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Progesterone significantly enhances the mobility of boar spermatozoa

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Abstract

Progesterone released from the cumulus cells of the oocyte causes a number of physiological responses in human sperm cells including hyperactivation, acrosome reaction and chemotaxis. We employed a validated sperm mobility assay, which involves measuring the ability of sperm to penetrate an inert cell separation solution over time, to assess the ability of progesterone to enhance the mobility of boar spermatozoa. Cells maximally penetrate the solution over 50 minutes. 100nM progesterone significantly ($P = 0.01$) increased the mobility of non-capacitated sperm cells causing a doubling in the rate at which the cells penetrated through the cell separation solution (control half maximal penetration rate [Km] = 18.0±2.2; +100nM progesterone Km = 8.8±0.8min). Similarly, capacitated cells penetrated at a rate (Km = 19.2±3.0 min) not significantly different from non-capacitated cells and 100nM progesterone also significantly increased the rate of penetration of capacitated cells (Km = 9.5±1.0 min, $P<0.05$). The T-type voltage gated calcium channel blocker mibefradil (30mM) significantly inhibited both the control and progesterone enhanced mobility in non-capacitated and capacitated sperm. Only capacitated cells showed a significant increase in the acrosome reaction in response to 100nM progesterone (control non-reacted = 75±4%, +100nM progesterone non-reacted = 47±10%). Western blot analysis confirmed that there was an increase in the total protein tyrosine phosphorylation levels in capacitated cells. In conclusion, we have demonstrated that 100nM progesterone accelerates the mobility of boar sperm cells through an inert cell separation solution in an extracellular calcium dependent manner.

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Introduction

Progesterone is released from cumulus cells surrounding the oocyte and has been demonstrated to act as a chemoattractant for human and rabbit sperm [1-4]. This chemoattractant property is thought to be necessary in order to guide the sperm to the oocyte, although appears to only occur in capacitated cells [5, 6]. The pattern of response of human sperm cells to progesterone has been suggested to involve a change in motility parameters with both positive and negative effects on hyperactivation depending on the temporal and spatial exposure [4, 7, 8]. At a molecular level progesterone induces a rapid rise in intracellular calcium [9, 10] that is due to activation of a sperm specific calcium ion channel, CatSper [11, 12]. Progesterone has also been demonstrated to induce a rapid rise in intracellular calcium in sperm in other species [13-15]. However, the percentage of sperm cells

undergoing the acrosome reaction also increases with exposure to increasing concentrations of progesterone [16-19]. Prematurely acrosome reacted sperm are unable to fertilise although this may serve to select against inferior sperm [20].

Studies examining the effects of progesterone on boar sperm cells have largely focussed on its effects on the acrosome reaction [21-23]. However, high (micromolar) concentrations have been reported to increase motility [24] and intracellular calcium levels [14] in capacitated cells. Generally, computer assisted sperm analysis (CASA) is employed to examine sperm motility. Changes in motility are examined by taking brief (approximately 1s) recordings followed by objective mathematical analysis of various motility parameters [25]. However there is little information as to how sperm respond in the presence of a chemoattractant over an extended period of time or if responsiveness correlates to a change in distance travelled. Here we used a simple mobility assay [26] to determine the effects of progesterone on the mobility (i.e. swim rate in a constant gradient) of non-capacitated and capacitated boar sperm cells over time.

Material and Methods

Semen supply

Semen was supplied commercially by The Pig Improvement Company (PIC) from their stock of boars (PIC337 Champion species). Semen was diluted 1:1 upon collection into TriXCell (IMV International, Minneapolis, MN, USA) semen extender according to the manufacturer instructions. Semen was delivered overnight in flatpacks at ambient temperature. Upon receipt, the diluted semen was then aliquoted into 15ml polypropylene centrifuge tubes and stored at 16°C in accordance with the manufacturer instructions until use. Assays were completed within 2 days of delivery. No information is available regarding their fertility.

Vitality test

Vitality testing using propridium iodide (PI) staining based on the method by Pintado and colleagues [27] was conducted on each batch of semen before conducting mobility assays. The final concentration of PI was 5 µg ml^{-1} when added to aliquot of cells in extender. The cells were incubated at room temperature for 5 min before being in 4% paraformaldehyde and placed between a glass slide and coverslip for cell counting under fluorescent microscope. A minimum of 200 cells were counted and non viable, membrane damaged cells showed clear fluorescence. Batches showing greater than 10% non viable cells were not used.

Acrosome staining

The acrosome status of sperm was investigated using the method of He *et al* [28]. Briefly, sperm cells were fixed and permeabilised in absolute ethanol. Fluoresceine isothiocyanate-labelled pisum sativum agglutinin (FITC-PSA) at 0.1mg/ml was used to determine acrosomal status. A minimum of 200 cells were counted per replicate and at least 4 replicates were analysed per reaction condition.

Mobility assay solutions

The mobility assay was based on the validated method of Vizcarra & Ford [26]. Two solutions were used to make up the final Accudenz solution for the mobility assay; the stock accudenz solution and mobility buffer that were mixed (1:5) to form a working Accudenz solution that was used for the spectrophotomeric analysis of sperm mobility. A stock Accudenz (Gentaur Ltd, London, UK) solution (pH 7.4) was prepared which consisted of 30% Accudenz (w/v), 3mM KCl and 5mM TES (N-tris[hydroxymethyl] methyl-2 aminoethansulfonic acid). The mobility buffer (pH 7.4) consisted of 111mM NaCl, 25mM glucose, 4mM $CaCl_2$ and 50mM TES. On a daily basis a working Accudenz (6% w/v) solution was made and aliquoted into 1.5ml polystyrene cuvettes and stored in the fridge at 4°C until used for the assay. For experiments involving progesterone and/or mibefradil, these compounds were added to the working Accudenz solution prior to adding to cuvettes.

Mobility assay

Cuvettes containing the working Accudenz solution were pre-warmed to 37°C. Data presented in each figure were derived using a split-ejaculate method such that cells under control and treatment conditions were taken from the same insemination dose. 1×10^8 live sperm cells (in maximally 150µl) were carefully pipetted onto the surface of the Accudenz solution and the change in absorbance (550nm) over time was monitored using a spectrophotometer. This treatment is hereafter called the mobility control. A negative control was prepared by first heating 1×10^8 sperm cells at 60°C for 60 minutes. A positive control was prepared by mixing 1×10^8 cells with 1.5ml working Accudenz solution prior to loading into a cuvette. Progesterone was added to the working Accudenz solution to a final concentration of 1, 10 or 100nM and used to monitor sperm penetration rate in response to progesterone concentration. Absorbance readings were recorded at time intervals over 50 minutes (1, 5, 10, 15, 20, 30, 40 and 50 minutes) and cuvettes were incubated at 37°C for the duration of this time period.

To construct data sets within a figure, multiple runs were taken from an individual boar sample and multiple boars were used as indicated in the figure legends. A total of 17 different boars were used by the end of the study.

Capacitating conditions

Capacitating media (CM) was composed of 5mM KCl, 1mM KH_2PO_4, 95mM NaCl, 5.55mM glucose, 25mM $NaHCO_3$, 2mM $CaCl_2$, 0.4% BSA and 2.5mM pyruvate (pH 7.4). Non-capacitating media (NCM) lacked calcium, bicarbonate and BSA and consisted of 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4, 137mM NaCl, 5.55mM glucose and 2.5mM pyruvate (pH 7.4). 1×10^8 cells were pelleted and resuspended in 1ml of either CM or NCM and incubated at 37°C for 3hours.

Following capacitation the samples were pelleted, resuspended in mobility buffer and the mobility assay ran as described for non-capacitated cells.

SDS-PAGE and Western Blotting

1×10^8 cells were incubated in NCM or CM for 3 hours, pelleted at 17000 × g then heated at 95°C in Laemmli sample buffer and frozen for subsequent use in SDS-PAGE. Sperm proteins were separated on 10% polyacrylamide gels and transferred to PVDF membranes by electrophoresis. The membranes were incubated overnight at 4°C with anti phospho-tyrosine primary antibodies (Cell Signaling Technology, UK) at 1:20 000 dilution in Tris-buffered saline (25mM Tris-HCl pH7.4, 150mM NaCl) containing 0.1%(v/v) Tween 20 and 5%(w/v) bovine serum albumin. The detection method involved the use of a WesternDot™625 western blot kit in accordance with the manufacturer instructions (Life Technologies, UK) and imaging with a Biorad Gel Doc XR system.

Data analysis

Data represented mean ± SEM for four ejaculates measured in quadruplet (Graphpad Prism). Curves are fitted using non-linear regression model and Km values (minutes) represent the time at which absorbance was half maximal (Graphpad Prism). $P < 0.05$ is taken as statistically significant.

To test for differences in the rate of mobility between control and progesterone treated sperm cells data were first transformed to linearize then an ANCOVA was carried out on regression plots of the linearized data using R (open source statistical software http://www.r-project.org/. R version 2.15.1 (2012-06-22) "Roasted Marshmallows". Copyright 2012 The R Foundation for Statistical Computing). Data were linearized using $\ln(y) = m*\ln(x)+\ln(c)$, where y = mean absorbance and x = 1/time (min). The effect of mibefradil on sperm mobility was analysed using ANOVA followed by Tukeys post-hoc test for three time points (10, 20 and 50 minutes).

Chemicals and reagents

Unless stated all chemicals and reagents were obtained from Sigma-Aldrich, UK.

Mibefradil was obtained from Tocris Bioscience, Bristol, UK.

Results

Non-capacitated boar sperm penetrated the working Accudenz solution with a Km = 18.0±2.2 min. Accudenz solutions containing increasing concentrations of progesterone (1, 10, 100nM) were prepared in order to monitor a change in mobility induced by this putative chemoattractant. Only 100nM progesterone treatment produced a significant increase in sperm mobility as measured by an increase in absorption over time in the sperm mobility assay (Figure 1). Analysis of the progesterone effect using ANCOVA required linearised plots so data from figure 1 was first transformed ($\ln(y) = m*\ln(x)+\ln(c)$ where y = mean absorbance and x = 1/time (min)) and the gradient and intercept of the regression lines were then compared. The independent variable in the regression analysis was ln(1/time), the dependent variable was ln(mean absorbance) and the factor was +/-progesterone treatment. Initial ANCOVA analysis revealed that there was no significant interaction between progesterone treatment and time ($p = 0.76$) and

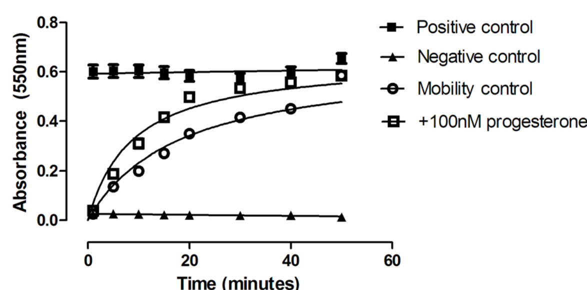

Figure 1. Progesterone accelerates the mobility of non-capacitated boar sperm. Under control conditions sperm penetrate the Accudenz solution with a Km = 18.0±2.2min (mobility control, 18 replicates). Sperm penetrate at a significantly (P=0.01) enhanced rate through the Accudenz solution containing 100nM progesterone (+100nM progesterone; Km = 8.8±0.8min, 20 replicates). Data sets are analysed as described in methods and results and are an average of 4 ejaculates from different boars with pooled replicates as indicated. Positive and negative control data sets are an average of 4 ejaculates measured in quadruplicate.

therefore progesterone treatment could be analysed for its effect alone on mobility. For non-capacitated sperm, the intercepts of the regression lines were significantly higher in the presence of 100nM progesterone. The intercept of the regression line for hormone treatment was -3.32 and for controls, the intercept was -3.49, (F= 8.997, p= 0.01). This result indicates progesterone significantly increases the rate of penetration of non-capacitated cells. Indeed 100nM progesterone caused over a two-fold increase in the rate of penetration rate (Km = 8.0±0.8min). There were no significant differences between the gradients for 100nM progesterone treated and control sperm for either capacitated (see below) or non-capacitated sperm indicating that there was no interaction between hormone treatment and time and that the relationship between absorbance and time could be modelled by a simple additive regression model without variable interaction.

CatSper is sensitive to the Ca_v blocker mibefradil [12]. Therefore, we examined the ability of this T-type calcium channel blocker to inhibit basal and progesterone-stimulated mobility. ANCOVA analysis revealed that the gradients of the regression line for non-capacitated and capacitated cells were significantly different (P<0.05) between control and mibefradil treatments suggesting an interaction between the factor i.e. treatment (+/- mibefradil) and the covariate (Ln(1/time) in addition to the main effect of the drug. Therefore these data were analysed using ANOVA followed by Tukeys post-hoc test for comparison of the effect of drug concentrations. Sperm failed to penetrate Accudenz solution containing 30μM mibefradil suggesting a complete abolition of mobility and hence the finding with the ANCOVA analysis. Statistical analysis indicated that penetration was significantly lower when compared to control from 10 minutes onward (P<0.05 at 10 minutes; Figure 2). At a concentration of 3μM, a significant difference was seen only after 50 minutes (Figure 2 and 3; P<0.001), suggesting a slower rate of penetration. Progesterone (100nM) could not reverse the mibefradil (30μM) mediated inhibition of mobility (P<0.001; Figure 3).

Capacitation is associated with alterations of the membrane of sperm [29] and may enhance sensitivity to progesterone [4]. Therefore we sought to repeat the experiments using capacitated boar sperm. Capacitated sperm cells penetrated the Accudenz solution with a Km value (19.2±3.0 min) that was not significantly different from non-capacitated cells. As with non-capacitated cells only 100nM progesterone caused a significant increase in cell mobility. For capacitated sperm ANCOVA analysis revealed again there was no significant interaction between treatment and time (p=0.35). Following ANCOVA analysis of the data as described above, the intercept of the regression line for 100nM progesterone treatment was -2.98 and for the controls the intercept was -3.33, (F= 5.75, p= 0.03) with the time taken to reach half maximal absorbance decreasing approximately two-fold in the presence of the hormone (Km = 9.5±1.0min; Figure 4). 30μM mibefradil significantly reducing the ability of the sperm to penetrate the accudenz solution from 20 minutes onwards (P<0.05 at 20 minutes; Figure 5 and 6) and progesterone could not reverse this inhibition of mobility of capacitated cells (Figure 6).

Progesterone (100nM) induced the acrosome reaction in significantly greater number of cells in capacitated cells only, although approximately half of the population remained unreacted (Figure 7). Finally, the capacitation status of the cells was confirmed by assessing the total protein tyrosine phosphorylation status (Figure 8).

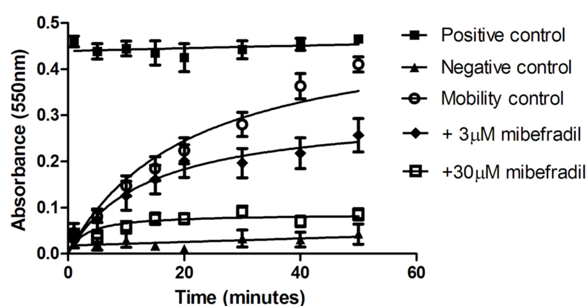

Figure 2. Mibefradil inhibits the mobility of non-capacitated boar sperm. Under control conditions sperm penetrate the Accudenz solution with a Km = 20.9±3.5 min (mobility control, 14 replicates). From 10 minutes onwards mibefradil (30μM) containing Accudenz solution significantly (P<0.05 at 10 minutes) inhibits cell mobility (17 replicates). Data are analysed using ANOVA followed by Tukeys post-hoc test. Data sets are an average of 4 ejaculates from different boars with pooled replicates as indicated. Positive and negative control data sets are an average of 4 ejaculates measured in quadruplicate.

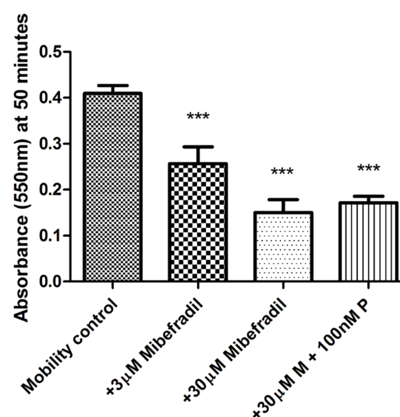

Figure 3. Progesterone (P) does not recover the mibefradil mediated inhibition of mobility of non-capacitated boar sperm cells. Maximum absorbance values at 50 minutes are given for sperm penetrating the Accudenz solution only (mobility control, 14 replicates) or containing mibefradil (M, +3/30μM, 19 and 17 replicates respectively) or 30μM mibefradil and 100nM Progesterone (+30μM M + 100nM P, 26 replicates). Data are analysed using ANOVA followed by Tukeys post-hoc test. Asterisks (***P<0.001) indicate significantly different from mobility control. Data sets are an average of 4 ejaculates from different boars with pooled replicates as indicated.

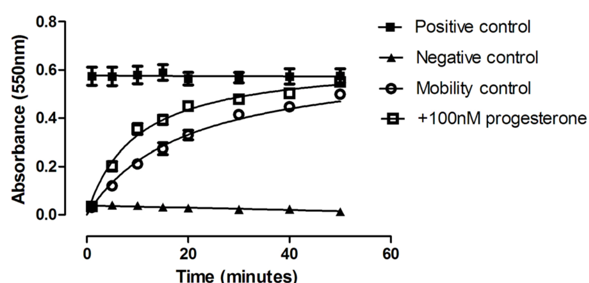

Figure 4. Progesterone accelerates the mobility of capacitated boar sperm. Under control conditions sperm penetrate the Accudenz solution with a Km = 19.2±3.0 min (mobility control, 16 replicates). Sperm penetrate at a significantly (P=0.03) enhanced rate through the Accudenz solution containing 100nM progesterone (+100nM progesterone; Km = 9.5±1.0 min, 20 replicates). Data sets are analysed as described in methods and results and are an average of 4 ejaculates from different boars with pooled replicates as indicated. Positive and negative control data sets are an average of 4 ejaculates measured in quadruplicate.

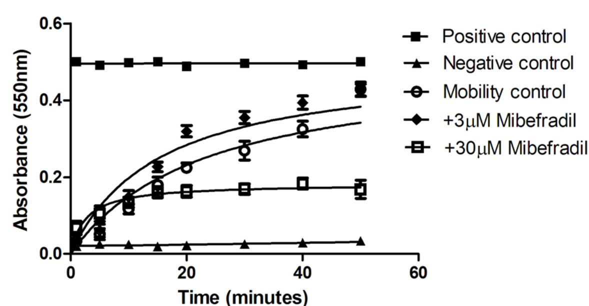

Figure 5. Mibefradil inhibits the mobility of capacitated boar sperm. Under control conditions sperm penetrate the Accudenz solution with a Km = 23.0±4.9 min (mobility control, 19 replicates). Mibefradil (30μM) containing Accudenz solution significantly inhibits cell mobility from 20 minutes onwards (P<0.05 at 20 minutes, 21 replicates). Data are analysed using ANOVA followed by Tukeys post-hoc test. Data sets are an average of 4 ejaculates from different boars with pooled replicates as indicated. Positive and negative control data sets are an average of 4 ejaculates measured in quadruplicate.

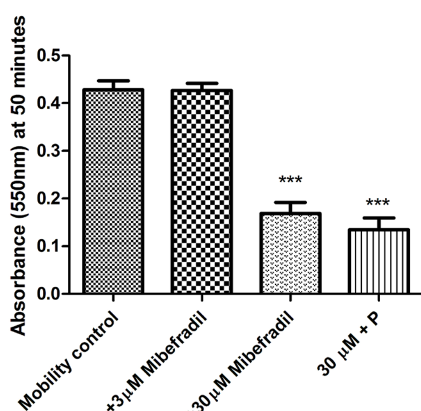

Figure 6. Progesterone (P) does not recover the mibefradil mediated inhibition of mobility of capacitated boar sperm cells. Maximum absorbance values at 50 minutes are given for sperm penetrating the Accudenz solution only (mobility control, 19 replicates) or containing mibefradil (M, +3/30μM, 14 and 21 replicates respectively) or 30μM mibefradil and 100nM Progesterone (+30μM M + 100nM P, 20 replicates). Data are analysed using ANOVA followed by Tukeys post-hoc test. Asterisks (P<0.001) indicate significantly different from mobility control. Data sets are an average of 5 ejaculates from different boars with pooled replicates as indicated. Positive and negative control data sets are an average of 4 ejaculates measured in duplicate.

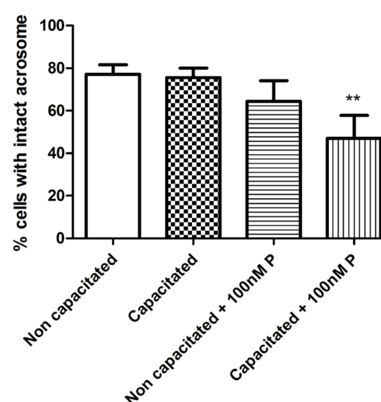

Figure 7. Effects of progesterone (P) on the acrosome reaction in non-capacitated and capacitated boar sperm cells. Cells were either left in non-capacitating or capacitating media for 3 hours followed by incubation with 100nM progesterone. Progesterone significantly increases the percentage of boar sperm cells undergoing spontaneous acrosome reaction. Data are analysed using paired students t-test.
** indicates significantly different from capacitated cells (P<0.05).

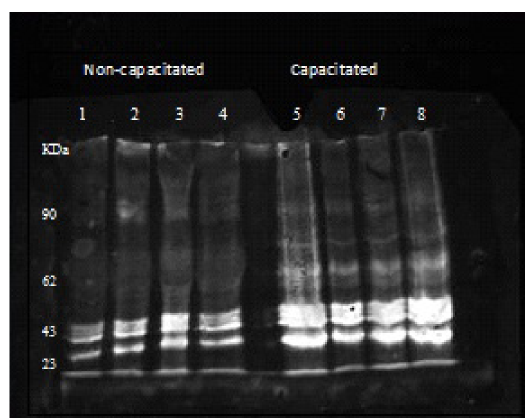

Figure 8. Sperm capacitation is associated with changes in protein phosphotyrosine content. Western blot demonstrating the change in protein phosphotyrosine pattern induced by capacitation of sperm from 4 different ejaculates from consecutive weeks from different boars. 1×10^8 sperm cells were incubated in either non-capacitating or capacitating conditions for 3 hours and subsequently pelleted at 17000×g then heated at 95°C in Laemmli sample buffer and frozen for subsequent use in SDS-PAGE/Western blot. Lanes 1&5, 2&6, 3&7 and 4&8 are paired.

Discussion

Progesterone has been demonstrated to induce a number of calcium dependent mechanisms in sperm including hyperactivation [7, 30], protein tyrosine phosphorylation [31, 32], acrosome reaction [33] and chemotaxis [34]. Calcium influx through the CatSper ion channel is likely to contribute to all of these processes [35-40] and recently progesterone has been demonstrated to directly activate this channel in human, but not mouse sperm [11, 12]. The characterisation of the effects of progesterone on boar sperm has been limited. Here we describe for the first time that progesterone can stimulate the mobility of both non-capacitated and capacitated boar sperm cells.

Previous studies examining the effect of progesterone

on human sperm cell motility have typically involved the use of CASA systems and report significant effects on hyperactivation [4, 7] that are probably linked to its chemotactic properties [8]. Capacitated boar sperm respond in a complex manner to a high concentration of progesterone but are clearly sensitive to this hormone and remain responsive over time [24]. The threshold sensitivity of boar sperm to progesterone has not been established; however in this study we show that 100nM progesterone significantly increases the rate of penetration in both non-capacitated and capacitated cells. This is notably higher than the picomolar levels that can stimulate human sperm activity [8, 34]. However due to the limitation of the mobility assay we would not expect to be able to demonstrate such sensitivity. Nevertheless, this concentration is well below the levels of progesterone seen in porcine follicular fluid regardless of season [41] suggesting that this response is likely to have physiological relevance.

As prematurely acrosome reacted sperm cannot undertake fertilisation [42] and progesterone, at micromolar concentrations, has previously been reported to cause the acrosome reaction in capacitated boar sperm [21, 22] we sought to assess if the enhanced swim rate would be compromised by a parallel increase in the acrosome reaction. We demonstrated that the acrosome reaction is increased in the population of capacitated cells treated with 100nM progesterone. Although this may suggest a compromise in function, approximately 50% of cells remain acrosome intact and it has been suggested that a hypersensitivity to progesterone may select against inferior sperm [20]. Therefore, we suggest that based on our experiments, cells that are responsive to progesterone and remain acrosome intact have a selective advantage for successfully winning the race to fertilise the egg.

The pattern of response to progesterone of boar sperm cells is interesting due to the molecular mechanism by which progesterone has been proposed to act in stimulating sperm motility. Progesterone potentiates CatSper-mediated currents in human but not mouse sperm, with the constitutive activity of CatSper being much higher in the latter species. CatSper is conserved between species [43] and has been cloned from pigs [44]. Therefore, it is likely to contribute to sperm movement in this species. We demonstrated that, as is similar to human sperm cells, the mobility of boar sperm cells is sensitive to progesterone and that this is a calcium dependent mechanism due to the ability of mibefradil to abolish progesterone-enhanced movement. This suggests the presence of a functional CatSper-like channel with properties analogous to that in human cells. In contrast, the ability of mibefradil to abolish basal mobility suggests a high degree of constitutive activity of this CatSper-like channel in boar sperm cells which is analogous to CatSper in mice cells. However, this clearly requires further investigation as the presence of different T-type voltage gated calcium channels or involvement of other calcium-permeable ion channels in boar sperm mobility cannot yet be ruled out [45, 46].

In summary, for the first time we have demonstrated that progesterone can sustainably increase the mobility of boar sperm cells in an extracellular calcium dependent manner and that the increased distance travelled with time presents a selective advantage for cells that remain acrosome intact.

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