Evaluation of stimulus-induced acrosome reaction by two-colour flow cytometric analysis*

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Acrosome status in human spermatozoa from 20 normozoospermic men was evaluated by flow cytometry following the induction of the acrosome reaction with the ionophore A23187. Dual fluorescence staining of methanol fixed spermatozoa incubated with and without (control) the ionophore A23187 was performed with probes which targeted the outer acrosomal membrane (OAM) (rhodamine-labelled Arachis hypogaea agglutinin) or constituents of the acrosomal vesicle (fluorescein-labelled Pisum sativum agglutinin). Flow cytometry analysis revealed two major subpopulations of cells: acrosome-intact and acrosome-reacted spermatozoa after induction of the acrosome reaction. The intensity of green and red fluorescence in acrosome-reacted spermatozoa was significantly lower than that of the acrosome-intact control spermatozoa ($P < 0.0001$). The intensity of green fluorescence in the acrosome-intact subpopulation of spermatozoa was significantly higher than that of the control population ($P < 0.002$). Exposure of spermatozoa to the ionophore A23187 resulted in reliable enhancement of the number of spermatozoa with very high intensity of green and/or red fluorescence compared with the control ($P < 0.03$). An inverse correlation between the number of acrosome-reacted spermatozoa and spermatozoa with a very high intensity of green and/or red fluorescence was demonstrated ($r = -0.631$, $P < 0.01$). This method provides an objective and efficient procedure for quantitative estimation of the acrosomal status of human spermatozoa.

Key words: acrosome reaction/Arachis hypogaea/flow cytometry/human spermatozoa/Pisum sativum

Introduction

The acrosome reaction (AR) of mammalian spermatozoa is associated with the activation and release of proteolytic enzymes from the acrosomal vesicle, specifically acrosin, which are thought to play a key role in facilitating the passage of spermatozoa through the zona pellucida (Yanagimachi, 1988). Acrosomal status of the native spermatozoa and of the spermatozoa responding to various inducers of AR is a very important functional characteristic which permits evaluation of sperm fertilizing potential (Liu and Baker, 1994). Many techniques have been developed to evaluate acrosomal status using staining with coloured dyes (Talbot and Chacon, 1981), variety of lectins (Cross et al., 1986; Mortimer et al., 1987; Centola et al., 1990; Graham et al., 1990; Holden et al., 1990; Nolan et al., 1992) and monoclonal antibodies (Wolf et al., 1985; Kallajoki et al., 1986; Moore et al., 1987; Fenichel et al., 1989; Cruz and Haas, 1992; Tao et al., 1993a). Aitken and Brindle (1993) suggested the simultaneous use of two fluorescent-labelled lectins for AR detection. These lectins specifically target the outer acrosomal membrane (OAM) (rhodamine-labelled Arachis hypogaea lectin) or the constituents of the acrosomal vesicle (fluorescein-labelled Pisum sativum lectin). The use of these stains permitted the fluorescent microscopy of certain sperm cells subpopulations at various AR stages and precise sequencing of events occurring during AR in human spermatozoa. However, the use of fluorescent microscopy for the quantitative evaluation of heterogeneity of spermatozoa in the process of AR is rather complicated and difficult. The objective of this study was to use flow cytometry for the identification and quantitative assessment of sperm cell subpopulations after AR induction.

Materials and methods

Patients and samples

Semen specimens were collected by masturbation from healthy donors and analysed soon after liquefaction according to the World Health Organization standards (WHO, 1987). Only antisperm antibody-negative specimens [mixed antiglobulin reaction (MAR) percentage = 0%] with sperm concentrations >60×10⁶ ml, motility rate >50% were used.

Mixed antiglobulin reaction test

The direct MAR test (Hinting et al., 1988) was performed by mixing on a microscope slide one drop (~10 µl in volume) of fresh semen, one drop of latex particles coated with immunoglobulin (Ig)G and one drop of rabbit anti-human IgG antiserum (SpermMar Kit, FertiPro, Gent, Belgium). The reactions were examined by phase contrast microscopy at ×400, and the percentage of motile spermatozoa carrying one or more latex particles was determined (MAR%) by the scoring of 100 motile spermatozoa. The results were assessed after 2–3 min and again after 10 min.


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Acrosome reaction

The acrosome reaction was induced by exposure of the spermatozoa to the ionophore A 23187 (Sigma, St. Louis, MO, USA). This material was prepared as a 10 mM stock solution in dimethyl sulfoxide.

Motile spermatozoa selected by swim-up technique were subsequently incubated at 37°C for 1 h with Medium 199 containing 0.5% bovine serum albumin (M199–0.5BSA) and 10 µM ionophore A23187. Spermatozoa incubated only in M199–0.5BSA with 0.1% dimethyl sulfoxide were used as control. After incubation spermatozoa were fixed by ice-cold methanol for 30 min. In some experiments, before fixation some cells were stained with propidium iodide (20 µg/ml) to estimate spermatozoa viability (Evenson et al., 1982), and the cells were analysed by flow cytometry. After fixation all the samples were washed twice in distilled water and the cell pellet was resuspended in 16 µl of Dulbecco’s phosphate-buffered saline (PBS), pH 7.4, supplemented with 20% of fetal calf serum (Gibco Laboratories, Grand Island, NY, USA) and incubated during 30 min at 23°C. A total of 2 µl of fluorescein isothiocyanate (FITC)-conjugated *P. sativum* lectin (Sigma) and 2 µl of tetramethylrhodamine isothiocyanate (TRITC)-conjugated *A. hypogaea* lectin (Sigma) was added to each sample at a final concentration of 0.025 and 2.5 mg/ml respectively. All the mixtures were then incubated at room temperature for 15 min. The cells were washed twice with distilled water, resuspended in 100 µl of distilled water and analysed by fluorescence microscopy and flow cytometry.

Fluorescence microscopy

Lectin binding to sperm samples was examined by fluorescence microscopy to determine the location of sperm-bound fluorescence and the proportion of labelled spermatozoa in the total population. A total of 100 spermatozoa were examined for each sample at ×400 magnification using a Leitz Laborlux S microscope (Leica, Germany). The following staining patterns were determined, as previously described (Aitken and Brindle, 1993): (a) acrosome-intact spermatozoa that were uniformly labelled over the acrosomal regions with both lectins; (b) spermatozoa that still appeared to be acrosome-intact when stained with *P. sativum* lectin which displayed dissipation of the *A. hypogaea* labelling; (c) spermatozoa in which the acrosomal contents appeared to be dispersing, as indicated by a reduction in *P. sativum* staining, while the labelling pattern observed with *A. hypogaea* was confined to the equatorial segment; (d) spermatozoa in which the labelling of the spermatozoa was confined to the equatorial segment with both lectins; and (e) spermatozoa characterized by an intense staining with *P. sativum* but lacking the target sites for *A. hypogaea*. In addition, spermatozoa with an intensely green fluorescent acrosomal region and red fluorescent equatorial segment were determined in our study.

Flow cytometry

All the samples were analysed by FACScan (Becton Dickinson Immunocytochemistry Systems, Mountain View, CA, USA). A gate was set on dot plot distributions of forward versus 90° scatter to exclude debris and clumps from the analysis of spermatozoa. The flow rate was 500 cells/s. Fluorescence data of 5000 cells were collected with logarithmic amplification for green and red fluorescence specific for lectin staining. To assess the fluorescent intensity the peak channel number (channel associated with the highest number of fluorescent cells) was determined for each subpopulation of spermatozoa. The data were analysed by CONSORT30 and LYSIS software from Becton Dickinson using a Hewlett Packard 300 series computer.

Statistical analysis

Comparison between groups was carried out using Student’s paired t-test. *P* < 0.05 was considered to be statistically significant.

Results

Typical fluorescence data of swim-up spermatozoa without labelling and spermatozoa stained only with FITC-labelled *P. sativum* lectin or only with TRITC-conjugated *A. hypogaea* lectin are shown in Figure 1A. Spermatozoa stained with both lectins are shown on Figure 1B. The majority of swim-up spermatozoa consisted of acrosome-intact spermatozoa, indicated by the high level of green and red fluorescence. Figure 2 shows the results of flow cytometry analysis of spermatozoa in one of 20 patients after the induction of AR by the ionophore A23187. The majority of control spermatozoa (Figure 2A) were acrosome-intact, with a high level of green and red fluorescence. After AR induction flow cytometry analysis revealed several distinct spermatozoa subpopulations (Figure 2B): acrosome-intact spermatozoa with a high level of green and red fluorescence (region 1); acrosome-reacted spermatozoa with a reduced level of green and/or red fluorescence (region 2); spermatozoa without green and red fluorescence (region 3); spermatozoa with a very high level of green fluorescence and a high or very high (region 4) or reduced level or absence (region 5) of red fluorescence. It is necessary to note that spermatozoa with a high level of red and a reduced level of green fluorescence were never observed.

After induction of AR the percentage of acrosome-reacted spermatozoa determined by flow cytometry was similar to that obtained by fluorescence microscopy (Figure 3; *P* > 0.05), and regression analysis indicated a significant correlation (*P* < 0.01, *r* = 0.861) between the two methods. The percentage of acrosome-reacted spermatozoa in the control population as determined by fluorescence microscopy (*n* = 10) was never >5%.

The percentage of the spermatozoa was determined in contour-plot regions in the control population and after induction of acrosomal reaction (Figure 2B, Table I). The percentage of acrosome-reacted spermatozoa in region 2 after induction of AR was 45.5 + 13.7% (mean + SD) and was not significantly different from that in the control group represented mainly by acrosome-intact cells (*P* = 0.06). No variation in the proportion of unlabelled spermatozoa (region 3) was detected (*P* = 0.4). A significant increase in the number of spermatozoa with very high intensity of green and/or red fluorescence (region 4) and with very high intensity of green and low intensity or absence of red fluorescence (region 5) was observed after induction of AR (*P* < 0.03 and *P* < 0.02 respectively). The inverse correlation between the number of acrosome-reacted spermatozoa (region 2) and of spermatozoa with a very high intensity of green and/or red fluorescence (region 4) was revealed (*r* = −0.631, *P* < 0.01) after induction of AR (Figure 4).

To assess the fluorescent intensity of spermatozoa in the control population and after induction of AR the peak channel number (channel associated with the highest number of fluorescent cells) was determined for each major subpopulation. (Table II). The interassay variation (10 different assays of the same sample) of the peak channel number of green and red fluorescence of acrosome-intact spermatozoa in control was <10%. The green fluorescence of the acrosome-intact sperma-
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Figure 1. Contour plot of green (x axis) and red (y axis) log fluorescence intensities of swim-up spermatozoa. (A) Spermatozoa without staining (a) or stained with fluorescein-labelled Pisum sativum (b) or rhodamine-labelled Arachis hypogaea (c) lectins. (B) Spermatozoa stained with both lectins.

toza after AR was significantly higher than that of the controls ($P < 0.002$). Acrosome-reacted spermatozoa were characterized by the decreased intensity of green and red fluorescence compared with the control ($P < 0.0001$). The relative fluorescence (percent of the fluorescent intensity of acrosome-intact spermatozoa in control) of acrosome-intact and acrosome-reacted spermatozoa after induction of AR was determined (Table II, Figure 5). Correlation between green relative fluorescence for acrosome-intact and that for acrosome-reacted subpopulation was found ($r = 0.626$, $P < 0.01$), while no correlation between red relative fluorescence for these subpopulations was detected ($P > 0.05$) (Figure 6). Correlation between green and red relative fluorescence for acrosome-intact and acrosome-reacted spermatozoa was not found ($r = 0.361$ and $r = 0.244$, $P > 0.05$ respectively).

The level of green relative fluorescence of acrosome-reacted spermatozoa did not change following 2 h incubation with ionophore A23187 and slightly decreased during a third hour of incubation (Figure 7). The red relative fluorescent intensity of acrosome-reacted spermatozoa decreased from 45 to 32% over 3 h. The level of green and red relative fluorescence for acrosome-intact spermatozoa was maximal after 1 h incubation with ionophore A23187. On further incubation a decrease in green and red relative fluorescence of acrosome-intact spermatozoa was found.

The number of acrosome-reacted cells (region 2) increased from 31.9 to 42.6% over 3 h incubation with ionophore A23187 and slightly decreased during a third hour of incubation (Figure 8) but on further incubation the cells number decreased to 6.6%. However, the number of cells permeable to propidium iodide increased during incubation from 24.6 to 58.0%, reflecting an increase in the number of dead spermatozoa.

Discussion

Flow cytometric detection of acrosome-reacted and acrosome-intact subpopulations of human spermatozoa using monoclonal antibodies (Fenichel et al., 1989; D’Cruz and Haas, 1992; Tao et al., 1993a) and lectins binding to acrosomal matrices (Miyazaki et al., 1990; Tao et al., 1993b; Uhler et al., 1993; Henley et al., 1994) has been reported by several authors. Evaluation of OAM content using peanut lectin showed two sperm subpopulations in mouse spermatozoa (Tao et al., 1993b), although these were not detected in human spermatozoa (Purvis et al., 1990; Engh et al., 1991). In our study we used simultaneous staining of cells populations with rhodamine-labelled A.hypogaea and fluorescein-labelled Psativum lectins. Two major sperm subpopulations were observed after AR induction in all sperm samples, both acrosome-reacted and acrosome-intact cells. We assessed the number of acrosome-reacted spermatozoa in whole sperm populations containing both live and dead cells. It has been shown previously that AR in live spermatozoa is significantly correlated with that in the total population of live and dead cells after 1 h incubation with the ionophore A23187 (Liu and Baker, 1996). This indicated the absence of any major difference resulting from whether the proportion of acrosome-reacted spermatozoa was expressed with respect to the whole sperm population or was restricted to the live population when motile spermatozoa were selected for the studies.

Our investigation was limited to semen samples obtained from healthy male donors with normal semen parameters. As it was reported that antisperm antibodies could be found in healthy donors (Clarke et al., 1986) and that ionophore A23187-induced AR could be blocked by antisperm antibodies (Lansford et al., 1990; Benoff et al., 1993), all the sperm...
Figure 2. FACScan analysis of spermatozoa stained with fluorescein-labelled *Pisum sativum* and rhodamine-labelled *Arachis hypogaea* lectins following 1 h incubation with and without (control) ionophore A23187. Top: Contour plot of green (x axis) and red (y axis) log fluorescence intensities of control (A) and ionophore A23187-treated (B) spermatozoa. Bottom: Log fluorescence histograms of green (C) and red (D) fluorescence intensities (x axis) of control (unimodal distributions) and the ionophore A23187-treated spermatozoa (multimodal distributions). The number of cells counted is on the y axis. Acrosome-reacted and acrosome-intact spermatozoa were discriminated by the channel value of the point with the lowest event count located between two peaks at the histograms of green and red fluorescence (Figure 2C, D). Region 1 (R1) = acrosome-intact spermatozoa with a high level of green and red fluorescence intensity (≥200% compared with the control); region 2 (R2) = acrosome-reacted spermatozoa with a lower intensity of green and/or red fluorescence; region 3 (R3) = unlabelled acrosome-reacted spermatozoa; region 4 (R4) = spermatozoa with a very high intensity of green and/or red fluorescence (>200% compared with the control); region 5 (R5) = spermatozoa with a very high level of green (>200% compared with the control) and a low intensity or absence of red fluorescence.

samples used in the experiment were tested by MAR test and only antisperm antibodies-negative samples were used.

The percentage of acrosome-reacted spermatozoa determined by flow cytometry was not significantly different from that obtained by fluorescence microscopy. Obviously, the main limitation of flow cytometry is that this method cannot provide such detailed information about spermatozoa at different stages of AR as the visual method. At the same time the quantitative estimation of the lectin binding with the AM and OAM by flow cytometry allowed us to estimate the level of both lectins during the acrosome reaction and to determine for the first time that the level of green fluorescence in the acrosome-intact subpopulation was higher than that in the control sperm population. In earlier studies (Nagae *et al.*, 1986), electron microscopic examination of spermatozoa showed that the initial AR stages are related to changes in the acrosomal matrix. From its initial homogenous and compact state, the matrix becomes swollen and diffused and the volume of acrosomal cap content between outer and inner membranes expands dramatically. It is possible that the enhancement of green fluorescence is related to the increase in the amount of matrix material. However, it is more likely that glycoproteins become more available for lectin binding as the matrix expands. Conceivably, increased *A. hypogaea* lectin binding is also related to the changed of OAM structures. We suggest that the enhanced lectin binding was due to the specific properties of dead acrosome-intact spermatozoa. However, the increased intensity of green and red fluorescence of acrosome-intact spermatozoa which was observed at the first stage of incubation with ionophore became less after a longer incubation period,
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Figure 3. Evaluation of the percentage of acrosome-reacted spermatozoa by flow cytometry and fluorescence microscopy after induction of acrosome reaction. The proportion of spermatozoa in region 2 (R2) and of spermatozoa with a reduction in *Arachis hypogaea* staining (spermatozoa with punctate labelling over the acrosome region or with labelling confined to the equatorial segment) was estimated (n = 10).

Table I. The number of spermatozoa stained with fluorescein-labelled *Pisum sativum* and rhodamine-labelled *Arachis hypogaea* lectins in the five regions of the fluorescence contour plot after 1 h incubation with, and without (control), ionophore A23187 (n = 20). Values are shown as mean ± SD; values in parentheses are ranges

<table>
<thead>
<tr>
<th>Number of cells (%)</th>
<th>Region 1</th>
<th>Region 2</th>
<th>Region 3</th>
<th>Region 4</th>
<th>Region 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>48.8 ± 21.6</td>
<td>35.7 ± 19.4</td>
<td>1.7 ± 1.8</td>
<td>10.6 ± 6.4</td>
<td>1.8 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>(8.5–76.8)</td>
<td>(7.6–71.0)</td>
<td>(0.1–6.4)</td>
<td>(1.3–21.3)</td>
<td>(0.1–6.0)</td>
</tr>
<tr>
<td>A23187</td>
<td>32.8 ± 13.2a</td>
<td>45.5 ± 13.7b</td>
<td>2.0 ± 2.4c</td>
<td>16.2 ± 12.4d</td>
<td>3.0 ± 3.2e</td>
</tr>
<tr>
<td></td>
<td>(13.4–63.5)</td>
<td>(20.4–70.7)</td>
<td>(0.1–9.6)</td>
<td>(2.0–43.9)</td>
<td>(0.1–10.8)</td>
</tr>
</tbody>
</table>

*a*Significantly lower than corresponding control value (P < 0.001).

*b*Not significant compared with corresponding control value (P = 0.06).

*c*Not significant compared with corresponding control value (P = 0.4).

*d*Significantly higher than corresponding control value (P < 0.03).

*e*Significantly higher than corresponding control value (P < 0.02).

Table II. The fluorescent intensity (mean ± SD) of acrosome-intact and acrosome-reacted spermatozoa stained with fluorescein-labelled *Pisum sativum* and rhodamine-labelled *Arachis hypogaea* after 1 h incubation with, and without (control), ionophore A23187 (n = 20)

<table>
<thead>
<tr>
<th>Sperm subpopulation</th>
<th>Fluorescence intensity</th>
<th>Percentage of control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak channel no.</td>
<td>Green</td>
</tr>
<tr>
<td>Control, acrosome-intact</td>
<td>151 ± 74</td>
<td>83 ± 40</td>
</tr>
<tr>
<td>A23187, acrosome-intact</td>
<td>185 ± 81a</td>
<td>88 ± 38b</td>
</tr>
<tr>
<td>A23187, acrosome-reacted</td>
<td>71 ± 46c</td>
<td>34 ± 19d</td>
</tr>
</tbody>
</table>

*a*Significantly higher than corresponding control value (P < 0.002).

*b*Not significant compared with corresponding control value (P = 0.2).

*c,d*Significantly lower than corresponding control value (P < 0.0001).

*e,f versus g, h versus i, j versus k, l versus m (P < 0.0001).

*e,f versus g, h versus i, j versus k, l versus m (P < 0.0001).

*g versus h (P = 0.1).*

though the number of dead cells increased dramatically. Meanwhile, the number of cells with a very high intensity of green and/or red fluorescence also decreased.
Figure 5. Relative fluorescence intensity (percentage of the fluorescent intensity of acrosome-intact spermatozoa in control) of acrosome-reacted and acrosome-intact spermatozoa subpopulations stained with fluorescein-labelled *Pisum sativum* and rhodamine-labelled *Arachis hypogaea* lectins following 1 h incubation with the ionophore A23187 (n = 20).

Figure 6. Correlation between green and red relative fluorescence intensity (percentage of the fluorescent intensity of acrosome-intact spermatozoa in the control population) of acrosome-reacted and acrosome-intact spermatozoa subpopulations stained with fluorescein-labelled *Pisum sativum* and rhodamine-labelled *Arachis hypogaea* lectins following 1 h incubation with the ionophore A23187 (n = 20).

Figure 7. The assessment of the relative fluorescence intensity (percentage of the fluorescent intensity of acrosome-intact spermatozoa in the control population) of acrosome-intact and acrosome-reacted spermatozoa subpopulations stained with fluorescein-labelled *Pisum sativum* and rhodamine-labelled *Arachis hypogaea* lectins following different periods of incubation with the ionophore A23187.

Figure 8. The number of dead spermatozoa as assessed by propidium iodide staining and spermatozoa in different regions of fluorescence contour plot after staining with fluorescein-labelled *Pisum sativum* and rhodamine-labelled *Arachis hypogaea* lectins following different periods of incubation with the ionophore A23187.

Obviously the OAM and AM partly disappeared after AR. The identical fluorescence intensity of acrosome-intact and acrosome-reacted spermatozoa may also indicate the changes of AM and OAM during AR which result in increased lectin binding, both in acrosome-intact and in acrosome-reacted spermatozoa.

The negative correlation between the number of acrosome-reacted spermatozoa and the number of cells with a very high intensity of green and/or red fluorescence suggests that the appearance of spermatozoa with higher intensity of green and/or red fluorescence preceded AR, and their accumulation may suggest a low AR rate. On the contrary, the number of these spermatozoa was low when the AR rate was high. We suggest that the addition of ionophore causes AM and OAM changes, which result in higher lectin binding in all spermatozoa, but which reaches a certain level only in those cells where AR was observed.

Much research has centered on the sequence of the AR-related changes (Nagae et al., 1986; Tesarik et al., 1988). Aitken and Brindle (1993) showed that the first stage of AR, which can be visualized with the help of double staining using
fluorescent microscopy, is associated with the appearance of a sperm population that still appears to be acrosome-intact when stained with FTTC-labelled Psatium lectin and yet displays dissipation of TRICT-labelled Alhypgaea lectin. Flow cytometry has revealed a small subpopulation of spermatozoa with a low level of red fluorescence and a very high level of green fluorescence. It is possible that these are spermatozoa at the first stage of AR which have intact acrosomal matrix but enhanced availability for lectin binding and dispersed OAM. It is also possible that disappearance of OAM (partial or whole) may have occurred in dead spermatozoa with an intact AM.

Tesari et al. (1988) have shown previously that at least one component of the acrosomal vesicle, acrosin, remains firmly bound to the inner acrosomal membrane, after the OAM has been lost. In the present study the positive correlation between the level of green, but not red fluorescence was shown has been lost. In the present study the positive correlation has been established between the level of green, but not red fluorescence was shown has been lost. In the present study the positive correlation has been firmly established between the level of green, but not red fluorescence was shown has been lost. In the present study the positive correlation has been firmly established between the level of green, but not red fluorescence was shown has been lost.

AR stimulation by ionophore A23187 is not a physiological process, but the AR to ionophore challenge (ARIC) test (Cummins et al., 1991) is recommended as a functional test in infertility diagnostics, as good correlations have been found between the response of human spermatozoa to calcium ionophore and their fertilizing ability (Tesari, 1996). Thus, development of new objective methods to evaluate ionophore A23187 induced AR is an expedient task. Of course the detection of AR induction using physiological inducers, such as follicular fluid and progesterone, is of great interest and will be the subject of our future studies.

Flow cytometry is a promising method allowing not only the detection of subpopulations of acrosome-reacted and acrosome-intact spermatozoa, but the performance of quantitative analysis of their structural, functional and metabolic parameters. Brook et al. (1996) demonstrated that two subpopulations of human spermatozoa with different intracellular pH appeared after AR induction in human spermatozoa by ionophore A23187. Differences in intracellular calcium were reported for two cell subpopulations after AR induction in bull and boar spermatozoa (Nolan et al., 1992; Harrison et al., 1993). It is evident that flow cytometry may have future applications in the study of AR mechanisms and AR abnormalities which compromise fertility.

Acknowledgements

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