ANDROLOGIA **32,** 335–343 (2000) ACCEPTED: OCTOBER 11, 1999

Review

Acrosome reaction: methods for detection and clinical significance

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Key words. Acrosome reaction—fertility—IVF outcome—spermatozoa

Summary. The present article reviews the methods for detection and the clinical significance of the acrosome reaction. The best method for the detection of the acrosome reaction is electron microscopy, but it is expensive and labour-intensive and therefore cannot be used routinely. The most widely used methods utilize optical microscopy where spermatozoa are stained for the visualization of their acrosomal status. Different dyes are used for this purpose as well as lectins and antibodies labelled with fluorescence. The acrosome reaction following ionophore challenge (ARIC) can separate spermatozoa that undergo spontaneous acrosome reaction from those that are induced, making the result of the inducible acrosome reaction more meaningful. Many different stimuli have been used for the induction of the acrosome reaction with different results. The ARIC test can provide information on the fertilizing capability of a sample. The ARIC test was also used to evaluate patients undergoing *in vitro* fertilization since a low percentage of induced acrosome reaction was found to be associated with lower rates of fertilization. The cut-off value that could be used to identify infertile patients is under debate. Therapeutic decisions can also be made on the basis of the value of the ARIC test.

Introduction

Fertilization in mammals requires the entry of the spermatozoon into the oocyte. The oocyte is surrounded by a biochemically complex structure called the zona pellucida. The spermatozoon must go through this layer before fusing with the oocyte plasma membrane. This process requires the acrosome reaction which is regulated by a number of ions (Fraser, 1998) and results in the exocytosis of digestive enzymes that will allow the spermatozoon to break through the zona pellucida. Fusion then occurs between the sperm plasma membrane and the oolemma. As a result, the spermatozoon is drawn into the oocyte thus forming the embryo (Kupker *et al.*, 1998).

In the past few years, new methods for evaluating the capacity of spermatozoa to carry out the acrosome reaction have been developed (Cross & Meizel, 1989). These methods are currently used to determine the fertilizing capability of a given sample. This review summarizes the information available on the methods for the detection of the acrosome reaction, the clinical significance of the result and the implication it will have on therapeutic decisions.

Biology of the acrosome reaction

After ejaculation the spermatozoon can not fertilize the oocyte immediately. A lag phase, during which capacitation takes place, is necessary in order for insemination to occur (Yanagimachi, 1994). It is generally believed that the stage of capacitation prepares the spermatozoon for the rest of its journey to the oocyte interior. The spermatozoon is deposited into the lower region of the female reproductive tract and must move a distance to the site of fertilization. It has been suggested that capacitation may actually prevent

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the spermatozoon from becoming fertile too quickly (Caswell & Hutchison, 1971).

The process of capacitation is poorly understood and many biochemical phenomena seem to take place. Some of the events that have been identified include: (i) the reorganization of membrane surface antigens that increase the fluidity of the membrane (Koehler & Gaddum-Rose, 1975); (ii) a change in the ratio of cholesterol/phospholipids due to the loss of cholesterol to an extracellular ligant such as albumin (Davis, 1981; Benoff *et al.*, 1993); (iii) protein phosphorylation as an important feature of both capacitation and the acrosome reaction (Naz *et al.*, 1991; Luconi *et al.*, 1995; Visconti *et al.*, 1995). The time necessary for capacitation to be completed varies among different species. In humans the time needed for sperm capacitation is not known for the *in vivo* situation and it varies widely *in vitro*.

When capacitation is completed the spermatozoon is ready for the acrosome reaction and the following steps occur:

Step 1: the spermatozoon must bind to the zona pellucida. The zona pellucida protein 3 (ZP3) was identified as the ligant where the sperm binds, and this observation was made using mouse gametes (Wassarman, 1994).

Step 2: the spermatozoon penetrates the zona pellucida due to the acrosome reaction, which, as mentioned, liberates hydrolytic enzymes due to exocytosis. The acrosome reaction is probably stimulated by the binding of the sperm head to ZP3 and the plasma membrane around the head and the outer acrosomal membrane fuse, leaving the equatorial segment intact. A number of separate steps can be identified on the spermatozoon, which were described by the terms 'poised', 'punctuate' and 'fenestrate' (Storey, 1991). Among the enzymes that are liberated are acrosin and hyaluronidase. These enzymes might exhibit trypsin action since treating the sperm with soybean trypsin inhibitor blocked zona–sperm penetration. (Liu & Baker, 1993; Llanos *et al.*, 1993). It has been suggested, however, that the enzyme acrosin can mediate the interaction of the spermatozoon and the zona pellucida (Tesarik *et al.*, 1988). It was also found that a large number of spermatozoa bound to the surface of the zona pellucida have already undergone the acrosome reaction (Aitken, 1990). The combination of these two findings possibly suggests another mechanism with which the first step is the acrosome reaction and the second step is the interaction between the spermatozoon and the zona pellucida (Aitken, 1990).

Step 3: the spermatozoon fuses with the oolema. The equatorial segment of the spermatozoon seems responsible for this fusion, and the acrosome reaction is involved in changes that make this phenomenon possible (Yanagimachi, 1994). The equatorial segment becomes more fusogenic, possibly due to the release of acrosin (Dravland & Meizel, 1982; Takano *et al.*, 1993). After the two membranes fuse, the genetic material of the spermatozoon is found in the oocyte, and the male pronucleus forms.

Methods for the detection of the acrosome reaction

Visualization of spermatozoa that have undergone the acrosome reaction and separation from those that have not can be achieved nowadays using many methods. The result of such testing could provide information on the fertilizing potential of a given sample. The most commonly used methods are described below.

1 Electron microscopy

The best method for detecting acrosome-reacted spermatozoa is electron microscopy, which can accurately identify the acrosomal status of the sample. The methods that are commonly used are compared with it for evaluation (Mack *et al.*, 1990; DasGupta *et al.*, 1993). Electron microscopy requires expensive equipment and trained personnel; in other words, it cannot be used routinely, and it is a valuable tool only for research purposes.

2 Bright-field light microscopy

The first method for assessing acrosomal status using light microscopy was developed by Talbot and Chacon (Talbot & Chacon, 1980; 1981). This method stains the acrosomal contents and the differential staining allows the separation between reacted and non-reacted spermatozoa. It is a triple-stain procedure that uses trypan blue to stain dead spermatozoa and Bismarck brown Y with Rose Bengal stain the post-acrosomal segment in viable spermatozoa. Other bright-field microscopy assays were also developed, including the method that uses Coomassie blue (Aarons *et al.*, 1993) and the method based on the use of silver staining (Gosalvez *et al.*, 1986). In the above-mentioned assays the sperm head membrane should become permeable to the probe that will detect the acrosomal material. This is achieved due to the harsh conditions of the fixing and staining process, which disrupt the sperm membrane.

Bright-field microscopic assays correlate well with electron microscopy (Mack *et al.*, 1990; Aarons *et al.*, 1993), and they are in use because

of their simplicity and relatively low cost. However, the accurate identification of acrosome-reacted spermatozoa is sometimes difficult.

3 Fluorescence microscopy using lectins

Fluorescent assays also use the light microscope for the separation of spermatozoa that have completed the acrosome reaction from those that have not. A wide variety of lectins that are labelled with fluorescence are used extensively since they give better differentiation between acrosome-reacted and acrosome-unreacted sperm heads. The use of different lectins can even separate the onset stage of the acrosome reaction from the latter stages where the acrosomal cup is lost (Amin *et al.*, 1996).

The most commonly used lectins are *Pisum sativum* agglutinin (Cross *et al.*, 1986) which binds to acrosomal contents and peanut agglutinin (Mortimer *et al.*, 1987) which binds to the outer acrosomal membrane. These probes detect the acrosomal contents when the spermatozoon has not yet undergone the acrosome reaction. Other methods were developed that detect the inner acrosomal membrane or exocytosed acrosomal contents; in other words, these methods stain spermatozoa that have completed the acrosome reaction. These methods use probes that are visualized again with fluorescence microscopy. The probes used include Concanavalin-A (Con-A) and soybean trypsin inhibitor (Holden *et al.*, 1990; Arts *et al.*, 1994). The use of different probes can possibly detect different stages of the acrosome reaction (Köhn *et al.*, 1997).

4 Fluorescence microscopy using antibodies

Antibodies have also been developed for the assessment of the acrosomal status such as antisperm antibody, antiacrosin antiserum, anti-outer acrosomal membrane antiserum as well as the monoclonal antibody HS21 (Wolf *et al.*, 1985; Sanchez *et al.*, 1991), which detect sperm cells that are intact. However, labelling with antibodies is more labour-intensive than labelling with lectins since exposure to a second fluoresceinated antibody is needed.

Another category of methods uses monoclonal antibodies GB24 and MH61 (Fénichel *et al.*, 1989). In this category the probes detect only reacted sperm heads that have exposed their inner acrosomal membrane or have exocytosed their acrosomal components, so there is no need for the sperm-head membrane to become permeable to the probe. The acrobead test uses polyacrylamide beads coated with anti-CD 46 monoclonal antibody to determine the acrosomal status and its use in clinical practice is being evaluated (Hershlag *et al.*, 1997; Sharma *et al.*, 1997).

5 Chlortetracycline fluorescence

This assay works on the principle that the compound chlortetracycline (CTC) and calcium ions bind to membranes and show highly enhanced fluorescence with the following four different patterns (Lee *et al.*, 1987; DasGupta *et al.*, 1993; Fraser *et al.*, 1996; Fraser, 1998).

Pattern 1: This pattern is characterized by a bright band of fluorescence in the post-acrosomal region and bright fluorescence on the midpiece. This is designated pattern EF (early fresh sperm) and is observed in human spermatozoa that were left to capacitate for 1 h after ejaculation.

Pattern 2: This pattern is characterized by bright fluorescence in the anterior portion of the head and the dark band in the post-acrosomal region and bright fluorescence on the midpiece. This is designated pattern DP (dark posterior head) and is observed in human spermatozoa that were left to capacitate for 3 h after ejaculation.

Pattern 3: This pattern is characterized by fluorescence over the entire head with bright perimeter and fluorescence on the midpiece. This is designated pattern CP (clear perimeter) and is observed in human spermatozoa that were left to capacitate for 10 h after ejaculation.

Pattern 4: This pattern is characterized by the lack of fluorescence on the head and fluorescence on the midpiece. This is designated pattern AR (acrosome-reacted) and is observed in human spermatozoa that were treated with ionophore A23187 (Lee *et al.*, 1987).

In conclusion, it can be noted that the CTC assay is easy to perform but the mechanisms by which it works are still unknown.

6 Flow cytometry

Sperm heads that are labelled with fluorescent probes can also be studied with the use of flow cytometry. This technique can measure the fluorescence of large numbers of cells in a sample in a very short time. The probes that are developed so far for this purpose include monoclonal antibodies (Fénichel *et al.*, 1989; Okabe *et al.*, 1990; Tao *et al.*, 1993), peanut agglutinin (Purvis *et al.*, 1990; Engh *et al.*, 1991) and *Pisum sativum* agglutinin (Henley *et al.*, 1994). A commonly used antibody is the antibody CD 46, which binds to the inner acrosomal membrane (D'Cruz & Haas, 1992; Carver-Ward *et al.*, 1994). In a recent study, CD 46 was used in conjunction with *Pisum sativum*

agglutinin for the distinction between acrosome reaction and acrosomal loss (Kawamoto *et al.*, 1999). Flow cytometry is very accurate and very sensitive, is not as labour-intensive as cell counting at the microscope and is less time consuming, but the cost of the flow cytometer is very high. As a result, the visual method will remain a useful and practical procedure for routine andrology (Uhler *et al.*, 1993).

7 Acrosome reaction following ionophore challenge (ARIC)

All of the above-mentioned assays can separate between spermatozoa that have completed their acrosome reaction from those that have not, in other words can only identify spontaneously reacted spermatozoa. Only acrosome intact spermatozoa will penetrate the cumulus oophorus and therefore the result of these assays will have little or no relationship to the fertilizing potential of a semen sample. Premature spontaneous acrosome reaction will prevent the binding of sperm to the zona pellucida. Tesarik (1989) emphasized the appropriate timing and conditions of the acrosome reaction and concluded that for an acrosome reaction test to be meaningful under specific circumstances it 'should therefore be designed to distinguish between spontaneous and induced acrosome reactions'.

This condition is met by the ARIC test (Cummins *et al.*, 1991). For this test, spermatozoa are prepared under capacitating conditions and are then treated with calcium ionophore A23187 to induce a calcium influx. Thereafter, spermatozoa are washed, spread on glass slides, permeabilized with ethanol and labelled with the fluorescent lectin *Pisum sativum*, which binds to the acrosomal matrix. The pattern of the fluorescence labelling is different between reacted and non-reacted sperm and this difference is used for the separation of spermatozoa.

The ionophore used in the ARIC test increases the concentration of calcium in the spermatozoon which responds to that change by carrying out the acrosome reaction. Spermatozoa are also left to capacitate before the ionophore challenge. Recent data suggest that none of the above-mentioned conditions are an absolute necessity since the acrosome reaction can occur without capacitation and the use of ionophore, although to a lesser extent (Bielfeld *et al.*, 1994).

The ARIC test presents a very useful tool in determining the inducibility of the acrosome reaction, after an appropriate stimulus is given to spermatozoa. A point of concern is the use of ionophore A23187, which differs from the physiological way that is needed to trigger the acrosome reaction in human spermatozoa. Calcium ionophore suffers the disadvantage of by-passing part of the transductive mechanisms that are needed for the calcium influx (Zaneveld *et al.*, 1993). Many other stimuli have been proposed as being responsible for the induction of the acrosome reaction in the fertilizing sperm. For example, progesterone was used for the induction of the acrosome reaction (Oehninger *et al.*, 1994) with only a slight decrease in the viability and motility of spermatozoa (Parinaud *et al.*, 1992). Although progesterone does increase intracellular calcium concentration (Emiliozzi *et al.*, 1996) contradictory results on its use as an acrosome reaction inducer also exist (Uhler *et al.*, 1992; Tomiyama *et al.*, 1995; Carver-Ward *et al.*, 1996). Other stimuli that have been suggested include the cumulus oophorus (Tesarik, 1985; Stock *et al.*, 1989), although this was not confirmed by others (Hoshi *et al.*, 1993), the cervical mucus (Perry *et al.*, 1996) and low temperature induction (Henkel *et al.*, 1993). Follicular fluid (Mortimer & Camenzind, 1989; Tarlatzis *et al.*, 1993; Liu & Baker, 1994; Zhu *et al.*, 1994) is widely used as an inducer of the acrosome reaction and produces results that correlate with those obtained with ionophore A23187 (Avrech *et al.*, 1997). These stimuli when used in the laboratory investigation of the acrosome reaction would produce a result closer to the naturally occurring reaction.

As the acrosome reaction occurs on the zona pellucida after binding of the sperm receptor with the protein ZP3, the use of zonae pellucida or their soluble products would have physiological significance if used as stimuli for the acrosome reaction. The zona pellucida was also found to induce the acrosome reaction (Liu & Baker, 1996a) and it was shown that the presence of antisperm antibodies can interfere with the acrosome induction (Francavilla *et al.*, 1997). The three-dimensional structure of the zona is very important. The acrosome reaction is greater when the whole zona pellucida is used rather than solubilized zonae (Liu & Baker, 1996a). It should also be noted that zona pellucida induction does not correlate with ionophore A23187 induction (Liu & Baker, 1996b). The contact of spermatozoa with the cervical mucus can also affect the acrosome reaction. When spermatozoa have passed through the cervical mucus, the zona-induced acrosome reaction is increased (Hoshi *et al.*, 1993). As spare zonae are hard to find and therefore difficult to use routinely, researchers have been working towards the synthesis of recombinant ZP3, which would be more useful as a stimulus for the diagnostic acrosome reaction test (Van Duin *et al.*, 1994).

Clinical significance of the acrosome reaction

The presence of the acrosome and its ability to function properly is essential for the fertilizing potential of the spermatozoon, and this was illustrated a number of years ago by the fact that spermatozoa without acrosomes are unable to penetrate the egg vestments (Schill, 1974). Lack of fertilization was also exhibited by spermatozoa that have a large percentage of round head forms (Jeyendran *et al.*, 1976). Furthermore it was shown that the fertilizing potential of a sample can be impaired due to defects of the structure or function of the acrosome (Schill, 1991).

The inducibility of the acrosome reaction can correlate with sperm penetration of the zona pellucida (Liu & Baker, 1996b). Based on the above, it can be concluded that the inducibility of the acrosome reaction can provide useful information for the management of infertility. For example, it can predict the fertilizing potential of a sperm sample (Tasdemir *et al.*, 1993). The contents of the acrosome can also be used to monitor the damage after freezing–thawing of a sample, since freezing of a sample can affect the incidence of intact acrosomes (Cross & Hanks, 1991). The inducibility of the acrosome reaction was even reduced in the sperm samples from smokers (El Mulla *et al.*, 1995). Only acrosome-reacted spermatozoa were selected with the use of antibody-labelled beads by Moutaffian & Parinaud (1995) and then used for evaluation of the acrosomal function (Sharma *et al.*, 1997).

Spermatozoa undergo spontaneous acrosome reaction during incubation in any medium, but the rate is very low (Fénichel *et al.*, 1991; Tarlatzis *et al.*, 1993). The physiological significance of the spontaneous acrosome reaction is not understood, but it seems that it represents an unwanted feature. In fact spontaneous premature acrosome reaction seems to be a deleterious feature (Takahashi *et al.*, 1992). Premature loss of the acrosome, which occurs after spontaneous acrosome reaction, possibly prevents the spermatozoon from binding to the zona pellucida.

On the contrary the inducible acrosome reaction, which is calculated by subtracting the total percentage of reacted spermatozoa from those that have spontaneously reacted, is a better approach to acrosome function (Cummins *et al.*, 1991; Fénichel *et al.*, 1991; Henkel *et al.*, 1993). Many attempts have been made to correlate the result of the inducible acrosome reaction with the outcome of *in vitro* fertilization and so add this test to the routine testing of the andrology laboratory along with sperm function testing (Francavilla *et al.*, 1995).

Many researchers have used the inducible acrosome reaction as a diagnostic test and correlated the result with the IVF outcome or the fertilizing ability of the semen sample. The cut-off value of the induction differs between studies, and which value will prove to have the greatest biological significance remains to be seen. Calvo *et al.* (1989) found that it was reduced in cases of unexplained infertility although the activity of acrosin may also be reduced (Koukoulis *et al.*, 1989). Fénichel *et al.* (1991) found a low response to calcium ionophore in cases of repetitive unexplained *in vitro* fertilization failure, when they used the discriminant threshold of 20% for induction of the acrosome reaction. Parinaud *et al.* (1995) predicted up to 83% of IVF results by combining parameters of motility and morphology with spontaneous acrosome reaction and the acrosome reaction in response to phorbol 12-myristate 13-acetate 4-*o*-methyl ether (TPA). However, there was no added predictive value with the use of calcium ionophore A23187.

Pampiglione *et al.* (1993) separated fertile from infertile men and found fertilization failure of 100% when the cut-off value for reacted spermatozoa was 31%. On the other hand, Cummins *et al.* (1991) used 5% reacted spermatozoa as the cut-off limit (fertilization was poor under this limit) and so the predictive value of the test was 90%. Henkel *et al.* (1993) suggested the value of 7.5% for induced reacted spermatozoa being indicative of subfertility.

Looking at the data, it becomes evident that consensus has not yet been reached and there are many reasons for this discrepancy in the literature. As already mentioned, the cut-off limit for spermatozoa that are induced to carry out the acrosome reaction differs widely between studies. In many cases the results cannot be compared since different methodologies are used either for the induction of the acrosome reaction or for the visualization of the result. The way patients are separated into fertile or infertile also varies widely and when fertilization rates are used the percentage of oocytes fertilized change.

The acrosomal status may also be related to abnormalities of sperm parameters. For example, in asthenozoospermia the rate of induced acrosome reaction is reduced (Pilikian *et al.*, 1992). In severe teratozoospermia the calcium influx, the spontaneous and the induced acrosome reactions are defective (Oehninger *et al.*, 1994). In teratozoospermic patients the ionophore-induced acrosome reaction is significantly related to fertilization rates (Liu & Baker, 1998). In cases of

teratozoospermia the induced acrosome reaction correlated with sperm morphology when the stimulus was solubilized zonae and not calcium ionophore (Franken *et al.*, 1997).

The acrosomal status can also help in therapeutic decisions. In cases of unexplained failure of *in vitro* fertilization, a defective acrosome reaction can suggest the mechanism and lead to the correct choice of treatment. The problems that are due to a defective acrosome reaction can be separated into two types:

(i) Acrosome reaction insufficiency. In this case spermatozoa do not acrosome react after incubation with an appropriate stimulus and the use of pentoxifylline (PF) is beneficial. It is known that the use of PF can increase the motility of spermatozoa (Dimitriadou *et al.*, 1995), increase the outcome of intrauterine insemination in selected cases (Matson *et al.*, 1995; Negri *et al.*, 1996) and minimize the damage caused to sperm by freezing and thawing (Esteves *et al.*, 1998). In cases of severe male infertility incubation of the sperm sample with PF can also increase the IVF outcome (Yovich *et al.*, 1988; Tesarik *et al.*, 1992; Tasdemir *et al.*, 1993; Tesarik & Mendoza, 1993; Tournaye *et al.*, 1994; Brandelli *et al.*, 1995). PF was reported to increase the interaction between gametes and also increase the binding of sperm to the zona pellucida as shown by the hypo-osmotic swelling test and competitive zona binding (Lambert *et al.*, 1992; Paul *et al.*, 1996). A response assay was recently proposed that would identify patients in whom the use of PF would be beneficial (Briggiler *et al.*, 1997).

(ii) Acrosome reaction prematurity. In this case, the percentage of spontaneously reacted spermatozoa is abnormally high. Treatment with egg yolk was shown to decrease the rate of spontaneously reacted spermatozoa (Tesarik & Mendoza, 1995). Since the percentage of ionophore-induced acrosome reactions is not altered, a significant increase in the percentage of acrosome-reacted sperm is documented. Treatment with egg yolk was also shown to increase the number of spermatozoa bound to the zona pellucida (Tesarik & Mendoza, 1995).

It becomes evident that, if the acrosome reaction cannot be induced in a sample, even though capacitation is normal, then intracytoplasmic injection (ICSI) should be performed for fertilization of oocytes to occur. It should be emphasized though that only when sperm is incapable of achieving normal fertilization *in vitro* (Fraser, 1998) should ICSI be performed and the inducible acrosome reaction can assist in the identification of cases where assisted fertilization must be preferred.

Conclusions

It becomes apparent by looking at the literature that the acrosome reaction and in particular the evaluation of the spontaneous acrosome reaction in relation to the induced can provide valuable information in the study of the infertile man. In an attempt to separate fertile from infertile men using the ARIC test, the difficulty lies in the appropriate selection of the stimulus as well as the cut-off value for the induced acrosome reacted spermatozoa and it is clear that the setting and general acceptance of such a limit is difficult to achieve. Although more work is needed before all questions can be answered the acrosome reaction can be of value in: (a) predicting the fertilizing ability of a sample, particularly when the induced acrosome reaction is calculated; (b) explaining the failure of fertilization in IVF cycles and improving the outcome with pharmacological agents; (c) deciding on cases where ICSI should be performed; and (d) assessing the possible damages after freezing–thawing of semen.

References

Aarons D, Boettger-Tong H, Biegler B, George G, Poirier GR (1993) The acrosomal status of human sperm evaluated by coomassie blue staining and electron microscopy. Mol Androl 5:31–37.

Aitken RJ (1990) Evaluation of human sperm function. Br Med Bulletin 46:654–674.

Amin AH, Bailey JL, Storey BT, Blasco L, Heyner S (1996) A comparison of three methods for the acrosome reaction in human spermatozoa. Hum Reprod 11:741–745.

Arts EG, Kuiken J, Jager S (1994) A new method to detect acrosome reacted spermatozoa using biotinylated soybean trypsin inhibitor. Fertil Steril 62:1044–1055.

Avrech O, Fisch B, Shalgi R (1997) Acrosomal status of human spermatozoa after follicular fluid or calcium ionophore challenge in relation to semen parameters and fertilizing capacity *in vitro*. Andrologia 29:97–101.

Benoff S, Hurley I, Cooper GW, Mandel FS, Rosenfield DL, Hershlag A (1993) Head specific mannose-ligant receptor expression in human cholesterol loss. Hum Reprod 8:2141–2154.

Bielfeld P, Anderson RA, Mack SR, De Johge CJ, Zaneveld LJD (1994) Are capacitation or ion influx required for the human sperm acrosome reaction? Fertil Steril 62:1255–1261.

Brandelli A, Miranda PV, Anon-Vazquez M, Marin-Briggiler CI, Sanjurjo C, Gonzalez-Echeverria F, Blaquier J, Tezon GJ (1995) A new predictive test for *in-vitro* fertilization based on the induction of sperm acrosome reaction by *N*-acetylglucosamine-neoglycoprotein. Hum Reprod 10:1751–1756.

Briggiler CI, Lancuba CL, Delcourt S, Gogorza S, Tricarico JM, Testa R, Cameo AS (1997) The acrosome reaction

response assay: a new way to indentify patients in whom pentoxifylline would improve the human follicular fluid-induced acrosome reaction. Int J Androl 20:96–103.

Calvo L, Vantaman D, Banks SM, Tezon J, Koukoulis GN, Dennison L, Sherins RJ (1989) Follicular fluid-induced acrosome reaction distinguishes a subgroup of men with unexplained infertility not identified by semen analysis. Fertil Steril 52:1048–1042.

Carver-Ward JA, Jaroudi KA, Einspenner M, Parhar RS, Al-Sedairy ST, Sheth KV (1994) Pentoxifylline potentiates ionophore (A23187) mediated acrosome reaction in human sperm: flow cytometric analysis using CD 46 antibody. Hum Reprod 9:71–76.

Carver-Ward JA, Hollanders JMG, Jaroudi KA, Einspenner M, Al-Sedairy ST, Sheth KV (1996) Progesterone does not potentiate the acrosome reaction in human spermatozoa: flow cytometric analysis using CD46 antibody. Hum Reprod 11:121–126.

Caswell AH, Hutchison JD (1971) Visualisation of membrane bound cations by a fluorescent technique. Biochem Biophys Res Commun 42:43–49.

Cross NL, Hanks SE (1991) Effects of cryopreservation on human sperm acrosomes. Hum Reprod 6:1279–1283.

Cross NL, Meizel S (1989) Methods for evaluating the acrosomal status of mammalian sperm. Biol Reprod 41:635–641.

Cross NL, Morales P, Overstreet JW, Hanson FW (1986) Two simple methods for detecting acrosome-reacted human sperm. Gamete Res 15:213–226.

Cummins JM, Pember SM, Jequier AM, Yovitch JL, Hartmann PE (1991) A test of the human sperm acrosome reaction following ionophore challenge; relationship to fertility and other seminal parameters. J Androl 2:98–103.

DasGupta S, Mills CL, Fraser LR (1993) Ca^{++} related changes in the capacitation state of human spermatozoa assessed by a chlortetracycline fluorescence assay. J Reprod Fertil 99:135–143.

Davis BK (1981) Timing of fertilization in mammals: sperm cholesterol/phospholipid ratio as a determinant of the capacitation interval. Proc Nat Acad Sci USA 78:7560–7564.

D'Cruz OJ, Haas GG (1992) Flow cytometry quantitation of the expression of membrane cofactor protein as a marker for the human sperm acrosome reaction. Fertil Steril 58:633–636.

Dimitriadou F, Rizos D, Mantzavinos Th, Arvaniti K, Voutsina K, Prapa A, Kanakas N (1995) The effect of pentoxifylline on sperm motility, oocyte fertilization, embryo quality, and pregnancy outcome in an *in vitro* fertilization program. Fertil Steril 63:880–886.

Dravland JE, Meizel S (1982) The effect of inhibitors of trypsin and phospholipase A2 on the penetration of zona pellucida hamster eggs by acrosome-reacted hamster sperm. J Androl 3:388–395.

El Mulla KF, Köhn FM, El Beheiry AH, Schill WB (1995) The effect of smoking and varicocele on human sperm acrosin activity and acrosome reaction. Hum Reprod 10:3190–3194.

Emiliozzi C, Cordonier H, Guerin JF, Ciara B, Benchaib M, Fénichel P (1996) Effects of progesterone on human spermatozoa prepared for *in-vitro* fertilization. Int J Androl 19:39–47.

Engh E, Clausen OP, Purvis K (1991) Acrosomal integrity assessed by flow cytometry in men with variable sperm quality. Hum Reprod 6:1129–1134.

Esteves CS, Rakesh KS, Thomas JA, Agarwal A (1998) Cryopreservation of human spermatozoa with pentoxifylline improves the post-thaw agonist-induced acrosome reaction rate. Hum Reprod 13:3384–3389.

Fénichel P, Hsi BL, Farahifar D, Donzeau M, Barrier-Delpech D, Yehy CJ (1989) Evaluation of the human sperm acrosome reaction using a monoclonal antobody, GB24, and fluorescence activated cell sorter. J Reprod Fertil 87:699–706.

Fénichel P, Donzeau M, Farahifar D, Basteris B, Ayraud N, Hsi B (1991) Dynamics of human sperm acrosome reaction: relation with *in vitro* fertilization. Fertil Steril 55:994–999. (1996)

Francavilla F, Romano R, Marrone V, Valenti M, Santucci R (1995) Relationship between acrosome reactions and hamster egg penetration after ionophore challenge in absence of teratozoospermia. Fertil Steril 63:1301–1305.

Francavilla F, Romano R, Santucci R, Marrone V, Properzi G, Ruvolo G (1997) Interference of antisperm antibodies with the induction of the acrosome reaction by the zona pellucida (ZP) and its relationship with the inhibition of ZP binding. Fertil Steril 67:1128–1133.

Franken DR, Bastiaan HS, Kidson A, Wranz P, Habenicht UF (1997) Zona pellucida mediated reaction and sperm morphology. Andrologia 29:311–317.

Fraser RL (1998) Sperm capacitation and the acrosome reaction. Hum Reprod 13 (Suppl 1):9–19.

Fraser RL, DasGupta S, Mills LC, Fertilization—calcium-dependent events. Letter to the editors. Fertil Steril 65: 1258–1259.

Gosalvez J, Lopez-Fernandez C, De La Torre J, Suja JA, Rufas JS (1986) A method for visualizing the acrosome by light microscopy. Stain Technol 61:227–230.

Henkel R, Müller C, Miska W, Gips H, Schill WB (1993) Determination of the acrosome reaction in human spermatozoa is predictive of fertilization *in vitro*. Hum Reprod 8:2128–2132.

Henley N, Bacon C, Roberts KD (1994) Flow cytometric evaluation of the acrosome reaction of human spermatozoa: a new method using a photoactivated supravital stain. Int J Androl 17:78–84.

Hershlag A, Paine T, Scholl GM, Rosenfeld DL, Mandel FS, Zhu JZ, Guhring P, Mecerod D, Benoff S (1997) Acrobeads test as a predictor of fertilization *in vitro*. Am J Reprod Immunol 37:291–299.

Holden CA, Hyne RV, Sathananthan AH, Trounson AO (1990) Assessment of the human sperm acrosome reaction using concanavalin A lectin. Mol Reprod Dev 25:247–257.

Hoshi K, Sugano T, Endo C, Yoshimatsu N, Yanagida K, Sato A (1993) Induction of the acrosome reaction in human spermatozoa by human zona pellucida and effect of cervical mucus on zona-induced acrosome reaction. Fertil Steril 60:149–153.

Jeyendran RS, van der Ven HH, Kennedy WP, Health E, Perez-Pelaez M, Sobrero AJ, Zaneveld LJ (1976) Acrosomeless sperm, a cause of primary male infertility. Andrologia 17:31–35.

Kawamoto A, Ohashi K, Kishikawa H, Zhu L, Azuma C, Murata Y (1999) Two-color fluorescence staining of lectin and anti-CD46 antibody to assess acrosomal status. Fertil Steril 71:497–501.

Koehler JK, Gaddum-Rose P (1975) Media induced alterations of the membrane associated particles of the guinea-pig sperm tail. J Ultrastruct Res 51:106–118.

Köhn FM, Mack SR, Schill WB, Zaneveld LJ (1997) Detection of human sperm acrosome reaction: comparison between methods using double staining, *Pisum sativum* agglutinin, concanavalin A and transmission electron microscopy. Hum Reprod 12:714–721.

Koukoulis GN, Vantman D, Dennison L, Banks SM, Sherins RJ (1989) Low acrosin in a subgroup of men with idiopathic infertility does not correlate with sperm density, percent

motility, curvilinear velocity, or linearity. Fertil Steril 52:120–127.
Kupker W, Diedrich K, Edwards RG (1998) Principles of mammalian fertilization. Hum Reprod 13:20–32.
Lambert H, Steinleitner A, Eisermann J, Serpa N, Cantor B (1992) Enhanced gamete interaction in the sperm penetration assay after coincubation with pentoxifylline and human follicular fluid. Fertil Steril 58:1205–1208.
Lee MA, Trucco GS, Bechtol KB, Wummer N, Kopf GS, Blasco L, Storey BT (1987) Capacitation and acrosome reactions in human spermatozoa monitored by a chlortetracycline assay. Fertil Steril 48:649–658.
Liu DY, Baker HWG (1993) Inhibition of acrosin activity with a trypsin inhibitor blocks human penetration of the zona pellucida. Biol Reprod 48:340–348.
Liu DY, Baker HWG (1994) Disordered acrosome reaction of spermatozoa bound to the zona pellucida: a newly discovered sperm defect causing infertility with reduced sperm-zona pellucida penetration and reduced fertilization *in vitro*. Hum Reprod 9:1694–1700.
Liu DY, Baker GHW (1996a) A simple method for the assessment of the human acrosome reaction of spermatozoa bound to the zona pellucida: lack of relationship with ionophore A23187-induced acrosome reaction. Hum Reprod 11:551–557.
Liu DY, Baker GHW (1996b) Relationship between the zona pellucida (ZP) and ionophore A23187-induced acrosome reaction and the ability of sperm to penetrate the ZP in men with normal sperm-ZP binding. Fertil Steril 66:312–315.
Liu DY, Baker GHW (1998) Calcium ionophore-induced acrosome reaction correlates with fertilization rates *in vitro* in patients with teratozoospermic semen. Hum Reprod 13:905–910.
Llanos M, Vigil P, Salgado AM, Morales P (1993) Inhibition of the acrosome reaction by trypsin inhibitors and prevention of penetration of spermatozoa through the human zona pellucida. J Reprod Fertil 97:173–178.
Luconi M, Bonaccorsi L, Krausz C, Gervasi G, Forti G, Baldi E (1995) Stimulation of protein phosphorylation by platelet-activating factor and progesterone in human spermatozoa. Mol Cell Endocrinol 108:35–42.
Mack SR, De Jonge C, Bielfeld P, Zaneveld LJ (1990) Acrosome reaction of human spermatozoa: comparative evaluation by triple stain and electron microscopy. Mol Androl 2:265–279.
Matson Ph, Yovich J, Edirisinghe W, Junk S, Yovich J (1995) An argument for the past and continued use of pentoxifylline in assisted reproductive technology. Hum Reprod 10:67–71.
Mortimer D, Camenzind AR (1989) The role of follicular fluid in inducing the acrosome reaction of human spermatozoa incubated *in vitro*. Hum Reprod 4:169–174.
Mortimer D, Curtis EF, Miller RG (1987) Specific labelling by peanut agglutinin of the outer acrosomal membrane of the human spermatozoon. J Reprod Fertil 81:127–135.
Moutaffian H, Parinaud J (1995) Selection and characterization of human acrosome reacted spermatozoa. Hum Reprod 10:2948–2951.
Naz RK, Ahmad K, Kumar R (1991) Role of membrane phosphotyrosine proteins in human spermatozoa function. J Cell Sci 99:157–165.
Negri P, Grechi E, Tomasi A, Fabbri E, Capuzzo A (1996) Effectiveness of pentoxifylline in semen preparation for intrauterine insemination. Hum Reprod 11:1236–1239.
Oehninger B, Blackmore T, Morsheli I, Sueldo C, Acosta AA, Alexander NJ (1994) Defective calcium influx and acrosome reaction (spontaneous and progesterone-induced) in spermatozoa of infertile men with severe teratozoospermia. Fertil Steril 61:349–354.
Okabe M, Nagira M, Kawai Y, Matzno S, Mimura T, Mayumi T (1990) A human sperm antigen possibly involved in binding and/or fusion with zona free hamster eggs. Fertil Steril 54:1121–1126.
Pampiglione JS, Tan SL, Campell S (1993) The use of the stimulated acrosome reaction test as a test of fertilizing ability in human spermatozoa. Fertil Steril 59:1280–1128.
Parinaud J, Labal B, Vieitez G (1992) High progesterone concentrations induce acrosome reaction with a low cytotoxic effect. Fertil Steril 58:599–602.
Parinaud J, Moutaffian H, Vieitez G, Richoiley G, Labal B (1995) Relevance of acrosome function in the evaluation of semen *in vitro* fertilizing ability. Fertil Steril 63:598–603.
Paul M, Sumpter JP, Lindsay KS (1996) The paradoxical effects of pentoxifylline on the binding of spermatozoa to the human zona pellucida. Hum Reprod 11:814–819.
Perry RL, Barratt CL, Warren MA, Cooke ID (1996) Comparative study of the effect of human cervical mucus and a cervical mucus substitute, Healonid, on capacitation and the acrosome reaction of human spermatozoa *in vitro*. Hum Reprod 11:1055–1062.
Pilikian S, Guerin JF, Adeleine P, Ecochard R, Czyba JC (1992) Spontaneous and ionophore induced acrosome reaction in asthenozoospermic infertile semen. Hum Reprod 7:991–992.
Purvis K, Rui H, Scholberg A, Hesta S, Clausen OP (1990) Application of flow cytometry to studies on the human acrosome. J Androl 11:361–366.
Sánchez R, Töpfer-Petersen E, Aitken RJ, Schill WB (1991) A new method for evaluation of the acrosome reaction in viable human spermatozoa. Andrologia 23:197–203.
Schill WB (1974) Quantitative determination of acrosin activity in human spermatozoa. Fertil Steril 25:703–712.
Schill WB (1991) Some disturbances of acrosomal development and function in human spermatozoa. Hum Reprod 6:969–978.
Sharma RK, Padron OF, Agrawal A (1997) Usefulness of the acrobead test in evaluating human acrosome function in fresh and cryopreserved sperm. J Urol 157:1692–1696.
Stock CE, Bates R, Lindsay KS, Edmonds DK, Fraser LR (1989) Human oocyte-cumulus complexes stimulate the human acrosome reaction. J Reprod Fertil 86:723–730.
Storey BT (1991) Sperm capacitation and the acrosome reaction. In: The Male Germ Cell: Spermatogonium to Fertilization. Robaire B (ed). NY Academy of Sciences, New York, pp 459–473.
Takahashi K, Wetzels AM, Bastiaans BA, Jansen HJ, Rolland R (1992) The kinetics of the acrosome reaction of human spermatozoa and its correlation with *in vitro* fertilization. Fertil Steril 57:889–894.
Takano H, Yanagimashi R, Ursh U (1993) Evidence that acrosin activity is important for the development of fusibility of mammalian spermatozoa with oolemma: inhibitors studies using the golden hamster. Zygotes 1:79–91.
Talbot P, Chacon RS (1980) A new procedure for rapidly scoring acrosome reactions of human sperm. Gamete Res 3:211–216.
Talbot P, Chacon RS (1981) A triple-stain technique for evaluating normal acrosome reactions of human sperm. J Exp Zool 215:201–208.
Tao J, Du JY, Critser ES, Critser JK (1993) Assessment of the acrosomal status and viability of human spermatozoa simultaneously using flow cytometry. Hum Reprod 8:1879–1885.
Tarlatzis BC, Pazaitou K, Billi H, Papadimas J, Lagos S, Spanos S, Mantalenakis S (1993) Growth hormone, oestra-

diol progesterone, testosterone concentrations in follicular fluid after ovarian stimulation with various regimes for assisted reproduction. Hum Reprod 8:1612–1616.

Tasdemir M, Tasdemir I, Kodama H, Tanaka T (1993) Pentoxifylline-enhanced acrosome reaction correlates with fertilization *in vitro*. Hum Reprod 8:2102–2107.

Tesarik J (1985) Comparison of acrosome reaction-inducing activities of human cumulus oophorus, follicular fluid and ionophore A23187 in human sperm populations of proven fertilizing ability *in vitro*. J Reprod Fertil 74:383–388.

Tesarik J (1989) Appropriate timing of the acrosome reaction is a major requirement for the fertilizing spermatozoon. Hum Reprod 4:95–98.

Tesarik J, Mendoza C (1993) Sperm treatment with pentoxifylline improves the fertilizing ability in patients with acrosome reaction insufficiency. Fertil Steril 60:141–148.

Tesarik J, Mendoza C (1995) Alleviation of acrosome reaction prematurity by sperm treatment with egg yolk. Fertil Steril 63:153–157.

Tesarik J, Drahorad J, Peknicova J (1988) Subcellular immunochemical localization of acrosin in human spermatozoa during the acrosome reaction and zona pellucida penetration. Fertil Steril 50:133–137.

Tesarik J, Mendoza C, Carreras A (1992) The effect of phosphodiesrerase inhibitors caffeine and pentoxifylline on spontaneous and stimulus induced acrosome reactions in human sperm. Fertil Steril 58:1185–1190.

Tomiyama T, Ohashi K, Tsutsui T, Saji F, Tanizawa O (1995) Acrosome reaction induced in a limited population of human spermatozoa by progesterone (Ca^{2+}-dependent) and ATP (Ca^{2+}-independent). Hum Reprod 10:2052–2055.

Tournaye H, Janssens R, Verheyen G, Devroey P, Van Steirteghem A (1994) *In vitro* fertilization in couples with previous fertilization failure using sperm incubated with pentoxifylline and 2-deoxyadenosine. Fertil Steril 62:574–579.

Uhler ML, Leung A, Chan SYW, Schmid I, Wang C (1992) Direct effects of progesterone and antiprogesterone on human sperm hyperactivated motility and acrosome reaction. Fertil Steril 58:1191–1198.

Uhler ML, Leung A, Chan SYW, Schmid I, Wang C (1993) Assessment of human sperm acrosome reaction by flow cytometry: validation and evaluation of the method by fluorescence-activated cell sorting. Fertil Steril 60:1076–1081.

Van Duin M, Polman JEM, De Breet ITM, Van Ginneken K, Bunschoten H, Grootenhuis A, Brindle J, Aitken RJ (1994) Recombinant human zona pellucida protein ZP3 produced by Chinese hamster ovary cells induces the human sperm acrosome reaction and promotes sperm-egg fusion. Biol Reprod 51:607–617.

Visconti PE, Moore GE, Bailey JL, Leclerc P, Connors SA, Pan D, Olds-Clarke P, Kopf GS (1995) Capacitation of mouse spermatozoa. II. Protein tyrosine phosphorylation and capacitation are regulated by a cAMP-dependent pathway. Development 121:1139–1150.

Wassarman PM (1994) Gamete interactions during mammalian fertilization. Theriogenology 41:31–44.

Wolf DP, Boldt J, Byrd W, Bechtol DB (1985) Acrosomal status evaluation in human ejaculated sperm with monoclonal antibodies. Biol Reprod 32:1157–1162.

Yanagimachi R (1994) Mammalian fertilization. In: The Physiology of Reproduction. Knobil E, Neill JD (eds). Raven Press, New York, pp 189–317.

Yovich J, Edirisinghe N, Cummins J, Yovich J (1988) Preliminary results using pentoxifylline in a pronuclear stage tubal transfer (PROST) program for severe male factor infertility. Fertil Steril 50:179–181.

Zaneveld LJ, Anderson RA, Mack SR, De Jonge CJ (1993) Mechanism and control of the human sperm acrosome. Hum Reprod 8:2007–2008.

Zhu J, Barratt CLR, Lippes J, Pacey AA, Cooke ID (1994) The sequential effects of human cervical mucus, oviductal fluid, and follicular fluid on sperm function. Fertil Steril 61:1129–1135.