

Analysis of the Responses of Human Spermatozoa to A23187 Employing a Novel Technique for Assessing the Acrosome Reaction

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ABSTRACT: Protocols for the use of A23187 in assessing the ability of human spermatozoa to acrosome-react and exhibit sperm-oocyte fusion have been developed and the results compared in two independent cohorts of infertile patients. Both bioassays were found to depend upon such factors as the dose and formulation of A23187, the duration of exposure, and the amount and type of protein used to supplement the medium. An optimal protocol for the hamster oocyte penetration test comprised a 3-hour exposure to 1.25–2.5 μ M ionophore and gave penetration rates of $93.2 \pm 3.2\%$ (11.26 ± 1.27 sperm/egg) for a group of 33 fertile donors compared with $63.0 \pm 5.4\%$ (4.73 ± 0.81 sperm/egg) for a cohort of 56 patients consulting for infertility ($P < 0.001$). Higher doses (5.0–10.0 μ M) of A23187 caused an inhibition of sperm-oocyte fusion in association with a loss of motility, although the integrity of sperm plasma membrane did not appear to be compromised and high rates (~80%) of acrosome reaction were observed. A protocol for assessing the abil-

ity of viable human spermatozoa to acrosome-react in response to A23187 was developed, employing a fluorescein-conjugated lectin in concert with the hypoosmotic swelling test, which gave values of $20.1 \pm 2.6\%$ and $13.6 \pm 1.6\%$ for groups of fertile donors ($n = 29$) and infertile patients ($n = 32$) respectively ($P < 0.05$). Although only acrosome-reacted spermatozoa were capable of fusing with zona-free hamster oocytes, there was no significant correlation between the proportion of acrosome-reacted cells and the levels of sperm-oocyte fusion observed in two independent groups of patients, indicating that these bioassays are measuring different aspects of human sperm function. These results have implications for the way in which the responses of human spermatozoa to ionophore treatment are quantified and interpreted.

Key words: A23187, acrosome reaction, oocyte fusion, human sperm.

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A major difficulty encountered in the design of sperm function tests for the diagnosis of male infertility is the inability of human spermatozoa to exhibit spontaneous, synchronized capacitation *in vitro*. This problem is exemplified by the low, variable rates of activity recorded in assays such as the zona-free hamster oocyte penetration test and the measurement of hyperactivated motility, both of which depend upon the ability of human spermatozoa to capacitate in simple balanced salt solutions *in vitro* (Yanagimachi et al, 1976; Yanagimachi, 1984; Aitken, 1986; Robertson et al, 1988). Furthermore, the rate at which human spermatozoa spontaneously acrosome-react in such media does not exceed 10%, even after 24 hours of incubation (Stock and Fraser, 1987). Although capacitation and the acrosome reaction rate can be enhanced by the addition of complex biological fluids such as maternal cord serum or follicular fluid (Suarez et al, 1986; Burkman, 1990), the uncontrolled nature of such

components precludes their use for the development of defined diagnostic systems. The preservation of human spermatozoa at low temperatures in Tris TES egg-yolk buffers has also been proposed as a mechanism for promoting the rates of sperm-oocyte fusion (Johnson et al, 1984). However, the permanent changes in membrane organization that occur as spermatozoa are taken below their phase transition temperature(s) (Holt and North, 1984) confounds the interpretation of test results obtained under such circumstances. Pharmacological approaches to the enhancement of sperm capacitation in the context of the hamster egg penetration test have focused on the use of phosphodiesterase inhibitors (Aitken et al, 1983a), calcium deprivation strategies incorporating strontium (Mortimer et al, 1986, 1988a), and raised osmolarity (Aitken et al, 1983b). Although such strategies certainly elevate the levels of sperm-oocyte fusion observed in this bioassay, uncertainties concerning the mechanism of action of such reagents may cause concern over their biological relevance. Moreover the diagnostic potential of such modified versions of the zona-free hamster oocyte penetration test has not yet been established in prospective clinical trials.

A more rational approach to the stimulation of human

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sperm function for diagnostic purposes, would be to try to emulate those cellular changes that are thought to precipitate the acrosome reaction *in vivo*, namely an influx of extracellular calcium and a rise in intracellular pH (Fraser and Ahuja, 1988; Garbers, 1989). Both of these changes can be induced artificially by the divalent cation ionophore A23187, which has been recognized as a potent stimulus for the activation of mammalian spermatozoa for more than a decade (Summers et al, 1976; Talbot et al, 1976).

Addition of A23187 to suspensions of human spermatozoa induces the acrosome reaction and high rates of sperm-oocyte fusion in the hamster egg penetration test (Russell et al, 1979; Aitken et al, 1984; Byrd and Wolf, 1986; Byrd et al, 1989; De Jonge et al, 1989; Mortimer et al, 1989; Ford et al, 1991). Furthermore, the rates of sperm-oocyte fusion observed in the presence of ionophore have been shown to correlate with the fertilizing capacity of human spermatozoa *in vivo*, in both a retrospective analysis of the fertility of donors employed in a donor insemination service (Irvine and Aitken, 1986) and a prospective study of the incidence of spontaneous pregnancy in untreated couples characterized by a normal female factor (Aitken et al, 1991). The diagnostic potential of sperm function tests involving A23187 has also been demonstrated in the context of *in vitro* fertilization therapy, using either the acrosome reaction or sperm-oocyte fusion as an end-point for such bioassays (Aitken et al, 1987; Cummings et al, 1991).

A major problem with A23187 is that it is cytotoxic at high doses and so the concentration employed has to be carefully regulated. It is also highly autofluorescent, necessitating the development of different formulations of A23187 for situations in which the biological responses of the spermatozoa are to be recorded in the presence of fluorescent probes to monitor second messengers, such as calcium or pH. Despite the diagnostic utility of A23187, there is considerable disparity in the existing literature with respect to the dose and formulation of this compound that should be used to stimulate spermatozoa, with values as extreme as 100 μM of the $\text{Ca}^{2+}/\text{Mg}^{2+}$ salt and 2 μM of the free acid being employed in different studies. An analysis of the factors influencing the bioactivity of A23187 in sperm function tests has therefore been undertaken and protocols for the use of this reagent in assays of sperm-oocyte fusion and the acrosome reaction described. Finally, the hypothesis that simple, possibly automated (Fenichel et al, 1989), assessments of the acrosome reaction might substitute for complex bioassays of sperm-oocyte fusion in monitoring the responses of human spermatozoa to A23187 (Byrd et al, 1989) has been investigated in independent cohorts of patients consulting for infertility and characterized by the absence of any detectable pathology in the female partner.

Materials and Methods

Sperm Preparation

Semen specimens were collected into sterile plastic containers and transported to the laboratory for analysis with 1 hour of production. The donor population included 33 fertile individuals who had fathered children within the past 3 years and a cohort of 56 patients who were characterized by infertility of at least 18 months duration despite a normal female partner, as assessed by history and examination, normal luteal phase progesterone levels and a diagnostic laparoscopy during which tubal patency was established by the passage of dye into the abdominal cavity (Aitken et al, 1991). The validity of the results obtained was confirmed in an independent study population, comprising 33 fertile donors and 32 infertility patients.

Initially, a conventional semen profile was constructed on each ejaculate using the criteria laid down by the World Health Organization (1987). The spermatozoa were then separated from the seminal plasma by discontinuous Percoll® gradient centrifugation using a two-step gradient comprising a 3-ml layer of 80% Percoll overlaid with 3 ml of 40% Percoll (Aitken and Clarkson, 1988). Isotonic Percoll® was created by supplementing 10 ml of 10 \times concentrated medium 199 (Flow Laboratories, Irvine, Scotland) with 300 mg bovine serum albumin (BSA) or the human serum albumin preparation 'Albuminar®' (Armour Pharmaceutical Co., Eastbourne, UK), 3 mg sodium pyruvate and 0.37 ml of a sodium lactate syrup and adding 90 ml of Percoll® (Pharmacia, Uppsala, Sweden). This preparation was designated 100% Percoll® (Lessley and Garner, 1983) and was subsequently diluted with HEPES-buffered medium BWB (Biggers et al, 1971; Aitken and Clarkson, 1988) supplemented with BSA or Albuminar®, as indicated. After a 20-min period of centrifugation at 500 \times g, the spermatozoa at the 40%:80% interface and at the base of the 80% fraction were collected separately, washed with a 5-ml volume of medium BWB and finally resuspended in BWB at a concentration of 20 \times 10⁶/ml.

Hamster Oocyte Penetration Test

The isolated spermatozoa were diluted 1:1 with the ionophore A23187 formulated as the free acid, the $\text{Ca}^{2+}/\text{Mg}^{2+}$ salt or the 4-bromo derivative. A23187-free acid and 4-bromo-A23187 were prepared as 100 mM stock solutions in DMSO and then diluted to 1 mM with BWB. Such 1-mM solutions of A23187 could be kept at 4°C for 4–6 weeks, without loss of bioactivity and were diluted to the final working concentration immediately before the initiation of each experiment. The $\text{Ca}^{2+}/\text{Mg}^{2+}$ salt was prepared immediately before use as an aqueous suspension, as described by Aitken et al. (1984). The spermatozoa were incubated with each formulation of ionophore for 3 hours at 37°C in an atmosphere of 5% CO_2 in air and were then pelleted by centrifugation at 500 \times g, resuspended in the same volume of fresh medium BWB, and distributed as 50- μl droplets under liquid paraffin.

Zona-free hamster oocytes were prepared as described in the original publication of Yanagimachi et al (1976) and dispensed into the droplets at 5 oocytes/drop and 15–20 oocytes/sample.

After a further 3 hours, the oocytes are recovered from the

droplets, washed free of loosely adherent spermatozoa, compressed to a depth of about 30 μm under a 22 \times 22-mm coverslip on a glass slide, and assessed for the presence of decondensing sperm heads with an attached or closely associated tail by phase-contrast microscopy. The number of spermatozoa penetrating each egg was assessed and the results expressed as the percentage of oocytes penetrated and the mean number of spermatozoa penetrating each oocyte (total number of penetrations/total number of oocytes). In order to compensate for differences in percentage motility between samples, the rates of sperm-oocyte fusion that would have been observed at a constant sperm concentration of $5 \times 10^6/\text{ml}$ were calculated using the Poisson distribution model introduced by Aitken and Elton (1984, 1986a,b).

Acrosome Reaction

At the end of the 3-hour incubation period with A23187, a 200- μl volume of the sperm suspension was removed, pelleted by centrifugation at $500 \times g$ for 5 minutes, and resuspended in the same volume of fresh BWW, prior to the assessment of sperm motility and the acrosome reaction. The protocol developed for assessing the acrosome reaction involved the use of a detection reagent targeting the acrosomal region of the sperm head, in conjunction with the hypoosmotic swelling test (Jeyendran et al, 1984) to monitor sperm viability. For this procedure, 50 μl of this sperm suspension was added to 500 μl of hypoosmotic swelling medium, comprising 7.35 g sodium citrate and 13.51 g fructose in 1 L of distilled water (Jeyendran et al, 1984), and incubated for 1 hour at 37°C. At the end of this period the spermatozoa were pelleted by centrifugation at $500 \times g$ for 5 minutes and resuspended in 50 μl of ice-cold methanol. Ten microliters of the fixed cells were subsequently pipetted onto the well of a four-spot Henley slide (C. A. Henley Ltd., Loughton, England) and allowed to dry. The wells were then overlaid with fluorescein-conjugated peanut lectin (*Arachis hypogaea*: Sigma Chem. Co., MO) at a concentration of 2 mg/ml and incubated for 15 minutes in the dark (Mortimer et al, 1988b). The excess lectin was then gently removed by washing the slides with Dulbecco's phosphate-buffered saline (Flow Laboratories, Irvine, Scotland) and visualized under a fluorescence microscope in the presence of an anti-quenching agent (Citifluor, London, UK). The spermatozoa were classified as non-acrosome-reacted, if the acrosomal region of the sperm head exhibited a uniform bright fluorescence and the sperm tails adapted the coiled configuration typical of viable cells (Fig. 1). However, if the acrosomal region of such viable cells exhibited a punctate labeling pattern or restriction of the fluorescence to the equatorial segment of the sperm head, then they were classified as undergoing the acrosome reaction (Mortimer et al, 1988b). Spermatozoa with straight tails were considered to be nonviable and were not included in the analysis (Fig. 1).

Statistics

All experiments were replicated at least 3 \times and the statistical significance of the results assessed by analysis of variance and, for independent groups, the *t*-test, using the Statview and Statworks program on an Apple Macintosh SE/30 computer. A value of $P < 0.05$ was considered statistically significant.

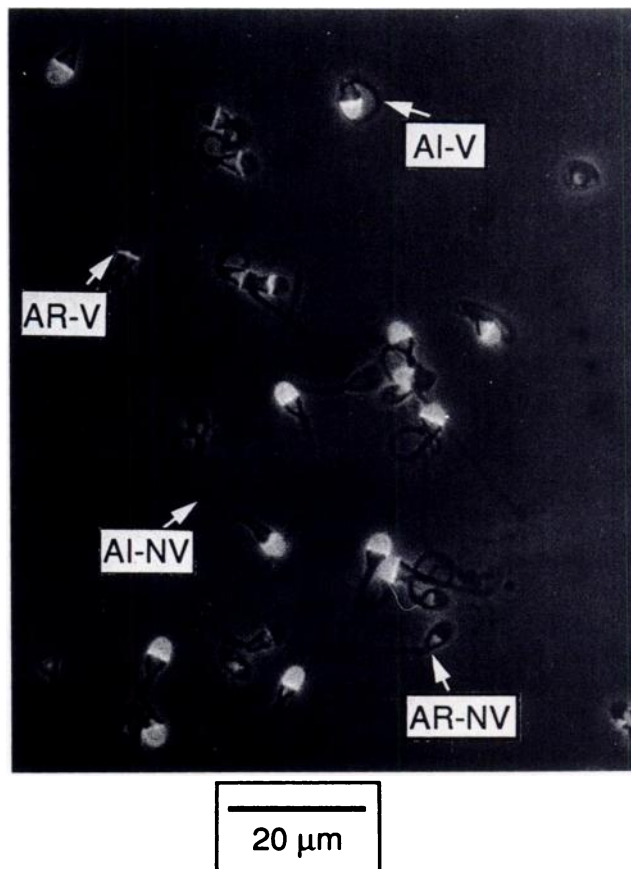


FIG. 1. Combination of a fluorescein-labeled lectin (*Arachis hypogaea*) and the hypoosmotic swelling test to monitor the acrosome reaction in populations of human spermatozoa. Four types of cells can be identified under these conditions: **AI V**, the acrosome is intact and exhibits bright fluorescence while the cell is viable, exhibiting a curled tail following exposure to hypoosmotic medium. **AR V**, the acrosome reaction is evident and the fluorescence is now confined to the equatorial segment of the sperm head or, occasionally, may have disappeared altogether; the curling of the sperm tail confirms the viability of the spermatozoa. Alternatively, the acrosome may be intact (**AI NV**) or have been lost (**AR NV**), but the cells are nonviable as indicated by the failure of the sperm tail to adopt a coiled configuration following exposure to hypoosmotic medium; such cells are excluded from the analysis when calculating an acrosome reaction rate.

Results

Hamster Oocyte Penetration Assay

A23187 Formulation and Dose Dependency—Previous studies had established that aqueous suspensions of A23187, formulated as the $\text{Ca}^{2+}/\text{Mg}^{2+}$ salt, were optimally active in this bioassay at concentrations of 50 and 100 μM (Aitken et al, 1984). Using this response to the A23187 salt as a guide, the influence of A23187 free acid on human sperm function was assessed in dose-dependent studies (Figs. 2, 3). The results revealed that although the free acid was competent to stimulate high levels of sperm-oocyte fusion, this response was highly concentration dependent. At doses above 5.0 μM both the motility of the

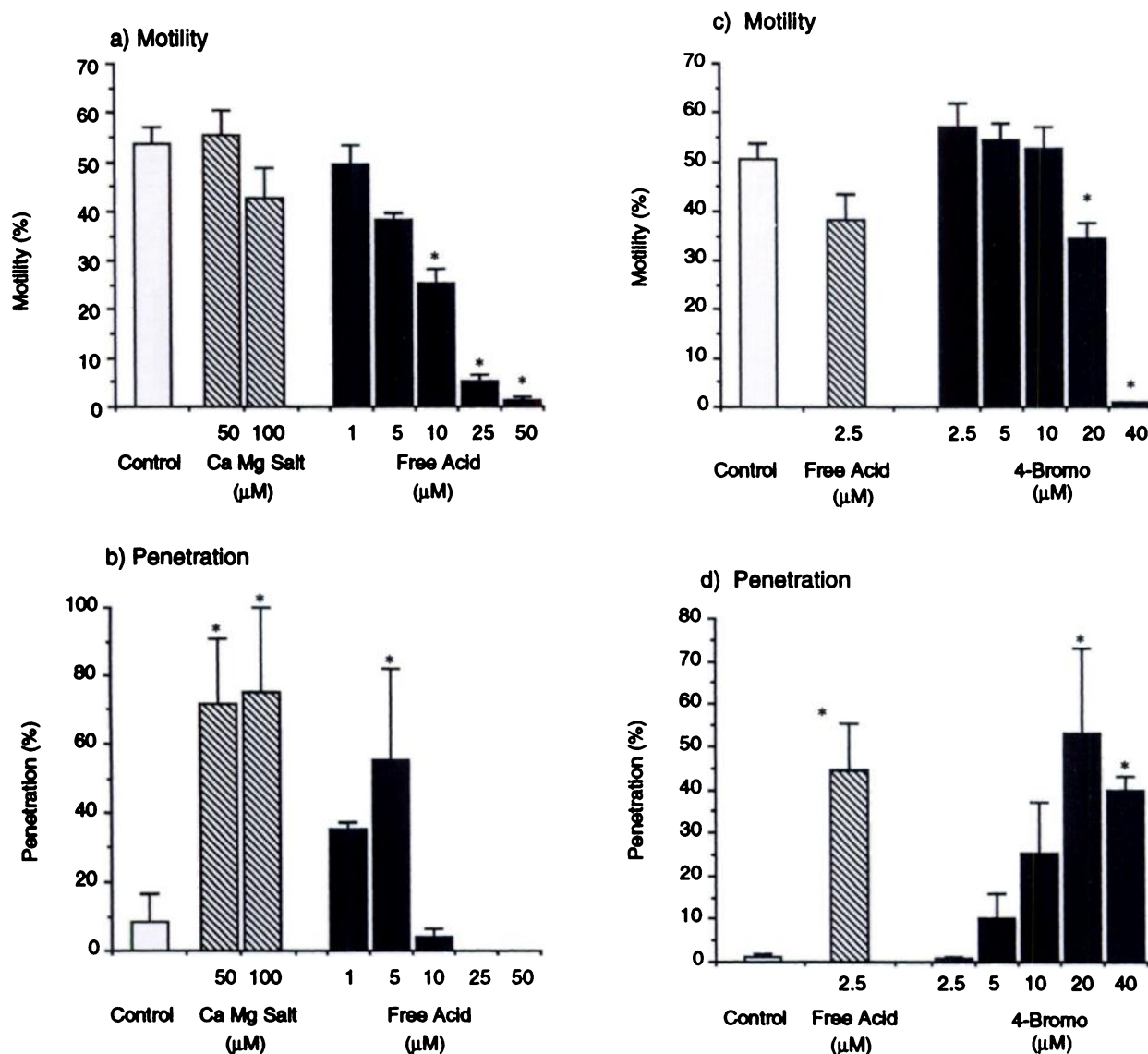


FIG. 2. Influence of A23187 formulation on the motility of human spermatozoa and their capacity for sperm-oocyte fusion. Comparison of the motility and percentage of hamster oocytes penetrated following stimulation with: (a, b) A23187 as the $\text{Ca}^{2+}/\text{Mg}^{2+}$ salt or the free acid and (c, d) A23187 as the free acid or 4-bromo formulation. * $P < 0.05$.

spermatozoa and their capacity for sperm-oocyte fusion were considerably diminished. At concentrations of 1 and 5 μM , the cytotoxicity of the free acid was less evident and the levels of sperm-oocyte fusion approached those observed with the $\text{Ca}^{2+}/\text{Mg}^{2+}$ salt (Fig. 2a,b). A more detailed dose-response analysis for the free acid established that the optimal concentration of reagent for the stimulation of sperm-oocyte fusion lay between 1.25 and 2.5 μM (Fig. 3a,b).

The decision to use the $\text{Ca}^{2+}/\text{Mg}^{2+}$ salt of A23187 in our original studies (Aitken et al, 1984) was based upon the fact that this particular formulation exhibited less autofluorescence than the free acid. This property was of value in studies where sperm-oocyte fusion was being assessed in the presence of fluorescent probes to monitor

intracellular second messengers, such as calcium. In the meantime, new, nonfluorescent formulations of A23187, such as the 4-bromo derivative, have been specifically designed with this application in mind. In view of the potential value of 4-bromo A23187 in studies of sperm cell biology, dose-dependent studies were conducted with this compound in the zona-free hamster oocyte penetration assay (Fig. 2c,d). These studies revealed that, on a molar basis, the 4-bromo formulation was approximately 10 \times less biologically active than the free acid, requiring a dose of 20 μM to generate a level of sperm-oocyte fusion equivalent to that observed with 2.5 μM A23187 free acid (Fig. 2d).

Influence of Protein Supplement—The nature of the protein source used to supplement the culture medium

was found to have a significant effect on the ability of A23187 to stimulate sperm-oocyte fusion. Hence, addition of human serum albumin to Albuminar®-supplemented medium to increase the protein concentration from 0.3 to 3.0%, decreased ($P < 0.001$) the penetration rates observed from $67.0 \pm 9.7\%$ (1.63 ± 0.48 sperm/egg) to $5.3 \pm 2.9\%$ (0.09 ± 0.05 sperm/egg). Furthermore, changing the protein supplement from Albuminar® to BSA resulted in a shift in the dose-response curve to the left (Fig. 3a,b), such that the optimal dose of $2.5 \mu\text{M}$ observed with Albuminar® shifted to $1.25 \mu\text{M}$ in the presence of BSA. The enhanced sensitivity of the spermatozoa to the cytotoxic effects of A23187 in BSA-supplemented media was also reflected in the motility of these cells, Albuminar® consistently supporting higher levels of motility than BSA (Fig. 3c).

Interindividual Variation in Dose-Responsiveness—In addition to the modulating influence of factors such as protein supplementation on the dose-response to A23187 within samples, some variation was also noted between samples in the concentration dependency of sperm-oocyte fusion. Hence in a cohort of 56 patients monitored with the hamster oocyte penetration assay at 1.25 and $2.5 \mu\text{M}$ A23187 free acid, the greatest response was obtained at the lower dose on 19 occasions and at the higher dose in the remaining 37, giving an optimal mean penetration rate of $63.0 \pm 5.4\%$ (4.73 ± 0.81 sperm/egg) for this group of patients. A cohort of 33 fertile donors screened under identical conditions gave the greatest response to the lower dose of A23187 on 10 occasions and produced an optimal mean penetration rate of 93.2 ± 3.2 (11.26 ± 1.27 sperm/egg), which was significantly greater ($P < 0.001$) than that observed in the patient population. In addition to revealing a significant difference between fertile donors and the patient population in the responsiveness of the spermatozoa to A23187, such results also emphasize the value of screening semen specimens at more than one dose of ionophore.

Acrosome Reaction

Dose and Time Dependency—Using the combination of fluorescein-conjugated lectin and hypoosmotic swelling test to monitor the acrosome reaction, time- and dose-dependent analyses of the responses to A23187 free acid were undertaken. At a fixed dose of $2.5 \mu\text{M}$, time-dependent analyses revealed a significant ($P < 0.05$) elevation of acrosome reaction rates within 30 minutes that plateaued after 1 hour. A dose-dependent analysis was then conducted employing a fixed exposure time of 3 hours, on the same samples that were used to establish the concentration dependency of hamster oocyte penetration (Fig. 3). In complete contrast to the oocyte penetration assay, where the loss of motility observed with doses of A23187 above $5 \mu\text{M}$ was associated with a dramatic decline in the

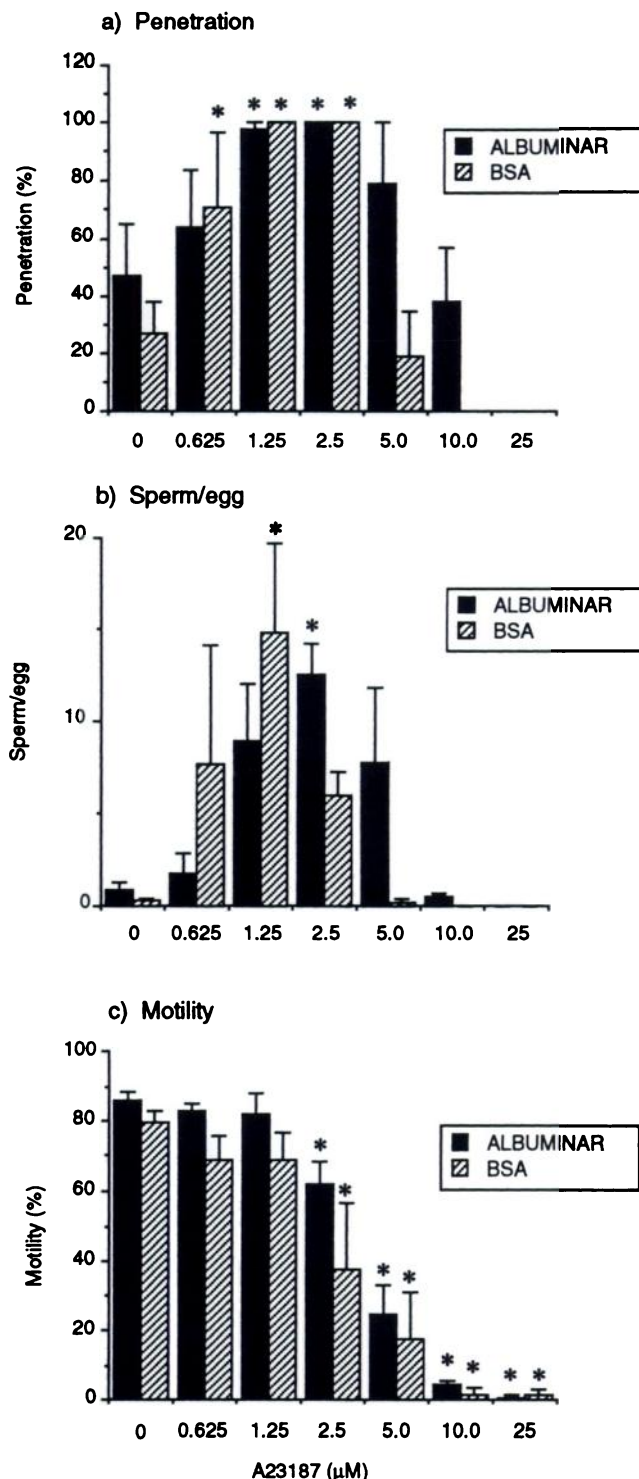


FIG. 3. Dose-dependent analysis of the influence of A23187 free acid on (a) the percentage of hamster oocytes penetrated, (b) the mean number of sperm penetrating each oocyte, and (c) percentage motility in media supplemented with either Albuminar® or BSA. * $P < 0.05$.

rates of sperm-oocyte fusion, the percentage of acrosome-reacted cells increased in a dose-dependent manner with concentrations of ionophore up to $10 \mu\text{M}$ (Fig. 4). At this point more than 80% of the spermatozoa had lost their

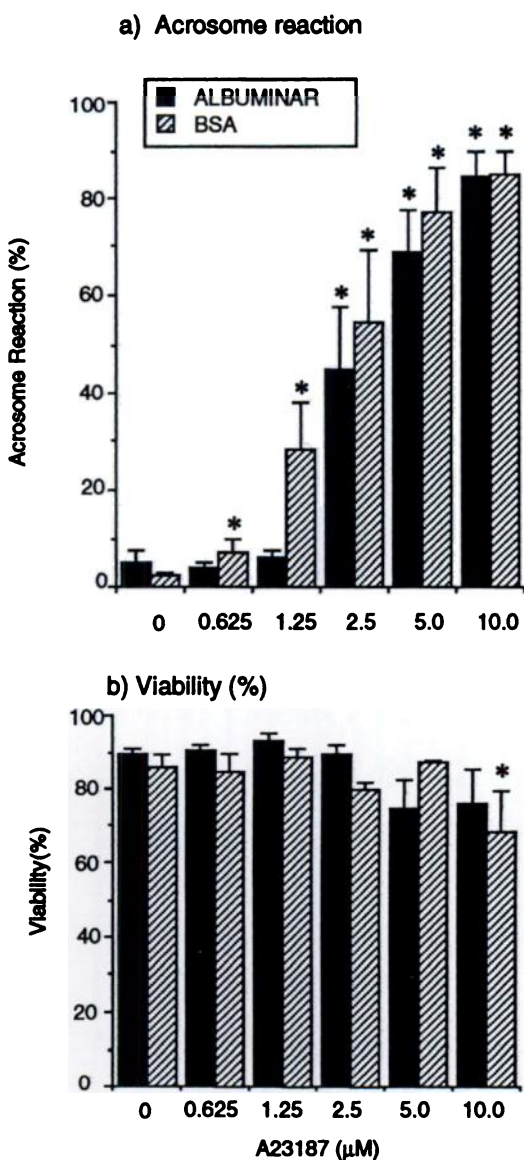


FIG. 4. Influence of A23187 free acid on acrosome reaction rates when the media were supplemented with either Albumin[®] or BSA. (a) Acrosome reaction assessed on only those cells that appeared viable according to the hypoosmotic swelling test. (b) Viability of the total sperm population according to this test. * $P < 0.05$.

acrosomes and, despite their lack of motility, had suffered no loss of structural integrity according to the hypoosmotic swelling test (Fig. 4).

Influence of Protein Supplement—The acrosome reaction rates observed in response to A23187 were, like sperm–oocyte fusion, influenced by the protein supplement used in the preparation of the medium, the presence of BSA shifting the dose–response to the left compared with Albumin[®] (Fig. 4). Hence in the presence of BSA, but not Albumin[®], a significant increase in acrosome reaction rates over control levels was observed with as little as 0.625 μM A23187. At 1.25 μM A23187, the acrosome reaction rate in the presence of BSA had increased

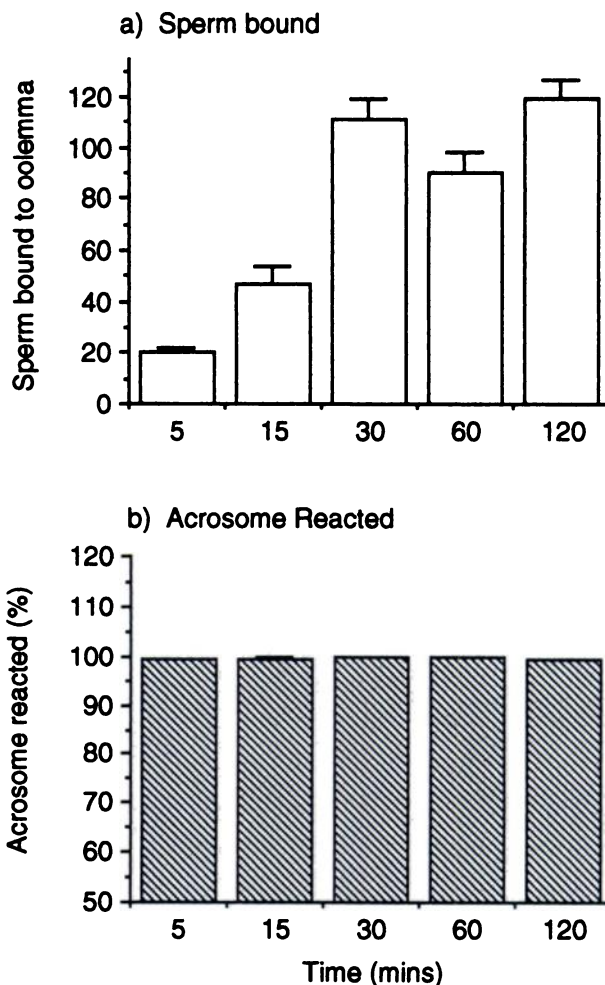


FIG. 5. Analysis of (a) the number of spermatozoa bound to the surface of zona-free hamster oocytes against time, and (b) the percentage of such spermatozoa that are acrosome-reacted.

to $28.3 \pm 9.5\%$ while, at the same dose, the rate supported by Albumin[®] supplementation ($6.0 \pm 1.1\%$) was not significantly different from the control. It was only at A23187 concentrations of 2.5 μM and above, that acrosome reaction rates in the presence of Albumin[®] also became significantly elevated (Fig. 4).

Acrosome Reaction and Hamster Oocyte Fusion

A relationship between the outcome of the hamster oocyte penetration assay and assessments of the acrosome reaction might be anticipated, since the original studies of Yanagimachi (Yanagimachi et al, 1976; Yanagimachi, 1984) had indicated that only acrosome-reacted spermatozoa could recognize and fuse with the vitelline membrane of the oocyte. Analysis of the acrosomal status of spermatozoa bound to the surface of 180 zona-free hamster oocytes in a time-dependent study clearly confirmed this point (Fig. 5).

Analysis of the relationship between acrosome reaction rate and the outcome of the hamster oocyte penetration test is presented in Fig. 6. These data were obtained in a cohort of 61 samples comprising 29 fertile donors and 32 patients characterized by a lack of detectable pathology in the female partner. In these samples, simultaneous assessments of acrosome reaction rates and hamster oocyte penetration following stimulation with A23187 were obtained on samples isolated from the 40%/80% Percoll® interface ($n = 28$) and pelleting at the base of the 80% Percoll® fraction ($n = 61$). Samples were screened at 1.25 and 2.5 μM A23187, and the optimal result was entered into the analysis.

Compared with the spermatozoa isolated on the 40%/80% Percoll® interface, the 80% Percoll® fractions were characterized by significantly elevated levels of sperm-oocyte fusion ($90.5 \pm 2.72\%$ vs. $34.01 \pm 7.01\%$; $P < 0.01$) and acrosome reaction ($17.04 \pm 1.56\%$ vs. $9.1 \pm 0.87\%$, $P < 0.01$). Within the 80% Percoll® fractions there was a significant difference between the fertile donors and the patients with respect to sperm-oocyte fusion ($93.2 \pm 3.6\%$ vs. $88.0 \pm 4.0\%$; $P < 0.001$) and acrosome reaction rates ($20.07 \pm 2.57\%$ vs. $13.6 \pm 1.6\%$; $P < 0.05$). Within the 40% fractions there was also a significant difference ($P < 0.001$) in the rates of sperm-oocyte fusion observed between the donor and patient populations ($48.5 \pm 11.7\%$ vs. $18.05 \pm 6.2\%$), although the acrosome reaction rates were very similar ($9.27 \pm 1.27\%$ vs. $8.5 \pm 1.3\%$). In neither Percoll® fraction was there a significant linear relationship between the capacity of a given sperm population to acrosome-react in response to A23187 and the subsequent rate of sperm-oocyte fusion (Fig. 6). The 80% Percoll® fraction, comprising the most motile, structurally normal spermatozoa in the ejaculate (Aitken and Clarkson, 1988), was characterized by samples that could give 100% sperm oocyte fusion despite acrosome reaction rates of less than 10%. Conversely nine samples from the 40%/80% Percoll® interface failed to fertilize any hamster oocytes whatsoever and yet exhibited acrosome reaction rates ranging from 1 to 13%. This lack of correlation between the capacity of human spermatozoa to acrosome-react and exhibit sperm-oocyte fusion was not due to the greater sensitivity of the latter to differences in sperm motility. If the outcome of the penetration assay was calculated in such a way as to compensate for any differences in sperm motility using the Poisson model (Aitken and Elton, 1984, 1986a,b), the lack of correlation with the acrosome reaction was still observed (Fig. 6c).

In order to verify these results, the relationship between the ability of human spermatozoa to acrosome-react and their capacity for sperm-oocyte fusion was reanalyzed using a different set of protocols in an independent study group comprising 33 fertile donors and 32 infertile patients. Using repeated ($3 \times$) centrifugation as the sperm

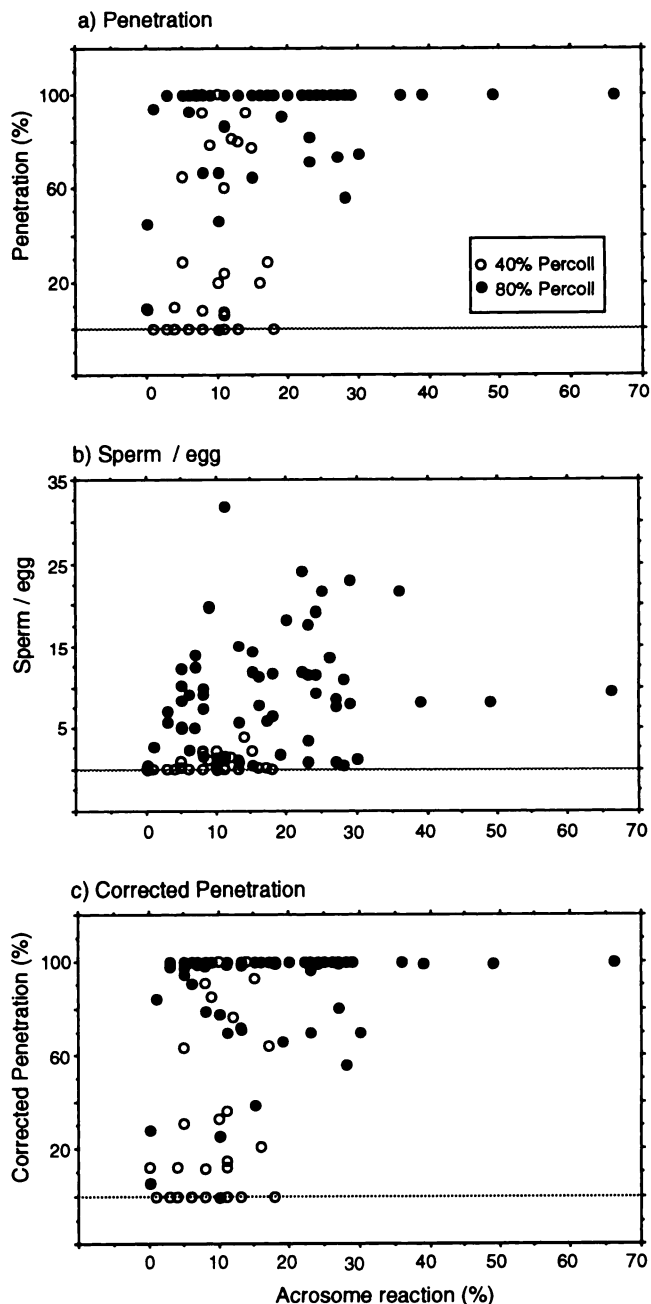


FIG. 6. Correlation between the percentage of spermatozoa undergoing the acrosome reaction and the capacity of the spermatozoa for sperm-oocyte fusion assessed as (a) the percentage of oocytes penetrated, (b) the mean number of spermatozoa penetrating each oocyte, and (c) percentage penetration corrected to a motile sperm concentration of $5 \times 10^6/\text{ml}$ (Aitken and Elton, 1984, 1986a,b).

preparation method, a 3-hour exposure to 50 μM A23187 ($\text{Ca}^{2+}/\text{Mg}^{2+}$ salt) as the stimulus, and the technique described by Wolf et al (1985) for assessing acrosomal status, no significant relationship was observed between the capacity of the spermatozoa for sperm-oocyte fusion and acrosome reaction rate ($r = 0.382$; $P > 0.05$). Hence, the variation between samples in their capacity to exhibit

sperm-oocyte fusion in response to A23187 cannot be accounted for on the basis of their capacity to acrosome-react, even though the latter is a prerequisite for fusion with the vitelline membrane.

Discussion

In light of recent results demonstrating the diagnostic potential of bioassays incorporating A23187 as a stimulant (Aitken et al, 1991), it is clearly important to define those factors influencing the biological potency of this compound toward human spermatozoa. The activity of A23187 in the bioassays featured in this study was shown to depend upon such factors as the dose and formulation of ionophore employed, the duration of exposure, and the type and concentration of protein used to supplement the medium. The influence of such variables on the performance of these bioassays should encourage individual laboratories to pay regard to such factors in validating their own optimal procedures. The modulating influence of several of these factors has previously been explored with respect to the acrosome reaction (Byrd and Wolf, 1986; Byrd et al, 1989; De Jonge et al, 1989), but it is worth repeating their importance in the context of the penetration test. For example, the ability of protein supplements to bind A23187 and thereby reduce its bioavailability has been clearly demonstrated by Byrd et al (1989). However, the present study has also emphasized the importance of the type of protein employed as well as its concentration, commercial preparations of Albuminar® being more active than BSA in this regard and necessitating the addition of higher concentrations of A23187 (2.5 instead of 1.25 μM) to induce a maximal biological response.

A comparative analysis of the potency of different A23187 formulations in the context of the zona-free hamster oocyte penetration assay has not previously been reported. For routine diagnostic purposes, maximal rates of sperm-oocyte fusion could be obtained with normal fertile specimens by employing a 3-hour exposure to 1.25 or 2.5 μM free acid, depending on whether Albuminar® or BSA was being used in the incubation medium. Higher doses of the free acid should not be used for diagnostic purposes because they have a profound effect on sperm motility over a 3-hour time course. If autofluorescence is to be avoided, because of a need to take simultaneous measurements of intracellular messengers using fluorescent probes, then alternative formulations, such as 4-bromo A23187, may be used. This material was found to be less biologically active than the free acid, necessitating an increase in the dose required to stimulate human spermatozoa from 2.5 μM to 20.0 μM . The development of the 4-bromo preparation obviates the need to use aque-

ous suspensions of the $\text{Ca}^{2+}/\text{Mg}^{2+}$ salt, which, although capable of stimulating human sperm function (Aitken et al, 1984; Mortimer et al, 1989), is difficult to suspend in a standardized manner and is subject to a batch-to-batch variation (Ford et al, 1991). This study has focused on A23187 because this particular ionophore is in common usage for the stimulation of human sperm function. However it should be noted that there are alternative reagents, such as ionomycin, which might also be worthy of detailed investigation, particularly because this compound does not suffer from the autofluorescence characteristic of A23187.

The influence of A23187 on acrosome reaction rates was examined using a novel protocol combining a fluorescent probe targeting the acrosomal membranes with the hypoosmotic swelling test to monitor sperm viability. The hypoosmotic swelling test provided a simple, convenient means of assessing the membrane integrity of spermatozoa that were being monitored for their acrosomal status. The need to assess the viability of spermatozoa, in order to differentiate physiological acrosome reactions from pathological acrosomal loss, has been recognized since the pioneering studies of Talbot and Chacon (1980) and Cross et al (1986). Both of these studies employed the principle of dye exclusion, trypan blue in the former and a DNA-sensitive fluorochrome in the latter, in order to assess membrane integrity. The hypoosmotic swelling test has a number of potential advantages over such dye exclusion techniques for monitoring sperm viability (Jeyendran et al, 1984; Schrader et al, 1986; Aitken, 1989), and it should provide a valuable alternative to more complex protocols involving the use of fluorochromes (Cross et al, 1986; Cross and Meizel, 1989) in monitoring the acrosome reaction.

In agreement with the results obtained by Moore et al (1987) the acrosome reaction was found to be a prerequisite for sperm binding to the vitelline membrane of the zona-free hamster oocyte. However, the variation between individuals in the acrosome reaction rates recorded following treatment was not correlated with the levels of sperm-oocyte fusion observed in the hamster oocyte penetration test. This lack of association did not appear to be due to the greater sensitivity of sperm-oocyte fusion to differences in sperm motility (Fig. 6c). Some samples, particularly those isolated from the low density Percoll fraction, were characterized by low rates of sperm-oocyte fusion despite acrosome reaction rates as high as 20%. In such cases there must be defects in the spermatozoa, possibly structural (Marsh et al, 1987), that prevent the acrosome-reacted spermatozoa from fusing with the vitelline membrane. Conversely, samples were recovered from the high density Percoll fraction that were competent to fertilize 100% of hamster oocytes, even though less than 10% of the sperm population had undergone the acrosome

reaction. In view of the high concentration of spermatozoa used in the hamster test ($10 \times 10^6/\text{ml}$) an acrosome reaction of 1% would still mean that the incubation medium contained 100,000 acrosome-reacted cells/ml. In this sense, the penetration assay is a more sensitive test of sperm function than the assessment of acrosome reactions, since the latter involves the sampling of only 100–200 cells, while the former is responding to the presence of acrosome-reacted, fusogenic spermatozoa in a 50- μl incubation droplet containing 500,000 cells.

The lack of a direct, linear correlation between acrosome reaction rates and the incidence of sperm–oocyte fusion observed in response to A23187 has important implications for the design of diagnostic tests to assess the functional competence of human spermatozoa. It is clear from the results presented here, and from results obtained using follicular fluid as the stimulant (Fukuda et al, 1989), that these two bioassays are measuring different aspects of human sperm function. The cytochemical assessment of acrosomal status is measuring the proportion of the viable sperm population that is competent to respond to the calcium and pH signals generated by A23187 by undergoing the acrosome reaction. In contrast, the penetration assay is assessing the capacity of the acrosome-reacted population to fuse with the vitelline membrane of the oocyte. Although the acrosome reaction is a prerequisite for sperm–oocyte fusion, the latter involves changes in the organization and biochemical composition of the plasma membrane that are necessary for sperm–oocyte fusion but are not reflected in the acrosome reaction rate *per se*. Hence, although the incidence of A23187-stimulated acrosome reactions has already been shown to be of significant prognostic value (Cummings et al, 1991), the diagnostic power of such tests would be significantly enhanced if coupled to the analysis of sperm–oocyte fusion.

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