Molecular BioSystems

RSC Publishing

PAPER

View Article Online
View Journal | View Issue

Unravelling the bull fertility proteome†

Cite this: *Mol. BioSyst.*, 2013, **9**, 1188

Alessio Soggiu,‡[a] Cristian Piras,‡[a] Hany Ahmed Hussein,[a] Michele De Canio,[bd] Alessandro Gaviraghi,[c] Andrea Galli,[c] Andrea Urbani,[bd] Luigi Bonizzi[a] and Paola Roncada*[c]

Received 31st October 2012,
Accepted 21st January 2013

DOI: 10.1039/c3mb25494a

www.rsc.org/molecularbiosystems

In the last few decades a negative association between the level of milk production and fertility has been observed. Currently, the most utilized method of measuring male fertility employed by the livestock industry is related to the Non-Return Rate (NRR). Through differential proteome analysis, this study evaluated changes in the expression of the protein profile of spermatozoa collected from 16 bulls with different levels of field fertility expressed as an estimated relative conception rate (ERCR). The main aim is to identify putative protein markers to be used as putative indices of fertility. Two dimensional electrophoresis coupled with mass spectrometry analysis was used for protein separation and identification. To improve differential proteome analysis among experimental groups, a part of shotgun MS analysis was also performed. Three protein spots showed a differential expression pattern among all ERCR classes. Alpha enolase was significantly down-regulated in the ERCR− group, while two other proteins, isocitrate dehydrogenase and triosephosphate isomerase, were up-regulated in ERCR− in comparison to ERCR+. Alpha-enolase and isocitrate dehydrogenase subunit alpha (IDH-alpha) have been described in the literature for having a potential role in bull fertility. The possibility of determining protein biomarkers for fertility is more useful and less expensive than ERCR for acquiring rapid estimation of fertility because it does not require the use of field insemination trials. Shotgun MS analysis conducted on the same samples revealed 7 proteins down-regulated in the ERCR− group and 1 protein up-regulated. Among these proteins, calmodulin, ATP synthase mitochondrial subunits alpha and delta, malate dehydrogenase and sperm equatorial segment protein 1 were shown to be linked with sperm fertility.

Introduction

Dairy herds have rapidly changed in the last few years. Milk production per cow has increased steadily due to a combination of improved management, better nutrition, and intense genetic selection. Genetic selection, through the use of polymorphism in genomic markers associated with milk production, body conformation, longevity, and other traits, has had a great impact on the economic return from dairy herds. On the other hand, it has been observed that there is a negative association between the level of milk production and fertility, which could both be linked to genetic factors (pleiotropy and inbreeding) and physiological factors (metabolic disease by high production).[1] Because of cow replacement, veterinary costs for the management of reproductive diseases, and the cost of frozen semen, dairy herds with high milk production and reproductive management account for a large part of production cost. In fact, when herd fertility decreases, the number of cows required to produce a given volume of milk increases, both in terms of the number of milk producing animals and in the number of pre-production female replacements. Many resources have been used to enhance female fertility but little studies and interventions have been made to increase male bovine reproductive efficiency. Some studies have shown that a significant percentage of reproductive failure is attributable to semen quality and not to cow problems.[2] Considering that the patterns of selection and reproductive management of dairy cattle are based on the use of artificial insemination (AI), it is easy to understand the importance, for

[a] *Dipartimento di Scienze veterinarie e sanità pubblica, Università Degli Studi di Milano, Milano, Italy*
[b] *IRCSS Fondazione S. Lucia, Roma, Italy*
[c] *Istituto Sperimentale Italiano "L. Spallanzani", Sezione di Proteomica c/o DIVET, Dipartimento di Scienze Veterinarie e Sanità Pubblica, Università degli Studi di Milano, Milano, Italy. E-mail: paola.roncada@guest.unimi.it; Fax: +39 02 50318171; Tel: +39 02 50318138*
[d] *Università di Roma "Tor Vergata", Dipartimento Medicina Sperimentale e Chirurgia, Roma, Italy*

† Electronic supplementary information (ESI) available. See DOI: 10.1039/c3mb25494a

‡ A.S and C.P. have equally contributed to this work.

the breeder, to assess the level of bull fertility. Unfortunately, neither a simple spermiogram nor the routine evaluation post-thaw will enable the determination *a priori* of the potential fertility level of the analyzed semen, particularly after AI. Currently, the most utilized method of measuring male fertility within the livestock industry is related to the Non-Return Rate (NRR).[3,4] The estimated relative conception rate (ERCR) is expressed as the percentage difference of the conception rate (non-return rate at 56 or 70 days) of a sire compared with other AI sires used in the same herd.[2] The main environmental factors, such as herds, month of insemination, age of cows, days in milk, and milk production of the mate, are taken into account. For ERCR calculation, only the first breeding in each cow's lactation is used, and in order to be accurate, a large number of inseminations per bull are required. Therefore, the ERCR is a phenotypic prediction (evaluation) of the bull's conception rate (CR), not a genetic evaluation.[5] Conception rates are influenced by a high number of different factors. Management and environmental factors are responsible for 96% of the variation in conception rates. Herd differences in nutrition, metabolic disorders, reproductive health, and climate can result in significant differences of conception rates. The remaining 4% of variation in conception rates is due to genetic factors of 3% for the cow and 1% for the service bull. For these reasons,[3,4] an accurate prediction of the fertility rate of a sire is still very difficult.

To overcome the static analysis of the bull genome, proteomics, by focusing on differential protein expression, could help the discovery of novel biomarkers that could be useful for the detection of the bull fertility rate.[5–14] Evaluating the differences in the proteome of the spermatozoa of bulls with different ERCRs could highlight some dynamic markers useful for the evaluation of the real fertility rate. The aim of this study is to evidence, through the differential proteome analysis, changes within the expression of the protein profile of the spermatozoa of bulls with different levels of fertility estimated by ERCR in order to identify possible protein markers to be used as putative indices of fertility and to enforce existing criteria that are commonly used.

Materials and methods

Animals and fertility data

All samples were grouped into 2 classes: ERCR+ (ERCR > 1) and ERCR− (ERCR < −1). Eight bulls were selected for each class according to the values of ERCR and calculated using data derived from functional controls of milk production (promoted by Italian Breeders Association, AIA) by Associazione Nazionale Allevatori Frisona Italiana (ANAFI). The measured data were the percentage of non-return at 56 days, adjusted for a number of variability factors (company, year, and month of insemination, birth order of the cow, bull semen production center, energy expended in the production of milk fat to 3.5% and 3.2% protein, *etc.*). The ERCR represents the effect of the bull on the percentage of cows inseminated in the non-return company, expressed as a difference from the average of the percentage of non-return obtained with the semen of other bulls.

Sperm sample preparation

Two commercial straws of semen of each bull were thawed in the water-bath at 37 °C for 1 minute. The content was centrifuged at 1000 × *g* for 10 minutes to get the sperm pellet. After the removal of the supernatant, 5 washes in PBS to eliminate the extender were performed. The pellet obtained was re-suspended in 200 μL of extraction buffer 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 30 mM Tris and sonicated (3 cycles of 20 seconds at full power on ice). After centrifugation to remove cellular debris (14 000 × *g*, 20 °C, 60 minutes), protein sample content was quantified using the 2D-Quant kit (GE Healthcare, Uppsala).

Two dimensional electrophoresis

The first dimensional analysis was performed using 7 cm strips in the pH range 4–8 (linear) and 13 cm strips in the pH range 3–10 (linear) (Euroclone, Italy). Samples were loaded by cup loading (100 μg of total protein). The IEF was performed using the Ettan IPGPhor III (GE Healthcare, Uppsala) until reaching 85 kV h^{-1} in total. After sequential equilibration of the strips with 1% dithioerythritol (DTE) and 2.5% iodoacetamide (IAA), the strips were transferred to SDS-PAGE 12% acrylamide for the separation in the second dimension on a Protean Tetra Cell (Biorad, USA). The gels were stained using colloidal coomassie staining.

Image analysis

Images were acquired using an ImageScanner III (GE Healthcare, Uppsala). Gel images were analyzed by Progenesis SameSpot v4.5 software (Nonlinear Dynamics, UK). Spot detection and normalization were performed using the automated tools of the software. Principal component analysis (PCA) was elaborated using the Stat module of this software.

In gel digestion

Differentially expressed spots were manually excised. Gel pieces were de-stained before digestion. After de-staining they were washed first with H_2O, then with 50 mM NH_4HCO_3–ethanol 1 : 1 (v/v). Gel plugs were dehydrated with pure ethanol, reduced with 10 mM dithiothreitol (DTT) in 50 mM NH_4HCO_3 (1 h at 37 °C) and alkylated with 55 mM IAA in 50 mM NH_4HCO_3 (30 min at room temperature). Plugs were washed with 50 mM NH_4HCO_3 and dehydrated completely. A solution of 0.01 μg μL^{-1} trypsin (Promega, Madison, WI) was added, and proteins were digested at 37 °C overnight. The reaction was stopped by adding 1% (v/v) trifluoroacetic acid (TFA) in H_2O.

MALDI-TOF MS analysis

Peptides were desalted using C_{18} ZipTips (Millipore, Billerica, MA) and co-crystallized with a solution of 0.5 mg mL^{-1} alpha-cyano-4-hydroxycinnamic acid dissolved in acetonitrile–0.1% (v/v) TFA in H_2O (1 : 1) on a Ground Steel plate (Bruker-Daltonics, Bremen, Germany) prespotted with a thin layer of

10 mg mL^{-1} alpha-cyano-4-hydroxycinnamic acid dissolved in ethanol–acetonitrile–0.1% (v/v) TFA in H_2O (49.5 : 49.5 : 1). Spectra were acquired with an Ultraflex III MALDI TOF/TOF spectrometer (Bruker-Daltonics, Bremen, Germany). A standard peptide mixture from Bruker-Daltonics (*m*/*z* 1046.54, 1296.68, 1347.73, 1619.82, 1758.93, 2093.08, 2465.19, 2932.59, 3494.65) was used for external calibration. Acquired spectra were processed by FlexAnalysis software v3.0 (Bruker Daltonics, Bremen, Germany).

Internal calibration was performed using autolysis peaks from porcine trypsin (*m*/*z* 842.509 and 2211.104). After exclusion of contaminant ions (known matrix and human keratin peaks), a database search was performed using the MASCOT 2.2.03 algorithm (www.matrixscience.com) against UniProtKB/Swiss-Prot bovine database release 2012_01 restricted to ''other mammalia'' taxonomy with carbamidomethylation of cysteines as fixed modification, and oxidation of methionines as variable modification, one missed cleavage site allowed for trypsin and 50 ppm as maximal tolerance. Protein scores greater than 66 were considered significant ($P < 0.05$).

MALDI-TOF MS/MS analysis was performed in LIFT mode. Precursor ions were selected manually. MS/MS spectra were acquired with a minimum of 4000 and a maximum of 8000 laser shots using the instrument calibration file. The precursor mass window was set automatically after the precursor ion selection. Spectra baseline subtraction, smoothing (Savitsky–Golay) and centroiding were performed by FlexAnalysis v3.0 software (Bruker Daltonics, Bremen, Germany). Database search was performed by setting the following criteria: maximum of one missed cleavage was allowed, the mass tolerance of precursor ions and fragments was set to 75 ppm and 0.5 Da respectively; the allowed fixed modification was carbamidomethylation on cysteine and the allowed variable modification was methionine oxidation. The taxonomy was restricted to other mammalia taxonomy. Individual ion scores >38 indicate identity or extensive homology ($p < 0.05$).

Shotgun MS, label free proteomics analysis by nLC-MSE

Bovine semen samples collected from 6 different bulls (3 samples for each group) were washed 5 times with PBS then spermatozoa were resuspended in 100 mM Tris/HCl pH 7.9 containing 6 M urea and lysed by sonication. Reduction and alkylation of proteins were performed by adding 100 mM DTT (1 h at 36 °C) and 200 mM iodoacetamide (1 h at R.T.). Protein samples, at a final concentration of 3 μg μl^{-1}, were digested with 1 : 20 (w/w) sequence grade trypsin (Promega, Madison, WI, USA) at 36 °C overnight. Reactions were stopped by adding 0.3% (v/v) TFA.

A total of 0.75 μg of digested protein was loaded on a nanoACQUITY UPLC System (Waters Corp., Milford, MA) coupled to a Q-Tof Premier mass spectrometer (Waters Corp., Manchester, UK). Digested enolase from *Saccharomyces cerevisiae* (Waters Corp. Manchester, UK) was added to samples as an internal standard at a final concentration of 100 fmol μL^{-1}. Tryptic peptides were preconcentrated and desalted onto a Symmetry C_{18} 5 μm, 180 μm × 20 mm precolumn (Waters Corp. Manchester, UK) and subsequently separated using a NanoEaseTM BEH C_{18} 1.7 μm, 75 μm × 25 cm nanoscale LC column (Waters Corp. Manchester, UK) operating at 35 °C. Mobile phases A and B were water with 0.1% formic acid and 0.1% formic acid in acetonitrile, respectively. Peptide separation was obtained by a gradient of 3–40% B over 150 min at a flow rate of 250 nL min^{-1}, followed by a gradient of 40–90% B over 5 min and a 15 min rinse with 90% B. The Q-Tof Premier mass spectrometer (Waters Corp. Manchester, UK) was operated in ''Expression Mode'' switching between low (4 eV) and high (15–40 eV) collision energies using a scan time of 0.8 s over 50–1990 *m*/*z* mass range. Samples were acquired in triplicate. Continuum LC-MS data were processed using ProteinLynx GlobalServer v2.4 (PLGS, Waters Corp. Manchester, UK). Protein identification was performed using the embedded ion accounting algorithm of the software and searching into the UniProtKB/Swiss-Prot bovine database release 2012_01 (Bos Taurus; 5879 entries) to which the sequence of enolase from *Saccharomyces cerevisiae* was appended. Parameters for database search were: automatic tolerance for precursor ions, automatic tolerance for product ions, minimum 3 fragment ions matched per peptide, minimum 7 fragment ions matched per protein, minimum 2 peptides matched per protein, 1 missed cleavage, carbamidomethylation of cysteines, and oxidation of methionines as modifications. The false positive rate (FPR) of the identification algorithm was set under 4%. Relative quantitative analysis was performed using the PLGS dedicated tool. Identified proteins were normalized against P00924 entry (enolase from *Saccharomyces cerevisiae*) while the most reproducible peptides for retention time and intensity derived from *Saccharomyces cerevisiae* enolase digestion (*m*/*z* 814.59; *m*/*z* 1159.61; *m*/*z* 1286.70; *m*/*z* 1288.70; *m*/*z* 1578.80; *m*/*z* 1840.93) were used to normalize the EMRTs table. The list of normalized proteins was screened according to the following criteria: proteins identified in at least 2 out of 3 runs of the same sample; proteins with $0 < p < 0.05$ or $0.95 < p < 1$, and proteins with a ratio of expression level of ±0.30 on a natural log scale (about 1.3 on a decimal scale). The analytical performances of shotgun analysis are also reported in the ESI† (Table S1 and Fig. S1).

Statistical analysis

1-Way ANOVA with a Tukey post-hoc test was used to evaluate the results from 2-DE gels (Progenesis Samespot 4.5, Nonlinear Dynamics, UK) FDR < 0.05 were considered significant. The Mann–Whitney test to analyse shotgun results was performed using the software Prism (version 5.02). *p*-Values <0.05 were considered significant.

Results and discussion

A representative 2D PAGE map of a cryopreserved semen sample is shown in Fig. 1. Only matched spots detected on all images were considered for image analysis. Image analysis by Progenesis Samespot revealed significant differences for three proteins between analyzed groups (Fig. 1 and 2a). One spot was significantly under-expressed ($P < 0.05$) in the ERCR− group

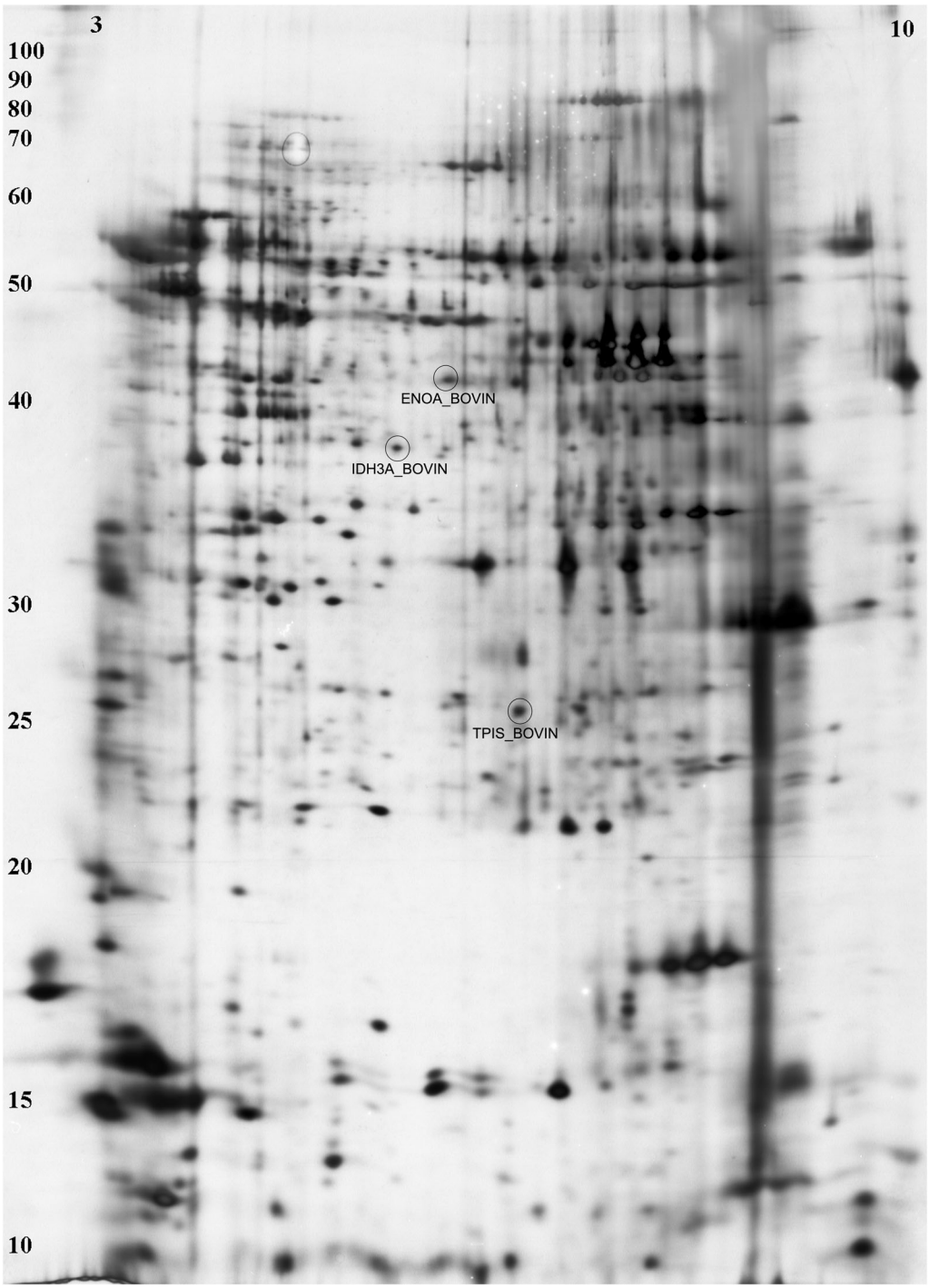

Fig. 1 Representative 2-DE map of a cryopreserved bull semen sample. Differentially expressed proteins are indicated in the map.

and was successfully identified through mass spectrometry (MS) analysis as *alpha enolase* (ENOA) (Fig. 1 and 2a). The remaining two spots identified through mass spectrometry (isocitrate dehydrogenase and triosephosphate isomerase) were predominantly overexpressed in the ERCR− group (Fig. 1 and 2a). Principal component analysis (PCA) was used to analyze 2-DE data of sperm samples (Fig. 2b). PCA results showed three different 2-D spots (326, 432 and 497) related to ERCR+ and ERCR−. Overexpression of spot 326 (alpha enolase) is strongly associated with the ERCR+ group, while overexpression of 432 (isocitrate dehydrogenase) and 497 (triosephosphate isomerase) are associated with the ERCR− group as the most significative loadings of PCA analysis (Fig. 2B, red numbers). Table 1 shows the proteins identified by tandem MS (MALDI-MS/MS). Shotgun MS analysis performed among the 2 groups of samples revealed the presence of 8 differentially expressed proteins. Seven of them were under-expressed in the ERCR− group and one of them was up-regulated (Fig. 3).

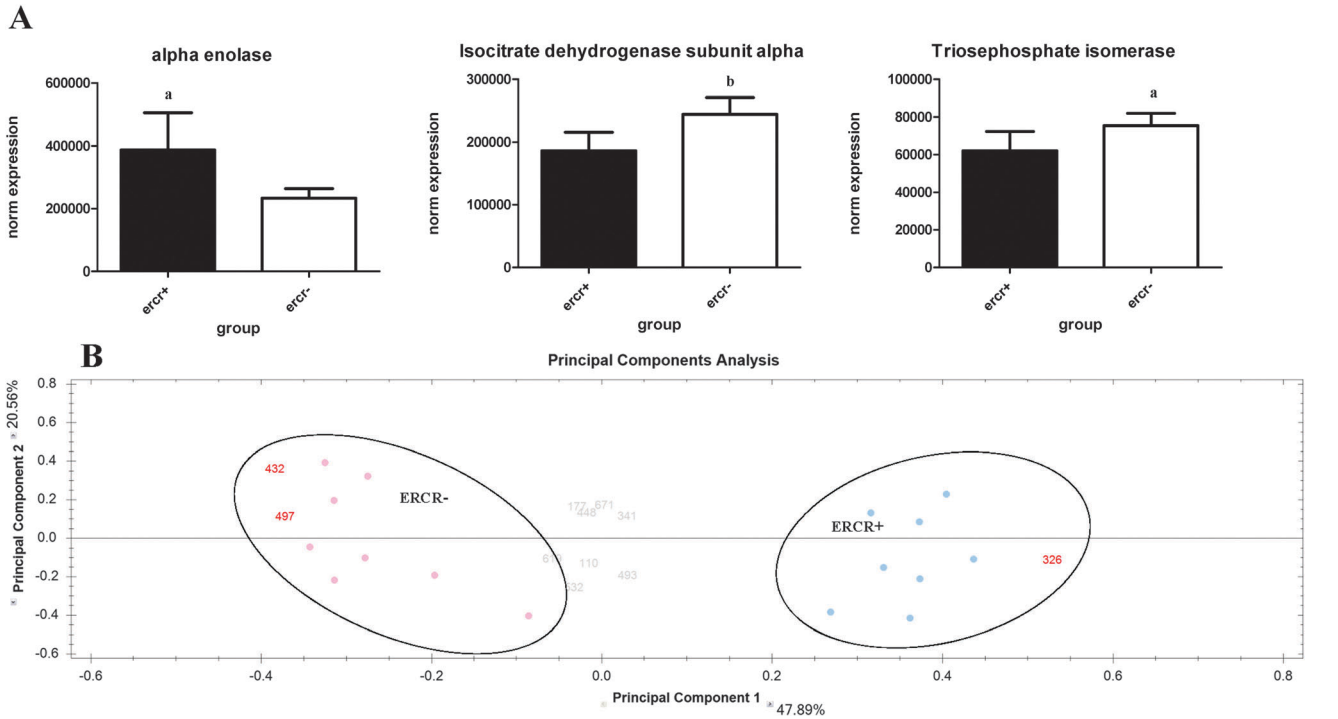

Fig. 2 (A) 2-DE differentially expressed proteins (a) $p \leq 0.05$; (b) $p \leq 0.01$). (B) PCA biplot with scores (colored spots) and most significative loadings (spot red numbers) of ERCR+ and ERCR− 2D samples. Spot 326 (alpha enolase), 432 (isocitrate dehydrogenase) and 497 (triosephosphate isomerase).

Table 1 Differentially expressed proteins identified by 2-DE/MS and confirmed by MS/MS

Uniprot accession code	Entry name	Protein name	Mascot PMF score	Protein mass (DA)	Peptide 1 = unique	Mascot MS/MS score	Peptide sequence
Q9XSJ4	ENOA_BOVIN	Alpha-enolase	78	47 639	0	46.21	AAVPSGASTGIYEALELR
P41563	IDH3A_BOVIN	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial	68	40 098	1	38.06	ENTEGEYSGIEHVIVDGVVQSIK
Q5E956	TPIS_BOVIN	Triosephosphate isomerase	69	26 901	1	50.77	DLGATWVVLGHSER
Q5E956	TPIS_BOVIN	Triosephosphate isomerase	69	26 901	1	45.59	VVLAYEPVWAIG

All analyzed samples were divided into 2 different groups according to the ERCR data (see the Results section). Collected samples were then analyzed through 2-DE coupled with mass spectrometry to highlight and identify differentially expressed proteins. Three protein spots showed a differential expression pattern among the two different groups. Alpha enolase, isocitrate dehydrogenase subunit alpha mitochondrial and triosephosphate isomerase are mainly involved in energy metabolism; in particular, glycolysis and TCA cycle. Among these differentially expressed proteins analyzed through shotgun MS, three are involved in the energy metabolism pathway and one of them in calcium homeostasis. All other proteins are mostly chaperon structural proteins involved in protection against stress response. It was particularly interesting to see a seminal plasma protein tending towards overexpression in ERCR− samples.

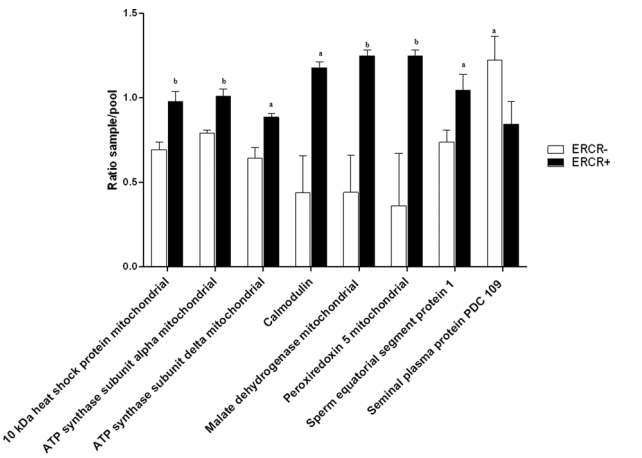

Fig. 3 Shotgun MS analysis results. Differential expression of proteins in ERCR+ and ERCR− groups: (a) $p \leq 0.05$; (b) $p \leq 0.01$.

2D electrophoresis proteins

As shown in Fig. 2a, alpha-enolase was down-regulated in ERCR−. This protein is an enzyme principally involved in the glycolysis and energy production and it is composed of three isozyme subunits, alpha, beta and gamma, which can form homodimers or heterodimers which are cell-type and development-specific.[15] Alpha-enolase in humans, but also in animals,

is an ubiquitary protein present in several isoforms due to differences in tissue expression or post-translational modifications.[16] It could be important during pathological conditions as well because of its ability to function as a heat-shock protein and to bind cytoskeletal and chromatin structures. Several experimental evidence noted how this enzyme plays a key role in the mechanisms involved in cellular motility,[17,18] interacts with microtubules and has a potential role in the regulation of energy metabolism.[19,20] The association of glycolytic enzymes, including alpha-enolase, seems to be important for human sperm motility.[17] For example it has been demonstrated that the high amount of ATP required in Chlamydomonas for flagella motility is supported by the presence of enolase in the flagellar microtubular compartment.[21] Moreover, alpha-enolase was identified as having a positive relationship with the first cycle conception rate in stallions.[22] In infertile men, sperm-specific enolase enzyme activity was elevated in normal sperm compared to abnormal sperm.[23] All these experimental evidence demonstrate how alpha-enolase expression could be linked with sperm motility and healthiness, and in this way could influence the ERCR rate. Elevated expression of alpha-enolase was found in bull sperm[24] and in fluid derived from the cauda epididymal of mature Holstein bulls in association with a high fertility profile.[7,25] So these results strengthen our results for alpha-enolase as a biomarker of bull fertility independent of the type of extender used.

As shown in Fig. 2a, isocitrate dehydrogenase subunit alpha (IDH-alpha) and triosephosphate isomerase (TPI) showed higher expression in the ERCR− group which is associated with a low score of fertility. NAD+-dependent isocitrate dehydrogenase (IDH), a key regulatory enzyme in the Krebs cycle, is a multi-tetrameric enzyme. Subunits 1/beta and 2/gamma are considered to be regulatory, while subunits 3,4/alpha are catalytic.[26] Moreover, this enzyme is known to modulate the transcription factor hypoxia-inducible factor-1 (HIF-1)[27] that plays important roles in development, physiology,[28] and many pathological processes including cancer biology[29] and fertility issues.[30] In human asthenozoospermic patients there exists a lower expression of isocitrate dehydrogenase subunit alpha[8] (IDH-alpha) but an increased expression of fumarate hydratase precursor (FHpre).[9] In stallion sperm three enzymes directly involved in the TCA cycle (citrate synthase, fumarate hydratase, and malate dehydrogenase) are overexpressed in samples with high fertility.[14] In this work there is an overexpression of IDH-alpha in sperm samples with low ERCR score (Fig. 2a). The explanation of this phenomenon can be found either in a possible modulation of hypoxia-inducible factor-1 in sperm cells due to several types of metabolic problems[30] or an increased necessity of NADPH in response to an increased oxidative stress. The latter possibility is largely supported by literature data. In effect, defective human spermatozoa show intense redox activity and oxidative stress has been associated with impaired sperm motility.[31] Also, sperm–oocyte fusion is inhibited by oxidative stress.[32]

Triosephosphate isomerase (TPI), another important glycolytic enzyme, is overexpressed in the ERCR− group in comparison to the other groups ($P < 0.05$, $p = 0.048$). Triosephosphate isomerase is an enzyme that if selectively blocked could bring mammalians to infertility.[33] It has been documented that ornidazole exerts a rapid and reversible antifertility effect in male rats.[34,35] In dogs, humans and rats the metabolites of ornidazole may produce inhibitors of the enzymes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and triosephosphate isomerase (TPI)[36,37] affecting the spermatozoa ability to obtain ATP by the glycolytic pathway. Triosephosphate isomerase was found in our experiment to be decreased linearly according to the ERCR score. As described above it represents one of the major important enzymes necessary for the glycolysis pathway necessary for sperm motility. Our results highlighted its overexpression in non-fertile bull sperm and its overexpression could be due to the deficient glycolysis step in the previous pathways. Obtained results showed, as previously described, a significant decrease of alpha-enolase that brings to a lack of pyruvate. The lack of pyruvate could be responsible for the activation of a feedback mechanism that enables a higher production of other glycolytic enzymes such as TPI. According to the authors' knowledge in literature, data are not present about this protein in bull sperm but similar profiles of expression are found in human sperm from asthenozoospermic (low motility sperm) patients.[6,8] So the results obtained in this study may contribute both to better understand the role of this protein in bull sperm and to insert this protein in the list of the putative biomarkers useful to predict the bull fertility rate without waiting for an ERCR score.

Shotgun MS analysis

Several immunocytochemical studies have demonstrated that CaM is present in the head and flagellum of mammalian sperm,[38–42] suggesting that CaM could be involved in functions occurring in the sperm head, tail, or both. Calmodulin is involved in sperm capacitation, the physiological changes that render mammalian sperm competent to fertilize oocytes, and is dependent upon exogenous Ca^{2+}. It has been documented how sperm treatment with calmodulin antagonists significantly decreases their fertilization competence in comparison to control sperm.[43] It has also been demonstrated that calmodulin function is directly related to tyrosine phosphorylation. Shotgun MS experiments demonstrated how the sperm group with low ERCR score presented lower levels of calmodulin. As previously described, Ca^{2+} concentration is important for sperm capacitation and an impaired calmodulin concentration could be linked to the low fertility rate as demonstrated. Energy such as ATP is necessary for sperm motility. The sperm cell uses both the ATP stored and the one newly synthesized during the motility phase. Therefore it is necessary for sperm fertility to have an efficient ATP production system. Both subunits have been found by Khan and colleagues in epididymal sperm,[44] and the results shown in Fig. 3 demonstrate that both are downregulated in the ERCR− group, underlining their putative function in promoting sperm fertility. Reactive oxygen species (ROS) can play a role in modulating signaling pathways required for human sperm activation. However, high levels impair sperm function, leading to infertility.[45] High levels of

ROS could be produced by spermatozoa themselves or by other cells present in the semen (*e.g.* leukocytes). High concentrations of ROS could lead to impaired sperm function and are positively correlated with male infertility.[46,47] For this reason the good function of the antioxidant system is necessary to protect sperm cells from ROS-dependent damage. An imbalanced antioxidant system could therefore bring infertility. Malate dehydrogenase mitochondrial (MDH2) is an enzyme of the TCA cycle that catalyzes oxidation of malate to oxaloacetate utilizing the NAD/NADH cofactor. This protein is involved in energy metabolism and several authors demonstrate that it is important in supplying reduction equivalents and energy during capacitation and acrosome reaction in cryopreserved bovine spermatozoa[48] and the expression of this protein correlates with high fertility in stallions.[14] Our shotgun results showed an overexpression of this protein in the ERCR+ group *vs.* the ERCR−. Acrosomal biogenesis is divided into four different steps, Golgi, cap, elongation (acrosome), and maturation phases.[49] Sperm equatorial segment protein 1 is present in all these phases of acrosome formation and seems to play a key role in sperm fertility.[50]

In cows (like other mammalia) fertilization is a process that requires a lot of steps and one of the more important is the fusion of the sperm with the egg plasma membrane; the so-called "acrosomal reaction". Precisely, fusion in mammals initiates in the equatorial segment and recently experimental evidence showed that there exists one specific equatorial protein that mediates fusion: the sperm equatorial segment protein 1 (SPESP1).[51] Anti-SPESP1 antibody inhibited sperm–egg fusion in the human sperm–hamster egg system showing the importance of this protein in modulation of mammalian fertility.[52] To date, there is not any experimental evidence about the overexpression of this protein in high fertility bull spermatozoa (ERCR+), as shown in this work, but similar results obtained in mice strongly confirm the importance of this protein to have fully fertile spermatozoa.[51]

Seminal Plasma protein, PDC-109, is the major protein of bovine seminal plasma and is important for sperm capacitation. Indeed, there are several experimental evidence that document how this protein has also got several analogies and functions as a chaperon protein. It has been recently demonstrated that its role in sperm capacitation could be due to its chaperon-like properties.[53] Obtained data demonstrated its over-representation in ERCR− sperm. This protein, according to the experimental design, should not be present in the analyzed sperm proteome because it is a seminal plasma protein. However, it is present because of its chaperone properties. Its presence could be due to its task as a chaperon protein in order to promote right protein folding and to prevent incorrect folding that could, for example, occur due to oxidative stress. In this case, its presence in major concentration in comparison to ERCR+ could be due to a higher necessity of ERCR− sperm to avoid incorrect protein folding.

Some recent experimental evidence documented how seminal plasma proteins are beneficial for sperm function and/or storage. However, on the argument there are a lot of controversies that describe how BSP proteins also induce changes in the sperm plasma membrane by stimulating cholesterol and phospholipid efflux. The continuous exposure of sperm to seminal plasma that contains BSP proteins is detrimental to the sperm membrane, which may render the membrane very sensitive to sperm storage in the liquid or frozen states.[54]

Conclusions

The present study provides the first evidence for protein variations linked to the ERCR values in the bull sperm proteome and demonstrates that 2-D gel electrophoresis coupled with mass spectrometry and bioinformatics is useful for the identification of biomarkers for evaluation of the level of fertility. Obtained data have indicated several possible candidate protein biomarkers for high and low ERCR. In particular alpha-enolase and isocitrate dehydrogenase subunit alpha (IDH-alpha) have been shown to play a key role in bull fertility. The decreased expression of alpha-enolase in ERCR− sperm cells (2D electrophoresis results) demonstrates, from the metabolic point of view, that ERCR− samples have an impaired glycolytic metabolism that could cause a lack of energy production and probably a lower motility of sperm cells. It has indeed been very well documented how energy production, and in particular the ability of sperm cells to rapidly produce energy such as ATP, is one of the most important tasks in order to avoid infertility. Many enzymes and proteins that have been found to be over-represented are mitochondrial. This could suggest that the fertility rate could be linked to the amount of mitochondria present in the sperm cells or to an impaired mitochondrial metabolism. Impaired mitochondrial metabolism could be due to the impaired glycolytic metabolism as demonstrated by our results that document the lack of alpha-enolase and, as effect, lower amounts of pyruvate available for mitochondria. Moreover, it has been observed that in ERCR− samples, there is a down-regulation of Calmodulin, Peroxiredoxin-5-mitochondrial, and sperm equatorial segment protein 1. Calcium and anti-oxidative mitochondrial metabolism are key elements linked to the fertility rate as well. The possibility of obtaining protein biomarkers for fertility could be more useful and less expensive than ERCR for rapid estimation of fertility because it does not require field insemination trials. Further investigations will be necessary to evaluate possible use of these markers in fast screening of bull semen (by immunological techniques) and to clarify the causes of bull infertility.

Acknowledgements

The authors are grateful to Associazione Nazionale Allevatori Frisona Italiana (ANAFI) for ERCR data. This work was supported by the PROZOO project.

Notes and references

1 A. Bach, N. Valls, A. Solans and T. Torrent, *J. Dairy Sci.*, 2008, **91**, 3259–3267.
2 J. S. Clay and B. T. McDaniel, *J. Dairy Sci.*, 2001, **84**, 1238–1245.

3 J. M. Feugang, N. Rodriguez-Osorio, A. Kaya, H. Wang, G. Page, G. C. Ostermeier, E. K. Topper and E. Memili, *Reprod. BioMed. Online*, 2010, **21**, 312–324.
4 N. Bissonnette, J. P. Levesque-Sergerie, C. Thibault and G. Boissonneault, *Reproduction*, 2009, **138**, 65–80.
5 A. Gaviraghi, F. Deriu, A. Soggiu, A. Galli, C. Bonacina, L. Bonizzi and P. Roncada, *Vet. Res. Commun.*, 2010, **34**(suppl 1), S33–S36.
6 A. B. Siva, D. B. Kameshwari, V. Singh, K. Pavani, C. S. Sundaram, N. Rangaraj, M. Deenadayal and S. Shivaji, *Mol. Hum. Reprod.*, 2010, **16**, 452–462.
7 A. A. Moura, C. E. Souza, B. A. Stanley, D. A. Chapman and G. J. Killian, *J. Proteomics*, 2010, **73**, 2006–2020.
8 C. Zhao, R. Huo, F. Q. Wang, M. Lin, Z. M. Zhou and J. H. Sha, *Fertil. Steril.*, 2007, **87**, 436–438.
9 J. Martinez-Heredia, S. de Mateo, J. M. Vidal-Taboada, J. L. Ballesca and R. Oliva, *Hum. Reprod.*, 2008, **23**, 783–791.
10 C. L. Barratt, *Hum. Reprod.*, 2008, **23**, 1240–1241.
11 A. K. Tomar, M. Saraswat, N. Chhikara, S. Kumar, V. K. Yadav, B. S. Sooch, T. P. Singh and S. Yadav, *Biomarkers Med.*, 2010, **4**, 905–910.
12 A. R. Suryawanshi, S. A. Khan, R. K. Gajbhiye, M. Y. Gurav and V. V. Khole, *J. Androl.*, 2011, **32**, 240–259.
13 D. Peddinti, B. Nanduri, A. Kaya, J. M. Feugang, S. C. Burgess and E. Memili, *BMC Syst. Biol.*, 2008, **2**, 19.
14 S. Novak, T. A. Smith, F. Paradis, L. Burwash, M. K. Dyck, G. R. Foxcroft and W. T. Dixon, *Theriogenology*, 2010, **74**, 956–967.
15 M. Peshavaria, G. B. Quinn, I. Reeves, L. J. Hinks and I. N. Day, *Biochem. Soc. Trans.*, 1990, **18**, 254–255.
16 G. Jagan Mohanarao and S. K. Atreja, *Anim. Reprod. Sci.*, 2011, **123**, 40–47.
17 V. M. Gitlits, B. H. Toh, K. L. Loveland and J. W. Sentry, *Eur. J. Cell Biol.*, 2000, **79**, 104–111.
18 A. Keller, J. Peltzer, G. Carpentier, I. Horvath, J. Olah, A. Duchesnay, F. Orosz and J. Ovádi, *Biochim. Biophys. Acta, Gen. Subj.*, 2007, **1770**, 919–926.
19 B. G. Vértessy, F. Orosz, J. Kovács and J. Ovádi, *J. Biol. Chem.*, 1997, **272**, 25542–25546.
20 C. Masters, *Int. J. Biochem.*, 1992, **24**, 405–410.
21 B. F. Mitchell, L. B. Pedersen, M. Feely, J. L. Rosenbaum and D. R. Mitchell, *Mol. Biol. Cell*, 2005, **16**, 4509–4518.
22 S. Novak, T. Smith, F. Paradis, L. Burwash, M. Dyck, G. Foxcroft and W. Dixon, *Theriogenology*, 2010, **74**, 956–967.
23 A. Force, J. L. Viallard, G. Grizard and D. Boucher, *J. Androl.*, 2002, **23**, 202.
24 Y. J. Park, W. S. Kwon, S. A. Oh and M. G. Pang, *J. Proteome Res.*, 2012, **11**, 4162–4168.
25 A. A. Moura, D. A. Chapman, H. Koc and G. J. Killian, *J. Androl.*, 2006, **27**, 534–541.
26 C. Weiss, Y. Zeng, J. Huang, M. B. Sobocka and J. I. Rushbrook, *Biochemistry*, 2000, **39**, 1807–1816.
27 S. Y. Kim and J. W. Park, *Biochimie*, 2010, **92**, 1908–1913.
28 G. L. Semenza, *Annu. Rev. Cell Dev. Biol.*, 1999, **15**, 551–578.
29 G. Melillo, *Cell Cycle*, 2004, **3**, 154–155.
30 C. Paul, S. Teng and P. T. Saunders, *Biol. Reprod.*, 2009, **80**, 913–919.
31 R. J. Aitken and M. A. Baker, *Reprod., Fertil. Dev.*, 2004, **16**, 581–588.
32 M. A. Baker and R. J. Aitken, *Mol. Cell. Endocrinol.*, 2004, **216**, 47–54.
33 W. Bone, N. Jones, G. Kamp, C. Yeung and T. Cooper, *J. Reprod. Fertil.*, 2000, **118**, 127–135.
34 R. M. McClain and J. C. Downing, *Toxicol. Appl. Pharmacol.*, 1988, **92**, 480–487.
35 R. M. McClain and J. C. Downing, *Toxicol. Appl. Pharmacol.*, 1988, **92**, 488–496.
36 D. Schwartz, J. C. Jordan, W. Vetter and G. Oesterhelt, *Xenobiotica*, 1979, **9**, 571–581.
37 A. Jones and T. Cooper, *Xenobiotica*, 1997, **27**, 711–721.
38 H. P. Jones, R. W. Lenz, B. A. Palevitz and M. J. Cormier, *Proc. Natl. Acad. Sci. U. S. A.*, 1980, **77**, 2772–2776.
39 J. Feinberg, J. Weinman, S. Weinman, M. P. Walsh, M. C. Harricane, J. Gabrion and J. G. Demaille, *Biochim. Biophys. Acta, Gen. Subj.*, 1981, **673**, 303–311.
40 P. B. Moore and J. R. Dedman, *J. Cell. Biochem.*, 2004, **25**, 99–107.
41 M. L. Kann, J. Feinberg, D. Rainteau, J. P. Dadoune, S. Weinman and J. P. Fouquet, *Anat. Rec.*, 1991, **230**, 481–488.
42 M. Moriya, C. Katagiri and K. Yagi, *Cell Tissue Res.*, 1993, **271**, 441–451.
43 Y. Si and P. Olds-Clarke, *Biol. Reprod.*, 2000, **62**, 1231–1239.
44 S. A. Khan, A. R. Suryawanshi, S. A. Ranpura, S. V. Jadhav and V. V. Khole, *Reproduction*, 2009, **138**, 81–93.
45 C. O'Flaherty and A. R. de Souza, *Biol. Reprod.*, 2011, **84**, 238–247.
46 A. Agarwal, S. Gupta and S. Sikka, *Curr. Opin. Obstet. Gynecol.*, 2006, **18**, 325–332.
47 R. J. Aitken and M. A. Baker, *Mol. Cell. Endocrinol.*, 2006, **250**, 66–69.
48 M. Cordoba, L. Pintos and M. T. Beconi, *Andrologia*, 2005, **37**, 40–46.
49 Y. Clermont and C. Leblond, *Am. J. Anat.*, 2005, **96**, 229–253.
50 M. J. Wolkowicz, J. Shetty, A. Westbrook, K. Klotz, F. Jayes, A. Mandal, C. J. Flickinger and J. C. Herr, *Biol. Reprod.*, 2003, **69**, 735–745.
51 Y. Fujihara, M. Murakami, N. Inoue, Y. Satouh, K. Kaseda, M. Ikawa and M. Okabe, *J. Cell Sci.*, 2010, **123**, 1531–1536.
52 M. J. Wolkowicz, L. Digilio, K. Klotz, J. Shetty, C. J. Flickinger and J. C. Herr, *J. Androl.*, 2008, **29**, 272–282.
53 R. S. Sankhala and M. J. Swamy, *Biochemistry*, 2010, **49**, 3908–3918.
54 P. Manjunath, A. Bergeron, J. Lefebvre and J. Fan, *Soc. Reprod. Fertil.*, 2007, **65**, 217.