Chapter 3

Evaluation of *in vitro* capacitation of stallion spermatozoa

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Abstract

The primary aim of this study was to establish a flow cytometric technique for determining the capacitation status of stallion spermatozoa. To this end, a flow cytometric technique that demonstrates changes in the plasma membrane fluidity, namely merocyanine 540 staining, was compared with the more conventional Ca\(^{2+}\) dependent fluorescence microscopic technique for assessing capacitation status, chlortetracycline (CTC) staining. In addition, the effect of bicarbonate/CO\(_2\) on the progress of capacitation and the acrosome reaction (AR) and on temporal changes in sperm motility with particular regard to hyperactivation, was analysed. For the study, fresh semen was washed and then incubated for 5 h in bicarbonate-containing or bicarbonate-free medium, with or without Ca\(^{2+}\)-ionophore to induce the AR, and at intervals during incubation aliquots were taken and analysed with respect to capacitation and acrosome status. The AR was assessed using both the CTC and FITC-PNA staining techniques with similar results. In brief it was found that merocyanine 540 detects capacitation related changes much earlier than CTC (0.5 h versus ~3 h) and that flow cytometry for evaluation of capacitation and AR was a quicker (10 sec per sample) and more accurate (10,000 cells counted) technique than fluorescence microscopy. Furthermore, it was observed that Ca\(^{2+}\)-ionophore could not induce the AR in the absence of bicarbonate, but that the ionophore synergies the bicarbonate-mediated induction of the AR as detected by CTC (although not significant when evaluated using FITC-PNA). The percentage of hyperactive sperm in each sample was not affected by time of incubation under the experimental conditions studied. In conclusion, merocyanine 540 staining is a better method for evaluating the early events of capacitation, for stallion spermatozoa incubated in vitro, than CTC staining. Furthermore, bicarbonate sperm activation clearly plays a vital role in the induction of the AR in stallion spermatozoa.

Introduction

"Capacitation" is a collective term for the changes that a spermatozoon undergoes when it comes into contact with the female reproductive tract. These changes include reorganisation of membrane proteins, metabolism of membrane phospholipids, a reduction in membrane cholesterol levels, and hyperactivation [1]. These changes, together with the subsequently induced acrosome reaction (AR), an irreversible exocytotic event, are essential if a sperm is to bind to and penetrate the zona pellucida and thereafter to fuse with the oocyte plasma membrane [1]. Capacitation is, thus, a critical event in the process of fertilisation. However, differentiating capacitated from non-capacitated spermatozoa remains inexact science, despite almost half a century having past since Chang [2] and Austin [3] first described the phenomenon of capacitation, and it is frustrating that a straightforward, validated and easy to interpret method for assessing capacitation is still not in common use.

On the other hand, chlortetracycline (CTC) staining has been used to assess the capacitation state of spermatozoa [4-7] and it is currently the assay of choice because it distinguishes
three different stages of sperm activation, viz. non-capacitated, capacitated acrosome-intact and capacitated acrosome-reacted. However, a clear understanding of how CTC interacts with the sperm surface at the molecular level is lacking and unfortunately the evaluation of CTC staining is performed on fixed sperm cells.

Merocyanine-540 staining is another technique that may be useful for assessing the capacitation status of spermatozoa. Merocyanine-540 is a hydrophobic dye that has been shown to stain cell membranes more intensely if their lipid components are in a higher state of disorder [8,9], as is the case for capacitated spermatozoa. In this latter respect, merocyanine-540 has recently been used to monitor alterations in the lipid architecture of the boar sperm plasma membrane during capacitation [10], a process that appears to be due to bicarbonate induced transbilayer scrambling of phospholipids [11]. One major advantage of merocyanine 540 over CTC is its suitability as a probe for assessing capacitation flow-cytometrically, since this latter technique should allow for more objective analysis of larger numbers of unfixed (and therefore relatively undamaged) sperm samples. Furthermore, using the flow cytometer, merocyanine-540 staining can be combined with the membrane impermeable DNA binding probe Yo-Pro-1 to allow coincident analysis of membrane lipid status and cell viability, while the acrosome reaction of living stallion spermatozoa, similarly can be assessed flow cytometrically using fluorescein isothiocyanate (FITC) conjugated peanut (Arachis hypogea) agglutinin (PNA) as a label [12,13].

Capacitation also involves changes in sperm motility, known as hyperactivation [1], which are thought to aid sperm progression up the oviduct by enabling spermatozoa to move away from the oviductal epithelium [14], and to provide the motive thrust needed for penetration of the zona pellucida [15].

The primary aim of this study was to establish a flow cytometric technique for assessing capacitation of stallion spermatozoa using a reporter probe with known sperm-binding characteristics rather than CTC which, though is empirically accepted but is laborious and its working mechanism is scientifically unexplained. To this end, we investigated whether CTC identification of capacitation and the AR in fixed sperm preparations correlated with flow cytometric detection of the same processes in unfixed sperm samples, where capacitation was indicated by changes in membrane lipid fluidity, as demonstrated by merocyanine 540 staining, and the AR was detected by FITC-PNA staining. In both cases capacitation was induced using a modified Tyrode’s medium, as described by Harrison et al.[10], in the presence of 15 mM bicarbonate/ 5% CO₂ (Tyr+bic) and the results were compared to control samples incubated in bicarbonate/ CO₂–free Tyrode’s medium (Tyr). Furthermore, Ca²⁺-ionophore was added to aliquots of both Tyr+bic and Tyr incubated sperm samples in an attempt to induce the AR. For all treatments, the alterations in CTC, merocyanine-540 and FITC-PNA labelling patterns were monitored over time and in addition, temporal changes in sperm motility and hyperactivation were assessed using a computer assisted sperm analysis (CASA) system.
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Fig. 1. The three patterns obtained for CTC-stained viable spermatozoa. A) Pattern A: whole sperm head shows bright fluorescence, with or without a brighter equatorial band; this is indicative of noncapacitated spermatozoa. B) Pattern B: the acrosomal region of the sperm head fluoresces brightly but the postacrosomal region does not; this denotes capacitated, acrosome-intact spermatozoa. C) Pattern C: the acrosomal region of the sperm head is nonfluorescent, with or without a fluorescent, postacrosomal region; this indicates capacitated, acrosome-reacted spermatozoa. Sperm head length ~7.0 µm. D) A bar chart demonstrating the mean (± sd) percentage of viable sperm fluorescing in the various CTC staining patterns after 0 h (unshaded) and 5 h (shaded) of incubation.

Materials and methods

Materials

CTC and Ca²⁺ ionophore A23187 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Merocyanine 540, ethidium homodimer (EthD-1), Yo-Pro-1 and propidium iodide (PI) were obtained from Molecular Probes Inc. (Eugene, OR, USA). FITC-PNA was purchased from EY Laboratories Inc. (San Mateo, CA, USA).

Media

A modified Tyrode’s medium was used for incubating sperm in “capacitating” conditions (Tyr+bic). The complete Tyrode’s medium contained 96 mM NaCl, 3.1 mM KCl, 2 mM CaCl₂, 0.4 mM MgSO₄, 0.3 mM KH₂PO₄, 50 µg kanamycin/ml, 20 mM Hepes, 5 mM glucose, 21.7 mM sodium lactate, 1 mM sodium pyruvate, 15 mM NaHCO₃ and 7 mg/ml BSA [16]. For incubating sperm in the control “non-capacitating” conditions, a modified Tyrode’s medium without bicarbonate (Tyr) was prepared. In both cases stock solutions minus the CaCl₂, BSA and pyruvate were prepared, filtered through a 0.2 µm filter and stored at 4°C [11]. The remaining three ingredients were added 20-24 h prior to the experiment and the medium was maintained in equilibrium with 5% CO₂ in air at 37°C until the time of the experiment. The pH and osmolality of both media were maintained at 7.4 and 300 mOsm/kg, respectively.
Semen collection and preparation

During the breeding season, semen was collected from three adult (4-10 years old) stallions once a day for three consecutive days (total: 3 ejaculates per stallion). All 9 ejaculates had a gel-free sperm concentration of \( >150 \times 10^6 \) cells/ml and a progressive sperm motility of \( >65\% \). The semen was collected using an artificial vagina, filtered through gauze to remove the gel and any large particles of debris and immediately processed for use. Ejaculates were processed separately. Two ml semen was transferred to a pre-warmed 15-ml tube, mixed with 6 ml Tyr and centrifuged at 900g for 10 min, to allow removal of seminal plasma. After removal of the supernatant, the pellet was resuspended with Tyr to a final volume of 2 ml (equal to the volume of semen used) and incubated at 37°C for 30 min for equilibration and subsequently divided into 2 equal portions: One of which was diluted with Tyr+bic and the other with Tyr, to final sperm concentrations of 25 x 10^6/ml. These samples were divided further into two portions, one of which was supplemented with Ca^{2+}-ionophore to a final concentration of 1µM [17]. All 4 samples were then incubated at 37°C [4] for 5 h. At 0, 0.5, 2, 3.5 and 5 h, aliquots of each sample were recovered and the state of capacitation and/or acrosome reaction was assessed using all three staining methods, as described below, and sperm motility was analysed with respect to the incidence of hyperactivation.

CTC/ EthD-1 staining

CTC staining was made freshly by dissolving CTC and L-cysteine in a chilled 20 mM Tris buffer supplemented with 130 mM NaCl to produce final concentrations of 0.75 mM CTC and 5 mM L-cysteine, respectively. The pH of the final solution was adjusted to 7.8 and it was kept in the dark at 4°C until it was used. For staining, a 100 µl aliquot of sperm suspension was mixed with 100 µl of a 2 mM solution of EthD-1 (a supra vital stain) in PBS and this mixture was incubated for 5 min before 100 µl of the CTC stain was added. Thereafter, the sample was fixed with 30 µl of 12.5% glutaraldehyde in 1 M Tris (pH = 7.0) to produce a final concentration of 1.1% fixative, and a 10 µl drop of the fixed sperm suspension was mixed with 5 µl of antifade on a glass microscope slide. Next, the droplet was covered with a cover-slip, and the slide was gently but firmly pressed under two folds of a tissue paper to absorb any excess fluid. The prepared slide was then stored in the dark until it was analysed, within 1 h of preparation. For analysis of the CTC staining an Epi-fluorescence microscope (BH-2; Olympus, Tokyo, Japan) equipped with a 458 ± 15 nm wavelength band-pass excitation filter, a 470 nm dichroic mirror and a 500 nm long-pass emission filter was used to assess at least 100 spermatozoa at a magnification of 400 x. This combination of filters enabled simultaneous identification of dead cells (EthD-1 positive) versus live cells (EthD-1 negative) and CTC fluorescence patterns. For more detailed visualisation CTC stained sperm were examined with a spectral confocal microscope (Leica TCS SP, Leica GmbH, Germany). The CTC and EthD-1 stains were excited with the 458 nm Argon laser line (emission selected at 495-535 nm) and resolution was optimised by using extended focus made up of 3 optical Z-sections (0.25 µm step size) (average of three X-Y scans per section) and recombined into one image.
Merocyanine540/Yo-Pro-1 staining

For flow cytometric analysis of capacitation status, sperm cells were incubated in Tyr+bic or Tyr containing 2.7 µM merocyanine-540 (a reporter probe for phospholipid scrambling; [10]), 25 nM Yo-Pro-1 (a membrane impermeable nucleic acid stain; [10]), 0.5mg/ml polyvinyl alcohol (PVA) and 0.5 mg/ml polyvinyl- pyrolidone (PVP). In vitro capacitation was performed in airtight sealed 5ml flow cytometer tubes (Becton Dickinson, San Jose, CA, USA) containing 3 ml medium. The tubes were flushed with air containing 5% CO₂ before closing, and incubated for approximately half an hour in a shaking water-bath at 37°C before flow cytometric analyses. Sperm cell analysis was performed using a flow cytometer (FACS Vantage SE; Becton Dickinson, San Jose, CA, USA). The system was triggered by the forward light scatter signal (FSC). The Yo-Pro-1 and merocyanine 540 probes were both excited by an argon ion laser (Coherent Innova, Palo Alto, CA) with 200 mW laser power at a wavelength of 488 nm. Fluorescence of the Yo-Pro-1 probe was then measured using a 520 ± 15 nm band pass filter (fluorescence detector equipped with a photo multiplier tube) while merocyanine 540 emission was deflected with a 560 nm short pass dichroic mirror in the emission pathway and measured using a 575 ± 15 nm band pass filter. Sperm cells were analysed at a rate of between 8,000 and 10,000 per second using PBS as sheath fluid, and for each sample 10,000 events were stored in the computer for further analysis with Cell-Quest software (Becton Dickinson, San Jose, CA). Sideward light scatter (SSC) and FSC were recorded so that only sperm cell specific events, which appeared in a typical L-shaped scatter profile, were selected for further analysis. During measurement, the sample input tube in the FACS Vantage SE was kept at 37°C and 5% CO₂ using a controlled temperature bath/circulator, to maintain constant incubation conditions during the whole analysis using a controlled temperature bath/circulator.

For visualisation of individual sperm cells, samples were placed in a life chamber at 37°C in which the bicarbonate/CO₂ equilibrium was maintained by continuous infusion of humidified air containing 5% CO₂, thus maintained under physiological conditions. The sperm were examined using an inverted spectral confocal microscope (Leica TCS SP, Leica GmbH, Germany) fitted with a 488 nm Argon laser line for exciting fluorescent probes. Yo-Pro-1 emission was detected using photo multiplier tube 1 which selected emissions with in the wavelength range of 500-550 nm while merocyanine 540 emission was detected using photo multiplier tube 2 (580-620 nm). Single scans were made to record the labelling patterns of motile sperm cells.

FITC-PNA/PI staining

The acrosomal status of the sperm was examined by staining the incubated samples with 5µg/ml FITC-PNA (as a marker for acrosomal leakage, [18]) and 1 µM PI (as marker for cell death [19]), and analysing the labelled sperm on a flow cytometer (FACScan; Becton Dickinson, San Jose, CA) as described before [18,20].
Evaluation of in vitro capacitation

For visualisation of individual sperm cells, samples were placed in a life chamber at 37°C in which the bicarbonate/CO₂ equilibrium was maintained by continuous infusion of humidified air containing 5% CO₂ in a spectral confocal microscope (Leica TCS SP, Leica GmbH, Germany) and excited with the 488 nm Argon laser line. The emission of PNA-FITC was detected using photo multiplier tube 1, focused for emissions in the 500-550 nm wavelength range, and the emission of PI was detected using photo multiplier tube 2 (600-700 nm). Single scans were made to capture the labelling patterns of (hyper) motile sperm cells.

Comparison of CTC, Merocyanine 540 and FITC-PNA staining

In a separate experiment, the proportion of viable cells with positive FITC-PNA staining were evaluated for 3 stallions (3 ejaculates per stallion analysed). The FITC-PNA staining was analysed under bicarbonate enriched (15 mM); and bicarbonate depleted (0 mM) conditions using either a flow cytometer or a confocal microscope (as described above). The labelled sperm samples were analysed in unfixed state as well as after 2% (w/v) paraformaldehyde fixation. The results obtained were compared with microscopic evaluations of CTC staining of the same incubated sperm samples (i.e. after fixation with 1.1 % w/v glutaraldehyde) and both stainings were also compared with merocyanine 540 staining (unfixed cells only).

**Fig. 2.** The two patterns obtained for viable sperm stained with merocyanine 540. A) The highly fluorescent sperm heads demonstrate capacitated spermatozoa, and the poorly fluorescent heads demonstrate non-capacitated spermatozoa. Sperm head length ~7.0µm. Scatter plots for the amount of fluorescence detected flow cytometrically for sperm from a single ejaculate stained with merocyanine-540 and Yo-Pro after 0 h (B) and 5 h (C) of incubation in Tyr+bic medium. The X-axis depicts the amount of fluorescence emitted by merocyanine-540 probe that was bound to individual sperm cells as measured in arbitrary units by the FL-1 detector, and the Y-axis depicts the amount of fluorescence in arbitrary units emitted by Yo-Pro-1 probe that was bound to individual sperm cells by the FL-3 detector. In both plots, sperm cells that fluoresced more intensely with Yo-Pro than the superimposed horizontal line were considered to be non-viable, whereas the viable sperm cells with higher merocyanine-540-depended fluorescence than the superimposed vertical line were considered to be capacitated.
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Hyperactivation

In order to examine the motility pattern of incubated spermatozoa, samples from each treatment and time point were examined using a Hamilton Thorne Research Motility Analyzer (HTM IVOS; model 8020, version 8.1, Beverly, MA). The Hamilton Thorne CASA system was programmed to the following settings: frames acquired = 20; frame rate = 30/sec; minimum contrast = 8; minimum size = 6; Low/High size = 0.5-1.8; Low/High intensity gates = 0.5-1.8; non-motile head size = 13; non-motile intensity = 25; Medium VAP value = 25; Low VAP value = 9; Slow cells motile = No; Threshold STR = 80. These values were selected on the basis of previous experience and their suitability was confirmed using the PLAYBACK option of the HTM. A 20 µm counting chamber (Cell Vision, Anthos Labtec BV, The Netherlands) maintained at 37°C was used for analysis, and samples were tested within 1 min of being taken out of the incubator. It took less than 5 minutes to analyse a sample on the HTM machine and particular attention was paid to the classic parameters of sperm hyperactivation, namely curvilinear velocity (VCL) and amplitude of lateral head displacement (ALH) [21,22]. A spermatozoon was designated as being hyperactive if it had a VCL 180 µm/sec and an ALH 12 µm.

Statistical analysis

Analysis was centred around identifying decreases in the percentages of non-capacitated or acrosome-intact spermatozoa. Statistical analysis was carried out using repeated measures ANOVA (n=3) to examine the effects of staining method, time and stallion. Since the sample size was only 3, normality could not be properly assessed and the data were therefore analysed untransformed. As an additional check the data were reanalysed after a normalising sine transformation and since this did not alter the outcome the assumption of normality was strengthened. In the case of the hyperactivation data, a one-way ANOVA was used to compare the motility of the 6 ejaculates. The statistical package used was SPSS 8.0 (SPSS for windows 1996, SPSS Inc. Chicago). Differences were taken to be statistical significant when $P < 0.05$.

Results

CTC staining patterns

CTC-stained viable spermatozoa (EthD-1 negative) showed 3 major fluorescence patterns. In the first of these, pattern-A, the whole of sperm head showed bright fluorescence, with or without a brighter equatorial band; this pattern was representative of non-capacitated spermatozoa (Fig 1 A). In pattern-B, the acrosomal region of the sperm head fluoresced brightly while the post-acrosomal segment was non-fluorescent; this pattern was indicative of capacitated but acrosome-intact spermatozoa (Fig 1 B). Finally, in pattern-C; the acrosomal region did not fluoresce while the post-acrosomal segment could be fluorescent.
or not; this pattern indicated capacitated and acrosome-reacted spermatozoa (Fig 1 C). With regard to the effect of time, incubation in Tyr+bic medium for 5 h resulted in a significant decrease in the average percentage of spermatozoa showing pattern-A (that is viable, non-capacitated sperm); 50.2±0.8% at 0 h of incubation and 22.8±4.9% at 5 h, and an increase in the percentage of spermatozoa displaying pattern-C (i.e. viable, capacitated and acrosome-reacted sperm); 9.5±3.2% at 0 h and 14.6±2.85% at 5 h.

**Merocyanine staining pattern**

Merocyanine-540 staining gave rise to two basic fluorescence patterns (Fig 2 A) in viable (Yo-Pro-1 negative) sperm cells, one of which was characterised by poorly and the other by relatively higher fluorescent sperm cells. Sperm cells with poorly fluorescent heads were considered to be non-capacitated while a highly fluorescent sperm head was considered to be a characteristic of a capacitated spermatozoon. These patterns were analysed flow cytometrically and the resulting data were presented in quadrants (Figs 2 B, C), where the X-axis represents increasing merocyanine-540 fluorescence and the Y-axis, shows increasing Yo-Pro-1 fluorescence. When incubated in Tyr+bic medium, 49.6±2.9% (mean ± SD) of the spermatozoa showed low merocyanine-540 fluorescence at 0 h. After the 5 h incubation, the proportion of poorly fluorescent sperm had decreased to 8.7±2.8% (Figs 2 B,C), thereby indicating a significant time-dependent decrease in the percentage of non-capacitated spermatozoa. Conversely, the percentage of spermatozoa with high merocyanine 540 fluorescence increased from 6.4% at 0 h to 25.6% at 5 h), demonstrating a corresponding increase in the percentage of capacitated live spermatozoa (Figs 2 B, C). The remaining sperm cells were labelled with Yo-Pro-1 and therefore non-viable.

**FITC-PNA staining pattern**

FITC-PNA staining differentiate the viable (PI negative) sperm cells into two distinct groups (Fig 3 A). The sperm were either not labelled with FITC-PNA, thereby demonstrating that their acrosomes were intact, or showed acrosomal FITC-PNA staining, which indicated that their acrosome was either reacting or reacted. Absence or presence of FITC-PNA labelling was also analysed flow cytometrically and the resulting data presented in quadrants (Figs 2 B, C), where the X-axis shows increasing FITC-PNA fluorescence and the Y-axis shows increasing PI fluorescence (Figs 3 B, C). At the onset of incubation in Tyr+Bic medium (0 h) 53.8±3.2% (mean ± SD) of the spermatozoa were not labelled with FITC-PNA. After 5 h of incubation, the proportion of unlabeled sperm cells had decreased to 38.9±4.1%, indicating a time dependent decrease in the percentage of acrosome-intact spermatozoa. On the other hand, the increase in the percentage of viable spermatozoa that were labelled with FITC-PNA between 0 h (2.2%) and 5 h (4.0%), demonstrated that only a slight increase in the percentage of acrosome-reacted live spermatozoa occurred. The remaining cells were labelled with PI and thus shown to be non-viable.
Fig. 3. The two different patterns obtained for viable sperm stained with FITC-PNA. (A) A green fluorescent sperm head denotes an acrosome-reacted/ reacting sperm a non-fluorescent sperm head indicates an acrosome-intact sperm. Sperm head length ~7.0µm. Scatter plots that depict the amount of fluorescence detected flow cytometrically per sperm for sperm cells stained with FITC-PNA and propidium iodide after incubation in Tyr+Bic for 0 h (B) and 5 h (C). The amount of fluorescence emitted by the FITC-PNA label, as detected by the FL-1 detector, is indicated in arbitrary units on the X-axis while the amount of fluorescence (in arbitrary units) emitted by the propidium iodide vital stain and detected by the FL-3 detector is indicated on the Y-axis. All sperm that had a PI dependent fluorescence value higher than the superimposed horizontal line were regarded as non-viable while the viable sperm cells with higher FITC-PNA dependent fluorescence than the superimposed vertical line were classified as acrosome reacted.

Comparison between capacitation state detected with merocyanine and CTC

At the onset of incubation in Tyr+bic medium (0 h), merocyanine 540 staining demonstrated that 49.6±2.9% of the spermatozoa had not undergone capacitation. Thereafter, the percentage of non-capacitated spermatozoa decreased initially rapidly to reach 21.5±5.0% after 0.5 h and then more gradually to reach a final value of 8.7±2.8% after 5 h of incubation (Fig 4 B). On the other hand, when capacitation status was monitored using CTC staining a less dramatic and more gradual decrease in the number of non-capacitated sperm, from 50.2±0.8% at 0h to 22.8±4.9% at 5 h, was observed (Fig 4 A).

Thus, although the proportion of non-capacitated sperm detected at the onset of incubation did not differ between the two techniques, the apparent rate of loss of non-capacitated sperm differed significantly ($P<0.05$). Therefore, if it is accepted that merocyanine-540 and CTC staining both follow changes in capacitation status then it is clear that CTC staining is very much slower to recognise these changes.
Evaluation of in vitro capacitation

Fig. 4. The mean (± sd) percentages of sperm cells that remained viable and demonstrated the indicated responses in the four incubation conditions tested, for nine ejaculates collected from three stallions. Percentages of sperm cells that remained viable and not capacitated, as detected by CTC (A) and merocyanine 540 (B) respectively. Percentages of total sperm cells that remained viable with intact acrosome as detected by CTC (C) and FITC-PNA (D), respectively. Solid line ( ), Tyrode’s medium; solid line ( ), Tyrode’s medium with 15 mM bicarbonate; broken line ( ), Tyrode’s medium with 1 µM Ca²⁺-ionophore; broken line ( ), Tyrode’s medium with 15 mM bicarbonate and 1 µM Ca²⁺-ionophore.

In the presence of Ca²⁺-ionophore and bicarbonate, a similar decrease in the percentage of non-capacitated spermatozoa, from 48.2±5.0% at time 0 h to 22.7±3.5% at 0.5 h time-point and 7.7±4.9% at 5 h, was observed with merocyanine-540 staining (Fig 4 B). In these latter conditions, CTC staining gave similar results to merocyanine-540 for the rate of decrease in the percentage of non-capacitated sperm since the values went from 45.2±3.5% at time 0 h, to 25.8±4.9% at 0.5 h and to 2.4±2.1% at 5 h, (Fig 4 A). Repeated measures analysis of variance did not show any significant difference between these two staining methods for the proportion of non-capacitated sperm detected at the various time points, when both bicarbonate and Ca²⁺-ionophore were included in the incubation medium.

In bicarbonate-free Tyrode’s medium (Tyr), only a very slight decrease in the percentage of non-capacitated sperm over time was observed with merocyanine-540 (51.5±2.9 % at 0 h to
42.2±8.9% at 5 h; Fig 4 B) and CTC (52.3±3.1% at 0 h and 45.6±7.5 % at 5 h; Fig 4 A) staining. Furthermore, incubation with Ca²⁺-ionophore had no apparent effect on the capacitation status of sperm, as detected with the merocyanine-540 and CTC stain (Fig 4 A, B). In contrast, the change in capacitation status of sperm was only fully (i.e. comparable to the effects monitored by the merocyanine-540 staining) detectable in presence of Ca²⁺-ionophore with the CTC stain (Fig. 4 A).

### Table 1: A comparison of the results obtained when stallion sperm were incubated for 5 h in Tyrode’s medium (with 15mM bicarbonate) before sperm plasma membrane integrity, capacitation status and acrosome integrity were analysed simultaneously using flow cytometry (FACS) or confocal microscopy (CLSM) on cells in a life chamber. The values in the table are the mean (± standard deviation) percentages of the viable (plasma membrane intact) cells which had either not undergone capacitation (CTC Cap and merocyanine) or were recorded to have an intact acrosome (CTC-AR and FITC-PNA), for nine ejaculates collected from three stallions. For each ejaculate CLSM counts were performed on 300 cells while FACS data were recorded for 10,000 sperm specific events. FACS analyses of CTC staining was not possible. ND: not detectable;

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Comparison of CTC and FITC-PNA staining for the detection of acrosomal integrity

The percentage of acrosome-intact cells as determined by CTC staining was taken to be the sum of the percentages of non-capacitated and capacitated acrosome-intact sperm (see Figs. 1 A, B). At the onset of incubation in Tyr+Bic medium, the percentage of acrosome-intact cells as detected by CTC staining was 55.2±1.6 %. During incubation the proportion of acrosome intact sperm cells decreased gradually, to 30.4±2.7 % after 5 h (Fig 4 C). These two values were similar to those observed with FITC-PNA staining for which the percentage of acrosome-intact cells dropped from 53.8±3.2 % at 0 h to 38.9±4.1 % after 5 h of incubation.
The addition of Ca\textsuperscript{2+}-ionophore to the Tyr+bic incubation medium resulted in a more rapid and pronounced decrease of acrosome-intact sperm cells, as detected by CTC and, to a lesser extent, by FITC-PNA stainings (Fig 4 C, D), thereby indicating that Ca\textsuperscript{2+}-ionophore induces the acrosome reaction in stallion sperm incubated in a bicarbonate containing medium. Although the decrease in the proportion of acrosome-intact cells was more prominent in CTC stained (55.6± 2.0% at 0 h to 5.6±3.7% at 5 h) than in FITC-PNA stained samples (53.3±4.4% at 0 h to 24.6±8.8% at 5 h) there was no significant difference between the data.

In the absence of bicarbonate, however, there was no time-dependent decrease in the percentage of acrosome-intact spermatozoa as detected by either CTC or FITC-PNA staining (Fig 4 C, D) and even addition of Ca\textsuperscript{2+}-ionophore to the Tyr medium did not induce AR in incubated spermatozoa (Fig 4 C, D).

In a separate experiment FITC-PNA staining was assessed in bicarbonate primed and control cells prior to and after 2% (w/v) paraformaldehyde fixation using flow cytometry and confocal laser scanning microscopy (Table 1). Flow cytometry and confocal laser scanning microscopy gave similar results. This was also the case for merocyanine-540 staining of the same sperm samples (only unfixed samples were analysed, Table 1). However, it was clear that fixation allowed FITC-PNA/PI binding to an extended sperm subpopulation (Table 1) both for control and bicarbonate stimulated samples. The CTC acrosome staining gave lower relative numbers of acrosome intact cells when compared with the FITC-PNA staining of unfixed sperm cells (Table 1). This effect may be attributed to fixation because FITC-PNA staining of paraformaldehyde fixed stallion sperm gave similar results as CTC staining of glutaraldehyde fixed cells (Table 1). Finally, the CTC capacitation staining gave lower relative amounts of capacitated cells when compared with merocyanine-540 staining (Table 1).

**Assessing the viability of sperm cells**

In this study the sperm cells stained with CTC, FITC-PNA or merocyanine-540 for the assessment of capacitation or acrosome status were counter–stained with EthD-1, PI and Yo-Pro-1, respectively for the simultaneous detection of sperm viability. At the onset of incubation in Tyr+bic, PI and Yo-Pro-1 staining identified approximately 43% of the cells as being dead whereas, EthD-1 staining recorded only 37% of the cells as dead, however, at all other times and for all treatments, the three viability stains gave nearly identical results for the proportions of dead cells. Thus, after 5 h of incubation, the percentage of non-viable cells as recorded by all three staining methods had increased to approximately 55% (the three methods gave non-significant differences), while the addition of Ca\textsuperscript{2+}-ionophore to the Tyr+Bic medium induced even greater cell death such that by 5 h of incubation more than 70% of the cells were non-viable.
In absence of bicarbonate, however, minimal cell death was observed such that by the end of the 5 h incubation period only 45% of cells were non-viable, irrespective of the presence or absence Ca\textsuperscript{2+}-ionophore. This demonstrated that, in the absence of bicarbonate, sperm cells do not lose their membrane integrity, even in the presence of Ca\textsuperscript{2+}-ionophore. By contrast, when exposed to the capacitating agent bicarbonate, the sperm became vulnerable to membrane disruption and cell death during the 5 h incubation period and these processes were facilitated by Ca\textsuperscript{2+}-ionophore.

**Sperm motility and hyperactivation**

Sperm motility with specific regard to hyperactivation was analysed for the spermatozoa of two stallions (3 ejaculates each) incubated in Tyr+bic medium. The average motility at 0 h was approximately 60% in all cases and had decreased to around 30% after 5 h of incubation. The addition of Ca\textsuperscript{2+}-ionophore had a negative effect on sperm motility and no spermatozoa were scored as motile after 3.5 h of incubation, despite the fact that

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**Fig. 5.** The scatter plots used to detect hyperactivation of the motile spermatozoa for sperm from one ejaculate incubated for 0 h (A) or 5 h (B) in Tyr+Bic medium without Ca\textsuperscript{2+}-ionophore. The amplitude of lateral head displacement (ALH) is shown on the X-axis and the curvilinear velocity (VCL) is represented on the Y-axis. Spermatozoa with a VCL > 180 µm/s and an ALH > 12µm were considered to be hyperactive. (C) The percentages of spermatozoa incubated in Tyr+bic medium for 5 h that fulfilled the criteria for hyperactivation at various time points during incubation. The values represent the mean percentages (± sd) for six ejaculates (two stallions).
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approximately 35% of the cells were at this time point still viable as determined with counter-staining using EthD-I as supra vital dye.

Hyperactivation was determined objectively by plotting the VCL and ALH of the motile cells on a two-dimensional scatter-graph [22]. Spermatozoa were considered to be hyperactive if their VCL was 180 and ALH 12 (i.e. they were in the upper right quadrant on figs 5 A, B). Using these parameters, 5.5 ± 2.4 % of the motile spermatozoa were considered to be hyperactive and the proportion of hyperactive cells did not change significantly with time (Fig 5 C). However, the presence of Ca²⁺-ionophore in the incubation medium resulted in the loss of about all hyperactive spermatozoa by 2 h and an almost complete motility loss within 3.5 h of incubation. It was clear then, that at any given time, the percentage of hyperactivated spermatozoa was lower than the percentage of capacitated spermatozoa as detected by either merocyanine-540 or CTC staining.

While assessing sperm motility visually it was observed that hyperactivated sperm (identified visually using Yanagimachi’s criteria [1]) showed a biphasic motility pattern. In short, hyperactive sperm tended to make vigorous non-progressive movements, but then become static for a while before moving again with the same vigorous non-progressive movement.

Discussion

Capacitation is a vital phenomenon that a spermatozoon must undergo before it can fertilise an oocyte. However, the lack of a reliable and easy method for assessing sperm capacitation has resulted in a rather incomplete understanding of this important process. At present, the only generally accepted capacitation assay is CTC staining where CTC is a fluorescent antibiotic that binds to the surface of sperm cells in a Ca²⁺ dependent manner [23]. In short, it appears that the CTC-Ca²⁺ complex binds preferentially to hydrophobic regions, such as the cell membrane [23,24], and capacitation induced changes in the sperm cell plasma membrane result in the changes of the CTC labelling pattern that are widely considered to reflect the attainment of the capacitated state [4,5,6,7]. However, the molecular basis of the interaction between CTC, Ca²⁺ and the sperm plasma membrane are far from clear and, moreover, CTC staining is a laborious technique, particularly because it cannot be analysed using a flow cytometer (Table 1). The reason for this flow cytometer incompatibility is that although the distribution of the CTC dye changes clearly (Fig 1), the total amount of CTC staining does not change between non-capacitated and capacitated cells, and it is this absolute change that a FACS machine would require for the differentiation of the cells. Furthermore, assessment of CTC staining implies fixation of cells and it is therefore important that the cells should be labelled with a membrane impermeable DNA stain for the discrimination of live and dead (fluorescent) cells before fixation, since this latter process introduces artefacts such as cellular and/or acrosomal degeneration (Table 1). A further drawback of CTC staining is its Ca²⁺ dependency which renders it useless for detecting
Ca\(^{2+}\) independent capacitation changes in sperm cells. This latter point was illustrated clearly in the present study by the fact that the capacitation induced changes detected by merocyanine-540 staining were only fully detectable by CTC staining when Ca\(^{2+}\)-ionophore was present (Fig 4). These drawbacks to the CTC technique underline the importance of developing flow cytometric assays to monitor capacitation dependent changes in, for example, membrane fluidity [11], and acrosome status [17,18] in live cells.

Flow cytometric detection of capacitation related changes in membrane architecture, using merocyanine-540 as a reporter probe, and acrosome status, using FITC-PNA staining, have some clear advantages over the all-comprising CTC staining technique. First, given the clear differences in the intensity of fluorescence between control and capacitated or acrosome reacted cells, flow cytometry allows for the very rapid and objective discrimination of the status of large numbers of sperm cells. For example, in the current study we analysed 10,000 sperm cells per data point in only a few seconds. Second, prior to analysis the sperm suspension requires only simultaneous addition of appropriate amounts of PI and FITC-PNA or Yo-Pro-1 and merocyanine-540, followed by a 10 min incubation for the completion of labelling. Third, the cells can be analysed in a flow cytometer in the unfixed state and under relatively physiological conditions (i.e. at 37°C and 5% CO\(_2\)). This ability to control the ambient conditions minimises the risk of cell deterioration, especially for the notoriously delicate capacitated sperm cells.

In the current study, we analysed the results in terms of the decrease in the percentage of viable non-capacitated or acrosome-intact spermatozoa, rather than by the increase in the percentage of viable capacitated or acrosome-reacted cells. It was considered that this represented a more realistic approach, because of the greater likelihood that the more delicate capacitated or acrosome-reacted cells would die for reasons other than incubation condition or treatment per se and thus be missed from the analysis.

The results of this study demonstrate clearly that CTC differs significantly to merocyanine-540 in terms of its ability to detect changes in the capacitation-state of spermatozoa incubated in Tyr+bic medium. Merocyanine-540, a marker for increased membrane fluidity, detected a rapid decrease in the number of non-capacitated sperm during the first 0.5 h of incubation, after which numbers tended to plateau. By contrast CTC detected the apparent loss of non-capacitated status much more slowly and a similar level of apparent capacitation was not seen until the end of the 3 h incubation.

It is proposed that these differences relate to the probability that the membrane fluidity related changes detected by merocyanine-540 precede the Ca\(^{2+}\) influx on which CTC binding depends. This conclusion is supported by the CTC and merocyanine-540 staining patterns in the presence of Ca\(^{2+}\)-ionophore. That is, a more pronounced and rapid decrease in the proportion of live non-capacitated sperm cells as detected by CTC staining but no change detected by merocyanine-540 staining when Ca\(^{2+}\)-ionophore was present. In summary, the ability of CTC staining to detect changes in capacitation status equalled that
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of merocyanine-540 only when Ca\(^{2+}\)-ionophore was included in the incubation medium, otherwise CTC was very slow at detecting changes in sperm membrane status.

It was also interesting that, in the absence of bicarbonate, capacitation whether measured by CTC or merocyanine-540 staining was minimal. In total then, the data presented in this paper indicate that bicarbonate induces a change in the lipid packaging of the plasma-membrane of stallion sperm that can be monitored by merocyanine-540, a marker for lipid ‘scrambling’ [11]. A further experiment (data not shown), has demonstrated that only a sub-population of the merocyanine-540 responsive cells show the CTC capacitation response and this presumably indicates that the rise in intracellular Ca\(^{2+}\) concentration necessary for CTC binding occurs later in sperm capacitation than bicarbonate mediated lipid scrambling. Similar results obtained for two-staining methods when Ca\(^{2+}\)-ionophore was included in the incubation medium is presumably because the ionophore allows the merocyanine-540 responsive cells to take up Ca\(^{2+}\) in a fashion that allows appropriate CTC binding because the ionophore makes the plasma membrane permeable to Ca\(^{2+}\) (2mM).

The other parameter commonly assessed using CTC is acrosome intactness [7]. The AR is a Ca\(^{2+}\) dependent process [1] and high intracellular Ca\(^{2+}\) is required for fusion of the sperm plasma membrane to the outer acrosome membrane. Thus the AR can proceed only after the intracellular Ca\(^{2+}\) levels of capacitated sperm cells have increased and it is only after this secretory event has occurred that FITC-PNA is able to bind to the appropriate epitope on the outer acrosomal membrane [12,18]. Therefore, like CTC, FITC-PNA staining is Ca\(^{2+}\) dependent, albeit indirectly and this may explain why AR assessments were similar for the two techniques. Interestingly, a moderate induction of the AR was observed when sperm were incubated in Tyr+bic whereas the AR induction was not observed in the absence of bicarbonate. Addition of Ca\(^{2+}\)-ionophore resulted in an even more widespread induction of the AR in Tyr+bic incubated sperm suspensions (not significant for FITC-PNA stained sperm), probably by facilitating the rise in intracellular Ca\(^{2+}\) required for this secretory event. Thus it appears that Ca\(^{2+}\)-ionophore synergies the effect of bicarbonate regarding the AR induction and, whereas bicarbonate alone is sufficient to induce the AR, Ca\(^{2+}\)-ionophore alone is not. It must be presumed that the bicarbonate-mediated increase in plasma membrane fluidity allows a subsequent increase in the permeability of that membrane to Ca\(^{2+}\).

With regard to sperm hyperactivation, it has been reported previously that this phenomenon occurs spontaneously in the majority of spermatozoa incubated under capacitating conditions in a medium that contains bicarbonate [25] and Ca\(^{2+}\) [see 16]. The suggestion was that bicarbonate activates adenylate cyclase, either directly or indirectly by causing Ca\(^{2+}\) influx (see [16]), and thereby elevates intracellular cAMP concentrations, which in turn induce sperm hyperactivation [26]. For this reason, we expected that in the current experiment the incubation of sperm in a medium containing bicarbonate and Ca\(^{2+}\) would lead to sperm hyperactivation, a phenomenon that is characterised by an increase in flagellar bending amplitude, that can be detected in a CASA system by an increase in ALH
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and VCL (27,28). However, the results of our study indicated that only a small proportion of the spermatozoa demonstrated characteristics of hyperactivity at any given time and, while it is likely that the percentage of hyperactive spermatozoa was underestimated because of the typical stop-start biphasic motility pattern recorded for hyperactive cells it was clear that hyperactivity is not a suitable parameter for accurately estimating the percentage of capacitated cells.

In conclusion, we have compared several methods for detecting sperm capacitation, acrosome status, sperm viability and particular characteristics of motility, under conditions where either sperm membrane fluidity was affected or the acrosome reaction was induced. The major aim of the study was to relate the CTC staining patterns obtained with fixed sperm cells, and used for simultaneous assessment of capacitation and acrosome reaction status, to the results of novel flow cytometric assays that detect membrane changes in a Ca²⁺ independent manner [10,11,18]. It is concluded that the merocyanine-540 and FITC-PNA assays are preferable to CTC staining because flow cytometric assays are easier to perform, quicker, more objective and more accurate than fluorescence microscope assays (CTC), and, in addition, because the washing and fixation steps that may interfere with apparent cell viability and integrity are not required. Moreover, the mechanisms of the molecular interaction between merocyanine-540/ FITC-PNA and the biomembranes and their relationship to the occurrence of capacitation and the AR are clear, whereas, the same cannot be said for CTC. It is, of course, important to simultaneously detect sperm viability using a membrane impermeable DNA stain to ensure that detected membrane changes are biologically relevant. In this respect, all three stains examined in the current study gave equally satisfactory results.

References
