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The identification of proteomic markers of sperm freezing resilience in ram seminal plasma

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ABSTRACT

The source and composition of seminal plasma has previously been shown to alter the ability of spermatozoa to survive cryopreservation. In the present study, the ionic and proteomic composition of seminal plasma from rams with high (HSP; n = 3) or low (LSP; n = 3) freezing resilient spermatozoa was assessed. 75 proteins were identified to be more abundant in HSP and 48 proteins were identified to be more abundant in LSP. Individual seminal plasma proteomes were established for each of the six rams examined. For each ram, correlations were conducted between previously recorded freezing resilience [1] and individual spectral counts in order to identify markers of freezing resilience. 26S proteasome complex, acylamino acid releasing enzyme, alpha mannosidase class 2C, heat shock protein 90, tripeptidyl-peptidase 2, TCP-1 complex, sorbitol dehydrogenase and transitional endoplasmic reticulum ATPase were found to be positively correlated ($r^2 > 0.7$) with freezing resilience. Cystatin, zinc-2-alpha glycoprotein, angiogenin-2-like protein, cartilage acidic protein-1, cathepsin B and ribonuclease 4 isoform 1 were found to be negatively correlated ($r^2 > 0.7$) with freezing resilience. Several negative markers were found to originate from the accessory sex glands, whereas many positive markers originated from spermatozoa and were part of or associated with the 26S proteasome or CCT complex.

1. Introduction

Seminal plasma is a complex, protein-dominated, fluid secreted from the testes, epididymides and male accessory sex glands [2]. In the ram, the protein component of seminal plasma has been shown to stabilise and reduce stress on the sperm membrane [3,4], prevent [5] or reverse [6] cold shock damage and increase the number of motile, viable cells when spermatozoa are frozen with cryoprotective agents [3,7]. However, other reports have portrayed a contradictory view of the effect of seminal plasma with no effect observed during chilled storage [8], and its supplementation even detrimental to epididymal [9] and sex-sorted ram spermatozoa [10]. Seminal plasma has also been used to improve the limited fertility of frozen-thawed spermatozoa following cervical artificial insemination [11], yet contradictory reports of its success also exist [12–14]. This variability in effect observed within the literature is hypothesised to be attributed to differences in seminal plasma composition (namely proteins) between individual males [3,4,12,15] brought about by disparate physiological, nutritional and/or seasonal conditions [4,15–19]. We have recently provided evidence to support this hypothesis and demonstrated that the source and composition of seminal plasma directly altered the ability of ram spermatozoa to survive cryopreservation [1]. Studies in the bull [20,21], buffalo [22] and ram [23] have attempted to link the expression of certain protein bands separated during electrophoresis, with individual freezing resilience. In the sheep, characterisation of such protein specific variation between rams has recently been undertaken based on liquid semen preservation ability. A number of proteins were found to be more or less abundant in high and low preservation ability groups [24]. Similar correlations between identified protein abundance and ram sperm freezing ability have not been performed. Goularte et al. [23] found correlations between unidentified protein bands and sperm motility prior to freezing. For example, motility was increased when a 15, 19 and 80 kDa band was present in ram seminal plasma. Likewise, Cardozo et al. [19] also compared the seminal plasma protein composition (2D-PAGE) to ram sperm motility over the course of a year. While such data provides encouraging evidence of the link between protein composition and freezing resilience, these proteins have yet to be identified or characterised via mass spectrometry.

Two low molecular weight protein candidates are the Ram Seminal Vesicle Proteins, RSVP14 and RSVP20, which are binders of sperm

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proteins (BSPs) belonging to the Fibronectin Type 2 family (Fn-2). These proteins bind to the membrane and protect sperm from cold shock when added prior to freezing [6,25,26]. Another potential protein candidate, which could indirectly influence cryosurvival, is Zinc-2-alpha glycoprotein (AZGP), a 43 kDa glycoprotein. In studies involving human spermatozoa, this protein has been labelled as a forward motility activator through its involvement in the cAMP pathway [27,28]. In addition to its complex protein interactions with albumin and semenogelin fragments [29], it has also been reported to regulate flagella movement [28,30]. Recently, AZGP was shown to have a biphasic effect on ram spermatozoa during liquid storage at 15 °C with an initial increase in motility (0 h) followed by a decrease after 24 h of storage [24]. Little is known of the effect of AZGP in the cryopreservation process.

The following study was conducted to quantitate and compare the proteomic and ionic differences between HSP and LSP to identify potential markers of freezing resilience. Such markers could be useful to screen rams and pre-empt freezing success.

2. Materials and methods

2.1. Chemicals

Unless otherwise stated all chemicals were supplied by Sigma-Aldrich (St Louis, MO, USA). Primary antibodies directed against the following proteins were purchased from Santa Cruz Biotechnologies (Santa Cruz, California, USA): Zinc alpha-2 glycoprotein (AZGP; 1/400, v/v; sc-11358), alpha enolase (ENO1; 1/500, v/v; sc-15343), sorbitol dehydrogenase (SORD; 1/400, v/v; sc-377200), TCP-1 complex zeta (CCT6A; 1/400, v/v; sc-271734), 26S Proteasome (PSMD13; 1/100, v/v; sc-73488). Primary antibodies directed against heat shock protein 70 (HSPA4L; 1/1000, v/v; 610607) were purchased from Becton Dickerson Transduction Laboratories (BD, Le Pont de Claix, France). Primary antibodies directed against Transitional endoplasmic reticulum ATPase (VCP; 1/2000, v/v; ab11433) were purchased from Abcam (Cambridge, UK).

The secondary antibody used for primary rabbit antibodies, was goat anti rabbit horseradish peroxidase (1/5000, A6154) purchased from Sigma Aldrich (Lyon, France). The secondary antibody used for primary mouse antibodies, was goat anti mouse horseradish peroxidase (1/5000, 172–1011; Biorad, Hercules, USA). The chemiluminescent, horse radish peroxidase substrate was SuperSignal West Pico and West Femto Substrate (Thermo Scientific, Rockford, USA).

2.2. Collection and preparation of seminal plasma

Ejaculates (n = 4/ram) were collected by artificial vagina (May–June 2012) from rams, previously identified to have spermatozoa with high (HSP; n = 3) or low (LSP; n = 3) resilience to freezing [1]. Identification of HSP and LSP rams was made as follows: ejaculates (n = 6/ram) were collected by artificial vagina from 17 rams of mixed breed (Merino, Poll Dorset, Finn X and Coopworth). Ejaculates were slowly diluted 1:4 (semen:diluent, v/v) with a tris-citrate-glucose cryoprotective diluent containing 15% egg yolk and 5% glycerol (v/v) [11], which had been prepared previously and frozen in aliquots, then thawed immediately prior to use. Concentration counts were performed on each sample using a haemocytometer (Neubauer Improved, Precicolor HBG, Giessen-Lützellinden, Germany) before further extension to a concentration of 200 × 10^6 spermatozoa/mL. Samples were then chilled to 5 °C over 2 h whereby they underwent pre freeze motility assessment and were then frozen via the straw method [11]. Briefly, 200 μL of sample were loaded into pre-chilled 0.25 mL straws (IMV, L'Aigle Cedex, France) and sealed with polyvinyl chloride (PVC) powder. Straws were randomly loaded onto a pre-cooled freezing rack before exposure to liquid nitrogen vapour 6 cm above the liquid nitrogen surface for 6 min. All straws were then submerged in liquid nitrogen and stored until assessment. Straws were thawed in a 37 °C water bath for 30 s with agitation. All samples were immediately diluted 1:1 with Androhep, adjusted to pH 7.3 (AH; Minitube Australia, Smythes Creek, VIC, Australia). Samples were incubated at 37 °C and aliquots taken at 0, 2 and 4 h post thaw for evaluation. Freezing resilience was determined by examining the change in total motility before (pre freeze) and after (0 h and 4 h post thaw) freezing. Freezing resilience for each ram was then ranked and the 3 rams which consistently (across 6 replicates) recorded small degrees of difference between pre-freeze and post-thaw motility (0 or 4 h) were deemed to have spermatozoa with high freezing resilience. Rams which consistently recorded large degrees of difference between pre-freeze and post-thaw motility (0 or 4 h) were deemed to have spermatozoa with low freezing resilience. Freezing resilience for each ram was assessed over six replicates (n = 6 ejaculates) and the mean calculated to ensure a true indication of inter-male variation in freezing resilience. Data collected on rams identified to have high or low resilience to freezing were subsequently analysed using a restricted maximum likelihood (REML) linear mixed model in Genstat (Version 16, VSN international, Hemel Hempstead, UK) and revealed significant differences in motility between each phenotypic group [1]. The pre-freeze and post-thaw motility of the three rams identified at each extreme end of the freezability spectrum are shown in Fig. 1. HSP rams clearly display a smaller decrease in sperm motility following freezing than LSP rams [1].

Seminal plasma from each high (HSP; n = 3) and low (LSP; n = 3) freezing resilience ram was obtained from 4 ejaculates acquired during the collection period (May–June 2012). Briefly, ejaculates were centrifuged at 16,000 ×*g* for 30 min, seminal plasma collected and spun again (16,000 ×*g*, 30 min) to remove any remaining sperm and cell debris. For each HSP or LSP ram, the seminal plasma from all ejaculates was pooled and frozen at −80 °C until needed. These multi-ejaculate individual pools constituted the HSP and LSP samples subjected to subsequent proteomic and ionic analysis.

2.3. Quantitative mass spectrometry analysis of HSP and LSP

2.3.1. 1D-SDS PAGE

The protein concentration of seminal plasma from all rams was determined using a Pierce bicinchoninic acid assay (BCA; Thermo Fisher Scientific, Rockford, USA). SDS-PAGE electrophoresis was carried out according to Laemmli's method [31]. For quantitative-MS based analysis (spectral counting), 20 μg of HSP (n = 3 rams) and LSP (n = 3 rams) seminal plasma were deposited in individual lanes on a 10% SDS

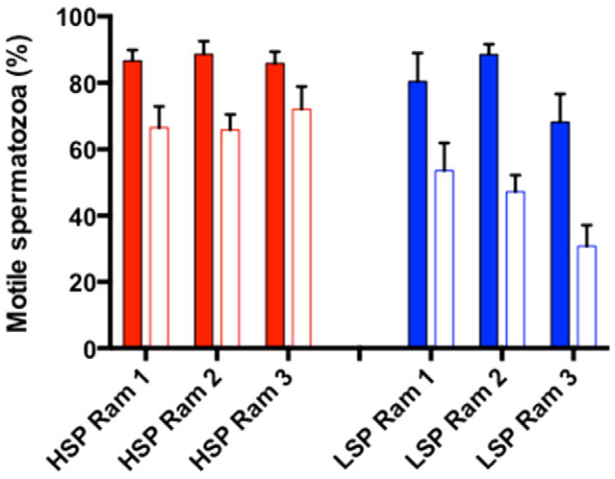

Fig. 1. Motility of individual rams (n = 6; rams 1–3 HSP; rams 4–6 LSP) prior to (filled in) and post freezing (0 h; unfilled). Data are actual means pooled over 6 ejaculates ± SEM [1].