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Limited Utility of a Test-Yolk Buffer Semen Extender for Preserving Human Semen Parameters for Use in a Diagnostic Setting

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ABSTRACT

Background: Identification of a preservation medium that accurately retains semen parameters would be advantageous for men requiring semen analysis by allowing them to produce sperm samples at home rather than on site or even facilitate 'mail order' semen analysis.

Objectives: To evaluate the utility of a commercially available refrigeration medium for preserving human semen parameters for use in a diagnostic setting.

Materials and Methods: In patients undergoing diagnostic semen analysis, extender was added to the remainder of each ejaculate following initial 'ground truth' testing. Samples were re-assessed to determine effects of incubation time, temperature, volume of extender and speed of addition.

Results: Total and progressive motility and vitality declined slowly at 2–5°C, with some motility remaining even after 4 days. The rate of motility and vitality decline was not consistent between samples, with samples outside the reference range being particularly prone to rapid decline. Varying extender conditions showed that even samples with initially good parameters exhibited inconsistent rates of decline in motility and vitality. This was partially ameliorated by incubation at 20–24°C compared to 2–5°C ($p < 0.001$), while varying the speed of extender addition (dropwise vs. rapid) or the ratio of extender to initial sample had no effect.

Discussion: Although semen refrigeration medium does allow sperm to retain some motility for several days, the high level of individual patient variability in the rate of decline precludes accurate reconstruction of initial semen parameters from extended samples. However, total sperm count can be accurately measured.

Conclusions: There are many commercially available kits for semen analysis; however, the reliability of the results is questionable. Semen extenders could potentially be used for triage for samples within the reference range but where measurements from extended samples fall outside reference ranges, confirmation must be obtained from a fresh sample assayed according to WHO guidelines before any diagnosis can be made.

Trial Registration: A Study Registration Number is not required as this was not a clinical trial

1 | Introduction

Manual semen analysis performed on a freshly produced sample is internationally regarded as the routine test of choice to diagnose male infertility [1, 2]; however, it is not always feasible for samples to be analysed shortly after ejaculation. Semen analysis examines individual sperm parameters including motility, vitality, concentration and morphology, as well as characteristics of the seminal fluid produced by the male accessory glands such as ejaculate volume, pH and viscosity, which provide essential information about male factor infertility. Performance of this test in accordance with best practice WHO guidelines [1, 2] ensures compliance with ISO 15189:2012 and ISO 23162:2021 and the most accurate and reliable results [3, 4]. To minimise variables that may affect the reliability of the result, WHO [1, 2] recommend samples to be analysed within 1 h of production to avoid deterioration of semen quality that can occur due to dehydration, changes in osmolarity, temperature fluctuations, energy depletion, oxidative stress, bacterial contamination and pH imbalances (reviewed by [5]). Therefore, specimen collection routinely occurs on site of the laboratory. In cases where men are unable to produce samples on site, they are instructed to deliver their sample to the laboratory within the hour. However, not all men have access to andrology laboratories in the immediate vicinity, rendering delivery of samples within 60 min impossible. There are many kits currently on the market designed for self-testing semen analysis. Some kits allow for the semen analysis itself to be conducted at home by use of a mobile phone application or a device to assess sperm count and motility [6]. By their very nature, these kits provide very limited information and are unable to provide accurate and reliable results [7, 8]. Furthermore, they may create more harm by the information that is lacking, providing a false sense of security that all is well if only one or two parameters appear to be within range. More recently, kits have been developed for sperm production at home which can be transported to a diagnostic laboratory, with a view to providing a more comprehensive and accurate test. However, sperm parameters may deteriorate significantly from 60 min of production [9], so these kits necessitate addition of a preservative to protect sperm parameters.

For many years, the integrity of sperm has been preserved using semen extenders for storage and transport of sperm for animal breeding such as artificial insemination for species including equines, roosters, dogs, pigs and cattle [10, 11]. Additionally, suitable conditions have been identified to maintain human semen in a variety of liquid media with a view to use in a human clinical setting to transport or store sperm for assisted reproduction [12–18]. In these settings, the need to maintain the original parameters as they were at production is unnecessary, so long as some viable sperm are available for use. However, this is unacceptable for diagnostic testing. Recently, a modified liquid preservative has been reported to maintain the integrity of the sample after several days' storage, sufficient for diagnostic testing of sperm parameters [19]. However, this report only included samples with parameters that fell within the fifth percentile for fertile men, maintained over a broad temperature range, using an undisclosed preservative that would be difficult to replicate. The aim of the current study was to test the hypothesis that a commercially available preservative, currently used for cool storage of human sperm prior to assisted conception, can be optimised to maintain sperm parameters sufficiently faithfully to allow for reliable diagnostic testing of semen after more than 24 h of storage.

2 | Materials and Methods

2.1 | Participant Recruitment

Men attending the Andrology Department at The Doctors Laboratory (TDL) from May 2021 to June 2022 for routine semen analysis, seminal fluid culture or a sperm DNA fragmentation test were recruited for the study. A total of 140 men gave informed consent to use the remains of their samples for this study. Studies have been performed according to the Declaration of Helsinki, and the procedures have been approved by The Faculty of Sciences Research Ethics Advisory Group for Human Participants from the University of Kent (No.: CREAG059-05-2021). Only men with a minimum age of 18 years were accepted for the study. To optimise reliability of measurands, samples were rejected if there was a history of febrile illness in the 3 months prior to analysis, or where the sexual abstinence period did not conform to 2–7 days. Samples classified as azoospermic were not included. In addition, hyperviscous samples were excluded as well as samples where microorganisms were clearly seen under microscopic observation at any time point pre- or post-extension. A total of 116 men were thus included in the various primary analyses. Some samples were split and used for more than one experiment. All participants produced ejaculates on-site so that semen samples were processed within 60 min. The distribution of semen parameters included those within and outside of the fifth percentile for fertile men (1) and ranged as follows: sperm count 0.4–249 million/mL (median 57.7; IQR 71.5), total motility 26%–85% (median 67, IQR 9), progressive motility 20%–85% (median 63; IQR 9) and vitality 46%–96% (median 75; IQR 12). Thirty-four samples were subject to morphology analysis and values ranged from 0% to 12% normal forms (median 3; IQR 3). The volume of all samples fell within WHO 2010 (1) reference range ($\geq$ 1.5 mL).

2.2 | Sample Handling

Samples were produced into batch tested pre-weighed containers. Manual semen analysis was conducted at TDL Andrology, a UKAS accredited laboratory (ISO 15189:20), according to WHO 2010 (1) as the study was initiated prior to the publication of the WHO 2021 recommendations [2]. Sperm count was performed on fixed samples using an improved Neubauer chamber. Vitality was assessed using Eosin-Y. Motility was assessed manually at 400 × microscopic magnification. Sperm morphology was performed on fixed samples stained with Papanicolaou stain and assessed blind by two operators. An initial evaluation of the raw specimen was performed within an hour of collection and subsequently after addition of extender. Validation and optimisation of the extender for preserving semen parameters was determined under different conditions.

2.3 | Effect of Time on Semen Parameters Using Manufacturer's Instructions for Addition of Extender

Semen samples were extended with a test-yolk buffer refrigeration medium (catalogue no. 90129; FUJIFILM Irvine Scientific,

CA, USA). In initial experiments, the extender was added dropwise to semen samples according to manufacturer's guidelines (1:1 v/v) and stored chilled at 2–5°C. Sperm count, total and progressive motility, vitality and morphology were assessed before (time 0) and after addition of extender at intervals up to 96 h. Samples were pre-warmed for 15 min at 37°C prior to the analyses to ensure standardisation of the samples prior to assessment.

2.4 | Temperature

Manufacturer's instructions recommend storage of extended samples at 2–5°C. To determine the most suitable storage temperature for this extender, samples were split and stored either at 2–5°C or 20–24°C (room temperature, RT) for up to 8 h. Semen analyses were performed before adding the extender and repeated hourly for both storage conditions. Specimens kept at 2–5°C were pre-warmed at 37°C for 15 min prior to analysis.

2.5 | Rate of Addition of Extender

The manufacturer's instructions recommend dropwise addition of extender. To test this variable, semen samples were split and semen parameters were compared between dropwise addition of an equal volume of extender or addition of the entire equal volume of extender all at once. Samples were mixed via swirling for 30 s until homogeneous prior to assessment. Semen analysis was performed prior to (time 0) and immediately after addition of the extender. Samples were then incubated at RT and repeat measurements obtained at varying time intervals thereafter. In comparing the extender addition protocols, replicate samples from the same ejaculate were always measured at the same time point(s); however, the time points chosen were deliberately variable between different ejaculates, to mimic the effect of unpredictable delivery times in a mail-based service.

2.6 | Varying Dilutions of Extender

Manufacturer's instructions recommend adding an equal volume of extender to semen.

To determine whether varying the volume of extender affects semen parameters, each semen sample was divided into four equal volumes. Addition of extender to each aliquot was as follows: semen:extender 1:1 v/v, 1:2 v/v, 1:4 v/v, 1:5 v/v. Semen parameters were compared between dilutions. Samples were mixed via swirling for 30 s until homogeneous prior to assessment.

2.7 | Statistical Analysis

Specimens were classified as normal if all parameters from the initial analysis (pre-extension) were within the fifth percentile for fertile men (1) reference ranges: Count $\geq$ 15 million/mL; total motility $\geq$ 40%; progressive motility $\geq$ 32%. Samples were classified as abnormal if one or more of these parameters were below range. At least 200 sperm (100 in duplicate) were counted for each parameter. Data were analysed by the paired *t*-test (paired two sample for means) to ascertain significance. Data were presented and considered significantly different if $p < 0.05$. Linear regression was carried out to determine the associations between measurements obtained following different methods of extender addition. Statistical analyses were performed using Excel and Graphpad Prism (2021, Version 9.2.0 for Windows, GraphPad Software, San Diego, California, USA).

3 | Results

3.1 | Validation Over Time According to Manufacturer's Instructions

The refrigeration medium used in this study was designed for short term preservation of human sperm for assisted reproduction procedures in a clinical laboratory setting, where total preservation of semen parameters is not mandatory. To determine whether this medium could also be used in a diagnostic laboratory setting for routine semen analysis, it was necessary to identify the maximum time that semen could be kept in this medium without significant alteration in the original semen parameters. Our initial experiment therefore examined 36 semen samples, measured daily for a total of 4 days in extender.

Upon initial analysis, prior to addition of extender, 15 samples were within the fifth percentile for fertile men [1, 2] and 21 showed at least one parameter outside of this range. All samples showed a decline in total motility (Figure 1A,B), progressive motility (Figure 1C,D) and vitality (Figure 1E,F) over the course of the experiment. The rate of decline in these three time-sensitive parameters was highly heterogeneous between different samples. Normal samples showed a decline of −0.238 / −0.249 /−0.111 percentage points per hour for total motility / progressive motility /vitality, with a standard deviation of 0.217 / 0.231 / 0.130 respectively. Abnormal samples showed a decline of −0.338 / −0.352 / −0.154 percentage points per hour for total motility / progressive motility /vitality, with a standard deviation of 0.187 / 0.196 / 0.230 respectively. Amongst the abnormal samples, seven showed especially rapid declines, falling from > 50% progressive motility at $t = 0$ to < 25% progressive motility within 24 h. This heterogeneity precluded any statistically rigorous determination of a 'correction factor' which would allow reconstruction of initial values from downstream time points.

Sperm morphology (Figure 1G,H) showed no consistent trends over the time course, and day-to-day fluctuations did not exceed binomial expectation. Morphology was therefore not assessed in subsequent experiments. All other measurements including sperm count (Figure 1I,J) also showed relatively high day-to-day fluctuations between time points for this experiment, which was tentatively ascribed to incomplete mixing and increased viscosity of many of these samples in this pilot experiment. In subsequent experiments, additional care was taken to ensure samples of normal viscosity were used.

The first follow-up experiment ($n = 13$ samples) focused on two questions: the initial effects of adding extender, and the time frame from 12 to 24 h, that is, the expected delivery window for rapid courier transport of a mail order sample. Figure 2 again demonstrates a high degree of heterogeneity in the response to

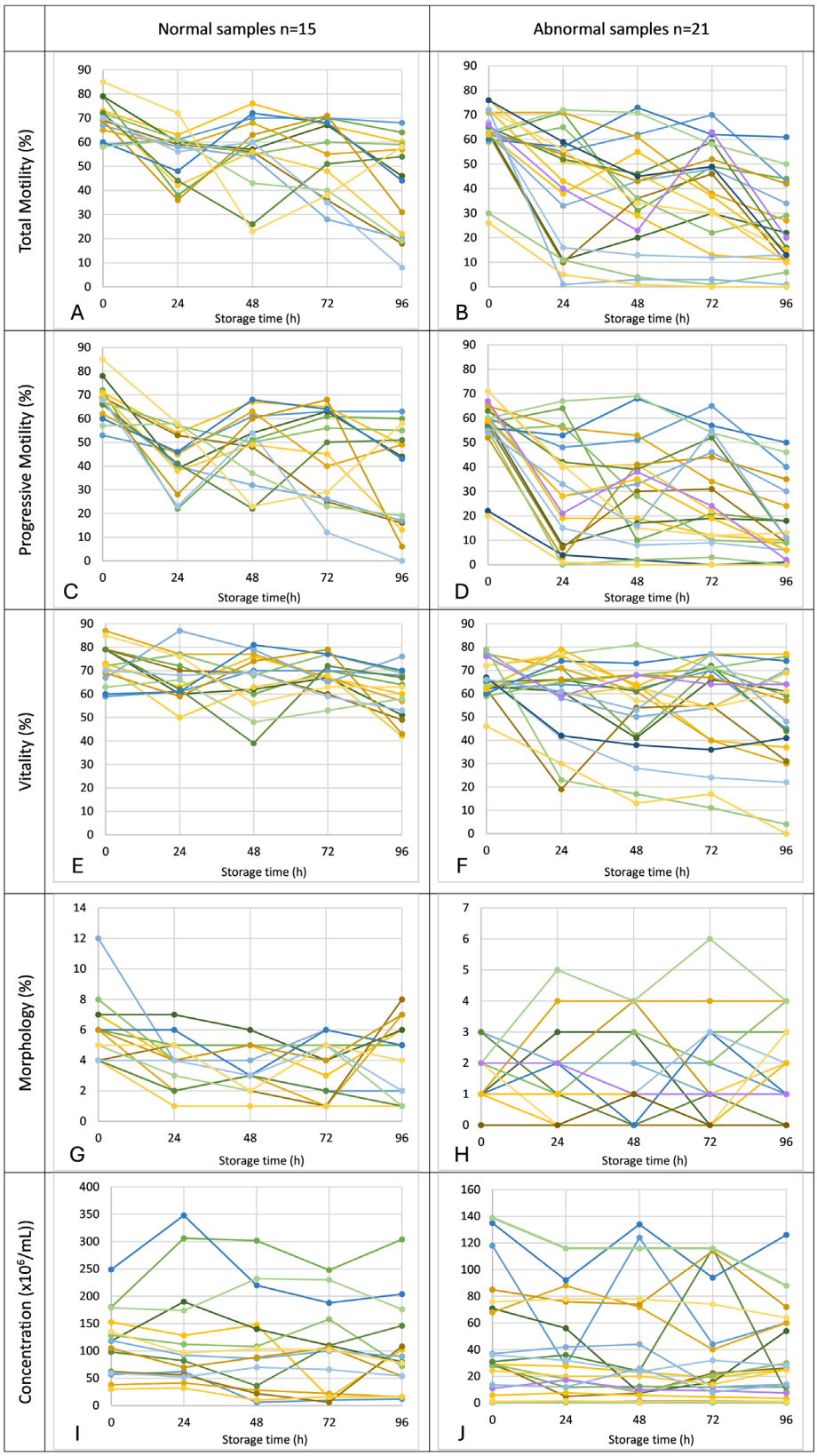

FIGURE 1 | Change in semen parameters over time after addition of extender. Parameters were analysed in raw semen (time 0). Extender was then added, and samples were incubated for up to 96 h at 2–5°C. Total motility (A/B), progressive motility (C/D), vitality (E/F), morphology (G/H) and concentration (I/J) were analysed in 24-h intervals for $n = 15$ normal and $n = 21$ abnormal samples. Normal samples were defined as having all parameters within the fifth percentile for fertile men. Abnormal samples were defined as having at least one semen parameter outside of the fifth percentile for fertile men [1, 2]. Data point and connecting line colours indicate repeat measurements on individual samples.

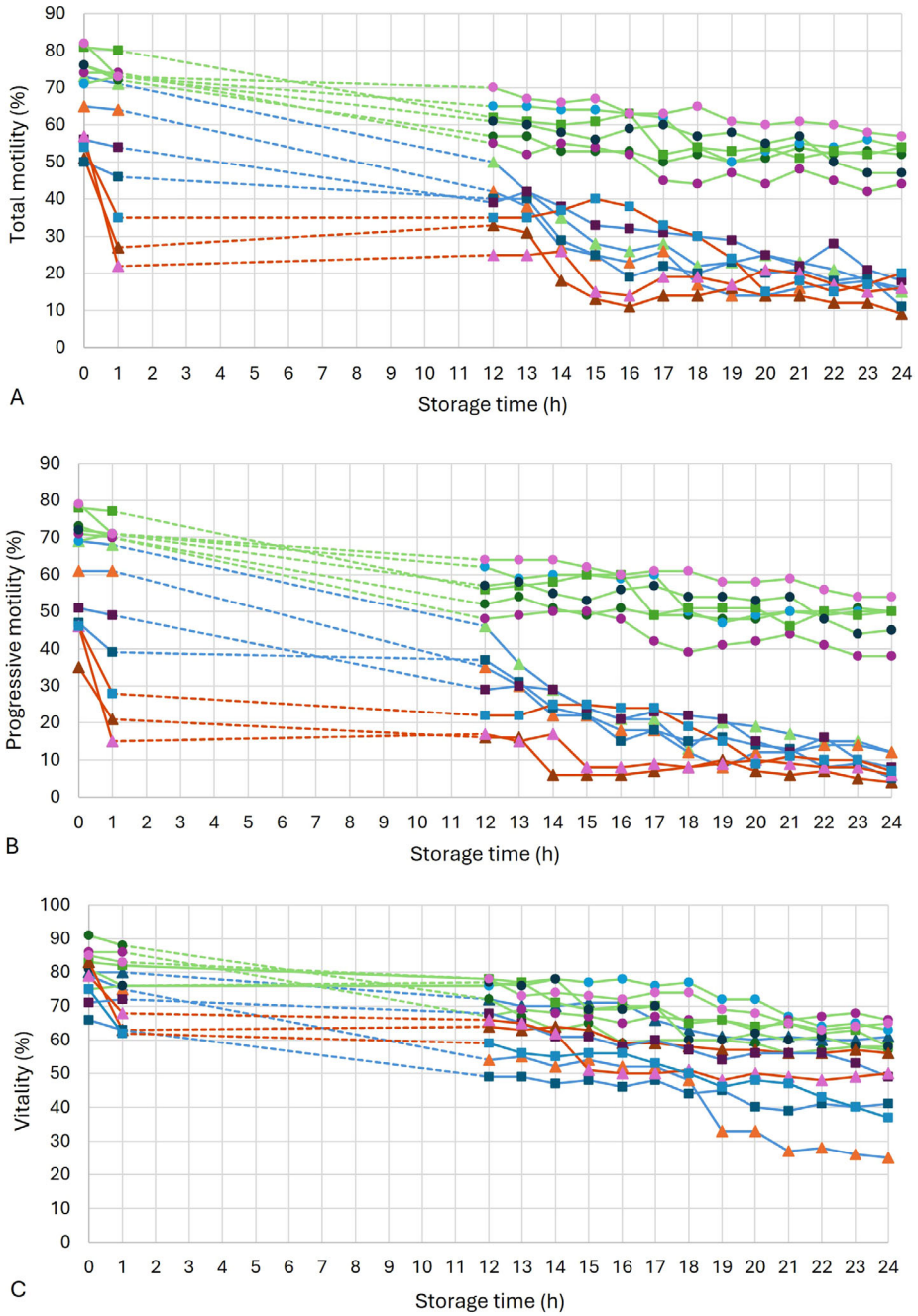

FIGURE 2 | Hourly decline in motility and vitality parameters in extended samples after 12–24 h. Shown are (A) total motility, (B) progressive motility and (C) vitality in $n = 13$ samples. Data point colours indicate repeat measurements on individual samples, with the shape of the point representing the starting sperm concentration (triangle < 15 million/mL, square 15–30 million/mL, circle > 30 million/mL). Connecting line colours group the samples into 'good responders' (green), 'rapid poor responders' (orange) and 'delayed poor responders' (blue).

extender. In this experiment using only samples with normal viscosity there was a clear bimodal distribution for motility parameters with seven samples showing rapid loss of motility (average decline 3.1 ± 0.1% over 24 h) and six showing better motility preservation (average decline 1.4 ± 0.1% over 24 h). Supporting Information Figure 1 shows the distribution of the rate of decline (percentage points per hour) across the sample set, and a bimodal distribution is evident for both total and progressive motility. Furthermore, although the number of samples was small in this experiment, there was an indication that there may be two different categories of 'poor responder' samples, with three samples showing a rapid loss of motility immediately on addition of extender, and four showing a more continuous decline over 24 h. Notably, this rapid decline was specific to the samples with poorer starting parameters, with both starting concentration and initial motility being lower in the 'poor responder' group than the 'good responder' group. In particular, the 'poor responder' samples all had low starting sperm concentrations (all seven equal or below 30 million/mL and four out of seven of them below the WHO reference limit of 15 million/mL), whereas five out of six samples exhibiting slow decline had a starting sperm concentration above 30 million/mL.

3.2 | Validation of Extender With Adjustments to the Manufacturer's Instructions

As the results indicated a deterioration in parameters over time in extender, including declines immediately on addition, a series of experiments were carried out to determine whether it was possible to improve conditions for preserving semen characteristics. The manufacturer's instructions recommend adding extender in a 1:1 (v/v) dilution, dropwise, slowly and maintaining samples in the extender at 2–5°C. To determine optimal handling conditions for extended samples, different storage and handling conditions were compared to the manufacturer's recommendations for preservation of semen parameters.

3.2.1 | Storage Temperature

Firstly, the effect of temperature was investigated, to determine whether the rapid declines within the first few hours were due to cold shock. Samples for this experiment (n = 15) were therefore split following addition of extender and stored either at 2–5°C (manufacturer's instructions) or at RT (RT = 20–24°C) with hourly measurement for 8 h. For all samples, a more pronounced decline was detected for samples stored at 2–5°C (average 4.8% per h for total motility (Figure 3A) and 5.0% for progressive motility (Figure 3C)) compared to RT storage (average 1.8% per h for total motility (Figure 3B) and 1.7% for progressive motility (Figure 3D)), and this difference was significant for both parameters by two-tailed t-test ($p < 0.001$). Vitality showed a mean hourly rate of decline of 3.7% for samples stored at 2–5°C (Figure 3E) and 2.1% for RT (Figure 3F) which was also found to be significant ($p < 0.001$). There was no difference in sperm concentration between storage at either temperature throughout the 8-h time course (Figure 3G/H). It was therefore concluded that for short time periods, storage of semen in extender at RT may preserve sperm parameters more efficiently than storage at 2–5°C as recommended by the manufacturer. Although semen parameters were preserved more efficiently at RT than at 2–5°C, there was still significant heterogeneity in the sample population, with two samples (both low starting concentration with one below 15 million/mL) showing relatively rapid declines in both progressive and total motility within 8 h even at RT despite initially good starting parameters. This could not be ascribed to cold shock. Supporting Information Figures 2 and 3 show the distribution of the rate of decline (percentage points per hour) across the sample set at 2–5°C and at RT, demonstrating the tighter distribution (more consistent rate of decline) for samples stored at RT.

3.2.2 | Addition of Extender

An investigation to probe the effects of osmotic shock was carried out by testing the rate of addition of extender. Figure 4 shows the comparison between slow, dropwise addition of extender according to manufacturer's instructions, versus fast addition by pouring and swirling to mix, all at a 1:1 ratio of semen:extender (n = 35). Most samples do not show any major change in any parameter immediately on extender addition; however, two samples reacted poorly to the extender and showed an immediate drop in total and progressive motility upon addition (Figure 4A/C, respectively). Importantly, any such initial drop was similar irrespective of the speed of extender addition. Scatter plots comparing motility and vitality for slow versus fast addition (Figure 4B,D,F) show these were highly correlated (R^2 = 0.930 for total motility, 0.941 for progressive motility and 0.739 for vitality). This indicates that both motility and vitality decline at the same rate irrespective of the method of addition of extender, for both high- and low-quality samples. We have shown that 1:1 (v/v) semen:extender dilutions have no significant effect on semen parameters. However, patients cannot be asked to measure their sample volume to add an equal volume of extender. We therefore also investigated the effects of 1:2, 1:4 and 1:5 semen:extender ratios (Table 1) and have seen high correlations between all ratios, suggesting that rapid addition of any extender volume does not significantly affect semen parameters.

TABLE 1 | Coefficient of determination (R^2) for motility parameters and vitality utilising various ratios of semen to extender.

	1:2 (n = 36)	1:4 (n = 24)	1:5 (n = 6)
Total motility	0.892	0.761	0.832
Progressive motility	0.906	0.799	0.955
Vitality	0.814	0.640	0.682

Numbers represent the R^2 value for the correlation between measurements obtained using a 1:1 dilution ratio versus those obtained using higher dilution ratios ranging from 1:2 to 1:5.

Importantly, while the rate of decline was not dependent either on the rate of addition of extender or on the ratio of extender to semen in this split-sample analysis, it remained highly variable between the individual samples. Supporting Information Figure 4 shows the rate of change of semen parameters for each individual sample at RT with dropwise addition. While there is a general tendency for motility to decline at ~1%–2% per h, some samples decline much more rapidly within the first 8 h, others decline slowly for 24 h and then rapidly thereafter, and others decline steadily over the full 48 h of the experiment. Understanding the source of this inter-sample variability remains a key goal for future research.

4 | Discussion

This study shows that in some samples, preservation of sperm motility and vitality can be achieved over an extended period using a test-yolk buffer designed for semen preservation. Furthermore, these parameters can be optimised by modification of the manufacturer's handling instructions to minimise the rate of decline in extender. However, the integrity of these parameters cannot be completely maintained, demonstrating that this preservative is unable to provide a direct and accurate measure of a man's raw semen parameters. Furthermore, it is not possible to adjust the results to take this deterioration into account due to the wide variation between samples in the response to the extender. Total sperm count is as expected, typically unaffected by the extension process, as observed in previous studies [13, 16], and both sperm motility and vitality decline following addition of extender. We observed high variability in the rate of decline

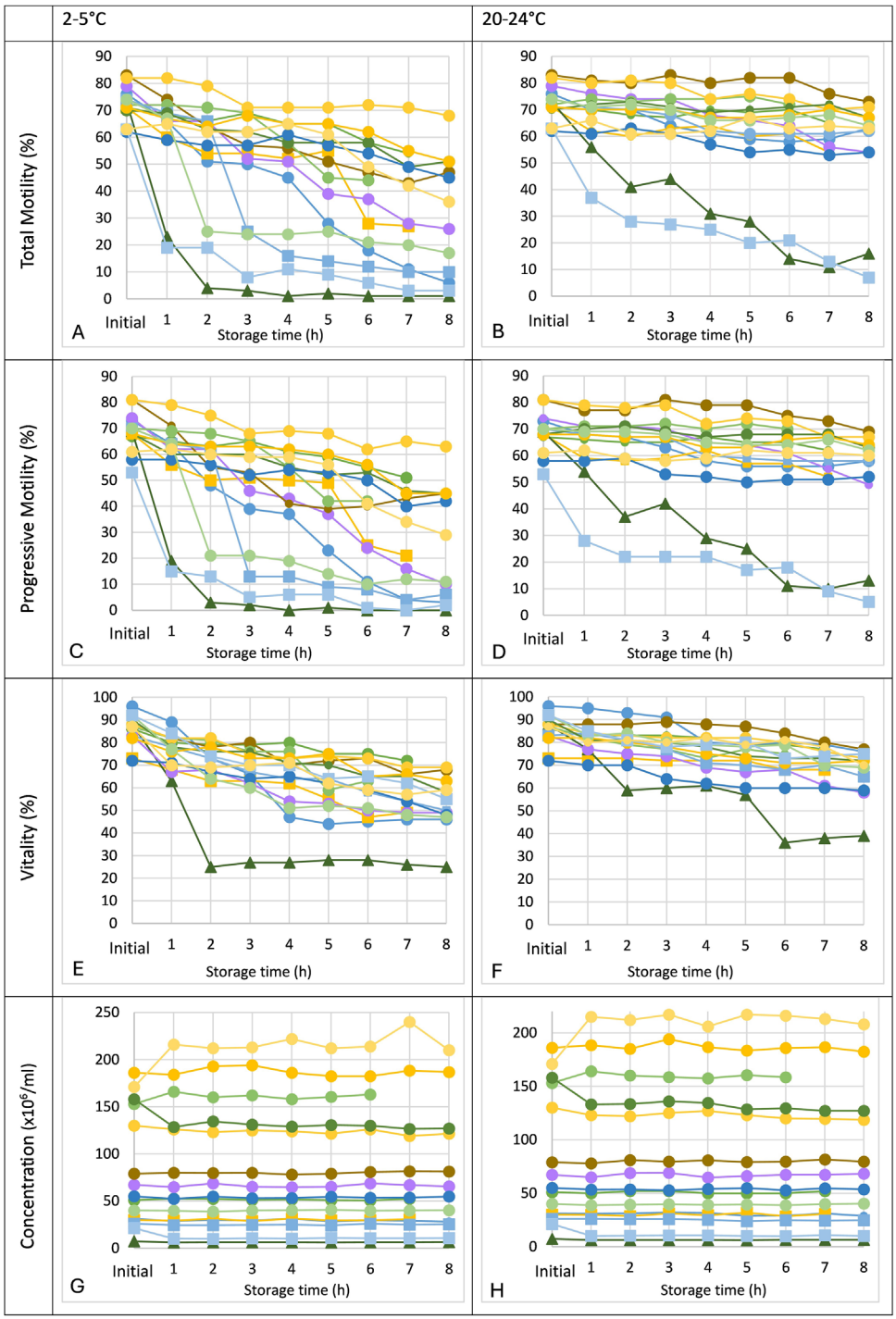

FIGURE 3 | Hourly decline in semen parameters in extended samples over the first 8 h at either 2–5°C (Left panel: A/C/E/G) or 20–24°C (Right panel: B/D/F/H). Shown are total motility (A/B), progressive motility (C/D), vitality (E/F) and concentration (G/H) in $n = 13$ samples. Data point and connecting line colours indicate repeat measurements on individual samples, with the shape of the point representing the starting sperm concentration (triangle < 15 million/mL, square 15–30million/mL, circle > 30million/mL).

in motility parameters. This applied at several stages, with some samples showing accelerated decline immediately upon addition of extender, some which initially tolerated the extender but that declined rapidly within 8 h, and others which tolerated short periods of extension but then declined rapidly at later time points (Supporting Information Figure 4). This is in contrast to a recent report of a modified preservation medium developed by Samplaski et al. [19], in which the sperm count remained stable over 52 h, but sperm motility declined gradually and consistently over time.

The difference in observations may be due in part to the difference between extender composition [5], and the method used for semen analysis; automated computer assisted sperm analysis [19] versus manual analysis according to WHO 2010 [1] criteria in this study. However, the most likely explanation

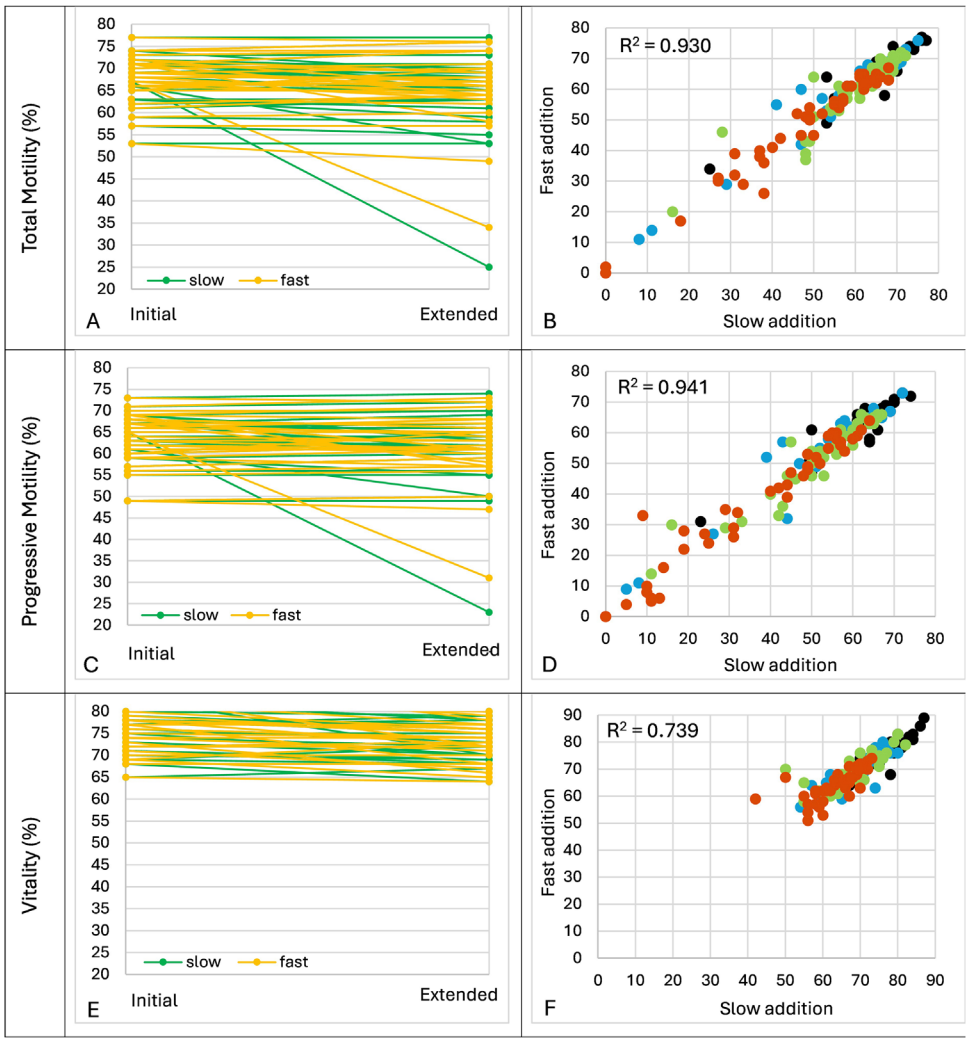

FIGURE 4 | Comparison of slow and fast addition of extender on semen parameters. Samples were stored at RT. Parameters were measured in the raw semen and then assessed at different time points up to 48 h following addition of extender added slowly dropwise or fast pouring. Data depicted as line graphs (A, C, E) and scatter plots (B, D, F) for fast versus slow addition in $n = 35$ samples. The left-hand panels A/C/E show the effect of the rate of addition of extender, with the unextended values shown on the left axis and extended values on the right axis. Green connecting line represents slow addition, yellow represents fast addition. The right-hand panels (B/D/F) are scatter plots comparing total motility (B), progressive motility (D) and vitality (F) for slow versus fast extender addition across all samples and time points. Scatter plot colours indicate measurements at different time points. Analyses were done immediately after extending the sample (black), within the first 8 h (blue), between 8–24 h (green) and 24–48 h post-extension (orange).

is the different patient population studied. Samplaski et al. [19] only used normozoospermic samples for their study, whereas samples used in the current study included a wide range of both normozoospermic as well as samples where parameters fell outside of the fifth percentile for fertile men. In this study it was consistently observed that samples which initially fell outside WHO reference boundaries for normal fertile males were more likely to show accelerated decline in motility in extender. However, this phenomenon was not solely restricted to abnormal samples, and even some samples initially classified as normal, showed unpredictable declines in motility over time, particularly samples with starting sperm concentrations towards the lower limit of normal variability (i.e., 15–30 million/mL).

Traditionally, cool storage in semen extender is the method of choice for prolonging the life of sperm for artificial insemination purposes in livestock [10, 11]. The benefits of cool storage include slowing down metabolism, controlling bacterial growth [5, 20] and protecting sperm from DNA damage [21]. Human sperm are relatively resistant to cold shock, due to the increased cholesterol content in their plasma membranes [22], allowing some degree of preservation of semen parameters at lower temperatures, and most manufacturers of extender, as well as mail-in and home kits on the market, currently use chilled extender approaches [5, 23]. This includes the manufacturer's handling instructions for the media tested in this study, which recommend cool storage of extended semen. However, set against this, sperm maintained at low temperatures are at significant risk of oxidative damage [24], as well as cold shock resulting from solute leakage across plasma membranes, caused by thermotropic membrane lipid phase transitions [22, 25, 26]. In 1996, in the first study on the development of extenders for the transport and storage of

human semen, Aitken et al. demonstrated significant improvement in sperm motility when samples were stored for 24 h at ambient temperature compared to 4°C, using a citrate-based egg yolk buffer semen extender [15]. This is consistent with multiple subsequent studies that indicate motility and vitality are better preserved when raw semen [27] or washed sperm [28–30] are stored at 20–23°C compared to storage at 4°C. Our results together with data from another study [31] using a TYB-based extender, further support these observations and further contextualise them. In particular, our data indicate that low-quality samples are less resilient to cool storage, and that for these samples in particular, storing samples at RT in extender may lead to superior preservation of sperm integrity. Logistically, the ability to preserve samples at RT without significant deterioration would be considerably more advantageous for transporting the samples to the laboratory than having to maintain a cold chain.

A further confounding factor is bacterial contamination, introduced into the sample either from normal skin flora, from genitourinary infections, or during the extending process itself, which will have detrimental consequences on the preservation of semen parameters [32]. While our study was carried out under strict laboratory conditions, ensuring the sterility of a home-produced sample will be exceptionally challenging. Bacteria adhere to spermatozoa and disrupt membrane integrity, not only causing a decline in vitality and motility, but also causing aggregation [33]. This applies to both RT incubation and cool storage. While higher temperatures are more permissive for bacterial proliferation, bacteria may also negatively affect semen parameters during cool liquid storage [12, 13, 19]. In this study, samples that were clearly infected were excluded from the primary analysis; however, it is not possible to determine presence of bacteria solely by microscopic observation, and some of the samples included in our study may have had undetected contamination. Samples with observed bacterial contamination tended to show accelerated declines in motility after 8 h or more of storage (data not shown). Bacterial proliferation can be reduced by incorporation of antibiotics into the extender, such as gentamycin used in the present study. However, not all bacteria will be sensitive to them and antibiotics often vary between extenders as do energy sources, membrane protectants, antioxidants and osmoprotectants [5] which may have different effects on the longevity of the sample in preservative.

The method of addition of extender is also an important practical factor when considering sample production at home. The manufacturer recommends adding an equal volume of extender to semen, slowly and dropwise. However, this is very difficult to control outside of a laboratory environment. Hence, the pouring of a fixed volume presents tremendous benefit for patients over dropwise addition of a calculated amount. In our hands neither the method of extender addition (dropwise vs. pouring) nor the amount of extender (1:1 ratio vs. excess extender) greatly affected the results. This contrasts with other observations using cryopreservative, which suggest that slow addition of preservative may actually protect against osmotic fluctuations that may damage sperm [15, 34]. This may be because the refrigeration medium used in this study does not contain glycerol as it is designed to maintain the osmolarity of the sample.

The accuracy of a semen analysis is affected by many variables irrespective of whether it is conducted in an accredited laboratory [2, 5, 32, 35, 36]. This also applies to extended semen. The data presented here were collected from samples produced at the laboratory; however, sample quality is increased in samples collected at home [37, 38] which may be a potential confounder. Results may be affected by increased viscosity and aggregation which are present after prolonged storage in extender, regardless of storage temperature. Hyperviscosity is not uncommon in semen samples and may play a significant role in undermining the accuracy of the results. Furthermore, the quality of the semen parameters influences the survival rate in liquid storage as the integrity of sperm is greater when sperm parameters are above the fifth percentile for fertile men [17]. Based on the results presented here, while short term storage of up to 8 h at RT may preserve semen parameters sufficiently for diagnosis in the majority of samples, past this time deterioration is less predictable, particularly for samples with poor pre-extension parameters, but also for some with pre-extension parameters that are low but still within the reference range.

At-home testing is therefore potentially a promising option for triage, since total count remains accurate irrespective of dilution, and any sample that remains within the reference range for motility at time of measurement *must* have been within the reference range at the time of production. However, poor semen motility in a post-extension sample does not necessarily imply poor motility prior to extension, and any diagnostic finding of poor semen quality requires confirmation with analysis of a freshly produced sample. A limitation of the current study is the relatively small sample size. Inclusion of a larger cohort of samples in future work may in principle permit development of an algorithm to overcome sample heterogeneity. In conclusion, until the causes of the high inter-sample variability are rigorously identified and subject to detailed validation and verification studies, it will not be possible to construct any correction factor for motility and vitality that would allow accurate diagnosis based on extended samples. To date, we are unaware of any commercial kits that sufficiently take this into consideration.

Author Contributions

Conceptualisation – Sheryl T. Homa; Formal analysis – Alessia Schadwell (lead), Peter J.I. Ellis; Funding acquisition – Sheryl T. Homa, Andrew Dawkins; Investigation and Validation – Alessia Schadwell (lead), Andrew Dawkins, Tina Holton; Project administration and Resources – Sheryl T. Homa; Supervision – Sheryl T. Homa (lead), Peter J.I. Ellis; Visualisation – Alessia Schadwell, Peter J.I. Ellis; Writing, original draft – Alessia Schadwell, Sheryl T. Homa; Writing, review and editing – Alessia Schadwell, Sheryl T. Homa, Peter J.I. Ellis; Writing, statistical analysis – Olivia Whiting.

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Ethics Statement

Approval for this project was granted by the Faculty of Sciences Research Ethics Advisory Group (CREAG) for Human Participants from the University of Kent (eighth of June 2021, Ref: CREAG059-05-2021). Patient data were therefore only collected when the consent forms reviewed and approved by the committee were signed by the participant prior to participation. Data were pseudoanonymised prior to analysis.

Conflicts of Interest

The corresponding author/researcher (Sheryl T. Homa) is consultant clinical lead for TDL Andrology Department.

Data Availability Statement

Data regarding any of the subjects in the study have not been previously published unless specified. Data will be made available to the editors of the journal for review or query upon request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting Figure S1: Distribution of rates of change after 24 h incubation at room temperature. Values given as percentage point decline per hour in (A) progressive motility (mean = −1.46, SD = 0.46, IQR = 0.63), (B) total motility (mean = −1.45, SD = 0.47, IQR = 0.58) and (C) vitality (mean = −1.12, SD = 0.44, IQR = 0.38). **Supporting Figure S2:** Distribution of rates of change after 8 h incubation at 2°C–5°C. Values given as percentage point decline per hour in (A) progressive motility (mean = −5.47, SD = 2.64, IQR = 5.13), (B) total motility (mean = −5.30, SD = 2.80, IQR = 5.16) and (C) vitality (mean = −4.21, SD = 1.70, IQR = 2.00). **Supporting Figure S3:** Distribution of rates of change after 8-h incubation at room temperature. Values given as percentage point decline per hour in (A) progressive motility (mean: -2.15, SD: 2.19, IQR: 1.47), (B) total motility (mean = −2.18, SD = 2.41, IQR = 1.13) and (C) vitality (mean = −2.46, SD = 1.28, IQR = 0.78). **Supporting Figure S4:** Effect of 2-day storage at room temperature on extended semen samples, measured at irregular intervals to mimic real world sample transport times. (A) Total motility, (B) Progressive motility and (C) Vitality for $n = 46$ samples.