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L-arginine alters the proteome of frozen-thawed bovine sperm during *in vitro* capacitation

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ABSTRACT

The aim of this work was to evaluate the proteomic changes that occurred in the frozen-thawed bovine spermatozoa after the addition of L-arginine (L-arg) during *in vitro* sperm capacitation. Aspects related to the sperm capacitation pattern like membrane integrity, mitochondrial activity, sperm motility and vigor, and the sperm proteome were determined. These were respectively assessed by chlortetracycline staining, H342/PI, JC-1, light microscopy, and the proteomic abundance by nUPLC-MS/MS analysis. Frozen-thawed sperm from three Nellore bulls were capacitated *in vitro* for 3 h in sp-TALP medium supplemented with 20 μg/mL heparin (Control) or with 20 μg/mL heparin plus 1 mM L-arg (L-arg group). Data were subjected to analysis of variance and means compared by SNK test at 5% probability. When compared to Control, the percentage of sperm motility was higher in the L-arg group ($P < 0.05$). For test data after 3 h of incubation, sperm capacitated with L-arg showed higher membrane integrity and mitochondrial potential when compared to Control ($P < 0.05$). Moreover, we observed an increase in the percentage of capacitated sperm pattern ($P < 0.05$). Protein abundance analysis identified 367 proteins. Forty proteins were differentially abundant between Control and L-arg group ($P < 0.05$), of which 11 were up-regulated, and 29 were down-regulated in L-arg group. In addition, we observed that one protein was uniquely abundant in the L-arg group. Our findings indicate that the addition of L-arg to the culture medium presented a differential protein abundance pattern and increased the bovine frozen-thawed sperm quality and the percentage of capacitated sperm. The proteomic changes observed may be linked to the molecular mechanisms involved in the action of L-arg on the *in vitro* sperm capacitation of cattle.

1. Introduction

The sperm capacitation is a process prior to fertilization and is of fundamental importance to generate a viable embryo of superior quality [1]. Nevertheless, this process still needs to be elucidated, to account for the spermatozoa molecular landscape in the female reproductive tract that progresses to a successful embryo production.

Capacitation relies on biochemical, biophysical, and molecular changes in the sperm cell to successfully fertilize the egg [2], both occurring *in vivo*, during its transit into the female tract, and *in vitro*, in the presence of a defined media. In mammals, these physiological changes in the sperm encompass an increase in the plasma membrane fluidity and hyperpolarization [3], together with the reorganization of the surface molecules. During capacitation, sperm also presents an increase in the intracellular concentration of Ca^{2+} [4] and HCO_3^- ions [5], intracellular pH increase [6], and protein

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phosphorylation, especially on tyrosine residues [7]. Furthermore, several regulatory second messengers and pathways that constitute the molecular basis for these changes have been described [1,8].

Reactive nitrogen species (RNS) participate in the sperm motility initiation by enhancing cAMP synthesis and protein phosphorylation [6]. RNS also act on the extracellular-regulated kinase (ERK) pathway which in turn contribute to capacitation, acrosome reaction and hyperactivation of sperm cells [1]. Nitric oxide (NO) is one of the most studied RNS in the sperm cell [9,10], acting as an intracellular messenger in cAMP and ERK pathways [11]. Previously, we have demonstrated in bovines [10,12,15] that NO increases the percentage of capacitated sperm in the presence of heparin, also increasing motility, membrane integrity, and mitochondrial activity during cultivation in a capacitating medium [12,13]. Moreover, we have already shown the importance of NO during capacitation for embryo production [14,15].

Biologically, L-arginine (L-arg) is the main precursor of the NO synthesis, in which its bioavailability is the factor necessary to maintain NO concentration both *in vivo* and *in vitro* [10,16]. The nitric oxide synthase (NOS) convert L-arg into L-citrulline and NO, in an oxidative reaction controlled by the presence of the cofactors NADPH, O_2, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) [16]. Although sperm cells are able to produce NO, we previously demonstrated in cattle that the exogenous use of L-arg during *in vitro* capacitation enhances NO synthesis at levels suitable for sperm motility, vigor, membrane integrity, mitochondrial activity, and penetration in homologous oocytes [10]. More recently, we have shown that 1 mM L-arg was the effective dose to improve these characteristics in cryopreserved/thawed sperm from bovines [14].

While much progress has been made in understanding the signaling pathways and their cross-talks during sperm capacitation, the molecular basis comprising these processes still requires elucidation. For decades, the sperm has been considered as an inert cell, *i.e.,* transcriptionally and translationally inactive cell [17]. However, sperm is a specialized cell with defined functions that are dependent on the activation of proteins [18]. Many processes prior to fertilization, such as sperm capacitation, are protein-dependent. In this way, post-translational modifications of proteins play a pivotal role in the capacitation mechanism [17]. Meanwhile, it has been shown that, during capacitation, the sperm could replace the degraded proteins or synthesize new proteins which are essential for both the capacitation and fertilization process [19].

To determine the dynamics of the protein profile in sperm capacitation, we used cryopreserved bovine spermatozoa as a model to characterize the changes induced by L-arg. Thus, our goal was to evaluate the proteomic changes that occurred in the frozen-thawed bovine spermatozoa after the addition of L-arg in the sperm capacitation medium *in vitro*. We also aimed to verify the effect of L-arg in the sperm quality and the percentage of the capacitated sperm in the presence of heparin after 3 h cultivation.

2. Material and methods

2.1. Sperm handling, selection, and capacitation

The experiments were conducted with commercial cryopreserved sperm from three Nellore bulls (*Bos taurus indicus*). For this reason, no ethical approval was required.

Briefly, sperm was thawed at 37 °C for 30 s and centrifuged at 600 × *g* for 15 min in a Percoll gradient 45/90% [20] to separate the sperm with higher viability. The pellet was washed in 400 μL with wash-TALP medium according to Chamberland et al. [21] and then centrifuged at 150 × *g* for 5 min.

We evaluated the sperm motility, vigor, and concentration through an optical microscope (NIKON - Eclipse E200, Melville, NY, USA). Mainly by the action of heparin in the capacitation medium, the lateral region of the sperm head agglutinates after hours. This process impairs the use of computerized methods to determine the progressive motility of sperm, since currently available software exclusively recognizes a predefined sperm size. Thus, despite the sperm agglutination, it is still possible to evaluate the sperm parameters by a subjective method [10].

For this purpose, a drop of sperm was placed between a slide and a cover slip. The motility was determined by the percentage of moving sperm with rectilinear movement, classified from 0 to 100% and, the vigor, was classified according to the strength of the flagellar beating, based on the quality of the straight-forward movement and speed, graded on a scale from 0 to 5 [22]. The motility and vigor were also investigated after 3 h of culture. The sperm concentration was determined by cell counting in a Neubauer chamber after dilution (1:20) of a sample of sperm in distilled water. The sperm concentration was adjusted to 15×10^6 sperm/mL for the sperm quality assessments and 10^7 sperm/mL for the protein investigation.

The selected sperm were transferred to 200 μL of capacitation medium. The *in vitro* sperm capacitation was induced for 3 h in an incubator at 38,5 °C in a humidified atmosphere of 95% air and 5% CO_2. The standard medium used in the capacitation was modified Tyrodes (sp-TALP) supplemented with 6 mg/mL BSA, fatty acids free, 100 IU/mL penicillin and 100 μg/mL streptomycin, and 20 μg/mL heparin [23], without (Control) or with 1 mM L-arg (L-arg group). The employed L-arg concentration was previously described by Leal et al. [14]. On the day of use, a solution of 1 M L-arg was prepared in sp-TALP. Afterward, a small aliquot was used for dilution in the culture medium of the treatment, reaching a final concentration of 1 mM.

All reagents used in these experiments were obtained from Sigma-Aldrich Brasil Ltda (São Paulo, Brazil) unless otherwise indicated.

2.2. Sperm quality assessment

2.2.1. Membrane integrity

Plasma membrane integrity of the sperm was assessed to verify sperm viability, which assumes that when the sperm is not functionally intact it has no fertilizing capacity [24].

To characterize the cell integrity [25], an aliquot of sperm was exposed to Hoechst 33342 (H342, 40 μg/mL) and propidium iodide (PI, 0.5 μg/mL) for 30 min. The sperm were investigated under an epifluorescence microscope (NIKON - Eclipse 80i, Melville, NY, USA) at 400 × magnification.

Four replicates (n = 12) were analyzed. For each analysis, at least 200 cells were counted and classified into two groups: (I) sperm with intact plasma membrane (stained with H342), and (II) sperm with damaged plasma membrane (stained with PI). PI binds to the DNA of cells with damage in the plasma membrane [26] and stains the nucleus red, while H342 can cross the membrane of intact cells, binding to the DNA of these cells and staining the nucleus blue [27].

2.2.2. Mitochondrial potential

Sperm motility correlates with mitochondrial activity. We use the 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1) to assess the mitochondrial functionality through the detection of inner mitochondrial membrane depolarization [24].

An aliquot of sperm was exposed to JC-1 (153 μg/mL) for 5 min to determine the mitochondrial potential [25]. This analysis was conducted in an epifluorescence microscope (NIKON - Eclipse 80i, Melville, NY, USA) at 400 × magnification.

Four replicates (n = 12) were analyzed. For each analysis, at least 200 cells were counted and classified into (I) sperm with low mitochondrial potential, and (II) sperm with high mitochondrial potential [25].

2.2.3. Sperm capacitation

In order to quantify the percentage of capacitated sperm, we used the fluorescent labeling technique of chlortetracycline hydrochloride (CTC, 0.75 mM) [28]. The sperm were evaluated in an epifluorescence microscope (NIKON - Eclipse 80i, Melville, NY, USA; 400 × magnification, excitation at 440 nm and emission at 470 nm).

Four replicates (n = 12) were evaluated. For each analysis, at least 200 cells were counted and classified into 3 groups, as described by Fraser [29]: F (fluorescent; intact and not capacitated sperm with positive fluorescence in the entire surface), C (capacitated; fluorescence loss in post-acrosomal region), and RA (reacted acrosome; fluorescence loss in post-acrosome and acrosome region and positive fluorescence exclusively in the middle and equatorial areas of the head).

2.3. Sperm proteome

2.3.1. Protein extraction

For total protein extraction, a previously described protocol was used with minor modifications [30]. Sperm cells from three biological replicates were washed with PBS and lysed by sonication on ice in an extraction buffer in five cycles of 10 s with 1-minute intervals. The extraction buffer consisted of 7 M urea, 2 M thiourea, 2% triton X-100, 1% dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride (PMSF), and 5 μM pepstatin. Protein concentration was measured using a 2-D Quant Kit (80-6483-56, GE Healthcare, Piscataway, NJ, USA).

2.3.2. Protein digestion

For protein digestion, 100 μg of protein was used. Before trypsin digestion, the protein samples were precipitated using methanol/chloroform to remove any remaining detergent [31]. Further, samples were resuspended in urea 7 M and thiourea 2 M buffer and desalted on Amicon Ultra-0.5 3 kDa centrifugal filters (UFC5003, Merck Millipore, Germany).

The methods used for protein digestion were previously described [32]. Briefly, samples were reduced by adding 2.5 μL of 100 mM DTT (17-1318-02, GE Healthcare, Piscataway, NJ, USA), alkylated with 2.5 μL of 300 mM iodoacetamide (RPN6302, GE Healthcare, Piscataway, NJ, USA), and digested with 50 ng/μL trypsin (V5111, Promega, Madison, WI, USA) at 37 °C overnight. Samples were then transferred to Total Recovery Vials (186000384C, Waters, Milford, CT, USA).

2.3.3. Mass spectrometry analysis

A nanoAcquity UPLC (nUPLC) connected to a Synapt G2-Si HDMS mass spectrometer (Waters, Manchester, UK) was used for ESI-LC-MS/MS analysis. The chromatography analysis was conducted by using 1 μL of digested samples (500 ng/μL). Mass spectrometry was performed in positive and resolution mode (V mode), with ion mobility, and in data-independent acquisition (DIA) mode. The transfer collision energy increased from 19 V to 55 V in high-energy mode and cone and capillary voltages of 30 V and 2750 V, respectively. This experiment was conducted with a source temperature of 70 °C. The human [Glu1]-fibrinopeptide B at 100 fmol/μL was used as an external calibrant and lock mass acquisition was performed every 30 s.

2.3.4. Bioinformatics

Spectra processing and database searching conditions were performed by Progenesis QI for Proteomics software v.2.0 (Nonlinear Dynamics, Newcastle, UK). The analysis used the following parameters: one missed cleavage, minimum peptide per protein equal to two, fixed modifications of carbamidomethyl (C) and variable modifications of oxidation (M) and phosphoryl (STY), default false discovery rate (FDR) value at a 4% maximum, peptide score higher than five, and maximum mass errors of 10 ppm. The analysis used the *Bos taurus* protein databank from UniProtKB (http://www.uniprot.org, access in July 2017). Label-free relative quantitative analyses were performed based on protein ion counts. The proteins that presented differential abundances were considered to be up-regulated if the fold-change (FC) was greater than 1.5 and down-regulated if the FC was less than −0.667, both tested with ANOVA and were considered significant when $P < 0.05$. Functional annotation was performed using Blast2Go software v.3.4 [33].

To verify the presence of mRNA transcripts, we blasted the corresponding sequences to Sequence Read Archive Nucleotide BLAST bovine sperm RNA-seq data from NCBI. The mRNA sequences from genes coding the differentially abundant proteins were determined by accessing UniProtKB databank (http://www.uniprot.org, accessed in October 2017). The mRNA sequence was recovered from UCSC Genome Browser (http://genome.ucsc.edu, accessed in October 2017). The SRA archives used for blast analysis were derived from bovine cryopreserved sperm transcriptome data (SRX158098), and sequencing of the high (SRX831208) and low fertility bull semen (SRX831206).

2.4. Statistical analysis

Data were analyzed using SAS software [34] through ANOVA to determine the effects of treatments in the studied characteristics in all evaluations. The results of the sperm quality assessment were submitted to the analysis of variance (GLM). Afterward, data (means ± SD) were compared by SNK test at 5% probability, to check de significance [35]. To exclude the individual effect, the means of each bull were tested and compared by the SNK test (data not shown), so that the total replicate number was the sum of all replicates.

3. Results

3.1. Sperm quality assessment

The percentage of sperm motility was higher in the L-arg group (67.5 ± 8.7%) when compared to Control (55.0 ± 8.0%, $P < 0.05$). Conversely, no difference was found in the vigor (Control: 2.5 ± 0.5 *vs.* L-arg: 2.7 ± 0.5, $P > 0.05$) (Table 1) after 3 h of cultivation.

We observed a significant increase in the percentage of capacitated sperm in the L-arg group when compared to the Control (70.3 ± 1.9% *vs.* 57.8 ± 2.2%, $P < 0.05$). In addition, the percentage of non-capacitated sperm (6,7 ± 1.7%) and the reacted acrosome (23.6 ± 1.4%) was significantly lower ($P < 0.05$) when compared to the Control (13.6 ± 0.6% and 27.9 ± 1.5%, respectively) (Table 1).

After 3 h of incubation, the sperm from L-arg group demonstrated approximately 15% higher intact plasma membrane when compared to the Control (57.2 ± 4.7% *vs.* 42.9 ± 4.7%, $P < 0.05$) (Table 1). Additionally, the percentage of sperm showing high mitochondrial potential was approximately 20% higher in L-arg group when compared to the Control (77.2 ± 5.0% *vs.* 57.7 ± 3.1%, $P < 0.05$) (Table 1).

3.2. Sperm proteomics investigation

Through the proteomic analysis, 367 proteins were identified in

Table 1
Sperm functional characteristics of frozen-thawed bovine sperm after *in vitro* capacitation for 3 h in Control (with heparin) and L-arg group (with 1 mM L-arginine + heparin).

Treatments	Sperm characteristics						
	Motility	Vigor	Non-capacitated	Capacitated	Acrosome-reacted	Intact PM	High MP
Control	55.0 ± 8.0[b]	2.5 ± 0.5[a]	13.6 ± 0.6[a]	57.8 ± 2.2[b]	27.9 ± 1.5[a]	57.2 ± 4.7[b]	57.7 ± 3.1[b]
L-arg	67.5 ± 8.7[a]	2.7 ± 0.5[a]	6.7 ± 1.7[b]	70.3 ± 1.9[a]	23.6 ± 1.4[b]	68.4 ± 6.5[a]	77.2 ± 5.0[a]

*Data are shown as means ± SD. Means followed by different letters in the column are different ($P < 0.05$) according to SNK test ($n = 12$, four replicates of three different bulls). Motility and vigor were assessed by light microscopy, capacitation status by chlortetracycline staining, plasma membrane (PM) integrity by H342/PI, and mitochondrial potential (MP) by JC-1.

the bovine sperm after heparin-induced *in vitro* sperm capacitation. From those, 40 were differentially abundant between Control and L-arg group. Additionally, one protein was uniquely abundant in the L-arg group. By comparing the protein profiles from the L-arg and Control groups, we identified 11 up-regulated and 29 down-regulated proteins. The protein abundance ranged from 1.5 to 73.7-fold in up-regulated proteins, and from 1.5 to 11-fold in down-regulated proteins.

The subcellular localization and the molecular function of the differentially abundant proteins are shown in Fig. 1a and b, and their description and linked biological process are listed in Table 2. Using the transcriptome databank, we found sperm mRNA sequences from 5 up-regulated and 24 down-regulated proteins (Table 2).

4. Discussion

To the best of our knowledge, this is the first study showing changes in the protein profile of frozen-thawed sperm after *in vitro* capacitation of cattle, using supplementation with L-arg. Our analyses revealed 40 differentially abundant proteins and one unique in L-arg group compared to Control. Many of these being associated with sperm capacitation and fertilization, such as A-kinase anchor protein (AKAP), a-disintegrin and a-metalloprotease (ADAM) family proteins, and Vitamin D binding protein. On the other hand, some proteins have not been previously identified or do not yet have a defined biological process (*e.g.,* probable RNA-binding EIF1AD and TPA: hypothetical protein LOC789612), requiring studies to evaluate their effect on the capacitation of bovine sperm. In this section, we will exclusively discuss the proteins that could be of importance for the capacitation process.

Few studies have depicted the synthesis of proteins in mature spermatozoa. Gur and Breitbart [19] observed for the first time that 55S mitochondrial ribosomes lead to the translation of nuclear-encoded proteins during human sperm capacitation. In humans, about 4% of the spots in the two-dimensional gel electrophoresis quantitatively changed after 3 h of incubation in capacitation media [36]. In this way, we could assume that protein synthesis is active in sperm during its transit in the female reproductive tract, being essential for the sperm motility, acrosome reaction, actin polymerization, *in vitro* fertilization (as in cattle [19]), and sperm-egg interaction (as in mice [37]).

Here we demonstrate that sperm treated with L-arg showed a distinct influence on the abundance of AKAP 3, a structural protein of the flagellum [38]. AKAPs play many roles in spermatozoa [39], being present in the fibrous sheath of the sperm tail and binding to the regulatory subunit of PKA [40]. During capacitation, AKAP3 is phosphorylated [41] and *S*-nitrosylated [40]. Furthermore, this protein may control the activities of other signaling molecules, such as calmodulin [42], calcineurin [43], and protein kinase C (PKC) [44,45], which take part of the capacitation process. AKAP3 tyrosine phosphorylation enhances sperm motility during capacitation of human sperm [46]. The presence of this protein in the group treated with L-arg solely strongly suggests that AKAP3 synthesis may have occurred during the capacitation of bovine spermatozoa *in vitro*, as reported before in human, mouse, bovine and rat [19,37]. In addition, the post-translational protein modifications being fully active in sperm during capacitation could have influenced its abundance. However, those hypotheses need to be addressed. Moreover, studies are required to assess whether AKAP is involved in the mechanisms by which L-arg increases sperm motility in bovines during the capacitation.

Through protein analysis, we found 5 up-regulated proteins in L-arg group that are important for sperm fertility and that could be categorized as (I) those related to energy production (NADP and nucleoside diphosphate kinase) [47], (II) binding/penetration of sperm in the oocyte (ADAMs family) [48], and (III) embryonic development (GLI3) [49].

NADP-dependent isocitrate dehydrogenase protein is found in the mitochondria and plays a role in the oxidative metabolic pathway [50]. In addition, in cryopreserved bovine sperm, NADP participates in the redox state, which is essential for capacitation and acrosome reaction [50]. Nucleoside diphosphate kinases also play a role in the ATP production by the spermatozoa, once it is part of the substrate cycling during glycolysis [51].

The ADAMs protein family are a class of membrane proteins [48] that have multiple domains. Its active metalloprotease site has protease activity, whereas its disintegrin domain acts on cell-cell and cell-matrix interactions [52]. In the reproductive landscape, ADAM protein family is critical for sperm to fertilize the oocyte, once its participation comprises sperm migration in the female reproductive tract [53], sperm-ZP binding [54], and sperm-sperm aggregation [55].

Transcriptional activator GLI3 isoform X1 was up-regulated in this study (44-fold). Gli is a transcription factor of vertebrates with three genes identified in humans and mice (Gli1, Gli2, and Gli3) that present a specific role in embryonic development [49].

Finally, we identified pleckstrin homology domain-containing family F member 1 (PLEKHF1) and an uncharacterized type I cytoskeletal 24 protein for the first time in bovine spermatozoa. Although their function is unknown, the abundance of both proteins in L-arg group were 73 times and 17 times higher when compared to Control samples, respectively. Future studies should investigate the role of both proteins in the L-arg function during heparin-induced *in vitro* sperm capacitation and the sperm quality.

From the 29 down-regulated proteins, more than half are associated with sperm motility and hyperactivation (*e.g.,* transforming protein RhoA, pyruvate dehydrogenase E1, ATP synthase subunit delta, dynein intermediate chain axonemal, acetyl-CoA synthetase, and glycerol-3-phosphate dehydrogenase). Other down-regulated proteins are associated with protection against pathogenic infections (*e.g.,* lysozyme 6), DNA packaging and regulation of cell shape (*e.g.,* histone H2A and fascin-3), intracellular concentration of polyamines, and coupling between the sperm head and tail control (*e.g.,* ornithine decarboxylase antizyme 3). Based on the literature, we did not detect any association between

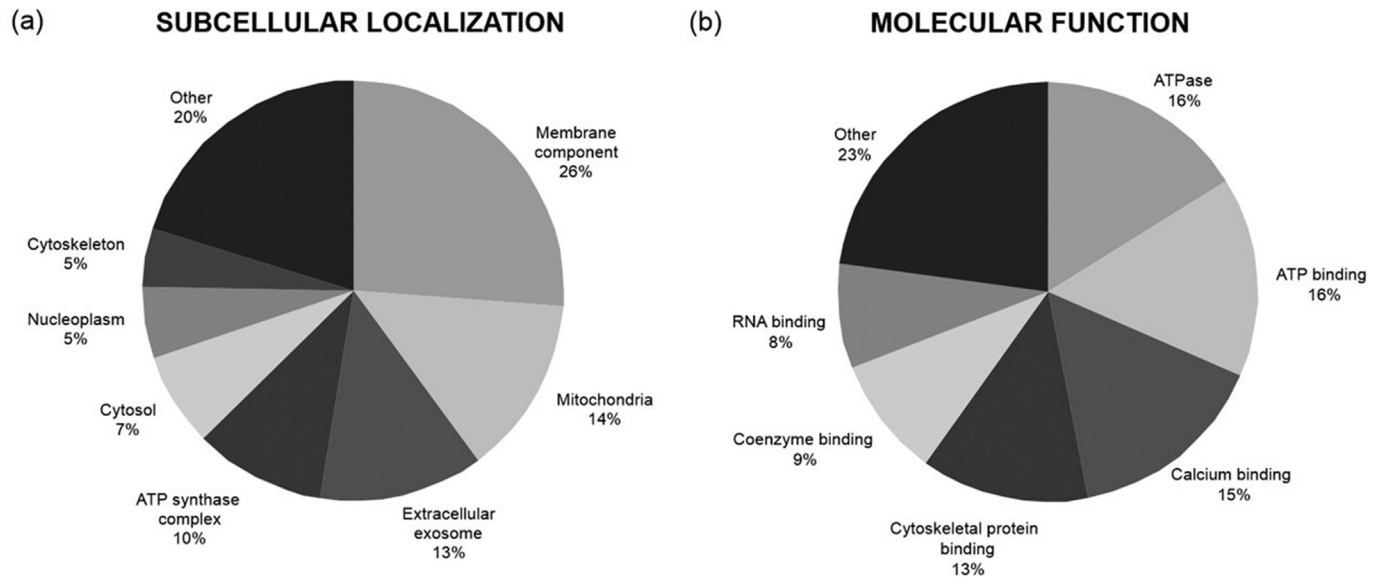

Fig. 1. Subcellular localization (a) and molecular function (b) of the differentially abundant (40) and unique abundant (one) protein in bovine spermatozoa after *in vitro* sperm capacitation with heparin and L-arginine.

these proteins and sperm capacitation.

Protein phosphorylation is a post-translational modification that is related to motility [9]. Furthermore, apart from phosphorylation, phosphopeptide abundance could also influence the expression of proteins [56] and thus decreasing its abundance. Based on the literature, Zhao et al. [37] suggested that the down-regulated proteins may be present on its phosphorylated form. Thus, although protein phosphorylation has not been evaluated, down-regulation of some motility-related proteins may have been caused by such modification or other biochemical changes.

The activation of PKA pathway negatively regulates RhoA signaling through phosphorylation and by the translocation from the membrane to the cytosol [57]. In this study, transforming RhoA abundance was down-regulated in the L-arg group. It can be suggested that AKAPs members, which binds to the regulatory subunit of PKA, could be associated with RhoA pathway members, leading directly to its phosphorylation [58]. However, more studies are needed to prove this hypothesis.

Pyruvate dehydrogenase E1 mitochondrial (PDHB) is a phosphotyrosine protein localized in the sperm flagella, which is correlated with the tricarboxylic acid cycle, adipocytokine, and insulin action [17]. In boar spermatozoa, a decrease in its abundance after capacitation is observed, from its involvement in energy production and metabolism for controlling sperm motility and hyperactivation [17]. Acetyl-CoA synthetase activates acetate to acetyl-CoA in the energy generation processes [59]. In gilthead sea bream acetyl-CoA phosphorylation initiates the sperm motility, decreasing acetyl-CoA abundance [59,60].

Tyrosine phosphorylation of glycerol-3-phosphate dehydrogenase 2 (GPD2) occurs during capacitation in hamster [61] and mouse [62] spermatozoa. For male fertility, GPD2 is related to the normal progression of hyperactivation due to its ability to synthesize ATP and generate ROS [62]. In boar, consumption of glycerol-3-phosphate by oxidation to dihydroxyacetone increases up to 60 min of incubation and drastically reduces its concentration [63].

The changes in protein abundance observed in this study are most likely consequence of a combination of protein synthesis and degradation [64], post-translational modifications such as phosphorylation [1] and *S*-nitrosylation [40], alterations in secondary or tertiary structure, and extracellular translocation. mRNA transcripts from genes that encode some of those proteins were already described in cryopreserved bull spermatozoa [65] being concordant with the hypothesis that at least some of these proteins could be synthesized during sperm capacitation. Moreover, according to the UniProt databank, we found three proteins that are related to transcription or translation, such as transcriptional activator GLI3 isoform X1, nucleoside diphosphate kinase B, and probable RNA-binding EIF1AD.

Lastly, we used sperm quality assessments to confirm the effects of treatment with L-arg during *in vitro* sperm capacitation of cattle. These effects were positive and in accordance with other studies of bovine [10,14,66,67], human [68], pig [69], and buffalo [70]. L-arg, a natural precursor of NO *in vivo* [16] and *in vitro* [10], was shown to maintain the sperm quality parameters after 3 h incubation in capacitating medium and the production of NO in our *in vitro* assays [14]. *In vivo*, the female reproductive tract [40] and the cumulus-oocyte complex (COC) [71,72] are the primary sources of NO for sperm capacitation, although sperm are also able to produce NO [10,14]. Unpublished results from our group demonstrate that the use of 1 mM L-arg led to a 31.8% increase in NO production (from 79.5 μM in Control group to 104.8 μM in L-arg). We suggest that the exogenous addition of L-arg could be leading to an increase in NO synthesis [10]. During the cultivation, this process could have improved the sperm quality. However, it does not rule out the hypothesis that L-arg may be acting in a different way, such as the polyamines pathway [73] or acting by itself on some other unknown reactions. These possible hypotheses should be further investigated.

In conclusion, our findings demonstrate that the action of L-arg on frozen-thawed sperm capacitation is directly related to changes in the abundance of specific proteins, up- or down-regulated, which are of importance for sperm capacitation, fertilization, and embryonic development. We suggest that the action of the differentially abundant proteins found in our study should be characterized in the clinical setting for fertility/infertility evaluation. Finally, our results allow us to presume that *in vivo*, the female reproductive tract metabolome may modulate the protein profile of the sperm during capacitation and further altering the fertility/pregnancy/birth rate in cattle.

Table 2
Proteins found to be differentially abundant in cryopreserved bovine sperm *in vitro* capacitated with L-arginine when compared to Control.

Accession	Peptide count	Description	Biological process	Fold Change T/C	TAG T/C	Reads of mRNA transcripts
F1MJS8	61	A-kinase anchor protein 3 isoform X1	regulation of protein kinase A signaling	–	Unique Control	+
A2VE19	2	pleckstrin homology domain-containing family F member 1	Positive regulation of intrinsic apoptotic signaling pathway; protein localization to plasma membrane	73,71	UP	–
F1N215	2	PREDICTED: transcriptional activator GLI3 isoform X1	regulation of transcription, DNA-templated; smoothened signaling pathway; embryonic organ development	44,57	UP	–
G5E6C5	4	disintegrin and metalloase domain-containing 20-like	integrin-mediated signaling pathway; proteolysis	31,67	UP	–
F1MFW9	2	type I cytoskeletal 24	–	17,28	UP	+
G3N3C2	4	disintegrin and metalloase domain-containing 21-like	proteolysis	7,15	UP	–
Q04467	2	isocitrate dehydrogenase [NADP] mitochondrial	glyoxylate cycle; isocitrate metabolic process; 2-oxoglutarate metabolic process; glutathione metabolic process; reductive tricarboxylic acid cycle	4,52	UP	+
F1MPL4	2	nucleoside diphosphate kinase B	CTP biosynthetic process; GTP biosynthetic process; nucleoside diphosphate phosphorylation; positive regulation of transcription from RNA polymerase II promoter	4,49	UP	+
Q58CY2	2	probable RNA-binding EIF1AD	regulation of translational initiation	4,27	UP	–
E1BKJ3	3	PREDICTED: calpain-8 isoform X1	proteolysis	3,12	UP	–
Q3MHN5	6	vitamin D-binding	vitamin transport; vitamin D metabolic process	1,66	UP	–
F1N6K8	4	TPA: hypothetical protein LOC789612	–	1,51	UP	+
Q3MHR0	3	acyl- thioesterase 1	phospholipid catabolic process; protein depalmitoylation; regulation of nitric-oxide synthase activity; fatty acid metabolic process; negative regulation of Golgi to plasma membrane protein transport	0,09	DOWN	+
A7E371	2	PROCA1 isoform X2	–	0,12	DOWN	+
Q32KX0	2	isochorismatase domain-containing mitochondrial	protein destabilization; metabolic process	0,13	DOWN	+
Q32P61	2	histone H2A	chromatin silencing; calcium ion-regulated exocytosis of neurotransmitter; vesicle fusion; regulation of calcium ion-dependent exocytosis	0,14	DOWN	+
Q3ZCA9	2	Hypothetical LOC509513	–	0,17	DOWN	+
F1MBE4	3	lysozyme 6	carbohydrate metabolic process.	0,18	DOWN	+
Q2YDE8	7	FAM71D isoform X2	–	0,19	DOWN	+
P61585	2	Transforming RhoA	polar body extrusion after meiotic divisions; negative chemotaxis; negative regulation of intracellular steroid hormone receptor signaling pathway; regulation of calcium ion transport; positive regulation of endopeptidase activity involved in apoptotic process; phosphatidylinositol-mediated signaling; embryonic cleavage; regulation of microtubule cytoskeleton organization; negative regulation of reactive oxygen species biosynthetic process; actin cytoskeleton reorganization; negative regulation of oxidative phosphorylation; stress-activated protein kinase signaling cascade; positive regulation of protein serine/threonine kinase activity	0,2	DOWN	+
Q2T9Y3	10	pyruvate dehydrogenase E1 component subunit testis-specific mitochondrial isoform X1	protein biosynthetic process; gluconeogenesis; glycolytic process; tricarboxylic acid cycle; mitochondrial acetyl-CoA biosynthetic process from pyruvate	0,2	DOWN	+
Q32LC5	3	CKLF-like MARVEL transmembrane domain-containing 2	–	0,28	DOWN	+
G3N0V3	9	fascin-3	spermatid development; actin filament organization	0,33	DOWN	–

Table 2 (*continued*)

Accession	Peptide count	Description	Biological process	Fold Change T/C	TAG T/C	Reads of mRNA transcripts
Q3SZ00	19	Trifunctional enzyme subunit mitochondrial	protein catabolic process; fatty acid beta-oxidation; tryptophan metabolic process; beta-alanine metabolic process; benzoate metabolic process.	0,39	DOWN	+
E1BHV9	4	ornithine decarboxylase antizyme 3	negative regulation of catalytic activity; spermatogenesis; ornithine catabolic process, by decarboxylation	0,45	DOWN	+
F1MWY9	15	enoyl-CoA delta isomerase 2, mitochondrial	cellular lipid metabolic process; fatty acid beta-oxidation; fatty acid catabolic process	0,45	DOWN	+
Q28851	2	ATP synthase subunit f mitochondrial	proton transport; ATP biosynthetic process	0,45	DOWN	+
Q2TA28	3	Signal-regulatory delta	–	0,46	DOWN	+
G3N357	3	FAM205A-like	–	0,48	DOWN	–
P0CH28	4	polyubiquitin-C	ubiquitin homeostasis	0,48	DOWN	+
P05630	3	ATP synthase subunit delta mitochondrial	oxidative phosphorylation; ATP synthesis coupled proton transport	0,53	DOWN	+
E1BPM9	3	dynein intermediate chain axonemal	outer dynein arm assembly	0,55	DOWN	–
P02722	5	ADP/ATP translocase 1	apoptotic mitochondrial changes; transmembrane transport; ribosome biogenesis; translation;	0,55	DOWN	+
Q862C2	3	similar to oligomycin-sensitivity conferral protein	ATP synthesis coupled proton transport	0,55	DOWN	–
Q148N0	11	2-oxoglutarate mitochondrial isoform X3	glycolytic process; tricarboxylic acid cycle; nervous system development; tryptophan metabolic process; lysine catabolic process.	0,56	DOWN	+
F1MQX0	12	acetyl-coenzyme A synthetase 2, mitochondrial	gluconeogenesis; glycolytic process; reductive tricarboxylic acid cycle; acetyl-CoA biosynthetic process from acetate	0,58	DOWN	+
Q2TA22	12	Acyl-CoA synthetase long-chain family member 6	long-chain fatty acid metabolic process	0,59	DOWN	+
A6QLU1	16	Glycerol-3-phosphate dehydrogenase, mitochondrial	glycerol-3-phosphate metabolic process; gluconeogenesis; glycerol catabolic process; cellular lipid metabolic process; oxidation-reduction process	0,6	DOWN	+
Q0VC58	4	trimeric intracellular cation channel type B	potassium ion transmembrane transport	0,61	DOWN	+
E1BKZ4	6	anthrax toxin receptor-like	signal transduction	0,66	DOWN	–
Q2NL19	6	MICOS complex subunit MIC60 isoform X2	mitochondrial calcium ion homeostasis	0,66	DOWN	+

T/C = Treatment/Control. + or - = presence or absence of mRNA reads retrieved from transcriptome databank (https://genome.ucsc.edu, accessed in October 2017).

Conflicts of interest

None of the authors have any conflicts of interest to declare.

Author contributions

V.L. Maciel Jr. conducted the experiment with the collaboration of V. Silveira, R.S. Reis, A.F.L. Rios, and C.S. Paes de Carvalho. Proteomic data were analyzed by V.L. Maciel Jr., M.C. Caldas-Bussiere and V. Silveira. M.C. Caldas-Bussiere and V. Silveira are the senior authors. M.C. Caldas-Bussiere is the project coordinator. All the authors participated in the experimental design and preparation of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.theriogenology.2018.06.018.

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