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Investigation of pig sperm plasma membrane reorganization using progesterone-albumin-fluorescein probes

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ABSTRACT

Objective: To relate semen susceptibility in cooling protocols to sperm plasma membrane properties. **Methods:** A series of experiments was performed using the fluorescent markers, progesterone-BSA-FITC and BSA-FITC. **Results:** These experiments indicated that both progesterone-BSA-FITC and BSA-FITC bound to specific sperm plasma membrane domains, thus producing four different binding patterns, revealing probable changes in membrane organization during capacitation and during cooling. Those patterns seem to make a sequence progressing from non-capacitated status to capacitated status. The proportion of each pattern was different during incubation than during cooling, showing the latter had a higher proportion of dead sperm than the former. **Conclusions:** At this stage, the association of sperm plasma membrane alterations was revealed by BSA-FITC probes and cryosensitivity remains unclear.

1. Introduction

The sperm plasma membrane is widely considered to be the main structure affected by cryopreservation protocols. Resistance or susceptibility to these procedures seems to be influenced by its biochemical composition and fluidity, and by other mechanisms including cytoskeletal proteins attached to intramembranous structures[1-2]. Watson has proposed that after freezing and thawing, spermatozoa enter a state which resembles capacitation, as if such processes trigger the mechanisms responsible for that physiological condition[3]. Capacitation implies increased fragility and readiness to fuse with other membranes, therefore freeze-thawing could be destabilizing the sperm membranes in a way which resembles the processes prior to fertilization. Maxwell and Johnson confirmed that capacitation-like effects, evaluated by the CTC test were induced by the cooling and cryopreservation of boar spermatozoa[4]. The effect of cooling may therefore be to induce premature capacitation and destabilization, with a consequently shortened sperm lifespan[5]. Green and Watson have shown that sperm capacitation-like state induced by cooling is not analogous to true capacitation[6]. The main differences were associated with changes in lipid bilayer fluidity and intracellular signalling pathways. There is evidence for the occurrence of a sperm plasma membrane progesterone receptor which seems to be laterally mobile in the membrane[7-9]. Two amino acid neurotransmitter receptor/Cl-channels (glycine receptor/Cl-channel and GABAA receptor-like/Cl-channel) have been identified in mammalian spermatozoa[9]. Those receptors are considered to play an important role in the acrosome reaction. GABAA receptor-like/Cl-channel is involved in the progesterone-initiated acrosome reaction[10]. Although there is no definite evidence for the presence of the non-genomic progesterone receptor in pig spermatozoa, Barboni *et al.* reported that this steroid accelerated or facilitated the process of capacitation without directly affecting the acrosome reaction[11]. Others however have observed that progesterone was able to stimulate the pig sperm acrosome reaction[9]. With the aid of a fluorescent progesterone-conjugate, different topographical distribution patterns have been detected on the human sperm membrane during capacitation. The proportion of those binding patterns changes with time in a temperature-dependent fashion, probably reflecting a

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membrane remodelling process. Tesarik *et al.* identified two binding patterns in live spermatozoa: one showing a uniform labelling of the whole acrosomal area, considered as acrosome-intact; and another showing restricted labelling to the equatorial segment, considered as acrosome-reacted spermatozoa[8]. Cheng *et al.* identified two patterns in live stallion spermatozoa: one restricted to the acrosomal area, similar to that obtained by indirect inmunofluorescence with a monoclonal antibody against the intracellular progesterone receptor; and other displaying various degrees of patchy fluorescence[12].

Different approaches to investigate the effect of progesterone on the sperm membranes have shown a common feature: only a proportion of the spermatozoa display a binding pattern or respond to its stimuli. Sabeur *et al.* used a mouse monoclonal antibody against the c-terminal steroid-binding domain of the human intracellular progesterone receptor to detect the plasma membrane progesterone receptor in human spermatozoa[13]. They observed that only 50% of the cells displayed immunoreactivity with such antibody, indicating that, probably, only capacitated spermatozoa display the putative receptor[13]. Cheng *et al.* observed that only 22% of stallion spermatozoa exhibited acrosomal cap immunoreactivity, using the same monoclonal antibody[12] . These authors suggested that the receptor may migrate from an inner compartment or become apparent after the removal of some proteins coating the spermatozoa.

The following series of experiments was based around the hypothesis that mobility of the progesterone plasma membrane receptor may be similar during capacitation and cooling; thus, the bigger the change from non-capacitated to capacitated status during incubation the higher sperm susceptibility to that cooling "capacitation-inducing" effect. In order to investigate this hypothesis, the sperm progesterone receptor was examined for its value as a probe of membrane organization and remodelling. The progesterone receptor was selected for study because previous studies showed that it would undergo redistribution after binding, an indication of mobility and membrane fluidity.

2. Materials and methods

2.1. Source and preparation of semen

Semen was obtained from fertile boars housed at the JSR Healthbred Ltd Centre (Yorkshire, England), diluted in Beltsville Thawing Solution (BTS), stored in plastic bottles, and sent to London in polystyrene containers.

Semen samples were washed through two-layer discontinuous Percoll density gradients (70% and 35%) to select the motile subpopulation[13]. Spermatozoa were then resuspended in Tyrode's complete medium, TALP[14], containing 5 mg/mL of unlabelled-BSA (Sigma Chemical Co., Poole, UK). Sperm suspensions were incubated at 39 °C in 5% (v/v) carbon dioxide and sampled when required.

2.2. Use of progesterone-BSA-fluorescein isothiocyanate (P-BSA-FITC) and BSA-fluorescein isothiocyanate (BSA-FITC) probes

Progesterone 3-(O-carboxymethyl)-oxime-BSA-FITC and BSA-FITC (Sigma, Poole Dorset, UK) solutions were prepared by dissolving 1 mg of each probe in 1 mL of deionised water each and were stored at 5 °C until required.

Either P-BSA-FITC or BSA-FITC solutions (5 μL) were added to 100 μL sub-samples of spermatozoa in Tyrode's medium (2.5×10^7 spermatozoa/mL) to give a final concentration of 50 g/mL BSA-FITC or P-BSA-FITC (*i.e.*, one percent of the concentration of unlabelled BSA). Spermatozoa were incubated for 10 min to allow binding of the probe.

Sperm suspensions were then smeared on slides and dried. After fixation for 30 min in ethanol, slides were allowed to dry and mounted with Citifluor (Citifluor Ltd., Leicester, UK). Slides were examined by epifluorescence microscopy, using a Zeiss Axioskop microscope; 200 cells were counted from each slide and classified according to the P-BSA-FITC and BSA-FITC probe binding patterns (from the first experiment).

2.3. Flow cytometry

The flow cytometer used in this work was a Coulter Epics XL instrument (Beckman Coulter Ltd, High Wickham, UK). The device was appropriately set up for signals and colour compensation, and at least 5 000 cells were assessed.

2.4. Acrosomal status assessment

Lectin solution (100 μL) from pisum sativum-tetramethylrhodamine conjugate (1 mg/mL; Sigma, Poole, UK) was flooded on each of the ethanol-fixed slides. They were incubated in the dark for 10 min, rinsed with deionized water, allowed to dry and mounted in Citifluor. Each cell was examined for both rhodamine and fluorescein fluorescence to assess the acrosomal status and binding pattern; that is, cells were double-stained. Acrosomes were classified as intact, reacted, or loose.

2.5. Disruption of the sperm plasma membrane

The method used was cold shock. Eppendorf tubes containing spermatozoa in TALP medium (500 μL) plus BSA-FITC, were submerged in a water-ice mixture at 0 °C for 20 min. Slides were prepared as mentioned.

2.6. Anti-BSA antibody technique

Spermatozoa were washed (Percoll gradients) and resuspended in Tyrode's medium. After 10 minutes incubation at 39 °C in 5% (v/v) carbon dioxide, unbound BSA was removed by passing the sperm suspensions into a Millipore filter unit (0.22 μm pore size). The filter was washed with Tyrode's medium lacking BSA to recover the spermatozoa. Spermatozoa were fixed in 4% formaldehyde in phosphate buffer (pH 7.3) for 30 min; after washing three

times in PBS by centrifugation the pellets were resuspended in either (i) monoclonal anti-BSA (mouse IgG_{2a} isotype; Sigma, Poole, UK) in PBS or (ii) PBS only, and incubated for 1 h at 39 °C. Spermatozoa were washed three times then resuspended in a mixture of normal rabbit serum (5%, v/v) and a blocking solution in PBS (0.1% fetal calf serum, v/v; 0.05% Tween, v/v, 10 g/L gelatine, 0.2 g/L glycine) to prevent non-specific second antibody binding, and left for 15 min at room temperature.

Spermatozoa were further incubated in FITC-labelled anti-IgG, washed and finally resuspended in antifade solution (Citifluor) and examined by fluorescence microscopy.

2.7. Isoelectric focusing technique

Six different sources of serum albumin were used in this experiment: (i) BSA-FITC (Sigma, Poole, UK), (ii) BSA-FITC (Molecular Probes, Leiden, The Netherlands), (iii) fatty acid-free BSA (Sigma, Poole, UK), (iv) Human serum albumin (Sigma, Poole, UK), (v) BSA-tetramethylrhodamine (Molecular Probes, Leiden, The Netherlands), and (vi) unlabelled BSA (Sigma, Poole, UK), using a concentration of 0.5 mg/mL in all cases.

The samples were run in a single phase 6% polyacrylamide gel, containing ampholines (pH range 3.0-10.0; Bio-Lyte 3/10 Ampholite, Bio Rad Hercules, CA). The gel was focused for 2 500-4 000 Vh for 17 h on a vertical electrophoretic apparatus (Cross Power 500 and Constapower 3500, Genetic Res. Inst. Dunmow, Essex).

2.8. Statistical analysis

Counts of spermatozoa displaying the different binding patterns were expressed as percentages, arcsine transformed and examined by Analyses of Variance (ANOVA), using Statistica for Windows (Statsoft, UK).

2.9. Experimental design and procedures

2.9.1. Localization of P-BSA-FITC and BSA-FITC probes on the pig sperm plasma membrane

Semen samples from four boars (replicates) were washed and resuspended in TALP medium. Sperm suspensions were incubated at 39 °C for 1 h in 5% (v/v) carbon dioxide in air.

Either P-BSA-FITC or BSA-FITC solution (5 μL) was added to 100 μL sub-samples taken from sperm in TALP after 15, 30 and 60 min of incubation, and further incubated for 10 min to allow binding of the probe.

Slides were prepared as described and examined for the topographical localization of P-BSA-FITC and BSA-FITC binding on the sperm head plasma membrane.

2.9.2. Dynamic changes of the proportion of P-BSA-FITC and BSA-FITC binding patterns on the pig sperm plasma membrane during incubation at 39 °C and cooling at 5 °C

Semen samples from eight boars (replicates) were washed and resuspended in TALP medium. Sperm suspensions were split into two aliquots, one was incubated at 39 °C for 3 h in 5% (v/v) carbon dioxide in air, the other was slowly cooled to 5 °C (0.2 °C/min) and stored at that temperature for 3 h.

Either Progesterone-BSA-FITC or BSA-FITC solution (5 μL) was added to 100 μL sub-samples taken from each aliquot of sperm in TALP: (i) just before incubation or cooling, (ii) after 90 min of incubation or cooling or (iii) after 180 min of incubation or cooling; and further incubated or kept cool at 5 °C for 10 min to allow binding of the probe. Slides were prepared as described and examined for the P-BSA-FITC and BSA-FITC binding patterns.

2.9.3. Flow cytometric examination of P-BSA-FITC and BSA-FITC binding to the sperm plasma membrane

The objectives of this experiment were as follows: (i) to use a sensitive quantitative technique for comparison of P-BSA-FITC and BSA-FITC in live cells, and (ii) to examine the comparison between live and dead cells. This was achieved using a BSA probe plus Propidium iodide (PI, Molecular Probes, Leiden, The Netherlands) for cell viability.

Semen samples from two boars (replicates) were processed as previously described. Spermatozoa in TALP medium were incubated for 90 min; at the end of that period either P-BSA-FITC or BSA-FITC was added. One aliquot of each treatment was cold-shocked by immersion in water-ice at 0 °C for 20 min to obtain a dead sperm population. Live (incubated) and dead (cold-shocked) sperm aliquots were then supplemented with PI (12 μmol/L), another two aliquots remained as control groups (without PI). All treatments were then examined by flow cytometry.

2.9.4. Acrosomal status of the BSA-FITC binding patterns after incubation at 39 °C

Spermatozoa from six boars (replicates) were incubated in TALP medium containing BSA, lactate, and pyruvate at 39 °C for 3 h in 5% (v/v) carbon dioxide. The sperm concentration was 2.5×10^6/mL.

BSA-FITC solution (5 μL) was added to 100 μL sub-samples taken from each boar sample: (i) before incubation, (ii) after 90 min and (iii) after 180 min of incubation. After 10 min further incubation at 39 °C with the probe, spermatozoa were smeared on slides and processed for acrosomal status assessment using the PSA-Rhodamine technique.

2.9.5. Alteration of the sperm plasma membrane to assess changes in the proportion of BSA-FITC binding patterns

The hypothesis behind this investigation was that since BSA-FITC binding patterns reflect properties of the sperm surface, stresses of various kinds would change the surface organization therefore changing the patterns; this may identify final points in the sequence of patterns.

Spermatozoa from four boars (replicates) were incubated in TALP medium at 39 °C; sub-samples were cold-shocked after 20 and 180 min. Slides were prepared after each cold shock treatment and from incubated, non-cold-shocked aliquots (control group). The slides were examined for the analysis of binding patterns.

2.9.6. Immunological localization of BSA bound to the sperm plasma membrane

Spermatozoa from two boars were incubated in TALP medium containing 5 mg/mL unlabelled BSA. The

suspending medium was replaced with TALP medium lacking BSA, and a monoclonal antibody against albumin was used to localize the BSA not removed by the sperm washing step. The localization of BSA on individual spermatozoa was assessed qualitatively.

2.9.7. Examination of different fluorescent-labelled BSA preparations for their sperm plasma membrane binding affinity

An isoelectric focusing experiment was carried out to see whether presence of the fluorochrome or the absence of lipids affected BSA electric charge, providing it with an electrostatic advantage over the unlabelled BSA contained in TALP medium.

Different sources of serum albumin: (i) BSA-FITC from Sigma, (ii) BSA-FITC from Molecular Probes, (iii) Fatty acid-free BSA, (iv) Human serum albumin, (v) BSA-Rhodamine, and (vi) BSA from Sigma, were assessed for their isoelectric point.

3. Results

3.1. Localization of P-BSA-FITC and BSA-FITC probes on the pig sperm plasma membrane

Several binding categories were observed on the sperm head plasma membrane with both fluorescent probes; however, four clearly distinguishable binding patterns were identified. The numbers were assigned regarding the abundance of each pattern in fresh ejaculates (Figure 1 A).

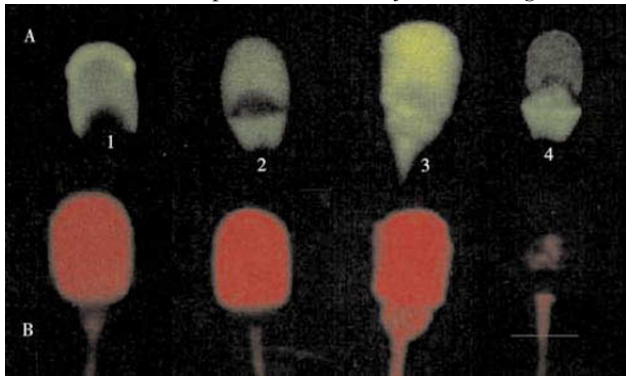

Figure 1. Micrographs showing (A) fluorescent progesterone-BSA-FITC and BSA-FITC binding patterns (1-4) on the pig sperm plasma membrane and (B) acrosome status of each pattern revealed by fluorescent PSA-rhodamine lectin.
Each pair of photographs was taken from the same double fluorochrome-loaded spermatozoon by changing the light filter. Scale bar represents 10 μm.

(i) Pattern 1. The fluorescent probe was localized on the acrosomal cap, covering two thirds of the head; in some cases the postacrosomal area was visible. (ii) Pattern 2. There was a brilliant and well defined line just below the equatorial segment, the acrosomal cap was visible. (iii) Pattern 3. A broad and brilliant band was present in the postacrosomal area, the acrosomal cap was very bright and irregular. (iv) Pattern 4. The postacrosomal segment was bright, the acrosomal cap was scarcely visible. These binding patterns were still present at the different sampling times, most of the sperm (92% approximately) showed one of such patterns.

3.2. Dynamic changes of the proportion of progesterone-BSA-FITC and BSA-FITC binding patterns on the pig sperm plasma membrane during incubation at 39 °C and cooling at 5 °C

Proportions of spermatozoa showing the various fluorescent binding patterns were the same with either P-BSA-FITC or BSA-FITC, at any time and temperature (Figure 2). However, there were differences in proportions of spermatozoa showing the various fluorescent binding patterns due to time (0, 90 and 180 min): after 90 min of incubation or cooling, there was a significant increase in the proportion of spermatozoa displaying patterns 2, 3 or 4, whereas sperm displaying pattern 1 decreased ($P<0.03$, Figure 3). None of these proportions changed at 3 h.

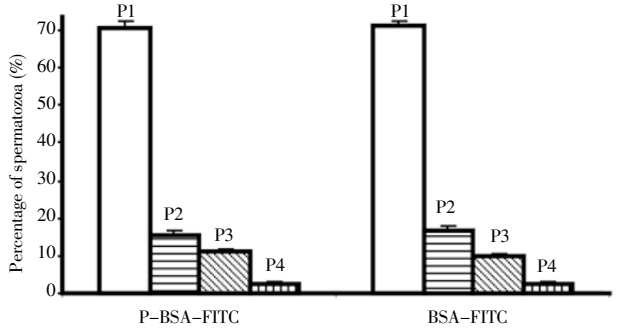

Figure 2. Progesterone-BSA-FITC (P-BSA-FITC) and BSA-FITC binding patterns on the pig sperm plasma membrane.
Values are overall means±SEM from three periods of time (0, 90, and 180 min) and two temperatures (incubation at 39 °C and cooling at 5 °C).

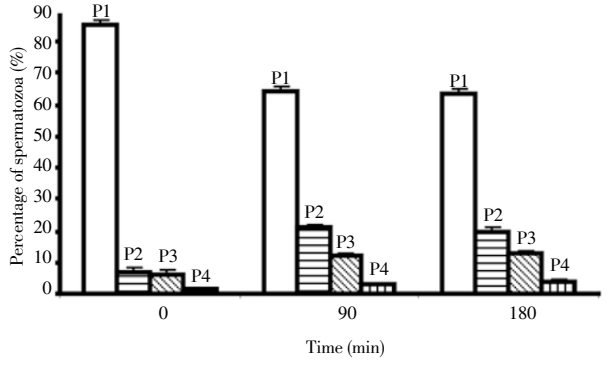

Figure 3. Effect of time progression on Progesterone-BSA-FITC and BSA-FITC binding patterns frequency.
Values are means±SEM of both probes at two temperatures (incubation at 39 °C and cooling at 5 °C).

Changes observed during incubation were different from those after cooling (Figure 4). During incubation, patterns 2 and 3 increased similarly, whereas during cooling pattern 2 increased remarkably: from 7.1% at time 0 to 19.0% after 90 and 180 min of cooling ($P<0.002$), but pattern 3 did not change.

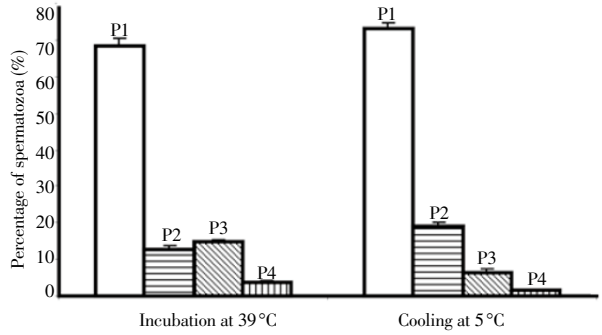

Figure 4. Effect of temperature on progesterone-BSA-FITC and BSA-FITC binding patterns frequency.
Values are means±SEM of both probes during three periods of time (0, 90, and 180 min).

3.3. Flow cytometric examination of P-BSA-FITC and BSA-FITC binding to the sperm plasma membrane

P-BSA-FITC fluorescence intensity was approximately 6 times greater than that from BSA-FITC in both live and dead spermatozoa. Fluorescence intensity was also greater in dead than in live spermatozoa for both probes (Table 1). These results confirmed the observations from the fluorescence microscope that dead sperm are brighter than live sperm. They also showed that P-BSA-FITC binding was more specific than that of BSA-FITC. Dot plot distribution data from the flow cytometer of both P-BSA-FITC and BSA-FITC was spread in a wide range of fluorescence, becoming more intense as they become PI positive. The wide fluorescence range suggested the presence of sperm subpopulations, presumably the described patterns. It is, however, important to note that the greater brightness produced by P-BSA-FITC cannot be detected visually by fluorescence microscopy. The presence of PI slightly quenched BSA-probe fluorescence in both live and dead spermatozoa. This effect was small but consistent; there was probably interference in the FITC and PI emission fluorescence spectra.

3.4. Acrosomal status of the BSA-FITC binding patterns after incubation at 39 °C

Most of the spermatozoa displaying pattern 1 had intact acrosomes and their value was constant along time: 75% at time 0, nearly 70% after 90 min, and around 80% after 180 min of incubation. Most of the spermatozoa displaying pattern 3 had reacted acrosomes (around 85%), and all spermatozoa displaying pattern 4 had loose acrosomes at any time of being sampled (Table 2, Figure 1B). These results suggested that pattern 1 may represent the uncapacitated acrosome-intact subpopulation; pattern 3 may represent capacitated acrosome-reacting spermatozoa; and pattern 4 could represent either the capacitated acrosome-reacted or the damaged deteriorated subpopulation. The sequence for these patterns seems to progress from pattern 1 to pattern 3 and then pattern 4. Other evidence shows that pattern 2 cells have damaged or leaky plasma membrane (see, next experiment results), so this pattern was not considered.

3.5. The effect of cold shock on the proportion of BSA-FITC binding patterns

After cold shock, the proportion of pattern 2 spermatozoa increased notably compared with the control (incubated, not cold-shocked) group, both after 20 min (70.6% vs 11.4%) and after 3 h (60.6% vs 15.9%). The increase was due mainly to a decrease in pattern number 1. The proportion of pattern number 3 after cold shock was 50% lower than the control group (Table 3).

3.6. Immunological localization of BSA bound to the sperm plasma membrane

The majority of spermatozoa exposed to anti-BSA antibody (most of them intact) displayed fluorescence over the whole acrosomal cap and the apical ridge; this resembled the BSA-FITC-probe pattern 1. In some spermatozoa the postacrosomal region and the tail were also fluorescent

Table 1
P-BSA-FITC and BSA-FITC mean fluorescence intensity on the plasma membrane of live and dead spermatozoa.

Fluorescent probes	Live spermatozoa		Dead spermatozoa	
	plus PI[a]	no PI[a]	plus PI	no PI
P-BSA-FITC	29.93	32.83	41.33	44.67
BSA-FITC	4.52	5.23	7.02	7.81
None	2.33	–	3.64	–

Data have been corrected for moles FITC/mole BSA (3 moles of FITC/mole P-BSA; 11 moles FITC/mole BSA). Fluorescence of P-BSA-FITC 3×10; Fluorescence of BSA-FITC 11×10. Fluorescence is expressed in arbitrary units. [a]Treatments were performed in the presence or absence of 12 μmol/L PI.

Table 2
Proportion of acrosome-intact spermatozoa (%) in each of the four BSA-FITC binding patterns sampled at various times during incubation.

Pattern	Time 0	Time 90 min	Time 180 min
Pattern 1	75.1±7.74[a]	69.4±3.4[a]	84.3±5.7[a]
Pattern 2	64.0±15.7[a]	34.2±9.4[b]	73.1±4.6[a]
Pattern 3	9.7±6.24[a]	7.7±2.7[a]	21.1±5.1[a]
Pattern 4	0.0	0.0	0.0

Values are means±SEM. Those bearing a different superscript in rows differ significantly ($P<0.05$) (six boars).

Table 3
Percentages of BSA-FITC binding patterns on the sperm plasma membrane after cold shock.

Pattern	Time 0	Time 20 min		Time 180 min	
BSA-FITC binding pattern	-	-	+	-	+
Pattern 1	84.4±1.9	66.6±4.3^{a**}	17.4±2.3^{b}	60.1±2.8^{a}	25.3±6.6^{b}
Pattern 2	5.8±1.9	11.4±5.6^{a**}	70.6±3.1^{b}	15.9±4.0^{a}	60.6±6.7^{b}
Pattern 3	9.6±2.1	19.4±5.8^{a*}	10.3±2.4^{b}	20.0±2.5^{a}	10.4±2.9^{b}
Pattern 4	0.3±0.1	2.6±1.5	1.8±0.3	4.0±1.3	3.8±1.5

Values are means±SEM. Those bearing a different superscript in rows differ significantly ($^{**}P<0.0001$, $^{*}P<0.02$) (four boars).

(Figure 5). These results confirmed that unlabelled BSA is indeed bound to the sperm surface and supports the view that probes based on BSA-FITC conjugates label the sperm surface through specific interactions involving the BSA itself.

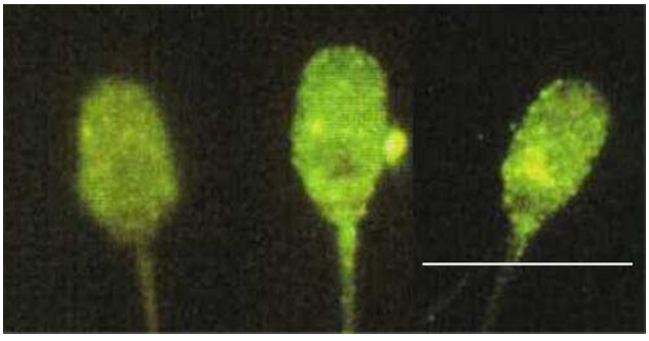

Figure 5. Micrograph showing fluorescent anti-BSA antibodies bound to the pig sperm plasma membrane during incubation conditions. Scale bar represents 10 μm.

3.7. Examination of different fluorescent-labelled BSA preparations for their sperm plasma membrane binding affinity

The isoelectric point of all six serum albumin preparations was similar; it was localized between pH 6.0 and 7.0. This showed that these compounds do not have net charge at physiological pH and that labelled BSA does not have any electrostatic advantage over unlabelled BSA.

4. Discussion

These experiments were set up to explore the possible use of a sperm plasma membrane marker to study semen susceptibility to cooling with regard to sperm plasma membrane properties such as fluidity. The first experiments (localization of P-BSA-FITC and BSA-FITC on the sperm head plasma membrane, and P-BSA-FITC and BSA-FITC binding with regard to changes of time and temperature) produced some doubt as to whether P-BSA-FITC was acting as a probe for the progesterone receptor. Thus, a more sensitive approach (flow cytometry) was used next to discriminate between P-BSA-FITC and BSA-FITC binding. Nevertheless, the subsequent experiments were designed to study the binding of BSA-FITC without progesterone at a number of events affecting the sperm plasma membrane, for example the acrosome reaction.

The BSA-FITC binding patterns on the pig sperm membrane were very distinctive considering that other reports, in human spermatozoa, describe BSA-FITC binding as being non-specific[7,8] or no binding at all in caprine spermatozoa[15]. The specificity and avidity of binding was clear, when it is considered that the spermatozoa were simultaneously exposed to a medium containing a hundred fold higher concentration of unlabelled BSA. However, the binding mechanism is unclear. One possibility is that BSA-FITC displaces non-conjugated BSA from the sperm membrane and binds to lipids on the outer bilayer; the BSA lipid-binding properties have been recognized[16,17]. If that were the case, the temporal and temperature changes in the sequence of BSA-FITC binding patterns could indicate a remodelling process (lateral phase distribution) occurring on the sperm membrane. We have established that BSA-FITC does not have an electrostatic advantage over the unlabelled BSA, since their isoelectric focusing points were not different; this supports the lipid-binding hypothesis. However, adding suspensions of cholesterol and other lipids (phosphatidylethanolamine, phosphatidylcholine) to the sperm incubation medium to saturate the BSA-FITC molecules did not interfere with the binding (data not shown). Another possible mechanism could include the specific binding of BSA-FITC to membrane proteins. Calvete *et al.* suggested two ways by which some seminal plasma proteins, spermadhesins, bind to the sperm plasma membrane: some may bind to lipids, while others may coat over them as aggregated molecules[18]. Perhaps BSA-FITC binds like the second group. In support of that view, BSA-binding to the sperm surface has been demonstrated by the use of anti-BSA antibodies which bind the whole head and part of the tail, resembling some of the BSA-FITC patterns. In this study it was shown that BSA-FITC does not bind to the acrosomal matrix after permeabilization; dissimilar patterns were observed when this probe was used simultaneously with PSA-rhodamine, which is known to bind to that site[19]. This evidence supports the idea that binding patterns are produced by BSA-FITC, since the microscopic examination of the sperm plasma membrane was not sensitive enough to detect any specific binding of P-BSA-FITC. However, flow cytometry results showed that fluorescence from P-BSA-FITC was higher than that from BSA-FITC, despite the lower content of FITC moles/mol of BSA (11, BSA-FITC vs. 3, P-BSA-FITC). These findings suggested that progesterone bound to specific sites on the sperm plasma membrane as reported in other species[12,13]. In support to this point of

view, Somanath and Gandhi reported that P-BSA-FITC-treated goat spermatozoa showed a single peak in flow cytometry (that is, the sperm population was homogeneous; more than 90% of the spermatozoa expressed the putative progesterone receptor)[15], in contrast to BSA-FITC treated spermatozoa in this study that showed a FITC negative peak. It is worth noting that in this study no changes were observed in the topographical localization of P-BSA-FITC with time. The evidence can be interpreted simply that patterns represent various stages of capacitation. In support of this view, fluorescent patterns produced by CTC, a probe regarded as a capacitation reporter[20,21], are similar to those seen with P-BSA-FITC and BSA-FITC. Wang *et al.*, who studied boar spermatozoa, found the same basic fluorescent CTC patterns identified in spermatozoa from other species; these similarities include both morphological binding and dynamic progression with time[21]. These experiments indicate that both P-BSA-FITC and BSA-FITC bind to the sperm plasma membrane, revealing probable changes in membrane organization during capacitation and during cooling. This approach could potentially be used as a marker for studying variations between membrane responses to cooling in different boars. The advantage of such an approach would be that individual spermatozoa and even membrane domains could be studied by simple methods.

At this stage, the association of plasma membrane alterations revealed by P-BSA-FITC and BSA-FITC probes during incubation and cooling and those produced during cryopreservation protocols remains unclear. However, these results are of sufficient interest to stimulate further research into this topic.

Conflict of interest statement

We declare that we have no conflict of interest.

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