0±1.4)%, respectively, among the P-pattern, G-pattern and normal morphologic groups. The mean (±SD) for ZIAR data of the P-pattern, G-pattern and normal morphologic groups were (14.1±0.6)%, (18.0±2.0)% and (25.0±2.3)%, respectively. Likewise, significant differences were recorded during the HZI, namely, (41.1±4.6)% (P-patterns), (60.9±4.7)% (G-patterns) and (77.0±6.2)% (normal patterns). The mean percentage spermatozoa showing hyperactivation after exposure to ZP were 2.39% (P-patterns), 3.56% (G-patterns) and 7.5% (normal patterns). Significant differences were also calculated between the three groups as far as sperm concentration and percentage motile spermatozoa are concerned. The spontaneous acrosome reaction values, however, did not differ between the groups. Sperm morphology correlated positively (r=0.93) and significantly (P<0.0001) with the ZIAR test among the 64 patients. Sperm-zona binding, as evaluated by the HZA and expressed as the hemizona index, correlated positively (r=0.92) and significantly (P<0.0001) with the percentage normal spermatozoa. Likewise, a positive (r=0.88) and significant (P<0.001) correlation was calculated between the ZIAR and HZI values.

The percentage spermatozoa showing hyperactivated motility differed significantly (P<0.001) among the three morphology groups in the ejaculates, after 3 hours capacitation as well as after ZP-exposure (Table 13). The percentage hyperactivated spermatozoa recorded for the normal morphology group increased from 1.44% in the ejaculates to 3.08% (2-fold increase, P<0.0001) after 3 hours capacitation and to 7.5% (5-fold increase, P<0.0001) after ZP-exposure. The G-pattern men had 1.04% hyperactivated spermatozoa in the ejaculates, 1.40% in the capacitated state and 3.56% (3-fold increase, P<0.0001)

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among the zona-exposed populations. Similarly, P-pattern semen had 0.44% hyperactivated spermatozoa in the ejaculates, 1.14% (3-fold increase, $P<0.0001$) in the capacitated state and 2.39% (6-fold increase, $P<0.0001$) among the zona-treated spermatozoa. In all cases, exposure to solubilized ZP caused a 4 to 6-fold increase in the percentage spermatozoa showing hyperactivated motility.

The mean (±SD) values for motion characteristics of hyperactivated spermatozoa among men with normal, G and P-pattern sperm morphology as recorded in ejaculates, after 3 hours capacitation and after exposure to solubilized ZP, are depicted in Table 14. The mean (±SD) values for motion characteristics of the non-hyperactivated spermatozoa population as recorded in the ejaculates, after 3 hours capacitation and following exposure to human ZP, are depicted in Table 15. The mean values for the motion characteristics, *i.e.*, VAP, VSL, VCL, ALH, BCF, STR and LIN, differ between ejaculates, capacitated and zona-exposed spermatozoa.

6.5 Discussion

Interaction between spermatozoa and the ZP is essential for fertilization. This biological step reflects the successful completion of multiple sperm functions, including the acquisition of capacitation, recognition and binding to specific ZP receptors, and induction of the physiological acrosome reaction (Oehninger 1992; Liu & Baker, 2000). Although diagnostic problems make it difficult to establish the extent of the male partner's contribution with certainty, a number of studies suggest that the male partner represents the most common single defined cause of infertility. Male related disorders are probably present in up to 40% of childless couples (Irvine, 1998). The suggested stratification approach to male infertility diagnostics (Oehninger *et al.*, 1997) has led to the development of bioassays that examine the various dynamic properties of the spermatozoon. Oehninger *et al.* (2000) suggested these tests should include (i) maturation and capacitation (Fraser, 1995); (ii) interaction with female tract components (Aitken, 1997); (iii) interaction with oocytes and surrounding cells (Liu *et al.*, 1988); and (iv) interaction with the ooplasma (Burkman *et al.*, 1988; Oehninger, 1992).

Clinically, the role of CASA has been difficult to define, since poor or no correlations could be found during in vitro fertilization treatment cycles (Oehninger *et al.*, 2000). Our understanding of the physiology of the normal human spermatozoon, let alone the dysfunctional spermatozoa, and its functions is limited. Therefore, basic studies investigating the fundamental mechanisms of sperm motion characteristics should be undertaken. The endpoint of the sperm capacitation process has been defined in terms of the acrosome reaction and hyperactivated motility (Fraser, 1995; Aitken, 1997). These fundamental studies should include

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understanding the role of physiological components such as follicular fluid, oviductal secretions and ZP on motility changes observed during the motion characteristic analyses of spermatozoa.

Cross *et al.* (1988) were the first to report that treatment of human spermatozoa in suspension with acid-disaggregated human ZP (2 to 4 ZP/μL) increased the incidence of acrosome-reacted spermatozoa. However, relatively few studies have addressed the role of the physiologic, homologous inducer of the acrosome reaction, the ZP, in human spermatozoa. This is probably due to the difficulty in obtaining human material (oocytes) to perform such experiments. Studies by Franken *et al.* (1996a) showed a dose-dependent effect of solubilized human ZP on the acrosome reaction in the range of 0.25 to 1 ZP/μL and also confirmed the involvement of G_i-proteins during the ZIAR of human spermatozoa.

The present study was carried out to assess ZP-induced changes in motility kinematics of human spermatozoa by a computer-aided sperm analyzer (Hamilton-Thorne Research; HTM-IVOS) and determine onset of hyperactivation and the acrosome reaction in ZP-treated spermatozoa. Semen samples from men classified in normal, G and P-pattern morphology groups were evaluated to determine differences in spermatozoal functions such as hyperactivation, sperm-zona binding, the acrosome reaction and spermatozoal motility characteristics. The hyperactivated spermatozoa in the three groups could be visualized on the monitor of the HTM-IVOS and they exhibited 'circling', 'thrashing', 'starspin' and 'helical' motility patterns.

Comparisons between the percentage hyperactivated spermatozoa in the ejaculates, after capacitation and after zona-exposure, revealed the morphology normal group, G-pattern samples, as well as P-pattern samples had a significant increase in the percentage hyperactivated spermatozoa after capacitation and zona-exposure compared to the ejaculates. The morphology normal group seems to be functional more competent compared to the P and G-pattern samples, since significant differences were found for the percentage hyperactivated spermatozoa, sperm-zona binding and ZIAR (Table 12). Sperm-zona binding and the ZIAR data showed a significant and positive correlation with the percentage normal spermatozoa. Sperm morphology reflects more than the morphometrical configurations of the “normal” cell, since it also correlates with specific functional aspects of the spermatozoon.

The present studies indicate that a normal spermatozoon not only has an enhanced acrosome reaction responsiveness when exposed to solubilized ZP, but it also show higher percentages of hyperactivated spermatozoa after ZP stimulation. Although the actual mechanism of ZP-induced hyperactivation is not known, it might be close correlated with the occurrence of the ZIAR. Tyrosine phosphorylation has

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recently been associated with capacitation and suggested as a regulator of sperm movement, especially characterizing hyperactivation (Bajpai & Doncel, 2003; Aquila *et al.*, 2003; Bajpai *et al.*, 2003; Yunes *et al.*, 2003).

Tyrosine phosphorylation is also closely related with the occurrence of the acrosome reaction and sperm-zona binding (Sakkas *et al.*, 2003). Phosphorylation increased significantly with capacitation and is localized mainly to the principal piece of human spermatozoa. Following binding to the ZP, the percentage of spermatozoa with phosphotyrosine residues localized to both the neck and the principal piece was significantly higher in bound spermatozoa than in capacitated spermatozoa in suspension (Sakkas *et al.*, 2003). Different compartments of human spermatozoa undergo a specific sequence of phosphorylation during both capacitation and upon binding to the ZP. Tyrosine phosphorylation in the principal and neck piece may be considered a prerequisite for fertilization in humans (Sakkas *et al.*, 2003).

The main tyrosine-phosphorylated proteins during the course of capacitation and fertilization are localized in the flagellum, although tyrosine phosphorylation of less abundant proteins may also be regulated in the sperm head (Urner & Sakkas, 2003). Spermatozoa bound to the ZP and fusing with the oocyte plasma membrane are characterized by a tyrosine-phosphorylated flagellum. Protein phosphorylation in the flagellum is linked to hyperactivated motility in spermatozoa, but may also regulate additional functions involved in sperm-oocyte fusion. Factors involved in the appearance of phosphorylation more likely arise from the milieu surrounding the spermatozoa, but their uptake and processing are likely to be regulated differentially at specific steps within the female genital tract and during penetration of the egg vestments (Sakkas *et al.*, 2003). One of these factors is glucose, the metabolic product of which (ATP and NADPH) appear to participate in signalling pathways by supporting a precise onset of tyrosine phosphorylation in the sperm flagellum, leading to successful fertilization (Urner & Sakkas, 2003).

Finally, CASA instruments, which capture a 'snapshot' of sperm trajectories in order to generate their data, may provide a poor measure of hyperactivated motility in a sperm population where hyperactivation is multiphasic in nature. Pacey *et al.* (1997) indicated that whilst this snapshot approach to quantifying hyperactivation could provide a figure for the percentage of hyperactivated spermatozoa within the sample window, this often inaccurately described the underlying behaviour of the population. Since there is very likely to be a significant amount of biological information contained within the nature of multiphasic behaviour, snapshot analysis is one which requires serious consideration by CASA manufacturers and medical researchers.

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TABLE 12

The mean (±SD) results for semen parameters, % ZIAR, % HZI and % Hyperactivation values as recorded for fertile sperm donors and andrology referrals.

	Morphology groups		
	P-pattern <4% normal (*n*=27)	**G-pattern 4-14% normal (*n*=31)**	**N-pattern >14% normal (*n*=6)**
Sperm concentration (10^6/ml)	86.9±24.3	146.0±21.2	182.0±60.6
Motility (%)	44.2±3.5	54.0±4.9	59.0±2.6
Number of hyperactivated sperm after ZP exposure (%)	56/2338[a] 2.39%	144/4042[b] 3.56%	374/5008[c] 7.5%
Morphology (%)	2.8±1.3[d]	8.0±1.7[e]	16.0±1.4[f]
HZI (%)	41.1±4.6[g]	60.9±4.7[h]	77.0±6.2[i]
Spontaneous AR (%)	11.2±1.1	11.3±0.3	14.1±0.4
% ZIAR	14.1±0.6[j]	18.0±2.0[k]	25.0±2.3[l]

Student's *t*-test:

a vs. b, $P<0.01$; a vs. c, $P<0.0001$; b vs. c, $P<0.0001$.

d vs. e, $P<0.05$; d vs. f, $P<0.05$; e vs. f, $P<0.05$.

g vs. h, $P<0.05$; g vs. i, $P<0.05$; h vs. i, $P<0.05$.

j vs. k, $P<0.05$; j vs. l, $P<0.05$; k vs. l, $P<0.05$.

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TABLE 13

The number of hyperactivated spermatozoa (HA) in ejaculates, after capacitation and following exposure to solubilized ZP.

	Normal		**G-pattern**		**P-pattern**	
	HA	**Non-HA**	**HA**	**Non-HA**	**HA**	**Non-HA**
Ejaculates	194[a] (1.44%)	13460	110[b] (1.04%)	10510	27[c] (0.44%)	6042
Capacitated	166[d] (3.08%)	5382	60[e] (1.40%)	4256	28[f] (1.14%)	2438
Zona-exposure	374[g] (7.5%)	4634	144[h] (3.56%)	4042	56[i] (2.39%)	2338

Comparison of proportions (Student's *t*-test):

a vs. b, $P<0.008$; a vs. c, $P<0.0005$; b vs. c, $P<0.0005$.

d vs. e, $P<0.0005$; d vs. f, $P<0.0005$; e vs. f, $P<0.4$.

g vs. h, $P<0.0001$; g vs. i, $P<0.0001$; h vs. i, $P<0.01$.

a vs. g, $P<0.0001$; b vs. h; $P<0.0001$; c vs. i, $P<0.0001$.

a vs. d, $P<0.0001$; b vs. e, $P<0.08$; c vs. f, $P<0.0001$.

d vs. g, $P<0.0001$; e vs. h, $P< 0.0001$; f vs. i, $P<0.002$.

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TABLE 14

The mean (±SD) values for motion characteristics of hyperactivated spermatozoa among men with normal, G and P-pattern sperm morphology as recorded in ejaculates, after 3 hours capacitation and after exposure to solubilized ZP.

Normozoospermic men (*n*=6)			
	Ejaculates	**Capacitated**	**Zona-exposed**
VAP (µm/s)	104.3±4[a]	107.2±4	100.1±4
VSL (µm/s)	56.4±10	62.3±4[e]	64.9±4
VCL (µm/s)	188.5±3[i]	182.1±20[j]	175.4±12[k]
ALH (µm)	11.3±2	9.7±2[p]	9.2±2
BCF (Hz)	16.9±2	17.9±2	18.5±3[v]
STR (%)	55.8±4[bb]	61.2±5	66.5±4
LIN (%)	30.9±3[hh]	34.7±1	37.3±2
G-pattern men (*n*=31)			
VAP (µm/s)	127.7±18[b]	96.1±9	89.4±10
VSL (µm/s)	54.1±9	49.8±7[f]	50.2±7[g]
VCL (µm/s)	191.7±30[l]	177.8±11[m]	177.0±11[n]
ALH (µm)	10.5±2[q]	8.8±2	9.8±3[r]
BCF (Hz)	19.9±4[w]	19.8±4[x]	18.9±3[y]
STR (%)	50.5±4[cc]	52.1±5[dd]	55.0±5[ee]
LIN (%)	25.0±3[ii]	28.6±4[jj]	29.1±4[kk]
P-pattern men (*n*=27)			
VAP (µm/s)	108.4±1	103.1±3[c]	129.9±17[d]
VSL (µm/s)	71.0±1	67.3±2	61.3±5[h]
VCL (µm/s)	157.6±2	187.9±1[o]	217.0±35
ALH (µm)	9.8±2[s]	7.5±0.3[t]	7.6±1[u]
BCF (Hz)	19.8±1[z]	21.5±2	18.1±2[aa]
STR (%)	49.6±1[ff]	57.2±2	52.4±4[gg]
LIN (%)	25.6±2[ll]	31.5±2[mm]	28.6±3[nn]

According to the Fischer's exact *t*-test, all values (except those listed below) in the ejaculates, after capacitation and following zona-exposure, for VAP, VSL, VCL, ALH, BCF, STR and LIN, differed significantly with $P \leq 0.05$ among normal, P and G-pattern men.

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The following values did not differ significantly:

a vs. c; b vs. d; e vs. h; f vs. g; i vs. l; i vs. o; j vs. m; j vs. o; k vs. m; l vs. o; m vs. n; p vs. r; p vs. s; q vs. s; r vs. s; t vs. u; v vs. x; v vs. aa; w vs. x; w vs. z; x vs. y; x vs. z; y vs. z; bb vs. ee; cc vs. ff; dd vs. gg; hh vs. mm; ii vs. ll; jj vs. kk; jj vs. nn; kk vs. nn.

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TABLE 15

The mean (±SD) values for motion characteristics of non-hyperactivated spermatozoa obtained from men with normal, G and P-pattern sperm morphology as recorded in ejaculates, after 3 hours capacitation and after exposure to solubilized ZP.

Normozoospermic men (*n*=6)			
	Ejaculates	**Capacitated**	**Zona-exposed**
VAP (µm/s)	75.1±20[a]	75.3±20	76.4±20
VSL (µm/s)	54.1±22	54.6±23[e]	54.9±22
VCL (µm/s)	83.2±31[i]	84.7±34[j]	88.2±36[k]
ALH (µm)	4.3±2	4.4±2[p]	4.6±2
BCF (Hz)	17.6±16	17.6±15	17.7±15[v]
STR (%)	42.3±24[bb]	42.9±25	43.8±24
LIN (%)	74.9±17[hh]	74.1±17	72.8±18
G-pattern men (*n*=31)			
VAP (µm/s)	92.6±10[b]	92.4±9	92.3±8
VSL (µm/s)	9.9±7	10±7[f]	10.8±9[g]
VCL (µm/s)	15.5±21[l]	15.9±22[m]	19±32[n]
ALH (µm)	0.9±2[q]	0.9±2	1.1±2[r]
BCF (Hz)	13.9±17[w]	13.7±17[x]	13.5±16[y]
STR (%)	9.2±7[cc]	9.3±7[dd]	10.3±10[ee]
LIN (%)	71.3±16[ii]	71.3±16[jj]	70.5±17[kk]
P-pattern men (*n*=27)			
VAP (µm/s)	92.4±9	92.5±9[c]	93.4±10[d]
VSL (µm/s)	9.7±7	10.1±8	10.6±10[h]
VCL (µm/s)	14.2±14	15.6±21[o]	18.1±33
ALH (µm)	0.9±2[s]	0.9±2[t]	1±2[u]
BCF (Hz)	13.7±17[z]	13.7±17	13.4±17[aa]
STR (%)	8.9±6[ff]	9.3±7	9.7±8[gg]
LIN (%)	71.6±16[ll]	71.5±16[mm]	71±16[nn]

According to the Fischer's exact *t*-test, all values (except those listed below) in the ejaculates, after capacitation and following zona-exposure, for VAP, VSL, VCL, ALH, BCF, STR and LIN, differed significantly with $P \leq 0.05$ among normal, P and G-pattern men.

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The following values did not differ significantly:

a vs. c; b vs. d; e vs. h; f vs. g; i vs. l; i vs. o; j vs. m; j vs. o; k vs. m; l vs. o; m vs. n; p vs. r; p vs. s; q vs. s; r vs. s; t vs. u; v vs. x; v vs. aa; w vs. x; w vs. z; x vs. y; x vs. z; y vs. z; bb vs. ee; cc vs. ff; dd vs. gg; hh vs. mm; ii vs. ll; jj vs. kk; jj vs. nn; kk vs. nn.

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CHAPTER 7

GENERAL DISCUSSION

Clinicians and scientists are still searching for semen parameter thresholds in the so-called "normal fertile populations" in order to be able to more accurately define fertility, subfertility and infertility. Fertility is dependent on a complex of events involving both male and female components. Normal sperm function involves many steps, including motility, capacitation, acrosome reactivity and, ultimately, fertilization of the oocyte (Yanagimachi, 1994). While male fertility is most often assessed by means of gross semen parameters (Zinaman *et al.*, 2000), infertility may also be caused by abnormal sperm function (Mackenna, 1995). Only by performing specific tests of this function, may the reasons for infertility become evident. Specific tests, which may be helpful, include semen analysis, sperm motility assessment, acrosome reactivity, sperm penetration assays and in vitro fertilization (IVF).

In the era of assisted reproductive technologies, the evaluation of semen and the functional capacity of its main cellular component, spermatozoa, have become quite detailed. Attempts to predict the fertility potential of spermatozoa has largely failed for several reasons: (i) the number of spermatozoa in the ejaculate is variable; (ii) there is no simple, accurate and reliable assay to predict the fertilization potential of spermatozoa; and (iii) unless IVF is utilized, evaluating fertility in human couples is very difficult because of potential male fertility factors (Mackenna, 1995). To date, there is no single assay available to assess the in vivo or in vitro fertilizing potential of spermatozoa as the process leading to that point is composed of numerous steps.

The development of a sequential, multistep diagnostic approach for the evaluation of the various morphological, dynamic and functional sperm characteristics is an important contribution to infertility diagnosis (Oehninger *et al.*, 1991). One of the cornerstones in the sequential diagnostic approach is the relevance of the AR during the fertilization process (ESHRE, 1996). Due to the development of new techniques for acrosome study, knowledge of acrosome function has greatly progressed during the past ten years (Tesarik, 1996). Basic physiological information has allowed clinical applications among humans and certain groups currently regard the assessment of acrosome function as a prognosticator for male fertility status (Tesarik & Mendoza, 1995).

The routine introduction of a simple and reliable assay for the evaluation of the physiologically induced AR as a component of the previously proposed sequential diagnostic workup program will assist in the identification of specific sperm defects and may allow the development of more directed therapies. Clinicians are sometimes faced with unexpected pregnancies among severely teratozoospermic patients,

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i.e., <4% morphologically normal spermatozoa, who were diagnosed as having a poor chance of achieving fertilization and/or pregnancy. This phenomenon may be caused by the presence of morphologically abnormal spermatozoa with normal function, *i.e.*, adequate zona binding and AR potential. According to Menkveld *et al.* (1996), a strong positive correlation exist between the acrosome index and IVF rates, therefore the acrosome index possibly may be regarded as an additional tool in the prediction of IVF outcome and especially may be of value in the group of severe teratozoospermia. The present results are the first steps towards the development of refined functional diagnostics, such as an ideal acrosome test, *i.e.*, acrosome responsiveness to ZP. Results indicate the clinical importance of using physiological inducers of the AR, since we obtained excellent correlations between sperm morphology and the ability of spermatozoa to undergo ARs when exposed to solubilized ZP. The physiological induced AR test, using homogenous ZP, differentiates between semen samples with normal parameters but with an impaired capacity to acrosome-react. The inclusion of clinically controlled ZP-mediated AR studies in the assisted reproductive areas will be a valuable additional indication for selected criteria's, eventually set for IVF/ICSI/GIFT/IUI patients.

In current clinical practice, treatment of patients by either standard IVF or ICSI in the first cycle is usually decided on semen analysis results. Standard IVF requires good sperm function, particular sperm-zona binding and penetration that are essential for fertilization. With ICSI, several sperm functions are not required for fertilization, especially those associated with sperm-zona interaction. Couples with severe spermatozoa defects such as teratozoospermia can be identified by routine semen analysis and ICSI is recommended for the first treatment. On the other hand, couples with unexplained infertility with normal semen analysis are usually treated with standard IVF. Previous studies have shown that between 10% and 25% (Esterhuizen *et al.*, 2001; Liu *et al.*, 2001) of these couples may have low ZIAR and are at risk of zero or very low fertilization rates in standard IVF. Although these cases can be treated with ICSI in the second cycle, there is a high cost to the patients both financially and emotionally. Failed attempts can also decrease the confidence of the patient in the therapy and therefore reduce the chances of success during future attempts.

The fundamental utility of the ZIAR test stems from its being a functional bioassay of sperm performance in relation to the human ZP. Together with tight binding to the ZP, the ZIAR potential of a sperm population can be seen as a requisite for IVF. The ZIAR results provide useful discrimination between men capable of achieving fertilization in vitro versus those who are unlikely to be successful. The results can become a valuable tool in the diagnostic scheme of the consulting clinician, because the outcome of the test points to IVF or ICSI as the most effective clinical option.

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The analysis of the inducibility of the AR aids the clinician in the management of male infertility. It is well recognized that the lack of information regarding the ZIAR in human spermatozoa is largely due to the paucity of human zonae available for diagnostic laboratory testing and research. Following the original elucidation of the cDNA sequence of the human ZP3 gene (Chamberlain & Dean, 1990), biologically active rhuZP3 has been recently expressed in Chinese hamster ovary cells (Van Duin *et al.*, 1994; Barratt & Hornby, 1995; Brewis *et al.*, 1996). It is accepted that, once available, biologically active rhuZP3 will be the ultimate agonist or trigger substance for human sperm AR. Such a test will most certainly become the basis of the ideal AR test (Tesarik, 1996). The implementation of acrosome assays using small volumes of human solubilized ZP (Franken *et al.*, 2000), biologically active rhuZP3 (Barrat & Hornby, 1995; Dong *et al.*, 2001) or active, synthetic ZP3 peptides (or analogs) (Hinsch *et al.*, 1994), will probably allow for the design of improved, readably available and physiologically oriented assays.

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CHAPTER 8
APPENDIX

8.1 Glossary

-spermia	Refers to the ejaculate.
Aspermia	No semen ejaculated.
-zoospermia	Refers to spermatozoa in the ejaculate.
Azoospermia	No spermatozoa in semen.
Asthenozoospermia	Poor motility ($\leq$20%) and/or forward progression ($\leq$2).
Normozoospermia	Normal ejaculate.
Oligozoospermia	Low sperm concentration ($\leq 10x10^6$/ml).
Teratozoospermia	Reduced % of morphologically normal spermatozoa ($\leq$14%).

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8.2 Standards for normal* semen parameters

Semen parameter	Infertile‡	Subfertile†	Fertile§
Concentration (x10^6/ml)	<2	2.0-9.9	≥10
Motility (% motile)	<10	10-29	≥30
Forward progression$^{\Phi}$ (0-4)	<1	1.0-1.9	≥2
Motility index	<20	20.0-49.9	≥50
Morphology (% normal)	<4 (P-pattern; poor prognosis)	5-14 (G-pattern; good prognosis)	≥15

*Tygerberg criteria

$^{\Phi}$Grading of forward progression:

0 No movement.

1 No forward movement.

1+ A few spermatozoa move now and then.

2 Movement slow and undirected.

2+ Movement slow and directly forward.

3- Movement fast and undirected.

3 Movement fast and directly forward.

3+ Movement very fast and directly forward.

4 Movement extremely fast and directly forward (wave-like).

§Fertile Optimal chance for conception.

†Subfertile Reduced chance for conception.

‡Infertile Very small chance for conception.

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8.3 Alphabetical list of abbreviations

A23187	calcium ionophore
ALH	amplitude of lateral head displacement
AR	acrosome reaction
ARIC-test	acrosome reaction ionophore challenge test
ASMA	automated sperm morphology analysis
BCF	beat cross frequency
BSA	bovine serum albumin
CASA	computer-assisted semen analysis
CI	confidence interval
CK	creatine kinase
DMSO	dimethylsulphoxide
FITC-PSA	fluorescein isothiocyanate *Pisum Sativum* agglutinin
GIFT	gamete intrafallopian transfer
G_i proteins	guanine nucleotide-binding regulatory proteins
HA	hyperactivated spermatozoa
HFF	human follicular fluid
HTF	human tubal fluid medium
HTM-IVOS	Hamilton-Thorne integrated visual optical system
HZA	hemizona assay
HZI	hemizona index
IBT	immunobead test
ICSI	intracytoplasmic/intracellular sperm injection
IUI	intrauterine insemination
IVF	in vitro fertilization
LIN	linearity
MAR	mixed antiglobulin reaction
PF	pentoxifylline
PT	pertussis toxin
PVP	polivinylpyrrolidone
rhuZP3	recombinant human ZP3
ROC	receiver operating characteristics curve analysis
ROS	reactive oxygen species

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STR	straightness
VAP	average path velocity
VCL	curvilinear velocity
VSL	straight line velocity
WHO	World Health Organization
ZIAR	zona pellucida-induced acrosome reaction
ZP	zona pellucida
ZP3	zona pellucida sulfated glycoprotein

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