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Calcium ionophore-induced acrosome reaction correlates with fertilization rates *in vitro* in patients with teratozoospermic semen

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The aim of this study was to determine the relationship between calcium ionophore A23187-induced acrosome reaction (AR) and sperm fertilizing ability. Semen samples remaining after preparation for standard IVF were studied in 109 patients who had sperm concentrations $\geqslant 20 \times 10^6$/ml. Ionophore-induced AR was performed on motile spermatozoa selected by centrifugation on a Percoll gradient. Semen analysis was performed using standard methods. Patients with higher (>50%, $n = 76$) fertilization rates had significantly higher ionophore-induced AR than patients with lower (<50%, $n = 33$) fertilization rates (49 ± 14 versus 38 ± 21%, $P < 0.05$). When the data from all patients were analysed by logistic regression, only the percentage sperm motility in insemination medium and ionophore-induced AR were significantly related to fertilization rates. Similar results were also obtained when the data from a subgroup of patients with poor (<15% normal) sperm morphology were analysed. However, when patients with normal sperm morphology ⩾15% were analysed separately, only sperm count and the percentage of spermatozoa with progressive motility in semen were significantly related to fertilization rates. In conclusion, ionophore-induced AR was significantly related to fertilization rates *in vitro* mainly in patients with teratozoospermic semen. Tests for ionophore-induced AR may provide additional information about sperm fertilizing ability but may not indicate specific defects of the physiological AR.

Key words: calcium ionophore/human acrosome reaction/IVF/sperm morphology

Introduction

The acrosome reaction (AR) is an important event during the process of human fertilization (Yanagimachi, 1994). Evidence suggests that the human physiological AR occurs on the surface of the zona pellucida (ZP) following sperm binding to the ZP (Cross *et al.*, 1988; Tesarik, 1989; Liu and Baker, 1994a, b). There is a strong correlation between ZP-induced AR and sperm–ZP penetration (Liu and Baker, 1996a). Disordered ZP-induced AR causes failure of sperm–ZP penetration and leads to infertility (Liu and Baker, 1994b). While the mechanism of ZP-induced AR is not completely understood, Ca^{2+} influx plays a major role in the signal transduction and effector processes of the AR (Yanagimachi *et al.*, 1994; O'Toole *et al.*, 1996a; Aitken, 1997; Breitbart and Spungin, 1997). Furthermore, protein kinase C appears to play an important role in both human ZP and progesterone-induced AR (O'Toole *et al.*, 1996b; Liu and Baker, 1997).

Assessment of the ability of spermatozoa to undergo the AR on the ZP is useful for detecting specific defects of sperm fertilizing ability (Liu and Baker, 1994b). However, routine assessment of ZP-induced AR is difficult because of the scarcity of human ZP material (Liu and Baker, 1994b). Although production of recombinant human ZP3 may in the future allow development of clinical tests for the human physiological AR, uniformly active material is not generally available (van Duin *et al.*, 1994; Barratt and Hornby, 1995; Brewis *et al.*, 1996, Whitmarsh *et al.*, 1996). Therefore, other biological and chemical stimuli such as progesterone, follicular fluid and calcium ionophore A23187 are still widely used for induction of the AR *in vitro* (Tesarik, 1985; Aitken *et al.*, 1993; Brucker and Lipford, 1995; ESHRE Andrology Special Interest Group, 1996, Tesarik *et al.*, 1996). Calvo *et al.* (1989; 1994a, b) reported that the ability of spermatozoa to undergo the AR following incubation with human follicular fluid was significantly correlated with fertilization rates *in vitro* and there was a clear difference in the distributions of AR induced by follicular fluid from fertile and infertile men. Because follicular fluid is a biological material which can have large variation in activity between different batches collected from different groups of patients (Morales *et al.*, 1992), it is less attractive than the chemical calcium ionophore A23187.

Although the AR induced by these stimuli may be different from the physiological AR induced by the ZP (Liu and Baker, 1996a), the ability of spermatozoa to respond to them may be useful for assessing certain aspects of sperm function. For example, ionophore A23187 induces the AR by causing Ca^{2+} influx. It has been reported that results of ionophore-induced AR may predict sperm fertilizing ability *in vitro* (Cummins *et al.*, 1991; Yovich *et al.*, 1994; Avrech *et al.*, 1996). We therefore investigated whether the ionophore-induced AR can provide additional information to that obtained by standard semen analysis in the prediction of fertilization rate *in vitro*.

Materials and methods

Patients

Investigations were performed on 109 couples who underwent in-vitro fertilization (IVF) at the Royal Women's Hospital between November 1994 and December 1995, where the wife's oocytes were

inseminated with the husband's spermatozoa. Couples with fewer than three mature oocytes inseminated, sperm concentration $<20\times10^6$/ml or sperm antibodies in either partner were excluded. Diagnoses were unexplained infertility ($n = 46$), male factor infertility ($n = 7$), tubal occlusion ($n = 39$) and endometriosis ($n = 4$). The remaining 13 patients had more than one problem in the following combinations: tubal occlusion with endometriosis ($n = 8$), tubal occlusion with male factor ($n = 3$) and endometriosis with male factor ($n = 2$). The diagnoses were determined before IVF treatment was performed.

During this study, new patients with severe semen defects, including most patients with abnormal morphology ⩾95% in pre-IVF semen analyses, were treated by intracytoplasmic sperm injection (ICSI). Patients with good fertilization rates in previous standard IVF cycles were usually continued with standard IVF despite abnormal semen analyses, particularly in cases of teratozoospermia. In these patients, higher concentrations ($2–9\times10^5$) than usual (1×10^5) of motile spermatozoa were used for insemination of oocytes.

IVF procedures

A stimulation protocol using a gonadotrophin releasing hormone analogue and human menopausal gonadotrophin (HMG) was used to induce follicular growth. Oocytes were collected by ultrasound guided ovarian puncture 36 h after the administration of HCG. Semen was collected by masturbation 2 h prior to the expected time of insemination and the spermatozoa for insemination of oocytes were prepared by swim-up or mini-Percoll as described previously (Ng *et al.*, 1992). Usually the spermatozoa were incubated with three oocytes in 0.6 ml of synthetic human tubal fluid (HTF, Irvine Scientific, Irvine, CA, USA) supplemented with 10% wife's serum in one well of a four-well multidish (Nunclon, Kamstrup, Roskilde, Denmark) at 37°C in 5% CO_2 in air. However, the number of spermatozoa inseminated varied between individuals, depending on the quality of semen and the number of motile spermatozoa present following swim-up or Percoll procedures. Fertilization was assessed at 18–20 h of incubation at 37°C in 5% CO_2 in air and embryos were transferred to the uterus 40–45 h after insemination. Oocytes which developed more than one pronucleus were regarded as fertilized.

Semen analysis

On the day of IVF, semen was obtained by masturbation after 2–3 days abstinence. Semen analyses were performed after liquefaction within 1–2 h of collection. Sperm concentrations were determined using a haemocytometer. Sperm motility and viability (eosin Y exclusion) were determined by standard methods (World Health Organization, 1992). Sperm morphology was assessed on stained smears prepared from semen after washing with 0.9% sodium chloride. The smears were stained with the Shorr method and 200 spermatozoa were assessed under oil immersion with magnification ×1000 and bright field illumination using strict criteria (Kruger *et al.*, 1988; Liu and Baker, 1992a, b). All slides were assessed by two observers and the mean of the two results was used for statistical analysis. There was a high correlation between the morphology results obtained by the two observers (Spearman $r = 0.838$, $n = 109$, $P < 0.001$).

Ionophore A23187-induced acrosome reaction

Motile spermatozoa were obtained by the mini-Percoll method from the semen sample used for IVF and resuspended in human tubal fluid (HTF) medium supplemented with 0.5% bovine serum albumin (Ng *et al.*, 1993). On the day of use, a frozen aliquot of 5 mM stock solution of calcium ionophore A23187 (Sigma Chemical Co., St Louis, MO, USA) in dimethyl sulphoxide (DMSO; Sigma) was diluted 10 times with protein-free HTF medium and 10 µl added to 0.5 ml of the motile sperm suspension (2×10^6/ml) making a final concentration of 10 µM A23187. As a control, 10 µl of 1:10 diluted DMSO was added to 0.5 ml of the same sperm suspension. Both test and control tubes were incubated for 1 h at 37°C in 5% CO_2 in air before the acrosomes were assessed. The ionophore-induced AR was calculated from the percentage acrosome intact spermatozoa exposed to DMSO alone minus percentage acrosome intact spermatozoa exposed to ionophore A23187.

In order to determine if expressing the ionophore-induced AR as a proportion of the live spermatozoa present gave a result different to that obtained when it was expressed as a proportion of the total sperm population (viable and non-viable), the acrosome status of live spermatozoa was determined with the hypo-osmotic swelling test in 35 sperm samples (Aitken *et al.*, 1993). After 1 h incubation in 10 µM A23187 as described above, the spermatozoa were recovered by centrifugation at 800 *g* for 5 min, resuspended in 1 ml of hypo-osmotic swelling medium (7.35 g sodium citrate and 13.51 g fructose in 1 litre of distilled water), and incubated for 1 h at 37°C. After incubation, the spermatozoa were washed with 5 ml of 0.9% NaCl by centrifugation at 800 *g* for 8 min and a smear prepared for acrosome assessment. The proportions of acrosome-reacted viable spermatozoa, as indicated by swelling and coiling of the tail, and acrosome-reacted total spermatozoa (non-viable and viable) were calculated for each sample.

Acrosome assessment

Acrosome status was assessed according to the method of Cross *et al.* (1986) with a slight modification. Sperm in semen or after Percoll selection were washed with 10 ml 0.9% sodium chloride twice with centrifugation at 800 *g* for 10 min and the sperm pellet was smeared on a glass slide (Liu and Baker, 1988). The smear was fixed in 95% ethanol for 30 min after air drying, washed in distilled water for 10 min with three to four changes and stained for 2 h with 25 µg/ml *Pisum sativum* agglutinin labelled with fluorescein isothiocyanate (PSA; Sigma) in Dulbecco phosphate buffered saline (pH 7.4; Commonwealth Serum Laboratory, Melbourne, Victoria, Australia) at 4°C. Finally, the slide was washed and mounted with distilled water and 200 spermatozoa were counted using a fluorescence microscope and oil immersion at a magnification of ×400. When more than half the head of a spermatozoon was brightly and uniformly fluoresced, the acrosome was considered to be intact. Spermatozoa without fluorescence or with a fluorescing band limited to the equatorial segment were considered to be acrosome-reacted. The person assessing the slides was unaware of the treatment of the sample.

Assessment of sperm–ZP binding and penetration in unfertilized oocytes

In patients with <25% fertilization rate, sperm–ZP binding and penetration were determined in oocytes which had failed to fertilize *in vitro*. Spermatozoa penetrating the ZP were counted after removing all the spermatozoa bound on the surface of the ZP using a pipetting technique described previously (Liu and Baker, 1994c).

Statistical analysis

The significance of differences between mean results of sperm tests for patients with ⩽50% IVF rate and >50% IVF rate was determined by Wilcoxon tests. This threshold was chosen subjectively to illustrate the ranges of the dates. Correlations between sperm test results were also performed using a non-parametric (Spearman) test. Relationships between sperm test results and IVF rates were examined by logistic regression analysis using the SPIDA statistical package (Macquarie University, Sydney, Australia).

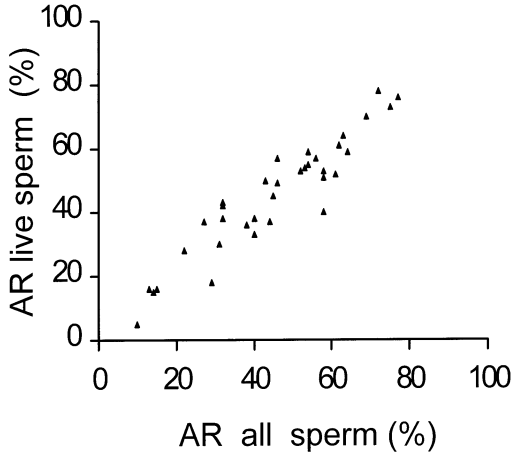

Figure 1. Correlation between results of ionophore-induced AR assessed on live spermatozoa and on all spermatozoa (viable and non-viable) ($n = 35$, $r = 0.922$, $P < 0.001$).

Table I. Sperm test results in patients grouped according to their fertilization rates (FR) ⩽50% ($n = 33$) or >50% ($n = 76$)

	FR ⩽50%	FR >50%
Fertilization rate (%)	24 ± 18 (0–50)	77±13 (53–100)
No. sperm inseminated (10^3/ml)	234 ± 159 (90–750)	229 ± 184 (75–900)
Motility (%, insem)	96 ± 5 (75–99)	96 ± 3 (85–99)
Count (10^6/ml, semen)	128 ± 131 (25–730)	119 ± 82 (21–495)
Motility (%, semen)	61 ± 10 (24–73)	62 ± 13 (25–79)
Viability (%, semen)	78 ± 8 (57–89)	78 ± 11 (27–93)
Normal intact acrosomes (%, semen)	63 ± 15 (18–91)	65 ± 16 (10–94)
Normal sperm morphology (%, semen)	14 ± 7 (3–28)	14 ± 8 (2–42)
Motility (DMSO, %)	78 ± 12 (54–96)	83 ± 12 (31–97)*
Motility (A23187, %)	28 ± 25 (0–74)	20 ± 18 (0–72)
Intact acrosome (DMSO, %)	76 ± 13 (39–91)	80 ± 13 (22–97)
Intact acrosome (A23187, %)	38 ± 19 (11–72)	32 ± 13 (7–71)
Ionophore-induced AR (%)	38 ± 20 (10–78)	49 ± 15 (5–76)*

*$P < 0.05$. Values are means ±SD.

Results

Sperm test and IVF results

The ionophore-induced AR was assessed on viable spermatozoa, determined by hypo-osmotic swelling, and on all spermatozoa (viable and non-viable) in the same 35 samples. The results were highly significantly correlated (Figure 1). The mean results were the same for viable or for viable plus non-viable spermatozoa (mean ± SD, 45 ± 18 versus 45 ± 18). Therefore for analysis of this study, ionophore-induced AR as assessed on all (viable and non-viable) spermatozoa has been used.

The results of sperm tests are summarized in Table I in patients grouped according to high (⩽50%) or low (>50%) fertilization rates. Although there was a wide range in results and numbers of spermatozoa inseminated, there was no significant difference between the two groups of patients except in the case of ionophore-induced AR. Patients with ⩽50% fertilization rate had significantly lower ($P < 0.05$) ionophore-induced AR than patients with >50% fertilization rate.

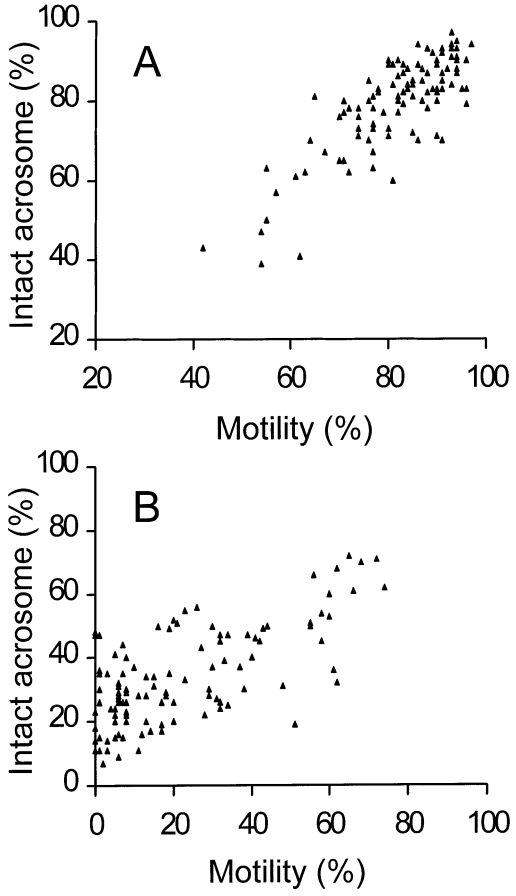

Figure 2. Correlation between percentage intact acrosomes and motility of sperm after incubation with (**A**) DMSO alone ($n = 109$, $r = 0.722$, $P < 0.001$) or (**B**) DMSO plus 10 μM A23187 ($n = 109$, $r = 0.593$, $P < 0.001$).

Correlations between ionophore-induced AR and sperm characteristics

Sperm morphology was negatively correlated with number of spermatozoa inseminated ($r = –0.539$, $P < 0.001$), since the policy was to increase the concentration of spermatozoa inseminated when normal morphology was <15%. Sperm morphology was significantly correlated with semen sperm concentration ($r = 0.364$, $P < 0.001$), and ionophore-induced AR ($r = 0.325$, $P < 0.001$). Ionophore-induced AR was negatively correlated with number of spermatozoa inseminated ($r = –0.378$, $P < 0.001$). Sperm motility and viability in semen were correlated ($r = 0.443$, $P < 0.001$), as were sperm motility in semen and in insemination medium ($r = 0.310$, $P = 0.001$). There was a positive correlation between percentage motility and intact acrosomes after incubation both with or without ionophore A23187 (Figure 2). There was a significant correlation between motility loss (DMSO–ionophore) and ionophore-induced AR (Figure 3).

Correlations between ionophore-induced AR and sperm–ZP binding and penetration

Sperm–ZP binding and penetration were examined in oocytes which failed to fertilize *in vitro* in 15 patients with low fertilization rates <25%. There was a mean of 8.4 (range 0.6–37) spermatozoa bound/ZP, 2.3 (range 0–8.9) spermatozoa

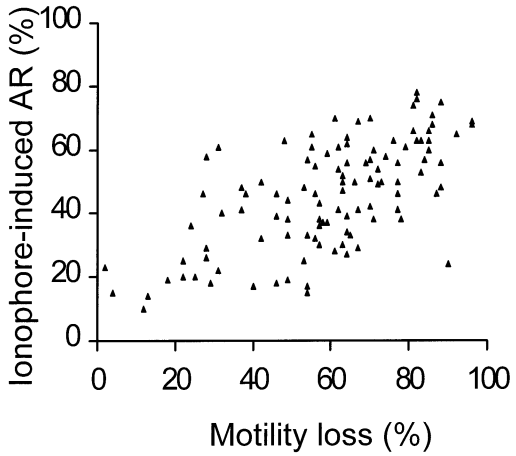

Figure 3. Correlation between ionophore A23187-induced AR (% intact acrosome spermatozoa in DMSO – % intact acrosome spermatozoa in ionophore) and motility loss (DMSO – ionophore) and, $n = 109$, $r = 0.637$, $P < 0.001$).

penetrating into each ZP and 57% (range 0–100%) of oocytes with one or more spermatozoa penetrating into the ZP. The ionophore-induced AR was correlated with the number of spermatozoa penetrating per ZP (Spearman $r = 0.575$, $P < 0.05$) and the proportion of ZP penetrated (Spearman $r = 0.570$, $P < 0.05$). The ionophore-induced AR was not significantly correlated to the number of spermatozoa bound per ZP.

Correlations between sperm tests and fertilization rate in vitro

When data from all patients were analysed, only ionophore-induced AR was significantly correlated with fertilization rates. However, the relationship was weak ($P < 0.05$) (Figure 4A). When data from the sub-group of patients with normal sperm morphology <15% (mean 9.1, range 0–14) were analysed, correlation of ionophore-induced AR and fertilization rates was more significant ($P < 0.01$) (Figure 4B).

To determine which sperm characteristics were independently related to IVF rates, all of the data were examined by logistic regression analysis. Only sperm motility in insemination medium and ionophore-induced AR were significantly related to fertilization rate. All other variables, including sperm morphology, were not significant (Table II). When ionophore-induced AR was excluded from the logistic regression analysis, the proportion of sperm with intact acrosomes in DMSO became significant. When data from the subgroup of patients ($n = 63$) with sperm normal morphology <15% were analysed, again only sperm motility in insemination medium and ionophore-induced AR were significantly related to fertilization rates (Table II). However, when data from the subgroup of patients ($n = 46$) with sperm normal morphology ⩾15% (mean 21.3, range 15–41) were analysed, only percentage motility and sperm concentration in semen were significantly related to fertilization rate. All other variables including ionophore-induced AR were not significantly related.

Discussion

The present study showed that ionophore A23187-induced AR was significantly related to fertilization rates in patients with teratozoospermic semen (normal morphology <15%). This result further confirms that ionophore-induced AR can provide additional information for prediction of fertilization rates *in vitro* in subfertile men as previously reported by others (Cummins *et al.* 1991; Yovich *et al.*, 1994; Avrech *et al.*, 1996). However, the mechanism of this relationship is not clear. Because ionophore A23187 induces the AR mainly through promoting extracellular calcium influx into the spermatozoa, it is tempting to believe that the ionophore-induced AR test reflects the ability of the spermatozoa to undergo the AR.

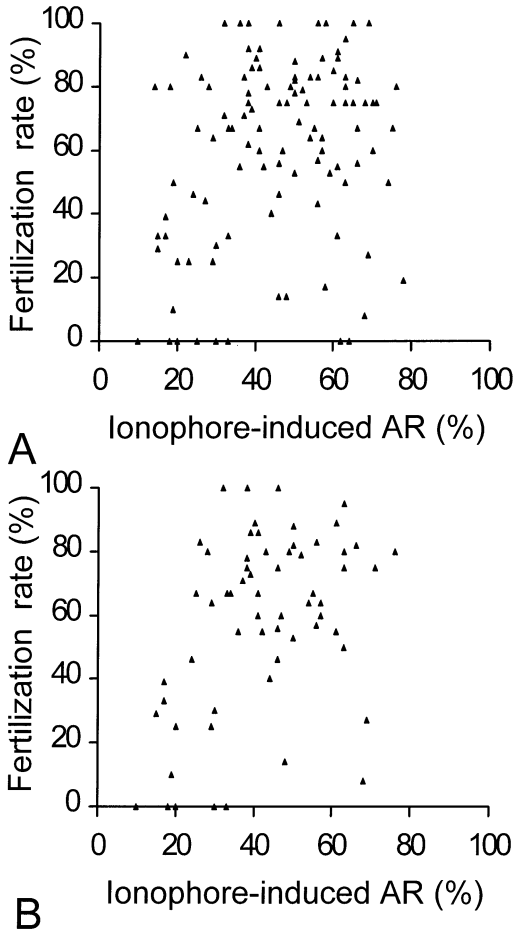

Figure 4. Relationship between fertilization rates *in vitro* and ionophore A23187-induced AR in (**A**) all patients studied ($n = 109$, $r = 0.206$, $P < 0.05$) or (**B**) in those patients with normal morphology <15% ($n = 63$, $r = 0.344$, $P < 0.01$).

Table II. Significant variables in logistic regression analysis of fertilization rates

Variables	b	SE	z	P
All patients ($n = 109$)				
Ionophore-induced AR (%)	0.016	0.004	4.0	<0.001
Motility (%, insem.)	0.060	0.018	3.3	<0.001
Patients with normal morphology <15% ($n = 63$)				
Ionophore-induced AR (%)	0.029	0.006	4.8	<0.001
Motility (%, insem.)	0.070	0.021	3.0	=0.001
Patients with normal morphology ⩾15% ($n = 46$)				
Count (10^6/ml, semen)	0.053	0.014	3.8	<0.001
Motility (% semen)	0.003	0.010	3.0	<0.01

b = regression coefficient, SE = standard error, z = ratio of b and SE.

The variability of response of ionophore-induced calcium influx between teratozoospermic and normal sperm samples may be related to the degree of both morphological and biochemical abnormalities of the acrosome. Oehninger *et al.* (1994) reported that severely teratozoospermic sperm had defective calcium influx and AR when induced by progesterone. Most severely teratozoospermic spermatozoa often have abnormal or small acrosomes which may be relatively unable to respond to calcium influx.

On the other hand, the ionophore-induced AR test may not reflect the ability of spermatozoa to undergo the physiological AR because our previous study showed no significant relationship between ZP and ionophore-induced AR in normozoospermic men with normal sperm–ZP binding (Liu and Baker, 1996a, 1996b). It is obvious that the mechanism of physiological AR induced by ZP is different from the AR induced by other stimuli, including ionophore and even progesterone (ESHRE Andrology Special Interest Group, 1996; Breitbart and Spungin, 1997). Breitbart and Spungin (1997) have recently provided a comprehensive review of the biochemical mechanism of physiological and chemically induced AR. The physiological AR involves activation of several signal transduction pathways, calcium influx and membrane fusion. Ionophore A23187-induced AR mainly involves a chemical effect on calcium influx. While the ionophore-induced AR test may detect patients with defective responses to calcium influx, it will not detect patients with disordered ZP-induced AR (Liu and Baker, 1994b).

In 15 patients with fertilization rate $<25\%$, ionophore-induced AR was significantly correlated with the number of sperm penetrating into the ZP and the proportion of ZP penetrated in oocytes which failed to fertilize. It is possible that this reflects defects of sperm–oocyte interaction not directly related to the AR such as reduced sperm–ZP binding associated with teratozoospermia or reduced penetration due to asthenozoospermia. The human ZP selectively binds morphologically normal spermatozoa, particularly those with normal shaped acrosome areas (Liu and Baker, 1992b, 1994d; Garrtte *et al.*, 1997). Acrosome morphology, including the shape and size, has been considered as one of the most important characteristics for classification of sperm normal morphology (Jeulin *et al.*, 1986; Liu and Baker, 1992a, b, 1994d; Ombelet *et al.*, 1995; Menkveld *et al.*, 1996). Correlations present between acrosome status, A23187 induced AR, sperm morphology and motility suggest that the latter characteristics may be involved in the relationship between A23187-induced AR and fertilization rate.

There was a strong correlation between ionophore-induced AR and ionophore-induced motility loss (Figure 3). This suggests that a common mechanism operates for A23187 causing both acrosome loss and sperm immotility. Thus the similarity of the results obtained when ionophore-induced AR is assessed on either viable or total (viable and non-viable) sperm populations indicates that it may be unnecessary to differentiate viable from non-viable sperm when a motile sperm suspension is prepared for testing. In contrast, human ZP or recombinant human ZP3 induces the AR without any effect on sperm motility (Liu and Baker, 1996b; Brewis *et al.*, 1996), reflecting another major difference between the ZP and A23187-induced AR.

Our previous study showed that the proportion of spermatozoa present with a normal intact acrosome was correlated with fertilization rates *in vitro* in patients with teratozoospermic semen (Liu and Baker, 1988). In this study we also found that the proportion of spermatozoa with an intact acrosome in DMSO was positively related to fertilization rates if A23187-induced AR was not included in the logistic regression model. A high frequency of spontaneous AR of spermatozoa in culture medium has been found to be negatively correlated with fertilization rates in patients with normal semen (Fenichel *et al.*, 1991; Tahahashi *et al.*, 1992; ESHRE Andrology Special Interest Group, 1996). Spermatozoa with premature loss of the acrosome are probably unable to bind to the ZP.

In this study, we found no correlation between sperm morphology and fertilization rates. Several factors may explain this unexpected finding. During the time of this study most new patients with severe sperm morphology defects ($\leqslant 5\%$ normal) were directly assigned to ICSI after pre-IVF semen analyses. Only eight patients had $\leqslant 5\%$ normal morphology and the average normal morphology was 10% for the 63 teratozoospermic ($<15\%$ normal morphology) patients. Most importantly, these teratozoospermic patients were treated by standard IVF because they had high fertilization rates in previous IVF treatments. Also higher concentrations of spermatozoa ($2–9\times10^5$) were used for insemination of oocytes in patients with normal sperm morphology $<15\%$. Increasing the concentration of spermatozoa for insemination of the oocytes in patients with sperm morphological abnormalities improves fertilization rates for some of the patients (Oehninger *et al.*, 1988). Therefore, a lack of relationship between sperm morphology and fertilization rates *in vitro* in this study may be explained by both selection of patients and from the increased number of spermatozoa in the insemination medium.

In conclusion, calcium ionophore-induced AR is correlated with fertilization rates *in vitro*, although not strongly. While significant in patients with teratozoospermia, ionophore-induced AR was not related to fertilization rate in patients with normal sperm morphology $>15\%$. It is possible that low ionophore-induced AR is more related to the presence of teratozoospermia than to specific defects of the AR.

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