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Proteomic comparison of detergent-extracted sperm proteins from bulls with different fertility indexes

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Abstract

Intrinsic factors such as proteins modulate the fertilising ability of male gametes. We compared detergent-extracted sperm protein composition of bulls with different fertility indexes in order to highlight putative fertility markers of sperm. Frozen semen from 23 Holstein bulls with documented fertility was used. According to their 'fertility solution' (SOL), as calculated by the Canadian dairy network, bulls were divided into four groups: high fertility (HF) (SOL > 3.0; $n=6$), medium-HF (2.9 > SOL > 2.0; $n=5$), medium-low fertility (−2.8 > SOL > −4.9; $n=8$) and low fertility (LF; SOL < −5.0; $n=4$), with a SOL = 0 being the average. Triton X-100 protein extracts from ejaculated spermatozoa were subjected to two-dimensional difference gel electrophoresis, and polypeptide maps were quantitatively analysed by ImageMaster software. Nine protein spots showed significant differences between the HF and LF groups, and eight of these proteins were identified by liquid chromatography–tandem mass spectrometry. T-complex protein 1 subunits ε and θ (CCT5 and CCT8), two isoforms of epididymal sperm-binding protein E12 (ELSPBP1), proteasome subunit α type-6 and binder of sperm 1 (BSP1) were more expressed in the LF group than in the HF group. On the other hand, adenylate kinase isoenzyme 1 (AK1) and phosphatidylethanolamine-binding protein 1 (PEBP1) were more expressed in the HF group than in the LF group. The presence and expression level of ELSPBP1, BSP1, AK1 and PEBP1 were confirmed by western blot. A linear regression model established that CCT5 and AK1 explained 64% ($P<0.001$) of the fertility scores. The reported functions of these proteins are in agreement with a putative involvement in defective sperm physiology, where lower or higher levels can jeopardise sperm ability to reach and fertilise the oocyte.
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Introduction

Male reproductive efficiency relies on the ability to mate with the female and fertilise the oocyte. Mating ability includes libido and physical capability to mount, achieve intromission and ejaculate (Parkinson 2004). In order to fertilise the oocyte, each spermatozoa must present motility, active mitochondria, intact membranes and a nucleus capable of proper decondensation and reorganisation (Graham & Moce 2005). These attributes will allow spermatozoa to reach, recognise, bind to and penetrate the oocyte in order to deliver its genome. In the dairy industry, where artificial insemination (AI) is the standard, male fertility is defined as the percentage of females inseminated that are not re-inseminated a defined number of days after the first insemination (Van Doormaal 1993). This fertility quantification is called non-return rate (NRR). In this context, NRR will only refer to the sperm fertilising ability. Moreover, in AI with cryopreserved semen, ability of the spermatozoa to survive the cryopreservation process is also part of the fertility definition (Collin *et al.* 2000).

Each of the single steps leading to fertilisation represents a critical point where defect could alter semen fertility. Based on their implication in particular steps of the fertilisation process, many sperm components such as lipids (Brinsko *et al.* 2007), proteins (Bellin *et al.* 1998, Parent *et al.* 1999), ions (Collin *et al.* 2000) and nucleic acids (Lalancette *et al.* 2008) have been proposed to vary in quantity or quality according to the male fertility status in many mammalian species. Levels of P25b, a bovine sperm membrane antigen, are lower in semen from subfertile bulls than in the semen from bulls with high fertility (HF) rates (Parent *et al.* 1999). P25b counterparts in human and hamster, P34H and P26h, are involved in zona pellucida (ZP) recognition (Berube & Sullivan 1994, Boue *et al.* 1994). A 30-kDa heparin-binding protein, namely fertility-associated antigen (FAA), characterised sperm membranes of beef bulls with greater fertility potential (Bellin *et al.* 1998). FAA was further identified as DNase I-like protein (McCauley *et al.* 1999).

The bovine sperm proteome is composed of or associated with hundreds of different proteins. Although the

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functions of the majority remain unknown, many of them must be involved in particular steps of fertilisation. From this point of view, absence, presence, under- or over-expression of specific proteins could alter sperm functions, jeopardising its fertilising abilities, thus lowering the semen fertility. The objective of the present study was to compare the proteome of sperm Triton X-100 extracts from fertile and subfertile bulls and quantify differences by the two-dimensional difference gel electrophoresis (2D-DIGE) technique in order to highlight putative subfertility explanations.

Results

An average of 861±45 protein spots were detected in the internal standard gel images (Fig. 1). Of these, 567±143 spots matched the master gel. Only matched spots detected on all the internal standard images were considered for DIGE analysis. None of these spots were exclusive to any of the four fertility bull groups, but DIGE analysis by ImageMaster revealed significant differences for nine of these spots between the HF and low fertility (LF) bull groups (Fig. 1).

Detection of protein differences

Two spots (686 and 740 in Fig. 1) were predominantly abundant in the HF group. Mass spectrometry (MS) analysis identified them as adenylate kinase isoenzyme 1 (AK1) and phosphatidylethanolamine-binding protein 1 (PEBP1) respectively. Both AK1 and PEBP1 were identified with 15 unique peptides with more than 95% confidence for sequence coverage of 68 and 84% respectively (Table 1). The seven other spots (214, 522, 531, 576, 582, 640 and 836 in Fig. 1) were predominantly abundant in the LF group. Spots number 214, 522, 576, 640 and 836 were identified as T-complex protein 1 subunits ε and θ (CCT5 and CCT8), epididymal sperm-binding protein E12 (ELSPBP1), proteasome subunit α type-6 (PSMA6) and binder of sperm 1 (BSP1) respectively. Spot 582 has also been identified as ELSPBP1. Only spot 531 was not identified, as it could not be visualised on the Coomassie blue-stained gel. All these spots were identified with at least nine unique peptides with more than 95% confidence, covering a minimum of 35% of amino acid sequences (Table 1). Table 1 shows liquid chromatography–tandem MS (LC–MS/MS) identification results and Fig. 2 shows graphical DIGE quantification for the eight differently abundant identified spots. According to the s.e.m. (Fig. 2), the LF group presented higher heterogeneity than the other groups, especially for ELSPBP1 spots and BSP1. The unidentified spot 531, which is not shown in Fig. 2, presented very similar abundance pattern to spots 576 and 582.

The major spot indicated by a black arrow next to BSP1 in Fig. 1 was identified by western blot as BSP3 (data not shown). Although not being statistically significant ($P=0.078$), BSP3 levels showed the same tendency as BSP1 with fertility for the abundance according to fertility group. The levels of both proteins were highly correlated ($r=0.92$; $P<0.001$; Fig. 3). By opposition, BSP1 was significantly and negatively correlated with PEBP1 ($r=-0.59$; $P=0.003$; Fig. 4).

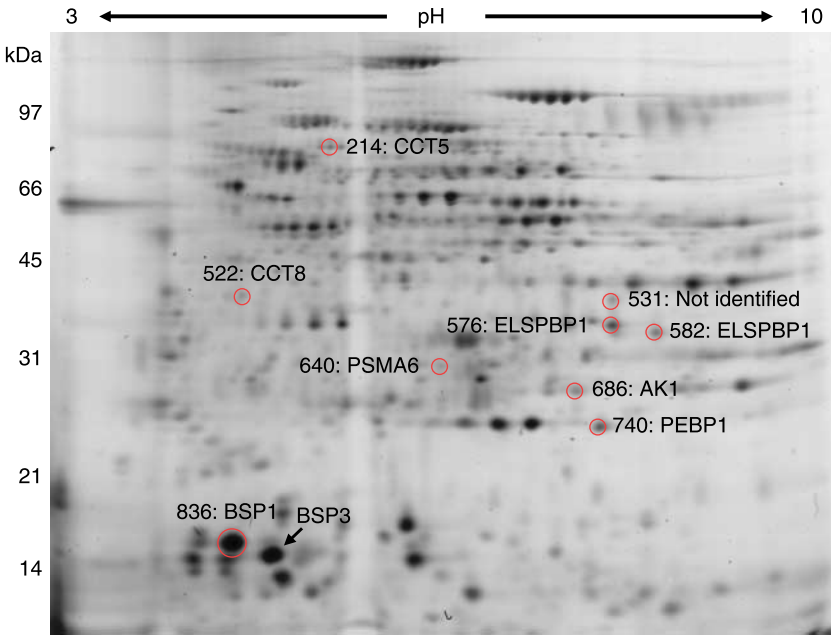

Figure 1 Triton X-100 protein extracts of bull spermatozoa submitted to two-dimensional SDS-PAGE. DIGE analysis by ImageMaster (GE Healthcare, Baie d'Urfé, Québec, Canada) revealed significant differences between high and low fertility bull groups for nine of these spots (identified by circle). Eight of these circled spots were analysed and identified by LC–MS/MS. The spot pointed by an arrow was recognised by BSP3 specific antibody.

Table 1 Sperm proteins associated with high and low fertility in Holstein bulls as determined by two-dimensional difference gel electrophoresis (2D-DIGE) analysis of sperm Triton X-100 1% protein extracts and identified by liquid chromatography–tandem mass spectrometry (LC–MS/MS)[a].

Spot number	Protein identification[b]		MW; pI[c]		Matched peptides[d]	Percentage of coverage[d]	LC–MS/MS	
	Name	Accession number	Theoretical	Experimental			Top two matched peptides[e]	Mascot e-value[e]
214	T-complex protein 1 subunit ε (CCT5)	Q3T115	60; 5.6	70; 5.5	29	47	IADGYEQAAR DVDFELIKVEGK	$2.7\,e^{-7}$ $1.4\,e^{-6}$
522	T-complex protein 1 subunit θ (CCT8)	Q3ZCI9	59; 5.4	35; 5	19	35	AIADTGANVVVTGGR KFAEAFEAIPR	$1.5\,e^{-10}$ $1.8\,e^{-7}$
531	Not identified			35; 8				
576	Epididymal sperm-binding protein E12 (ELSPBP1)	XP_ 600577	26; 8.3	32; 8	15	65	YCETNEYGGNSFSKPCIFPATYR ENLLWCATSYNYDR	$8.9\,e^{-7}$ $6.6\,e^{-6}$
582	Epididymal sperm-binding protein E12 (ELSPBP1)	XP_ 600577	26; 8.3	31; 8.5	13	54	YCETNEYGGNSFSKPCIFPATYR QWCSVTSSFDEKQQWK	$3.1\,e^{-7}$ $1.1\,e^{-6}$
640	Proteasome subunit alpha type-6 (PSMA6)	Q2YDE4	27; 6.4	28; 6.5	13	53	ITENIGCVMTGMTADSR AINQGGLTSVAVR	$1.9\,e^{-7}$ $1.4\,e^{-7}$
686	Adenylate kinase isoenzyme 1 (AK1)	P00570	22; 8.4	25; 7.5	15	68	IAQPTLLLYVDAGPETMTK KVNAEGSVDNVFSQVCTHLDALK	$6.6\,e^{-10}$ $4.4\,e^{-7}$
740	Phosphatidylethanolamine-binding protein 1 (PEBP1)	P13696	21; 7.4	22; 7.8	15	84	GNNISSGTVLSDYVGSGPPK YGGAEVDELGK	$1.7\,e^{-9}$ $6.1\,e^{-7}$
836	Binder of sperm 1 (BSP1)	P02784	13; 5.1	16; 5	9	48	HFDCTVHGSLFPWCSLDADYVGR IGSMWMSWCSLSPNYDKDR	$1.8\,e^{-8}$ $5.0\,e^{-6}$

[a]2D-DIGE indicates two-dimensional difference gel electrophoresis; LC–MS/MS indicates liquid chromatography–tandem mass spectrometry. [b]Spot numbers refer to those shown in bull sperm protein 2D map shown in Fig. 1. [c]Theoretical molecular weight and pI were calculated by the Compute pI/Mw tool from ExPASy Proteomics Server (http://ca.expasy.org/) based on the bovine amino acid sequence. Experimental values were deduced from the bull sperm protein 2D map shown in Fig. 1. [d]LC–MS/MS results as given by Scaffold software. [e]The two matched peptides with the highest Mascot e-value.

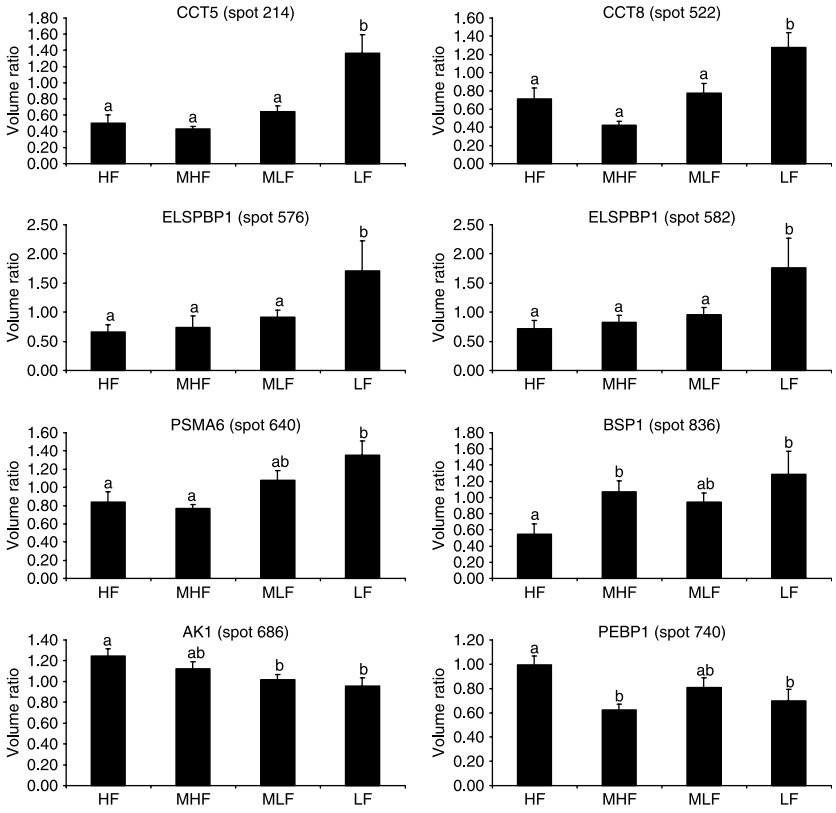

Figure 2 Volume ratio (±S.E.M.) of protein spots identified as CCT5, CCT8, ELSPBP1 isoforms, PSMA6, BSP1, AK1 and PEBP1 on two-dimensional maps of 1% Triton X-100 sperm extracts from Holstein sires and quantified by DIGE. Bulls were grouped based on their fertility solution (SOL): high fertility (HF; SOL > 3.0; $n=6$), medium-high fertility (MHF; 2.9 > SOL > 2.0; $n=5$), medium-low fertility (MLF; −2.8 > SOL > −4.9; $n=8$) and low fertility (LF; SOL < −5.0; $n=4$). Values followed by different letters are significantly different ($P<0.05$).

Western blot confirmation

Antibodies against BSP1, ELSPBP1, AK1 and PEBP1 were used to validate the LC–MS/MS identifications. Western blot analysis visually showed the same tendencies as DIGE results for the 23 bulls, which confirm LC–MS/MS identification. Figure 5 shows an example of western blot detection of BSP1, ELSPBP1, AK1 and PEBP1 with corresponding quantification by DIGE for three bulls from the HF group and three bulls from the LF group.

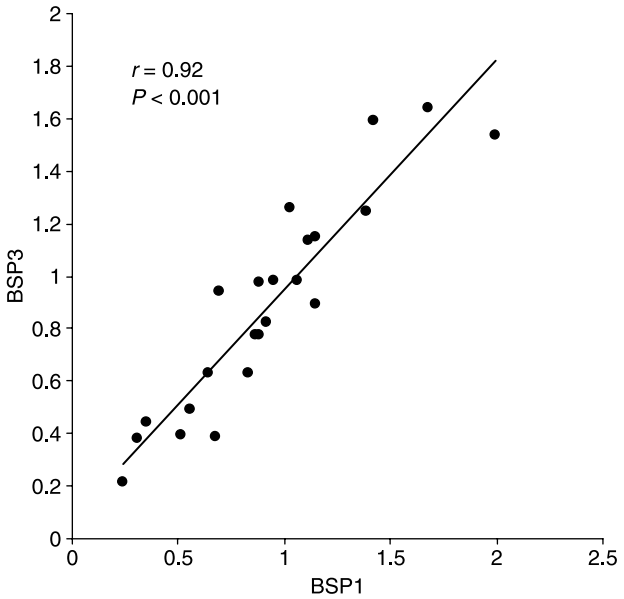

Figure 3 Correlation between BSP3 and BSP1 levels as determined by DIGE quantification.

Linear regression models

In order to assess the relationship between bull fertility and the abundance of the seven identified proteins, a regression model using fertility solution (SOL) as a dependent variable and the seven proteins as independent variables was established using Statistical Analysis Systems (SAS) software (Toronto, Ontario, Canada). The STEPWISE approach was used to select independent variables with the highest determination coefficient (R^2). The regression model established that CCT5 and AK1 explained a significant proportion of the variation in fertility scores ($R^2=0.6394$; $P<0.001$). Using the RSQUARE approach to select independent variables, addition of a third protein to the model increased R^2 by about 2%, which is negligible. A regression model taking into account the seven proteins increased R^2 by only 10%. Table 2 shows regression models established with both CCT5 and AK1 and with the seven proteins. Finally, since bulls used in this study do not have the same reliability (REL) for their SOL, a balanced regression

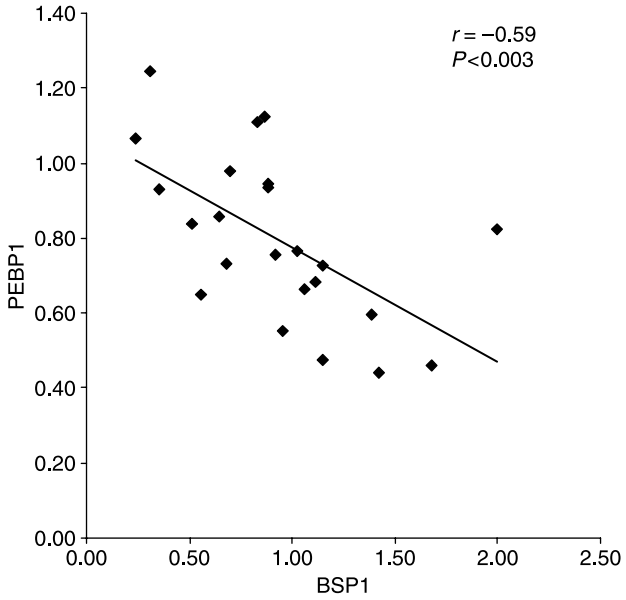

Figure 4 Correlation between PEBP1 and BSP1 levels as determined by DIGE quantification.

model with the REL variable was established in order to give more weight to bulls with a high REL. Addition of the individual REL variable gave a similar model to the one established with AK1 and CCT5.

Presence of the identified proteins in the motile and immotile sperm subpopulations

Following sperm separation on Percoll discontinuous gradient, both immotile and motile subpopulations were assayed for the presence of α-tubulin, ELSPBP1, PSMA6, AK1, PEBP1 and BSP1 by western blot (Fig. 6). Abundance of AK1 did not change between the two subpopulations. However, ELSPBP1 and BSP1 were in higher abundance in the immotile subpopulation, and PEBP1 was in higher abundance in the motile subpopulation. PSMA6 was found in higher abundance in the immotile subpopulation for two bulls, but was not detectable for the third one.

Since ELSPBP1, PEBP1, BSP1 and PSMA6 are predominant either in the immotile or in the motile subpopulation, we correlated their abundance on sperm with the percentage of live spermatozoa in the whole population using propidium iodide (PI) staining and flow cytometry. ELSPBP1 did not significantly correlate with the percentage of viable spermatozoa (Fig. 7A). Although not significant, PEBP1 showed a positive tendency with the percentage of viable spermatozoa (Fig. 7B). On the contrary, BSP1 showed a negative and highly significant correlation with the percentage of viable spermatozoa (Fig. 7C). PSMA6 presented a negative and significant correlation with the percentage of viable spermatozoa (Fig. 7D).

Discussion

Using the 2D-DIGE technique, we found that nine protein spots, from which eight were identified, were differently abundant on sperm from HF and LF proven bulls. Although significant, these differences did not characterise every single bull for every single protein, which highlight the multifactorial nature of male subfertility/infertility. Also, most of the differences identified were significant only between the HF and LF bull groups. This suggests that imbalance in sperm protein abundance greatly affects sperm fertilising ability.

Semen samples used in our study have been cryopreserved and straws from the same ejaculate batches were used for field inseminations in the Canadian dairy production industry. As highlighted by

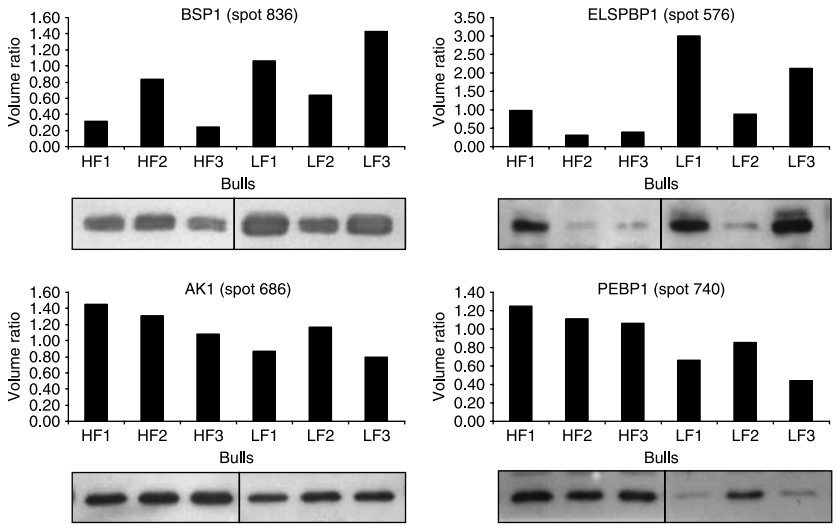

Figure 5 Western blots confirmation of BSP1 (spot 836), ELSPBP1 (spot 576), AK1 (spot 686) and PEBP1 (spot 740) for three bulls from the high fertility (HF) and low fertility (LF) groups. Equal amounts of protein for each sample were loaded on gel. Histograms show the corresponding spot quantification by DIGE for each of the bulls.

Table 2 Regression models of bull fertility based on volume ratio of spots in two-dimensional difference gel electrophoresis protein maps of sperm Triton X-100 1% extracts.

Regression equations	R^2	*P* value
SOL = −9.23346 − 4.95663 × (CCT5) + 10.67058 × (AK1)	0.6394	$P<0.001$
SOL = −11.26895 + 11.16030 × (AK1) − 4.02233 × (CCT5) − 0.21990 × (ELSPBP1) + 4.13624 × (BSP1) + 2.54656 × (PEBP1) + 0.03277 × (CCT8) − 4.82768 × (PSMA6)	0.7328	$P=0.0257$

SOL indicates the fertility solution, a normalised non-return rate; CCT5, volume ratio of spot 214; CCT8, volume ratio of spot 522; ELSPBP1, volume ratio of spot 576; PSMA6, volume ratio of spot 640; AK1, volume ratio of spot 686; PEBP1, volume ratio of spot 740; BSP1, volume ratio of spot 836 as shown in Fig. 1.

Van Doormaal (1993), fertility data (NRR or SOL) do not reflect the inherent fertility of bulls, but rather reflect the fertility of their semen after processing, diluting, freezing and thawing. Therefore, the use of frozen semen for such a study reflects the field reality. Indeed, a multiple linear regression model using proteins identified in this study explains a high and significant proportion of fertility variation ($R^2=0.64$; $P<0.001$). Recently, similar studies using fluids from cauda epididymis and accessory sex glands instead of sperm have identified proteins that correlate with fertility data in dairy bulls (Moura *et al.* 2006*a*, 2006*b*). These previous reports suggest that epididymal environment and post-ejaculation events regulated by accessory sex gland components affect sperm physiology and have further implication in sperm fertilising ability. These organs could be the source of the sperm differently expressed proteins identified in the present study.

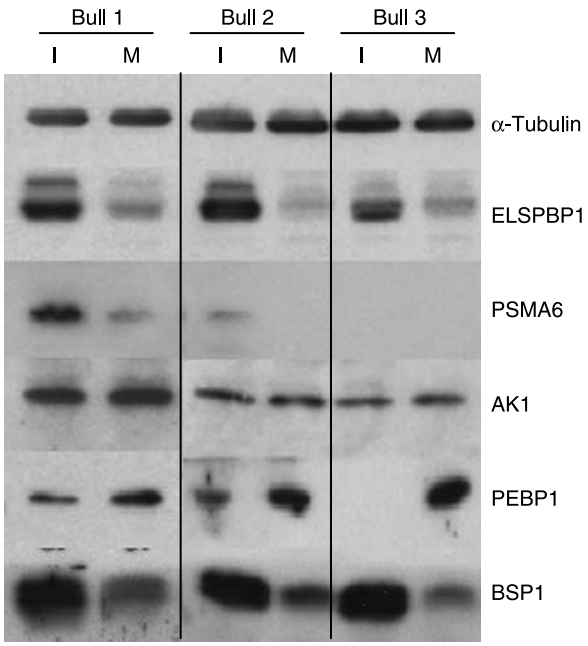

Figure 6 Detection of α-tubulin, ELSPBP1, PSMA6, AK1, PEBP1 and BSP1 in the immotile (I) and motile (M) sperm subpopulations of frozen–thawed semen from three bulls. Immotile and motile sperm subpopulations were separated following centrifugation of frozen–thawed semen on discontinuous Percoll gradient. Qualitative sperm motility was assessed visually. Each line contains protein extracts from 3×10^6 cells.

Although several limitations and shortcomings of standard 2D electrophoresis are overcome by 2D-DIGE, several disadvantages still remain. Even if the CyDye DIGE fluors offer great sensitivity, the technique will only allow the visualisation of 1000–2000 of the most abundant proteins on a single 2D gel (Marouga *et al.* 2005). In addition, many of the large, hydrophobic, extremely acidic or basic proteins will have difficulty in entering the gel during the first dimension and will be under-represented (Van den Bergh *et al.* 2003*b*). This is the case for integral membrane proteins, which are highly hydrophobic and rarely detected on 2D gels (Van den Bergh *et al.* 2003*a*). Moreover, lysine being required for protein staining, the technique is not applicable to those proteins without lysine. Although several proteins could have been lost during the sample preparation steps, the proteomic analysis was performed on proteins from the plasma membrane, the cytosolic fraction and the acrosomal and mitochondrial matrices, as well as from the remaining cytoplasmic droplet. Hence, differences could exist in other sperm subcellular compartments.

The subunits 5 and 8 of the T-complex protein 1 (CCT), a member of the class II chaperonins, were found in higher concentration in the LF sperm samples. CCTs share the common structure of all chaperonins, i.e. a large oligomeric cylinder formed from two rings placed back to back and composed of ∼60-kDa proteins (reviewed by Gomez-Puertas *et al.* (2004)). CCT rings are constructed from eight different subunits (CCT-α, -β, -γ, -δ, -ε, -ζ, -η and -θ; Rommelaere *et al.* 1993), and expression levels of these subunits are tightly co-regulated to maintain a constant ratio between them (Kubota *et al.* 1999). Chaperonins are involved in the proper folding of other proteins, with actin and tubulin being the major substrates for CCT (Sternlicht *et al.* 1993). In rat spermatids, cytoplasmic CCT is localised in centrosomes and microtubules of the manchette during spermatogenesis and would be discarded in residual bodies at spermiation, albeit a subpopulation remains in mature cells (Souès *et al.* 2003). Since the presence of three CCT subunits, including CCT5, has been reported in ejaculated sperm from fertile men (de Mateo *et al.* 2007), it is not surprising to find CCT5 and CCT8 subunits in bull-ejaculated sperm. However, since CCT is supposed to be discarded at spermiation, high levels

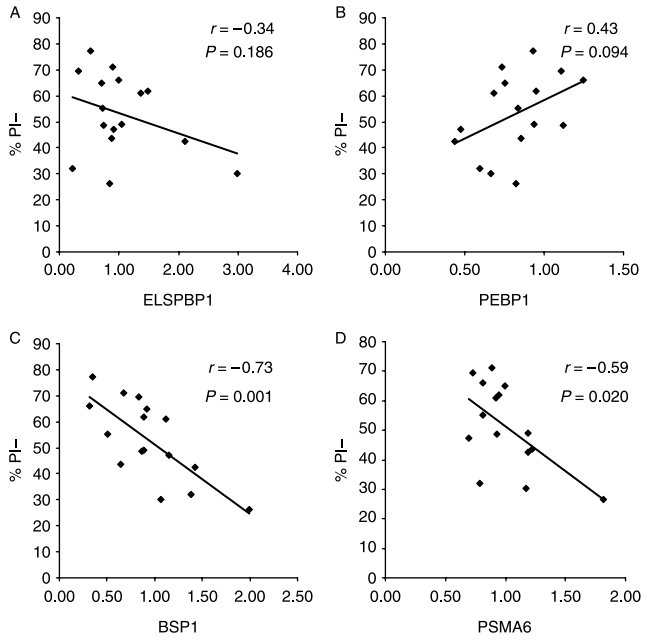

Figure 7 Correlation between ESLPBP1 (A), PEBP1 (B), BSP1 (C) and PSMA6 (D) as quantified by DIGE and percentage of propidium iodide (PI)-negative spermatozoa following thawing. PI-negative cells were considered to have a fully functional membrane and to be alive.

of CCT subunits in sperm samples from LF bulls may reflect incomplete processes involving these proteins during spermatogenesis.

PSMA6, another protein found in higher concentration in sperm from LF bull group, is part of the proteasome multicatalytic protease that degrades polyubiquitinated proteins into amino acids and small peptides (reviewed by Glickman & Ciechanover (2002)). Proteasome activity has been detected in sperm from many mammals, including bovine and human (Pizarro *et al.* 2004). Immunofluorescence assays localised proteasome in the acrosomal region in all species studied (Rawe *et al.* 2008). Although some discrepancies across species are reported, all studies report an implication of proteasome in events related to the acrosomal reaction (Morales *et al.* 2003, Sutovsky *et al.* 2004, Rawe *et al.* 2008). Proteasomes have also been detected at the connecting piece between head and tail in bovine and human sperm (Wojcik *et al.* 2000, Rawe *et al.* 2008). It has been suggested that they play different roles during the post-fertilisation disassembly of the sperm tail connecting piece, sperm aster formation, pronuclear development and apposition (Rawe *et al.* 2008). Proteomic analysis of human sperm using 2D gel electrophoresis revealed several proteasome subunits, including PSMA6 (de Mateo *et al.* 2007). The same study associated high levels of PSMA6 with DNA fragmentation using the TUNEL assay. To explain the latter observation, high degree of correlation has been found between sperm ubiquitination and DNA defects in bulls (Sutovsky *et al.* 2002), which is in accordance with proteasome catalytic activity of ubiquitinated proteins and might explain the significant negative correlation between the percentage of viable spermatozoa and amount of PSMA6. Another proteasome subunit, PSMB3, was found to be more abundant on sperm from patients presenting asthenozoospermia (Martinez-Heredia *et al.* 2008). Our results thus suggest that proteasomes, or some of their subunits, are involved in bull subfertility as in men.

AK1, which was found to be more abundant on sperm from HF bull group, is a ubiquitous enzyme contributing to the energy homeostasis of the cell by catalysing the reaction: $2ADP \leftrightarrow ATP + AMP$ (Atkinson 1968). Its presence in bovine and murine sperm flagella has been reported, strongly suggesting a function in motility (Schoff *et al.* 1989, Cao *et al.* 2006). The fluctuation of nucleotide concentrations in normal and metabolically stimulated sperm suggests that AK1 is mostly active when the cell is highly motile (Schoff *et al.* 1989). According to these authors, AK1 would ensure the availability of additional energy in times of increased demand, i.e. when spermatozoa undergo hyperactivation. Indeed, Ho *et al.* (2002) showed that more ATP is required by the dynein ATPase in the axoneme to produce the deep flagellar bends characteristic to hyperactivation. Hyperactivation may be critical to the success of fertilisation, because it enhances the ability of sperm to detach from oviductal epithelium and to penetrate the ZP (reviewed by Suarez & Ho (2003)). Moreover, using IVF as a biological end point, Sukcharoen *et al.* (1995) identified hyperactivation as the most important attribute of movement in predicting the fertilising potential of human spermatozoa. Hence, AK1 relationship with male fertility could be to sustain energy needs during hyperactivated motility of spermatozoa.

PEBP1, also known as RKIP, was found in higher abundance in sperm from HF bulls. The roles of PEBP1 in sperm remain to be established, but recent studies in mice suggest that it has an inhibitory effect on sperm capacitation. It acts either as a decapacitation factor that is released during capacitation (Nixon *et al.* 2006) or as a membrane-bound, glycophosphatidylinositol (GPI) anchored receptor for a decapacitation factor (Gibbons *et al.* 2005). This anti-capacitation effect is further supported by the observation that sperm from PEBP1$^{-/-}$ mice are precociously capacitated compared with their wild-type counterparts (Moffit *et al.* 2007). Although PEBP1$^{-/-}$ male mice remain fertile, data from mating experiments indicate significant decreased reproduction rates between crosses with either heterozygous or PEBP1$^{-/-}$ females, but interestingly, not with wild-type females.

BSP1, BSP3 and BSP5, formerly known as BSP-A1/A2, BSP-A3 and BSP-30 kDa respectively (Manjunath *et al.* 2009), are the three major proteins found in the bovine seminal plasma and are collectively called binder of sperm, previously known as bovine seminal plasma

(BSP) proteins. BSP3 has previously been found to be more abundant on sperm membrane from subfertile Nellore bulls (Roncoletta *et al.* 2006). Similarly, BSP5 was found in higher concentration in accessory sex gland fluid from subfertile Holstein bulls (Moura *et al.* 2006*b*). Upon ejaculation, BSP proteins bind to spermatozoa via choline phospholipids (Desnoyers & Manjunath 1992). It has been shown that BSP proteins induce cholesterol and phospholipids efflux from epididymal sperm in a dose- and time-dependent manner (Thérien *et al.* 1998, 1999). These effluxes are expected to provoke destabilisation of the membrane. Low-density lipoproteins (LDLs) from egg yolk (EY) are though to be the major cryoprotective factors of EY-based semen extender (Moussa *et al.* 2002, Amirat *et al.* 2004). BSP proteins form stable complex with LDLs (Manjunath *et al.* 2002), which decrease binding to sperm, hence preventing lipid efflux from the membrane (Bergeron *et al.* 2004). As proposed by Bergeron & Manjunath (2006), negative effects of BSP proteins on sperm fertility could be through a higher destabilisation of the membrane prior to dilution, which renders sperm more sensitive to cryopreservation. This is in accordance with the fact that abundance of BSP1 in sperm detergent extract significantly correlates with the percentage of live spermatozoa following cryopreservation ($r=-0.73$; $P=0.001$). Moreover, since BSP1 is clearly more abundant in the immotile subpopulation, this suggests that these spermatozoa are more receptive to BSP proteins.

It is noteworthy that PEBP1 abundance on sperm was significantly and negatively correlated with BSP1 ($r=-0.59$; $P=0.003$). So, it is not surprising to find PEBP1 in higher abundance in the motile subpopulation and to observe a positive tendency with the percentage of viability ($r=0.43$; $P=0.094$). Since BSP proteins are expected to destabilise membranes by promoting lipid efflux, it is possible that membrane proteins normally discarded during capacitation (Nixon *et al.* 2006) are susceptible to be released when the membrane is destabilised and reorganised. Therefore, the lower amount of PEBP1 in sperm could be a consequence of higher levels of BSP1 associated with sperm.

As a group, BSPs share functional and biochemical properties. They are all characterised by two tandemly repeated fibronectin type 2 (Fn2) domains (Esch *et al.* 1983, Seidah *et al.* 1987, Calvete *et al.* 1996). BSP1 Fn2 domains are single binding sites for either choline phospholipids or heparin (Moreau *et al.* 1998). Although of epididymal origin (Saalmann *et al.* 2001), ELSPBP1 is a four-Fn2 domain-containing protein, which suggests biochemical properties similar to those of BSPs. Its affinity for choline phospholipids has been established, and sequence alignment reveals that the key amino acid residues involved in choline phospholipids binding are highly conserved (Ekhlasi-Hundrieser *et al.* 2007). As BSPs alone can promote cholesterol and phospholipids efflux, ELSPBP1 could bind to sperm membrane and stimulate by itself lipid effluxes, hence destabilising membranes. Although ELSPBP1 was found in higher abundance in the immotile sperm subpopulation, ELSPBP1 amount in sperm detergent extract did not correlate significantly with the percentage of viability ($r=-0.34$; $P=0.186$). ELSPBP1 could be associated with the immotile but still alive sperm subpopulation.

Ejaculated frozen sperm from HF and LF bulls present proteomic differences and account for a significant proportion in the variation of bull fertility indexes. Their physiological functions or biochemical properties sustain plausible implications in sperm functionality defects, where lack or over-expression can jeopardise sperm chances to reach and fertilise the oocyte. Proteins presented in this study are starting points for further studies in our quest for understanding male infertility or subfertility in animal species or even human.

Materials and Methods

Animals and fertility data

Frozen semen from 23 Holstein bulls with proven fertility was generously provided by l'Alliance Boviteq Inc. (Saint-Hyacinthe, Quebec, Canada). Bulls were housed at Centre d'insémination artificielle du Québec (CIAQ; Saint-Hyacinthe, Quebec, Canada) and at Gencor (Guelph, Ontario, Canada). Fertility indexes for each bull were evaluated by the Canadian dairy network as the NRR. NRRs are based on the number of cows that do not return to service 56 days after the first insemination. Ninety-five to 31 905 first inseminations were used to calculate individual NRRs, giving an accuracy of the fertility indexes ranging from 29 to 99%. This accuracy index is called REL. Twenty of these bulls had a REL above 60%. NRRs are adjusted by a linear statistical model to include effects of age of the inseminated cows, month of insemination, technician, herd and price of the bull semen (Van Doormaal 1993). NRRs were converted to another index called the 'SOL', where zero is the average of the population at a given location for a specific period of time. According to their SOL, bulls were divided into four groups: HF (SOL$>$3.0; $n=6$), medium-HF (MHF; 2.9$>$SOL$>$2.0; $n=5$), medium-LF (MLF; $-2.8>$SOL>-4.9; $n=8$) and LF (SOL<-5.0; $n=4$). Nineteen bulls were from the local progeny testing programme and four were commercially used bulls. There was a commercially used bull in each of the four fertility groups. Average age at collection was 965 days $\pm$759 for the HF group, 1279 days $\pm$1180 for the MHF group, 771 days $\pm$962 for the MLF group and 841 days $\pm$788 for the LF group. Such heterogeneity in age at collection for each fertility group should avoid any bias regarding effect of age with fertility data.

Sperm samples preparation

Semen straws stored in liquid nitrogen were thawed in a water bath at 37 °C for 1 min. Frozen–thawed spermatozoa were first washed by centrifugation at 850 ***g*** for 20 min on an isotonic 45% (v/v) Percoll in PBS (137 mM NaCl, 3 mM KCl, 8 mM

Na_2PO_4 and 1.5 mM KH_2PO_4, pH 7.3). This procedure did not allow any sperm selection according to their viability, motility or cell integrity. Sperm pellets were washed twice by centrifugation at 850 ***g*** for 10 min in PBS. Proteins were extracted by resuspending sperm pellets in 1% Triton X-100 (w/v) in water (containing protease inhibitors: 5 μg/ml pepstatin A, 10 μg/ml leupeptin and 1 mM phenylmethylsulphonyl fluoride) for 15 min at room temperature, which allowed the extraction of proteins from the plasma membrane, the cytosolic fraction and the acrosomal and mitochondrial matrices, as well as from the remaining cytoplasmic droplet (Sullivan & Bleau 1985). Extracted spermatozoa were pelleted by centrifugation at 4000 ***g*** for 5 min, and protein concentration in the supernatant was quantified by the Bradford assay (Bio-Rad). Proteins were precipitated for 2 h at −20 °C with nine volumes of ice-cold acetone and pelleted by centrifugation for 15 min at 8000 ***g***, air dried and resuspended in lysis buffer (7 M urea, 2 M thiourea, 30 mM Tris and 4% CHAPS (w/v), pH 8.5) to a final concentration of 2 μg/μl for two-dimensional electrophoresis and in Laemmli sample buffer (2% SDS, 2% β-mercaptoethanol, 50 mM Tris and 20% glycerol) to a final concentration of 1 μg/μl for one-dimensional electrophoresis. Samples were kept frozen at −80 °C until used.

Two-dimensional difference gel electrophoresis and gel analysis

Experimental design

2D-DIGE development by Ünlü *et al.* (1997), has made bidimensional electrophoresis more accurate and reliable to monitor differences in protein abundance between two distinct biological samples. 2D-DIGE allows multiplexing, i.e. co-migration of up to three different samples on the same 2D gel. This is made possible by prelabelling each extract with one of the three spectrally distinct, charge- and mass-matched cyanine fluorescent dyes known as Cy2, Cy3 and Cy5. The sample labelled with Cy2 serves as an internal standard and is made from an equal protein amount of all biological samples evaluated in an experiment. Thus, each spot from individual biological samples can be measured as a ratio to its corresponding spot in the internal standard, significantly reducing the effects of gel-to-gel variations on protein spot quantification (Alban *et al.* 2003, Van den Bergh *et al.* 2003*a*). Moreover, CyDye DIGE fluors offer sensitivity as low as 0.025 ng and a dynamic range above 3.6 orders of magnitude (Marouga *et al.* 2005), giving a great accuracy to protein quantification. In the present study, samples from bulls with SOL > 0 and SOL < 0 were alternatively labelled with Cy3 and Cy5 to prevent dye-specific protein labelling.

Protein labelling with CyDye DIGE fluors

Prior to labelling, equal amounts of proteins from two to three ejaculates were pooled together for a total of 50-μg proteins per bull. Each sample was labelled with the CyDye DIGE fluors developed for fluorescence 2D-DIGE technology according to the manufacturer's instructions (GE Healthcare, Baie d'Urfé, Quebec, Canada). Briefly, 50 μg of each sample were minimally labelled by incubation with 400 pmol of amine-reactive cyanine dye, Cy3 or Cy5, on ice for 30 min in the dark. At the same time, the internal standard sample was generated by combining equal amounts of proteins from each bull and labelled with Cy2 for a ratio of 50 μg protein/400 pmol Cy2. Reactions were quenched by incubation with 1 μl of 10 mM lysine/50 mg protein (Sigma–Aldrich). Samples labelled with Cy3 were mixed with samples labelled with Cy5 and 50 μg of Cy2-labelled internal standard, and rehydration solution was added (7 M urea, 2 M thiourea, 2% CHAPS (w/v), 2% pharmalyte 3–10 (Pharmacia Biotech) and 0.28% dithiothreitol (DTT) (w/v)) to a final volume of 250 μl.

Two-dimensional SDS-PAGE

Precast immobilised pH gradient strips (pH 3–10 l, 13 cm) were used for isoelectric focusing, which was carried out on an IPGphor II IEF system (GE Healthcare). Gels were subjected to 30 V for 12 h, 250 V for 30 min, 500 V for 1 h, 1000 V for 1 h, 3000 V for 1 h and 8000 V until 16 000 V was reached. Strips were equilibrated in SDS equilibration buffer (6 M urea, 57 mM Tris–HCl, pH 8.8, 29.3% glycerol, 2% SDS (w/v) and trace of bromophenol blue) containing 10 mg/ml DTT for 15 min and then in SDS equilibration buffer containing 25 mg/ml iodoacetamide for 15 min and were sealed to 4% acrylamide stacking gels using 0.7% agarose in standard Tris–glycine electrophoresis buffer. Second dimension 12.5% SDS-PAGE was run at 35 mA/gel until the tracking dye had run off the gel.

Gel imaging and data analysis

After SDS-PAGE, CyDye-labelled proteins were visualised by scanning with specified excitation and emission filter wavelengths using an Ettan DIGE Imager (GE Healthcare). All gels were scanned at a resolution of 100 μm. Gel images were processed using ImageMaster 2D Platinum 6.0 DIGE enabled (GE Healthcare), which allowed gel matching and spot quantification. A master gel was generated by the software, which is one of the internal standard images with the most and best resolved spots. Gel images were automatically matched to the master gel by ImageMaster and were manually verified. Only spots whose match was beyond any doubt were considered for DIGE analysis. Spots were quantified as their volume ratio, i.e. volume of the spot divided by its corresponding volume spot on the internal standard co-migrated on the same gel. After DIGE analysis, gels were stained with Coomassie brilliant blue R-250 (Bio-Rad). Spots presenting significant differences between fertile and subfertile bull groups were manually excised, trypsin digested and identified by LC–MS/MS as described below.

Statistical analysis

Differences in protein abundance among the four groups (HF, MHF, MLF and LF) were evaluated by a one-way ANOVA followed by the Duncan statistical test. Differences were considered significant at $P<0.05$. Spots presenting significant differences between HF and LF groups were identified by LC–MS/MS and used as independent variables in regression models (Statistical Analysis Systems, version 9.1.3).

Protein identification by mass spectrometry

The in-gel digest and MS experiments were performed by the Proteomics platform of the Eastern Quebec Genomics Center, Quebec, Canada.

In-gel protein digestion

Tryptic digestion of protein-excised spot was performed on a MassPrep liquid handling robot (Waters, Milford, MA, USA) using Shevchenko's protocol (Shevchenko *et al.* 1996) modified by Havlis *et al.* (2003). Briefly, proteins were reduced with 10 mM DTT and alkylated with 55 mM iodoacetamide. Protein digestion was performed using 105 mM of modified porcine trypsin (Sequencing grade, Promega) at 58 °C for 1 h. Digestion products were extracted with 1% formic acid, 2% acetonitrile followed by 1% formic acid and 50% acetonitrile. The recovered extracts were pooled, vacuum centrifuged, dried, and then resuspended into 8 µl of 0.1% formic acid and were analysed by MS.

Mass spectrometry

Peptide samples were separated by online reverse-phase nanoscale capillary LC and analysed by electrospray MS (ES MS/MS). The experiments were performed with a Thermo Surveyor MS pump connected to a linear ion trap quadrupole mass spectrometer (Thermo Electron, San Jose, CA, USA) equipped with a nanoelectrospray ion source (Thermo Electron). Peptide separation was done on a PicoFrit column BioBasic C18, 10 cm×0.075 mm internal diameter (New Objective, Woburn, MA, USA), with a 2–50% linear gradient of solvent B (acetonitrile and 0.1% formic acid) in 30 min at 200 nl/min (obtained by flow splitting). Mass spectra were acquired using a data-dependent acquisition mode using Xcalibur software version 2.0. Each full scan mass spectrum (400–2000 m/z) was followed by collision-induced dissociation of the seven most intense ions. The dynamic exclusion (30-s exclusion duration) function was enabled, and the relative collisional fragmentation energy was set to 35%.

Database searching

All MS/MS samples were analysed using Mascot (Matrix Science, London, UK; version 2.2.0). Mascot was set up to search the bovine Uniref100 database (release 13.2) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.50 kDa and a parent ion tolerance of 2.0 kDa. Iodoacetamide derivative of cysteine was specified as a fixed modification, and oxidation of methionine was specified as a variable modification. Two missed cleavages were allowed.

Criteria for protein identification

Scaffold (version Scaffold-2_00_06, Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted at a 95.0% probability as specified by the Peptide Prophet algorithm (Keller *et al.* 2002). Protein identifications were accepted if they could be established at >95.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the protein prophet algorithm (Nesvizhskii *et al.* 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Motile and immotile sperm separation

Immotile and motile sperm subpopulations were separated on Percoll discontinuous gradient according to Parrish *et al.* (1995). Briefly, 0.5-ml aliquots of thawed semen were layered on a discontinuous gradient of 45 and 90% (v/v) isotonic Percoll. The gradient consisted of 2 ml of 45% Percoll and 2 ml of 90% Percoll in a 15-ml conical plastic tube. Sperm suspensions were layered on the top of the gradient and centrifuged at 700 ***g*** for 20 min. Immotile spermatozoa were recovered at 45–90% Percoll interface, while the motile spermatozoa were recovered in the pellet at the bottom of the gradient. Qualitative sperm motility was assessed visually. Separated subpopulations were washed by centrifugation in PBS and were then resuspended in Laemmli sample buffer, heated for 5 min and centrifuged, and the supernatant was kept frozen until used in 12.5% SDS-PAGE, and immonublots were probed for α-tubulin, ELSPBP1, PSMA6, AK1, PEBP1 and BSP1. Laemmli sample buffer was used in order to extract α-tubulin, which was used as a housekeeping protein. ELSPBP1, AK1, PEBP1 and BSP1 were fully extracted by the Triton X-100 treatment, while PSMA6 was partly extracted by Triton X-100 (data not shown). Thus, PSMA6 was extracted with Triton X-100 as described above.

One-dimensional gel electrophoresis and western blot analysis

As neither β-actin nor α-tubulin, which are usually used as ubiquitous proteins, is not extracted uniformly when sperm suspensions are treated with Triton X-100, protein concentrations in the sample buffer were quantified by amido black staining of dot blots (Chapdelaine *et al.* 2001). For Triton X-100 protein extracts, 15 µg of protein/sample were loaded on gel and proteins from 3×10^6 cells were loaded when extracted directly with Laemmli sample buffer. Proteins were separated by 12.5% SDS-PAGE and transferred onto nitrocellulose membrane. Nitrocellulose membranes were stained with Ponceau Red to verify uniformity of transfers. Membranes were blocked for 1 h in PBS 0.1% Tween-20 (PBS-T) containing 5% (w/v) defatted milk. Membranes were incubated with 1/500 rabbit anti-CE12-3 (ELSPBP1; generously provided by C Kirchhoff from the Institute for Hormone and Fertility Research, Hamburg, Germany (Saalmann *et al.* 2001)), 1/5000 anti-BSP-A1/A2 (BSP1; generously provided by Dr P Manjunath from Université de Montréal, Montréal, Canada (Manjunath & Sairam 1987)), 1/200 goat anti-AK1 (S-16) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), 1/200 rabbit anti-RKIP (FL-187) (PEBP1; Santa Cruz Biotechnology Inc.) and 1/200 rabbit anti-PSMA6 (H-135) (PSMA6; Santa Cruz Biotechnology Inc). When proteins were directly extracted in Laemmli sample buffer, α-tubulin monoclonal antibody

(Sigma) was used as the housekeeping protein. Membranes were then incubated with HRP-conjugated goat anti-rabbit, rabbit anti-goat or goat anti-mouse IgG (dilution 1/5000) in 5% (w/v) defatted milk in PBS-T for 1 h at room temperature. Immunological complexes were visualised using the ECL detection kit (Amersham Biosciences).

Membrane integrity

Membrane integrity of spermatozoa from 16 of the 23 bulls initially used in this study was measured by flow cytometry using PI. Two batches of semen samples were analysed for each bull used for the DIGE analysis. After thawing, spermatozoa were washed twice by centrifugation (350 ***g***; 5 min) in sp-TALP (Parrish *et al.* 1988). Sperm concentration was adjusted to 2×10^6 cells/ml and then incubated with 2.4 μM PI for 10 min at 34.5 °C. Measurements were made with LSRII (Becton Dickinson, San Jose, CA, USA). A minimum of 10 000 spermatozoa were analysed for each reading, and PI-negative cells were considered to have a fully functional membrane and to be alive.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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