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Development of a novel home sperm test

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BACKGROUND: The majority of men find the production of a semen sample an embarrassing and stressful experience. Consequently, the availability of an over-the-counter home sperm test, which would reliably and accurately allow the patient to obtain an assessment of fertility potential at their convenience, would be a major benefit. Our objective was to develop and evaluate a home sperm test that provides a visual estimate of the concentration of progressively motile sperm in a semen sample. METHODS: Three particular challenges are described (i) developing a visualization system; (ii) optimization of the detection limit; and (iii) controlling variation due to changes in ambient temperature. The accuracy of the device was tested against two reference methods: computer-assisted sperm analysis (CASA) and a hyaluronate migration test (HMT). RESULTS: In 129 semen samples, where both reference methods agreed (positive or negative), the accuracy of the device was 95%. The observed likelihood ratio of 8.8 indicated that a sample showing a red line in the device was over eight times more likely to have a positive (normal) result in CASA and HMT than a sample without a red line. CONCLUSIONS: The final device provides a visual estimate of the concentration of progressively motile sperm in a semen sample using a test that is completed within approximately 1 h of production of the sample and can be used by the man in the comfort of his own home.

Key words: cervical mucus/home sperm device/hyaluronic acid/sperm function test/sperm penetration test

Introduction

Semen analysis is the cornerstone of infertility investigation in the male. On the basis of the result, the couple are provided with prognostic and diagnostic information to assist in their management. Many men find the production of a semen sample an embarrassing and stressful experience. In addition, there is often a significant waiting time for an initial appointment and an additional delay before the results are available. All these factors heighten the anxiety associated with infertility investigations. Consequently, the development of an over-the-counter home sperm test has been a primary objective in andrology for a number of years. An effective home sperm test would allow the patient to obtain an assessment of fertility potential at their convenience.

The concentration of progressively motile sperm is one of the most predictive parameters for estimating natural fertility in both subfertile (Ayala *et al*., 1996; Tomlinson *et al*., 1999) and normal couples (Larsen *et al*., 2000; Zinaman *et al*., 2000). For example, Larsen and colleagues examined 358 normal couples planning to conceive with no previous history of subfertility. The concentration of motile sperm (curvilinear velocity >25 μm/s) was the most predictive parameter for *in vivo* conception and, not surprisingly, differences in the concentration of motile sperm in the low range of the scale made the largest difference with regard to fertility (Larsen *et al*., 2000). Our objective was to develop a simple and reliable test for the concentration of progressively motile cells. In addition, we wanted an assay that would provide an assessment of the functional capacity of the sperm as a small proportion of men with normal semen parameters (e.g. $>10 \times 10^6$ progressively motile sperm/ml semen) have dysfunctional sperm (Schats *et al*., 1984; Mortimer *et al*., 1986; Barratt *et al*., 1989).

Sperm penetration into human cervical mucus *in vitro* is known to provide important predictive information about sperm function (Barratt *et al*., 1989; Eggert-Kruse *et al*., 1989; Abu-Heija *et al*., 1996; Eggert-Kruse *et al*., 1996). Hyaluronic acid has been used extensively as a human cervical mucus substitute and penetration of sperm into it is highly correlated with semen characteristics (Mortimer *et al*., 1990; Neuwinger *et al*., 1991; Tang *et al*., 1999) and sperm function testing (Aitken *et al*., 1992). For example, Aitken and colleagues compared sperm penetration into hyaluronic acid with quantitative motility and the zona-free hamster penetration assay (Aitken *et al*., 1992). Seventy five per cent of the variation in quantitative motility and 65% of the variation in the zona-free hamster penetration assay could be accounted for by penetration into hyaluronic acid and, importantly, patients with zero oocyte penetration could be identified. In addition, Tang and colleagues demonstrated that antisperm antibodies significantly impair sperm penetration into hyaluronic acid (Tang *et al*., 1999). In summary, penetration into hyaluronic acid is regarded as a simple and objective means of measuring the functional competence of sperm and was chosen as the basis of our assay.

Figure 1. Home test device for progressively motile sperm.

In this study we describe brief details of the scientific principles employed in the development of the assay based on the separation of progressively motile sperm and detection using a lateral flow nitrocellulose strip, and the performance of the final device on 150 semen samples.

Materials and methods

Development of the device was a multidisciplinary collaborative project managed by Genosis Ltd with contributions from The Birmingham Women's Hospital (UK), The University of Birmingham (UK), Pearson Matthews Design (UK), British BioCell International Ltd (Wales) and Plextek Ltd (UK). The final device that was evaluated—the home sperm test (trade name Fertell; Figure 1)—was provided by Genosis Ltd (UK).

Principles of the assay

The device can be regarded as performing two distinct operations (Figure 2). The first operation involves separation of progressively motile sperm from liquefied semen by a direct swim-up through hyaluronic acid. During this step, the temperature of the hyaluronic acid buffer is maintained at 37 ± 3°C by a thermostatically controlled heating collar. In the second operation and detection step, the sperm fraction of the swim-up is reacted with a monoclonal antibody conjugated with colloidal gold and the labelled sperm then collect at the interface of a nitrocellulose lateral flow strip, while the free, unreacted, conjugate is washed clear. The appearance of the clear red line (test result) due to the colloidal gold label on the antibody-bound sperm is indicative of a concentration of progressively motile sperm in the semen sample of 10×10^6/ml or greater. To ensure that the device is functioning properly, at various stages, feedback is provided to the consumer by means of a simple light emitting diode (LED). The LED is controlled by a microprocessor integral to the device.

Development of the assay

Three specific challenges were encountered in the development process: visualization of sperm, optimization of the detection limit, and robustness to variation in ambient temperature.

Visualization of sperm

A major challenge of any home test system is the visual detection of sperm without the use of any specialized equipment, thus allowing the man to obtain results in the comfort of his own home. The device uses a lateral-flow nitrocellulose strip using anti-CD59 antibody conjugated to colloidal gold to generate a visible red line when sperm are present.

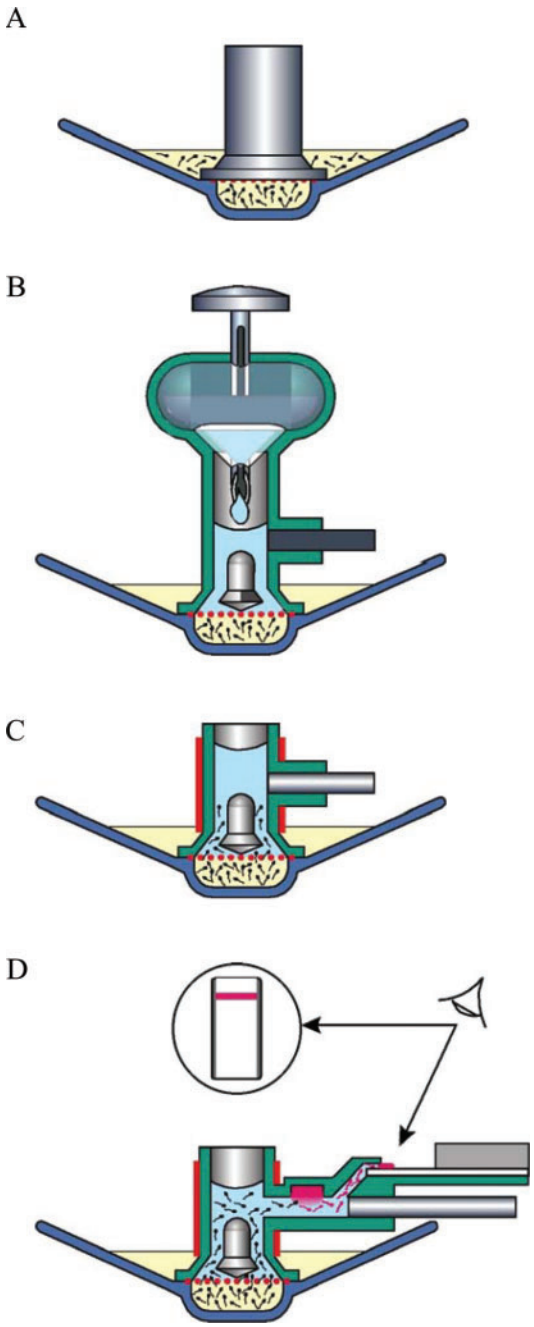

Figure 2. (A) Schematic drawing of the test device with 600 µl at the bottom sealed off by a swim-up chamber with mesh at the top of the semen volume. The figure shows the situation after the lid has been put in place following 30 min of liquefaction. (B) The swim-up process is initiated by the depression of a button which releases a hyaluronate (hyaluronic acid) solution (blue) on top of the mesh at the semen surface. (C) During the half-hour swim-up phase, the swim-up chamber is heated with a heating collar (red line) to 37°C. (D) After the swim-up phase, progressively motile sperm in the hyaluronic acid solution are released into capillary channel, labelled with anti-CD59 colloidal gold conjugate (red sperm) and trapped on the nitrocellulose strip, where a visible line is formed if sufficient numbers of progressively motile sperm have migrated into the hyaluronic acid solution.

Following the separation of progressively motile cells in hyaluronic acid, an aliquot is moved by capillary action under an absorbent polyester pad impregnated with anti-CD59 antibody labelled with colloidal gold (see below). Wetting of this pad releases the conjugate, which reacts with the sperm in the capillary channel. As the reaction proceeds, the fluid is carried onto the surface of a lateral-flow nitrocellulose membrane at the end of the capillary channel. The labelled sperm become trapped at the interface of this membrane while the free, labelled antibody is washed clear and drawn along the nitrocellulose by an absorbent wick in fluid contact with the nitrocellulose. The appearance of a clear red test line is formed on the surface of the nitrocellulose in the presence of a defined concentration of sperm.

Anti-CD59 antibody, which detects a 18–20 kDa GPI-linked glycoprotein thought to play a role in protecting cells (sperm) from attack by complement (Rooney *et al*., 1993), was used to label the sperm. Anti-CD59 binds to spermatids in the human testis (Simpson and Holmes, 1994), to both acrosome-intact and reacted sperm (D'Cruz and Hass, 1992; Fenichel *et al*., 1994; Simpson and Holmes, 1994) and, following mild detergent treatment, to internal structures. Preliminary experiments showed that anti-CD59 bound to practically all sperm (>95%) and no noticeable difference was observed between men from different ethnic groups (data not shown). CD59 is also present on white blood cells, immature and dead sperm and in seminal plasma (Rooney *et al*., 1993). Separation of progressively motile sperm, which was an integral part of the assay, therefore allowed the detection of only progressively motile cells on the nitrocellulose strips, avoiding potentially erroneous results, e.g. from non-progressively motile cells or seminal plasma (i.e. azoospermic men).

Optimization of detection limit

It was important to maximize the number of progressively motile sperm recovered (yield) in order to optimize the detection limit. To increase sperm yield, an inverted conical swim-up chamber was used with an internal space filler. This maximizes the interface between hyaluronic acid and semen (i.e. collisions of sperm in semen with hyaluronic acid) and, by reducing the volume of hyaluronic acid, increases the concentration of sperm. Preliminary experiments tested aspirating sperm from several different heights (0.65, 1 and 2 cm) above the semen–hyaluronic acid interface. A height of 0.65 cm was finally used as this provided the most effective differentiation between normal and abnormal (definition from WHO, 1999) semen samples (Ivic *et al*., 2002).

Robustness to variation in ambient temperature

Temperature control is a particularly significant challenge as progressive sperm motility is highly dependent on temperature (Milligan *et al*., 1978; Ford *et al*.,1992) and the home test environment by definition introduces significant variation in ambient temperature. To address this, a thermostatically controlled heating coil was devised that covered the outer surfaces of the swim-up chamber. The temperature at the height where the sperm are automatically aspirated is maintained at 37 ± 3°C. Conduction of heat warmed the liquefied semen sample to 30 ± 2°C when ambient temperature was ≥20°C. At any ambient room temperature between 18° and 30°C, the swim-up chamber would equilibrate to 37 ± 3°C within 5 min.

The assay was designed to provide a reflection of the concentration of progressively motile sperm in semen as this is the primary diagnostic and prognostic semen parameter (see Introduction). A reference value (cutoff point) of 10×10^6 progressively motile sperm/ml semen, which reflected two of the three primary WHO criteria of normality (20×10^6 sperm/ml and 50% progressive motility; WHO, 1999), was chosen.

Study population

The results of the present study incorporate testing of the final device in 150 men selected from research donors (n = 132), subfertile males (n = 7) and post-vasectomy donors (n = 11). The subfertile males had an abnormal semen analysis, with a concentration of $<10 \times 10^6$ progressively motile sperm per ml of semen. The clinical diagnosis of subfertility was based on unsuccessful attempts of the couple to achieve a pregnancy within 12 months of unprotected sexual intercourse, but no data on the medical status of the female was known. Semen samples were obtained from research donors and from patients attending the andrology laboratory at the Birmingham Women's Hospital, Birmingham, UK [Human Fertilisation and Embryology Authority (HFEA) centre 0119], as part of fertility investigations. Consent for the use of semen samples were taken in accordance with the HFEA Code of Practice. Local ethical approval from South Birmingham Research Ethics Committee (0472) was obtained. During the development period, prior to testing the final device reported here, we analysed over 3000 semen specimens. Semen samples were obtained from Caucasian, Asian and black African men and men of Middle Eastern origin.

In order to achieve the desired distribution of specimens with high and low concentrations of progressively motile sperm, respectively, the research donors were screened in a previous semen analysis and post-vasectomy subjects were included in order to increase the proportion of negative (concentration of progressively motile sperm below the test cutoff) subjects. It was intended that a target minimum of 25% of the subjects should have progressively motile sperm concentrations below the test cutoff. In the final analysis of the results, only data for samples with concordant reference method results (see below) were included.

Reference methods

The concentration of progressively motile sperm, obtained by computer-assisted sperm analysis (CASA) (Hamilton Thorne, Beverley, MA, USA), and a modified Kremer test [hyaluronate migration test (HMT)] was performed on each semen sample. Due to the expected variability in the reference methods, not least the HMT due to the relatively low numbers of cells assessed for samples close to the calculated cutoff, the results from the device were compared with those for samples where the reference methods were concordant [i.e. both showing low values (negative) or both showing high values (positive)].

Quantitative motility

CASA. Quantitative sperm motility was assessed using a Hamilton-Thorne IVOS, version 10.9. The CASA equipment was operated using the standard setup (37°C working temperature; cell depth 20 μm; 30 frames acquired at 60 Hz; starting analysis at least 3 mm from opening of chamber; magnification calibrated with micrometer scale on microscope slide; average path velocity low cutoff 5.0 μm/s and high cutoff 25.0 μm/s). High-concentration samples could not be quantified by the instrument when the system warned that the concentration of progressively motile cells was too high for accurate assessment. This typically occurred for samples with progressively motile concentrations exceeding $60–70 \times 10^6$/ml. Therefore, all the samples with a very high concentration of progressively motile sperm were verified by observation on the TV display and classified as positive. All analyses were performed at 37°C. Preheated (37°C) MicroCell 20 μm fixed-depth chambers were filled with 3 μl well-mixed semen. At least five fields with 200 motile sperm (defined by standard settings of the CASA equipment) were assessed. The IVOS software calculated the concentration of progressively motile sperm. Concentrations of progressive and motile sperm were recorded together with donor code and time of analysis. A concentration of progressively motile sperm of 10×10^6/ml or above was designated as a positive result.

Hyaluronate Migration Test. Microslide capillaries (0.4 × 4.0 mm internal diameter; VitroCom, Mountain Lakes, NJ, USA) were filled with the same hyaluronate solution (hyaluronic acid) as used in the final device, sealed at one end with Cristaseal (Hawksley, Scientific laboratory supplies, Nottingham) and heated to 37°C in a humid chamber with air containing 5% CO_2. Migration tests were performed in duplicate: two capsules (BEEM Capsules, Agar Scientific, Stanstead, Essex) were filled with 50 µL semen and one capillary was placed vertically into each capsule so that the hyaluronic acid was in direct contact with the semen. Capsules with capillaries were then placed in an incubator (37°C, 5% CO_2) for 1 h, after which the capillaries were removed from the capsules and placed on graded microscope slides and assessed immediately. Assessment was done at ×200 total magnification using phase-contrast microscopy and a calibrated reticulum in the ocular (Ivic *et al*., 2002). The number of sperm per mm^2 was assessed at 20 mm from the lower end of the capillary. The sperm in four microscopic fields were counted and the average number of sperm per mm^2 was calculated. To decrease the uncertainty of the results near the calculated cutoff point (61 sperm/mm^2), eight fields in total were assessed when the number of sperm in any of the initial four fields was less than 21. A concentration of 61 sperm/mm^2 or above was designated as a positive result.

Semen collection and processing

Semen samples were produced in a room adjacent to the laboratory and collected in a specimen pot of the type used in diagnostic andrology and the therapeutic assisted reproductive procedures (60 ml Biotite Container, A1 LW5465; Alpha Laboratories, Eastleigh, Hampshire UK,). In order to mimic the home environment, the samples were allowed to liquefy at ambient temperature (20°C) and then carefully mixed before two 3 µl droplets were sequentially withdrawn for CASA assessment, followed by two 50 µl aliquots for the HMT. The remaining semen was transferred to the test device. From this point the handling of the test device followed the instructions of the device.

Statistical analysis

Contingency tables were analysed with the Fisher exact test. All calculations were performed using GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com).

Results

Of the 150 subjects enrolled, five were excluded due to insufficient volume and a further two due to technical problems. For the comparison with the combined reference method (CASA and HMT), 14 subjects were excluded because the results of the two reference tests were not concordant, i.e. not valid. The device gave a positive, red test line in 95 of the 129 valid experiments and no test line in the remaining 34. Of the 95 positive results, 91 (95.8%) also showed positive results with the reference tests and of the 34 negative device results, 32 (94.1%) were negative in the reference tests. Thus, the device gave results with high sensitivity and specificity (Table I). The observed likelihood ratio of 8.8 indicated that a sample rendering a red line in the device was over eight times more likely to have a positive (normal) result in CASA and HMT than a sample without a red line.

The accuracy of the device in relation to the reference methods was very high (accuracy 95.3%; Fisher's exact test, $P < 0.0001$).

Table I. Results of the device in comparison with concordant reference methods [computer-assisted sperm analysis (CASA) and hyaluronate migration test (HMT)] in a selected population of men ($n = 129$)

		95% confidence interval
Accuracy	95.3%	90.2–98.3
Sensitivity	97.8	92.5–99.7
Specificity	88.9	73.9–96.9
Likelihood ratio	8.8	

Samples with reference results close to the cutoff were excluded (see text) to decrease random influence due to uncertainty in reference methods.

Discussion

The objective of this work was to develop and evaluate the effectiveness of an over-the-counter home sperm test. In this study, which analysed 129 semen samples with valid reference method results, the overall accuracy was 95%. To our knowledge this is the first time that such a device, providing a visual signal representing the concentration of progressively motile cells in the semen, has been developed and evaluated.

Semen analysis is known to have many sources of variation. For the investigation of the analytical reliability of a new diagnostic device it is essential to use appropriate reference methods. To avoid errors due to uncertainty in results from the reference methods, measures must be taken to reduce their imprecision. Here we optimized the numbers of cells assessed in the reference methods (Kvist and Björndahl, 2002) and we excluded samples where the reference methods gave contradictory results. Using such measures, we estimate that we have eliminated, as far as possible, errors in the reference methods. Therefore, in the present study ($n = 129$) the reliability of the device could be rigorously tested.

A key aspect of this study was the establishment of a robust and effective visualization system. We used a novel rapid test system utilizing a nitrocellulose strip and a conjugate (anti-CD59 antibody conjugated to immunogold) dried down on a polyester pad. Numerous modifications to this system were evaluated in order to allow (i) the automatic transfer of fluid onto the nitrocellulose strip, (ii) the correct releasing system for the anti-CD59 conjugate, and (iii) effective fluid flow within the device. As CD59 is also present on other cells in semen as well as free in seminal plasma [possibly associated with proteosomes (Rooney *et al*., 1993)], it was essential to separate progressively motile sperm from other cells and seminal plasma in order to avoid erroneous results. Separation of the sperm based on their motility, i.e. penetration through hyaluronic acid, ensured the effective separation of progressively motile sperm. Hyaluronic acid was chosen as the sperm separation media as it is widely used in andrology and is regarded as an effective alternative to human cervical mucus and an objective means of measuring the functional competence of sperm (Aitken *et al*., 1992). Our initial experiments used several different media for potential sperm separation. Some of these gave very good separation of sperm, e.g. methyl cellulose 4000 c.p.i. (concentration 10 mg/ml) (Ivic *et al*., 2002) but were unsuitable for use in a rapid home sperm test. Hyaluronate was an effective method to separate

progressively motile sperm and allowed rapid visualization of the strip test results.

Infertility is a couple problem and as advancing age has a significant negative impact on future fecundity it is essential that couples attempting conception have rapid and reliable information about their potential fertility at the earliest opportunity. This device has a significant number of advantages. Firstly, as a home test it allows the man to examine his potential fertility rapidly and in the comfort and privacy of his own home. Secondly, it provides the man with a potential warning sign (concentration of progressively motile sperm in semen less than 10×10^6/ml) so that he can seek detailed investigations and potential treatment at the earliest opportunity. Thirdly, it allows the patients to take an active part in their management and potentially allows them to make a more informed choice about the use of putative self-administered drug therapy, e.g. antioxidant treatment (Lenzi *et al*., 2004). However, although our results are very encouraging and in home trials (unpublished data) patients have found the device easy to use, this device should only be used as a screening test and should not be seen as an alternative to a detailed, high-quality, comprehensive semen assessment.

In summary, the final device provides an accurate, rapid and easily visualized estimate of the concentration of progressively motile sperm in a semen sample that can be used by a man in the comfort of his own home.

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Conflict of interest

C.L.R.B. is a member of the Scientific Advisory Board of Genosis Ltd.

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