Human Reproduction, Vol.28, No.4 pp. 866–876, 2013
Advanced Access publication on February 12, 2013 doi:10.1093/humrep/des467

human reproduction

ORIGINAL ARTICLE *Andrology*

The clinical significance of calcium-signalling pathways mediating human sperm hyperactivation

Wardah Alasmari[1,4], Christopher L.R. Barratt[1,2,*], Stephen J. Publicover[3], Katherine M. Whalley[2], Erica Foster[1,5], Vanessa Kay[2], Sarah Martins da Silva[1,2], and Senga K. Oxenham[1]

[1]Reproductive and Developmental Biology, Medical School, Ninewells Hospital, University of Dundee, Dundee DD1 9SY, UK [2]Assisted Conception Unit, NHS Tayside, Ninewells Hospital, Dundee DD1 9SY, UK [3]School of Biosciences, University of Birmingham, Birmingham B152TG, UK
[4]Present address: Department of Anatomy, School of Medicine, Umm Al Qura University, Kingdom of Saudi Arabia
[5]Present address: Complete Fertility Centre, Princess Anne Hospital, Level G, Mailpoint 105, Coxford Road, Southampton SO16 5YA, UK

*Correspondence address. E-mail: c.barratt@dundee.ac.uk

Submitted on September 25, 2012; resubmitted on December 10, 2012; accepted on December 20, 2012

STUDY QUESTION: What is the prevalence of defects in the Ca^{2+}-signalling pathways mediating hyperactivation (calcium influx and store mobilization) among donors and sub-fertile patients and are they functionally significant, i.e. related to fertilization success at IVF?

SUMMARY ANSWER: This study identifies, for the first time, the prevalence of Ca^{2+} store defects in sperm from research donors, IVF and ICSI patients. It highlights the biological role and importance of Ca^{2+} signalling (Ca^{2+} store mobilization) for fertilization at IVF.

WHAT IS KNOWN ALREADY: Sperm motility and hyperactivation (HA) are important for fertility, mice with sperm incapable of HA are sterile. Recently, there has been significant progress in our knowledge of the factors controlling these events, in particular the generation and regulation of calcium signals. Both pH-regulated membrane Ca^{2+} channels (CatSper) and Ca^{2+} stores (potentially activating store-operated Ca^{2+} channels) have been implicated in controlling HA.

STUDY DESIGN, SIZE, AND DURATION: This was a prospective study examining a panel of 68 donors and 181 sub-fertile patients attending the Assisted Conception Unit, Ninewells Hospital Dundee for IVF and ICSI. Twenty-five of the donors gave a second sample (~4 weeks later) to confirm consistency/reliability of the recorded responses. Ca^{2+} signalling was manipulated using three agonists, NH_4Cl (activates CatSper via pH), progesterone (direct activation of CatSper channels, potentially enhancing mobilization of stored Ca^{2+} by CICR) and 4-aminopyridine (4-AP) (effect on pH equivalent to NH_4Cl and mobilizes stored Ca^{2+}). The broad-spectrum phosphodiesterase inhibitor 3-isobutyl-1-methyxanthine (IBMX), a potent activator of HA was also used for comparison. For patient samples, an aliquot surplus to requirements for IVF/ICSI treatment was examined, allowing direct comparison of Ca^{2+} signalling and motility data with functional competence of the sperm.

MATERIALS, SETTING, METHODS: The donors and sub-fertile patients were screened for HA (using CASA) and changes in intracellular Ca^{2+} were assessed by loading with Fura-2 and measuring fluorescence using a plate reader (FluoStar).

MAIN RESULTS AND THE ROLE OF CHANCE: The relative efficacy of the stimuli in inducing HA was 4-AP >> IBMX > progesterone. NH_4Cl increased $[Ca^{2+}]_i$ similarly to 4-AP and progesterone but did not induce a significant increase in HA. Failure of samples to generate HA (no significant increase in response to stimulation with 4-AP) was seen in just 2% of research donors but occurred in 10% of IVF patients ($P = 0.025$). All donor samples generated a significant $[Ca^{2+}]_i$ increase when stimulated with 4-AP but 3.3% of IVF and 28.6% of ICSI patients failed to respond. Amplitudes of HA and $[Ca^{2+}]_i$ responses to 4-AP were correlated with fertilization rate at IVF ($P = 0.029$; $P = 0.031$, respectively). Progesterone reliably induced $[Ca^{2+}]_i$ responses (97% of donors, 100% of IVF patients) but was significantly less effective than 4-AP in inducing HA. Twenty seven per cent of ICSI patients failed to generate a $[Ca^{2+}]_i$ response to progesterone ($P = 0.035$). Progesterone-induced $[Ca^{2+}]_i$ responses were correlated with fertilization rate at IVF ($P = 0.037$) but induction of HA was not. In donor samples examined on more than one occasion consistent responses for 4-AP-induced $[Ca^{2+}]_i$ ($R^2 = 0.97$) and HA ($R^2 = 0.579$) were obtained. In summary, the data indicate that defects in Ca^{2+} signalling leading to poor HA do occur and that ability to undergo Ca^{2+}

-induced HA affects IVF fertilizing capacity. The data also confirm that release of stored Ca^{2+} is the crucial component of Ca^{2+} signals leading to HA and that Ca^{2+} store defects may therefore underlie HA failure.

LIMITATIONS, REASONS FOR CAUTION: This is an *in vitro* study of sperm function. While the repeatability of the $[Ca^{2+}]_i$ and HA responses in samples from the same donor were confirmed, data for patients were from 1 assessment and thus the robustness of the failed responses in patients' needs to be established. The focus of this study was on using 4AP, which mobilizes stored Ca^{2+} and is a potent inducer of HA. The *n* values for other agonists, especially calcium assessments, are smaller.

WIDER IMPLICATIONS OF THE FINDINGS: Previous studies have shown a significant relationship between basal levels of HA, calcium responses to progesterone and IVF fertilization rates. Here, we have systematically investigated the ability/failure of human sperm to generate Ca^{2+} signals and HA in response to targeted pharmacological challenge and, related defects in these responses to IVF success. $[Ca^{2+}]_i$ signalling is fundamental for sperm motility and data from this study will lead to assessment of the nature of these defects using techniques such as single-cell imaging and patch clamping.

STUDY FUNDING/COMPETING INTEREST(S): Resources from a Wellcome Trust Project Grant (#086470, Publicover and Barratt PI) primarily funded the study. The authors have no competing interests.

Key words: calcium signalling / sperm / male fertility / hyperactivation / sperm motility / IVF.

Introduction

Sperm dysfunction (lacking 'normal' function) has consistently been identified as the single most common cause of male infertility (Hull *et al*., 1985; Irvine, 1998). Men can produce sperm which are dysfunctional even when their semen parameters are 'normal' (Aitken *et al*., 1991). Currently, there are no drugs a man can take, or add to his spermatozoa *in vitro*, to treat sperm dysfunction. The only option is assisted reproductive technology (ART), which comprises a range of treatments, all of which are invasive. The particular treatment selected depends on the severity of the condition, i.e. intrauterine insemination for mild, IVF for moderate and ICSI for men with severe sperm dysfunction. The development of non-invasive, pharmacological treatment alternatives has been severely hampered by our limited understanding of the cellular and molecular workings of the mature spermatozoon (reviews Aitken and Henkel, 2011; Barratt *et al*., 2011). There are, however, some areas where progress has been made. One that has received considerable attention, ignited by the creation and characterization of CatSper knockout mice, concerns the generation and regulation of calcium signals that control sperm motility, in particular hyperactivation (HA) (see below and Publicover *et al*., 2007; Costello *et al*., 2009; Lishko *et al*., 2011; Publicover and Barratt, 2011a, b; Strünker *et al*., 2011; Barratt and Publicover, 2012).

HA is critical to sperm function, playing a key role in the ability of sperm to successfully ascend the female reproductive tract and reach the site of fertilization. For example, HA may facilitate sperm migration through the highly visco-elastic oviductal mucus and enable penetration of the layers surrounding the oocyte (Suarez *et al*., 1991; Suarez and Dai, 1992; Stauss *et al*., 1995; Ren *et al*., 2001; Carlson *et al*., 2003; Quill *et al*., 2003). Additionally, experimental studies show that HA may be required to detach sperm from the oviduct epithelium in animals (Demott and Suarez, 1992; Gwathmey *et al*., 2003) and in humans (Pacey *et al*., 1995). Although data are limited in humans, clinical studies on HA generally suggest that (i) the percentage of hyperactivated sperm correlates with fertilization rate *in vitro* (e.g. Sukcharoen *et al*., 1995) and (ii) there are significant differences in the proportion of hyperactivated cells (spontaneous and in response to physiological or artificial stimulants) between men with normal semen parameters and sub-fertile patients (Burkman, 1984; Tesarik *et al*., 1992; Peedicayil *et al*., 1997; Munire *et al*., 2004).

As the spermatozoon ascends the female tract, its motility must be finely regulated by cues from the female tract and cumulus–oocyte complex, in order that the cell can deploy behaviour appropriate to its environment (Olson *et al*., 2011). The central regulator is Ca^{2+}, elevated $[Ca^{2+}]_i$ being required both for the initiation and maintenance of hyperactivated motility. There are at least two sources of Ca^{2+} that contribute to HA in mammalian sperm: firstly, entry of Ca^{2+} via pH-dependent CatSper channels in the plasma membrane of the flagellar principal piece. Sperm from mice null for CatSper are motile but do not hyperactivate, rendering them unable to migrate to or within the oviduct and unable to fertilize oocytes even by IVF (Ren *et al*., 2001; Carlson *et al*., 2003; Quill *et al*., 2003; Ho *et al*., 2009). In bovine sperm elevation of pHi with NH_4Cl, to activate CatSper, induces HA and similar results have been reported in mouse sperm (Marquez and Suarez, 2007; Chang and Suarez, 2011). Secondly, induction of HA has been reported in bovine, mouse and human cells upon mobilization of calcium stored in the neck/midpiece region (Ho and Suarez, 2001, 2003; Marquez *et al*., 2007; Costello *et al*., 2009). In human sperm thimerosal (which activates intracellular Ca^{2+} channels, releasing stored Ca^{2+}) potently induces HA Alasmari *et al.* (2013) whereas stimulation of CatSper with progesterone (Lishko *et al*., 2011; Strünker *et al*., 2011) or by raising pHi have little effect Alasmari *et al.* (2013). 4-Aminopyridine (4-AP), a particularly potent inducer of HA (Bedu-Addo *et al*., 2008; Gu *et al*., 2004; review Costello *et al*., 2009), both stimulates release of stored Ca^{2+} and raises pHi (Ishida and Honda, 1993; Grimaldi *et al*., 2001; Navarro *et al*., 2007; Bhaskar *et al*., 2008; Chang and Suarez, 2011; Alasmari *et al*., 2013). However, equivalent cytoplasmic alkalinization induced with NH_4Cl or trimethylamine hydrochloride fails to cause HA and pharmacological block of CatSper does not inhibit 4-AP-induced HA Alasmari *et al*., (2013). Therefore it is unlikely that the increase in pHi produced by 4-AP explains its effect and we can conclude that it stimulates human sperm HA primarily or fully through its action on stored Ca^{2+}.

A key question therefore is: do functional defects occur in the sperm Ca^{2+}-signalling apparatus that prevent regulation of HA and

cause sperm dysfunction? To investigate this a panel of donors and sub-fertile patients were screened for HA and changes in intracellular Ca^{2+} in response to targeted agonists namely (i) NH_4Cl (pH-induced activation of flagellar CatSper channels); (ii) progesterone (direct activation of flagellar CatSper channels, potentially enhancing the mobilization of stored Ca^{2+} by calcium-induced calcium release (CICR) Harper *et al*., 2004) and (iii) 4-AP (raises pHi (similarly to NH_4Cl) and mobilizes stored Ca^{2+} in the neck/midpiece of human sperm). 3-Isobutyl-1-methylxanthine (IBMX) was used to stimulate HA via the cAMP pathway which does not induce an immediate calcium influx in human sperm cells (Strunker *et al*., 2011). Additionally, the relationship between HA (spontaneous and induced), intracellular stimulus-induced Ca^{2+} responses and fertilization rates at IVF was investigated. The primary aims of this study were to examine (i) the incidence of defects in the Ca^{2+}-signalling pathways that mediate HA in sperm from donors and from sub-fertile patients; (ii) if these defects (assessed by measuring HA/calcium signalling) were related to IVF success.

Materials and Methods

Reagents

4-AP (Sigma Aldrich, Catalog number 275875-5G, UK), progesterone (Sigma Aldrich, Catalog number P8783-5G, UK), IBMX (Calbiochem, Catalog number 410957, UK) and ammonium chloride (NH_4Cl) (Sigma Aldrich, Catalog number 4316230J, UK) were dissolved in distilled water, ethanol, dimethyl sulphoxide (DMSO) and distilled water, respectively. Aliquots were diluted and added to the sperm suspensions to achieve a final concentration of 2 mM, 3.6 μM, 100 μM and 25 mM, respectively, volumes were chosen so that the maximum concentration of solvent was 1% (v/v). Fura-2 acetoxymethyl ester (Fura-2/AM) (Molecular Probes, Invitrogen, OR, USA) was dissolved in DMSO and used at a final concentration of 1 μM.

Media used for donor samples

Synthetic tubal fluid (STF; based upon Mortimer, 1986) was used as capacitating media (CM) for donor samples. It consisted of 4.7 mM KCl, 3 mM $CaCl_2$, 1 mM $MgSO_4$, 106 mM NaCl, 5.6 mM D-glucose, 1.5 mM NaH_2PO_4, 1 mM Na pyruvate, 41.8 mM Na lactate, 25 mM $NaHCO_3$, 1.33 mM glycine, 0.68 mM glutamine, 0.07 mM taurine, non-essential amino acids (1: 100 dilution in STF) and 30 mg/ml bovine serum albumin. A non-capacitating HEPES-buffered medium (NCM) adapted from the above but lacking in both albumin and bicarbonate (5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4 \cdot 7H_2O$, 116.4 mM NaCl, 5.6 mM D-glucose, 1.0 mM NaH_2PO_4, 2.7 mM Na pyruvate, 41.8 mM Na lactate and 25 mM HEPES) was also used.

Ethical approval

Written consent was obtained from each patient in accordance with the Human Fertilisation and Embryology Authority (HFEA) Code of Practice (version 8) under local ethical approval (08/S1402/6) from the Tayside Committee of Medical Research Ethics B. Similarly, volunteer sperm donors were recruited in accordance with the HFEA Code of Practice (version 8) under the same ethical approval.

Study subjects

Semen samples were obtained from three groups: 100 semen samples from 68 young healthy research donors (mainly students with no known fertility problems, aged 20–35 and with a normal sperm concentration and motility according to WHO criteria 1999), 181 sub-fertile patients who underwent IVF (170 patients) or ICSI (11 patients) treatment at the Assisted Conception Unit (ACU), Ninewells Hospital, Dundee, Scotland, between April 2009 and August 2011.

Semen samples

Semen samples from donors and patients were collected by masturbation into a sterile plastic container after 2–3 days of sexual abstinence. The samples were used for analysis after liquefaction of the semen at 37°C for approximately 30 min and within 1 h of production. Semen samples obtained from patients were assessed for the semen profile by clinical embryologists. Patients were selected for IVF or ICSI according to clinical indications and semen quality. For the latter, although it was no't always the case, men with approximately 1×10^6 progressively motile cells post-preparation were allocated to IVF and below this limit to ICSI.

With respect to the patient samples, to eliminate inter ejaculate-variation, the surplus of the clinical sample used in the IVF or ICSI treatment process was taken for analysis of HA and where possible intracellular Ca^{2+}. IVF fertilization rates were obtained in order to examine the potential functional relationship between stimulation with agonists known to act on different components of the Ca^{2+}-signalling system and IVF.

Density gradient centrifugation

For the donors, spermatozoa were isolated from seminal plasma by density gradient centrifugation (DGC) using PureSperm (Nidacon, Molndal, Sweden) diluted with NCM. After centrifugation (300*g*, 20 min), the supernatant was discarded and pellet was washed (500*g* for 10 min) resuspended in CM, and incubated for ~2 h at 37°C in a 5% CO_2 incubator. This incubation period in CM was chosen because in general, no further notable change was observed in the percentage of spontaneous/induced HA (see Supplementary data, Fig. S1) or in agonist-stimulated intracellular Ca^{2+} level. In the ACU commercially available media was used for sperm preparation. The spermatozoa were separated from semen by DGC using PureSperm diluted with Cook Sydney IVF Gamete Buffer, a HEPES-buffered solution (Cook Sydney IVF Limited, National Technology Park, Ireland, UK). After centrifugation, the pellet was washed by centrifugation at 500*g* for 10 min in 4 ml of Cook Gamete Buffer. If the samples were assigned for IVF, following centrifugation, the supernatant was discarded and pellet resuspended in Cook Sydney IVF fertilization medium, a bicarbonate-buffered medium similar to STF, containing 25 mM $NaHCO_3$ (Moseley *et al*., 2005; data not presented). If the sample was allocated for ICSI, the cells were washed in Cook Gamete Buffer. Following this, IVF samples were gassed with CO_2 and kept at room temperature (up to 4 h) until 1 hur before the insemination, at which point the sample was incubated at 37°C in a 5% CO_2 incubator. The ICSI samples were kept at room temperature (up to 4 h) until the time of the injection procedure. Following insemination or injection the remaining portion of the sample was analysed for basal and agonist-induced HA and if there was a sufficient yield of sperm available, for intracellular Ca^{2+} (see below). ICSI samples were prepared in the IVF clinic (in Cook Gamete Buffer™ which does not support the completion of capacitation; Moseley *et al*., 2005). These samples were only assessed for intracellular Ca^{2+} and were re-suspended in CM as part of the Fura loading protocol (see below).

Assessment of basal level of HA and sperm motion characteristics by CASA

A Hamilton Thorn CEROS machine (version 12) attached to an external microscope was used to assess motion characteristics. The concentration

of prepared spermatozoa from donors or patients was adjusted between 5 and 25 × 10^6/ml with CM. Samples with a concentration ≤2 × 10^6/ml were generally excluded from the study as the number of cells was insufficient to obtain data comparable to the other samples. The samples were mixed to ensure a homogenous concentration of spermatozoa. Hamilton-Thorn 2X-Cel chambers (20 μm depth) (Dual Sided Sperm Analysis Chamber, Hamilton Thorn Biosciences, Beverly, MA, USA) were pre-warmed at 37°C on a heated stage of an Olympus CX41 microscope (Olympus Corporation, Tokyo, Japan) after which 4 μl of sperm suspension was placed onto each slide chamber and then covered by a pre-warmed cover slip (22 mm × 22 mm). Slides were maintained at 37°C for ∼2 min prior to the start of data acquisition. For HA and motion characteristics were assessed under a negative phase contrast objective (×10) at a final magnification of ×100. Four different samples were assessed for each ejaculate and at least 200 motile cells were counted on randomly selected fields in each sample so that a minimum of approximately 800 motile cells were assessed in total. The percentage of hyperactivated cells was assessed using standard criteria to identify HA, namely VCL ≥150 μm/s, linearity ≤50%, and ALH ≥7 μm (Mortimer *et al*., 1998).

Assessment of hyperactivated motility in response to different agonists

Agonist stimulation was achieved by adding 1 μl of agonist to 99 μl of sperm suspension, giving final concentrations of 2 mM 4-AP, 100 μM IBMX, 3.6 μM progesterone or 25 mM NH_4Cl. Sperm HA and other kinematic parameters were then assessed as described above.

Twenty-one of the 68 donors produced more than one sample. The basal and 4-AP-induced HA for donors who produced two samples is presented in Supplementary data, Fig. S1. Importantly, the HA response to 4-AP was consistent between the assessments in all 21 donors with 20/21 donors showing a normal significant response in both assessments and 1/21 showing a poor response in both samples (Supplementary data, Fig. S2a).

Fertilization rate at IVF

Oocytes were considered normally fertilized when two pronuclei (2PN) and two distinct or fragmented polar bodies were observed. In IVF, the fertilization rate (FR) was calculated from the number of oocytes normally fertilized divided by the total number of inseminated oocytes. In order to reduce the influence of minimal egg numbers the data used for fertilization rates in this analysis are only those cases where at least four mature eggs were inseminated for IVF ($n = 145$). Fertilization rates where ICSI was the designated treatment were not taken into account as ICSI bypasses any functional requirement needed for a sperm to bind and penetrate the egg vestments. The median female age of IVF patients ($n = 145$) was 34 (31–37 25 and 75th centile, respectively). The median number of eggs recovered in the IVF patients ($n = 145$) was 10.5 (8–14; 25 and 75th centile, respectively).

Measurement of intracellular Ca^{2+}

After sperm preparation (either in the research laboratory for healthy donors or at the IVF laboratory for patients), 500 μl aliquots of sperm suspension (concentration adjusted to ∼8–20 × 10^6/ml with CM) were loaded with Fura-2 by incubating them with 1 μM Fura-2-AM for 12 min at 37°C under 5% CO_2 in the dark (the DMSO concentration was 0.2% (v/v)), then centrifuged at 500*g* for 15 min. The supernatant was removed and the pellet resuspended in 100 μl of medium. Since loading with Fura-2 required a centrifugation step followed by resuspension, samples from all three groups (donors, IVF, ICSI) could be resuspended in the same medium (CM). The intracellular Ca^{2+} response is rapidly developed with capacitation (Baldi *et al*., 1991; Bedu-Addo *et al*., 2005). Fluorescence measurements were carried out on a FLUOstar Omega (BMG Labtech Offenburg, Germany) using alternating excitation wavelengths of 340 nm and 380 nm and recording emission at 510 nm. Stimulus-induced increments in the ratio of emission intensities (at 340 and 380 excitation) were used to quantify changes in $[Ca^{2+}]_i$ concentration. Aliquots of 50 μl were pipetted into a 96-well plate and 100 s of control data (20 readings) were acquired (resting level (*R*)). Five microlitres of agonist (4-AP or progesterone) were then added and the response was recorded. Usually, a minimum of ∼2 million cells per well (50 μl) were required for robust results. In a number of cases (primarily those involving ICSI samples) this could not be achieved and no measurement of intracellular Ca^{2+} response was reported.

To examine the consistency of different samples from the same donors, eight donors were tested on two occasions with an interval of at least 1 month between donations. Responses to progesterone were very similar between samples ($R^2 = 0.972$) (Supplementary data, Fig. S2b). One donor who showed a poor response was tested on several different occasions with consistent results (Supplementary data, Fig. S2b).

Definition of failed HA and Ca^{2+} responses among donors and sub-fertile patients

A failed HA response was recorded when agonist stimulation did not induce a significant change in the % of hyperactivated cells compared with control (basal) level (assessed by one-way analysis of variance (ANOVA) and non-parametric ANOVA on ranks Kruskal–Wallis test on four different samples from each ejaculate, $P < 0.05$).

To define failed Ca^{2+} responses a normal range was determined from the distribution of response amplitudes (agonist-induced increments in the 340/380 ratio) in donor populations stimulated with 4-AP and progesterone (Supplementary data, Fig. S3). Upper and lower limits were set to include 99% of the distribution (mean ± 2.58 × SD; see the Statistical analysis section below for normalizing procedure). Using this approach, the cut-off values for a failed Ca^{2+} response were increments in the 340/380 ratio of ≤0.1 upon addition of 4-AP or progesterone.

Statistical analysis

Normality of data was assessed according to the Kolmogorov–Smirnov test. Results are expressed as the mean ± SD (standard deviation), median and range for HA. Statistical comparisons were made using the ANOVA if the data were either originally normally distributed or normalized after transformation by square root. However, some HA and intracellular Ca^{2+} data were not normalized by transformation. Thus, the statistical comparisons for these data were examined by non-parametric ANOVA Kruskal–Wallis one-way ANOVA on ranks. The correlation between HA and intracellular Ca^{2+} in response to agonists (4-AP and progesterone) was examined using Pearson's correlation coefficient. The correlations between HA and intracellular Ca^{2+} in the basal level and in response to agonists with IVF fertilization rates were examined by Spearman's correlation coefficient (because the data were not normalized after transformation) and Pearson's correlation coefficient, respectively.

To define a cut-off value for a failed Ca^{2+} response, the data were log-transformed and cut-off values were calculated based on mean and SD. The differences in proportions of failed responders between the populations (donors and IVF patients) and between agonists were examined using a χ^2 test. $P < 0.05$ was considered significant. All statistical analyses were performed using the SigmaStat 10 statistical package (Systat Software Inc., Chicago, IL, USA).

Results

Induction of HA in donor and IVF patient samples

Of the four agonists used (4-AP, NH_4Cl, progesterone, IBMX), 4-AP was the most potent inducer of HA in sperm from donors, the magnitude of the effect being significantly greater than that of all other agonists ($P < 0.001$; Fig. 1). IBMX was the next most effective agonist, followed by progesterone (Fig. 1). As reported in detail elsewhere Alasmari *et al.* (2013), 25 mM NH_4Cl was not an effective inducer of HA (NS; Fig. 1) though in a minority of samples, primarily those with low basal HA ($\leq$10% HA), there was a detectable response ($P < 0.05$).

The relative efficacy of the four different agonists on IVF patient samples was similar to that seen with donor samples (Fig. 1 compare left and right panels), but for the three effective stimuli (4-AP, IBMX and progesterone) the percentage of hyperactivated cells after treatment was significantly lower in IVF patients ($P < 0.05$) (Fig. 1 and Supplementary data, Fig. S4). The basal level of HA was significantly lower in IVF patients compared with donors ($P < 0.05$).

Intracellular calcium responses to 4-AP and progesterone in donors and patients

Spermatozoa from 37 donors, 68 IVF and 11 ICSI patients were assessed for their $[Ca^{2+}]_i$ responses to 4-AP and progesterone. The 340/380 ratio for fluorescence of fura-2 (R) in resting cells differed between individuals. To facilitate comparison the data from each individual were normalized to the pre-stimulus value (Fig. 2). Descriptive statistics of the raw 340/380 ratio data are presented in Supplementary data, Table SI. The basal Ca^{2+} was significantly lower in spermatozoa from ICSI patients, compared with research donors and IVF patients ($P < 0.05$) (Supplementary data, Table SI).

Both 4-AP and progesterone induced a biphasic elevation of intracellular Ca^{2+} in donor sperm, comprising a $[Ca^{2+}]_i$ transient followed by a sustained phase. However, the shape of these responses was clearly different, the peak ratio increase in the transient induced by progesterone typically being higher than that induced by 4-AP, whereas the $[Ca^{2+}]_i$ increase induced by 4-AP was sustained for longer (Fig. 2; Supplementary data, Fig. S5). Both agonists were effective in raising $[Ca^{2+}]_i$ in sperm from the two patient groups, but whereas responses in IVF patients were similar to those in donor sperm, the magnitude of the response was much smaller in ICSI patients (Fig. 2, $P < 0.05$). There was a significant difference between the ratio at the sustained phase in response to 4AP between donor and ICSI patients ($P < 0.001$) and between IVF and ICSI patients ($P = 0.007$).

Failed HA and Ca^{2+} responses among donors and sub-fertile patients

To further characterize the differences between donor and patient groups we assessed the occurrence of HA and $[Ca^{2+}]_i$ signal 'failures' (as defined in the Materials and Methods section). In response to stimulation with 4-AP only 2% of the donor samples gave a 'failed' HA response, whereas HA failure occurred in $\approx$10% of the IVF patients ($\chi^2 = 7.9$, $P = 0.025$). Due to practical and technical limitations it was not possible to assess intracellular Ca^{2+} for all patients but of those who could be tested a failed response was recorded in 2/61 ($\sim$3%) and 2/7 (28.6%) of IVF and ICSI patients, respectively ($P = 0.048$). In contrast, none of 37 donor responses fell below the threshold defining failure (see Materials and Methods). The two IVF patients who showed a failed intracellular Ca^{2+} response to 4-AP did not hyperactivate in response to 4AP and had poor IVF fertilization rates (39%, 6%).

Stimulation with progesterone induced a significant $[Ca^{2+}]_i$ response in >97% of samples from donors and all IVF patients. However, as with 4-AP, ICSI patients showed a higher failure rate (3/11 men, 27.3%; $P = 0.035$, cf. donors). Failure of significant HA was much more common in response to stimulation with progesterone compared with 4-AP, 51% of donors and 62% of IVF samples failing to respond. In some of the samples where there was no significant induction of HA there were still detectable effects on motility. Changes in one or more kinematic parameters (VCL, ALH or LIN) were observed in 56% (19/34 samples) of the donors who failed to hyperactivate and 40% (23/57) of the IVF patients. Twenty of 65 (31%) of IVF samples screened for both HA and intracellular Ca^{2+} showed a normal Ca^{2+} response to progesterone but no significant change in HA or kinematics as measured by CASA.

In most cases failure of HA was not stimulus-specific, the majority of samples that showed a failed HA response to 4-AP showing no significant response to IBMX, progesterone or NH_4Cl (Supplementary data, Table SII).

Correlation between HA and intracellular $[Ca^{2+}]_i$

To determine whether or not there was a relationship between the magnitude of agonist-induced intracellular Ca^{2+} elevation (absolute increase in 340/380 ratio) and HA (absolute increase % cells), we examined responses in samples from IVF patients to 4-AP ($n = 57$) and progesterone ($n = 65$). There was a significant correlation for responses induced by 4-AP ($R = 0.35$, $P = 0.009$). Figure 3 shows data from four representative IVF patient samples upon stimulation with 4-AP. There was no significant relationship between the Ca^{2+} and HA responses induced by progesterone ($R_s = -0.002$, $P = 0.98$), an observation consistent with the great disparity in $[Ca^{2+}]_i$ and HA responses seen with this agonist.

Relationship between agonist-induced HA, $[Ca^{2+}]_i$ and IVF fertilization rates

To assess the clinical significance of $[Ca^{2+}]_i$ and HA responses, basal and 4-AP-induced $[Ca^{2+}]_i$ and HA in cells from IVF patients were examined in relation to fertilization rates. Analysis of $[Ca^{2+}]_i$ data showed a significant relationship between basal intracellular Ca^{2+} (340/380 ratio before stimulation) and fertilization rates ($R = 0.3$, $P = 0.025$). Additionally, there was a significant relationship between the increment in $[Ca^{2+}]_i$ induced by 4-AP (52 patients) and progesterone (57 patients) and fertilization rates ($R = 0.28$, $P = 0.047$, $R = 0.280$, $P = 0.037$, respectively).

Fertilization rate was significantly related both to the basal level of HA ($R_s = 0.19$; $P = 0.02$) and to the 4-AP-induced increment in HA ($R_s = 0.18$, $P = 0.029$) (Fig. 4A). When the data were separated into four groups according to the fertilization rates achieved, FR1

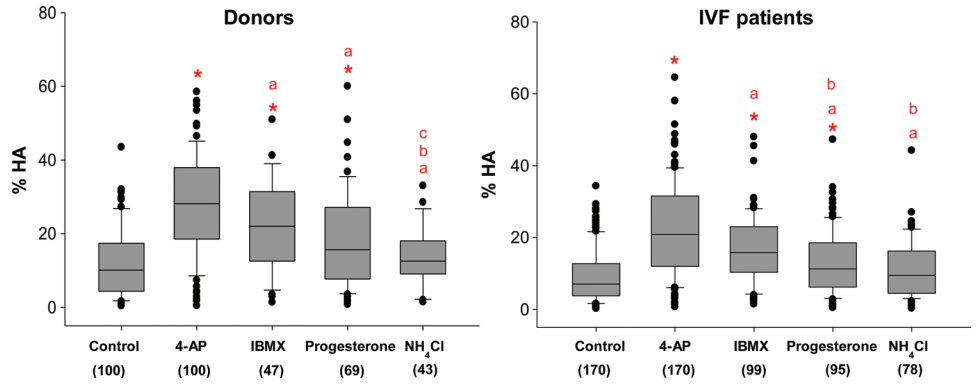

Figure 1 Comparison of four different agonists on HA) in two populations (donors and IVF patients). Box and whisker plots illustrating the data distribution for HA in the baseline (control) and samples treated with 4-AP, IBMX, progesterone and NH_4Cl from donors and IVF patients. The boxes represent the interquartile range and lines within them are the medians. The number in brackets is the sample size. *Highlights that the agonist-induced HA is significantly different to baseline, (**a**) highlights a significant difference between responses to 4-AP and all other agonists (**b**) highlights a significant difference between responses to IBMX compared with progesterone and NH_4Cl and (**c**) highlights a significant difference between responses to progesterone and NH_4Cl. Significance was considered as $P < 0.05$ assessed by one-way ANOVA and non-parametric ANOVA on ranks Kruskal–Wallis test. Not all samples were tested with each of the agonists.

≤25%, FR2 25%–50%, FR3 50–75%, FR4 >75%, it was clear that sperm samples giving the highest fertilization rate were more likely to exhibit a large increment in HA when stimulated with 4-AP (Fig. 4B). Incidence of failure to respond to 4-AP was similarly related to fertilization success. There was no significant relationship between fertilization rates and increment in HA in response to progesterone ($n = 84$) or to the other agonists IBMX ($n = 89$) and NH_4Cl ($n = 68$) (data not shown).

Discussion

The primary aims of this study were to examine the prevalence of defects in Ca^{2+}-signalling pathways mediating HA among donors and sub-fertile patients and the significance of such defects for IVF success. The most effective inducer of HA, in both donors and IVF patients, was 4-AP and this compound was therefore the focus of this study. Failure of HA in response to stimulation with 4-AP was significantly more common in IVF patients than in donors and failure of Ca^{2+} signalling was a common observation in ICSI patients. Importantly, both 4-AP-induced intracellular Ca^{2+} responses and consequent induction of HA were significantly related to IVF fertilization rate. It has been established previously that there are differences in HA (spontaneous and in response to physiological or artificial stimulants) between men with proven fertility and sub-fertile patients (Burkman, 1984; Tesarik *et al.*, 1992; Peedicayil *et al.*, 1997; Munire *et al.*, 2004). The data reported here document, for the first time, the biological significance of responses to direct, targeted manipulation of the Ca^{2+}-signalling apparatus in human spermatozoa.

This study not only employed a large sample size (compared with other clinical studies on HA and $[Ca^{2+}]_i$ signalling) but responses to agonist stimulation were also assessed in samples that were used for IVF treatment, permitting direct comparison of functional responses with fertilization success. In achieving this it was necessary to analyse the IVF samples in keeping with the clinical protocols. We believe this had a minimal effect on the results as (i) the donors and IVF samples were prepared using similar techniques and in comparable media supporting capacitation (Supplementary data, Fig. S6, Moseley *et al.*, 2005) and (ii) though IVF samples were incubated in CM for longer than donor samples, preliminary experiments showed that 4--AP-induced HA was not modified by varying incubation time (2–4 h) under capacitating conditions (Supplementary data, Fig. S1). (iii) Though ICSI samples were prepared and incubated in non-capacitating conditions in the IVF laboratory, transfer to capacitating conditions restores $[Ca^{2+}]_i$ responses within minutes (Bedu-Addo *et al.*, 2005) and as such calcium assessments are robust and allow comparison between donors, IVF and ICSI patients. The robust nature of the data reported here is illustrated by the consistency in agonist-induced $[Ca^{2+}]_i$ and HA responses between ejaculates (Supplementary data, Fig. S2).

Differing potency of the agonists used

4-AP was the most effective inducer of HA in both donor and IVF cells. Cytoplasmic alkalinization by 25 mM NH_4Cl is equivalent to the effect on pHi of 2 mM 4-AP Alasmari *et al.* (2013). NH_4Cl induced Ca^{2+} influx under both capacitating and non-capacitating conditions (data not presented), yet there was negligible effect on HA. These data confirm the pivotal role of stored Ca^{2+} in HA of human sperm and demonstrate that Ca^{2+}-influx induced by elevated pHi alone is not sufficient to induce robust levels of HA (Fig. 1). Progesterone induced an intracellular calcium response in >97% of donors and all IVF patients yet HA was weak. It has been suggested that the large progesterone-induced $[Ca^{2+}]_i$ transient (up to 40 s) is accompanied by a burst of HA in some cells (Gakamsky *et al.*, 2009; Servin-Vences *et al.*, 2012). This brief effect is hard to detect using standard CASA and in our study HA was not significantly different between 1 and 5 min of treatment with progesterone (data not presented). Thus, Ca^{2+} influx (through CatSper) induced by progesterone, similar to cytoplasmic alkalinization, was not sufficient to induce sustained

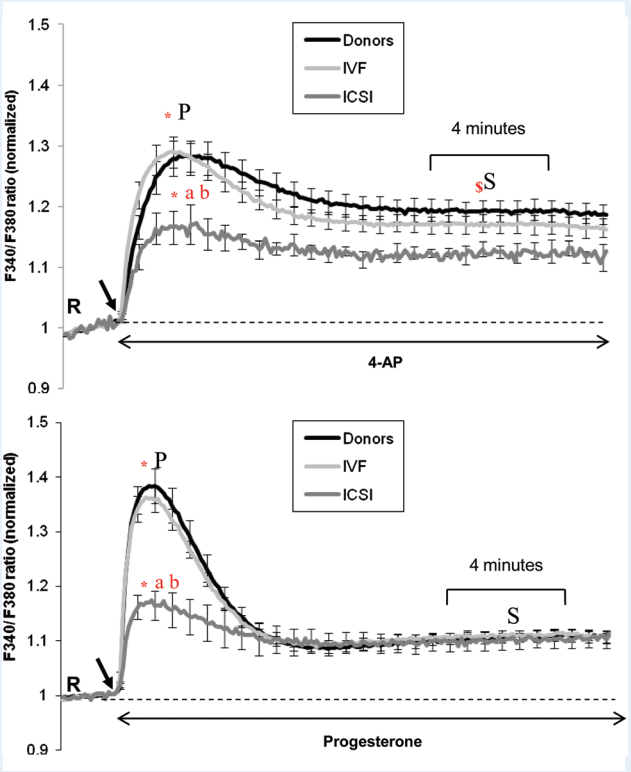

Figure 2 Ca^{2+} ratio in response to 4-AP and progesterone in three populations (donors, IVF and ICSI patients). Intracellular Ca^{2+} responses induced by 4-AP (upper panel) and progesterone (lower panel) in donors (4-AP $n = 36$, progesterone $n = 37$), IVF (4-AP $n = 61$, progesterone $n = 68$) and ICSI patients (4-AP $n = 7$, progesterone $n = 11$). Each trace shows mean of n fluorimetric (population) responses ± SE. Agonists were added (indicated by black arrow) at 100 s after acquisition of 20 readings at resting level (R). The data for each sample were normalized to pre-stimulus (R) level to facilitate comparison. *Significant difference between the ratio at the peak (P) and the initial resting level (R), (**a**) significant difference of ratio at peak between donor and ICSI patients, (**b**) significant difference of ratio at peak between IVF and ICSI patients. $^{\$}$Significant difference between the ratio at the sustained phase (S) between donor and ICSI patients ($P < 0.001$) and between IVF and ICSI patients ($P = 0.007$). There was no significant difference in the sustained response with progesterone between the groups. Significance was considered as $P < 0.05$ assessed non-parametric ANOVA on ranks Kruskal–Wallis test.

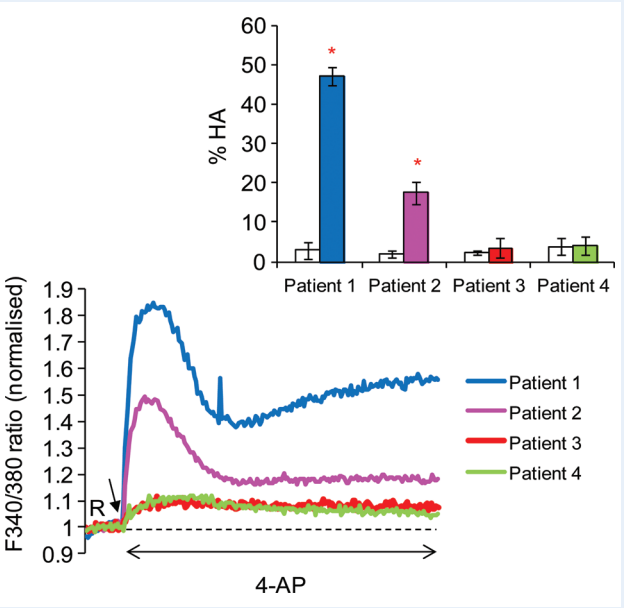

Figure 3 Intracellular Ca^{2+} in response to 4-AP from four IVF patients including two with a failed Ca^{2+} response. Inset shows % HA from the same patients labelled with the same colours; white bars show % HA in the baseline (control) and coloured bars show % HA in samples treated with 4-AP. Patients 3 and 4 showed a failed intracellular Ca^{2+} and HA responses to 4-AP (IVF fertilization rates for patients 3 and 4–39% and 6% respectively). 4-AP was added to suspensions at 100 s after acquisition of 20 readings at resting level (R) indicated by black arrow. The HA data (inset) are the mean ± SD. *Values are significantly different to baseline ($P < 0.05$).

changes in kinetic parameters and consistently reach the threshold criteria to identify HA. While CatSper channels are essential for HA in mice (Ren *et al.*, 2001; Carlson *et al.*, 2003; Quill *et al.*, 2003), their role in humans remains largely unknown (Brenker *et al.*, 2012). Genetic studies have indicated that mutations in human CatSper channels are significant in rare cases of male infertility (Avidan *et al.*, 2003; Avenarius *et al.*, 2009), but HA was not examined. Our observations clearly do not preclude involvement of CatSper in human HA. CatSper may become activated during capacitation, supporting spontaneous HA Alasmari *et al.* (2013) and when strongly activated (as occurs during the progesterone transient) CatSper may trigger activation of Ca^{2+} stores or store-operated channels (Lefievre *et al.*, 2012). It is also possible that more sophisticated sperm function tests may be required to determine the effect of direct CatSper activation such as penetration into a viscous media (Ivic *et al.*, 2002) or more sophisticated analysis of flagella motion—3-dimensional tracking (Su *et al.*, 2012) and high-speed videomicroscopy (Kirkman-Brown and Smith 2011; Curtis *et al.*, 2012).

IBMX was used to stimulate HA by strong activation of the cAMP pathway, which does not induce an immediate Ca^{2+} influx in human sperm cells (Strunker *et al.*, 2011). The majority of the samples from donors and IVF patients responded to IBMX with a significant increase in HA, consistent with other studies on human sperm, which have reported a specific influence of phosphodiesterase inhibitors such as pentoxyfilline on the kinematic parameters defining HA (Tesarik *et al.*, 1992; Kay *et al.*, 1993; Tournaye *et al.*, 1994). These observations are in contrast to other studies on bull sperm which have documented that HA occurred upon elevation of intracellular Ca^{2+} and did not respond to the AC/cAMP/PKA-signalling pathway (Ho *et al.*, 2002; Marquez and Suarez, 2004, 2008). The difference in findings between studies on human and bull sperm may simply reflect species variation in the requirements for HA.

Differences in HA and calcium between donor and patient groups

4-AP-induced HA was correlated with the amplitude of the $[Ca^{2+}]_i$ response, confirming that 4-AP-induced HA was mediated by this

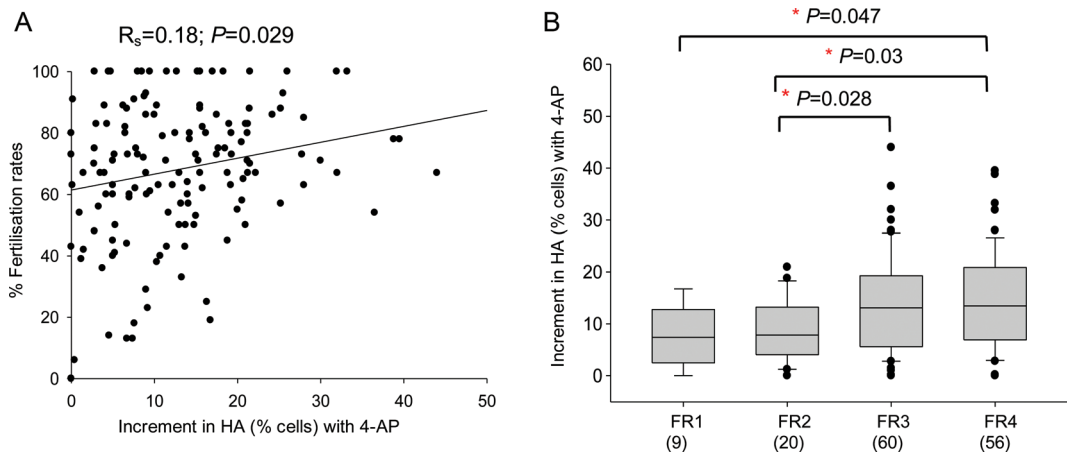

Figure 4 Relationship between increment in HA (% cells) stimulated by 4-AP and IVF fertilization rate. (**A**) 4-AP-induced increment in HA (% cells) was significantly correlated to fertilization rate ($R_s = 0.18$; $P = 0.031$, $n = 145$). (**B**) Expression of these data in four defined groups according to fertilization rate: FR 1 ≤25%, FR2 >25–≤50%, FR3 >50–≤75%, FR4 >75%. Box and whisker plots show the 4-AP-induced increment in HA for the samples from patients in each group. The boxes represent the inter-quartile range and lines within them are the medians. The number in brackets is the sample size. Significance was assessed by one-way ANOVA.

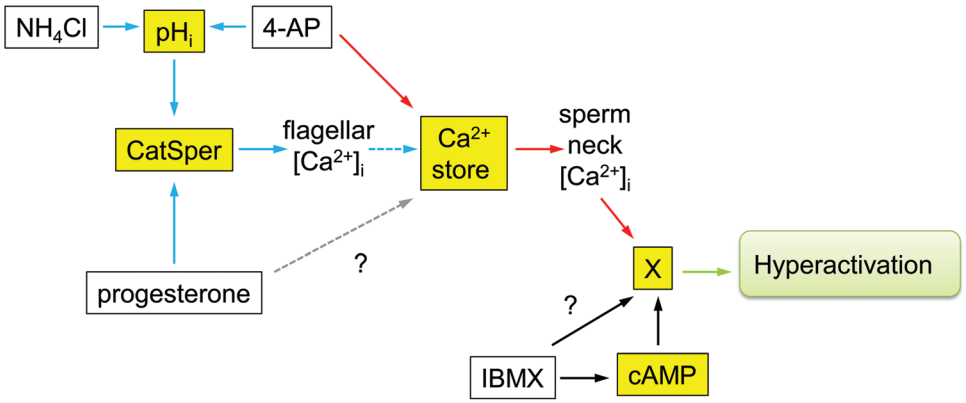

Figure 5 Model of the calcium-signalling cascade in the human spermatozoon: key points in the pathway affected in sub-fertile men (adapted from Barratt and Publicover, 2012). White boxes show compounds used to manipulate pH_i (NH_4Cl, 4AP), stored calcium (4AP) and cAMP (IBMX). Yellow boxes show signalling components activated by these compounds (pHi, Ca^{2+}, cAMP). Blue and red arrows show Ca^{2+}-signalling pathways involving CatSper and the calcium store, respectively. The Ca^{2+}-store pathway may involve activation of SOCs (not shown; Lefievre *et al.*, 2012). Dashed blue arrow shows the mobilization of stored Ca^{2+} downstream of CatSper activation by CICR. This occurs in a minority of the cells, is sensitive to modulation (e.g. by capacitation) and is responsible for CatSper-mediated hyperactivation. Dashed grey arrow shows possible modulation of Ca^{2+}-store mobilization by progesterone through CatSper-independent mechanisms (Sagare-Patil *et al.*, 2012). X represents unknown target mechanism/pathway whereby following mobilization of stored Ca^{2+} hyperactivation is stimulated. ? represents potential pathway subject to further experimentation. The amplitude of the calcium transient (stimulated by both 4AP and progesterone) and the hyperactivation response (to 4AP) were significantly related to IVF fertilization rates suggesting the occurrence of important abnormalities in the Ca^{2+}-signalling pathways mediated by CatSper (blue) and the calcium store (red). The calcium signal (4AP and progesterone) in the ICSI patients was significantly lower than in the donors or the IVF patients (~25%; Fig. 2), providing further evidence of abnormalities in the CatSper functioning/operating complex and calcium store in male infertility. Examination of individual cases demonstrated ~10% of men undergoing IVF had defective calcium hyperactivation. Although the data are limited, a number of these men did not show a hyperactivation response to IBMX (probably through cAMP, black arrows, ?). Previous studies have indicated that, in humans, increases in cAMP are do not lead to changes in $[Ca^{2+}]_i$ (Brenker *et al.*, 2012), thus these men may suffer from a specific defect in hyperactivation as opposed to a Ca^{2+}-signalling deficit (Supplementary data, Table SII).

pathway. The proportion of samples in which 4-AP failed to induce normal HA and $[Ca^{2+}]_i$ responses was significantly greater in IVF samples than in donors showing that malfunction of the Ca^{2+}-signalling pathway activated by 4-AP is likely to be responsible for a proportion of cases of male-factor sub-fertility. Additionally, there was a clear difference in $[Ca^{2+}]_i$ responses between ICSI patients and other

populations (donors and IVF patients) demonstrating increased incidence of $[Ca^{2+}]_i$ abnormalities in the former. Interestingly, the basal Ca^{2+} was significantly lower in spermatozoa from ICSI patients (Supplementary data, Table SI; $P < 0.05$). This suggests that these cells have a reduced ability to initiate the Ca^{2+}-signalling cascade. The reasons for this are unclear but may be due to (i) low expression or abnormalities in CatSper leading to reduction in Ca^{2+} influx across the plasma membrane and consequently poor recruitment of intracellular Ca^{2+} stores and/or (ii) the sperm cytoplasm does not become sufficiently alkalinized to activate CatSper due to abnormality in the expression or regulation of HV1; and/or (iii) defects in internal store channels such as RyRs, IP3R or SOCs.

Samples that gave a failed HA response to 4-AP usually failed to respond to IBMX, progesterone or NH_4Cl. Though the lower efficacy of these stimuli as inducers of HA (particularly progesterone and NH_4Cl) makes interpretation complex, it appears many HA failures involve a signalling node or late stage in a cascade such that alternative stimulation (such as increasing cAMP) does not bypass the lesion (Fig. 5). HA failure may reflect either failure of Ca^{2+} signalling itself or of events downstream of Ca^{2+}, independent of the $[Ca^{2+}]_i$ signal amplitude. Analysis of HA and $[Ca^{2+}]_i$ in 4-AP-stimulated cells showed that the amplitudes of the two responses were significantly correlated and that in those samples where $[Ca^{2+}]_i$ signal failure occurred (≈3%), there was also a failure of HA. Thus it is likely that failed HA in sub fertile men can reflect failure of Ca^{2+} signalling. Potential defects include impaired capacitation, which may affect activity of CatSper channels (Lishko *et al.*, 2011; Strunker *et al.*, 2011), resulting in a low resting $[Ca^{2+}]_i$ and consequent effects on emptying and filling of the internal stores. It has been suggested that Src kinase located in the neck/midpiece and in the post-acrosomal region of human sperm head regulates Ca^{2+} store mobilization during capacitation (Varano *et al.*, 2008). Further investigation of Ca^{2+} store filling and mobilization in sperm is required to clarify this.

Stimulus-induced $[Ca^{2+}]_i$ elevation, HA and fertilization success

Fertilization rate of the IVF samples was correlated with basal HA, a finding that is concordant with data from other studies (Burkman, 1984; Wang *et al.*, 1993; Sukcharoen *et al.*, 1995). More significantly, the ability of 4-AP to raise $[Ca^{2+}]_i$ and to induce HA was correlated significantly with IVF outcomes, providing clear evidence of a biological role of the sperm Ca^{2+}-signalling apparatus (almost certainly store mobilization; see above) in human sperm (Fig. 4). Amplitude of the $[Ca^{2+}]_i$ transient induced by progesterone, probably reflecting activation of CatSper (Lishko *et al.*, 2011; Strünker *et al.*, 2011), was similarly correlated to fertilization rates at IVF, consistent with other studies (Krausz *et al.*, 1996). However, progesterone was not a potent inducer of HA (Fig. 1) and targeted activation of CatSper is not sufficient to induce HA in human sperm Alasmari *et al.* (2013), thus there was no significant correlation between progesterone-induced HA and IVF success. Defective Ca^{2+} responses to progesterone were not seen in IVF patients and occurred in one donor and three ICSI patients. This low incidence of functionally significant CatSper defects, as manifested by failed $[Ca^{2+}]_i$ responses, suggests that such failures are relatively rare. Patch clamping studies will be required to confirm abnormalities in CatSper function (Kirichok and Lishko, 2011; Lefievre *et al.*, 2012).

In summary, this study highlights the biological role and importance of Ca^{2+}-signalling (and neck/midpiece Ca^{2+} store mobilization) for fertilization at IVF and identifies for the first time, the incidence of store defects among donors, IVF and ICSI patients. Further studies are required to investigate the nature of these defects. Such studies will act as a platform for a potential drug-based screening programme to augment/modulate calcium mobilization as a possible rational treatment for sperm dysfunction.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Acknowledgements

The authors are very grateful to all members of the Assisted Conception Unit at Ninewells Hospital for their invaluable assistance in obtaining donor and patient samples for research and training purposes, in particular the embryologists (Ellen, Sylvia, Anne and Philip) and nurses. We are also grateful to all the patients and donors who took part in this study. The authors acknowledge other members of the laboratory, in particular Dr Lindsay Tulloch, Dr Steve Tardif and Steven Mansell for their continual helpful advice and comments, and Evelyn Barratt for assisting with the recruitment of patients and donors. We also acknowledge the statistical support of Professor Steve Hubbard, Professor C De Jonge for reading and commenting on the manuscript and Timo Struenker for advice regarding FLUOstar.

Authors' roles

W.A. performed the sperm function assays, prepared the cells for analysis, analysed the initial data and wrote, the first draft of the manuscript. S.K.O. was involved in the experimental design and interpretation of data. C.L.R.B., S.J.P. and V.K. designed the study and obtaining funding for the experiments. S.M.D.S., V.K. and S.K.O. were involved in the recruiting and consenting of patients. E.F. screened some patients for the HA assay. K.M.W. was involved in the IVF, experimental design and delivery of ART samples. C.L.R.B. and S.J.P. were responsible for and wrote the final drafts of the manuscript. All authors contributed to the editing of the manuscript.

Funding

Resources from a Wellcome Trust Project Grant (# 086470, S.J.P.C.L.R.B. PI) primarily funded the study. Work in the authors' laboratories is funded by The Wellcome Trust, TENOVUS (Scotland), University of Dundee, MRC (Developmental Pathway Funding Scheme), Ministry of Higher Education—Kingdom of Saudi Arabia (Ph.D. studentship to W.A.), NHS Tayside and Scottish Enterprise. Resources from a Wellcome Trust Project Grant (principal investigators C.L.R.B. and S.J.P. (grant # 086470)) primarily funded the data presented in this study. Funding to pay the Open Access Publication charges for this article was provided by the Wellcome Trust.

Conflict of interest

None declared. The Excel spread sheets with all the primary data are available from c.barratt@dundee.ac.uk.

References

Aitken RJ, Henkel RR. Sperm cell biology: current perspectives and future prospects. *Asian J Androl* 2011;**13**:3–5.

Aitken RJ, Irvine DS, Wu FC. Prospective analysis of sperm-oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. *Am J Obstet Gynecol* 1991;**164**:542–551.

Alasmari W, Costello S, Correia J, Oxenham SK, Morris J, Fernandes L, Kirkman-Brown J, Michelangeli M, Publicover S, Barratt CLR. Ca^{2+} signalling through CatSper and Ca^{2+} stores generate different behaviours in human sperm. *J Biol Chem* 2013;**288**:6248–6258.

Avenarius MR, Hildebrand MS, Zhang Y, Meyer NC, Smith LL, Kahrizi K, Najmabadi H, Smith RJ. Human male infertility caused by mutations in the CATSPER1 channel protein. *Am J Hum Genet* 2009;**84**:505–510.

Avidan N, Tamary H, Dgany O, Cattan D, Pariente A, Thulliez M, Borot N, Moati L, Barthelme A, Shalmon L *et al.* CATSPER2, a human autosomal nonsyndromic male infertility gene. *Eur J Hum Genet* 2003;**11**:497–502.

Baldi E, Casano R, Falsetti C, Krausz C, Maggi M, Forti G. Intracellular calcium accumulation and responsiveness to progesterone in capacitating human spermatozoa. *J. Androl* 1991;**12**:323–330.

Barratt CL, Publicover SJ. Sperm are promiscuous and CatSper is to blame. .. *EMBO J* 2012;**31**:1624–1626.

Barratt CL, Mansell S, Beaton C, Tardif S, Oxenham S. Diagnostic tools in male infertility-the question of sperm dysfunction. *Asian J Androl* 2011; **13**:53–58.

Bedu-Addo K, Lefièvre L, Moseley FL, Barratt CL, Publicover SJ. Bicarbonate and bovine serum albumin reversibly 'switch' Bicarbonate and bovine serum albumin reversibly 'switch' capacitation-induced events in human spermatozoa. *Mol Hum Reprod* 2005;**11**:683–691.

Bedu-Addo K, Costello S, Harper C, Machado-Oliveira G, Lefievre L, Ford C, Barratt CL, Publicover S. Mobilisation of stored calcium in the neck region of human sperm—a mechanism for regulation of flagellar activity. *Int J Dev Biol* 2008;**52**:615–626.

Bhaskar A, Subbanna PK, Arasan S, Rajapathy J, Rao JP, Subramani S. 4-aminopyridine-induced contracture in frog ventricle is due to calcium released from intracellular stores. *Indian J Physiol Pharmacol* 2008;**52**:366–374.

Brenker C, Goodwin N, Weyand I, Kashikar D, Naruse M, Krahling M, Muller A, Kaupp B, Strunker T. The CatSper channel: a polymodal chemosensor in human sperm. *EMBO J* 2012;**31**:1654–1665.

Burkman LJ. Characterization of hyperactivated motility by human spermatozoa during capacitation: comparison of fertile and oligozoospermic sperm populations. *Arch Androl* 1984;**13**:153–165.

Carlson AE, Westenbroek RE, Quill T, Ren D, Clapham DE, Hille B, Garbers DL, Babcock DF. CatSper1 required for evoked Ca^{2+} entry and control of flagellar function in sperm. *Proc Natl Acad Sci USA* 2003;**100**:14864–8.

Chang H, Suarez SS. Two distinct Ca^{2+} signaling pathways modulate sperm flagellar beating patterns in mice. *Biol Reprod* 2011;**85**:296–305.

Costello S, Michelangeli F, Nash K, Lefievre L, Morris J, Machado-Oliveira G, Barratt CL, Kirkman-Brown J, Publicover S. Ca^{2+}-stores in sperm: their identities and functions. *Reprod* 2009;**138**:425–437.

Curtis MP, Kirkman-Brown JC, Connolly TJ, Gaffney EA. Modelling a tethered mammalian sperm cell undergoing hyperactivation. *J Theor Biol* 2012;**309**:1–10.

DeMott RP, Suarez SS. Hyperactivated sperm progress in the mouse oviduct. *Biol Reprod* 1992;**46**:779–785.

Gakamsky A, Armon L, Eisenbach M. Behavioral response of human spermatozoa to a concentration jump of chemoattractants or intracellular cyclic nucleotides. *Hum Reprod* 2009;**24**: 1152–1163.

Grimaldi M, Atzori M, Ray P, Alkon DL. Mobilization of calcium from intracellular stores potentiation of neurotransmitter-induced calcium transients and capacitative calcium entry by 4-aminopyridine. *J Neurosci* 2001;**21**:3135–3143.

Gu Y, Kirkman-Brown JC, Korchev Y, Barratt CL, Publicover SJ. Multi-state, 4-aminopyridine-sensitive ion channels in human spermatozoa. *Dev Biol* 2004;**274**:308–317.

Gwathmey TM, Ignotz GG, Suarez SS. PDC-109 (BSP-A1/A2) promotes bull sperm binding to oviductal epithelium *in vitro* and may be involved in forming the oviductal sperm reservoir. *Biol Reprod* 2003;**69**:809–815.

Harper CV, Barratt CL, Publicover SJ. Stimulation of human spermatozoa with progesterone gradients to simulate approach to the oocyte. Induction of $[Ca^{2+}](i)$ oscillations and cyclical transitions in flagellar beating. *J Biol Chem* 2004;**279**:46315–46325.

Ho HC, Suarez SS. An inositol 1,4,5-trisphosphate receptor-gated intracellular $Ca(^{2+})$ store is involved in regulating sperm hyperactivated motility. *Biol Reprod* 2001;**65**:1606–1615.

Ho HC, Suarez SS. Characterization of the intracellular calcium store at the base of the sperm flagellum that regulates hyperactivated motility. *Biol Reprod* 2003;**68**:1590–1596.

Ho HC, Granish KA, Suarez SS. Hyperactivated motility of bull sperm is triggered at the axoneme by Ca^{2+} and not cAMP. *Dev Biol* 2002; **250**:208–217.

Ho K, Wolff CA, Suarez SS. CatSper-null mutant spermatozoa are unable to ascend beyond the oviductal reservoir. *Reprod Fertil Dev* 2009; **21**:345–350.

Hull MG, Glazener CM, Kelly NJ, Conway DI, Foster PA, Hinton RA, Coulson C, Lambert PA, Watt EM, Desai KM. Population study of causes, treatment, and outcome of infertility. *Br Med J (Clin Res Ed)* 1985;**29**:1693–1697.

Irvine DS. Epidemiology and aetiology of male infertility. *Hum Reprod* 1998(Suppl 1);**13**:33–44.

Ishida Y, Honda H. Inhibitory action of 4-aminopyridine on $Ca(^{2+})$-ATPase of the mammalian sarcoplasmic reticulum. *J Biol Chem* 1993; **268**:4021–4024.

Ivic A, Onyeaka H, Girling A, Brewis IA, Ola B, Hammadieh N, Papaioannou S, Barratt CL. Critical evaluation of methylcellulose as an alternative medium in sperm migration tests. *Hum Reprod* 2002; **17**:143–149.

Kay VJ, Coutts JR, Robertson L. Pentoxifylline stimulates hyperactivation in human spermatozoa. *Hum Reprod* 1993;**8**:727–731.

Kirichok Y, Lishko PV. Rediscovering sperm ion channels with the patch-clamp technique. *Mol Hum Reprod* 2011;**17**:478–499.

Kirkman-Brown JC, Smith DJ. Sperm motility: is viscosity fundamental to progress? *Mol Hum Reprod* 2011;**17**:539–544.

Krausz C, Bonaccorsi L, Maggio P, Luconi M, Criscuoli L, Fuzzi B, Pellegrini S, Forti G, Baldi E. Two functional assays of sperm responsiveness to progesterone and their predictive values in in-vitro fertilization. *Hum Reprod* 1996;**11**:1661–1667.

Lefievre L, Nash K, Mansell S, Costello S, Punt E, Correia J, Morris J, Kirkman-Brown J, Wilson SM, Barratt CL *et al.*. 2-APB-potentiated channels amplify CatSper-induced Ca^{2+} signals in human sperm. *Biochem J* 2012;**448**:189–200.

Lishko PV, Botchkina IL, Kirichok Y. Progesterone activates the principal Ca^{2+} channel of human sperm. *Nature* 2011;**471**:387–391.

Marquez B, Suarez SS. Different signaling pathways in bovine sperm regulate capacitation and hyperactivation. *Biol Reprod* 2004;**70**:1626–1633.

Marquez B, Suarez SS. Bovine sperm hyperactivation is promoted by alkaline-stimulated Ca^{2+} influx. *Biol Reprod* 2007;**76**:660–665.

Marquez B, Suarez SS. Soluble adenylyl cyclase is required for activation of sperm but does not have a direct effect on hyperactivation. *Reprod Fertil Dev* 2008;**20**:247–252.

Marquez B, Ignotz G, Suarez SS. Contributions of extracellular and intracellular Ca^{2+} to regulation of sperm motility: release of intracellular stores can hyperactivate CatSper1 and CatSper2 null sperm. *Dev Biol* 2007;**303**:214–221.

Mortimer D. Elaboration of a new culture medium for physiological studies on human sperm motility and capacitation. *Hum Reprod* 1986; **1**:247–250.

Mortimer ST, Swan MA, Mortimer D. Effect of seminal plasma on capacitation and hyperactivation in human spermatozoa. *Hum Reprod* 1998;**13**:2139–2146.

Moseley FLC, Jha KN, Björndahl L, Brewis IA, Publicover SJ, Barratt CL, Lefièvre L. Induction of human sperm capacitation varies between incubation media; an effect that is not associated with protein kinase A activation. *Mol Hum Reprod* 2005;**11**:523–529.

Munire M, Shimizu Y, Sakata Y, Minaguchi R, Aso T. Impaired hyperactivation of human sperm in patients with infertility. *J Med Dent Sci* 2004;**51**:99–104.

Navarro B, Kirichok Y, Clapham DE. KSper, a pH-sensitive K+ current that controls sperm membrane potential. *Proc Natl Acad Sci USA* 2007;**104**:7688–7692.

Olson SD, Fauci LJ, Suarez SS. Mathematical modeling of calcium signaling during sperm hyperactivation. *Mol Hum Reprod* 2011;**17**:500–510.

Pacey AA, Davies N, Warren MA, Barratt CL, Cooke ID. Hyperactivation may assist human spermatozoa to detach from intimate association with the endosalpinx. *Hum Reprod* 1995;**10**:2603–2609.

Peedicayil J, Deendayal M, Sadasivan G, Shivaji S. Assessment of hyperactivation, acrosome reaction and motility characteristics of spermatozoa from semen of men of proven fertility and unexplained infertility. *Andrologia* 1997;**29**:209–218.

Publicover SJ, Barratt CL. Sperm motility: things are moving in the lab!. *Mol Hum Reprod* 2011a;**17**:453–456.

Publicover SJ, Barratt CL. Reproductive biology: progesterone's gateway into sperm. *Nature* 2011b;**471**:313–314.

Publicover SJ, Harper CV, Barratt CL. [Ca^{2+}]i signalling in sperm—making the most of what you've got. *Nat Cell Biol* 2007;**9**:235–242.

Quill TA, Sugden SA, Rossi KL, Doolittle LK, Hammer RE, Garbers DL. Hyperactivated sperm motility driven by CatSper2 is required for fertilization. *Proc Natl Acad Sci USA* 2003;**100**:14869–14874.

Ren D, Navarro B, Perez G, Jackson Ac, Hsu S, Shi Q, Tilly JL, Clapham DE. A sperm ion channel required for sperm motility and male fertiliy. *Nature* 2001;**413**:603–609.

Sagare-Patil V, Galvankar M, Satiya M, Bhandari B, Gupta SK, Modi D. Differential concentration and time dependent effects of progesterone on kinase activity, hyperactivation and acrosome reaction in human spermatozoa. *Int J Androl* 2012;**35**:633–644.

Servin-Vences R, Tatsu Y, Ando H, Guerrero A, Yumoto N, Darszon A, Nishigaki T. A caged progesterone analog alters intracellular Ca^{2+} and flagellar bending in human sperm. *Reproduction* 2012;**144**:101–109.

Stauss CR, Votta TJ, Suarez SS. Sperm motility hyperactivation facilitates penetration of the hamster zona pellucida. *Biol Reprod* 1995; **53**:1280–1285.

Strünker T, Goodwin N, Brenker C, Kashikar ND, Weyand I, Seifert R, Kaupp UB. The CatSper channel mediates progesterone-induced Ca^{2+} influx in human sperm. *Nature* 2011;**471**:382–386.

Su TW, Xue L, Ozcan A. High-throughput lensfree 3D tracking of human sperms reveals rare statistics of helical trajectories. *Proc Natl Acad Sci USA* 2012;**109**:16018–16022.

Suarez SS, Dai X. Hyperactivation enhances mouse sperm capacity for penetrating viscoelastic media. *Biol Reprod* 1992;**46**:686–691.

Suarez SS, Katz DF, Owen DH, Andrew JB, Powell RL. Evidence for the function of hyperactivated motility in sperm. *Biol Reprod* 1991; **44**:375–381.

Sukcharoen N, Keith J, Irvine DS, Aitken RJ. Predicting the fertilizing potential of human sperm suspensions *in vitro*: importance of sperm morphology and leukocyte contamination. *Fertil Steril* 1995; **63**:1293–1300.

Tesarik J, Thebault A, Testart J. Effect of pentoxifylline on sperm movement characteristics in normozoospermic and asthenozoospermic specimens. *Hum Reprod* 1992;**7**:1257–1263.

Tournaye H, Wieme P, Janssens R, Verheyen G, Devroey P, Van Steirteghem A. Incubation of spermatozoa from asthenozoospermic semen samples with pentoxifylline and 2-deoxyadenosine: variability in hyperactivation and acrosome reaction rates. *Hum Reprod* 1994; **9**:2038–2043.

Varano G, Lombardi A, Cantini G, Forti G, Baldi E, Luconi M. Src activation triggers capacitation and acrosome reaction but not motility in human spermatozoa. *Hum Reprod* 2008;**23**:2652–2662.

Wang C, Lee GS, Leung A, Surrey ES, Chan SY. Human sperm hyperactivation and acrosome reaction and their relationships to human *in vitro* fertilization. *Fertil Steril* 1993;**59**:1221–1227.

World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interactions,* 4th edn. Cambridge: Cambridge University Press, 1999.