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Complex CatSper-dependent and independent $[Ca^{2+}]_i$ signalling in human spermatozoa induced by follicular fluid

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Running Title: CatSper activation by human follicular fluid

Study question: Does progesterone in human follicular fluid (hFF) activate CatSper and do other components of hFF modulate this effect and/or contribute separately to hFF-induced Ca^{2+} signaling?

Summary answer: hFF potently stimulates CatSper and increases $[Ca^{2+}]_i$, primarily due to high concentrations of progesterone, however other components of hFF also contribute to $[Ca^{2+}]_i$ signaling, including modulation of CatSper channel activity and inhibition of $[Ca^{2+}]_i$ oscillations.

What is known already: CatSper, the principal Ca^{2+} channel in spermatozoa, is progesterone-sensitive and essential for fertility. Both hFF and progesterone, which is present in hFF, influence sperm function and increase their $[Ca^{2+}]_i$.

Study design, size, duration: This basic medical research study used semen samples from >40 donors and hFF from >50 patients who were undergoing surgical oocyte retrieval for IVF/ICSI.

Participants/materials, setting, methods: Semen donors and patients were recruited in accordance with local ethics approval (13/ES/0091) from the East of Scotland Research Ethics Service REC1. Activities of CatSper and KSper were assessed by patch clamp. Sperm $[Ca^{2+}]_i$ responses were examined in sperm populations and single cells. Computer-assisted sperm? analysis (CASA) parameters and penetration into viscous media were used to assess functional effects.

Main results and the role of chance: hFF and progesterone significantly potentiated CatSper currents. Under quasi-physiological conditions, hFF (up to 50%) failed to alter membrane K^+ conductance or current reversal potential. hFF and progesterone (at an equivalent concentration) stimulated similar biphasic $[Ca^{2+}]_i$ signals both in sperm populations and single cells. At a high hFF concentration (10%), the sustained (plateau) component of the $[Ca^{2+}]_i$ signal was consistently greater than that induced by progesterone alone. In single cell recordings, 1% hFF induced $[Ca^{2+}]_i$ oscillations similarly to progesterone but with 10% hFF generation of $[Ca^{2+}]_i$ oscillations was suppressed. After treatment to 'strip' lipid-derived mediators, hFF failed to significantly stimulate CatSper currents but induced

small $[Ca^{2+}]_i$ responses that were greater than those induced by the equivalent concentration of progesterone after stripping. Similar $[Ca^{2+}]_i$ responses were observed when sperm pre-treated with 3 μM progesterone (to desensitise progesterone responses) were stimulated with hFF or stripped hFF. hFF stimulated viscous media penetration and was more effective than the equivalent does of progesterone.

Large scale data: N/A

Limitations, reasons for caution: This was an in-vitro study. Caution must be taken when extrapolating these results in vivo.

Wider implications of the findings: This study directly demonstrates that hFF activates CatSper and establishes that the biologically important effects of hFF reflect, at least in part, action on this channel, primarily via progesterone. However, these experiments also demonstrate that other components of hFF both contribute to the $[Ca^{2+}]_i$ signal and modulate the activation of CatSper. Simple in-vitro experiments performed out of the context of the complex in-vivo environment need to be interpreted with caution.

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Key words: follicular fluid / patch clamp electrophysiology / CatSper / potassium channel / spermatozoa

Introduction

Human follicular fluid (hFF) affects various important functions of human spermatozoa, including hyperactivated motility, chemotaxis and acrosome reaction (Baldi *et al.,* 1998). Almost 30 years ago Thomas and colleagues demonstrated that hFF stimulated a rapid influx of Ca^{2+} in human spermatozoa (Thomas and Meizel 1988). Subsequently, progesterone (P4) was shown to have effects on sperm function similar to those of hFF and was found to be the component of hFF that was primarily responsible for induction of Ca^{2+}-influx (Osman *et al.,* 1989; Thomas and Meizel 1989). In 2011, Lishko and Strunker independently showed that induction of Ca^{2+} influx by P4 was via the sperm specific channel CatSper (Lishko *et al.,* 2011; Strunker *et al*. 2011) which is now known to be stimulated by a wide range of small organic molecules (Brenker *et al.,* 2012). P4, at high concentrations (~µM range), also inhibits KSper channels (Mannowetz *et al.,* 2013). It has been proposed that high concentrations of P4 encountered in the vicinity of the oocyte and its vestments achieve full activation of CatSper through a combination of CatSper activation and depolarisation of membrane potential due to KSper inhibition (Mannowetz *et al.,* 2013).

As P4 is a primary component of hFF, a logical assumption is that exposure of human spermatozoa to hFF in vivo activates CatSper. However, the 'clean' stimuli that are used for in-vitro investigations, such as those by which the action of P4 on CatSper was established, differ greatly from the complex environment of the reproductive tract (Mortimer *et al*. 2013; Sakkas *et al.,* 2015). hFF is a complex fluid (Revelli *et al.,* 2009; O'Gorman *et al.,* 2013) and, in its presence, sperm are simultaneously exposed to multiple ligands, potentially leading to multiple separate effects and/or interactions. Significantly, pre-treatment with oestrogen ($17\beta E_2$), which elevates $[Ca^{2+}]_i$ in spermatozoa apparently by a mechanism independent of CatSper (Luconi *et al.,* 1999; Lishko *et al.,* 2011; Mannowetz et al, 2017), reduced the Ca^{2+} response to subsequent stimulation with P4 (Luconi *et al,* 1999). Consequently, two fundamental questions are. (i) Does hFF act on CatSper in a manner consistent with the previously described effects of its principal component P4, or are there synergistic or even

antagonistic effects on CatSper upon exposure to these complex mixtures? (ii) Do other components of hFF contribute significantly, but separately, to hFF-induced Ca^{2+} signalling?

Materials and Methods

Experimental solutions

Details for HEPES buffered saline, bicarbonate buffered capacitating medium, supplemented Earle's balanced salt solution (sEBSS), standard bath solution (patch seals and quasi-physiological recording), standard pipette solution (quasi-physiological recording), Cs^+-based pipette and bath solutions (monovalent CatSper currents) and bath (Ba^{2+}) and pipette solutions for CatSper tail currents are given in Supplementary File 1 .

Selection and preparation of spermatozoa

Semen samples were from donors with normal sperm concentration and motility (WHO 2010). Samples were obtained by masturbation after 2-3 days sexual abstinence. After liquefaction, sperm were isolated by either swim up or density gradient centrifugation (electrophysiological studies) and left to capacitate (37°C, 6% CO_2) for 3-5 hours (Alasmari *et al*, 2013a). Samples were obtained and analysed in line with suggested guidance for human semen studies and variations identified (Bjorndahl *et al*., 2016).

Human Follicular Fluid

Oocytes were retrieved by transvaginal aspiration 36 hours after injection of r-hCG. Most (90%) of these oocytes were in metaphase II. Human follicular fluid (hFF) without blood contamination from the largest follicles of each ovary was centrifuged at 2500g for 10 min to separate cellular components and the supernatant (0.22 µm filtered) was either used on the day for experimentation or stored (at -20°C) until use (<1 week). hFF progesterone (in whole and dextran-coated charcoal-stripped samples) was assayed before use (Siemens ADVIA Centaur®XP competitive Immunoassay System).

Stripping of steroids, prostaglandins and other lipid-derived components from hFF.

Steroids and prostaglandins were removed from hFF by adapting the dextran-coated activated charcoal method for removal of steroids from serum (product information sheet C9157; Sigma Aldrich, UK; Supplementary File 1).

Electrophysiology

Currents were recorded from sperm isolated by density gradient using whole cell patch clamp (Mansell *et al.,* 2014). To investigate K^+ channel function, cells were studied under quasi-physiological conditions (standard pipette and bath solutions) using a ramp protocol (-92 to 68 mV over 2500 ms). Membrane potential was held at -92mV between ramps (Brown *et al.,* 2016). Reversal potentials (E_{rev} – to estimate resting Vm) and membrane conductance (Gm) were calculated as previously described (Brown *et al.,* 2016). Monovalent CatSper currents were recorded using Cs^+-based divalent-free pipette and bath solutions. Currents were evoked by a ramp protocol (−80 to 80 mV over 1 s). Membrane potential was held at 0 mV between ramps. Divalent (Ba^{2+}) CatSper tail currents (Lishko *et al*., 2011) were evoked by 400 ms pulses followed by stepping to -150mV (200 ms). Vm was held at -70mV between sweeps (Lishko *et al*, 2011). Tail current amplitudes were used to plot voltage activation (G-V) curves. Data were sampled at 2 kHz, filtered at 1 kHz. Tail current data were leak subtracted using pClamp P/4 protocol to minimise the impact of membrane resistance (PClamp 10 software, Axon instruments).

Assessment of $[Ca^{2+}]_i$ signals

Population recordings. Following swim-up, sperm (≈6 million/ml) were capacitated (3-5 h) then loaded with 4.5 μM Fluo4 for 30 min, washed twice (700 g for 10 min) and resuspended in sEBSS. $[Ca^{2+}]_i$ was assessed using a FLUOstar microplate reader (BMG Labtech Offenburg, Germany) with 488nm (excitation) and 520nm (emission) filters. After a control period, (30-60 s) stimuli were added using a multichannel pipetter as described by Strunker *et al* (2011). To compare $[Ca^{2+}]_i$ responses to hFF and equivalent [P4] aliquots from the same fluo4 loaded sample, tests were performed in parallel. Emission was background corrected and normalized to the control (pre-stimulus) amplitude. To compare duration of P4 and hFF-induced transients, the half-duration (midpoint of the rising phase to midpoint of decay) was calculated. In desensitization experiments, cells were first stimulation with 3 μM

P4 then, after a delay of 300 s, a second 'test' stimulus was applied in the continued presence of the desensitising P4.

Single cell recordings. Recordings were made as described previously (Nash et al, 2010) but using Fluo4. All experiments were performed at 25±0.5 °C in a continuous flow of medium. Images were captured at 0.2 Hz using a 40x oil objective and Andor Ixon 897EMCCD camera controlled by iQ software (Andor Technology, Belfast, UK). Fluorescence from the sperm posterior head/neck was background-corrected and normalized to give % change in intensity (Nash *et al*, 2010).

To assess $[Ca^{2+}]_i$ oscillations, paired experiments were conducted using cells from the same sample exposed to hFF or P4. Traces were examined by eye for the occurrence of cyclical $[Ca^{2+}]_i$ oscillations following the initial $[Ca^{2+}]_i$ transient.

Assessment of sperm function

Viscous media penetration test and CASA were carried out as previously described (Alasmari *et al,* 2013a; Williams *et al.,* 2015).

Ethical approval

Written consent was obtained from each IVF patient in accordance with the Human Fertilization and Embryology Authority (HFEA) Code of Practice (V8) under local ethics approval (13/ES/0091) from the East of Scotland Research Ethics Service REC1. Similarly, volunteer sperm donors were recruited under the same ethical approval in Dundee and ethical approval number ERN-12-0570R at the University of Birmingham.

Data analysis

Data were analyzed using Microsoft Excel™ or GraphPad Prism™ (version 5, GraphPad Software Inc.). Statistical significance was determined using Student's paired/unpaired t-test or analysis of variance (ANOVA) and adjusted using the Holm-Bonferroni correction (Gaetano, 2013) as appropriate. Percentage data were ArcSine converted before testing. Data are presented as mean±SEM with $P<0.05$ indicative of statistical significance. All sets of experimental repeats include sperm and hFF samples from more than one donor. Values of 'n' for patch clamp experiments are given in Tables 1-6 and show the number of cells

patched. Unless stated otherwise, the values of 'n' for $[Ca^{2+}]_i$ and motility assessments provided in text and figure legends show the number of experiments used for statistical analysis.

Results

hFF and ion channel currents

Effects of hFF on CatSper current

Since P4 is an activator of CatSper, we first used whole-cell patch clamp electrophysiology to examine the effect of hFF on CatSper currents ($I_{CatSper}$). hFF (diluted 1%) potently potentiated both inward and outward monovalent CatSper currents (Fig 1a,b; Table 1; P<0.01). P4 potentiates CatSper currents primarily by shifting channel activation to more negative voltages (Lishko *et al.,* 2011). Assessment of voltage-sensitivity of CatSper activation (using Ba^{2+} tail currents) showed that 1% hFF shifted the G-V curve to more negative voltages (Fig 1c), significantly changing the V_{50} (table 2; *P*<0.001). Similarly, 500nM P4 caused a negative shift of the CatSper G-V curve (Fig. 1d, Table 2; *P*<0.01) as demonstrated previously (Lishko *et al.,* 2011).

Effects of steroid stripping on hFF-stimulation of CatSper currents

hFF contains, in addition to P4, prostaglandins (Lishko *et al*, 2011) and other ligands that may influence $[Ca^{2+}]_i$ signalling. To examine the effect of depleting lipid derived agonists (steroids and prostaglandins), samples of FF were 'stripped' using dextran-coated charcoal. This procedure reduced [P4] by 98.6±0.13% (n=31; suppl. Fig 1). Spermatozoa were exposed first to 1% charcoal-stripped hFF (ShFF) then to 1% hFF from the same sample incubated similarly but without dextran-coated charcoal. ShFF failed to stimulate $I_{CatSper}$, (both inward and outward currents were smaller; Fig 2a; Table 3; P<0.05), but subsequent application of hFF potentiated both inward and outward currents amplitude (Fig 2a; Table 3; P=0.05; P<0.01 respectively). Similarly, when tail currents were used to assess CatSper activation, hFF but not ShFF shifted voltage sensitivity to less positive potentials (Fig 2b; Table 4; P<0.01). The concentration of P4 present in 1% ShFF is 2-3 nM, which has been reported to increase CatSper currents (Lishko et al, 2011). We therefore assessed whether we could detect this effect under our recording conditions. Both using standard Cs^+ saline

recording (P4 added directly to Cs^+ saline before perfusion of the recording chamber) and also when progesterone was first dissolved in a mixture of 1% standard bath solution (containing 2 mM Ca^{2+}, 0.7 mM Mg^{2+}) and 99% Cs^+ saline (to mimic ionic conditions in ShFF experiments), superfusion of sperm with 2 nM P4 significantly increased both outward and inward currents (Table 5). Finally, we increased the concentrations of divalent chelators (EGTA, EDTA) in our Cs^+ recording saline to 9 mM of each to chelate any residual Ca^{2+} and Mg^{2+} from the hFF. Under these conditions, we observed a response to ShFF in some cells (Suppl. Fig 2) and mean inward and outward currents were increased, but this effect was not significant (Table 5; $P>0.1$). Examination of [P4] concentrations showed that detectable effects of ShFF occurred only with with hFF samples where the [P4] was unusually high (Suppl. Fig 2).

Effect of hFF on membrane potential and K^+ current

To investigate the possible effects of hFF on membrane potential, cells were challenged with hFF (1%; 10% and 50% dilution) under quasi-physiological conditions (see methods). hFF did not alter resting membrane potential or outward membrane conductance indicating that hFF did not modulate/suppress K^+ channel function at these dilutions (Fig. 3; Table 6). Stimulation with P4 significantly depolarised membrane potential and reduced conductance at 30 μM but at 10 μM effects were not significant (Table 6).

hFF and sperm $[Ca^{2+}]i$

hFF-induced $[Ca^{2+}]_i$ signals in sperm populations

In agreement with previous reports hFF, similarly to P4, caused a dose-dependent, biphasic elevation of $[Ca^{2+}]_i$ consisting of a transient followed by a plateau (Fig. 4a,b). Using hFF samples in which the P4 concentration had been determined we directly compared $[Ca^{2+}]_i$ signals induced by hFF (diluted to 10%, 1%, 0.1% and 0.01%) and by an equivalent concentration of P4 alone (using aliquots of sperm from the same batch of Fluo-4 loaded

sperm cells run in parallel). Analysis of these data pairs showed that at low concentrations of hFF (0.01-1%) the amplitudes of signals induced by hFF and P4 were similar (Fig 4c,d). However, at the highest hFF concentration (10%) the $[Ca^{2+}]_i$ plateau induced by hFF (assessed 10 min after stimulus application) was consistently greater than that induced by an equivalent concentration of P4 (mean amplitude sample ratio=1.6±0.1; Fig 4d red symbols; *P*=0.001; n= 7;). In cells stimulated with 10% hFF the $[Ca^{2+}]_i$ transient also appeared longer than in cells from the same the same batch of Fluo-4 loaded sperm cells stimulated with an equivalent concentration of P4 (Fig 4a,b). Assessment of the transient 'half-duration' (latency from midpoint of the rising phase to midpoint of decay) confirmed that this was the case (*P*=0.0005; n=7).

hFF-induced $[Ca^{2+}]_i$ signals in single cells

Similarly to population measurements, single cell imaging of $[Ca^{2+}]_i$ at the posterior head/neck showed transient responses in the vast majority of cells exposed to hFF, which resembled those induced by P4 alone (Fig 5a,b). In P4-stimulated cells the initial Ca^{2+} transient was often followed by $[Ca^{2+}]_i$ oscillations (not synchronised and therefore detectable only in single cell records; Kirkman-Brown *et al*, 2004; Harper *et al*, 2004; Fig 5a). In cells stimulated with hFF, oscillations were observed but their occurrence was markedly concentration dependent. 1% hFF, similarly to 300 nM P4 (estimated equivalent [P4]) induced oscillations in ≈25% of cells (Fig 5c; P=0.47; n=10). However, whereas 3μM P4 was similarly effective (19% of cells; e.g. Fig 5a), 10% hFF induced oscillations in only 4% of cells (Fig 5b,d,e; P=0.002, n=10).

$[Ca^{2+}]_i$ responses to charcoal-stripped hFF

Since the ability of 1% hFF to potentiate CatSper currents was removed by stripping of steroids/prostaglandins with dextran-treated charcoal (Fig 2a), we examined whether hFF-induced $[Ca^{2+}]_i$ signals were similarly affected. Surprisingly, $[Ca^{2+}]_i$ responses were always

detected in cell populations stimulated with 1% ShFF, with the $[Ca^{2+}]_i$ transient amplitude being 36.8±1.8% of that in the parallel control (1% hFF) experiments (Fig 6a; Suppl. Fig 3; $P=3.2*10^{-12}$; n=21). In 28 experiments where parallel recordings were carried out with ShFF and [P4] equivalent to that in ShFF, $[Ca^{2+}]_i$ transient amplitudes were similar (P=0.14). However, the subsequent $[Ca^{2+}]_i$ 'plateau' was significantly greater with ShFF (43±9% for the period 30-240 s post-stimulus; $P=4.8*10^{-6}$; Fig 6b). The 'non-P4' component, isolated by subtraction of traces (ShFF-equivalent [P4]), showed activation later than the $[Ca^{2+}]_i$ signal induced by P4 and peaked 60-100 s after stimulation (Fig 6b).

In single cell imaging experiments where immobilised sperm were superfused with 1% ShFF or equivalent [P4], cells failed to generate the $[Ca^{2+}]_i$ transient seen in the equivalent population experiments and instead we observed a slow $[Ca^{2+}]_i$ ramp (Fig 7 a). This reduced efficacy of stimuli delivered by perfusion is due to binding of progesterone to the perfusion tubing (see discussion). The mean increase in $[Ca^{2+}]_i$ was greater in the ShFF-treated cells, but the effect was highly variable and the difference was not significant (Fig 7a,b; P=0.14). After 5-10 min exposure to 1% ShFF or equivalent [P4], oscillations developed in approximately 20% of cells (Fig 7c,d), resembling the response to P4 ramps (Harper *et al*, 2004).

Effects of P4 desensitisation on $[Ca^{2+}]_i$ response to hFF

Component(s) of hFF not removed by charcoal stripping contribute significantly to late/sustained components of hFF-induced $[Ca^{2+}]_i$ signals (Fig 6b). To further investigate this, we tested the effect of desensitisation of the P4 response on the $[Ca^{2+}]_i$ signal induced by hFF. As previously described (Aitken *et al*, 1996; Schaefer *et al*, 1998), when sperm were pre-stimulated with 3 μM P4 complete desensitisation occurred (Fig 6c). However, when P4-desensitised cells were stimulated with hFF there was a clear response (13.8±0.9% of that evoked by the preceding, desensitising P4 stimulus; $P=3.2*10^{-5}$ compared to second stimulation with 3 μM P4; n=10; Fig 6d,f). Since P4 and prostaglandins stimulate CatSper by

separate mechanisms that do not cross-desensitise (Schaefer *et al*, 1998), this could reflect a small contribution of prostaglandins to the hFF-induced $[Ca^{2+}]_i$ transient. We therefore investigated whether the desensitisation-resistant component of hFF was removed by charcoal stripping. In six experiments ShFF always induced a $[Ca^{2+}]_i$ response (11.5±2.0% of that evoked by the desensitising 3 μM P4 stimulus) which was significantly greater ($P=2.8*10^{-5}$) than the response to a second stimulation with 3 μM P4; Fig 6e,f).

hFF and sperm motility

To assess functional effects of hFF on motility, we assessed hyperactivation and penetration into viscous medium. Both hFF (1%, 10%) and equivalent [P4] significantly stimulated penetration ($P<0.005$; n=6) but the effect of hFF was significantly greater (Suppl Fig 4). hFF also induced a dose-dependent increase in hyperactivation, whereas the effect of equivalent [P4] was small and not significant ($P<0.05$; n=6; Suppl Fig 5a). Analysis of the kinematics (VCL, ALH, LIN) indicated this effect of hFF was primarily due to increased curvilinear velocity ($P<0.01$; Suppl Fig 5b).

Discussion

Our findings clearly show that CatSper is activated by hFF and that this is the primary contribution to hFF-induced $[Ca^{2+}]_i$ signalling in human sperm. However, by direct comparison of responses to hFF and to equivalent [P4], charcoal-stripping of hFF and desensitisation of the P4 response, we identified clear differences between the responses to hFF and to P4 which indicate that regulation of $[Ca^{2+}]_i$ by hFF is considerably more complex than simple activation of CatSper.

Modulation of ion channel activity and $[Ca^{2+}]_i$ by hFF

The electrophysiological data clearly show that hFF, similarly to P4, enhances CatSper currents and shifts CatSper voltage sensitivity to less positive potentials (Fig. 1; Tables 1 and 2). Mannowetz and colleagues (2013) reported that high concentrations of P4 also inhibit KSper (I_{50}≈7 μM), depolarising the membrane potential and potentially augmenting activation of CatSper. We could detect no effect of hFF on conductance or resting Vm even with 50% hFF (containing 10-15 μM progesterone; Fig. 3). In positive control experiments with P4, we saw no significant effect with 10 μM but clear inhibition of conductance with 30 μM P4 (equivalent [P4] to 100% hFF; Table 6). Thus effects of hFF on KSper may occur at higher concentrations than those used in this study, potentially in very close proximity to the oocyte.

$[Ca^{2+}]_i$ signals induced by hFF

$[Ca^{2+}]_i$ transients induced by treatment of human sperm suspensions with hFF were similar in amplitude to those induced by an equivalent [P4] and activation of CatSper by P4 is apparently the primary determinant of this response. However, when sperm were stimulated with 10% hFF, the sustained $[Ca^{2+}]_i$ signal was >60% greater than that induced by an equivalent [P4]. Recently Mannowetz and colleagues reported that endogenous steroids

other than P4 also modulate activity of CatSper in human sperm. 17beta-estradiol and hydrocortisone, both present in hFF, inhibit the stimulatory action of 1 μM P4 (IC_{50} = 833 nM and 153 nM respectively) and their actions might be expected to result in a response to hFF smaller than that of an equivalent [P4] (Mannowetz et al. 2017). The concentration of P4 in hFF (typically >30 μM) may be high enough for these inhibitory effects to be outcompeted (Mannowetz et al., 2017), but the stimulatory effects observed with 10% hFF indicate that other components of hFF, when present at sufficient concentration, either activate (or suppress inactivation of) CatSper or activate other $[Ca^{2+}]_i$ signalling components that contribute to the sustained $[Ca^{2+}]_i$ signal (see below).

Single cell $[Ca^{2+}]_i$ responses to P4 resemble population responses (transient and plateau phase; Kirkman-Brown *et al*, 2000) but some cells then generate repetitive oscillations (Fig 5a; Harper *et al*, 2004; Kirkman-Brown *et al*, 2004) that may regulate motility and/or acrosome reaction (Harper *et al*, 2004; Bedu-Addo *et al*, 2007; Alasmari *et al*, 2013; Sánchez-Cárdenas *et al.,* 2014). In paired experiments, 1% hFF and 300 nM progesterone (equivalent concentration) both induced repetitive $[Ca^{2+}]_i$ oscillations in approximately 20% of cells (Fig 5c), while 1% ShFF and matched [P4], (after a latency of 5-10 min) were similarly effective. However, when challenged with 10% hFF, just 4% of sperm generated oscillations compared to 19% with 3 μM (equivalent) progesterone (Figs 5d,e), again suggesting that substances within hFF modulate human sperm Ca^{2+} signalling by mechanisms other than CatSper activation. Darszon and colleagues assessed $[Ca^{2+}]_i$ and acrosomal status and concluded that calcium oscillations suppress the acrosome reaction (Sánchez-Cárdenas *et al.,* 2014). If the sperm encounters high concentrations of hFF on approaching the cumulus-oocyte complex, this may inhibit $[Ca^{2+}]_i$ calcium oscillations and 'disinhibit' acrosome reaction.

Charcoal stripping and evidence for presence of an active 'cocktail' in hFF

To further investigate the relative contributions of P4 and other components to the observed effects of hFF, samples were treated with dextran-coated charcoal to 'strip' lipid-derived agonists (steroids/prostaglandins), removing almost 99% of P4. In fluorimetric experiments the $[Ca^{2+}]_i$ transients evoked by ShFF were consistent with a response to the residual P4, but the subsequent sustained $[Ca^{2+}]_i$ signal was significantly greater (Fig 6b). Furthermore, when we pretreated sperm with P4 to desensitise the P4-induced $[Ca^{2+}]_i$ signal (Aitken et al, 1996; Schaefer et al, 1998), we found that a small, sustained response persisted whether stimulating with hFF or ShFF (Fig 6c-f). These observations indicate that hFF includes factors that contribute to and/or regulate Ca^{2+}-signalling that are resistant to stripping with dextran-coated charcoal and are therefore unlikely to be steroids or prostaglandins.

Though the $[Ca^{2+}]_i$ transient induced by 1% ShFF appeared to be primarily a response to residual P4 (see above), when we investigated effects on patch-clamped sperm we observed no stimulation of CatSper currents, suggesting that other components of hFF modulate the response to P4. Two factors should be taken into account in interpreting these data. Firstly, P4 applied by perfusion binds to the plastic perfusion tubing (as evidenced by reduced efficacy of P4 in our imaging experiments and also observed by others; T Strunker personal communication), thus comparison with fluorimetric $[Ca^{2+}]_i$ assessment, where direct addition of ShFF to the well induced a significant $[Ca^{2+}]_i$ response (Fig 6), is misleading. This is particularly significant since the inhibitory of hFF was masked at higher [P4] (Suppl Fig 2). Secondly, divalent cations in hFF (2.2 mM Ca, 0.68 mM Mg; Chong et al, 1977; Ng et al, 1987) may be inadequately buffered, masking any stimulatory effect (IC_{50} for Ca^{2+} ~100nM; Lishko et al., 2011). However, (i) in 'supplemented' control experiments where Ca^{2+}/Mg^{2+} was present at equivalent levels to that in ShFF, responses to 2 nM P4 resembled those seen in 'divalent-free' controls (Table 5) and (ii) increased divalent cation buffering (calculated $[Ca^{2+}]+[Mg^{2+}]$ with 1% ShFF=2.14 nM) failed to rescue stimulation of CatSper currents to ShFF (Table 5; Suppl. Fig 2). We conclude that residual P4 in 1% ShFF (a [P4] sufficient to activate CatSper in 'supplemented' control recordings (Table 5)), when delivered by perfusion tubing, failed significantly to potentiate CatSper current and propose that other

substances present in hFF, resistant to charcoal stripping, partially inhibit the response of the channel to low (nM) concentrations of progesterone. Thus the slowly-developing ShFF-induced $[Ca^{2+}]_i$ ramp seen in imaging experiments (Fig 7a,c) is apparently induced independently of CatSper activation. The complexity of hFF, even after charcoal stripping, is such that discussion of the nature of such an effect can only be speculative. However, the effects on human sperm $[Ca^{2+}]_i$ of kisspeptin (Pinto *et al,* 2012) and leutenising hormone (López-Torres *et al,* 2017), suggest that activation G-protein coupled receptors by protein or peptide hormones might exert such an effect.

Functional effect of hFF

We reported previously that stimulation of penetration into artificial mucus was mediated by activation of CatSper whereas manoeuvres designed to mobilise stored Ca^{2+} strongly stimulate hyperactivation (Alasmari *et al.,*2013). Analysis of motility showed that hFF potently stimulated penetration into viscous medium and also induced a small but significant increase in hyperactivation. Both these effects exceeded those of equivalent [P4], consistent with the significantly greater effects of hFF on $[Ca^{2+}]_i$ signalling and the likelihood that hFF recruits stored Ca^{2+} in addition to activation of CatSper . These data suggest that stimulation by hFF may contribute significantly to sperm penetration of the cumulus matrix.

In conclusion, the assumption that hFF stimulates CatSper similarly to progesterone is correct but a comparison of responses to hFF and P4, particularly at high hFF concentrations or using charcoal-stripped samples, reveal supplementary and modulatory effects of other, unidentified components of hFF. Thus the mixtures/fluids that the sperm encounters in vivo appear to have subtly different and more complex effects than those observed in single agonist, in-vitro experiments. To understand modulation of sperm function by the reproductive tract, we will need to study more physiological systems.

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Role of the authors

S.G.B. performed patch clamp experiments. S.C and M.K. performed fluorimetry/imaging and sperm function experiments. M.R. and S.M.d.S were involved in recruiting patients and seeking informed consent. E.D. processed the follicular fluid samples. S.G.B, and S.J.P. performed analysis of electrophysiological data. S.J.P., S.G.B., M.R, S.M.d.S and C.L.R.B. designed the study. S.J.P., S.G.B and C.L.R.B. obtained funding for the study. The manuscript was drafted by C.L.R.B, S.G.B and S.J.P. All authors contributed to the construction, writing and approval the final manuscript.

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Conflict of interest

C.L.R.B. is EIC of MHR and Chair of the WHO ESG on Diagnosis of Male infertility. The remaining authors have no conflict of interest.

Figures

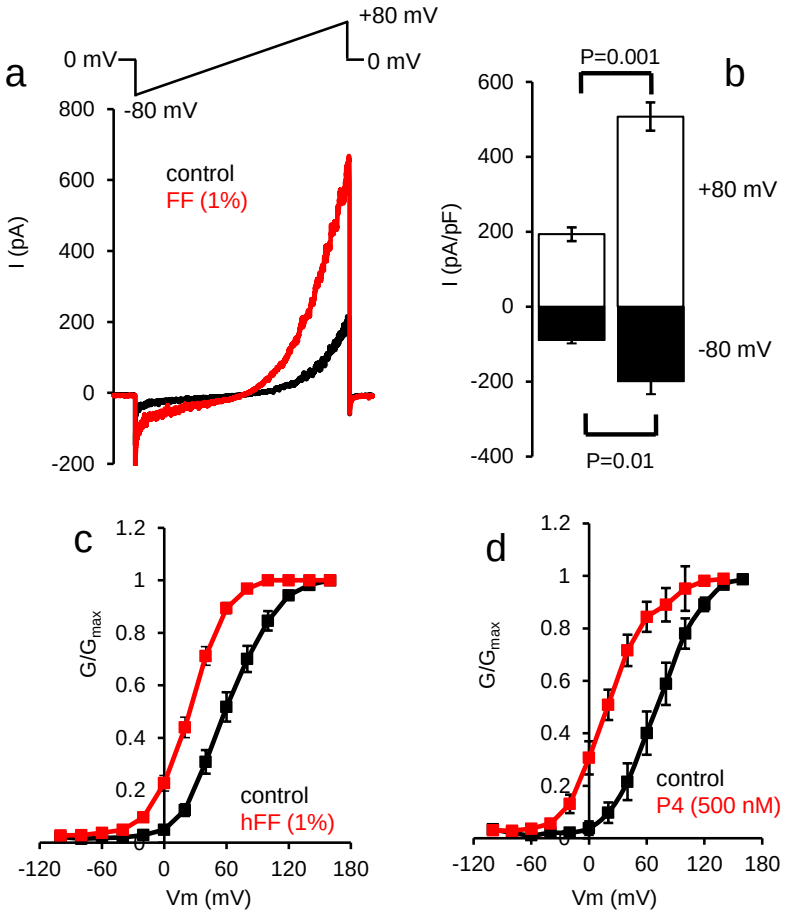

Figure 1. Human follicular fluid potentiates CatSper currents and shifts the voltage sensitivity to less depolarised potentials. **a:** Representative Cs^+-mediated CatSper current in the absence (black) and presence (red) of 1% hFF. Voltage protocol imposed is shown above. **b**: Mean amplitudes (±SEM) of CatSper currents recorded in the absence (left) and presence (right) of 1% hFF (n = 8 hFF samples). White bars show inward current (-80mV), black bars show outward currents (80mV; n=13). **c** and **d** show conductance-voltage (G-V) relationships for Ba^{2+}-mediated CatSper tail currents in the absence and presence of 1% hFF (c, n = 12) and 500nM P4 (d, n = 4)

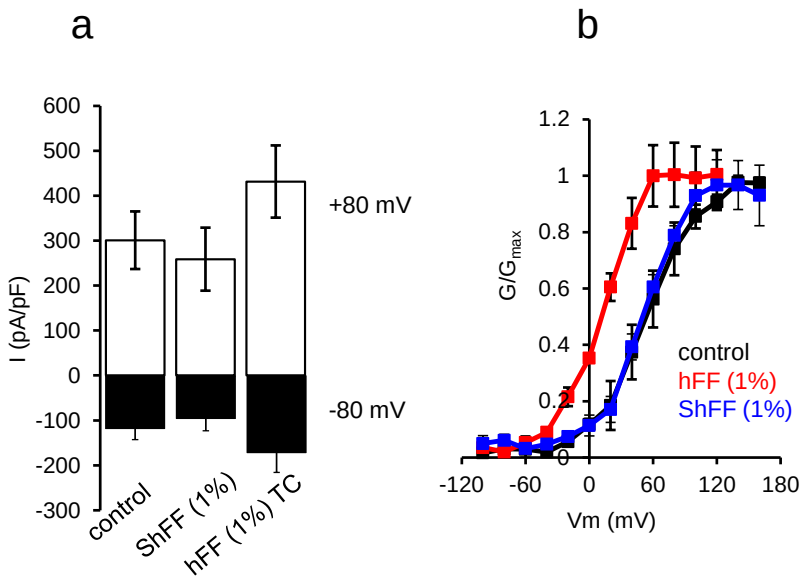

Figure 2. Charcoal-stripped hFF (ShFF) does not potentiate CatSper currents. **a.** Mean±SEM inward CatSper currents at -80mV (black) and outward currents at 80mV (white; n = 8 cells) under control conditions, in presence of 1% stripped hFF (ShFF) and 1% time-control (hFF; 7FF samples). ShFF reduced current amplitude ($P<0.05$) but subsequent application of control hFF potentiated both inward and outward currents ($P<0.01$). **b:** 1% stripped hFF (ShFF) failed to alter CatSper voltage sensitivity but subsequent application of control follicular fluid (hFF) caused a significant leftward shift in voltage sensitivity (V_{50} $P<0.01$ compared to control and ShFF). n = 4 cells, 4 hFF.

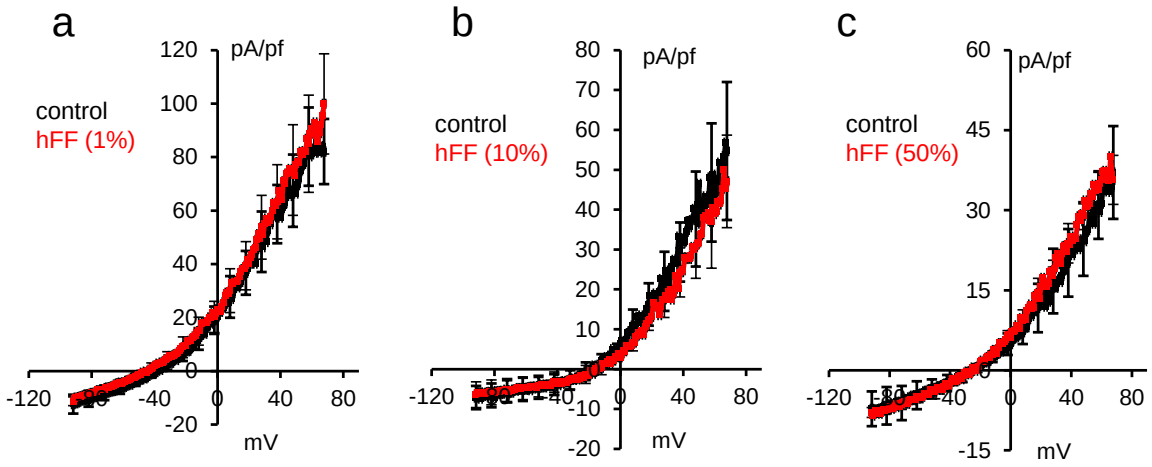

Figure 3. hFF does not affect K^+ channel activity recorded under quasi-physiological conditions. In each panel, black trace shows mean (±SEM) control current and red trace shows mean (±SEM) of currents recorded after exposure to hFF. (**a**) 1% hFF; n = 6 cells, 4 hFF tested; (**b**) 10% hFF. n = 3 cells, 3 hFF tested; (**c**) 50% hFF. n = 3 cells, 3 hFF.

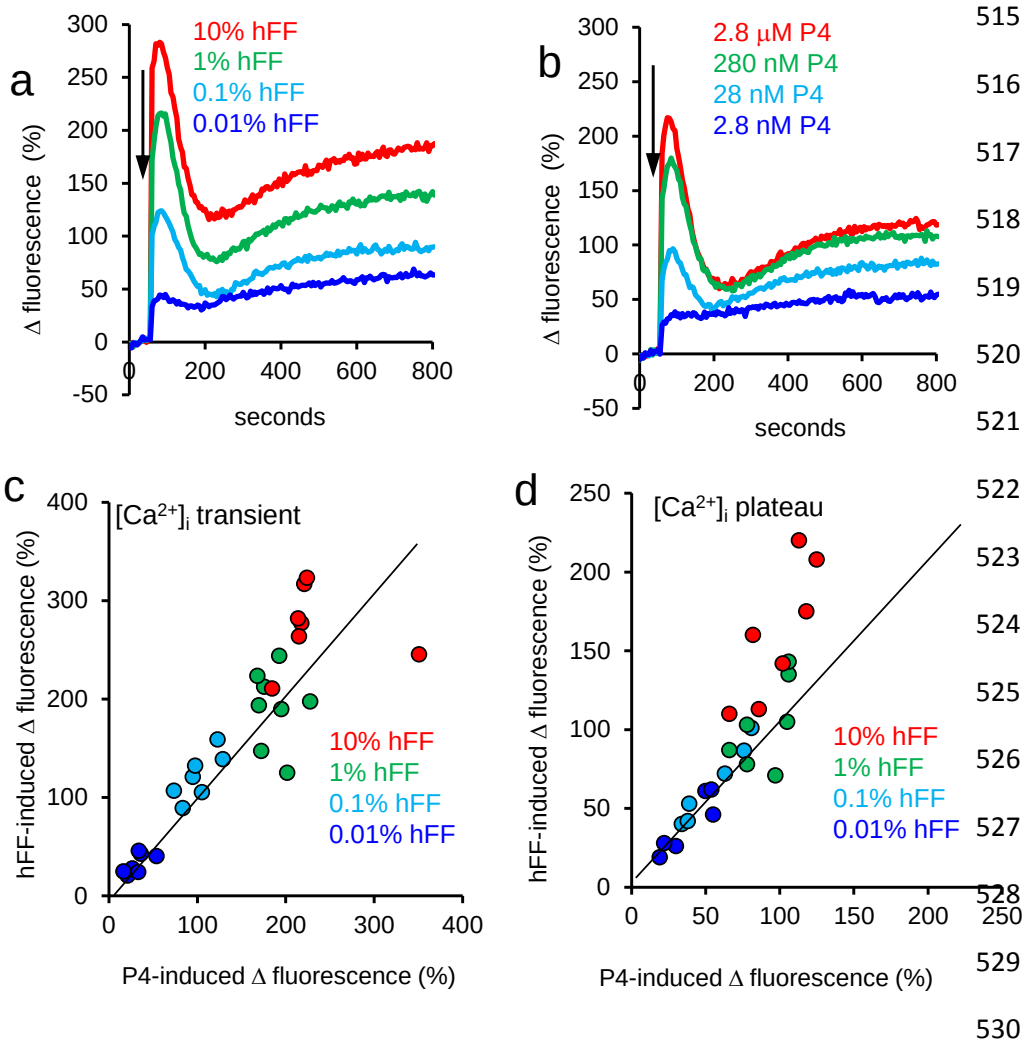

Figure 4. $[Ca^{2+}]_i$ responses to hFF and progesterone are similar but not identical. **a** and **b** show an example of $[Ca^{2+}]_i$ responses induced in paired experiments using (**a**) four dilutions of hFF (dark blue=0.01%, light blue=0.1%, green=1%, red=10%) and (**b**) P4 at concentrations equivalent to those in the hFF dilutions (dark blue=2.8 nM, light blue=28 nM, green=280 nM, red=2.8 μM). **c** and **d** show relative amplitudes (Δ fluorescence (%)) of the $[Ca^{2+}]_i$ transients (c) and $[Ca^{2+}]_i$ plateau (d, assessed 10 min post-stimulation) induced in seven sets of experiments, each using four dilutions of hFF (0.01%=dark blue, 0.1%=light blue, 1%=green, 10% =red) and P4 at concentrations equivalent to those in the hFF dilutions. Six different hFF samples were used. Line in each graph marks position of equal response amplitude. At the highest hFF concentration used (10%; red symbols), plateau responses are consistently larger than those of equivalent [P4] (P=0.001).

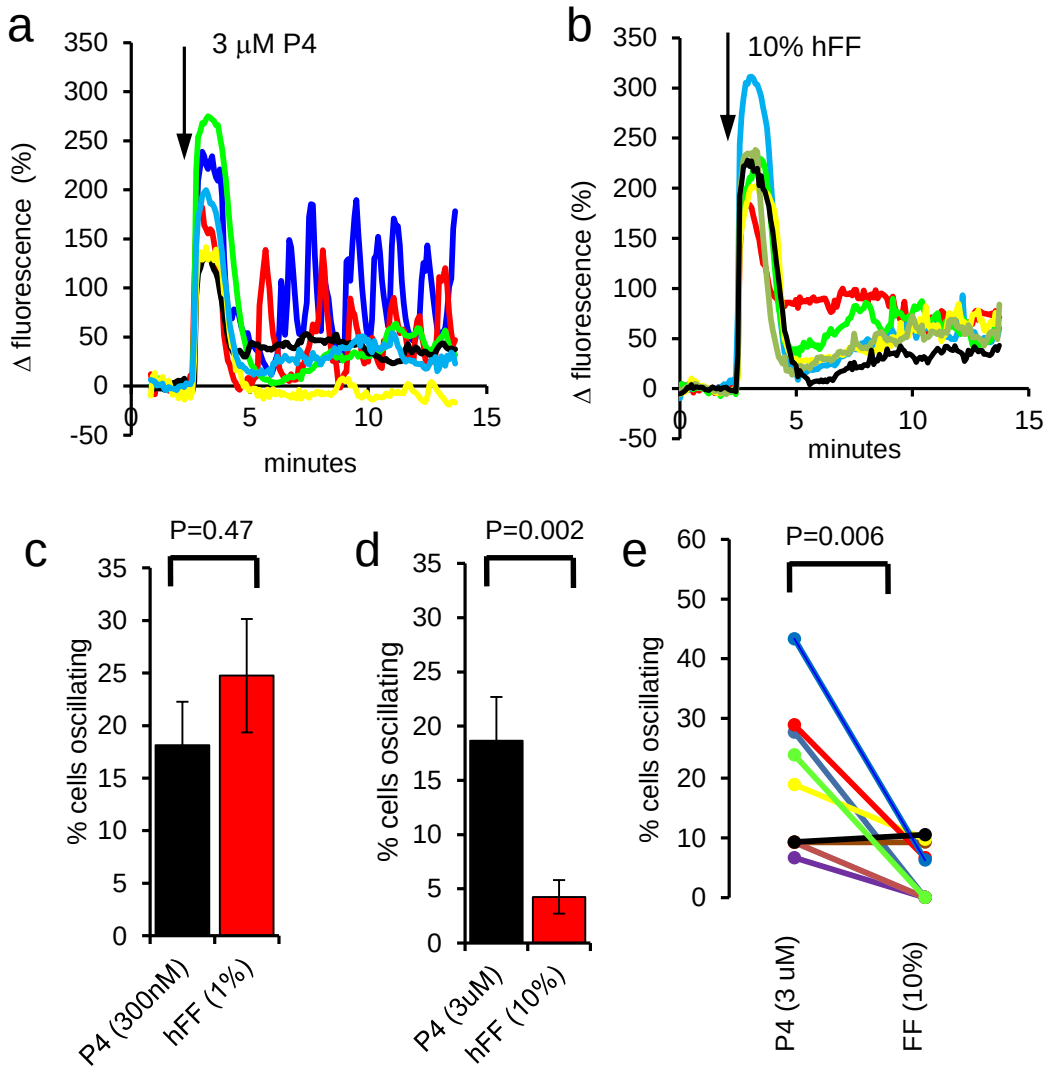

Figure 5. Single cell $[Ca^{2+}]$i responses to hFF. **a** and **b** show examples of $[Ca^{2+}]_i$ responses in a paired experiment in which cells from the same sample were exposed to 3 μM P4 (a) and 10% hFF (b). Panel **c** shows mean±SEM percentage of cells in which $[Ca^{2+}]_i$ oscillations occured after stimulation of sperm (from the same sample) with 300 nM P4 (black) or 1% hFF (red); n=10 paired experiments. Panel **d** shows results from a similar series of 10 paired assessments using 3 μM P4 (black) and10% hFF (red; P<0.01). **e** shows data from the 3 μM P4/10% hFF experiments (panel d with paired experiments joined and shown in same colour.

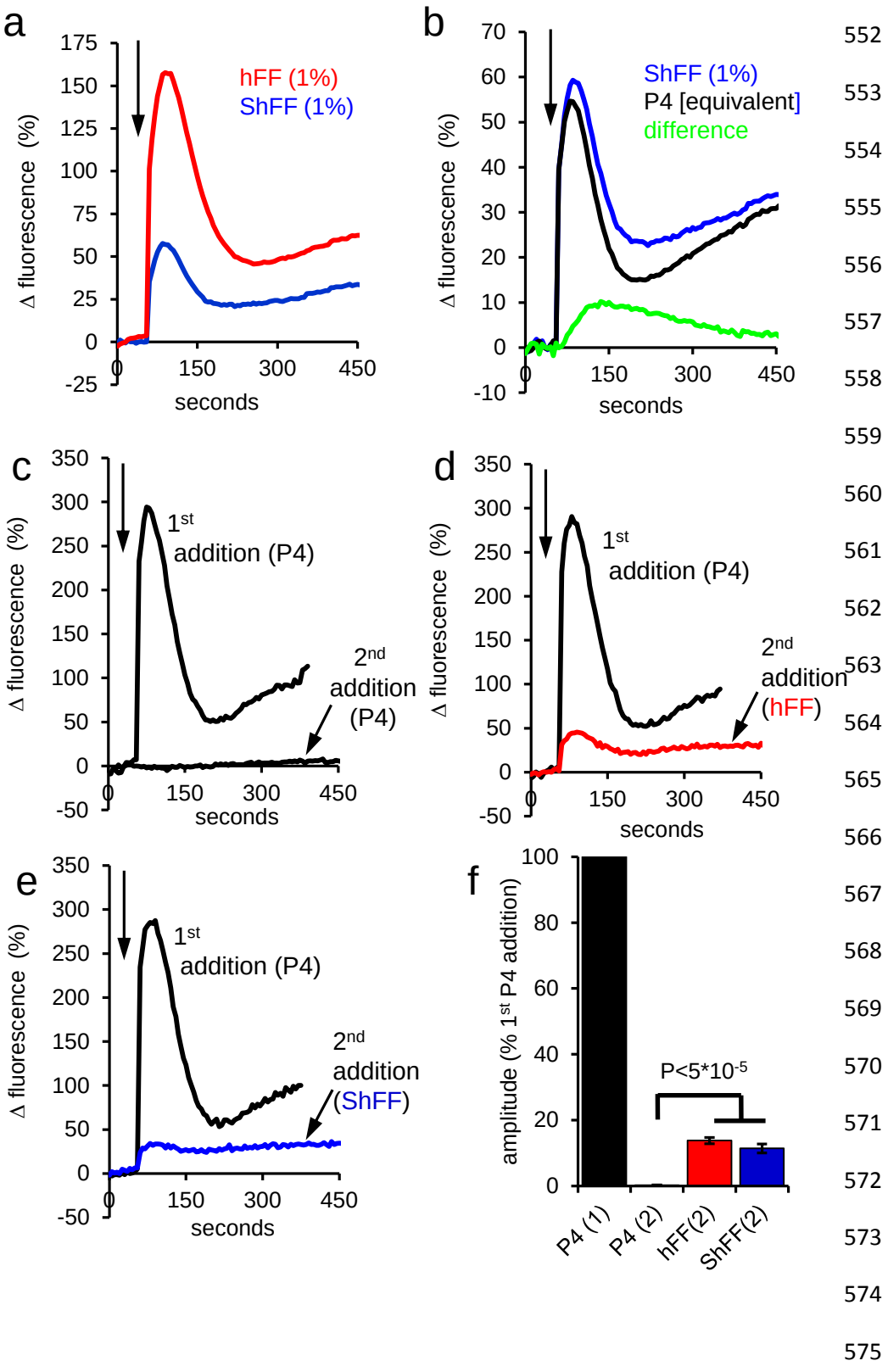

Figure 6. Components of the hFF-induced $[Ca^{2+}]_i$ signal are resistant to P4 desensitisation and charcoal stripping. **a:** Mean $[Ca^{2+}]_i$ response from 21 experiments (5 different hFF used) in which aliquots from the same sperm sample treated with 1% hFF (red) and 1% ShFF (blue). **b**: Mean $[Ca^{2+}]_i$ response from 28 paired experiments (9 different hFF used) in which

aliquots from the same sperm sample were treated with 1% ShFF (blue) or the equivalent concentration of P4 (black). Green shows the 'non-P4' component obtained by subtraction of traces. **c** to **e:** Examples of $[Ca^{2+}]_i$ responses in three parallel recordings where sperm were first stimulated with 3 μM P4 (1st addition-black traces) then, after an interval of 5 min, exposed to either a second 3 μM P4 stimulus (6 μM P4 total; **c**, 2nd addition-black trace), 1% hFF (**d**, 2nd addition-red trace) or 1% ShFF (**e**, 2nd addition-blue trace). In each panel the responses to the first (3 μM P4) stimulus and to the second stimulus are overlaid (arrow at top left shows time of additions). When 3 μM P4 was followed by a second P4 stimulus the second response was negligible (desensitisation). However, when either 1% hFF or 1% ShFF was added as the second stimulus there was a small transient followed by a plateau. **f**: Mean amplitude (±SEM) of $[Ca^{2+}]_i$ transients evoked by the first 3 μM P4 stimulus (P4(1) black) and by a second addition of P4 (P4(2); n=7; black), hFF (hFF(2); n=10; red) or stripped hFF (ShFF(2); n=6; blue). All amplitudes are normalised to that induced by the first P4 addition in that experiment.

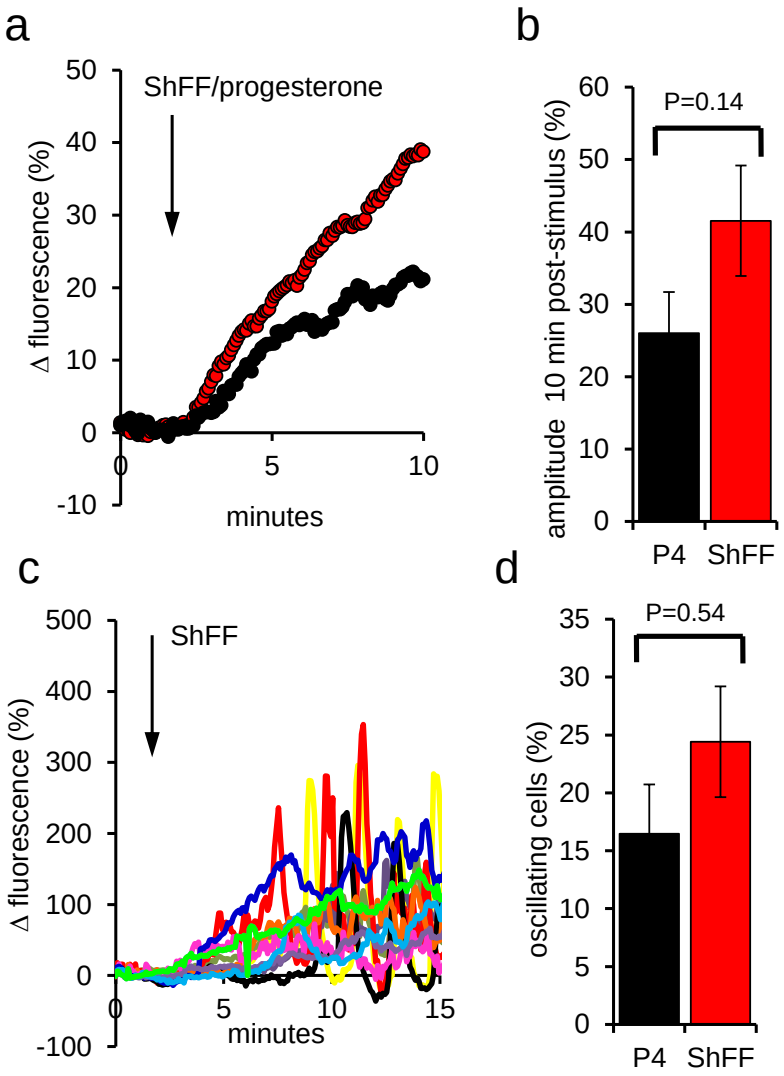

Figure 7. Single cell $[Ca^{2+}]_i$ responses to 1% ShFF. **a** shows mean responses to 1% ShFF (red; n=10 experiments; 826 cells) and equivalent [P4] (black; n=6 experiments; 447 cells), arrow marks stimulus addition. Both stimuli induced a $[Ca^{2+}]_i$ ramp rather than the biphasic response seen in fluorimetric experiments. **b** shows mean (±SEM) amplitude (Δ fluorescence) 9 min after stimulus application. **c** shows responses of 12 individual cells stimulated with ShFF, arrow marks stimulus addition. Red, yellow and black cells developed oscillations 5-10 min after stimulation. **d** shows proportions of cells generating $[Ca^{2+}]_i$ oscillations after stimulation with 1% ShFF (red; n=10 experiments; 826 cells) or equivalent [P4] (black; n=6 experiments; 447 cells).

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Supplementary File 1

A. Salines

HEPES buffered saline solution consisted of (in mM): $CaCl_2$, 1.8; KCl, 5.4; $MgSO_4 7H_2O$, 0.8; NaCl, 116.4; NaH_2PO_4, 1; D-glucose, 5.5; sodium pyruvate, 2.73; sodium lactate, 41.75; HEPES, 25; BSA, 0.3% (w/v); pH adjusted to 7.4 using NaOH.

Bicarbonate buffered capacitating medium consisted of (in mM): $CaCl_2$, 1.8; KCl, 5.4; $MgSO_4 7H_20$, 0.8; NaCl, 116.4; NaH_2PO_4 1; D-glucose, 5.5; sodium pyruvate, 2.73; sodium lactate, 41.75; sodium bicarbonate, 26; BSA, 0.3% (w/v); pH adjusted to 7.4 using NaOH.

Supplemented Earle's balanced salt solution (sEBSS) contained (in mM): NaH_2PO_4, 1.02; KCl, 5.4; $MgSO_4$, 0.811; D-glucose, 5.5; Na pyruvate, 2.5; Na lactate, 19.0; $CaCl_2$,1.8; $NaHCO_3$,,25.0; NaCl, 118.4 and HEPES, 15 (pH 7.4), supplemented with 0.3% (w/v) BSA.

Standard bath solution consisted of (in mM): NaCl, 135; KCl, 5; $CaCl_2$, 2; $MgSO_4$, 1; HEPES, 20; Glucose, 5; Na pyruvate, 1; Lactic acid, 10; pH adjusted to 7.4 with NaOH which brought $[Na^+]$ to 154 mM.

Standard pipette solution consisted of (mM): NaCl, 10; KCl, 18; K gluconate, 92; $MgCl_2$, 0.5, $CaCl_2$, 0.6; EGTA, 1; HEPES, 10; pH adjusted to 7.4 using KOH which brought $[K^+]$ to 114 mM and $[Ca^{2+}]_i$ to 0.1 µM. $[Ca^{2+}]$ in buffered solutions was calculated using MaxChelator (Maxchelator.stanford.edu).

Cs^+-based pipette solution contained Cs-methanesulphonate, 130 mM; HEPES, 40 mM; Tris-HCl, 1 mM; EGTA, 3 mM; EDTA, 3 mM, pH adjusted to 7.4 with CsOH.

Cs^+-based bath solution contained Cs-methanesulphonate, 140 mM; HEPES, 40 mM; EGTA, 3 mM; EDTA, 3 mM pH adjusted to 7.4 with CsOH.

CatSper tail current (Ba^{2+}) bath solution contained 10 mM $BaCl_2$, 140 mM NMDG, 100 mM HEPES, pH 7.4 with $HMeSO_3$.

CatSper tail current pipette solution contained 145 mM NMDG, 100 mM HEPES, 10 mM BAPTA, 0.5 mM TrisHCl, pH 7.4 with $HMeSO_3$.

B. Dextran-coated charcoal solution

Dextran-coated charcoal was prepared by mixing 4C charcoal (0.25% w/v) and dextran T-70 (0.0025% w/v) in a solution containing 1.5mM $MgCl_2$, 10mM HEPES and 0.25M sucrose, pH 7.4 with NaOH and kept at 4°C. A volume of dextran-coated charcoal mixture double that of the volume of hFF to be steroid stripped was centrifuged to pellet the charcoal. The supernatant was removed and replaced with hFF. The charcoal was mixed with the hFF and incubated overnight at 4°C. To remove the charcoal, the hFF/charcoal mix was centrifuged at 1000g for 5 minutes and the hFF was removed and filtered using a 0.22 µm filter. A paired sample of the same hFF not incubated with dextran-coated charcoal was also left overnight at 4°C (referred to as time control).

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Tables

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Table 1. Effect of hFF on monovalent (Cs+) CatSper current amplitude

		-80mV			80 mV		
stimulus	n	control (pA)	treated (pA)	P	control (pA)	treated (pA)	P
1%hFF	13	-89.4±8.3	-199±33.6	0.01	193.3±18.4	507.3±37.7	0.001

Table 2. Effect of hFF on CatSper V50

stimulus	n	control (mV)	treated (mV)	P
1% hFF	12	61.8±5.2	25.1±2.7	<0.001
500 nM P4	4	71.7±8.0	15.1±6.1	<0.01

Table 3. Effect of stripped hFF (ShFF) on monovalent (Cs+) CatSper current amplitude

		-80mV			80 mV		
stimulus	n	control (pA)	treated (pA)	P	control (pA)	treated (pA)	P
1%ShFF	8	-130.3±28.9	-105.6±32.2	0.013	300.8±68.6	258.7±74.9	0.07
1%hFF	8	-130.3±28.9	-189.9±52.0	0.05	300.8±68.6	431.5±85.8	0.008

Table 4. Effect of stripped hFF (ShFF) on CatSper V50

stimulus	n	control (mV)	treated (mV)	P
1%ShFF	4	54.0±10.8	51.0±8.8	NS
1%hFF	4	54.0±10.8	9.3±4.0	0.01

Table 5. Is failure of 1% ShFF to potentiate CatSper currents due to contamination with divalent cations?

		-80mV			80 mV		
stimulus	n	control (pA)	treated (pA)	P	control (pA)	treated (pA)	P
2 nM P4	4	-60.3±13.5	-90.0±18.9	0.02	193.4±23.7	237.4±36.7	0.046
2 nM P4 with Ca/Mg	5	-62.1±16.7	-111.9± 21.7	0.002	156.6± 22.1	213.2 ±16.0	0.012
ShFF with 9 mM EGTA, 9 mM EDTA	17	-98.9±14.4	-125.6± 21.7	0.12	214.6± 24.7	223.9 ±31.7	0.62

Table 6. Effect of hFF on K+ current reversal potential and conductance

		Erev (mV)			Gm (ns/pF)		
stimumlus	n	control (pA)	treated (pA)	P	control (pA)	treated (pA)	P
1%hFF	6	-34.6 ± 4.4	-36.5± 6.6	>0.05	1.02 ± 0.17	1.12 ± 0.21	>0.05
10%hFF	3	-22.0 ± 9.0	-22.8 ± 9.1	>0.05	0.79 ± 0.20	0.72 ± 0.25	>0.05
50%hFF	3	-23.95 ± 3.8	-24.0 ± 4.0	>0.05	0.64 ± 0.06	0.57 ± 0.04	>0.05
10μM P4	3	-28.2 ± 2.8	-18.28 ± 4.6	0.09	0.51 ± 0.06	0.41 ± 0.03	0.32
30μM P4	4	-41.4 ± 3.5	-21.0 ± 5.5	0.023	0.68 ± 0.08	0.25 ± 0.06	0.026

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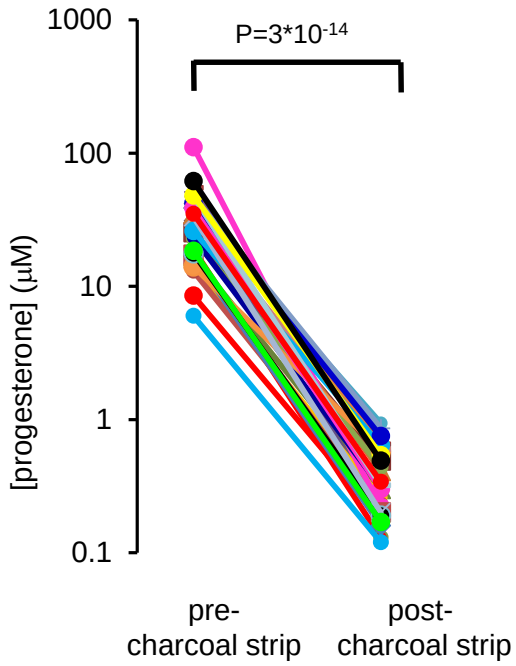
Supplementary fig 1



Supplementary Figure 1. Concentrations of P4 in 31 hFF samples assessed before (left) and after (right) stripping of lipid-derived molecules with dextran-coated charcoal.

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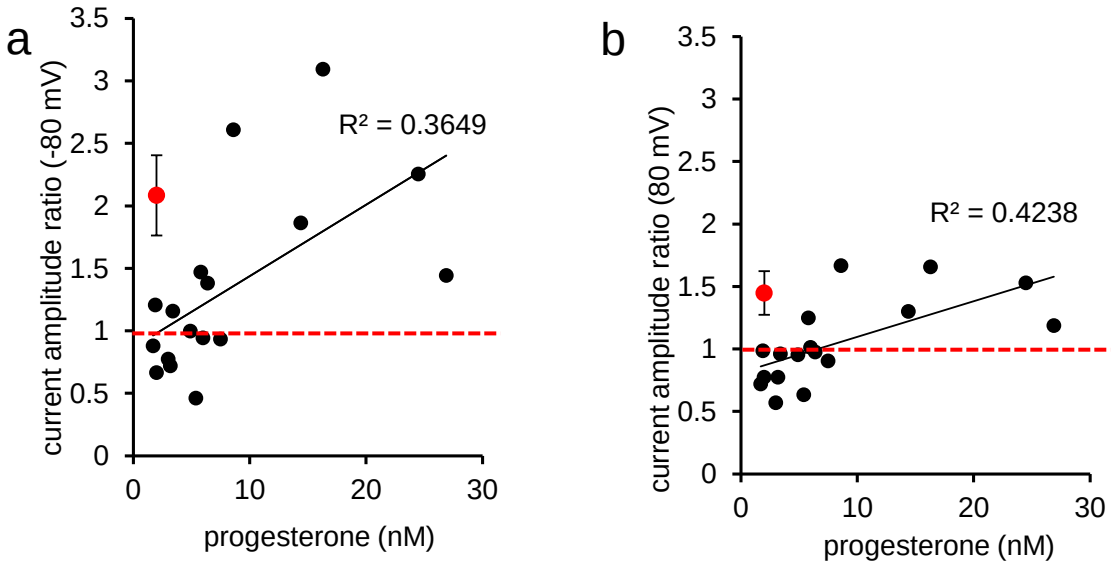
Supplementary fig 2



Supplementary Figure 2. Relationship between concentration of progesterone in ShFF and current amplitude ratio (stimulated amplitude:control amplitude). Inward currents (-80 mV) and outward currents (80 mV) are shown in panels a and b show respectively. Progesterone concentrations shown (17 different hFF used) are after dilution to 1% as used in the experiment. Black symbols show responses of cells treated with 1% ShFF diluted in Cs^+ recording saline containing 9 mM EDTA and 9 mM EGTA, red symbols shows mean±sem for 5 experiments where ShFF was replaced with standard bath solution (containing 2 mM Ca^{2+}, 0.7 mM Mg^{2+} and 200 nM progesterone). Fitted linear regressions are shown on each plot.

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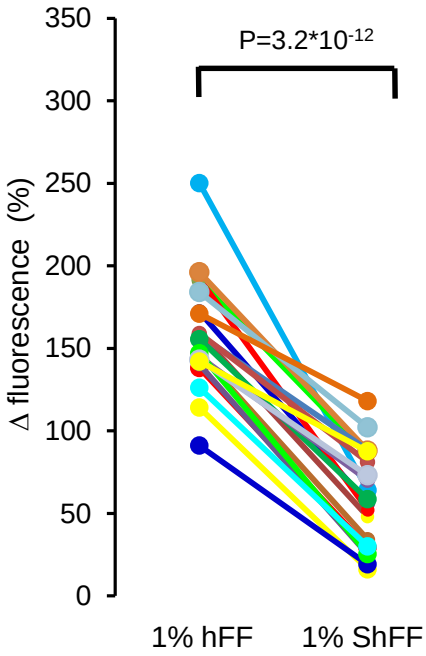
Supplementary fig 3



Supplementary Figure 3. Amplitude of $[Ca^{2+}]_i$ transient induced by 1% hFF before (left) and after (right) 'stripping' with dextran-coated charcoal. Data from 21 paired recordings using 5 different hFF samples are shown. $P=3.2*10^{-12}$ (paired t-test)

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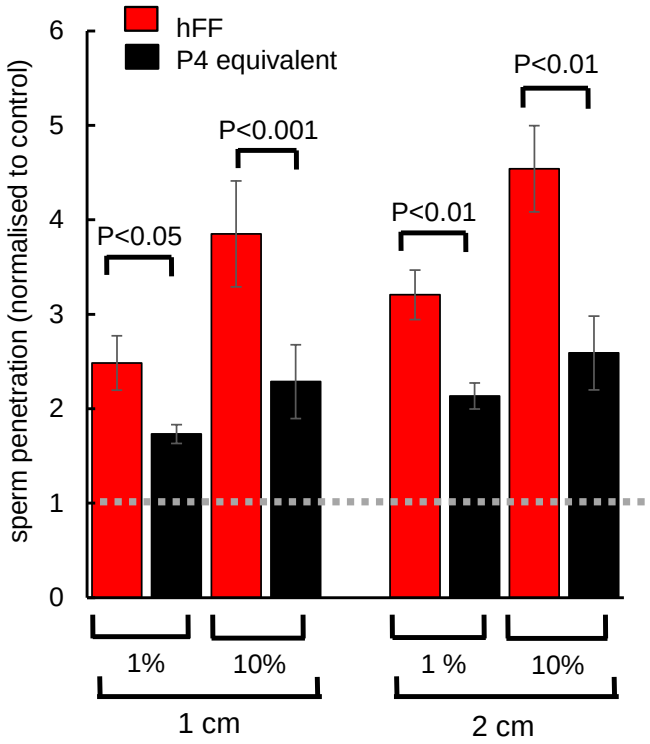
Supplementary fig 4



Supplementary fig. 4. Effects of exposure to hFF or equivalent P4 concentration on sperm penetration into viscous medium. Data for number of sperm penetrating to 1 cm (left panel) and 2 cm (right panel) have all been normalised to parallel, untreated control (control response indicated by grey dashed line). All bars show mean±SEM of 6 experiments using 5 different hFF. Red bars show hFF (1% and 10% as marked below x-axis), black bars show progesterone (P4) at equivalent dose to 1% hFF. All treatments were significantly different from control (P<0.005). Statistical significance markers indicate comparison of effect of hFF with equivalent dose of P4.

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