Validation of Merocyanine 540 staining as a technique for assessing capacitation-related membrane destabilization of fresh dog sperm

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Abstract

The aim of this study was to determine whether flow cytometric evaluation of combined merocyanine 540 and Yo-Pro 1 staining (M540-YP) would identify viable dog sperm that had undergone membrane stabilization known to be associated with capacitation in other species, and whether such destabilization is detected earlier than when using the tyrosine phosphorylation and ethidium homodimer stain combination (TP-EH) with epifluorescence microscopy. Semen from nine dogs was collected and incubated in parallel in bicarbonate-free modified Tyrode's medium (-BIC), in medium containing 15 mM bicarbonate (+BIC), in dog prostatic fluid (PF), and in phosphate buffered saline (PBS). Aliquots for staining were removed at various time points during incubation of up to 6 hours. Staining with M540-YP allowed the classification of dog sperm as viable without destabilized membranes, viable with

destabilized membranes, non-viable without destabilized membranes or non-viable with destabilized membranes. The percentage of viable sperm detected using EH (83.5 ± 1.37%; mean ± SEM) was higher than when using YP (66.7 ± 1.37%: $P < 0.05$; n=54 semen samples). On the other hand, M540-YP identified a higher percentage of viable sperm with destabilized membranes than TP-EH (75 ± 1.76% vs. 35 ± 1.70%: $P < 0.05$; n=54 semen samples). Staining with M540-YP indicated a rapid increase in the percentage of viable sperm with destabilized membranes, reaching a maximum during the first 30 min of incubation in +BIC. For all other treatments (i.e. -BIC, PF, and PBS) the peak in the percentage of viable sperm with destabilized membranes was reached as much as 90 to 210 minutes later than incubation in +BIC. The lowest percentage of viable sperm showing signs of capacitation was recorded during incubation in PBS. We conclude that YP identifies sperm committed to cell death earlier than EH, and that the M540-YP stain combination identifies membrane destabilization known to be associated with capacitation in other species earlier than the TP-EH stain combination.

Keywords: dog semen, ethidium homodimer, tyrosine phosphorylation, Yo-Pro 1, capacitation, membrane destabilization

1. Introduction

The ability to accurately evaluate the quality and estimate the fertilizing potential of dog semen has increased in importance as a result of the more widespread use of artificial insemination [1].

Before they are capable of fertilizing an oocyte, ejaculated sperm need to undergo biochemical modifications within the female reproductive tract [2]. The collective term for the modifications that enable sperm to fertilize is ‘capacitation’ and in most species examined, bicarbonate plays an essential role in initiating critical aspects of capacitation [3]. For example, bicarbonate induces sperm surface changes, including the loss of coating glycoproteins, and thereby induces an increase in membrane fluidity and membrane destabilization, which are important early events in the capacitation process [4, 5].

The phosphorylation of protein tyrosine residues is critical to the regulation of various cellular functions including ion transfer and receptor affinity [8, 9]. In the mouse, a protein kinase pathway induces protein tyrosine phosphorylation approximately 60 minutes after introducing sperm into bicarbonate-containing medium, making it a late but meaningful indicator of capacitation [6, 7].

In vivo, capacitation is delayed by exposure of the sperm to components of the male accessory sex gland secretions during ejaculation, which suppress bicarbonate-induced sperm surface changes [10]. *In vitro*, maintaining sperm in a bicarbonate-free simple medium such as phosphate buffered saline (PBS) can also be used to delay the progression of capacitation [11]. In this respect, it has also been shown that the membrane destabilization induced by bicarbonate leads to reduced sperm longevity, as a result of accelerated progress towards cell death [3].

Both, the assessment of viability and the early detection of capacitation-related changes in dog sperm are considered useful indicators of the fertilizing potential of a fresh or stored semen sample [12]. Semen samples that show a large population of capacitated cells without prior incubation in capacitating conditions are more likely to have reduced longevity that may compromise fertility. The fluorescent viability probe,

ethidium homodimer (EH) [13], is able to detect sperm during the late stages of cell death whereas the semi-permeable DNA-binding probe Yo-Pro 1 (YP) [14] detects earlier stages of cell death. EH is a non-permeable fluorophore that is unable to penetrate living cells, but that binds to the nucleic acid in membrane-damaged cells with high affinity, facilitating a simple staining procedure [13]. Yo-Pro 1 'leaks into' cells when they become destabilized via increased permeability of pannexin-gated channels in the cell membrane prior to complete loss of integrity when EH would be able to enter [14, 15, 16, 17]. Both, EH and Yo-Pro 1 have been used in species such as the ram [18], stallion [19, 20, 21], and boar [14, 22], while only EH has been reported previously for the dog [23].

Anti-phospho-tyrosine antibodies can be used to immune-fluorescently detect phosphorylation of tyrosine residues in the tail of capacitated sperm [6, 7, 24–34]. Tyrosine phosphorylation (TP) has been reported to detect significant changes in the percentage of capacitated dog sperm after 90 minutes of incubation in bicarbonate-containing medium, with the increase accompanied by changes in the motility pattern indicative of hyperactivation, another hallmark of capacitation [28, 30].

Merocyanine 540 (M540) detects bicarbonate-induced changes in lipid packaging and distribution within the sperm plasma membrane [34], which are thought to be very early changes in the capacitation process [5]. As membrane fluidity increases, more M540 is able to intercalate into the membrane, thereby acting as a useful marker for membrane destabilization [5, 11, 35–39]. Moreover, increased M540 staining occurs within a few minutes after exposure of boar sperm to bicarbonate, and much earlier than TP staining is able to detect capacitation in stallion sperm [5, 35]. M540 staining indicates an early stage of sperm capacitation in the boar [5, 11], bull [38, 39], and stallion [35] but has yet to be validated for the dog.

Dog prostatic fluid (PF) contains components that mask the progesterone receptors on the sperm plasma membrane, postponing initiation of the capacitation process until removal [10, 40, 41]. A similar ability to delay capacitation has been observed when PBS is used as an incubation medium to assess the capacitation status of boar sperm [11].

The aim of this study was to compare two different fluorescent-staining techniques for determining the viability and capacitation status of fresh dog sperm and, in particular, to validate the use of M540 staining of dog sperm as a means of detecting membrane destabilization as has been associated with capacitation in sperm of other species.

The two stain combinations used were: (i) tyrosine phosphorylation staining and ethidium homodimer using epifluorescence microscopy as analysis tool (TP-EH) and (ii) M540 and Yo-Pro 1 (M540-YP) using flow cytometry analysis. In the remainder of this paper we refer to sperm with destabilized membranes, irrespective of whether they have completed the process of capacitation or not.

2. Materials and methods

2.1 Experimental animals

All experimental procedures were approved by the Animal Use and Care Committee of the University of Pretoria (Project number V059/11).

Nine intact male dogs (5 beagles, 3 Greyhounds and 1 Dalmatian) were used in the study, with informed consent of the owners. All animals were vaccinated annually against distemper, parvovirus, parainfluenza virus, adenovirus and rabies. Only

medium-sized to large dogs were used, in order to ensure recovery of sufficient sperm per ejaculate to perform all the incubations. Semen donors were subject to a breeding soundness examination prior to the study, and only animals considered to have met breeding soundness requirements were included in the study [42].

2.2 Reagents

All media components and the ethidium homodimer stain were purchased from Sigma-Aldrich (Kempton Park, South Africa). The merocyanine and Yo-Pro 1 stains were purchased from Molecular Probes Inc. (Eugene, Oregon, USA; via Invitrogen). The primary anti-phosphotyrosine antibody (Clone 4G10) and the lyophilized secondary antibody-Cy3 conjugate (isotype IgG2βκ) for detecting anti-phosphotyrosine binding were purchased from Millipore (290 Concord Road, Billerica, MA 01821 USA).

2.3 Media used to inhibit or stimulate capacitation

A modified Tyrode's medium was used for incubating sperm under capacitating and control conditions. Bicarbonate-free Tyrode's medium (-BIC) contained 90–120 mM NaCl, 3.1 mM KCl, 0.4 mM $MgSO_4$, 20 mM Hepes, 5.0 mM glucose, 21.7 mM sodium lactate, 0.3 mM NaH_2PO_4, and 1.0 mM sodium pyruvate. Fifteen mM $NaHCO_3$ was added to provide capacitating conditions (+BIC). The pH and osmolarity of both media was maintained at 7.4 and 300 mOsm/kg. Media were prepared and stored at 4 °C until use. On the day of use 5 mg/mL bovine serum

albumin (BSA) and 2.0 mM $CaCl_2$ were added to the capacitating medium (+BIC) and 1 mM pyruvate was added to both media. Subsequently the -BIC medium was maintained at 37 °C, and the +BIC medium at 37 °C in 5% CO_2 in air [43].

In addition to −BIC and +BIC, sperm were also incubated in PF and PBS, which are both known to delay capacitation.

2.4 Semen collection and processing

The sperm-rich fraction of one ejaculate, henceforth referred to as the "ejaculate", from each dog was collected, by means of digital massage of the penis, via a warm glass funnel into a graduated 15 mL tissue culture tube (Elkay Products, Shrewsbury, MA, USA). Immediately thereafter, the post-sperm fraction (prostatic fluid, PF) was collected into a separate tube. The PF from all dogs from which semen was recovered on a given day was pooled, centrifuged and only the sperm-free supernatant used as an incubation medium. Prior to processing, the volume of the ejaculate, sperm concentration and sperm motility were evaluated and recorded.

Each freshly collected ejaculate was diluted 1:2 with PBS and washed by centrifugation through a two-layered Percoll gradient of 4 ml of 35% Percoll-saline and 2 ml of 70% Percoll-saline [43]. After separation, the supernatant was carefully removed, leaving the sperm pellet in approximately one millilitre of 70% Percoll. To remove the residual Percoll, the pellet was washed three times with -BIC. After the last wash, the pellet was resuspended in 1000 µL of -BIC and the sperm concentration determined using a haemocytometer. The sperm suspension was divided and transferred into four separate tubes. The sperm in each tube were diluted to 10 x

10^6/mL with -BIC, +BIC, PF or PBS, respectively. All tubes were incubated at 37 °C in a 5% CO_2 incubator, with the +BIC tube left open and the others closed.

Aliquots of 500 µL were removed at the following times:

- 0, 2, 4, and 6 hours of incubation in -BIC for staining with TP-EH
- 2, 4, and 6 hours of incubation in +BIC for staining with TP-EH
- 0, 0.5, 1, 2, 4, and 6 hours of incubation in -BIC, PF and PBS for staining with M540-YP
- 0.5, 1, 2, 4, and 6 hours of incubation in +BIC for staining with M540-YP.

2.5 Staining

2.5.1 Ethidium homodimer and Tyrosine phosphorylation (TP-EH) staining

The following working solutions were prepared: Fifty micrograms of the primary anti-phosphotyrosine antibody Clone 4G10 was suspended in 1000 µL distilled H_2O and stored at 4 °C until further use. The lyophilized and conjugated secondary antibody Cy3 was diluted to 1 mg/mL in distilled H_2O and stored at 4 °C until further use [29, 30].

Five hundred microlitres of the sperm suspension to be stained with TP-EH was centrifuged at 600 x g for 5 min, after which the supernatant was removed and the pellet resuspended in 500 µL PBS.

Fifty microlitres of EH (1.25 µM) were added to the sperm suspension which was then incubated at 37 °C for 3 min. Forty microlitres of single-stranded DNA solution (1 mg/mL in PBS) was then added and the sample incubated for another 2 min at 37

°C. Next, 500 µL PBS was added to the sample, followed by centrifugation at 600 x g for 5 min, after which the supernatant was removed. The wash and centrifugation step was repeated twice more. The resulting pellet was resuspended in 200 µL PBS and 200 µL of 2% paraformaldehyde and the suspension was incubated for 30 min at room temperature. After a further wash with 1000 µL PBS, the pellet was resuspended in 200 µL Triton X-100 (0.1%) and incubated for 10 min. The sample was then centrifuged once with 500 µL PBS, and incubated for 10 min in 200 µL BSA (1% BSA in PBS) before being centrifuged and washed twice with 200 µL of 1% BSA in PBS. The sample was then incubated overnight at 4 °C with 200 µL of the primary antibody (Clone 4G10; dilution 1:200 in 1% BSA). The next day the sample was centrifuged and washed twice with 500 µL of 1% BSA in PBS and incubated with 100 µL of the second antibody (Cy3; dilution 1:200 in 1% BSA in PBS) for one hour in the dark at room temperature. Thereafter the sample was centrifuged and washed twice with 500 µL of 1% BSA in PBS and resuspended in 100 µL PBS. Five microlitres of the sample was then removed and mixed with 3 to 5 µL of DAPCO (Sigma-Aldrich, D2522) to attenuate the fading of fluorescence, put on a microscope slide, covered with a cover slip of which the edges were sealed with nail varnish. Two smears were prepared and stored at 4 °C in the dark until they were evaluated during the same day. For analysis, 200 sperm were visualised per smear under an epifluorescence microscope (BH2-RFCA, Olympus, Tokyo, Japan) equipped with a DMU set of filters containing a 470-nm band pass excitation filter, a 505-nm dichroic mirror, and a 520-nm long-pass emission filter at a magnification of at least x 400. The filters used enabled the simultaneous identification of non-viable- (EH positive) and viable cells (EH negative), as well as the tyrosine phosphorylation fluorescence patterns.

2.5.2 M540 and Yo-Pro 1 staining

The following working solutions were prepared: The YP stock solution (1 mM in DMSO) was diluted 1:99 in phosphate buffered saline (PBS) to obtain a 10 μM working solution, while the M540 stock solution (54 mM) was diluted 1:39 in PBS to obtain a 1.35 mM working solution [5, 11].

One hundred microlitres of sperm suspension destined for staining with M540-YP was centrifuged at 600 x g for 5 min, the supernatant removed, and the pellet resuspended in 500 μL PBS with 1 mg/mL of polyvinyl alcohol. To achieve a final concentration of 25 nM YP, 1.25 μL of YP working solution was added to the sperm suspension. The sample was incubated for 10 min in the dark after which 1.0 μL of M540 (final concentration of 2.7 μM) was added and the sample was analyzed using a flow cytometer (FC500 flow cytometer (Beckman Coulter, Brea, CA, USA).

The stained cells were excited by a 15 mW 488 nm laser. Green fluorescence (YP) was collected using the FL-1 detector (525 ± 10 nm band-pass filter), while red fluorescence (M540) was collected using the FL-3 detector (620 ± 10 nm band-pass filter). Data from 10,000 cells were collected per analysis. Post-acquisition analyses were done using the Kaluza flow cytometry analysis software (Beckman Coulter, Miami, USA). On FL1/FL3 (YP/M540) dot plots, regions were set to differentiate viable sperm with intact membranes (YP negative and M540 negative); viable sperm with destabilized membranes (YP negative and M540 positive); non-viable sperm with intact membranes (YP positive and M540 negative); and non-viable sperm with destabilized membranes (YP positive and M540 positive).

Samples incubated in non-capacitating medium at time 0 were used to set the regions.

2.6. Statistical analysis

2.6.1 Comparing EH and YP as viability stains

The percentage of viable sperm in a sample was transformed to the arcsine of the square root of the percentage of viable sperm and these values were subjected to repeated measurements ANOVA with Dog (subject), Stain (EH or YP), Treatment (-BIC or +BIC), and Time (2, 4 or 6 h), as well as the interactions between factors included in the model.

2.6.2 Comparing TP-EH and M540-YP as stains to identify viable sperm that were capacitated

The data obtained for the status of membrane destabilization and capacitation status of viable sperm stained with M540-YP and TP-EH was expressed as the percentage of viable sperm that showed a staining pattern consistent with membrane destabilization and capacitation. The arcsine of the square root of the percentage of viable sperm with destabilized membranes and of sperm that were capacitated was subjected to repeated measurements ANOVA. Dog (subject), Time (2, 4 and 6 h), Treatment (-BIC and +BIC), and Stain (TP-EH and M540-YP), and the interactions between factors were included in the model.

2.6.3 Comparison of the effect of incubation in various media on the percentage of viable sperm that were capacitated after staining with M540-YP

The data obtained for viable sperm with destabilized membranes stained with M540-YP and incubated in various media was expressed as the percentage of viable sperm with destabilized membranes. The arcsine of the square root of the percentage of viable sperm with destabilized membranes was subjected to repeated measurements ANOVAs. The first ANOVA included Dog (subject) and Treatment (–BIC, PF and PBS) and was used to compare the time zero values for membrane stabilization of the three treatment groups for which membrane stabilization was assessed at that time. In the second ANOVA, Dog (subject), Time (0, 0.5, 1, 2, 4 and 6 h), Treatment (-BIC, +BIC, PF and PBS), and the Treatment by Time interaction were included in the model. In the second ANOVA, the cell for +BIC at Time 0 was empty and excluded from the model.

For each ANOVA, pair-wise comparisons of means were done by means of Bonferroni's method, with the overall α for all pairwise comparisons pertinent to each ANOVA set at 0.05. Although the statistical analyses were performed on the transformed data, means ± S.E.M. are reported for the non-transformed values to facilitate interpretation.

Statistical analyses were performed using the NCSS statistical software package (Kaysville, Utah, USA) and STATA 11 (StataCorp, LP, College Station, Texas, USA).

3. Results

3.1 Staining patterns observed in dog sperm with M540-YP and EH-TP

Sperm changes indicated through M540 staining will be referred to as sperm with destabilized membranes rather than sperm which are capacitation.

Staining of sperm with M540-YP gave rise to four major populations of sperm as shown in the four quadrants of each of the dot plots in Figure 1: viable sperm without destabilized membranes (YP negative and M540 negative); viable sperm with destabilized membranes (YP negative and M540 positive); non-viable sperm without destabilized membranes (YP positive and M540 negative); and non-viable sperm with destabilized membranes (YP positive and M540 positive).

In contrast to the patterns observed with M540-YP, non-viable sperm as detected with the EH staining method did not show any staining with the TP stain to identify capacitated sperm.

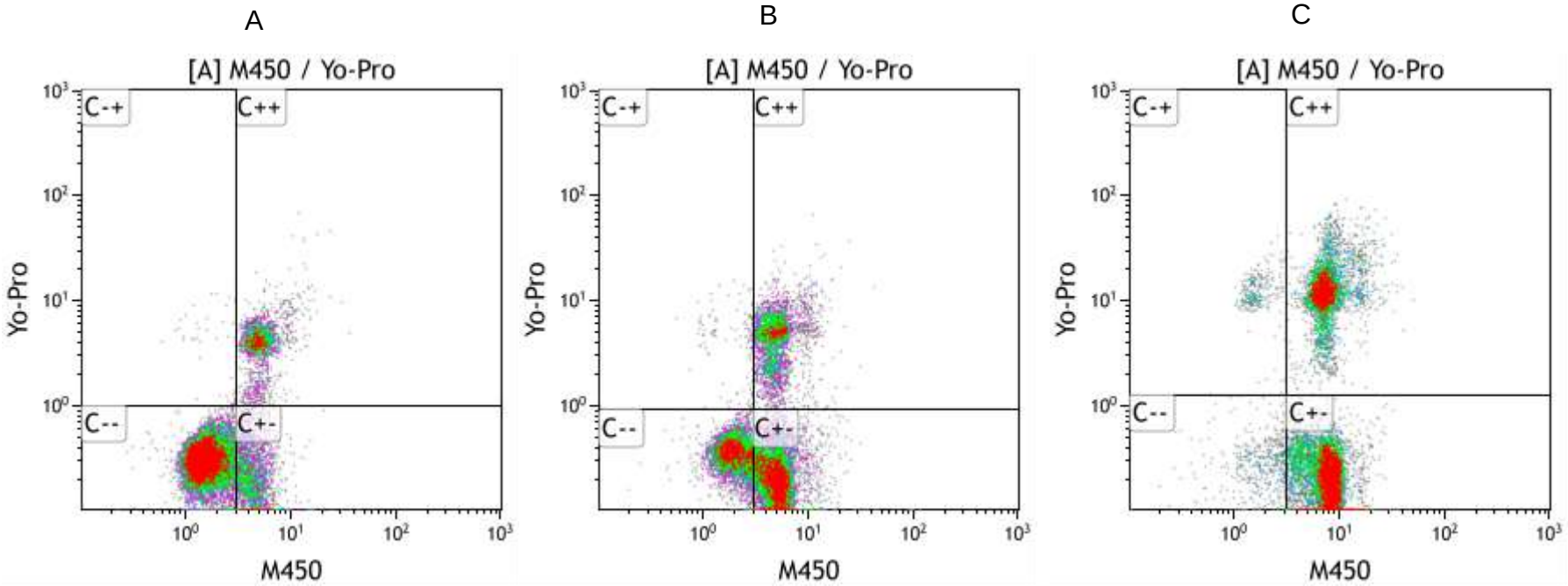

Figure 1. Flow cytometric dot plot of M540-YP stained dog sperm populations, obtained from one ejaculate of each of nine dogs, incubated in modified Tyrode's medium without 15mM bicarbonate at Time 0 (A) and Time 0.5, or with 15mM bicarbonate at Time 0.5 (B). C-+ indicates M540 negative and YP positive sperm, C++ indicates M540 positive and YP positive sperm, C-- indicates M540 negative and YP negative sperm and C+- indicates M540 positive and YP negative sperm.

3.2 Comparison of viability assessed using EH versus YP

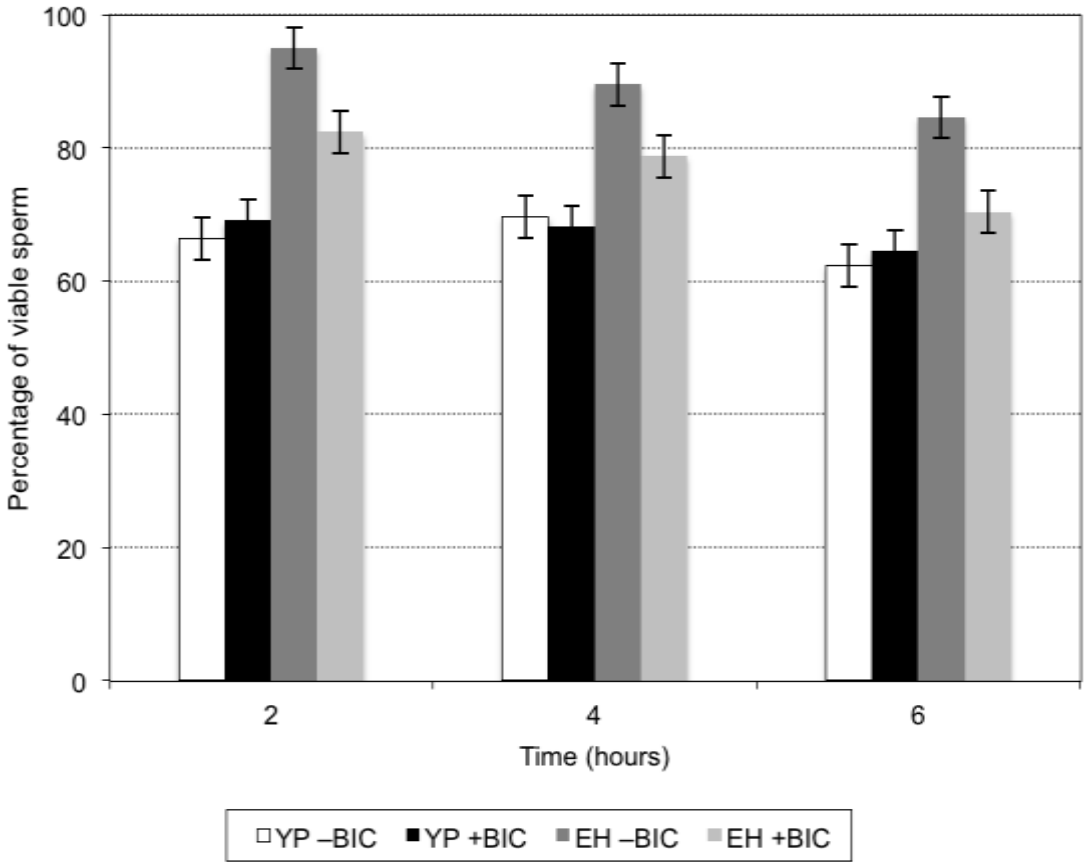

Figure 2. Percentage of viable dog sperm, obtained from one ejaculate of each of nine dogs, identified by Ethidium homodimer versus Yo-Pro 1 staining of dog sperm incubated in modified Tyrode's medium with (+BIC) or without (-BIC) 15mM bicarbonate and assessed after 2, 4 and 6 hours (mean +/- S.E.M).

Figure 2 shows the mean (+/- S.E.M.) percentage of viable sperm for each viability stain with -BIC and +BIC at each time.

Stain, incubation medium and incubation time significantly affected the percentage of sperm characterized as viable ($P \leq 0.001$). EH identified a higher percentage of sperm as viable than did YP, while incubation in +BIC resulted in a lower percentage of sperm retaining viability than did incubation in -BIC (Table 1). The percentage of viable sperm changed little between two and four hours of incubation but then declined markedly between four and six hours (Table 1). Stain interacted with incubation medium ($P < 0.0005$): EH indicated a higher mean

Table 1

Mean percentage of dog sperm, obtained from one ejaculate of each of nine dogs,a characterized as viable by staining with Ethidium homodimer (EH) or Yo-Pro 1 (YP) and after incubation in modified Tyrode's medium with 15mM bicarbonate (+BIC) or without (-BIC) for up to 6 hours

Factors	n	Mean (± S.E.M.)
Stain		
EH	54	83.5±1.37[a]
YP	54	66.7±1.37[b]
Medium		
-BIC	54	78.0±1.37[a]
+BIC	54	72.3±1.37[b]
Time		
2 hours	36	78.2±1.67[a]
4 hours	36	76.6±1.67[a]
6 hours	36	70.5±1.67[b]

Within a main effect (Stain, Medium or Time) means marked [a] differ from means marked [b] ($P < 0.05$)

percentage of viable sperm in -BIC than in +BIC (89.7 ± 1.93 compared to 77.3 ±1.93) whereas YP did not (66.2 ± 1.93 compared to 67.3 ± 1.93).

3.3 Comparison of capacitation in viable sperm assessed with M540-YP and TP-EH

Figure 3 shows the mean (+/- S.E.M.) percentage of viable sperm with destabilized membranes (after staining with M540-YP) or viable sperm that were capacitated (after staining with TP-EH) for -BIC and +BIC at each time.

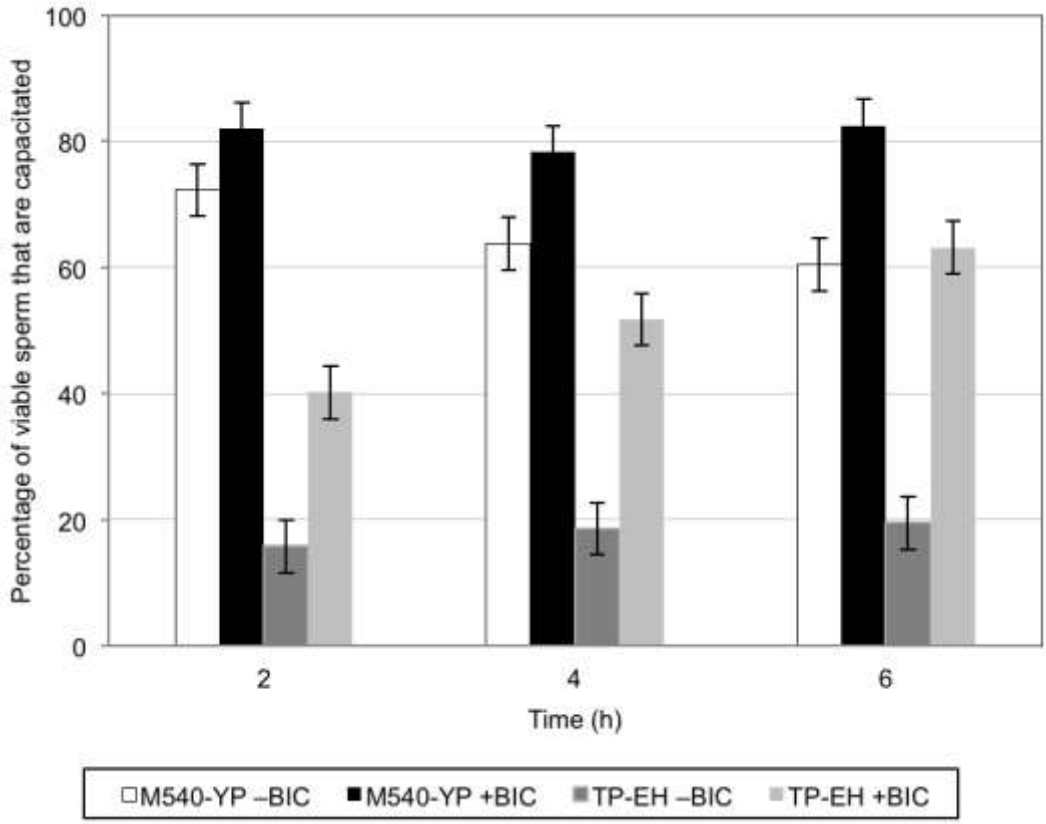

Figure 3. Percentage of viable dog sperm, obtained from one ejaculate of each of nine dogs, that are capacitated or viable dog sperm with destabilized membranes as identified by TP-EH or M540-YP staining, respectively, after incubation in modified Tyrode's medium with (+BIC) or without (-BIC) 15mM bicarbonate and assessed after 2, 4 and 6 hours (mean +/- S.E.M).

The percentage of viable sperm that showed membrane destabilization or were capacitated significantly depended on the stain combination and the incubation medium used ($P<0.0001$) but not on time in incubation ($P>0.5$). When M540-YP was used, approximately 75% of the viable spermatozoa showed membrane destabilization, more than double the 35% of capacitated sperm indicated by TP-EH staining (Table 2). On average, 68% of viable spermatozoa incubated in +BIC showed membrane destabilization (M540-YP) or were capacitated (TP-EH) compared to 42% of the viable spermatozoa that were incubated in -BIC (Table 2).

Table 2

The effects of stain, medium and time of incubation on the mean percentage of viable dog sperm, obtained from one ejaculate of each of nine dogs, that showed membrane destabilization and were capacitated

Group	n	Mean (± S.E.M.)
Stain		
TP-EH	54	34.9 ± 1.93[a]
M540-YP	54	75.1 ± 1.93[b]
Medium		
-BIC	54	41.7 ± 1.93[a]
+BIC	54	68.2 ± 1.93[b]
Time		
2 hours	36	52.7 ± 2.36
4 hours	36	55.4 ± 2.36
6 hours	36	56.9 ± 2.36

[a, b] Within a Main effect (Stain, Medium or Time) means marked [a] differ from those marked [b] ($P<0.05$)

Stain interacted with medium ($P<0.005$). Staining with TP-EH suggested a larger difference between the percentages of viable sperm that were capacitated when they were incubated in -BIC versus +BIC (17.2 ± 2.73% compared to 52.6 ± 2.73%) than was the case for sperm showing destabilized membranes for M540-YP staining (66.3 ± 2.73% compared to 83.9 ± 2.73%). Stain also interacted with time ($P<0.05$). The mean percentage of viable sperm with destabilized membranes or that were capacitated increased over time after two hours for sperm stained with TP-EH (from 27.9 ± 3.44% at Time 2 h to 35.3 ± 3.44% at Time 4 h and to 41.5 ± 3.4% at Time 6 h) but not for sperm stained with M540-YP, which had yielded the maximum level already by two hours (77.4 ± 3.34% at Time 2 h, 75.5 ± 3.34% at Time 4 h and 72.3 ± 3.34% at Time 6h), (n = 18 in each group).

3.4 The effect of incubation in various media with known effects on capacitation over time on the percentage of viable sperm that are capacitated as detected by M540-YP

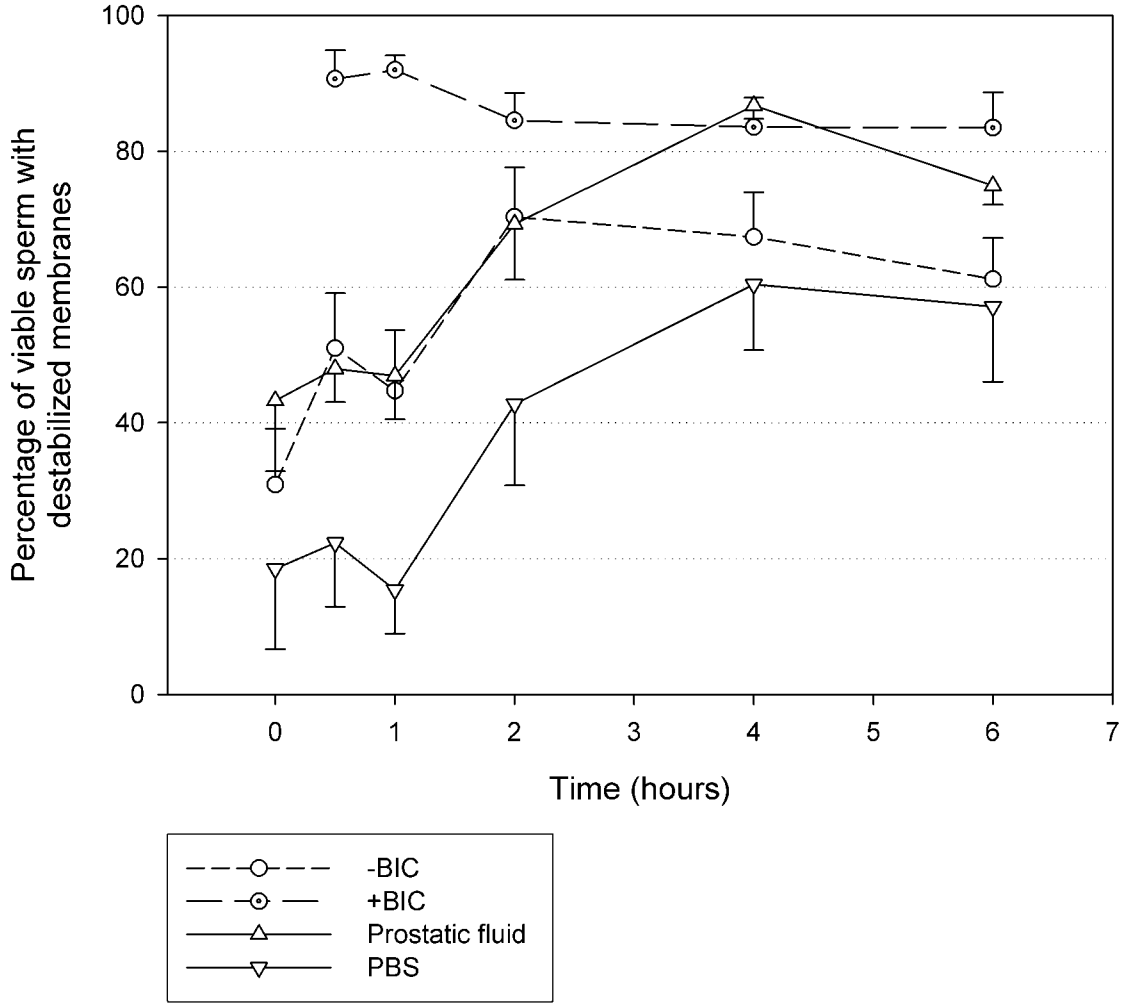

Figure 4. Change in the mean percentage of viable dog sperm, obtained from one ejaculate of each of nine dogs, with destabilized membranes using M540-YP and after incubation in various media (mean +/- S.E.M.).

Figure 4 shows the percentages of viable spermatozoa with destabilized membranes in the four incubation media during incubation.

At time zero the percentages of viable sperm with destabilized membranes were similar (P = 0.29) in –BIC (30.9 ± 8.2%), PF (43.3 ± 10.4%) and PBS (18.5 ± 11.9%).

Incubation medium and duration of incubation affected the percentage of viable sperm with destabilized membranes when stained with M540-YP ($P<0.0001$).

The percentages of viable sperm with destabilized membranes were higher in +BIC and PF than in PBS (Table 3). The percentage of viable sperm with destabilized membranes remained similar from the onset of incubation until one hour thereafter, then increased until 2 hours and remained higher until 6 hours after the onset of incubation (Table 3). Incubation medium and time interacted ($P = 0.0006$). This interaction was absent when +BIC was removed from the model ($P = 0.32$). Figure 4 shows that this interaction is because the percentages of viable sperm with destabilized membranes showed a rising trend between one

Table 3

Mean percentages of viable dog sperm, obtained from one ejaculate of each of nine dogs, with destabilized membranes after 6 h of incubation in non-capacitating medium (-BIC), PF and PBS and stained with M540-YP

Factors	n	Mean (± S.E.M.)
Medium		
-BIC	54	54.3 ± 2.66
+BIC	45	86.8 ± 1.84[a]
PF	53	61.7 ± 2.69[a]
PBS	48	38.0 ± 2.88[b]
Time		
0 hours	26	31.4 ± 6.00
0.5 hours	35	53.4 ± 3.31
1 hour	35	50.1 ± 3.31
2 hours	35	67.1 ± 3.31[c]
4 hours	35	74.9 ± 3.31[c]
6 hours	34	70.4 ± 3.36[c]

[a,b] Means with different superscripts differ ($P < 0.05$)
[c] The mean differs from the mean for 0 hours, which served as the control group

hour and 2 hours of incubation in –PBS, PF and PBS whereas the same did not occur for +BIC.

4. Discussion

Similar to other species [5, 35] four staining outcomes could be observed using flow cytometry to assess staining of dog sperm with M540-YP, allowing the simultaneous classification of dog sperm as viable sperm without destabilized membranes, viable sperm with destabilized membranes, non-viable sperm without destabilized membranes membranes, and non-viable sperm with destabilized membranes.

Prior to the current study the capacitation status of sperm as determined by TP has only been reported in viable sperm [29, 31], leaving it unclear whether TP does stain dead sperm that had capacitated or not. The current study showed that non-viable sperm as detected with the EH staining method did not show any staining with the TP stain, rendering it impossible to identify sperm that had capacitated before they died. This restricted the study to the comparison of the ability of EH-TP and M540-YP to identify capacitation of viable cells only.

The current study demonstrated that EH identifies a smaller percentage of sperm as non-viable than YP does, and supports previous studies that showed that YP identifies sperm early in the process of cell deterioration and death, whereas EH only enters sperm in a more advanced state of membrane deterioration [14, 21]. This is most likely caused by the silencing of a multidrug transporter that is able to actively pump YP out in intact cells but not any more in subviable cells [14, 17]. In subviable cells in which the plasma membrane has

destabilized, the cells lack appropriate amounts of ATP to transport YP back out of the cell [14, 17]. In this respect, it appears that YP penetrates the plasmalemma of sperm committed to cell death via specific pannexin channels and stains sperm DNA before complete membrane disruption would allow entrance of other 'classic membrane impermeant' DNA stains [14, 15, 16, 17]. YP may thus be a useful indicator of impending cell death in dog sperm and M540-YP might therefore be a more sensitive indicator than EH-TP to monitor the viability of sperm after processing or cryopreservation.

In both, -BIC and +BIC media, a significant decline in the percentage of viable sperm was detected by both EH and YP between four and six hours of incubation. Irrespective of whether the sperm were incubated in a non-capacitating medium or a capacitating medium, they could thus maintain their integrity for as long as four hours of incubation at 37 ^{o}C.

Incubation in bicarbonate-containing Tyrode's medium induced changes in the sperm plasma membrane consistent with an early stage of capacitation and, at the same time, caused the cells to become more susceptible to damage and subsequent death [5]. This explains the higher percentage of non-viable (EH-positive) sperm observed in samples incubated in +BIC compared to those incubated in -BIC. Even though bicarbonate had no significant effect on the percentage of viable sperm in samples stained with YP, the overall percentage of viable sperm was lower in samples stained with YP than it was in samples stained with EH. This may be due to an ability of YP to penetrate and stain cells at a relatively early stage of the degeneration process and, in this study, stain cells that were already compromised before being exposed to bicarbonate. YP is a semi-permeable DNA-binding probe and can leak into a cell only after destabilization and increased permeability of the membrane, under conditions where EH does not.

Although M540-YP is an accepted marker for sperm membrane destabilization and subsequent sperm capacitation in other species [5, 11, 35, 45] the current study is the first to validate its use in the dog. This study shows that M540-YP identifies a significantly and substantially larger percentage of viable sperm with destabilized membranes than viable sperm that are capacitated as TP-EH does (means of 75% and 35%, respectively). M540-YP detects a relatively rapid increase in the percentage of viable sperm with destabilized membranes in bicarbonate-containing medium, reaching a maximum during the first two hours of incubation, after which the percentage plateaus. In contrast, the rise in the percentage of viable sperm that are capacitated using TP-EH is slower, and fails to reach similar levels to those obtained with M540-YP by 6 hours after the onset of incubation. We propose that these differences are related to the fact that M540 should detect early changes in membrane fluidity, which precede the increase in protein tyrosine phosphorylation of the sperm tail, as detected by TP-EH.

The studies by Petrunkina and co-workers [28, 30] are the only available research on the tyrosine phosphorylation status of fresh dog sperm incubated in bicarbonate-containing medium, and their results differ from those of the current study. However, because Petrunkina *et al.* considered the tyrosine phosphorylation status of all sperm, not only that of viable sperm as we did in the current study, it is not feasible to compare the results of the studies. In the stallion, Pommer *et al*. [29] reported a low percentage of phosphorylated stallion sperm after one hour of incubation in capacitating medium and no significant difference until three hours of incubation. Differences in time course between our study and the one conducted by Pommer *et al*. may be due to differences between species but appear to indicate that capacitation is slow in the dog.

Although all the dogs used in the current study were breeding sound, with at least 150 x 10^6 spermatozoa in their ejaculates of which >75% were progressively motile and 80%

morphologically normal, we found a large variation among dogs in the percentages of viable sperm that were capacitated at the onset of incubation in our study. This variation may be due to variation in the age of the animals, ejaculation frequency, or due to variation in the susceptibility of their ejaculates to handling, processing such as centrifugation, and incubation under different conditions that may not be apparent during a routine breeding soundness examination. The large variation in the percentage of viable sperm with destabilized membranes or are capacitated among subjects may result in variation in fertility because semen samples with a large percentage of viable sperm with destabilized membranes or are capacitated at collection are likely to exhibit reduced sperm longevity and, as a result, reduced fertility [3].

In this study, M540-YP staining detected a rapid increase in the percentage of viable sperm with destabilized membranes, reaching a maximum by 30 min of incubation in medium containing bicarbonate with no further change over six hours of incubation. A similar finding has been reported in the stallion [34]. In the boar, an increase in the M540-YP stainability has been reported as early as 100 seconds after exposure to bicarbonate [5]. The ability of M540-YP to detect sperm with destabilized membranes in dog sperm incubated under capacitating conditions earlier than other stains, such as the chlortetracycline stain [35] and TP, and its use in flow cytometry makes it a valuable tool for the evaluation of activation status of dog sperm.

In contrast to bicarbonate-containing medium, the average percentage of viable sperm with destabilized membranes after one hour of incubation in all other media examined, reached a peak between 1½ and 3½ hours later than incubation in bicarbonate-containing medium. Although incubation in bicarbonate-free medium is not supposed to stimulate sperm from undergoing capacitation [6, 7], and in contrast to other studies [35, 45], we detected a rise in the percentage of viable sperm with destabilized membranes between one and two hours of

incubation. While changes in the membrane fluidity and membrane destabilization of the sperm plasma membrane induced by bicarbonate have been shown to be immediate [5], it may be possible that dog sperm are more susceptible to prolonged periods of incubation, inducing membrane destabilization as detectable by M540-YP even in the absence of bicarbonate. Recent studies on stallion sperm have shown that an increase in the pH of the incubation medium induces capacitation (46). Unfortunately the pH of the media used in the current study was not monitored in order to detect a change therein. The same potential effect of an increase in pH may apply to incubation in PF and PBS, which showed a similar response to that observed in bicarbonate-free medium. On the other hand, in the boar, addition of seminal plasma to bicarbonate-free medium resulted in a low percentage of viable cells acquiring high merocyanine staining [5]. As in the pig, seminal plasma also impedes capacitation of dog sperm. Dog prostatic fluid contains glycoproteins, such as arginine esterase, acid phosphatase, amylase, β-glucoronidase, fibrinogase, and traces of alkaline phosphatase, that coat the sperm plasma membrane and thereby delay capacitation [10, 40, 41]. This is in agreement with the low percentage of viable sperm with destabilized membranes we found during the first hour of incubation of sperm in PF. In addition to capacitation-delaying glycoproteins dog prostatic fluid also contains small amounts of bicarbonate (approximately 1.7 mmol/L; [47]). Harrison *et al.* [5] found that the concentration of bicarbonate used in the incubation medium directly affected the percentage of live boar sperm with high merocyanine staining. Bicarbonate induces the unmasking of glycoproteins on the sperm membrane of boars and rams within two to three hours of incubation [4, 3]. This may be one explanation why the percentage of viable sperm with destabilized membranes while incubated in PF reached the same high level as those in bicarbonate-containing medium by four hours. PF may be able to postpone membrane

destabilization and capacitation at first but this effect may wear of over time and capacitation may be induced in the long run.

PBS is a simple water-based salt solution without bicarbonate, a source of energy, or macromolecules. By contrast, the bicarbonate-free medium used in the current study contained glucose as an energy source but no macromolecules, whereas prostatic fluid contains bicarbonate, glucose as an energy source and macromolecules such as the enzymes arginine esterase, amylase, acid phosphatase, β-glucornidase, and fibrinogenase [5, 47]. These differences may be responsible for the significantly lower percentage of viable sperm with destabilized membranes in PBS compared to bicarbonate-free medium and PF. Hagen *et al.* [48] reported the importance of macromolecules in sperm preservation as sperm motility and velocity, and fertility was lower for sperm in PBS alone than for sperm in PBS and bovine serum albumin. Fertility of rabbit sperm could be restored by adding bovine serum albumin. Although at a lower level, PBS is still able to follow a trend over time to that of the other two treatments. Due to its lowest percentage of viable sperm with destabilized membranes, compared to bicarbonate-free medium and PF, and its simple composition PBS may thus be a good medium to use as a control medium when researching sperm capacitation.

Conclusion

The results of this study indicate that the M540-YP stain combination can identify viable sperm with destabilized membranes as an early indicator of sperm capacitation. The results also indicate that it can do so earlier than the TP-EH stain combination. Merocyanine staining allows for a much faster staining and a rapid and objective assessment of a large sperm population via flow cytometry.

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