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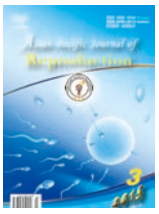

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The preparation and culture of washed human sperm: A comparison of a suite of protein-free media with media containing human serum albumin

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ABSTRACT

Objective: To compare two suites of culture media (one with HSA and one protein-free (PF) supplemented with methylcellulose) for washing human sperm in IVF. **Methods:** Semen samples (n=41) underwent parallel density gradient preparation using PF or HSA-supplemented culture medium and subsequent yield, survival, morphology and motility were compared. **Results:** The PF medium resulted in a significantly higher sperm yield (P<0.0001), but similar sperm morphology (P=0.822) and 24-hour survival (P=0.11). There was, however, a lower percentage of progressively motile sperm (P<0.0001) and a higher proportion of sperm demonstrating non-progressive motility (P<0.0001) in the PF medium when observed on a Makler Chamber, apparently an artefact as a similar sperm motility index was measured using a Sperm Quality Analyser (P=0.83). Attachment of sperm in PF medium to the glass chamber reduced with time and any differences had disappeared after 6 minutes on the counting chamber. **Conclusion:** These results support the use of PF media supplemented with methylcellulose as an alternative to HSA, although a modification to the manufacturer's protocol of 6-minutes pre-incubation before assessing sperm motility must be used. Further studies should investigate the function of such sperm prepared in PF medium.

1. Introduction

The desire to use serum-free medium exists in a number of industries, ranging from the pharmaceutical industry where there is a need to produce biopharmaceutical products free of infectious agents[1, 2], to the area of toxicity testing, in which novel protein biomarkers of cells in culture need to be monitored with minimal background contamination[3]. But with over 70 000 treatment cycles and 13 000 babies born each year in Australia alone following the culture of human embryos in vitro[4], assisted reproductive technology (ART) is a major user of medium which, when used to culture preimplantation embryos for less than one week after fertilisation, can have profound effects on the health and characteristics of the foetus and children later in life[5-9].

IVF medium must be safe and effective[10], and over the years has contained protein supplemented in the form of patient serum through to human serum albumin (HSA) as a means of improving the growth of the embryos[11]. Nevertheless, the various protein supplements have each presented their own problems regarding safety with pooled patient serum having resulted in the transmission of hepatitis B to recipients[12] and a batch of HSA prepared from donated blood having been withdrawn because of a donor having been found to be infected with Creutzfeld-Jakob disease[13]. Whilst albumin can have significant batch-to-batch variability[14], HSA is beneficial in improving fertilisation, cleavage and pregnancy rates[15] but even current day practices of HSA purification still

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results in the presence of a large variety of non-declared proteins of unknown consequences to the health of patients and future offspring [16, 17]. Given the aforementioned limitations and risks of the use of human serum albumin in ART, some interest has been shown in the identification and/or development of more stable and safer alternatives. There are several macromolecules derived from non-animal sources that have been investigated for use as a supplement for ART culture media[18-20], though many of these alternatives are yet to be incorporated into commercially available culture media and adopted into routine clinical use. One exception, however, is methylcellulose: a non-toxic, non-allergenic, non-carcinogenic, non-infectious, plant-derived macromolecule with antioxidant and osmolytic properties[21]. A formulation of IVF medium which contains methylcellulose and is truly protein-free has shown excellent results in preliminary trials[22-24], although experience with a commercially-available version has not been so encouraging[25].

Whilst a commercially-available protein-free ART medium would have merit, the ability of such a medium to support the many steps involving gametes and embryos in an IVF procedure must be demonstrated, and given the apparent lack of investigations into same, a systematic appraisal of the various individual steps is warranted. The aim of the present study was, therefore, to assess the effectiveness of a commercially-available protein-free medium and its associated density gradient system when applied to sperm preparation in terms of sperm yield, motility, morphology and motility maintenance in culture, and to compare this to a commercially-available suite of culture media supplemented with HSA as they might be used in a clinical setting.

2. Materials and methods

2.1. Study design

The present study involved the analysis and parallel preparation of semen samples from men attending Fertility North for fertility assessment between August and December 2013. The study design ensured that each sample was prepared in an identical fashion using both media types to allow direct comparison, using only one sample per man to avoid pseudo-replication. The study was approved by the Joondalup Health Campus Human Research Ethics Committee and the Ethics Committee of Edith Cowan University, and all participants provided informed consent for their samples to be utilised in the research.

2.2. Collection of semen

Semen samples were obtained over the full period of the study from a total of 41 men (average ± SEM age of 35.4±1.0 years). Each man was instructed to abstain from ejaculation for 2-5 days and then produce a semen sample by masturbation into a wide-mouthed, 60 mL sterile specimen container (TechnoPlas, Australia). After production, each sample was held in a warming oven (Memmert, Germany) at 37 ℃ for a maximum of 60 minutes until analysis and processing.

2.3. Semen and sperm assessment

All semen samples underwent routine semen analysis following protocols employed routinely by the clinic, which are based upon the methods described in the World Health Organisation Guidelines [26]. Washed sperm were assessed using the same methodology. To reflect clinical practice, sperm concentration was determined for each sample at a dilution of 1:20 using an Improved Neubauer Haemocytometer. A 50 μL aliquot of neat semen or the final sperm suspension was added to 950 μL of water and mixed well to ensure the suspension was evenly distributed. Then 10 μL of this diluted suspension was pipetted under the cover slip of each chamber of the haemocytometer and allowed to settle for 5 minutes in a humidified chamber, before the sperm were counted. Sperm morphology was evaluated by making duplicate air-dried smears and staining with DiffQuick® (Bactolaboratories, Australia). A minimum of 200 sperm were assessed at x400 magnification under brightfield illumination, and the proportion of sperm with normal morphology expressed as a percentage of the total number of sperm assessed. For the assessment of sperm motility, 10 μL of neat semen or the final sperm suspension was aliquoted onto the surface of a Makler chamber (Sefi Medical Instruments Ltd, Israel) and the cover slip put in place. At least 200 sperm were observed at 200x using phase-contrast microscopy, and their motility categorised as rapid progressive ("A"), slow progressive ("B"), non-progressive ("C") or immotile ("D"). In addition, sperm motility was assessed in some samples using an automated Sperm Quality Analyser (SQA IIB, Medical Electronic Systems, Israel). This form of assessment uses deep chambers and the instrument detects movement of sperm suspended in medium by measuring light scatter and calculating a sperm motility index (SMI) [27].

2.4. Sperm preparation and culture

Two suites of culture media were used, one supplemented with HSA (Quinn's Advantage HSA [#3001] and Quinn's Advantage Medium with Hepes [#1023], Sage Biopharma, USA; Puresperm® #PS100-250, Nidacon, Sweden) and the other being protein-free (CellCura® PF Protein Free GradiART™ Upper and lower Layer Medium [#GP21050 and #GP31050], PF Protein Free Gamete

Handling Medium with HEPES and Gentamicin [#GP700500], CellCura, Norway). All preparations were performed "blind" so the scientist doing the work was unaware which preparation was HSA or PF.

Following routine semen analysis, all 41 semen samples underwent parallel density gradient preparation in each suite of media. Depending on the volume of semen available, between 0.5 and 1.0 mL semen was applied to discontinuous gradients of equal volumes (1ml each) of 95% and 50% Puresperm or Upper and Lower CellCura gradients in conical centrifuge tubes (#352095, Falcon Corning, USA). The same volume of semen was applied to each of the two types of gradients to avoid discrepancies. Each gradient tube was centrifuged (#C28A, Boeco, Germany) at 350 g for 15 minutes. The resulting pellet was removed and resuspended in a clean Falcon tube (352095, Falcon Corning, USA) in 2 mL of the corresponding culture media: Sage (HSA) or CellCura (PF). Each suspension was washed by centrifugation, at 500 g for 5 minutes. The supernatant was removed by aspiration with a clean Pasteur pipette (PPB-150-100-PL, Hunter, Australia) and resuspended with another 2 mL of the corresponding culture media: HSA or PF. Each suspension then underwent a final wash, at 500 g for 5 minutes. The supernatant was removed and the pellet resuspended in 0.3 mL of HSA or PF media and the suspension was examined microscopically at 200x (phase-contrast, 20x objective, 10x ocular) (#BX50F, Olympus, Australia) and resulting sperm motility and concentration then manually assessed.

Following the trial preparation described above, 20 of the duplicate preparations were cultured in Falcon tubes (#352095, Falcon Corning, USA) overnight at room temperature. Twenty-four hours later, sperm motility and the calculated motility maintenance (= final progressive motility *100% initial progressive motility) of the sperm in each culture media were manually assessed.

2.5. Exposure to anti-static measures

Following trial preparation, the HSA and PF suspensions of 11 samples were manually assessed for sperm motility on microscope slides (Knittel, Germany) that had been subjected to antistatic treatments in the 5 minutes preceding the motility assessment, namely (i) wiped slides had been treated with an anti-static Superfine Fibre Cloth (GGS Photographic Equipment Company, China), and (ii) slides had been treated with Distinct Anti-Static Screen Cleaning fluid (E3! Style Product Specialists, Brisbane, Australia).

2.6. Statistical analysis

The differences between the experimental and control groups were compared for statistical significance with the Paired Student's T-Test using the StatistXL (StatistXL, Western Australia, 2009) add-in to Excel (Microsoft Office, Microsoft Corporation, 2010). Differences were considered significant if $P<0.05$.

3. Results

3.1. Initial density gradient preparations sperm culture

The overall results of the semen preparation using HSA-supplemented and PF media for all 41 samples are shown in Table 1. Analysis of the total number of sperm recovered from each media suite showed that PF preparation resulted in significantly higher numbers of sperm compared with the HSA suite ($P<0.05$) and consequently a higher yield from the semen ($P<0.05$). A slight but significant improvement in sperm morphology was noted for both gradients compared to the original semen (both $P<0.05$) although they did not differ between themselves. Compared to the semen, both gradients resulted in higher proportions of sperm with progressive (A+B) motility and fewer immotile (D) sperm (both $P<0.05$). However, there did appear to be a difference in the motility profiles of the sperm recovered from the two different media suites, with PF suspensions demonstrating a lower proportion of sperm with "A+B" type motility and a greater proportion of "C" type motile sperm. The proportion of immotile sperm was not significantly different between the two preparation types.

A subset of 20 prepared pairs of samples were cultured at room temperature overnight and the motility and percentage sperm motility maintenance were assessed as shown in Table 2. The proportion of immotile sperm was the same for both systems, but once again the proportion of "A+B" motile sperm at 24 hours was greater in those suspensions prepared using the HSA suite of media whilst there was more "C" type motility observed in the PF suspensions. Whilst the survival of sperm seemed to be superior in the HSA suspensions, this difference was not statistically significant.

3.2. Factors affecting sperm motility profile

The increased proportion of sperm showing "C" grade motility in the PF medium, as described above, was eventually noticed to be associated with sperm sticking to the glass of the Makler chamber. In an attempt to explain this, sperm motility was further assessed in a sub-set of 9 sperm suspensions using the SQA II automated analyser as shown in Table 3. Despite the samples processed using the PF gradient again showing the same pattern of lower progressive and higher non-progressive motility than the HSA gradients preparations when manually assessed on the Makler chamber, the SQA demonstrated no significant difference in the SMI between the two media suites ($P=0.83$).

Table 1

The preparation of washed sperm by discontinuous gradients with suites of media either containing human serum albumin (HSA media; Medicult medium and Puresperm) or being protein-free (PF media: CellCura media and gradient). Values are mean ± sem for 41 samples from 41 men.

Sperm parameter		Original semen	Final preparation	
			HSA media	PF media
Sperm concentration ($\times 10^6$/mL)		87.8±10.2	-	-
Total sperm loaded/recovered ($\times 10^6$)		77.2 ± 9.0	14.9 ± 2.1	23.1 ± 2.8*
Normal forms (%)		10.7±1.3	13.4±2.7	13.6±2.3
Motility (%)	A+B	57.2±2.2	83.9 ± 2.6	71.3 ± 2.4*
	C	7.6±0.7	8.1 ± 1.3	24.1 ± 2.2*
	D	34.3±1.9	8.4 ± 2.3	5.1 ± 1.2
	Yield (%)	-	20.5 ± 2.6	31.7 ± 4.0*

*P<0.05 between the HSA and protein-free medium.

Table 2

The motility and motility maintenance (mean ± sem) of washed sperm cultured for 24hrs following preparation in HSA-containing and protein-free media. A total of 20 samples from 20 men were processed, and sperm survival in culture was defined as the final motility x 100 / initial motility.

Sperm parameter	HSA media		PF media	
	0 hrs	24 hrs	0 hrs	24 hrs
Motility: A+B (%)	83.9±2.6	57.3±4.9*	71.3±2.4	39.6±5.9*
C (%)	8.1±1.3	16.6±1.9*	24.1±2.2	35.5±4.5*
D (%)	8.4±2.3	25.8±4.1*	5.1±2.5	26.6±5.5*
Survival (%)[1]	-	68.0±5.3	-	57.7±7.5

[1]Survival = final motility ×100/initial progressive motility (motility maintenance), *Results for the same preparation method after 24 hours in culture are significantly different (P<0.05)

Table 3

The motility (mean ± SEM) of sperm prepared using HSA-containing and protein-free media when assessed both manually and with the Sperm Quality Analyser (n=9 semen samples from 9 men).

Motility assessment (%)		Media suite	
		HSA	Protein-free
Manual	A+B	90.8±2.9	73.1±4.4*
	C	4.9±1.9	23.2±3.3*
	D	4.2±1.9	3.8±1.6
SQA analyser	Sperm motility index	452.4±43.9	470.1±36.8

*P<0.05 between the HSA and protein-free medium.

To endeavour to determine whether static electricity may have caused adherence of sperm to the glass microscope slide during motility assessment, and hence an apparent increase in "C" type motility, the slides used to assess 11 pairs of washed preparations were exposed to 2 antistatic measures as shown in Table 4. The results indicate that the antistatic spray and wipes both increased the proportion of sperm with "C" type motility in the HSA-containing medium, and did not improve the situation in the PF medium.

Table 4

Motility of sperm (mean ± SEM) prepared using HSA-containing and protein-free media on slides when assessed on microscope slides pre-treated with anti-static spray or wipes (N=11).

Motility grade	Spray pre-treatment		Wipe pre-treatment	
	HSA	Protein-free	HSA	Protein-free
A+B (%)	53.1±3.4	41.0±4.2*	55.3±3.6	43.0±4.4
C (%)	36.0±2.9	45.8±4.4	36.0±3.1	49.8±3.8*
D (%)	9.8±2.1	11.1±3.1	9.6±1.2	7.3±1.6

*significant P<0.05

Sperm motility in PF medium was opportunistically noticed to improve in PF medium when viewed on a microscope slide left on the bench. In a final attempt to ensure the difference in motility patterns between the two different media was not a simple artefact, motility was manually assessed at 0, 3 and 6 minutes after loading of the glass slides in a subset of 11 samples as shown in Table 5. While statistically significant differences between patterns of sperm motility are apparent in the different media types at the early time intervals, these differences appear to resolve over time. Samples therefore processed on the two sets of gradients and assessed 6 minutes after loading on to the Makler chamber, as shown in Figure 1, were similar for both media types.

Table 5

The assessment of motility (mean ± SEM) at different times after loading on to a microscope slide. Sperm from 11 ejaculates produced by 11 men were prepared and cultured in both HSA-containing and protein-free media.

Time (mins)	HSA media			PF media		
	A+B	C	D	A+B	C	D
0	84.7±3.7	7.5±2.4	7.9±3.5	74.9±2.7	21.1±3.0*	3.6±1.0
3	85.6±4.1	8.1±2.6	7.1±3.7	71.0±3.4*	25.4±3.6*	3.6±1.2
6	85.2±3.5	7.0±2.1	8.3±3.2	86.8±2.0	9.4±1.9	3.4±0.9

*P<0.05 comparing HSA-containing and protein-free media.

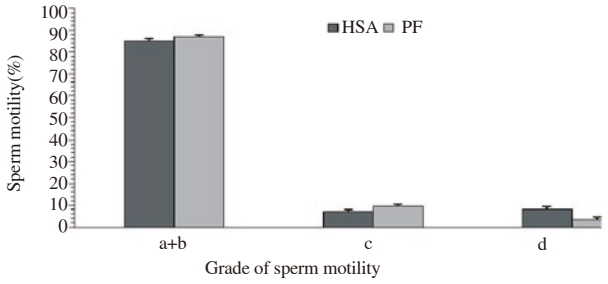

Figure 1. The motility of sperm from 11 semen samples prepared using density gradients and resuspended in medium either containing human serum albumin (HSA) or being protein-free (PF). Sperm motility was assessed 6 minutes after loading on to the microscope slide.

4. Discussion

The use of serum to supplement the culture of mammalian embryos dates back to the 1960s when it was discovered that the addition of rabbit or bovine serum to culture media significantly increased the proportion of blastocysts formed[28], and the early work on human IVF used patient serum as a supplement to the medium[29]. Serum is thought to help create an environment that more closely resembles that of the female reproductive tract that may improve the rate of cleavage, encourage activation of the embryonic genome and enhance the embryos' implantation potential[30], and because of its complex composition, human serum added to culture media can act as a buffer, helping to minimise fluctuations in pH, whilst the amino acids contained within it may supplement the activity of those amino acids already separately added to culture media and it may also help bind any heavy metals[31]. In addition to its effect on embryos, the presence of serum appears to facilitate the process of capacitation and/or the acrosome reaction of sperm, which is essential to enable them to fertilise an oocyte in vitro[32, 33]. Serum in culture also appears to reduce the incidence of DNA damage in sperm and increase the rate of cleavage in human embryos[34]. At a practical level, serum also prevents gametes and embryos from becoming sticky and difficult to handle in culture, thus facilitating their manipulation in culture and preventing them from adhering to the plastic and glassware used[35].

The use of serum has been criticised because, being a biological solution, its composition is difficult to define, can be subject to significant variation, and may include components that impact on embryonic development such as hormones, vitamins, fatty acids and ions[14, 36]. When used as a supplement in assisted reproductive technology, there is the compounding problem that the maternal serum from some women, such as those with endometriosis, may contain elements that are embryotoxic[37]. Perhaps more importantly, the use of serum, both from animal and human origins, has also been criticised because of the risk of disease transmission. In spite of donor screening processes, in 1991 in the Netherlands, 79 IVF patients contracted Hepatitis B following the culture of their embryos in culture media containing pooled serum that had been contaminated by the Hepatitis B virus[12] and, more recently, it was revealed that a commercially-available HSA product had potentially been contaminated following the death of an albumin donor due to the infectious Creutzfeldt Jakob Disease (CJD) prion[13].

In the quest to remove serum from IVF culture medium, a version of medium without serum has been used[38]. However, human serum albumin in one form or another, but still derived from blood, is predominantly used to supplement ART culture media in the hope that it provides a better defined medium and elicits improved results[15, 30, 39–41], although there is some concern that serum supplementation yields better results than protein supplementation alone and the latter may be suitable only in conjunction with additional components[42]. Recombinant albumin has been used in cell culture because of the concerns over transmissible spongiform encephalopathies[2], and it has also been used in a general IVF setting[43] as well as the special case of Jehovah's witnesses[44]. Nevertheless, the use of non-protein macromolecules has been investigated for use as a supplement for ART culture media in an attempt to avoid the problems associated with blood products, such as the addition of polyvinyl alcohol (PVA)[45], polyvinylpyrolidone (PVP)[46] and dextran[18] to aid with the potential handling differences when HSA is not incorporated into culture media. However, the teratological safety of the likes of PVA and PVP is uncertain[47].

Methylcellulose has been shown to be a suitable macromolecule to support gametes and embryos[22, 23] and following patenting of a formulation[21] this has now been placed in to commercial production and marketed by Cellcura®. The availability of such a medium would appear to be a step forward but a recent clinical trial had to be cancelled due to poor performance of the medium[25]. It was unclear whether this was due to issues with the formulation, manufacture or shipment, but the satisfactory performance of the formulation reported earlier[22–24] cannot be ignored. The usefulness of the medium is best confirmed by a systematic evaluation of the methylcellulose-supplemented medium in the key steps of the ART process, rather than just an overview of the overall process. The aim of the present study, therefore, was to independently assess the effectiveness of the medium using density gradients, a routine sperm preparation technique employed by many clinics worldwide, in terms of sperm yield, motility, survival and morphology. One suite of media used was supplemented traditionally with HSA; the other with the plant-derived macromolecule, methylcellulose.

The results of the present study confirmed that the yield of sperm using the PF suite of gradients and medium was satisfactory. However, the main limitation was the artefact in assessing sperm motility. There was an apparent increase in the proportion of non-progressively motile sperm but this was not apparent with the deep

chamber used with the SQA sperm analyser, and resolved itself on glass slides by simply waiting for 6 minutes and allowing the sperm to detach themselves from the glass surface. As mentioned above, the "stickiness" of cells is relieved by the inclusion of protein[35], but equally a revised protocol using the PF medium was effective. However, regulatory authorities (eg the Therapeutic Goods Administration, TGA, in Australia) require such a modification to be documented in the manufacturer's Instructions for Use (see https://www.tga.gov.au/publication/australian-regulatory-guidelines-medical-devices-argmd) and this was not provided. The use of the suite of PF media under sub-optimal conditions will bias the findings of laboratories and may result in erroneous conclusions on the suitability of the media for clinical use. These findings raise the question of whether modifications are required at other stages of gamete culture, and so further systematic studies on different aspects of use of the media with sperm are warranted.

In summary, the present study has confirmed that the use of a commercially-available protein-free suite of media can result in good preparations of washed sperm, but that a modified protocol must be used to assess sperm motility in order to avoid the artefact of sperm sticking to the microscope observation chamber. While these results support the use of methylcellulose as an alternative to HSA, albeit it with a modified protocol, further studies on the functional capacity of the sperm are required.

Conflict of interest statement

The authors KP, PR, and PM declare no competing interests. JA is a scientific consultant to CellCura and the inventor of the PF medium.

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