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Complex CatSper-dependent and independent [Ca2+]i signalling in human spermatozoa induced by follicular fluid

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DOI: 10.1093/humrep/dex269

Document Version Peer reviewed version

Citation for published version (Harvard):

Browm, S, Costello, S, Kelly, M, Ramalingan, M, Drew, E, Publicover, S, Barratt, C & Martins da Silva, S 2017, 'Complex CatSper-dependent and independent [Ca2+]i signalling in human spermatozoa induced by follicular fluid: CatSper activation by human follicular fluid', *Human Reproduction*, vol. 32, no. 10, pp. 1995-2006. https://doi.org/10.1093/humrep/dex269

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2	spermatozoa induced by follicular fluid
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20	Running Title: CatSper activation by human follicular fluid
21	

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
 Ca²⁺ 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.

29 What is known already: CatSper, the principal Ca²⁺ channel in spermatozoa, is

30 progesterone-sensitive and essential for fertility. Both hFF and progesterone, which is

31 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
 hvF/r021

34 IVF/ICSI.

35 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

37 Ethics Service REC1. Activities of CatSper and KSper were assessed by patch clamp.

38 Sperm [Ca²⁺]_i responses were examined in sperm populations and single cells. Computer-

assisted sperm? analysis (CASA) parameters and penetration into viscous media were usedto assess functional effects.

Main results and the role of chance: hFF and progesterone significantly potentiated 41 CatSper currents. Under quasi-physiological conditions, hFF (up to 50%) failed to alter 42 membrane K⁺ conductance or current reversal potential. hFF and progesterone (at an 43 equivalent concentration) stimulated similar biphasic [Ca²⁺], signals both in sperm 44 populations and single cells. At a high hFF concentration (10%), the sustained (plateau) 45 component of the [Ca²⁺], signal was consistently greater than that induced by progesterone 46 alone. In single cell recordings, 1% hFF induced [Ca²⁺], oscillations similarly to progesterone 47 but with 10% hFF generation of [Ca²⁺], oscillations was suppressed. After treatment to 'strip' 48 lipid-derived mediators, hFF failed to significantly stimulate CatSper currents but induced 49

small $[Ca^{2+}]_i$ responses that were greater than those induced by the equivalent concentration

of progesterone after stripping. Similar [Ca²⁺]_i responses were observed when sperm pre-

52 treated with 3 μM progesterone (to desensitise progesterone responses) were stimulated

53 with hFF or stripped hFF. hFF stimulated viscous media penetration and was more effective

54 than the equivalent does of progesterone.

55 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.

58 Wider implications of the findings: This study directly demonstrates that hFF activates

59 CatSper and establishes that the biologically important effects of hFF reflect, at least in part,

60 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

62 the activation of CatSper. Simple in-vitro experiments performed out of the context of the

63 complex in-vivo environment need to be interpreted with caution.

64 Study funding/competing interest(s): Funding was provided by MRC (MR/K013343/1,

65 MR/012492/1) (SGB, SJP, CLRB) and University of Abertay (sabbatical for S.G.B.).

66 Additional funding was provided by TENOVUS SCOTLAND (S.Md.S.), Chief Scientist

67 Office/NHS Research Scotland (S.Md.S). C.L.R.B. is EIC of MHR and Chair of the WHO

68 ESG on Diagnosis of Male infertility. The remaining authors have no conlicts of interest.

69

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

71 spermatozoa

Introduction

Human follicular fluid (hFF) affects various important functions of human spermatozoa, 74 including hyperactivated motility, chemotaxis and acrosome reaction (Baldi et al., 1998). 75 Almost 30 years ago Thomas and colleagues demonstrated that hFF stimulated a rapid 76 influx of Ca²⁺ in human spermatozoa (Thomas and Meizel 1988). Subsequently, 77 progesterone (P4) was shown to have effects on sperm function similar to those of hFF and 78 was found to be the component of hFF that was primarily responsible for induction of Ca2+-79 influx (Osman et al., 1989; Thomas and Meizel 1989). In 2011, Lishko and Strunker 80 independently showed that induction of Ca²⁺ influx by P4 was via the sperm specific channel 81 82 CatSper (Lishko et al., 2011; Strunker et al. 2011) which is now known to be stimulated by a 83 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 84 that high concentrations of P4 encountered in the vicinity of the oocyte and its vestments 85 86 achieve full activation of CatSper through a combination of CatSper activation and depolarisation of membrane potential due to KSper inhibition (Mannowetz et al., 2013). 87

88

As P4 is a primary component of hFF, a logical assumption is that exposure of human 89 spermatozoa to hFF in vivo activates CatSper. However, the 'clean' stimuli that are used for 90 in-vitro investigations, such as those by which the action of P4 on CatSper was established, 91 differ greatly from the complex environment of the reproductive tract (Mortimer et al. 2013; 92 Sakkas et al., 2015). hFF is a complex fluid (Revelli et al., 2009; O'Gorman et al., 2013) and, 93 94 in its presence, sperm are simultaneously exposed to multiple ligands, potentially leading to multiple separate effects and/or interactions. Significantly, pre-treatment with oestrogen 95 $(17\beta E_2)$, which elevates $[Ca^{2+}]_i$ in spermatozoa apparently by a mechanism independent of 96 CatSper (Luconi et al., 1999; Lishko et al., 2011; Mannowetz et al, 2017), reduced the Ca²⁺ 97 98 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 99 100 previously described effects of its principal component P4, or are there synergistic or even

- 101 antagonistic effects on CatSper upon exposure to these complex mixtures? (ii) Do other
- 102 components of hFF contribute significantly, but separately, to hFF-induced Ca²⁺ signalling?
- 103
- 104

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Materials and Methods

107 **Experimental solutions**

Details for HEPES buffered saline, bicarbonate buffered capacitating medium, supplemented Earle's balanced salt solution (sEBSS), standard bath solution (patch seals and quasiphysiological recording), standard pipette solution (quasi-physiological recording), Cs⁺-based pipette and bath solutions (monovalent CatSper currents) and bath (Ba²⁺) and pipette solutions for CatSper tail currents are given in Supplementary File 1.

113 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% CO₂) 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).

120 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).

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

129 Steroids and prostaglandins were removed from hFF by adapting the dextran-coated 130 activated charcoal method for removal of steroids from serum (product information sheet 131 C9157; Sigma Aldrich, UK; Supplementary File 1).

132 Electrophysiology

Currents were recorded from sperm isolated by density gradient using whole cell patch 133 clamp (Mansell et al., 2014). To investigate K⁺ channel function, cells were studied under 134 quasi-physiological conditions (standard pipette and bath solutions) using a ramp protocol (-135 92 to 68 mV over 2500 ms). Membrane potential was held at -92mV between ramps (Brown 136 et al., 2016). Reversal potentials (E_{rev} – to estimate resting Vm) and membrane conductance 137 (Gm) were calculated as previously described (Brown et al., 2016). Monovalent CatSper 138 currents were recorded using Cs⁺-based divalent-free pipette and bath solutions. Currents 139 were evoked by a ramp protocol (-80 to 80 mV over 1 s). Membrane potential was held at 0 140 mV between ramps. Divalent (Ba²⁺) CatSper tail currents (Lishko et al., 2011) were evoked 141 by 400 ms pulses followed by stepping to -150mV (200 ms). Vm was held at -70mV between 142 sweeps (Lishko et al, 2011). Tail current amplitudes were used to plot voltage activation (G-143 V) curves. Data were sampled at 2 kHz, filtered at 1 kHz. Tail current data were leak 144 145 subtracted using pClamp P/4 protocol to minimise the impact of membrane resistance (PClamp 10 software, Axon instruments). 146

147

148 Assessment of [Ca²⁺]_i signals

149 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 150 in sEBSS. [Ca2+], was assessed using a FLUOstar microplate reader (BMG Labtech 151 Offenburg, Germany) with 488nm (excitation) and 520nm (emission) filters. After a control 152 period, (30-60 s) stimuli were added using a multichannel pipetter as described by Strunker 153 et al (2011). To compare [Ca²⁺]_i responses to hFF and equivalent [P4] aliquots from the 154 same fluo4 loaded sample, tests were performed in parallel. Emission was background 155 corrected and normalized to the control (pre-stimulus) amplitude. To compare duration of P4 156 and hFF-induced transients, the half-duration (midpoint of the rising phase to midpoint of 157 decay) was calculated. In desensitization experiments, cells were first stimulation with 3 µM 158

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

170 Assessment of sperm function

171 Viscous media penetration test and CASA were carried out as previously described 172 (Alasmari *et al*, 2013a; Williams *et al.*, 2015).

173 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.

179 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.

<u>Results</u>

192 **hFF and ion channel currents**

193 Effects of hFF on CatSper current

194 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 195 potentiated both inward and outward monovalent CatSper currents (Fig 1a,b; Table 1; 196 P<0.01). P4 potentiates CatSper currents primarily by shifting channel activation to more 197 198 negative voltages (Lishko et al., 2011). Assessment of voltage-sensitivity of CatSper activation (using Ba²⁺ tail currents) showed that 1% hFF shifted the G-V curve to more 199 negative voltages (Fig 1c), significantly changing the V_{50} (table 2; *P*<0.001). Similarly, 200 500nM P4 caused a negative shift of the CatSper G-V curve (Fig. 1d, Table 2; P<0.01) as 201 202 demonstrated previously (Lishko et al., 2011).

203

204 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 205 may influence [Ca²⁺], signalling. To examine the effect of depleting lipid derived agonists 206 (steroids and prostaglandins), samples of FF were 'stripped' using dextran-coated charcoal. 207 208 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 209 incubated similarly but without dextran-coated charcoal. ShFF failed to stimulate I_{CatSper}, 210 (both inward and outward currents were smaller; Fig 2a; Table 3; P<0.05), but subsequent 211 application of hFF potentiated both inward and outward currents amplitude (Fig 2a; Table 3; 212 213 P=0.05; P<0.01 respectively). Similarly, when tail currents were used to assess CatSper 214 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 215 reported to increase CatSper currents (Lishko et al, 2011). We therefore assessed whether 216 we could detect this effect under our recording conditions. Both using standard Cs⁺ saline 217

recording (P4 added directly to Cs⁺ saline before perfusion of the recording chamber) and 218 also when progesterone was first dissolved in a mixture of 1% standard bath solution 219 (containing 2 mM Ca²⁺, 0.7 mM Mg²⁺) and 99% Cs⁺ saline (to mimic ionic conditions in ShFF 220 experiments), superfusion of sperm with 2 nM P4 significantly increased both outward and 221 inward currents (Table 5). Finally, we increased the concentrations of divalent chelators 222 (EGTA, EDTA) in our Cs⁺ recording saline to 9 mM of each to chelate any residual Ca²⁺ and 223 Mg²⁺ from the hFF. Under these conditions, we observed a response to ShFF in some cells 224 (Suppl. Fig 2) and mean inward and outward currents were increased, but this effect was not 225 significant (Table 5; P>0.1). Examination of [P4] concentrations showed that detectable 226 227 effects of ShFF occurred only with with hFF samples where the [P4] was unusually high (Suppl. Fig 2). 228

229

230 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 μ M but at 10 μ M effects were not significant (Table 6).

237

238 hFF and sperm [Ca²⁺]i

239 <u>hFF-induced [Ca²⁺], signals in sperm populations</u>

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 $[Ca^{2^{2+}}]_i$ 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 245 hFF (0.01-1%) the amplitudes of signals induced by hFF and P4 were similar (Fig 4c,d). 246 However, at the highest hFF concentration (10%) the [Ca²⁺], plateau induced by hFF 247 (assessed 10 min after stimulus application) was consistently greater than that induced by 248 an equivalent concentration of P4 (mean amplitude sample ratio=1.6±0.1; Fig 4d red 249 symbols; P=0.001; n= 7;). In cells stimulated with 10% hFF the $[Ca^{2+}]_i$ transient also 250 251 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 252 'half-duration' (latency from midpoint of the rising phase to midpoint of decay) confirmed that 253 254 this was the case (P=0.0005; n=7).

255

256 <u>hFF-induced [Ca²⁺], signals in single cells</u>

Similarly to population measurements, single cell imaging of [Ca²⁺], at the posterior 257 258 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 Ca²⁺ 259 transient was often followed by [Ca2+] oscillations (not synchronised and therefore 260 detectable only in single cell records; Kirkman-Brown et al, 2004; Harper et al, 2004; Fig 5a). 261 In cells stimulated with hFF, oscillations were observed but their occurrence was markedly 262 concentration dependent. 1% hFF, similarly to 300 nM P4 (estimated equivalent [P4]) 263 induced oscillations in ≈25% of cells (Fig 5c; P=0.47; n=10). However, whereas 3µM P4 was 264 similarly effective (19% of cells; e.g. Fig 5a), 10% hFF induced oscillations in only 4% of 265 cells (Fig 5b,d,e; P=0.002, n=10). 266

267

268 [Ca²⁺]; 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 hFFinduced $[Ca^{2+}]_i$ signals were similarly affected. Surprisingly, $[Ca^{2+}]_i$ responses were always

detected in cell populations stimulated with 1% ShFF, with the [Ca²⁺], transient amplitude 272 being 36.8±1.8% of that in the parallel control (1% hFF) experiments (Fig 6a; Suppl. Fig 3; 273 P=3.2*10⁻¹²; n=21). In 28 experiments where parallel recordings were carried out with ShFF 274 and [P4] equivalent to that in ShFF, [Ca²⁺], transient amplitudes were similar (P=0.14). 275 However, the subsequent [Ca²⁺], 'plateau' was significantly greater with ShFF (43±9% for the 276 period 30-240 s post-stimulus; P=4.8*10⁻⁶; Fig 6b). The 'non-P4' component, isolated by 277 subtraction of traces (ShFF-equivalent [P4]), showed activation later than the [Ca²⁺], signal 278 279 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 280 or equivalent [P4], cells failed to generate the [Ca²⁺], transient seen in the equivalent 281 population experiments and instead we observed a slow $[Ca^{2+}]_i$ ramp (Fig 7 a). This reduced 282 efficacy of stimuli delivered by perfusion is due to binding of progesterone to the perfusion 283 tubing (see discussion). The mean increase in [Ca²⁺], was greater in the ShFF-treated cells, 284 but the effect was highly variable and the difference was not significant (Fig 7a,b; P=0.14). 285 After 5-10 min exposure to 1% ShFF or equivalent [P4], oscillations developed in 286 approximately 20% of cells (Fig 7c,d), resembling the response to P4 ramps (Harper et al, 287 2004). 288

289

290 Effects of P4 desensitisation on [Ca²⁺]_i response to hFF

Component(s) of hFF not removed by charcoal stripping contribute significantly to 291 late/sustained components of hFF-induced [Ca²⁺]; signals (Fig 6b). To further investigate 292 this, we tested the effect of desensitisation of the P4 response on the [Ca²⁺]_i signal induced 293 by hFF. As previously described (Aitken et al, 1996; Schaefer et al, 1998), when sperm were 294 pre-stimulated with 3 µM P4 complete desensitisation occurred (Fig 6c). However, when P4-295 desensitised cells were stimulated with hFF there was a clear response (13.8±0.9% of that 296 evoked by the preceding, desensitising P4 stimulus; $P=3.2*10^{-5}$ compared to second 297 stimulation with 3 µM P4; n=10; Fig 6d,f). Since P4 and prostaglandins stimulate CatSper by 298

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 μ M P4 stimulus) which was significantly greater (P=2.8*10⁻⁵) than the response to a second stimulation with 3 μ M P4; Fig 6e,f).

305

306 **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

315 316

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.

323

324 Modulation of ion channel activity and [Ca²⁺]_i by hFF

The electrophysiological data clearly show that hFF, similarly to P4, enhances CatSper 325 currents and shifts CatSper voltage sensitivity to less positive potentials (Fig. 1; Tables 1 326 and 2). Mannowetz and colleagues (2013) reported that high concentrations of P4 also 327 328 inhibit KSper (I₅₀~7 µM), depolarising the membrane potential and potentially augmenting activation of CatSper. We could detect no effect of hFF on conductance or resting Vm even 329 with 50% hFF (containing 10-15 µM progesterone; Fig. 3). In positive control experiments 330 with P4, we saw no significant effect with 10 µM but clear inhibition of conductance with 30 331 µM P4 (equivalent [P4] to 100% hFF; Table 6). Thus effects of hFF on KSper may occur at 332 higher concentrations than those used in this study, potentially in very close proximity to the 333 oocyte. 334

335

336 [Ca²⁺]_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 342 hydrocortisone, both present in hFF, inhibit the stimulatory action of 1 μ M P4 (IC₅₀ = 833 nM 343 and 153 nM respectively) and their actions might be expected to result in a response to hFF 344 345 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 346 (Mannowetz et al., 2017), but the stimulatory effects observed with 10% hFF indicate that 347 348 other components of hFF, when present at sufficient concentration, either activate (or suppress inactivation of) CatSper or activate other [Ca²⁺], signalling components that 349 contribute to the sustained $[Ca^{2+}]_i$ signal (see below). 350

Single cell [Ca²⁺], responses to P4 resemble population responses (transient and plateau 351 phase; Kirkman-Brown et al, 2000) but some cells then generate repetitive oscillations (Fig 352 5a; Harper et al, 2004; Kirkman-Brown et al, 2004) that may regulate motility and/or 353 acrosome reaction (Harper et al, 2004; Bedu-Addo et al, 2007; Alasmari et al, 2013; 354 Sánchez-Cárdenas et al., 2014). In paired experiments, 1% hFF and 300 nM progesterone 355 (equivalent concentration) both induced repetitive [Ca²⁺], oscillations in approximately 20% of 356 cells (Fig 5c), while 1% ShFF and matched [P4], (after a latency of 5-10 min) were similarly 357 effective. However, when challenged with 10% hFF, just 4% of sperm generated oscillations 358 359 compared to 19% with 3 µM (equivalent) progesterone (Figs 5d,e), again suggesting that substances within hFF modulate human sperm Ca²⁺ signalling by mechanisms other than 360 CatSper activation. Darszon and colleagues assessed [Ca²⁺], and acrosomal status and 361 concluded that calcium oscillations suppress the acrosome reaction (Sánchez-Cárdenas et 362 al., 2014). If the sperm encounters high concentrations of hFF on approaching the cumulus-363 oocyte complex, this may inhibit [Ca²⁺], calcium oscillations and 'disinhibit' acrosome 364 reaction. 365

366

367 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 368 effects of hFF, samples were treated with dextran-coated charcoal to 'strip' lipid-derived 369 agonists (steroids/prostaglandins), removing almost 99% of P4. In fluorimetric experiments 370 the $[Ca^{2+}]_i$ transients evoked by ShFF were consistent with a response to the residual P4, 371 but the subsequent sustained $[Ca^{2+}]_i$ signal was significantly greater (Fig 6b). Furthermore, 372 when we pretreated sperm with P4 to desensitise the P4-induced [Ca²⁺], signal (Aitken et al, 373 1996; Schaefer et al, 1998), we found that a small, sustained response persisted whether 374 stimulating with hFF or ShFF (Fig 6c-f). These observations indicate that hFF includes 375 factors that contribute to and/or regulate Ca²⁺-signalling that are resistant to stripping with 376 377 dextran-coated charcoal and are therefore unlikely to be steroids or prostaglandins.

Though the [Ca²⁺]_i transient induced by 1% ShFF appeared to be primarily a response to 378 residual P4 (see above), when we investigated effects on patch-clamped sperm we 379 observed no stimulation of CatSper currents, suggesting that other components of hFF 380 381 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 382 reduced efficacy of P4 in our imaging experiments and also observed by others; T Strunker 383 personal communication), thus comparison with fluorimetric [Ca²⁺]_i assessment, where direct 384 addition of ShFF to the well induced a significant [Ca²⁺], response (Fig 6), is misleading. This 385 is particularly significant since the inhibitory of hFF was masked at higher [P4] (Suppl Fig 2). 386 Secondly, divalent cations in hFF (2.2 mM Ca, 0.68 mM Mg; Chong et al, 1977; Ng et al, 387 1987) may be inadequately buffered, masking any stimulatory effect (IC_{50} for Ca^{2+} ~100nM; 388 Lishko et al., 2011). However, (i) in 'supplemented' control experiments where Ca²⁺/Mg²⁺ 389 was present at equivalent levels to that in ShFF, responses to 2 nM P4 resembled those 390 seen in 'divalent-free' controls (Table 5) and (ii) increased divalent cation buffering 391 (calculated [Ca²⁺]+[Mq²⁺] with 1% ShFF=2.14 nM) failed to rescue stimulation of CatSper 392 currents to ShFF (Table 5; Suppl. Fig 2). We conclude that residual P4 in 1% ShFF (a [P4] 393 394 sufficient to activate CatSper in 'supplemented' control recordings (Table 5)), when delivered 395 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 396 the channel to low (nM) concentrations of progesterone. Thus the slowly-developing ShFF-397 induced [Ca²⁺], ramp seen in imaging experiments (Fig 7a,c) is apparently induced 398 independently of CatSper activation. The complexity of hFF, even after charcoal stripping, is 399 400 such that discussion of the nature of such an effect can only be speculative. However, the effects on human sperm [Ca²⁺], of kisspeptin (Pinto *et al*, 2012) and leutenising hormone 401 (López-Torres et al, 2017), suggest that activation G-protein coupled receptors by protein or 402 peptide hormones might exert such an effect. 403

404

405 Functional effect of hFF

406 We reported previously that stimulation of penetration into artificial mucus was mediated by activation of CatSper whereas manoeuvres designed to mobilise stored Ca²⁺ strongly 407 stimulate hyperactivation (Alasmari et al., 2013). Analysis of motility showed that hFF 408 409 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 410 with the significantly greater effects of hFF on [Ca²⁺], signalling and the likelihood that hFF 411 recruits stored Ca²⁺ in addition to activation of CatSper . These data suggest that stimulation 412 by hFF may contribute significantly to sperm penetration of the cumulus matrix. 413

414

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.

423 Acknowledgements

We are very grateful to all members of the ACU for their invaluable assistance in particular the embryologists (Kath, Sylvia, Philip), lab practitioners (Hannah, Rachel, Lynsey, Steven, David) and nurses. We also thank all the patients and donors who took part in this study and Steven Gellatly and Evelyn Barratt for their continual support of our research studies.

428

429 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.

437

438 Funding

Funding was provided by MRC (MR/K013343/1, MR/012492/1) (SGB, SJP, CLRB) and
University of Abertay (sabbatical for S.G.B.). Additional funding was provided by TENOVUS
SCOTLAND (S.Md.S.), Chief Scientist Office/NHS Research Scotland (S.Md.S).

442

443 Conflict of interest

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



Figure 1. Human follicular fluid potentiates CatSper currents and shifts the voltage sensitivity 465 to less depolarised potentials. a: Representative Cs⁺-mediated CatSper current in the 466 absence (black) and presence (red) of 1% hFF. Voltage protocol imposed is shown above. 467 468 **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 469 470 show outward currents (80mV; n=13). c and d show conductance-voltage (G-V) relationships for Ba²⁺-mediated CatSper tail currents in the absence and presence of 1% 471 hFF (c, n = 12) and 500nM P4 (d, n = 4) 472



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 (V₅₀ P<0.01 compared to control and ShFF). n = 4 cells, 4 hFF.

hFF (1%)

-120

-40 -20

mV

-120



hFF (10%)

-20

-120

mV

-15

-40

mV

hFF (50%)



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** 531 show an example of $[Ca^{2+}]_i$ responses induced in paired experiments using (a) four dilutions 532 of hFF (dark blue=0.01%, light blue=0.1%, green=1%, red=10%) and (b) P4 at 533 concentrations equivalent to those in the hFF dilutions (dark blue=2.8 nM, light blue=28 nM, 534 green=280 nM, red=2.8 μ M). **c** and **d** show relative amplitudes (Δ fluorescence (%)) of the 535 [Ca²⁺], transients (c) and [Ca²⁺], plateau (d, assessed 10 min post-stimulation) induced in 536 seven sets of experiments, each using four dilutions of hFF (0.01%=dark blue, 0.1%=light 537 blue, 1%=green, 10% =red) and P4 at concentrations equivalent to those in the hFF 538 dilutions. Six different hFF samples were used. Line in each graph marks position of equal 539 response amplitude. At the highest hFF concentration used (10%; red symbols), plateau 540 541 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 543 in a paired experiment in which cells from the same sample were exposed to 3 μ M P4 (a) 544 and 10% hFF (b). Panel **c** shows mean±SEM percentage of cells in which [Ca²⁺]_i oscillations 545 occured after stimulation of sperm (from the same sample) with 300 nM P4 (black) or 1% 546 hFF (red); n=10 paired experiments. Panel d shows results from a similar series of 10 paired 547 548 assessments using 3 µM P4 (black) and 10% 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 549 colour. 550



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

580 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 581 traces. **c** to **e**: Examples of $[Ca^{2+}]_i$ responses in three parallel recordings where sperm were 582 first stimulated with 3 µM P4 (1st addition-black traces) then, after an interval of 5 min, 583 exposed to either a second 3 μ M P4 stimulus (6 μ M P4 total; **c**, 2nd addition-black trace), 1% 584 hFF (d, 2nd addition-red trace) or 1% ShFF (e, 2nd addition-blue trace). In each panel the 585 responses to the first (3 µM P4) stimulus and to the second stimulus are overlaid (arrow at 586 top left shows time of additions). When 3 µM P4 was followed by a second P4 stimulus the 587 588 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**: 589 Mean amplitude (±SEM) of $[Ca^{2+}]_i$ transients evoked by the first 3 μ M P4 stimulus (P4(1)) 590 black) and by a second addition of P4 (P4(2); n=7; black), hFF (hFF(2); n=10; red) or 591 stripped hFF (ShFF(2); n=6; blue). All amplitudes are normalised to that induced by the first 592 P4 addition in that experiment. 593



Figure 7. Single cell [Ca²⁺], responses to 1% ShFF. **a** shows mean responses to 1% ShFF. 613 (red; n=10 experiments; 826 cells) and equivalent [P4] (black; n=6 experiments; 447 cells), 614 arrow marks stimulus addition. Both stimuli induced a [Ca²⁺], ramp rather than the biphasic 615 response seen in fluorimetric experiments. **b** shows mean (\pm SEM) amplitude (Δ 616 617 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 618 oscillations 5-10 min after stimulation. **d** shows proportions of cells generating $[Ca^{2+}]_{i}$ 619 oscillations after stimulation with 1% ShFF (red; n=10 experiments; 826 cells) or equivalent 620 621 [P4] (black; n=6 experiments; 447 cells).

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745

747 Supplementary File 1

- 748 <u>A. Salines</u>
- 749 <u>HEPES buffered saline</u> solution consisted of (in mM): CaCl₂, 1.8; KCl, 5.4; MgSO₄7H₂O, 0.8;
- 750 NaCl, 116.4; NaH₂PO₄, 1; D-glucose, 5.5; sodium pyruvate, 2.73; sodium lactate, 41.75;
- 751 HEPES, 25; BSA, 0.3% (w/v); pH adjusted to 7.4 using NaOH.
- 752 Bicarbonate buffered capacitating medium consisted of (in mM): CaCl₂, 1.8; KCl, 5.4;
- 753 MgSO₄7H₂0, 0.8; NaCl, 116.4; NaH₂PO₄ 1; D-glucose, 5.5; sodium pyruvate, 2.73; sodium
- ⁷⁵⁴ lactate, 41.75; sodium bicarbonate, 26; BSA, 0.3% (w/v); pH adjusted to 7.4 using NaOH.

755 <u>Supplemented Earle's balanced salt solution (sEBSS)</u> contained (in mM): NaH₂PO₄, 1.02;

756 KCl, 5.4; MgSO₄, 0.811; D-glucose, 5.5; Na pyruvate, 2.5; Na lactate, 19.0; CaCl₂,1.8;

- 757 NaHCO₃, 25.0; NaCl, 118.4 and HEPES, 15 (pH 7.4), supplemented with 0.3% (w/v) BSA.
- 758 <u>Standard bath solution</u> consisted of (in mM): NaCl, 135; KCl, 5; CaCl₂, 2; MgSO₄, 1; HEPES,
- 20; Glucose, 5; Na pyruvate, 1; Lactic acid, 10; pH adjusted to 7.4 with NaOH which brought
 [Na⁺] to 154 mM.
- 761 <u>Standard pipette solution</u> consisted of (mM): NaCl, 10; KCl, 18; K gluconate, 92; MgCl₂, 0.5,
- 762 CaCl₂, 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 μ M. $[Ca^{2+}]$ in buffered solutions was calculated using MaxChelator

- 764 (Maxchelator.stanford.edu).
- <u>Cs⁺-based pipette solution</u> 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.

767 <u>Cs⁺-based bath solution</u> contained Cs-methanesulphonate, 140 mM; HEPES, 40 mM;

- EGTA, 3 mM; EDTA, 3 mM pH adjusted to 7.4 with CsOH.
- 769 <u>CatSper tail current (Ba²⁺) bath solution</u> contained 10 mM BaCl₂, 140 mM NMDG, 100 mM
- HEPES, pH 7.4 with HMeSO₃.
- 771 <u>CatSper tail current pipette solution</u> contained 145 mM NMDG, 100 mM HEPES, 10 mM
 772 BAPTA, 0.5 mM TrisHCl, pH 7.4 with HMeSO₃.
- 773

774 B. Dextran-coated charcoal solution

775 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 MgCl₂, 10mM HEPES and 0.25M sucrose, pH 776 7.4 with NaOH and kept at 4°C. A volume of dextran-coated charcoal mixture double that of 777 the volume of hFF to be steroid stripped was centrifuged to pellet the charcoal. The 778 779 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 780 at 1000g for 5 minutes and the hFF was removed and filtered using a 0.22 µm filter. A paired 781 sample of the same hFF not incubated with dextran-coated charcoal was also left overnight 782 at 4°C (referred to as time control). 783

784

785

<u>Tables</u>

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788

Table 1. Effect of hFF on monovalent (Cs+) CatSper current amplitude									
-80mV 80 mV									
stimulus	n	control (pA)	treated (pA)	Р	control (pA)	treated (pA)	Р		
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)	Р	
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)	Р	control (pA)	treated (pA)	Р
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 <u>+</u> 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 C	atSper V50					
stimulus	n	control (mV)	treated (mV)	Р			
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 pote	ntiate	CatSper currents	s due to contamir	nation with d	ivalent cations	?	
			-80mV		80 mV		

			001111			001110	
stimulus	n	control (pA)	treated (pA)	Р	control (pA)	treated (pA)	Р
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)			Gm (ns/pF)			
stimumlus	n	control (pA)	treated (pA)	Р	control (pA)	treated (pA)	Р		
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		

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.

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 Ca²⁺, 0.7 mM Mg²⁺ and 200 nM progesterone). Fitted linear regressions are shown on each plot.

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)

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.