

# Improved methodology for the detection and quantification of the acrosome reaction in mouse spermatozoa

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**Summary.** This study evaluates the use of two fluorescein-labelled (FITC) plant lectins, *Pisum sativum* (edible pea) agglutinin (PSA) and *Arachis hypogaea* (peanut) agglutinin (PNA), in order to determine the most accurate and reliable method to experimentally detect and assess the acrosome reaction in mouse spermatozoa.

PNA-FITC labelling was restricted to the acrosome and was not influenced by the fixation procedure; either absolute methanol or paraformaldehyde. In contrast, PSA-FITC not only labelled the acrosome, but also the whole head and the flagellum. This aspect was especially marked after methanol fixation. The cytoplasmic droplet, when present, was also stained by PSA-FITC. Incubation with the calcium ionophore ionomycin induced a concentration and time-dependent increase in the number of acrosome reactions. Compared to spotted preparations, smear samples exhibited a high proportion of spermatozoa with damaged acrosome.

In conclusion, PNA-FITC labelling was more accurate than PSA-FITC labelling to detect the acrosome of mouse spermatozoa. The fixation method (methanol vs. paraformaldehyde) had no influence on the staining pattern of PNA-FITC labelling, but spotted preparations are recommended to avoid mechanical damage to the acrosome. Ionophore challenge confirmed the existence of a calcium-dependent acrosome reaction in mouse spermatozoa and validated the use of PNA-FITC to quantify this physiological process. The present study illustrates important methodological considerations which need to be taken into account in order to design a

reliable and reproducible protocol for the study of the acrosome reaction.

**Key words:** Acrosome, Lectin, Spermatozoa, Ionomycin

## Introduction

Mammalian fertilization involves capacitation of the spermatozoon and acrosome reaction (AR), the latter process being a major event for sperm penetration through the zona pellucida (ZP) and subsequent fusion with the egg membrane (Guraya, 2000). The mechanisms involved in the spermatozoa acrosome reaction are still under debate. This irreversible physiological process relates to endogenous, calcium-dependent, molecular events occurring at the level of the acrosomal cap membrane. In addition, the zona pellucida is considered to be the physiological initiator of the acrosome reaction in mammalian sperm (Guraya, 2000). *In vitro*, several agonists, such as soluble ZP proteins, follicular fluid, steroid hormones or calcium ionophores can initiate the acrosome reaction. The *in vitro* induction of acrosome reaction has been demonstrated to exhibit a significant correlation with fertility in different species (Whitfield and Parkinson, 1992, 1995; Pampiglione et al., 1993). Besides evaluation of semen quality, assessment of the acrosome status is also used in andrology research for development of male contraception (Suri, 2005) and to detect the gonadotoxic effects of food or drugs (Kumi-Diaka and Townsend, 2003). Hence, the precise assessment and quantification of the acrosomal loss is of high clinical significance when evaluating sperm fertilizing capacity or damage (Ortloff et al., 2006).

The presence or absence of acrosome can be

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assessed by several methods, including electron and conventional microscopy. Electron microscopy delivers detailed information on the acrosomal status but requires an expensive and specialized equipment, as well as a skillful, trained staff. Several techniques have also been described to visualize the acrosome by conventional microscopy. In different laboratory species, the acrosome can be observed by phase contrast microscopy. The triple stain technique (Talbot and Chacon, 1981) and Coomassie Blue labelling (Larson and Miller, 1999) have also been widely used. Combinations of classical stains are, however, extremely time consuming and are difficult to run in parallel with other immunohistochemical methods. In contrast, fluorescence techniques allow simultaneous acrosome detection and labelling of spermatozoan structures (Maier et al., 2003). The use of fluorescent labelled plant lectins (Aviles et al., 1997) or antibodies raised against acrosomal proteins (Yamashita et al., 2007) are procedures frequently described for the evaluation of the acrosomal status. However, many variations in the experimental protocols are encountered in the scientific literature, and species-related differences in the suitability of each technique have been reported (Larson and Miller, 1999). In particular, assessment of the acrosome of rodent sperm is considered as being especially difficult due to the acrosome thinness and the sperm head morphology.

The present study was conducted to reevaluate the use of two plant lectins, *Pisum sativum* (edible pea) agglutinin (PSA) and *Arachis hypogaea* (peanut) agglutinin (PNA), at detecting the acrosome status in rodent spermatozoa. The experimental procedure was also assessed in terms of sperm preparation onto microscope slides, fixation method and choice of lectin in order to obtain an easy, convenient and reproducible technique most suitable to evaluate the spontaneous and induced-acrosome reaction in mouse.

## Materials and methods

### Animals

C57BL/6J and CBA mice were purchased from Charles River Laboratories (Brussels, Belgium). Male F1 breeders (5 months old) were used for spermatozoa collection. The animals were housed in a climate-controlled room with a 12h light: 12h dark photoperiod schedule in the animal care facility. They were fed standard rodent chow and water "ad libitum". The protocol, approved by the Ethical and Animal Welfare Committee of the Université Libre de Bruxelles (ULB), was followed in all experimental procedures.

### Sperm collection

Mice were killed by cervical dislocation. The epididymides and vasa deferentia were dissected free and rinsed with gassed (95% O<sub>2</sub>/5% CO<sub>2</sub>) Hanks

medium (NaCl 137 mM, KCl 5.37 mM, CaCl<sub>2</sub> 1.26 mM, MgSO<sub>4</sub> 0.81 mM, Na<sub>2</sub>HPO<sub>4</sub> 0.34 mM, KH<sub>2</sub>PO<sub>4</sub> 0.44 mM, NaHCO<sub>3</sub> 4.17 mM, pH 7.4). Under a binocular microscope, the vas deferens, cauda and corpus region from the epididymis were separated from fat tissue and placed in 1 ml of a gassed (95% O<sub>2</sub>/5% CO<sub>2</sub>) modified Krebs-Ringer bicarbonate capacitating medium (NaCl 94.6 mM, KCl 4.78 mM, MgSO<sub>4</sub> 1.19 mM, Na lactate 21.58 mM, Na pyruvate 0.5 mM, NaHCO<sub>3</sub> 25.07 mM, CaCl<sub>2</sub> 1.71 mM, KH<sub>2</sub>PO<sub>4</sub> 1.19 mM, D-glucose 5.56 mM, Streptomycin 50 µg/ml, Penicillin 50 UI/ml, Bovine Serum Albumin 4 mg/ml, pH 7.4) (Miyoshi et al., 1997; Kawai et al., 2000). The cauda epididymis and vas deferens were gently pressed to extrude spermatozoa. The corpus epididymis was diced to allow spermatozoa to swim out into the capacitating medium. The capacitating medium containing the spermatozoa was transferred to a non-treated 4-wells multidish (Nunc, Roskilde, Denmark).

### Experiment 1: Evaluation of PSA and PNA for labelling of the acrosome

Spermatozoa in the capacitating medium were incubated for 30 min at 37°C under 5% CO<sub>2</sub>. The spermatozoa suspension was then transferred to microtubes and centrifuged at 500g for 5 min at room temperature. The supernatant was discarded and phosphate buffer saline (PBS) solution (NaCl 145 mM, NaH<sub>2</sub>PO<sub>4</sub> / Na<sub>2</sub>HPO<sub>4</sub> 10 mM, pH 7.2) was added to the sperm pellet. The tube was centrifuged again at 500g for 5 min, the supernatant discarded and replaced by PBS. Ten µl of sperm suspension were spotted twice onto superfrost microscope slides (Menzel, Braunschweig, Germany) and spread out with a glass pipette. Alternatively and more conventionally, preparations were made by smearing, with a second glass slide, 20 µl of sperm suspension over a slide. Sperm samples were allowed to air-dry for 15 min.

Spermatozoa preparations were then fixed by immersion in either absolute methanol (Merck, Darmstadt, Germany) for 15 min or 4% paraformaldehyde (PAF) (Sigma-Aldrich, Steinheim, Germany) in PBS for 20 min. The slides were rinsed twice in PBS for 5 min before incubation with the different lectin solutions. *Pisum sativum* (edible pea) agglutinin: PSA-FITC (Sigma-Aldrich, Steinheim, Germany) or *Arachis hypogaea* (peanut) agglutinin: PNA-FITC (Vector Laboratories, Burlingame, USA) was applied onto the spermatozoa sample at a final concentration of 15 µg/ml in PBS and the slides were incubated for 30 min at room temperature. PSA-FITC slides were then rinsed in distilled water for 15 min and mounted with an aqueous mounting medium (Prolong<sup>®</sup>Gold antifade with DAPI, Molecular Probes, Oregon, USA). DNA was counterstained with DAPI included in the mounting medium. PNA-FITC labelled slides were rinsed with PBS for 15 min before mounting

## Acrosome quantification in spermatozoa

as described above.

Slides were examined with a Nikon Eclipse 50i microscope equipped with an epi-fluorescence device and a camera (Nikon Instruments, Badhoevedorp, The Netherlands).

### *Experiment 2: Validation of PNA-FITC labelling for the evaluation of the acrosome reaction*

Spermatozoa in capacitating medium were transferred to a non-treated 4-wells multidish (Nunc, Roskilde, Denmark). The spermatozoa were allowed to capacitate for 30 min at 37°C under 5% CO<sub>2</sub>. Ionomycin (1 or 10 µM final concentration) (Calbiochem, EMD Biosciences, La Jolla, USA), dissolved in DMSO, was added to the sperm suspension to induce the acrosome reaction and the incubation period was extended for either 30 or 60 min. DMSO was added to the control samples. The different samples were then transferred to microtubes, centrifuged and rinsed twice with PBS as described previously. Ten µl of sperm suspension were spotted onto superfrost slides, spread with a glass pipette and air-dried. The sperm slides were fixed by immersion in absolute methanol for 15 min and rinsed in PBS before incubation with PNA-FITC (15 µg/ml) for 30 min. Finally, the slides were rinsed in PBS and mounted with Prolong<sup>®</sup>Gold antifade with DAPI. Visualization and image recording were carried out as described above. For each slide, images of adjacent fields, under a x100 magnification, were recorded to achieve a sperm count of minimum 100 spermatozoa. Spermatozoa were classified as being in one of 3 categories of PNA-labelling, i.e.: I = presence of intact acrosome, II = absence or perforated acrosome, III = doubtful. For each experimental condition, a minimum of 3 slides were examined and quantified.

Experiments have been repeated on spermatozoa collected from different mice.

### *Statistical analysis*

The percentage of acrosome-reacted sperm was calculated as [number of spermatozoa with absence or perforated acrosome / (total number of spermatozoa - the doubtful)] x 100. Data were expressed as means ± SEM. The percentages of acrosome-reacted sperm after a 10 µM ionomycin treatment for 30 or 60 min were compared using a two-factor-ANOVA procedure. The percentages of acrosome-reacted sperm after 1 or 10 µM ionomycin treatment for 60 min were compared by regression analysis and a one-factor-ANOVA procedure followed by a Bonferroni post hoc test.

The percentage of damaged spermatozoa was calculated as [number of damaged spermatozoa / (total number of spermatozoa - the doubtful)] x 100 and was quantified on spotted or smear preparations after both fixation conditions (methanol or PAF). Data were expressed as means ± SEM and compared using a two-

factor-ANOVA procedure.

## Results

### *Evaluation of PSA and PNA-FITC for labelling of the acrosome*

Labelling spermatozoa with PSA-FITC, after methanol fixation, revealed an intense staining of the whole spermatozoa (Fig. 1A). In the head region, the acrosomal cap and the equatorial segment were intensely marked (Fig. 1A). The flagellum was also labelled (Fig. 1A). Spermatozoa fixed by paraformaldehyde, instead of methanol, and incubated with PSA-FITC also exhibited a diffuse labelling of the head and flagellum, although the intensity of the staining was systematically lower (Fig. 1B). At the head level, the acrosomal cap, when present, was preferentially labelled (Fig. 1B).

In another series of experiments, the spermatozoa fixed either by methanol or by paraformaldehyde were incubated for 30 min with PNA-FITC. Under such experimental conditions, and whatever the fixation procedure, PNA-FITC labelling was clearly restricted to the head and the acrosomal cap (Fig. 1C,D). The flagellum was not stained (Fig. 1C,D). This experimental approach, using PNA-FITC, further disclosed various patterns of acrosome staining. The acrosomal caps appeared complete and intact (Fig. 2, white arrow) or displayed irregularities with multiple perforations (Fig. 2, white arrowhead). Some spermatozoa exhibited only remnants of acrosomal caps (Fig. 2, red arrow). A few were observed with no fluorescent labelling on the sperm head (Fig. 2, red arrowhead).

Incidentally, and when present, the PSA-FITC labelling revealed the presence of the cytoplasmic droplet on the spermatozoa flagellum (Fig. 1A,B arrows). Such a staining was visible after methanol or paraformaldehyde fixation (Fig. 1A,B, arrows). In contrast, the cytoplasmic droplet could not be clearly evidenced after PNA-FITC labelling (Fig. 1C,D).

In a third series of experiments, we compared two procedures to lay down the spermatozoa onto the glass slides. The sperm suspension was either spotted gently and spread out with a glass pipette or “more conventionally” was smeared throughout the glass slide by means of a second glass slide. Smear preparations displayed a relatively low density of spermatozoa in the center of the preparation (Fig. 3A,C) and a higher concentration at the periphery. In contrast, sperm suspensions spotted onto the microscope slides manifested a homogeneous distribution of spermatozoa (Fig. 3B,D).

Under high magnification (x100), numerous spermatozoa heads appeared damaged in the smear preparations. A “bubble” of faint fluorescent staining was observed alongside the acrosomal cap of PSA-FITC labelled spermatozoa (Fig. 4A, arrowheads). Impaired acrosomal cap could also be evidenced on PNA-labelled

### Acrosome quantification in spermatozoa

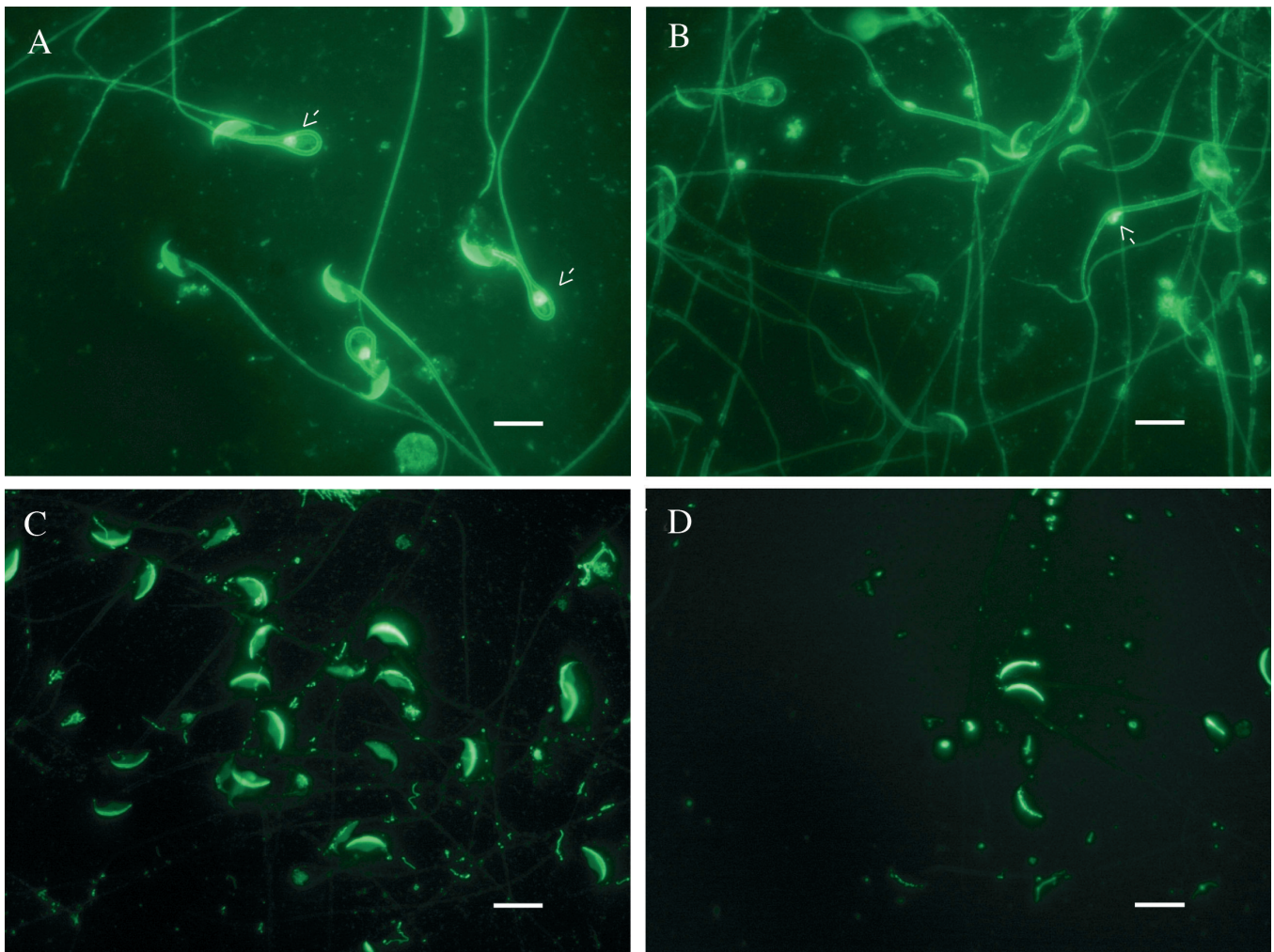
spermatozoa from smear preparations (Fig. 4B, arrowhead). Spotted preparations, after methanol or paraformaldehyde fixation, resulted in  $6.3\pm 1.2\%$  and  $9.2\pm 1.7\%$  of damaged spermatozoa, respectively (N.S.). Smear preparations led to a higher percentage of damaged spermatozoa:  $27.7\pm 6.1\%$  after methanol and  $24.5\pm 2.9\%$  after paraformaldehyde fixation ( $P<0.001$ ).

#### Validation of PNA-FITC labelling for the evaluation of the acrosome reaction

Quantitative changes in acrosome status were assessed in capacitated spermatozoa incubated in the absence or presence of the  $\text{Ca}^{2+}$  ionophore ionomycin.

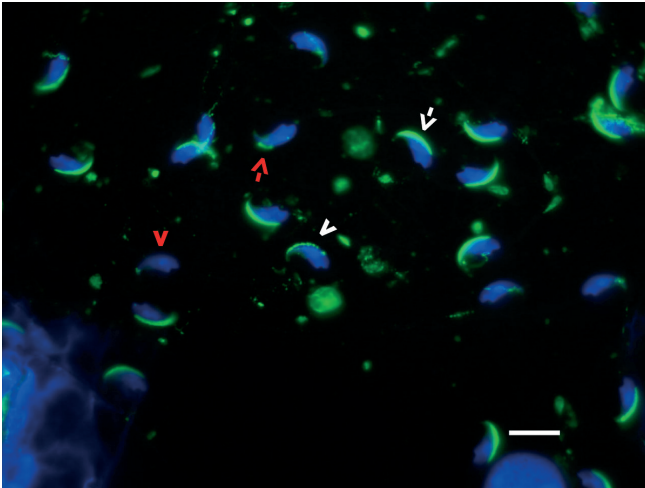
The percentage of acrosome-reacted sperm in control experiments averaged  $49.4\pm 2.7\%$  after a 30 min incubation period and  $47.2\pm 3.7\%$  after a 60 min incubation period (N.S.) (Fig. 5A). Incubation with  $10\ \mu\text{M}$  ionomycin for 30 min resulted in  $67.5\pm 2.5\%$  of acrosome reacted sperm (Fig. 5A) whilst a 60 min incubation with  $10\ \mu\text{M}$  ionomycin led to  $91.1\pm 0.8\%$  of acrosome-reacted sperm (Fig. 5A). The stimulatory effect of ionomycin was highly significant under the different experimental conditions ( $P<0.001$ ) and the ionomycin-induced acrosome reaction was more pronounced after a 60 than a 30 min incubation period ( $P<0.001$ ).

The percentage of spermatozoa classified as



**Fig. 1.** Fluorescein-labelled (FITC) plant lectins, *Pisum sativum* agglutinin (PSA) and *Arachis hypogaea* (peanut) agglutinin (PNA), to detect the acrosome in mouse spermatozoa. Upper panels: PSA-FITC. Absolute methanol fixed sperm preparation (A) and paraformaldehyde 4% fixed sperm preparation (B). Spermatozoa exhibit a diffuse labelling of the head and flagellum. The intensity is lower for preparations fixed with paraformaldehyde and, when present, the acrosomal cap is also labelled. White arrows refer to PSA-FITC labelled cytoplasmic droplets. Lower panels: PNA-FITC. Absolute methanol fixed sperm preparation (C) and paraformaldehyde 4% fixed sperm preparation (D). Whatever the fixation procedure, PNA-FITC labelling is clearly restricted to the acrosomal cap. Scale bar: 10  $\mu\text{m}$ .

## Acrosome quantification in spermatozoa



**Fig. 2.** Spotted sperm preparations fixed with absolute methanol. Acrosome labelled with PNA-FITC (green) and nuclei counterstained with DAPI (blue). Different patterns of acrosome staining are visible: intact acrosome (white arrow), perforated acrosome (white arrowhead), absent acrosome (red arrowhead), remnants of acrosome (red arrow). Scale bar: 10  $\mu$ m.

“doubtful” was not statistically different between each population (absence or presence of ionomycin after either 30 or 60 min incubation, <7.2% in each case).

The addition of increasing concentrations of ionomycin in the incubation media led to a dose-dependent increase in the acrosome reaction (Fig. 5B). After the addition of 1  $\mu$ M and 10  $\mu$ M ionomycin, the percentage of acrosome-reacted sperm averaged  $61.3 \pm 2.4\%$  ( $P < 0.01$ ) and  $81.9 \pm 2.2\%$  ( $P < 0.001$ ), respectively. In control experiments, the acrosome reaction rate amounted to  $47.0 \pm 4.3\%$ .

As noticed above, the percentage of “doubtful” spermatozoa was similar under the different experimental conditions (N.S.).

## Discussion

The acrosome reaction is of fundamental importance in the chain of events leading to the fertilization of the oocyte. Acrosome evaluation is a valuable tool in modern andrology for both research purposes and diagnosis of male infertility (Esteves et al., 2007). Moreover, the *in vitro* induction of the acrosome reaction has been shown to be of prognostic value as to the sperm fertilization capacity (Whitfield and Parkinson, 1992, 1995; Pampiglione et al., 1993).

The use of lectins to label the acrosome has been initially described by Talbot and Chacon (1980). A wide variety of lectins; *Ricinus communis* agglutinin I (RCA I), *Pisum sativum* agglutinin (PSA), *Arachis hypogaea* (peanut) agglutinin (PNA), concanavalin A (ConA), soybean agglutinin (SBA) have been evaluated for their ability to label the acrosome of spermatozoa from

several species (Tao et al., 1993; Kinger and Rajalakshmi, 1995; Cheng et al., 1996; Martí et al., 2000; Ozaki et al., 2002; Esteves et al., 2007) and such a technical approach has been compared to other methods, including triple stains, chlortetracyclins staining, antibodies labelling, phase contrast or electron microscopy (Lee and Ahuja, 1987). The main advantage of using fluorescent lectins resides in the possibility of combining this technique with fluorescent antibody labelling of spermatozoal proteins and/or with supravital stains (Cross et al., 1986; Mortimer et al., 1990; Tao et al., 1993; Kinger and Rajalakshmi, 1995; Martí et al., 2000; Ozaki et al., 2002; Esteves et al., 2007). PNA-FITC and PSA-FITC are the most routinely used lectins to label the acrosome. PNA lectin has been shown to bind Gal $\beta$ (1-3)Gal NAC residues (Varki et al., 1999; Sharon, 2007) located on the outer acrosomal membrane (Mortimer et al., 1987; Martínez-Menárguez et al., 1992; Avilés et al., 1997) whilst PSA has been reported to recognize  $\alpha$ -methyl mannoside residues from complex oligosaccharide structures, localized within the acrosome contents (Cross et al., 1986; Varki et al., 1999; Sharon, 2007).

Sperm preparations are classically fixed by absolute methanol or paraformaldehyde treatment. Methanol fixation is known to permeabilize the plasma and acrosomal membranes over the entire sperm surface (Haas et al., 1988). PAF fixation, however, does not permeabilize the membranes and maintains, to some extent, the morphologic integrity of the acrosomal and plasma membranes (Haas et al., 1988). Therefore, the fixation method might influence the labelling pattern of both lectins.

In the present study, we did not observe any difference when labelling the acrosome by PNA-FITC after either methanol or PAF fixation. In contrast, PSA-FITC labelling of the acrosome was clearly affected by the fixation procedure. Spermatozoa fixed with methanol exhibited an almost uniform PSA-FITC staining of the whole spermatozoa together with a labelling of the acrosome. This staining pattern is consistent with previous observations (Mendoza et al., 1992). After PAF fixation, a less diffuse and slightly more precise PSA-FITC acrosomal labelling was observed.

Our data clearly show that, whatever the fixation procedure, the labelling of the spermatozoa by PNA-FITC was restricted to the acrosomal region, whereas PSA-FITC labelled the acrosome and the whole head, as well as the flagellum. The PSA-FITC labelling appeared less selective and it was sometimes hard to discriminate between the presence or the absence of acrosome.

Incidentally, the use of PSA-FITC allowed the labelling of the cytoplasmic droplet, when present. Hence, the present data further suggest that PSA-FITC, although being less selective for detecting the acrosome than PNA-FITC, can be recommended for tracking the cytoplasmic droplet, a transient spermatozoal organelle (Okó et al., 1993). These authors previously reported a

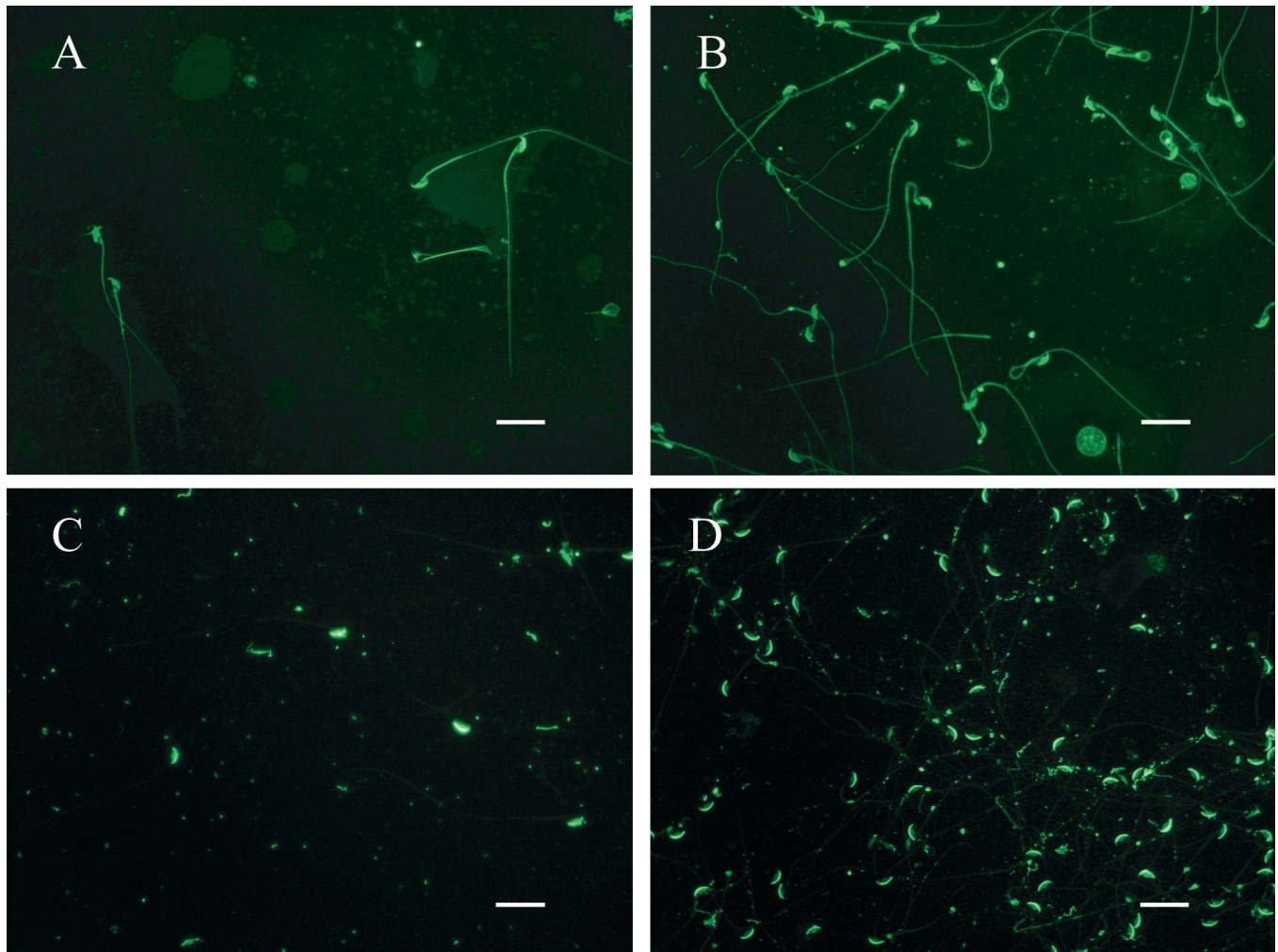
### Acrosome quantification in spermatozoa

lectin labelling of saccular elements of the cytoplasmic droplet by colloidal gold linked- Ricinus communis agglutinin I (RCA I) and *Helix pomatia* lectin (HPL) (Oko et al., 1993). Therefore, it is not surprising that the acrosome and the cytoplasmic droplet, both organelles derived from the Golgi apparatus (Abou-Haila and Tulsiani, 2000), might share some lectin specificity.

It should be mentioned, however, that our investigation focused on the acrosome detection and did not include any viability assessment of the spermatozoa. Further investigations should combine acrosome lectin labelling with a procedure of viability determination to detect the acrosome reaction resulting from a physiological event or a secondary degenerative process. Several methods have been previously described for such a purpose, including the use of conventional vital

stain, such as yellowish eosin (Ozaki et al., 2002), labelling with fluorescent DNA-binding supravital stain, such as Hoechst 33258 (Tao et al., 1993; Kingler and Rajalakshmi, 1995) or propidium iodide (Martí et al., 2000) and the hypo-osmotic swelling test (Esteves et al., 2007).

The present technical approach also compared spermatozoa suspensions either smeared or spotted and then spread onto glass slides. Smear preparations clearly gave rise to major drawbacks. Spermatozoa were concentrated on the edges of the slides with a relatively small number in the center. Moreover, a marked proportion of spermatozoa evidenced damaged acrosomes, and such an aspect can affect the subsequent quantification of true acrosome-reacted sperm. A sperm suspension spotted and spread gently onto glass slides



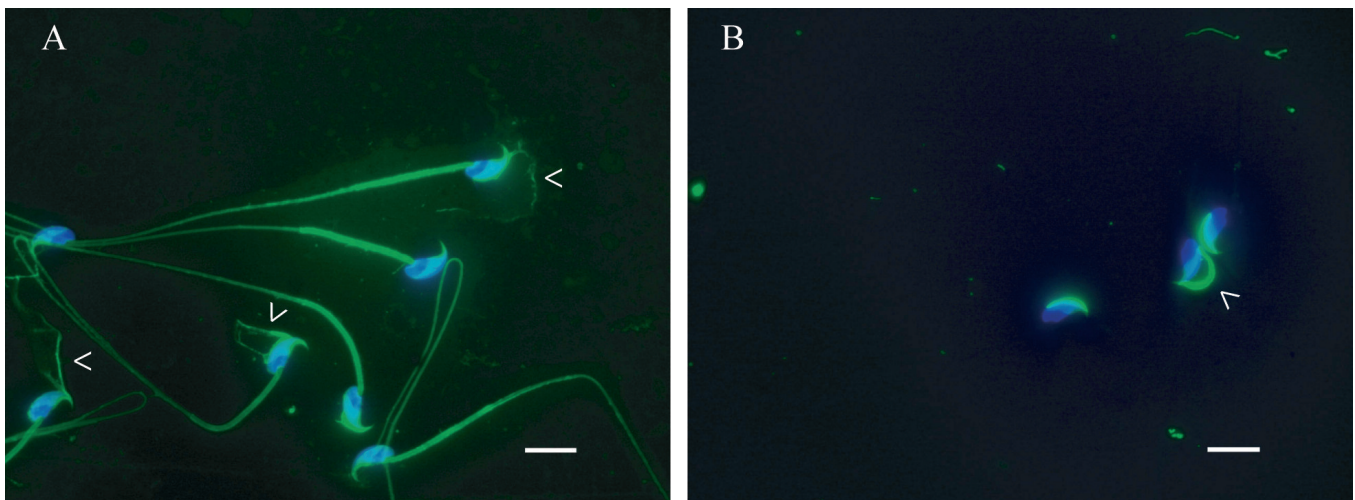
**Fig. 3.** Acrosome detection by PSA-FITC and PNA-FITC on different types of sperm preparation. Upper panels: PSA-FITC. Absolute methanol fixed. Smear sperm preparation (A) and spotted sperm preparation (B). Lower panels: PNA-FITC. Absolute methanol fixed. Smear sperm preparation (C) and spotted sperm preparation (D). Smear preparations (A and C) display a relatively low density of spermatozoa in the center of the preparation. Sperm suspensions spotted and spread gently onto the microscope slide (B and D) exhibit a homogenous distribution of spermatozoa. Scale bar: 25  $\mu$ m.

### Acrosome quantification in spermatozoa

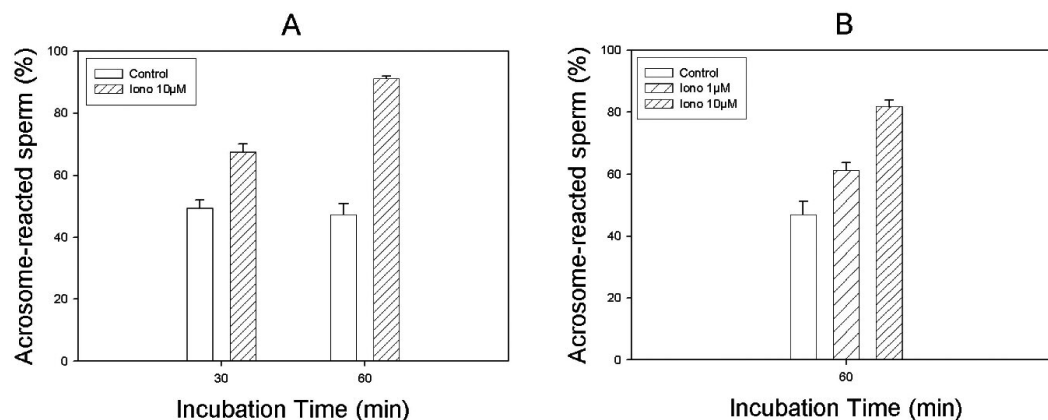
was more homogeneously distributed over the microscope slides and generated less damaged spermatozoa. Therefore, the latter procedure appears preferable to the more usual smear preparation, which should be avoided.

Lastly, our results indicate that the calcium ionophore ionomycin (Fasolato and Pozzan, 1989) provoked a time- and concentration-dependent increase in the acrosome reaction from mouse spermatozoa labelled with PNA-FITC. These findings emphasize the role of  $Ca^{2+}$  inflow in the induced - acrosome reaction and further validate our histological procedure to detect and quantify this physiological process. We also provided evidence that the percentage of “doubtful” spermatozoa was not influenced by the ionomycin challenge, indicating the reliability of the present methodology.

It is worth noting that “Inducability”, the difference between “induced” and “spontaneous” acrosome reaction, has been reported to be of prognostic value for the sperm fertilization capacity (Köhn et al., 1997). Moreover, the morphological changes of spermatozoa following a calcium ionophore treatment have been reported to be similar to those occurring during spontaneous or physiologically-induced acrosome reaction (Russell et al., 1979). Hence, the quantification of acrosome reaction in ionomycin-treated mouse spermatozoa, which is a simple and reliable test allowing a functional evaluation of the sperm, would help to predict the fertilization ability of semen (Cummins et al., 1991; Fenichel et al., 1991; Pampiglione et al., 1993; Yovich et al., 1994). The acrosome reaction test completes the list of other specialized sperm function tests, like the zona-free sperm penetration assay, IVF,



**Fig. 4.** Smear sperm preparations (absolute methanol fixed). Acrosome staining by PSA-FITC (A) or PNA-FITC (B) (green). Nuclei are counterstained with DAPI (blue). Numerous spermatozoa heads appear damaged (arrowheads). Scale bar: 10 µm.



**Fig. 5.** Quantification of the acrosome reaction induced by ionomycin. **A.** Acrosome reaction following a 30 or a 60 min incubation period in the presence of ionomycin (iono) 10 µM. **B.** Acrosome reaction following a 60 min incubation period in the presence of ionomycin (iono) 1 or 10 µM.

hemizona assay, creatine kinase or examination of DNA fragmentation (Esteves et al., 2007).

In conclusion, the present study clearly evidences that PNA-FITC staining was more specific than PSA-FITC labelling to detect the acrosome in mouse spermatozoa. The fixation method had no influence on the PNA-FITC staining pattern. Moreover, our data indicate that sperm suspensions should be spotted and gently spread rather than smeared onto glass slides in order to avoid mechanical damage to the acrosome.

The easy, reproducible and convenient method described here allows the precise quantification of the "induced"-acrosome reaction and, besides helping to evaluate the sperm fertilization capacity of semen, should be considered as a valuable tool to investigate mouse acrosomal physiology.

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