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Original article

Comparative characteristics of spermatozoa harvested and cryopreserved in culture and cryoprotectant media with or without donor serum proteins

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ABSTRACT

The objectives of this study is to evaluate the efficacy of protein-free media in the preparation, holding and crypreservation of spermatazoa for use in ART. Normozoospermic semen samples ($N = 71$) were used to compare the effects of media on the survival and quality of spermatozoa when washed and cultured with different media with and without added proteins at 4 °C, 15 °C, 22 °C and 37 °C for 0, 4–7 and 24 h. Survival and quality of spermatozoa were assessed after freeze-thaw with synthetic cryoprotectant with and without proteins. Ethics/IRB approval was obtained (Ref. 1073.52). Spermatozoa parameters were similar in all media after washing and culture for 24 h. Post-thaw survival and quality of spermatozoa was not significantly different 24 h after thawing of samples frozen in all cryoprotectant medium. In conclusion synthetic protein-free culture and cryoprotectant media are equal in efficacy to protein-containing media in culture and cryopreservation of spermatozoa . Use of these synthetic media are anticipated to significantly reduce the risk, potentially associated with conventional protein-containing media, of transmission of disease and possibly harmful undeclared proteins to the patient, baby and the healtcare worker. Synthetic media also ensure consistency of quality between batches of media.

1. Introduction

In ART donor serum proteins [DSPs] or human serum albumin [HSA] are usually added to culture and cryoprotectant media as a source of fixed nitrogen and as surfactant [1]. However, strict sterilization of DSPs and HSA may not exclude with certainty all pathogens particularly prions that are known to cause a range of neurogenerative diseases including mad cow disease and its human equivalent Creutzfeld–Jacob disease vCJD [2,3]. Incidents of disease transmission or accidents involving use of vCJD prion or hepatitis virus-tainted serum have been reported [4–7]. Serum may also contain gamete and embryo-toxic factors [8,9], and batch variations affect the viability and quality of cultured gametes and embryos [10]. Recently a variety of unknown potentially harmful protein contaminants have been found in unconditioned commercial embryo culture media [11]. Clearly, use of protein-free medium [PFM] in the practice of ART is safer than use of DSP containing media.

The European Union recommends [EU Tissue Directive No.2004/23/EU] the avoidance of the use of non-uniform biological components in healthcare products.

As early as the mid-1980s [12] attempts were made to discard DSPs in human ART culture media with limited success because, although the embryo culture media were protein-free, the spermatozoa preparation media needed protein to ensure it retained its fertilizing capability. Consequentlyly the use of DSPs or

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HSA has persisted to the present time in spite of the hazards and disadvantages associated with its use. Indeed the dictum that HSA is essential for spermatozoa survival [12], capacitation and hyperactivation [13,14] has persisted to this day. The present study compares the effects on spermatozoa quality [motility and DNA fragmentation level] when spermatozoa are washed and cryopreserved in commercial synthetic medium or protein containing medium. Spermatozoa quality at the time of insemination influences subsequent embryonic and fetal development [15–17].

2. Materials and methods

2.1. Materials

2.1.1. Semen collection and analysis

Normozoospermic semen samples were obtained from 71 men undergoing semen assessment at the University of Malaya Fertility Center (UMFC), the University of Malaya Medical Center (UMMC) Polyclinic, National Population and Family Development Board (NPFDB) and Hospital Kuala Lumpur (HKL) . . . BLINDED . . . from August 2014 through January 2015. Ethics/IRB approval was obtained [Ref. 1073.52] and written informed consent was collected from each patient who agreed to participate in the study. The patients produced semen samples by masturbation in a private room close to the laboratory after an abstinence 2–3 days from sexual activity.

2.1.2. Spermatozoa handling and cryopreservation media utilized

The commercial protein-containing media selected for this study were: the Multipurpose Handling Medium™ [Catalog ID: 9016], Continuous Single Culture Medium™ [Catalog ID: 90164], and Human Serum Albumin Solution [HSA] [Catalog ID: 9988], which were obtained from IRVINE SCIENTIFIC [USA]. The commercial synthetic protein-free media used in the study are: the Protein-Free IUI Medium with HEPES and Gentamycin [Catalog ID: GP40050], Protein-Free Human Embryo Culture Medium with Gentamycin [Catalog ID: EM10050], Protein-Free GradiART Lower Layer [Catalog ID: GP2105] and the Protein-Free GradiART Upper Layer [Catalog ID: GP31050] from CELLCURA ASA [Norway]. The Minimum Essential Medium [MEM] with HEPES and the MEM without HEPES are obtained from BIOWEST SAS [France]. The spermatozoa cryoprotectant media used were [i] synthetic protein-free spermatozoa cryoprotectant [SCA; described by Ali [18], [ii] DSP-containing conventional cryoprotectant made in-house [CCM; described by Mortimer [19] or [iii] the commercial DSP-containing conventional cryoprotectant medium [CCI; Irvine Scientific, USA].

2.2. Methods

2.2.1. Spermatozoa washing and preparation

Semen analysis was performed according to the WHO 2010 Standard Format [20].

Semen samples were washed and spermatozoa harvested by the standard density gradient centrifugation [DGC] technique [21–25].

2.2.2. Evaluation of spermatozoa motility and vitality

A modified Neubauer Chamber [Hawksley, Lancing, England] was used to assess spermatozoa motility in compliance with the norms stated in the WHO Semen Analysis Manual 2010 [20]. Observations were made using a light microscope at 200x magnification. The results were expressed as a percentage of motile spermatozoa. Due to the propensity of spermatozoa heads to stick to the surface of the counting chamber, a holding time of 5–7 min was required after charging the Neubauer chamber to enable the spermatozoa to detach themselves as recommended by the Inventor of the PFM. It is noted that this behavior was observed only in PFM but not in CPC and MEM as was also reported by Peirce et al. [26]. Therefore, all samples were held for 5–7 min prior to the motility evaluation in order to standardize the method and render our treatment identical for all specimens irrespective of the washing or culture media used.

Spermatozoa VitalStain™ [Nidacon International AB, Mölndal, Sweden] was used to give an indication of live and dead spermatozoa in each treatment. Equal amounts of the VitalStain™ solution and spermatozoa sample [50 μl SVS + 50 μl spermatozoa sample] were mixed well in an Eppendorf micro tube *and* the mixture was kept for 30 s at room temperature prior to observation. A total of 200 spermatozoa were counted per observation.

2.2.3. Evaluation of spermatozoa plasma membrane integrity

The hypo-osmotic swelling [HOS] test was performed as described by its originator [27]. It is suggestive of the functionality and integrity of spermatozoa plasma membrane and thus an assumption of its viability. Spermatozoa were treated with a hypo-osmotic solution. A modified *Neubauer Chamber* [Hawksley, Lancing, England] was filled with the treated spermatozoa and allowed to stand for at least 1 min before observations were made under the phase-contrast microscope at 400× magnification. A total of 200 spermatozoa were observed for swellings:

$$\text{Membrane IntegrityLevel}\,[\%] = \frac{\text{Number of Swollen Spermatozoa} \times 100}{\text{Total Number of Spermatozoa Counted}}$$

Response to the hypo-osmotic solution was observed as a swelling or small enlargement present at the tip of the tail or at the junction of the midpiece. A curved, shortened or thickened tail as descibed by Jeyendran et al. [27] was also recorded as a response.

2.2.4. Spermatozoa DNA fragmentation assay

Spermatozoa DNA fragmentation [SDF] level was measured using the *Halosperm® G2* kit [Halotech DNA, Madrid, Spain] [28]. The assay was performed according to the instructions of the manufacturer to measure spermatozoa DNA strand breaks [29]. The presence of a halo of chromatin decondensation around the heads of the treated spermatozoa is an indication of their DNA integrity. The absence of a halo is indicative of fragmented DNA. Observations were made under the bright field light microscope at 400× magnification. A total of 200 spermatozoa were counted for each treatment and results were expressed as the percentage of spermatozoa with intact DNA. A control consisting of sibling spermatozoa not treated with denaturing solution was included alongside each sample tested. All control spermatozoa responded to the stain and showed a halo of chromatin dispersion around the head

$$\text{Spermatozoa DNA Integrity Level}\,[\%] = \frac{\text{Number of Spermatozoa with Halo} \times 100}{\text{Total Number of Spermatozoa Counted}}$$

2.2.5. Freeze–thaw protocol

The specimens were exposed to individual cryoprotectant medium as per the protocol supplied by the manufacturers or inventors [18,19]. In Experiment 4 the equilibrated semen specimen were apportioned into 1.8 ml cryo vials [NUNC, Denmark] and frozen according to standard methods in which the vials were held at 14 cm above surface of LN_2 in a LN_2 tank for 20mins and then at 7 cm also for 20 min. At the end of this period