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ORIGINAL ARTICLE *Andrology*

Viable acrosome-intact human spermatozoa in the ejaculate as a marker of semen quality and fertility status

Dorte Louise Egeberg Palme[1], Anders Rehfeld[1], Anne Kirstine Bang[1], Kristiana Alexandrova Nikolova[1], Søren Kjærulff[2], Morten Rønn Petersen[3], Janni Vikkelsø Jeppesen[3], Martin Glensbjerg[2], Anders Juul[1], Niels E. Skakkebæk[1], Søren Ziebe[3], Niels Jørgensen[1], and Kristian Almstrup[1,*]

[1]University Department of Growth and Reproduction, Section GR-5064, Copenhagen University Hospital, Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen, Denmark [2]ChemoMetec A/S, Gydevang 43, DK-3450 Allerød, Denmark [3]The Fertility Clinic, Section 4071, Copenhagen University Hospital, Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen, Denmark

*Correspondence address. University Department of Growth and Reproduction, GR-5064, Rigshospitalet, Blegdamsvej 9, DK-2100 Copenhagen, Denmark. Tel: +45-35-45-66-39; Fax: +45-35-45-60-54; E-mail: kristian@almstrup.net

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STUDY QUESTION: Is it possible, in an unbiased and clinical relevant way, to determine the number of viable acrosome-intact human spermatozoa in ejaculates and to use this as a measure of fertility chances?

SUMMARY ANSWER: Image cytometry enables easy and unbiased quantification of viable acrosome-intact spermatozoa and it correlates with semen quality and fertility status.

WHAT IS KNOWN ALREADY: The presence of the acrosome and its ability to respond to physiological inducers (e.g. progesterone) in the female reproductive tract at the appropriate time and place is required for fertilization. However, the available assays are labor intensive and therefore not used clinically.

STUDY DESIGN, SIZE, DURATION: Washed semen samples and capacitated swim-up fractions from volunteers were used to develop the assay. Subsequently washed ejaculates from patients in fertility treatment ($n = 156$), proven fertile men ($n = 54$) and volunteers ($n = 10$) were assessed to evaluate the number of acrosome-intact spermatozoa in the ejaculate (acrosomal status) and compared to other semen parameters, fertility status, fertility treatments and pregnancy rates.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Image cytometry was used to assess the fluorescence intensity of *Pisum sativum* agglutinin and Propidium iodide.

MAIN RESULTS AND THE ROLE OF CHANCE: The assay was validated by inducing the acrosome reaction in swim-up-purified and capacitated spermatozoa with progesterone and ionomycin, and in repeated acrosomal status measurements of washed ejaculates a small coefficient of variation (3.7%) was observed. Men with poor semen quality had fewer viable acrosome-intact spermatozoa in the ejaculate ($P = 0.0012$; median 32.6% vs. 49.3%). A large proportion (44%) of normozoospermic men from infertile couples had less than the observed median fraction (46%) of viable acrosome-intact spermatozoa in the ejaculate. Furthermore, the total number of viable acrosome-intact spermatozoa was significantly lower among men with male factor infertility compared to fertile men (median 35 vs. 97 mill, $P = 1 \times 10^{-7}$). Men from couples going through one or more ICSI cycles had significant fewer viable acrosome-intact spermatozoa than men from couples who only underwent IUI ($P = 0.002$; 44.4% vs. 62.0%) and the fraction of viable acrosome-intact spermatozoa appeared better than classical semen parameters in classifying whether or not couples needed ICSI. A positive, although non-significant, tendency toward ongoing pregnancy with an increasing number of viable acrosome-intact spermatozoa was observed ($P = 0.2$).

LARGE SCALE DATA: N/A.

LIMITATIONS, REASONS FOR CAUTION: Even larger cohorts of infertile couples are needed to substantiate the clinical application of the assay in regard to estimation of fertility potential of an individual.

WIDER IMPLICATIONS OF THE FINDINGS: The presented assay makes it possible to measure the number of acrosome competent spermatozoa in an ejaculate in a standardized manner and hence may serve as a new biomarker for male fertility. Few spermatozoa in an ejaculate are acrosome competent and it might be a valuable measure when evaluating male reproductive function.

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Key words: acrosome / image cytometry / human semen quality / fertilization / infertility

Introduction

Analysis of semen quality is an essential procedure during clinical work-up of infertile couples, and standardized procedures have been recommended by the World Health Organization (WHO) (World Health Organization, 2010). The actual function of the spermatozoa in the female reproductive tract, e.g. ability to respond to female factors, is however rarely assessed in the clinical work-up. Only human spermatozoa that have an intact acrosome and are capable of undergoing acrosome reaction can penetrate the zona pellucida (Yanagimachi *et al.*, 1976; Aitken *et al.*, 1993; Esterhuizen *et al.*, 2001; Bastiaan *et al.*, 2002; Liu and Baker, 2003; Vogiatzi *et al.*, 2013). Hence, the presence of an acrosome (the acrosomal status) and the ability of the spermatozoa to release the acrosomal content (the acrosomal responsiveness) and expose zona pellucida binding sites in response to female factors correlates with fertilization rates (Cummins *et al.*, 1991; Falsetti *et al.*, 1993; Oehninger *et al.*, 1994; Krausz *et al.*, 1995, 1996; Liu and Baker, 1998). Thus, the determination of the acrosomal status as well as the responsiveness of spermatozoa in the ejaculate may be indicative of fertility chances. Acrosomal status and responsiveness are, however, rarely assessed in the clinic because it is difficult to measure in a standardized manner.

So far, acrosome reaction has mainly been assessed either by microscopy (Cross *et al.*, 1986; Cross and Meizel, 1989; De Jonge *et al.*, 1989; Aitken *et al.*, 1993; Jaiswal *et al.*, 1999; World Health Organization, 2010) or flow cytometry (Cooper and Yeung, 1998; Nikolaeva *et al.*, 1998). Both procedures usually take advantage of fluorescently conjugated lectins that distinctly label acrosomal compartments. *Pisum sativum* agglutinin (PSA), a lectin from peas, binds to glycoproteins in the acrosomal matrix and has mostly been used to label intact acrosomes of fixed and permeabilized cells (Cross *et al.*, 1986; Fierro *et al.*, 1996). Recently, it was further demonstrated that PSA can be used to label living cells undergoing acrosome reaction (Zoppino *et al.*, 2012). PSA can enter the acrosomal interior via the pores that forms between the cell membrane and the outer acrosomal membrane of spermatozoa undergoing acrosome reaction and in this way label living cells that undergo acrosome reaction (Zoppino *et al.*, 2012). However, as cell death entails membrane disruption it is important to include a viability marker in the assay to ensure that only viable spermatozoa are labeled (Cross *et al.*, 1986), and many assays do not make this distinction, including the procedure recommended by WHO (World Health Organization, 2010). Furthermore, manual counting limits the number of cells that can be analyzed.

Image cytometry builds on principles from both fluorescence microscopy and flow cytometry and provides numerical data based on thousands of analyzed cells. Here we show that image cytometry can be used to measure the acrosomal status and responsiveness by the use of PSA and propidium iodide (PI). We further show that the fraction of viable acrosome-intact spermatozoa in the washed ejaculate correlates with other semen parameters and differs between fertile men and men with a male factor infertility as well as the type of fertility treatment recommended for the infertile couples.

Materials and Methods

Initially, we developed an assay to assess the acrosome reaction of viable spermatozoa after induction (acrosomal responsiveness). Acrosomal responsiveness was performed after recovery of motile spermatozoa by swim-up and capacitation, which are laborious procedures. In order to develop an assay for routine semen analysis, we replaced the swim-up and capacitation steps with a simple wash, and measured the fraction of viable spermatozoa with an intact acrosome (acrosomal status). The assays were initially developed using ejaculates from volunteers and subsequently measured in ejaculates from men with different fertility statuses.

Semen samples

We included ejaculates ($n = 17$) from six volunteers for development and validation of the acrosomal responsiveness assay. Four of these samples were also used for the acrosomal status assay (see below). Ejaculates from volunteers are normally used for internal quality control (QC) at the Department of Growth and Reproduction, Rigshospitalet (GR). Only self-reported healthy men with unknown fertility status were included and were only selected by previously having two ejaculates of normozoospermic quality (concentration >15 mill/ml, progressive motile spermatozoa >32%, morphologically normal spermatozoa >4%). Only volume and concentration were determined for ejaculates from volunteers.

In the clinical study, we included ejaculates from 156 men from infertile couples and from 54 men in couples who obtained pregnancy by natural intercourse (fertile men). Furthermore, volunteers also provided ejaculates ($n = 10$, 4 were also used for method development; see above) for assessment of acrosomal status. The 156 men from infertile couples were included when they had their ejaculates analyzed during clinical work-up. The samples were included during a period of 16 months and only samples

with a volume >1.5 ml and spermatozoa concentration >4 mill/ml were included. Both partners from infertile couples had undergone extensive examination including semen analysis, hormone measurements, physical examination and information on life-style factors and medical history. Based on the clinical evaluation written in the patient records, the infertile couples were stratified according to their primary cause of infertility: female factor (e.g. polycystic ovaries, tuba occlusion, anovulation) $n = 23$, male factor (e.g. oligozoospermia, oligoteratozoospermia) $n = 62$, mixed factor: $n = 18$, unknown (i.e. if neither the male or female partner had any reproductive health issues) $n = 53$. The 54 fertile men all participated in an ongoing study of testis function of fertile men. They had been invited to participate when attending nuchal translucency with their pregnant partners and were only included if the pregnancy had been conceived spontaneously.

Handling of semen samples

All semen samples were produced by masturbation and ejaculated into clean, wide-mouthed plastic containers. The samples were collected in the privacy of a room near the laboratory ($n = 207$) or at home ($n = 26$, only from infertile men). The ejaculates were allowed to liquefy for at least 30 min at 37°C. A basic semen analysis including assessment of semen volume by weighing, motility and morphology was performed according to WHO guidelines with the modifications described by Jorgensen *et al.* (2012). Sperm concentration of all samples was determined by image cytometry as described in Egeberg *et al.* (2013).

Swim-up and capacitation of spermatozoa

Motile spermatozoa were recovered from ejaculates from volunteers by swim-up separation in human tubular fluid (HTF^+) medium containing (in mM): 72.8 NaCl, 4.69 KCl, 0.2 $MgSO_4$, 0.37 KH_2PO_4, 2.04 $CaCl_2$, 0.33 sodium pyruvate, 21.4 sodium lactate, 2.78 glucose, 21 HEPES, and 25 $NaHCO_3$, adjusted to pH 7.4 with NaOH for 1 h at 37°C with 10% CO_2 in air, as described elsewhere (Strunker *et al.*, 2011). After two washes in HTF^+ medium, the samples were adjusted to 10×10^6 spermatozoa/ml and capacitated for 3 h at 37°C with 10% CO_2 in the air and in the presence of 3 mg/ml (3% [v:v]) human serum albumin (HSA, Irvine Scientific, CA, USA) as described by WHO (World Health Organization, 2010).

Assessment of acrosomal responsiveness by image cytometry

A well-mixed suspension of swim-up recovered and capacitated spermatozoa was divided into equal aliquots and mixed with a staining solution containing (final concentrations): 5 µg/ml fluorescein isothiocyanate conjugated *Pisum sativum* agglutinin (FITC-PSA, Sigma-Aldrich, MO, USA), 0.5 µg/ml propidium iodide (PI, ChemoMetec A/S, Allerød, Denmark), and 10 µg/ml Hoechst-33342 (H342, ChemoMetec) in HTF^+. To induce acrosome reaction, ionomycin or progesterone (both Sigma-Aldrich, MO, USA) was added to the capacitated spermatozoa in final concentrations of 2 and 10 µM, respectively, and incubated for 30 min at 37°C. As a negative control 0.2% dimethyl sulfoxide (DMSO) was added. After induction, samples were thoroughly mixed by pipetting and a 50 µl aliquot was drawn and mixed with 100 µl of an immobilizing solution containing 0.6 M $NaHCO_3$ and 0.37% (*v/v*) formaldehyde in distilled water. This solution was mixed by pipetting and immediately loaded into a two-chamber NC-Slide A2™ slide (ChemoMetec). The loaded slide was subsequently analyzed by image cytometry using a NucleoCounter® NC-3000™ (ChemoMetec). In brief, this instrument combines large field-of-view and depth-of-focus optics facilitating high content cell analysis with a magnification of ~2X. Further details on the image cytometer NC-3000™ can be found at www.chemometec.com. The accompanying NucleoView™ software facilitates automated image acquisition and analysis, data visualization, and enables quantification and gating of subpopulations. The FlexiCyte™ module enables the user to construct assays, including choice of excitation/emission filters and minimum number of cells (events) to be analyzed. Here, FITC-PSA fluorescence was detected using peak excitation at 475 nm and emission at 560/35 nm (exposure time 3000 ms), PI was detected using peak excitation at 530 nm and emission at 675/75 nm (exposure time 500 ms), and the minimum number of analyzed cells was set to 5000. H342 staining was used for cell segmentation (only H342-positive cells were included in the image analysis), and aggregates of more than five cells were excluded. PI intensity as a function of FITC-PSA intensity was plotted on bi-exponential scales and specific quadrant gates were used to distinguish four groups.

Assessment of acrosomal status in washed semen samples

Spermatozoa (3 mill) were washed twice in Dulbecco's phosphate-buffered saline (PBS, ThermoFisher scientific) ($500 \times g$, 10 min), and the spermatozoa pellet was re-suspended in the staining solution (final concentrations: 5 µg/ml FITC-PSA, 0.5 µg/ml PI, and 10 µg/ml H342 in PBS) and assessed by image cytometry as described above.

Assay validation

Quantification of spectral overlap between PSA and PI and definition of quadrant gates were carried out on two capacitated and two washed ejaculates from volunteers using cells only stained with H342 or in combination with either PSA or PI. The obtained compensation matrix was applied to all measurements.

To establish the optimal incubation and induction time, initial time course experiments were conducted. Samples in staining solution were incubated at 37°C either in the presence of DMSO or progesterone and measured after ~5, 15, 30, 60 and 120 min of incubation (Supplementary Fig. S1).

To validate the staining patterns, the remaining of labeled samples were concentrated by centrifugation ($700 \times g$, 10 min), re-suspended in 10 µl immobilizing solution, transferred to a glass slide, and immediately evaluated by manual inspection on a fluorescence microscope (Olympus BX61, Olympus, Denmark) by two operators. Spermatozoa with an acrosome brightly stained by PSA and negative for PI staining were evaluated as viable acrosome-reacting spermatozoa.

To describe the robustness of the acrosomal responsiveness assay, 16 swim-up recovered and capacitated samples were measured and the progesterone or ionomycin induced acrosome responses were plotted relative to the DMSO control.

Variability of the acrosomal status assay was determined by six simultaneous measurements of four washed ejaculates from men from infertile couples. The coefficient of variation (CV) was calculated as the standard deviation divided by the mean.

Moreover, leukocytes were isolated from whole blood with the Erythrocyte Lysis Buffer (Qiagen, Denmark) as described by the manufacturer, and spiked in different numbers to determine the influence of leukocytes in ejaculates on the measured values (Supplementary Fig. S2).

IUI, IVF and ICSI procedures

Of the 156 men infertility treatments, 82 couples received treatment at the Fertility clinic at Rigshospitalet. The remaining received treatment elsewhere and we did not have access to their records. The fertility treatment procedures are described elsewhere (Freiesleben *et al.*, 2008; Lemmen *et al.*, 2016). The standard practice is to offer couples with no tubal factor infertility and with a minimum of 2 million progressive motile spermatozoa (after density gradient centrifugation (DGC)), up to three cycles of intrauterine insemination. IVF was used for cases with no severe male factor

infertility or one or more failed IUI cycles. Couples with <2 million progressive motile spermatozoa after DGC or couples or with one or more failed IVF cycles were offered ICSI. Overall, the couples underwent a total of 283 cycles (87 IUI cycles, 68 IVF cycles, 94 ICSI cycles and 34 frozen embryo replacements from December 2010 to May 2017. One couple used oocyte donation (2 IVF cycles), and one couple was offered prenatal genetic diagnostics (1 ICSI cycle). Ongoing (intrauterine) pregnancies with minimum one fetus and cardiac activity were confirmed by transvaginal ultrasonography at gestational week 6 and 7. There were 49 couples who experienced at least one positive ultrasonography (3 couples experienced two positive results).

Statistical analysis

Measurements were entered into a database and analyzed in the statistical software R version 3.3.3 (http://cran.r-project.org/). A paired *t*-test was used to compare responses after incubation with progesterone and ionomycin relative to the DMSO control. Qq plots (Supplementary Fig. S3) indicated normal distribution of spermatozoa concentration and total number of viable acrosome-intact spermatozoa after log-transformation, whereas square root transformation was used for motility and morphology. Linear regression of the relationship between viable acrosome-intact spermatozoa and semen parameters as well as fertility treatments was obtained from (parametric) linear models (the lm function) and plotted using ggplot2. A non-parametric Wilcoxon rank sum test was used to compare the number of viable acrosome-intact spermatozoa with the cumulative number of spermatozoa defects, the total number of viable acrosome-intact spermatozoa (log-transformed) in the different groups of men and between the different kinds of fertility treatments. The levels of significance were set at $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***) and $P < 0.0001$ (****).

Confidence intervals (CI) and Pearson's correlation coefficients were obtained from the Rmisc and cor function. Receiver operating characteristic (ROC) curves were plotted with the pROC package and smoothed Kernel density estimates with the ggpubr package. Kernel density estimation represents a smoothed version of a normal histogram. In all boxplots, the box corresponds to the first and third quartiles, and the band inside corresponds to the median. The whiskers mark 1.5× the inter-quartile range.

Ethical approval

Collection and analysis of ejaculates from patients and fertile men as well as collection of clinical information were approved by the Research Ethics Committee of the Capital Region of Denmark (H-4-2010-138, H-KF-289428, H2-2012–091, H-16036581). The healthy volunteers belonged to corps of semen donors used for internal quality control and received 500 DKK per sample.

Results

Assay validation

Application of image cytometry to study acrosomal responsiveness

Addition of PSA and the membrane impermeable PI to capacitated swim-up recovered spermatozoa from volunteer ejaculates allowed us to simultaneously distinguish viable acrosome-reacted cells (PSA-positive and PI-negative) from dead acrosome-reacted cells (PSA- and PI-positive) (Fig. 1A). Initial time course experiments showed that progesterone-induced induction of acrosome reaction reached a steady-state level compared to DMSO after 30 min of incubation (Supplementary Fig. S1). When either 10 µM progesterone or 2 µM ionomycin were added to the samples, the percentage of PSA-positive and PI-negative cells (viable acrosome-reacted cells) increased (Fig. 1B and C). The acrosomal responsiveness after 30 min of induction with progesterone or ionomycin on 16 different samples (from six volunteers) showed a consistent proportional increase in the percentage of spermatozoa undergoing acrosome reaction (Fig. 1C), indicating robustness of the assay. In the DMSO, progesterone and ionomycin treated samples, the mean fraction of viable acrosome-reacted cells were 9.0 ± 1.2% (mean ± 95% CI), 16 ± 2.9% and 31 ± 4.6%, respectively. The relative proportion of viable spermatozoa that had undergone acrosome reaction after induction with progesterone or ionomcyin was significant when compared to DMSO (Prog: 1.8 ± 0.15%, $P = 1.4 \times 10^{-9}$ relative to DMSO, Iono: 3.4 ± 0.49%, $P = 9.3 \times 10^{-9}$ relative to DMSO). A 2- and 3-fold induction by progesterone and ionomycin is in line with what is reported by WHO and others (De Jonge *et al.*, 1989; Jaiswal *et al.*, 1999; Bonaccorsi *et al.*, 2001; World Health Organization, 2010; Tamburrino *et al.*, 2014). Moreover, the treatment with progesterone or ionomycin had no significant effect on the viability of the spermatozoa (data not shown) and correlated well with manual counting (data not shown). Taken together, these data indicate that image cytometry can be applied to evaluate the ability of spermatozoa to undergo acrosome reaction (the acrosomal responsiveness).

Application of image cytometry to assess acrosomal status in washed ejaculates

Measurement of acrosomal responsiveness as described above requires a considerable amount of sample pretreatment. We therefore sought to omit the swim-up and capacitation steps and questioned whether image cytometry could be applied to measure viable acrosome-intact spermatozoa in a washed ejaculate. In concordance with the swim-up recovered spermatozoa, addition of PI and PSA to washed ejaculates from men from infertile couples showed the same spermatozoa-specific staining patterns as described above (Fig. 1D). Figure 1E show examples of scatter plots produced by the image cytometer on washed ejaculates. The plots contain more noise (populations scatter more) compared to the swim-up recovered samples (Fig. 1B), but the same four cell populations can be identified. Only H342-positive objects are included in the image analysis, whereby some of the noise in the washed samples is eliminated (e.g. the large round cell and the background staining, possibly originating from cell debris evident in Fig. 1D, top row and bottom row, respectively). To describe the assay variability, four washed samples were repeatedly measured ($n = 6$) and the mean CV of the assay was determined to be 3.7% (range: 2.7–4.3%) (Fig. 1F). Taken together these results indicate that the image cytometry assay can be used to determine the number of viable acrosome-intact spermatozoa in a washed ejaculate (the acrosomal status) with quite good precision (a low CV).

Clinical application

Acrosomal status and standard WHO assessed semen quality

We next investigated whether the fraction of viable acrosome-intact spermatozoa was correlated with the classical semen parameters: spermatozoa concentration, motility and morphology. To determine this, the acrosomal status of washed ejaculates from 156 men from infertile couples was measured. Linear regression between the fraction of viable acrosome-intact spermatozoa and spermatozoa concentration, motility and morphology (Fig. 2A–C, respectively) indicated a

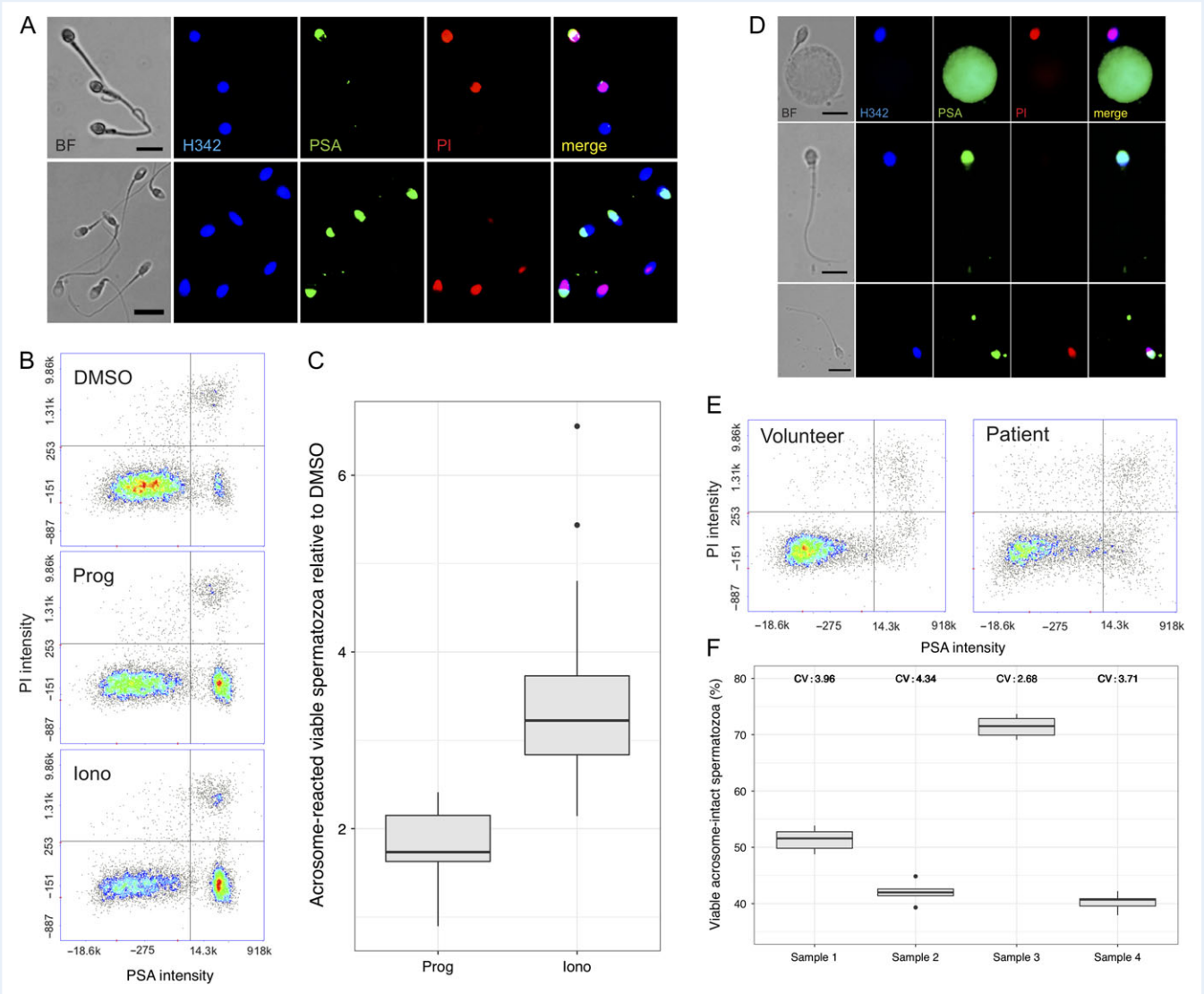

Figure 1 Assessment of acrosomal responsiveness and acrosomal status by image cytometry. Swim-up recovered and capacitated spermatozoa (for assessment of acrosomal responsiveness) or washed ejaculates (for assessment of acrosomal status) were mixed with Hoechst-33342 (H342), propidium iodide (PI), and FITC conjugated PSA. For assessment of acrosomal responsiveness, spermatozoa were induced to undergo acrosome reaction with either 10 µM progesterone (Prog) or 2 µM ionomycin (Iono). Addition of vehicle (DMSO) served as a negative control. (**A**) Images of fluorescently labeled spermatozoa obtained by bright field (BF) and fluorescence microscopy. Merge indicates the overlap of fluorescence images. Scale bar = 10 µm. (**B**) Examples of scatter plots produced by the image cytometer with PSA intensity plotted against PI intensity. Each dot in the plot represents a H342-positive spermatozoon. The cells in the lower left quadrant are the viable acrosome-intact spermatozoa (PI- and PSA-negative) whereas the cells in the lower right quadrant are viable acrosome-reacted (PI-negative and PSA-positive). The PI-positive acrosome-reacted dead spermatozoa are located in the upper right quadrant. (**C**) Box plot representation of 16 independent assessments of acrosome reaction showing the proportion of viable acrosome-reacted spermatozoa after induction with progesterone (Prog, 1.8 ± 0.15% (mean ± 95%CI), $P = 1.4 \times 10^{-9}$ relative to DMSO) or ionomycin (Iono, 3.4 ± 0.49%, $P = 9.3 \times 10^{-9}$ relative to DMSO) relative to the negative control DMSO. (**D**) Images from BF and fluorescence microscopy of a washed ejaculate from a patient. Image segmentation ensures that only H342-positive objects are included in the image cytometry analysis. Scale bar = 10 µm. (**E**) Examples of scatter plots, similar to (B), but obtained from measurement on washed ejaculates. (**F**) CV of four samples where assessments of acrosomal status were repeated six times on the same samples. The mean CV was 3.7%.

significant association between progressive motility ($P = 1.4 \times 10^{-6}$, Pearson's correlation coefficient: 0.38) and morphology ($P = 0.0044$, Pearson's: 0.25) but a non-significant association to concentration ($P = 0.10$, Pearson's: 0.1). The fraction of viable acrosome-intact spermatozoa, however, varied substantially independently of the level of motility (residual standard error: 1.7) and morphology (residual standard error: 0.97). Moreover, the fraction of viable acrosome-intact spermatozoa in relation to the cumulative number of semen parameters below WHO reference values for semen characteristics (Cooper *et al.*, 2010) showed that ejaculates from men with no semen parameters below WHO reference levels had 17% more viable acrosome-intact spermatozoa than ejaculates from men

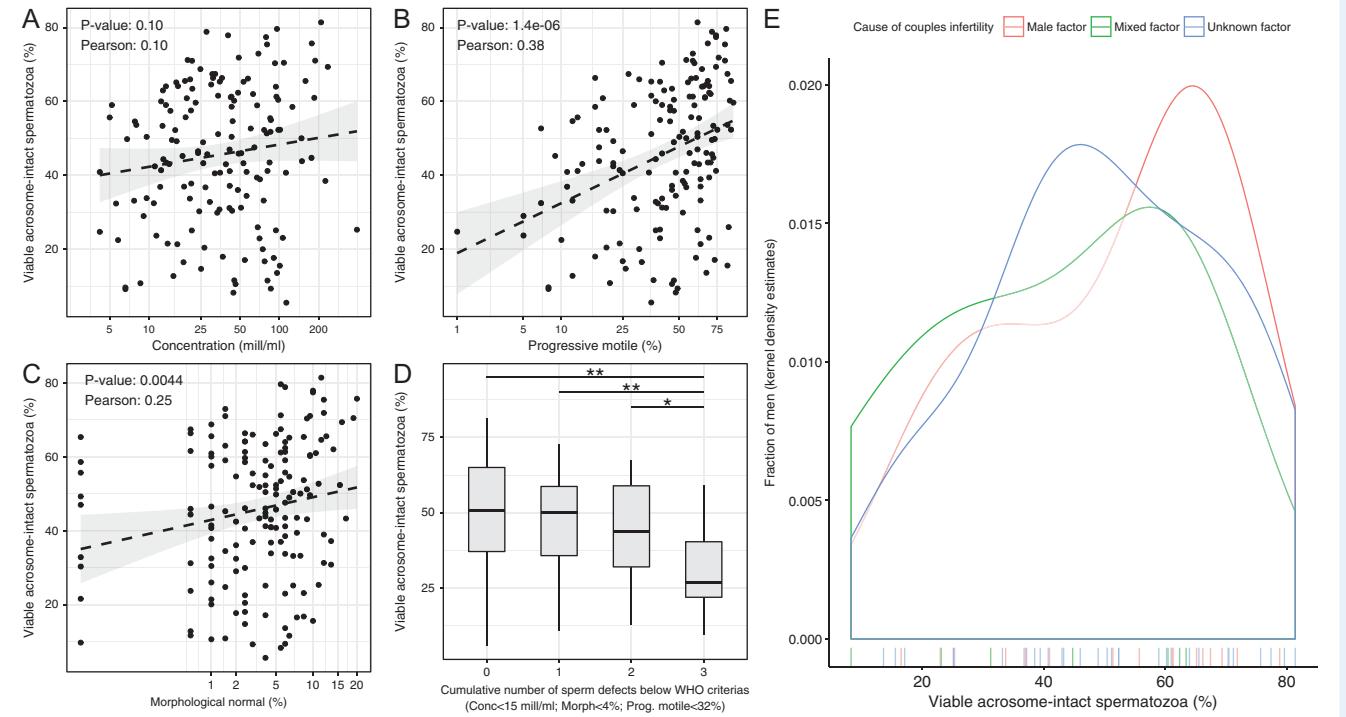

Figure 2 Assessment of acrosomal status in washed ejaculates and correlation to classical WHO semen parameters. The fraction of viable acrosome-intact spermatozoa of 156 ejaculates from men attending the outpatient clinic due to couple infertility was measured and correlated to spermatozoa concentration (**A**), motility (**B**) and morphology (**C**). To obtain a better normal distribution concentration was log-transformed, motility and morphology were square root transformed. Pearson's correlation coefficient (Pearson) and the *P*-value from linear regression is indicated on each plot. The shaded area around the line indicates the confidence interval. (**D**) The fraction of viable acrosome-intact spermatozoa in relation to the cumulative number of semen parameters below WHO reference values for semen characteristics (Cooper *et al.*, 2010). The samples with a cumulative number of three spermatozoa defects all have a concentration (Conc) below 15 mill/ml, fewer than 4% morphological normal forms (Morph) and <32% progressive motile (Prog. motile) spermatozoa, whereas the samples with zero spermatozoa defects have all values above the indicated criteria. A *denotes a *P*-value < 0.05, and **a *P*-value of < 0.01. (**E**) Smoothed distribution (kernel density estimates) of the fraction of viable acrosome-intact spermatozoa in the ejaculate among normozoospermic men from infertile couples and stratified by the infertility factor. Normozoospermic men from couples with an unknown ($n = 31$), mixed ($n = 7$) and male ($n = 16$) factor infertility were included in the plot whereas couples with a female factor infertility ($n = 16$) were excluded. Note that a substantial proportion of normozoospermic men from infertile couples were found in the lower end of the distribution.

with all three semen parameters below the WHO levels (median of 49% vs. 33%) (Fig. 2D). In general, the more semen parameters below the WHO reference values, the smaller a fraction of viable acrosome-intact spermatozoa an ejaculate contained ($P = 0.0012$, $P = 0.0019$, $P = 0.018$, between zero, one, and two sperm parameters vs. all three sperm parameters, respectively). Among the men from infertile couples with no semen parameters below WHO ranges (infertile normozoospermic men, $n = 70$), 31 men (or 44%) had <46% (the median of all 156 men form infertile couples) viable acrosome-intact spermatozoa in their ejaculate. The distribution (using kernel density estimation) of viable acrosome-intact spermatozoa among infertile normozoospermic men also showed that many of these men had few acrosome-intact spermatozoa in their ejaculate (excluding female factor ($n = 16$), Fig. 2E). A lower fraction of viable acrosome-intact spermatozoa seemed to more prevalent among normozoospermic men from couples with a mixed ($n = 7$) or unknown ($n = 31$) factor infertility than men from couples with a male factor infertility ($n = 16$; Fig. 2E).

Acrosomal status and cause of infertility

To address the clinical value of the acrosomal status measurement, we investigated the total number of viable acrosome-intact spermatozoa in ejaculates from men from infertile couples ($n = 156$, stratified according to the cause of couples infertility), from fertile men ($n = 55$), and from volunteers ($n = 10$) as outlined in Table I. Across all groups of men, the total number of viable acrosome-intact spermatozoa was median: 76, mean: 124 and range: 2.1–858 mill and the distribution best fitted normality after log-transformation (Supplementary Fig. S3). Comparison of the total number of viable acrosome-intact spermatozoa between the groups (Fig. 3, Table I) showed significant fewer viable acrosome-intact spermatozoa in ejaculates from men with male factor infertility when compared to fertile men (median 35 vs. 97 mill, $P = 1.1 \times 10^{-7}$) and volunteers (median 35 vs. 203 mill, $P = 8.5 \times 10^{-6}$). Also, a significant difference was observed between ejaculates from men from couples where the infertility factor was unknown and men from couples with an isolated male factor infertility (median 86 vs.

Table I Summary statistics of semen samples used in the clinical part of the study. Only samples from patients with a volume >1.5 ml and a concentration >4 mill/ml were selected for analysis.

	Volunteers	Fertile men[a]	Men from infertile couples with female factor[a]	Men from infertile couples with unknown factor[a]	Men from infertile couples with mixed factor[a]	Men from infertile couples with a male factor[a]
Number of samples	10	54	23	53	18	62
Age (years)	Unknown	35 (28–45)	33 (25–47)	34 (25–47)	36 (24–47)	34 (25–50)
Abstinence (days)	ND	4 (2–34)	4 (0—15)	4 (1–16)	4 (2–15)	4 (0–8)
Concentration (mill/ml)	92 (53–352)	86 (18–276)	71 (10–188)	44 (5–393)	43 (4–176)	24 (4–235)
Morphology (% normal)	ND	8 (2–26)	6 (2–14)	6 (0–20)	4 (0–14)	3 (0–16)
Motility (% progressive)	ND	68 (26–93)	62 (31–81)	56 (5–88)	41 (1–72)	41 (7–84)
Volume (ml)	3 (2–6)	4 (2–9)	4 (2–10)	4 (2–9)	4 (2–7)	4 (2–7)
Fraction viable acrosome-intact	68% (50–80)	41% (4–80)	50% (6–78)	49% (14–81)	38% (8–65)	46% (10–79)
Total number viable acrosome-intact (mill)	203 (88–596)	97 (14–858)	101 (21–521)	86 (5–577)	48 (2–316)	35 (2–733)

Note: Data are presented as median (range).
ND, not determined.
[a]Data obtained from the routine examination of patient or project participant semen samples.

35 mill, $P = 8.3 \times 10^{-5}$). In addition, a significant difference was observed between fertile men and men from couples with a mixed factor infertility (median 97 vs. 48 mill, $P = 0.02$) as well as between the volunteers and men from all the other groups. When only considering the fraction of viable acrosome-intact spermatozoa, only the differences between the volunteers and men from the other groups remained significant (data not shown).

Acrosomal status and fertility treatment

Based on the clinical evaluation of both male and female, the infertile couples had been referred to different types of fertility treatment at the fertility clinic. Comparison of the fraction of viable acrosome-intact spermatozoa in washed ejaculates (not the same ejaculate used for the fertility treatment procedures) showed a significantly ($P = 0.002$, Wilcoxon rank sum test) higher level among men where the couple ($n = 11$) only were treated with IUI in comparison to couples ($n = 43$) that at some point were referred to ICSI (independent of whether IUI or ICSI treatments resulted in live birth or not) (median 44% vs 62%; Fig. 4A). No significant differences were observed compared to men from couples ($n = 28$) referred to IVF (not ICSI but may have prior IUI treatments) albeit the median level (52%) was between that of couples referred to IUI only and ICSI (Fig. 4A). Using regression analysis, similar significant levels ($P = 0.005$) were obtained for IUI only vs. ICSI groups and showed that this was independent of the sperm concentration but showed an interaction with morphology and motility. Furthermore, ROC curve analysis of the fraction of viable acrosome-intact spermatozoa with respect to fertilization process (IUI only vs. ICSI) showed a median specificity of 63% (95% CI: 36–91) and sensitivity of 93% (95% CI: 84–100) at a threshold of 61% viable acrosome-intact spermatozoa (Supplementary Fig. S4). The area under the curve (AUC) was 79% (95% CI: 63–96), 66% (48–84), 72% (56–88), and 71% (54–88) for the fraction of viable acrosome-intact spermatozoa, sperm concentration, morphology and motility, respectively (Supplementary Fig. S4). Using the best predictive threshold for each measure (Supplementary Fig. S4), the accuracy (sum of true positive and true negative/total population) of classifying couples into whether they received ICSI or not was 87, 69, 61 and 74% based on the fraction of viable acrosome-intact spermatozoa, sperm concentration, morphology and motility, respectively.

When couples with female infertility were excluded, we observed a negative correlation between the number of IVF cycles and the fraction of viable acrosome-intact spermatozoa (unadjusted for other semen parameters; Supplementary Fig. S5). Finally, plotting the chance of an ongoing pregnancy in Week 7 (pregnancy confirmed by ultrasonography per total number of treatment cycles for each couple) against the fraction of viable acrosome-intact spermatozoa for 59 couples (female factor infertility excluded) showed a positive, although not significant ($P = 0.2$), correlation (Fig. 4B).

Discussion

We have developed an assay of human spermatozoa acrosome reaction, which makes it possible to use the acrosomal status as a parameter in the routine evaluation of men with fertility problems. It is well recognized that the acrosome reaction is a crucial step in the fertilization process along with other processes like capacitation and hyperactivation. However, tests for acrosome reaction are rarely used in clinical settings. The WHO-recommended assay is based on a tedious microscopic

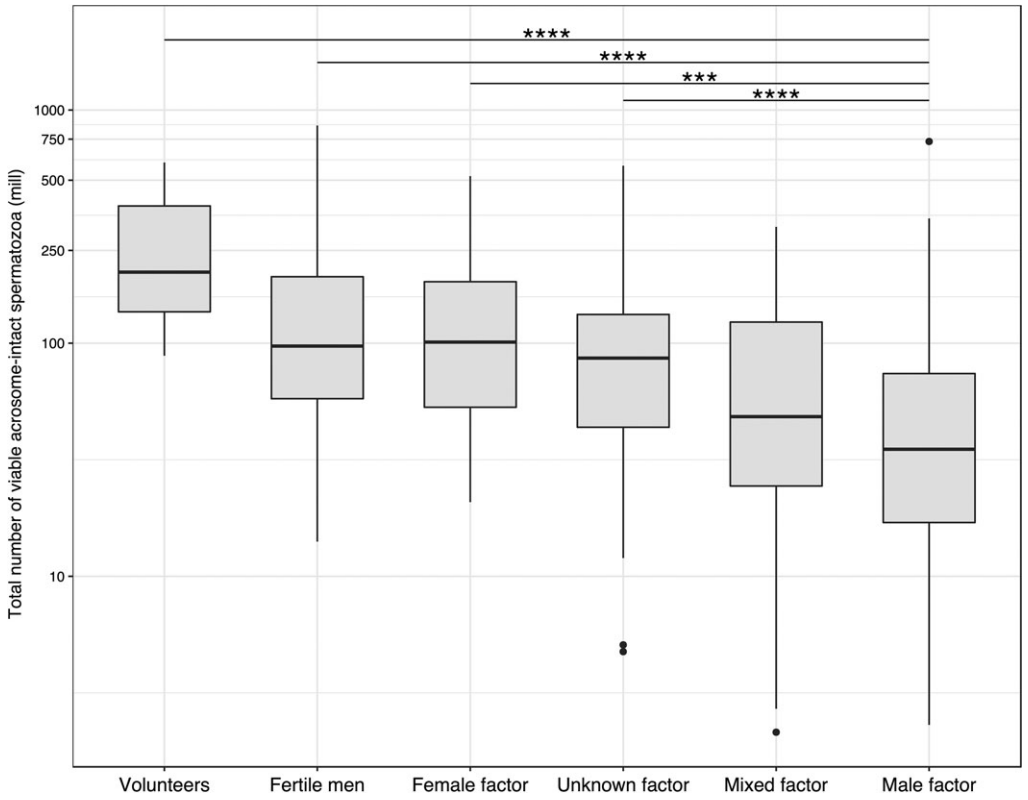

Figure 3 Acrosomal status and cause of infertility. Acrosomal status was measured on 221 washed ejaculates. The total number of viable acrosome-intact spermatozoa (calculated as concentration × volume × % viable acrosome-intact spermatozoa/100) in ejaculates was measured and the men were grouped according to their fertility status and/or the primary cause of the couple infertility (volunteers: $n = 10$, fertile men: $n = 55$, female factor: $n = 23$, unknown factor: $n = 53$, mixed factor: $n = 18$, and male factor: $n = 62$). The distribution best fitted normality after log-transformation. A ***denotes a P-value < 0.001 and ****a P-value of < 0.0001.

judgment of staining patterns that are not always easily distinguishable. In contrast, assessment of acrosomal status on washed ejaculates by image cytometry, is quite simple and does not require much sample preparation besides a quick spin. With image cytometry, acrosomal status in an ejaculate can be measured within 60 min (with ~10 min hands-on work) and the estimated cost is approximately one-third that of the manual procedure (Supplementary Table SI). Moreover, the image cytometer may, in addition, be used to measure many other aspects of semen quality, like viability and concentration (Egeberg *et al.*, 2013; Egeberg Palme *et al.*, 2017). Measurement of acrosomal responsiveness require preparation of capacitated spermatozoa and therefore takes considerably more time (~5.5 h). This is a nevertheless valuable tool in the research laboratory (Rehfeld *et al.*, 2017).

The presence of non-spermatozoa (round) cells and debris can vary substantially between samples and might disturb the read-out. The image segmentation, however, ensures that only H342-positive objects are counted and spiked leukocytes were found only to influence the read-out minimally (Supplementary Fig. S2). Moreover, the addition of PI to the assay allows differentiation between dead and viable acrosome-reacted spermatozoa. Image cytometry also provides superior counting statistics compared to manual evaluation by counting at least 12 times as many spermatozoa than normally reported for manual counting. In line with this, we found a very low coefficient of variation of measurements, indicating a high precision of the determined level of acrosomal status.

Our results indicate that between men there is a great variation in the number of spermatozoa with an intact acrosome. Among men with male factor infertility, the mean fraction of spermatozoa that retains an intact acrosome after ejaculation was as low as 45% (mean of all men with male factor infertility) compared to 68% among the volunteers. This indicates that besides the well-known impairment of the classical semen parameters among men with male factor infertility, less than half of their spermatozoa is equipped with an intact acrosome that can assist in penetration of the zona. Median values for our group of men with male factor infertility were a concentration of 24 mill/ml, 2.5% morphological normal forms and 41% progressive motile indicating that in the worst case, <0.5% (0.11 mill/ml) of the spermatozoa could have the capacity to fertilize the oocyte. Moreover, among the spermatozoa with intact acrosomes, we found, in line with others (Falsetti *et al.*, 1993; Bonaccorsi *et al.*, 2001; Tamburrino *et al.*, 2014), that <20% are able to undergo progesterone-induced acrosome reaction. Theoretically, this indicates that <0.1% of the total number of spermatozoa are capable of fertilizing an oocyte. Among the men in our fertile group this number was ~17 times higher (1.9 mill/ml).

A correlation between *in vitro* fertilization success rates and acrosomal responsiveness has been described previously (Calvo *et al.*, 1994;

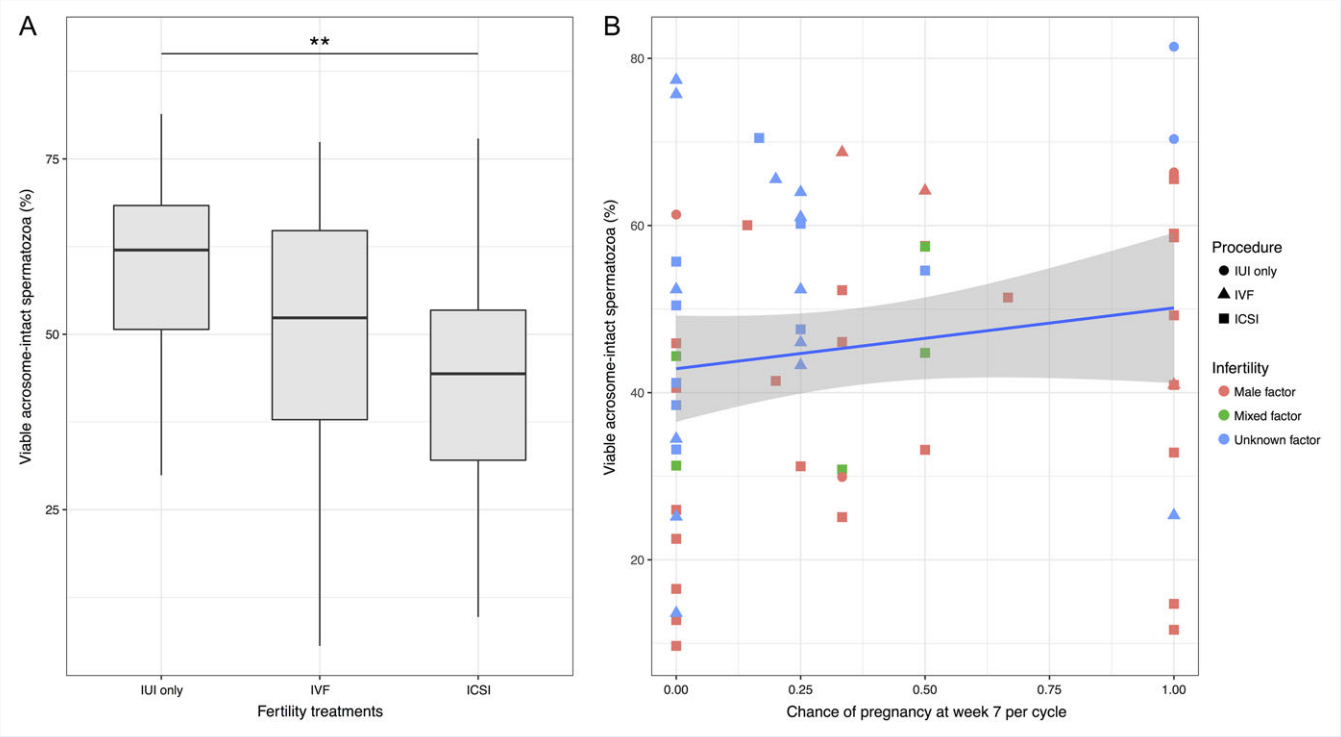

Figure 4 Acrosomal status and fertility treatment. (**A**) Box plot showing which type of fertility treatment the couples had been referred to in relation to the fraction of viable acrosome-intact spermatozoa. IUI (n = 11) only includes couples referred to IUI and not IVF or ICSI. IVF (n = 28), included couples referred to IVF, and not ICSI, but may have had prior IUI treatments. ICSI (n = 43) includes all couples who were referred to ICSI and may have had prior IUI and/or IVF treatments. Grouping was independent of whether the treatment resulted in a live birth or not. (**B**) Non-significant correlation between the fraction of viable acrosome-intact spermatozoa and the chance of an ongoing pregnancy in Week 7 (confirmed by ultrasonography) per total number of treatment cycles for each couple. Couples with a female factor infertility were excluded. The couples were stratified according to their primary infertility cause (red = Male factor (n = 30), green = mixed factor (n = 5), blue = unknown (n = 24)) and the type of treatment (circle = ICSI (n = 38), triangle = IUI only (n = 5), square = IVF (n = 16)). The blue line indicates the linear fit and the gray shaded area the confidence interval. A **denotes a P-value < 0.01.

Krausz *et al.*, 1995, 1996; Esterhuizen *et al.*, 2001; Liu and Baker, 2003). In our study, we further observed that men from couples with male factor infertility as well as men from couples referred to ICSI had significant lower numbers of viable acrosome-intact spermatozoa compared to fertile men and men from couples referred to IUI, respectively. This even tended to correlate with chances of ongoing pregnancy. Our data are, however, based on relative few infertile couples (n = 82) and larger cohorts of men are needed to firmly link the proportion of acrosomal-competent spermatozoa in ejaculates directly to fertility. Moreover, there was an interaction between motility and morphology, but not concentration, on the fraction of viable acrosome-intact spermatozoa. This was evident from the correlation with semen parameters and also in the regression analysis of fertility treatments. This indicates that motility and morphology are partly influenced by the acrosomal status or vice versa. Our data nevertheless indicate that acrosomal status is better at classifying whether ICSI will be needed during the fertility treatment than classical semen parameters. When we compared ROC curves of IUI only vs. ICSI, acrosomal status had a larger AUC and accuracy than concentration, morphology and motility. This could potentially help fertility clinics, for example in recommending ICSI early in the process. However, decisions on whether or not ICSI were used in our study was based on many parameters and the true (cross-classified) predictive values should be tested in a standardized setup. Among the normozoospermic infertile men, 44% had fewer acrosome-intact spermatozoa than the median of all men from infertile couples. This could imply that a proportion of the infertile, but otherwise normozoospermic, men might be infertile due to low numbers of acrosome-intact spermatozoa in their ejaculate. Using the optimal cut-off from the ROC curve analysis indicated that out of the 54 normozoospermic men (female factor excluded), 35 or 65% should be directed towards ICSI. Future lines of research should, however, include much larger cohorts of men from couples in fertility treatment in a carefully designed setup to firmly deduce the value of the acrosomal status. Moreover, the possible influence of life-style parameters like diet, stress, age and cell phone usage should be assessed along with measurement of e.g. time-to-pregnancy in large cohorts.

Conclusion

We have developed a new assay based on image cytometry that allows robust assessment of the spermatozoa acrosome. Measurement of the total number of viable acrosome-intact spermatozoa in the washed ejaculate could distinguish men with male factor infertility from fertile

men. The fraction of viable acrosome-intact spermatozoa in the ejaculate could be used to stratify whether infertile couples were referred to ICSI rather than IUI. The assay is easy and fast and therefore has the potential to become a useful tool in the clinical evaluation of semen quality.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

D.L.E.P., K.A., N.J., S.K., N.E.S., M.G. and A.J. conceived the study. D.L.E.P., A.R. and K.A. designed the experiments. D.L.E., A.R. and K.A.N. conducted all the experiments. D.L.E.P., A.K.B., J.V.P., M.R.P. and S.Z. obtained data from the semen analysis, fertility status and fertility treatment. D.L.E.P., A.R., A.K.B. and K.A. analyzed the data. D.L.E.P. and K.A. wrote the manuscript. All authors approved the final version of the manuscript.

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Conflict of interest

Two of the co-authors (M.G. and S.K.) currently work at the company ChemoMetec A/S, which produces the image cytometers used in the study. All work has been performed independently of ChemoMetec A/S at the Dept. of Growth and Reproduction and ChemoMetec has not biased the experiments or interpretation of the results. M.G. holds shares in ChemoMetec. The other authors have no conflict of interest.

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