Proteomics to investigate bull's semen freezability

Proteômica para investigar a congelabilidade do semen bovino

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Abstract

The present study was undertaken the protein composition in 2D-electrophoretic pattern (2DE) of the seminal plasma (SP) can interfere in the semen bull freezability, and if we can use that for predicting semen bull freezability. Samples were obtained of 20 bulls (different breeds) with a minimum of 3 years history semen production in commercial semen collection Center. All animals ranged between 2 - 7 years of age. The semen freezability was calculated by # of thawed and approved ejaculates / # of ejaculates submitted to cryopreservation (after semen evaluation and approved to submitted to freeze). The bulls were divided in 3 groups: HIGH (=>80% ejaculates approved); MEDIUM (>60% and <79% ejaculates approved); LOW (=<59% ejaculates approved); the pattern and criteria were the same used in the routine of the commercial semen Center. 68 gels were carried through by 2DE of SP samples indicated 225 detected spots with protein different amount (VION) comparing. Comparing bull´s semen freezability and VION of each spot found difference among 2 spots from High and Low, even considering just spots with % of detection frequency bigger than 75%. The taurine bulls demonstrated more homogeneous profile when comparing with zebu bulls, considering number and frequency of appearance of spots. The results showed that proteomics can be a useful tool to predict the semen freezability, but we´ll need to study better the interactions between sperm membrane, seminal plasma and extender to comprehend better which proteome phenotype interfere positive or negatively in the semen freezability.

Keywords: Cattle. Proteins. Seminal Plasma. Cryopreservation.

Resumo

O objetivo do presente estudo foi avaliar se a composição proteica de plasma seminal, verificada por eletroforese bidimensional (2DE), pode interferir na congelabilidade do sêmen de touros, e se existe a possibilidade de predizer tal característica em touros doadores de sêmen. Amostras de 20 touros (diferentes raças) com mínimo de três anos de histórico de produção em Central de Coleta de sêmen foram coletadas. Todos os touros tinham de 2 a 7 anos de idade. A congelabilidade do sêmen foi calculada por nº de ejaculados aprovados pós-descongelação / nº de ejaculados submetidos à congelação (depois do crivo do padrão de qualidade da empresa para aprovar o ejaculado para ser submetido a congelação). Os touros foram divididos em três grupos: ALTA (=>80% aproveitamento de ejaculados encaminhados à congelação); MÉDIA (>60 a 79% ejaculados aprovados) e BAIXA (=<59% ejaculados aprovados). Sessenta e oito corridas de géis 2DE foram avaliadas nas amostras de plasma seminal, que detectaram 225 *spots* com quantidade de proteína diferente entre as mesmas (VION). Pela estatística utilizada, comparando-se a congelabilidade do sêmen com quantidade de proteína detectada de cada *spot* (VION), contatou-se que 2 *spots* apresentaram-se com quantidade significativa de proteína diferente entre os dois grupos de congelabilidade do sêmen, considerando *spots* que foram detectados em mais de 75% das amostras corridas. Os touros taurinos apresentaram perfil proteico mais homogêneo quando comparado com os zebuínos, considerando número e frequência de *spots*. Tais resultados demonstram um potencial para uso da proteômica como ferramenta preditiva da congelabilidade do sêmen de touros, porém com necessidade de maiores estudos, principalmente para melhor compreensão de interações proteicas entre membrana espermática, plasma seminal e diluidores de sêmen utilizados, já que podem interferir no fenótipo proteico avaliado e na congelabilidade do sêmen, seja de forma positiva ou negativa.

Palavras-chave: Bovino. Proteínas. Plasma Seminal.Criopreservação.

Introduction

The semen cryopreservation can be considered a crucial stage for the success of the artificial insemination (AI) as a tool to accelerate the genetic progress and consequently increment the livestock

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productivity. The capacity of semen to freeze and thaw well, keeping viable sperms is an individual characteristic, and with a difficulty estimation using regular parameters to evaluate the semen. The prediction of this potential, of animals used as semen donors, has a considerable impact on reproductive effectiveness and consequently on the cattle productive success. However we have a long journey to get that. The difficult to predict the semen freezability is based on qualify the several factors that can interfere in the physiological events involved in cryopreservation events. The cryopreservation process promotes several structural and functional alterations of the semen or sperm cells, that could commit the viability of the cells post-thawing, including protein profile modifications[1,2,3,4,5,6,7]. In that concern, our hypothesis was, if we can determine which proteomic pattern of seminal plasma proteins of High Semen Freezability Bulls and Low Semen Freezability, we can use the 2DE technique as a prediction tool to choose the semen donors bulls.

Material and Methods

Animals

A total of 20 bulls (twelve Nelore - Bos taurus indicus and 08 Angus – Bos taurus taurus), all with a history in commercial semen collection Center. All animals ranged between 2 and 7 years of age.

Freezability

To rank the bull's by semen freezability, it was used a history of semen production in Commercial Center, no minor than 3 years of collection and semen evaluation. The semen freezability of each Bull was calculated by # of thawed and approved ejaculates / # of ejaculates submitted to cryopreservation (after semen evaluation and approved to submitted to freeze). The bulls were divided in 3 different groups – HIGH (=>80% of ejaculates approved); MEDIUM (>60% and <79% of ejaculates approved); LOW (=<59% of ejaculates approved); the pattern and criteria were the same used in the routine of the commercial semen Center, and resumed in the semen collection and evaluation criteria item. The table 1 described the % of approve of ejaculates of each bull and groups.

Semen collection and evaluation criteria

Semen was collected using artificial vagina. Sperm concentration was determined by a cell counter, while motility was evaluated visually under a phase contrast microscope (x200), equipped with a warm stage (37ºC). Samples with over 60% motility and less than 30% morphological alterations were cryopreserved. Samples were submitted to a quality control proce-

Table 1 – Groups of bull's by semen freezability based on % of thawed and approved ejaculates (in average for minimal of 3 years of semen collection) The patterns to approved or not approved the ejaculates were based on the routine of the AI Center. Bulls breeders are Nel = Nelore; Gil = Gir Leiteiro; BBB = Belgian Blue; HOP = Holstein; SIM = Simenthal; BLA = Black Angus

Freezability Groups	Bull Code	% of thawed and approved ejaculates
Low	Nel 01	39.75
	Nel 03	21.61
	Nel 05	33.33
	Nel 08	46.89
	Nel 09	51.03
	Gil 04	00.00
	Gil 05	25.75
	Gil 06	20.00
Medium	Gil 03	80.00
	Nel 07	76.68
	BBB 01	78.26
	BLA 01	79.63
High	Gil 01	92.78
	Gil 02	84.56
	Hop 01	99.00
	Hop 02	100.00
	Hop 03	84.73
	Hop 04	96.50
	Sim 01	90.78
	Bla 01	88.06

dure at the AI Center, which consisted in evaluating viability characteristics of the frozen-thawed semen. These were: post-thaw motility (0h), motility and acrosome integrity (PIA3 – % intact acrosomal membrane or apical ridge) following three hours of incubation at 37ºC. The allowed minimum results for sample approval were: motility at 0h > 30%; Motility after 3h incubation at 37ºC > 20%; PIA3 > 45%. The semen samples were diluted in an egg yolk-TRIS-citrate extender to a final concentration of $30x10^6$ cells/straw. Following dilution, samples were pre-cooled to 4ºC for 2h, kept incubated at 4ºC for 4h, and put in the 0.5mL French straws submitted to cryopreservation curve in an IMV *digitcool*™ machine.

Seminal plasma samples (SP)

From each semen bath, 01mL of fresh semen was taken to get seminal plasma and sperm membrane samples. A minimal of 3 semen bath was used to get seminal plasma and sperm membrane samples for each bull. To obtained seminal plasma samples, the fresh semen was centrifuged at 3.500g for 20 minutes at 4ºC, and the supernatant was freezed in liquid nitrogen for future use. After thawing, these samples was centrifuged for 30 minutes at 10.000g at 4ºC to eliminated cell *debris*. The supernatant was used as sample to quantification of total protein according to Lowry et al.[8], and to 2D electrophoresis according to O'Farrel[9].

2D-Electrophoresis (2DE)

Two-dimensional electrophoresis of samples (seminal plasma and sperm membrane crude) was performed by methods previously described O'Farell[9] with some modifications described above. To the Isoeletric focusing (IEF), a Multiphor II Electophoresis Unit (Amersham Biosciences) was used and the focusing was performed in individual IPG dry strips[1] 3mm wide and 180mm long, with a pH range from 4-7 for seminal plasma proteins and from 3-10 for sperm membrane proteins. Prior to IEF, IPG dry strip were rehydrated overnight with a solution containing 40mM TRIS, 8M urea, 2% CHAPS, 0.25% DTT and 0.2% IPG buffer (Amersham Biosciences). The rehydrated strips were then placed into the cooling plate of an Electro-focusing chamber, covered with mineral oil[1] and sample cups were placed on the surface of the gel strips. Samples were diluted to 75µg of total protein in 100µl of solution containing 40mM TRIS, 9M urea, 2% CHAPS, 0.25% DTT and 0.2% of a specific IPG buffer (pH 4-7 or 3-10). A low voltage gradient was applied (0-3500 V) for 90 minutes, and then 3500V for 7 hours. Following IEF the strips were equilibrated in 2.5ml of 1.5M TRIS-HCl pH 8.8, 6M urea, 30% glycerol and 2% SDS for 25 minutes. Then, the equilibrated IPG gel strips were loaded on top of vertical SDS-PAGE with 17% of polyacrilamide for seminal plasma samples. The equilibrated IPG gel strips were embedded in a sealing solution (0.5% agarose in 25mM TRIS, 192mM Glycine and 0.1% SDS). The molecular weight standard used was range to 90-14 kDa. Vertical setups were used for 10 large gel plates (Hoefer DALT – Amersham Biosciences) with 20x25cm and 1,5mm wide, using 90V constant overnight. The silver staining of the gels was performed as suggested by Roncoletta et al.[10]. The stained gels were kept in a 10% glycerol 40% ethanol solution to posterior analyses.

Gel analyses

Each gel was scanned with an Image Scanner System, interfaced with software Phoretix (Amersham Biosciences). Previously the scanner was calibrated by color intensity (pixels), according with manufacturer. After identify the spots, the area (mm^2) and volume (mm^2 x pixels intensity) of each one was obtained, standardized by molecular weight (MW) and isolectric point (pI), and normalized by the experiment created into the software to analyzes these data. A randomized choice get a reference gel into the experiment, used to compare the spots in the different gels. Parameters like frequency of detection, quantity (normalized volume - VION) and standard deviation

of each spot was considered to evaluation and to compare with the semen freezability groups.

Statistical Analysis

The ImageMaster DATABASE1 created an average gel with average normalized spot volume (VION) for each group of semen freezability (HIGH, MEDIUM and LOW). The imposed norms to create the average gel had been free standard deviation (SD). The means of each spot was compared by Tukey's Procedure by group of fertility.

Results

Sixty eight gels were carried through, and it was statistically considered 225 spots and the amount of protein of each spot. Spots were identified by numbers, MW (molecular weight in KDa) and pI (Isoeletric point). The amount of protein could be detected by normalized average volume (VION), standard deviation (SP) and frequency of spot detection (%F) into the 3 different groups HIGH, MEDIUM and LOW, considering semen freezability. The statistical analysis, comparing bull's semen freezability and VION of each spot found difference among 2 spots from High and Low, even considering just spots with % of detection frequency bigger than 75%. Visually, the taurine bulls demonstrated more homogeneous profile when comparing with zebu bulls, considering number and frequency of appearance of spots. The tables 2 and 3 can demonstrate that results and Figure 01 shows an example of the 2DE-gels of seminal plasma samples used in this study.

Discussion

The prediction of semen freezability had been study for a long time, but until now we don't have any kind of test to estimate that pattern, may be the proteomics can show a different way to do that prediction. The results showed differences between the HIGH and LOW semen freezability groups. We prefer not consider protein profile for each ejaculate, because the enormous numbers of variation factors that can interfere in the amount and profile protein (like nutrition, and environment), even that they are controlled in the Center. Our hypothesis was looking for a lab technique that can predict the semen freezability, an individual characteristic, not a ejaculate characteristic. The complicated point here is obtained bull's semen donors in sufficient number to compose the groups, just because animals with low semen freezability don't stay so much time in a commercial Center; only 04

Table 2 – The difference between amounts of protein of the spot # SP220, that presented 18,20 (molecular weight KDa) and 5,3pI (Isolectric point). The amount of that spot can be detected by normalized average volume (VION), standart deviation (SP) and frequency of spot detection (%F) between the semen freezability groups (High, Medium and Low) and between subspecies

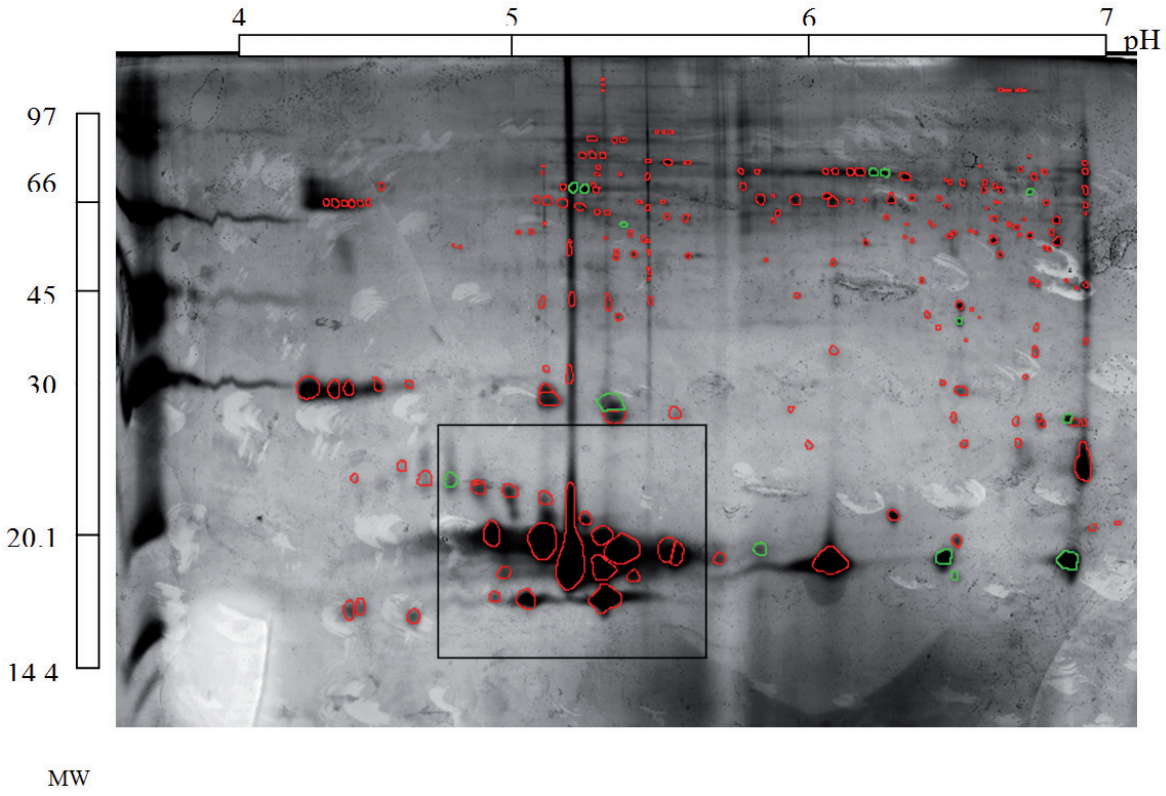
	Groups	VION	SD	%F
zebu	HIGH	22547,80[a]	3.003,30	100
	MEDIUM	25645,50[a]	4.107,90	100
	LOW	13515,80[b]	849,40	100
taurine	HIGH	29872,39[a]	3.131,83	100
	MEDIUM	33781,49[a]	2.626,19	100
	LOW	.	.	0

Source: Roncoletta, M.18
Bills: (a, b means statistical diference by Tuckey Test p<0,01)

Table 3 – The difference between amounts of protein of the spot # SP213, that presented 17,90 (molecular weight KDa) and 4,8pI (Isolectric point). The amount of that spot can be detected by normalized average volume (VION), standart deviation (SP) and frequency of spot detection (%F) between the semen freezability groups (High, Medium and Low) and between subspecies

	Groups	VION	SD	%F
Zebu	HIGH	17660,40[a]	1608,70	100
	MEDIUM	5279,10[b]	1467,60	100
	LOW	1681,10[c]	1679,20	100
Taurine	HIGH	11462,15[a]	1361,12	100
	MEDIUM	11452,30[a]	1415,53	100
	LOW	.	.	0

Source: Roncoletta, M.18
Bills: (a, b, c means statistical diference by Tuckey Test p<0,01)



Source: Roncoletta, M.[18]

Figure 1 – This figure is an example of a 2DE gel of seminal plasma samples. One hundred micrograms of total protein, electro focusing in dry strips gels with pH in 3–10 range. SDS-PAGE was conducted in plates 20 x 25 cm and 1.5mm thick at 13% acrylamide. The MW standard used was ranged to 90–14 kDa. The MW was provided on *Y*-axis and p*I* (3–10) provided on the *X*-axis of this figure. The silver staining detected 225 spots, with the numbered spots being the spots associated with semen freezability groups, SP213 and SP220

bulls were classified in the LOW group. An important result found was the variation of protein profile in seminal plasma samples comparing zebu and taurine bulls, may be because the variety on genetic status (oldest *versus* newest breeds). This is a first step to shows that the proteomics can be an important tool to predict the semen freezability, as the bull fertility that had been study too. The next steps can be considering the proteomics of samples like sperm membrane collected in the fresh semen and after dilution with extenders. The dilution and cryopreservation process promotes several structural and functional alterations of the semen or sperm cells, that could commit the viability of the cells post-thawing, including protein profile modifications[1,2,3,4,7]. The physiologic role of the proteins involves metabolic events that can suffer alteration during the sperm cryopreservation process. Soluble and structural proteins have an important role in the spermatozoa metabolism, which can interferes in the male fertility[11,12], in the bull's fertility[13,14,15,16,17,18,19,20] and semen's freezability[10,21,22].

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Abbreviations: 2DE, Two-dimensional gel electrophoresis; AI, artificial insemination; MW, molecular weight; SP, seminal plasma, VION, normalized volume of spots (amount of protein), pI (isolectric point).

References

1. OLLERO, M.; CEBRIAN-PEREZ, J. A.; MIIÑO-BLANCO, T. Improvement of cryopreserved ram sperm heterogeneity and viability addition of seminal plasma**. Journal of Andrology**, v. 18, n. 6, p. 732-739, 1997.
2. OLLERO, M.; BESCOS, O.; CEBRIAN-PEREZ, J. A.; MIIÑO-BLANCO, T. Loss of plasma membrane proteins of bull spermatozoa through the freezing-thawing process. **Theriogenology**, v. 49, n. 3, p. 547-555, 1998.
3. DELEEUW, F. E.; COLENBRANDER, B.; VERKLEIJ, A. J. The role membrane damage plays in cold shock and freezing injury. In: INTERNATIONAL CONFERENCE ON BOAR SEMEN PRESERVATION, 2., Beltsville. **Proceedings…** 1990. p. 95-104.
4. MUER, S. K.; ROY, S. B.; MOHAN, G.; DHOBLE, R. L. Cryogenic changes in seminal protein of cattle and buffalo. **Theriogenology**, v. 30, n. 5, p. 1005-1011, 1988.
5. MAXWELL, W. M. C.; JOHNSON, L. A. Membrane status of boar spermatozoa after cooling or cryopreservation. **Theriogenology**, v. 48, v. 2, p. 209-219, 1997.
6. THAKKAR, J. K.; EAST, J.; SEYLER, D.; FRANSON, R. C. Surface-active phospholipase A_2 in mouse spermatozoa. **Bioquimistry et Biophisycs Acta**, v. 745, n. 1, p. 44-50, 1983.
7. DICK, M. K.; BUHR, M. M. Phospholipase A_2 activity in fresh and criopreserved spermatozoa from boars. **Canadian Journal of Animal Science**, v. 74, n. 1, p. 59-64, 1993.
8. LOWRY, O. H.; ROSEBROUGH, N. J.; FARR, A. L.; RANDALL, J. R. Protein measurement with the Follin phenol reagent. **Journal Biological Chemistry,** v. 193, p. 265-275, 1951.
9. O'FARREL, P. H. High resolution two-dimensional electrophoresis of proteins. **Journal of Biology Chemistry**, v. 250, n. 10, p. 4007-4021, 1975.
10. RONCOLETTA, M.; FRANCESCHINI, P. H.; ESPER, C. R. Description of alternative silver stain protocol for detection of proteins in large two-dimensional gels. In: INTERNATIONAL MEETING ON PROTEOME ANALYSES. PROTEOMIC FORUM, 2003, Munich, Germany.
11. LAFLAMMME, B. A.; WOLFNER, M. F. Identification and function of proteolysis regulators in seminal fluid. **Molecular Reproduction Development,** v. 80, n. 2, p. 80-101, 2013.
12. SALVOLINI, E.; BULDREGHINI, E.; LUCARINI G.; VIGNINI, A.; LENZI, A.; DI PRIMO, R.; BALERCIA, G. Involvement of sperm plasma membrane and cytoskeletal proteins in human male infertility. **Fertility and Sterility**, v. 99, n. 3, p. 697-704, 2013. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0015028212023862>. Acesso em: 30 mar, 2012.
13. KILLIAN, G. J.; CHAPMAN, D. A.; ROGOWSKI, L. A. Fertility-associated proteins in Holstein bull seminal plasma. **Biology of Reproduction,** v. 49, v. 6, p. 1202-1207, 1993.
14. MANJUNATH, P.; NAUC, V.; BERGERON, A.; MENÁRD, M. Major proteins of bovine seminal bind to the low-density lipoprotein fraction of Hen's egg yolk. **Biology of Reproduction,** v. 67, n. 4, p. 1250-1258, 2002.
15. GERENA, R. L.; IRIKURA, D.; URADE, Y.; EGUCHI, N.; CHAPMAN, D. A.; KILLIAN, G. J. Identification of a fertility associated protein in bull seminal plasma as lipocalin-type prostaglandin D synthase. **Biology of Reproduction,** v. 58, n. 3,p. 826-33, 1998.
16. GERENA, R. L.; IRIKURA, D.; EGUCHI, N.; URADE, Y.; KILLIAN, G. J. Immunocytochemical localization of lipocalin-type prostaglandin D synthase in the bull testis and epididymis and on ejaculated sperm. **Biology of Reproduction**, v. 62, n. 3, p. 547-556, 2000.
17. NAUC, V.; MANJUNATHM, P. Radioimmunoassays for bull seminal plasma proteins (BSP-A1/A-2, BSP-A3 and BSP-30 kDa), and their quantification in seminal plasma and sperm. **Biology of Reproduction,** v. 63, n. 4, p. 1058-1066, 2000.
18. RONCOLETTA, M. **Perfil em SDS-PAGE das proteínas de plasma seminal e membrana de espermatozóides e sua relação com a congelabilidade do sêmen de touros doadores de sêmen**. 1999. [956 f]. Dissertação (Mestrado em Patologia Animal) - Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista "Julio de Mesquita Filho", Jaboticabal, 1999.

19. D'AMOURS, O.; FRENELLE, G.; FORTIER, M.; LECLERC, P.; SULLIVAN, R. Proteomic comparasion of detergent-extracted sperm proteins form bulls with different fertility indexes. **Reproduction**, v. 139, n. 3, p. 345-556, 2010.

20. GAVIRAGHI, A. L.; DERIU, F.; SOGGIU, A.; GALLI, A.; BONACINA, C.; BONIZZI, L.; RONCADA, P. Proteomics to investigate fertility in bulls. **Veterinary Research Communication**, v. 34, p. S33-S36, 2010. Supplement, 1

21. RONCOLETTA, M.; MORANI, E. S. C.; FRANCESCHINI, P. H. 14 kDa seminal plasma protein identification and it relation with bull semen freezability. **Theriogenology**, v. 57, n. 1, p. 479, 2002.

22. RONCOLETTA, M.; MORANI, E. S. C.; ESPER, C.; BARNABE, W. H.; FRANCESCHINI, P. H. Fertility Associated proteins in Nelore Bulls. **Animal Reproduction Science**, v. 91, n. 1/2, p. 77-87, 2006.