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Cryopreservation and egg yolk medium alter the proteome of ram spermatozoa

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ABSTRACT

Cryopreservation causes significant lethal and sub-lethal damage to spermatozoa. In order to improve freezing outcomes, a comprehensive understanding of sub-lethal damage is required. Cryopreservation induced changes to sperm proteins have been investigated in several species, but few have employed currently available state of the art, data independent acquisition mass spectrometry (MS) methods. We used the SWATH LC-MS method to quantitatively profile proteomic changes to ram spermatozoa following exposure to egg yolk and cryopreservation. Egg yolk contributed 15 proteins to spermatozoa, including vitellogenins, apolipoproteins and complement component C3. Cryopreservation significantly altered the abundance of 51 proteins. Overall, 27 proteins increased (e.g. SERPINB1, FER) and 24 proteins decreased (e.g. CCT subunits, CSNK1G2, TOM1L1) in frozen thawed ram spermatozoa, compared to fresh spermatozoa. Chaperones constituted 20% of the proteins lost from spermatozoa following cryopreservation. These alterations may interfere with both normal cellular functioning and the ability of frozen thawed spermatozoa to appropriately respond to stress. This is the first study to apply SWATH mass spectrometry techniques to characterise proteins contributed by egg yolk based freezing media and to profile cryopreservation induced proteomic changes to ram spermatozoa.

Significance: This study profiles changes to the sperm proteome induced by exposure to egg yolk based media and the process of cryopreservation, and the biological consequences are discussed.

1. Introduction

The process of cryopreservation has been shown to inflict considerable damage to the sperm cell through cellular dehydration, osmotic stress and intracellular ice formation [1–4]. As a result, frozen thawed spermatozoa have reduced membrane and acrosome integrity [5,6], in addition to exhibiting reorganisation and disruption of important lipid-protein associations within the plasma membrane [1,7–10]. During cooling, freezing and rewarming, spermatozoa undergo important alterations to membrane phospholipids [11], sustain significant DNA damage [12], produce high amounts of reactive oxygen species [13] and often show hallmarks of capacitation [14]. Cryopreservation also significantly decreases tolerance to stressors such as reactive oxygen species [15,16] and osmotic shock [17,18]. While cryoprotectants such as egg yolk generally help to minimise damage, the full scope of their effects on spermatozoa are not well characterized. Thus while cryopreservation can clearly be lethal, there is an element of sub-lethal damage which may significantly affect those spermatozoa which remain viable post thaw.

While frozen thawed semen is currently employed in a range of animal industries, as well as human reproductive medicine, improvements in freezing outcomes would no doubt be welcomed. This is of particular interest in sheep, where the use of cryopreserved spermatozoa is limited due to its reduced fertility following cervical insemination [19,20], a symptom of the diminished ability of frozen thawed ram spermatozoa to transit the ovine cervix. Minimising both lethal and sub-lethal damage to spermatozoa during cryopreservation is key to improving insemination outcomes, but in order to do this, we must comprehensively understand what this damage entails. In particular, the freezing induced loss of functionally important proteins is of significant interest. In addition, the modification of the sperm proteome by cryoprotective agents, particularly ubiquitously used egg yolk,

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requires further investigation. With the advent of comparative proteomics, there has been investigation into protein based changes caused by cryopreservation of spermatozoa from man [21,22], bull [23], ram [24], boar [25], rooster [26], carp [27], sea bream [28] and trout [29]. However despite these encouraging developments, many of these studies have used mass spectrometry to identify differentially abundant spots from 2D gel electrophoresis (2DGE). While an effective approach, 2DGE is not without its limitations, such as limited sensitivity of the densitometry analysis, poor detection of proteins with very high or low pI, under representation of small (< 10 kDa) or large (> 100 kDa) proteins, and limited detection of hydrophobic proteins. Labelled (e.g. iTRAQ) or label free quantification of whole sperm lysates, as previously published for boar [25] and human [22] spermatozoa, offers a more comprehensive assessment of proteomic changes due to cryopreservation, including changes to low abundance proteins not detectable by 2DGE. However, these methods are yet to be applied to ram spermatozoa to compare proteomic changes due to cryopreservation, or to investigate proteins which may be conferred by freezing media containing egg yolk.

One such method is a non-labelled, quantitative acquisition technique, involving data-independent sequential window acquisition of all theoretical mass spectra (SWATH-MS; [30]). This technique has been employed in recent years to investigate proteomic changes in bodily fluids and somatic cells from a wide range of species with high success [31]. SWATH based data-independent acquisition facilitates simultaneous high throughput scanning and fragmentation in specified *m/z* blocks, identifying all peptides within a given mass range. Spectra are then compared back to an ion library which computationally generates objective and reliable quantification [32]. To date, Perez-Patiño et al. [33] is the only study to apply SWATH-MS in a reproductive context, investigating proteomic differences between portions of the boar ejaculate. Therefore, applying this technique to ram spermatozoa offers a novel, accurate method of quantifying potentially small but biologically relevant proteomic differences between fresh and frozen thawed ram spermatozoa.

The aim of the current study is to utilise LC-MS/MS employing SWATH acquisition to develop a quantitative picture of the proteomic differences between fresh and frozen thawed ram spermatozoa, as well as any proteins which are contributed to spermatozoa by an egg yolk based medium. We hypothesise that the sperm proteome will be significantly altered both by exposure to egg yolk and cryopreservation, and that proteins which are lost or gained may have important functional roles, including altering the ability of frozen thawed ram spermatozoa to successfully traverse the ovine cervix.

2. Materials and methods

2.1. Experimental design

Changes due to exposure to egg yolk media and cryopreservation were investigated using LC-MS/MS employing SWATH acquisition. In order to investigate these effects in parallel, ejaculates were collected from 3 rams and aliquots from the same ejaculate were processed either as “fresh”, ‘fresh + EY’ or “frozen”. Two treatment comparisons were made; fresh versus fresh + EY and fresh versus frozen.

2.2. Chemicals

Unless otherwise stated, all reagents were sourced from Sigma Aldrich (Castle Hill, Australia).

2.3. Animals

Mature merino rams (n = 3) and ewes (n = 2) used for semen collection were housed at the University of Sydney, Camperdown campus. Animals were maintained on a chaff based diet, supplemented with lupins. All procedures were approved by the University of Sydney animal ethics committee (approval 2013/5854).

2.4. Collection and preparation of spermatozoa

Ejaculates (n = 2/ram) were collected by artificial vagina (June 2016) from Merino rams (n = 3) in the presence of a teaser ewe. Ejaculates were immediately assessed for wave motion (data not shown), and only accepted if wave motion scored 4 or higher out of 5. Samples were slowly extended 1 + 3 (semen + diluent; v/v) with either warmed (37 °C) tris, citrate, fructose solution (“fresh”; 308 mM tris, 104 mM citric acid, 28 mM D-fructose, pH 7.3), a tris, citrate, fructose solution supplemented with 15% (v/v) egg yolk (“fresh + EY”) or tris, citrate, glucose solution supplemented with 15% (v/v) egg yolk and 5% (v/v) glycerol (“frozen”, [34]). Frozen samples were chilled to 5 °C and frozen by the pellet method (250 µL, Evans and Maxwell, 1987). Briefly, 250 µL of sample was deposited on a block of dry ice for 3 min, after which the pellet was submerged in liquid nitrogen. Pellets were thawed in a dry glass tube by agitating for 2 min in a 37 °C water bath.

Immediately post dilution (fresh and fresh + EY) or thawing (frozen), samples were washed free of seminal plasma and freezing diluent by a “swim up” procedure. Briefly, 500 µL aliquots of sample were layered under 3 mL of warmed phosphate buffered saline (PBS) and incubated for 1 h at 38.5 °C. Post incubation, the top 2 mL was removed and centrifuged (900 × *g*; 10 min; room temperature). The supernatant was discarded and the concentration of the resultant pellet was determined using a haemocytometer (Neubauer Improved, Precicolor HBG; Giessen-Lützellinden, Germany) before being re-suspended to 500 × 10^6 spermatozoa/mL with PBS. An aliquot was taken to assess sperm viability. Briefly, samples stained for 10 min with SYBR-14 (final concentration 100 nM) and propidium iodide (final concentration 6 µM) were assessed using an Accuri C6 flow cytometer (Becton Dickson) equipped with a standard argon laser (488 nm) and suitable detectors (533/30 nm BP, > 670 nm LP), reading a minimum of 10,000 spermatozoa per sample.

The remainder of each sample was centrifuged again (900 × *g*; 10 min; room temperature) before resuspension in lysis buffer (62.5 mM tris, 2% (w/v) sodium dodecyl sulphate (SDS) and cOmplete protease inhibitor cocktail; 1:1.5 v/v). Lysates were standardised to contain approximately 200–300 × 10^6 total spermatozoa. Samples were vortexed for 2 min before being left to stand at room temperature for 1 h, vortexing every 15 min. Lysed samples were then centrifuged (7, 500 × *g*; 15 min; room temperature), the supernatant collected and stored at −80 °C until further use.

2.5. Digestion and preparation of samples for mass spectrometry

SDS was removed from sperm lysates using a chloroform/methanol precipitation as previously described by Wessel and Flügge [35]. Protein concentration was determined using a Qubit protein assay (2.0 fluorometer; Invitrogen, Carlsbad, CA, USA) as per manufacturer's instructions and was consequently standardised to 100 µg of total protein with 50 mM ammonium bicarbonate. Samples were reduced (10 mM TCEP; 1 h; 37 °C), alkylated (50 mM iodoacetamide; 30 min; room temperature in the dark) and digested overnight with trypsin (final ratio 1:50 (v/v) trypsin: substrate; 37 °C; Promega; Madison, WI, USA). Digested samples were then desalted using a C18 Oasis HLB column (Waters; Elstree, Herts, UK) and vacuum dried prior to mass spectrometry. Samples for LC-MS/MS were resuspended in 3% (v/v) acetonitrile, 0.1% (v/v) formic acid (1 µg injection). A global standard was created to enable the generation of an ion spectral library. Equal amounts of protein from each sample (n = 9) were pooled, dried down and resuspended in 90% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid (10 µg injection; 2D LC-MS/MS).