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Variations of protein profiles and calcium and phospholipase A_2 concentrations in thawed bovine semen and their relation to acrosome reaction

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Abstract

Just as calcium plays an integral role in acrosome capacitation and reaction, several spermatozoon proteins have been reported as binding to the ovum at fertilization. We examined the relationship between thawed bovine semen protein profiles, seminal plasma calcium ion concentration, spermatozoon phospholipase A_2 (PLA_2) activity and acrosome reaction. Electrophoretic profile analysis of spermatozoa and bovine seminal plasma proteins (total and membrane) revealed qualitative and quantitative differences among bulls. Variations in PLA_2 and seminal plasma calcium concentration indicated genetic diversity among individuals. A 15.7-kDa membrane protein was significantly correlated (r = 0.71) with acrosome reaction, which in turn has been associated with *in vivo* fertility.

INTRODUCTION

Mammalian fertilization is one of the most intricate, regulated cell-to-cell interactions, with ions and proteins playing an important role in the binding of spermatozoa and ovum. Calcium has a fundamental role in acrosome capacitation and reaction (Yanagimachi, 1988). Calcium flux control through the spermatozoal membrane is essential to fertilization (Bailey and Buhr, 1993). The complementary nature of spermatozoon zona-pellucida receptors linked to ZP3 glycoprotein is not well known, e.g., ZP3 may interact and/or activate a particular sperm binding protein/receptor to permit spermatozoon binding, subsequently inducing production of intracellular second messengers leading to acrosome reaction. Or ZP3 may interact and activate one sperm receptor type, subsequently activating another receptor. Then, again ZP3 interaction with sperm surface may occur in a multivalent fashion. Thus, different sperm binding proteins/receptors could together constitute a functional ZP3 receptor complex capable of translating intracellular signals to modulate acrosome reaction (Kopf *et al*., 1995). Several proteins found in spermatozoa have already been described as ovum-binding proteins: D-manosidase (human, 135 kDa; Tulsiani *et al*., 1990), HIS-50 and HIS-100 (rat, 66 and 100 kDa; Dyson and Orgebin-Crist, 1973), ZRK-receptor tyrosine kinase (human, 14, 18, 51, 63 and 95 kDa; Leyton and Saling, 1989), receptor galactosyl (human, 50 kDa; Abdullah *et al*., 1991), sp56 (mouse, 56 kDa; Bookbinder *et al*., 1994) and phospholipase A_2 (bovine, 16 kDa; Weinman *et al*., 1986). Artificial insemination, embryo transfer and *in vitro* fertilization are increasingly used for animal genetic improvement in bovine reproduction, with success depending on the quality of thawed semen used (Renard, 1984). We studied bovine semen alterations that could influence fertility (Karabinus, 1991), by analyzing proteins, ions and enzymatic activity in thawed semen that, in turn, may influence the acrosome reaction, which has been associated with *in vivo* fertility (Feliciano Silva *et al*., 1996).

MATERIAL AND METHODS

Thirty-seven samples of cryopreserved bull semen were evaluated, 14 from *Bos taurus*, 15 from *Bos indicus* and eight from cross-bred animals. The breeds studied were: Caracu (1), Gir (7), Nelore (4), Guzera (2), Danish (1), Holstein (6), Aberdeen Angus (1), Limousin (2), Simmental (4), and Girolandesa (Holstein-Gir breed) (8). An additional sample, consisting of a 5-bull semen pool with different morphological alterations (evaluation of sperm size, shape and appearance characteristics: normal forms, abnormal head, abnormal tail and immature germ cells), was included to eliminate abnormal proteins from the analysis as well as to allow comparison of proteins, ions and enzymatic activity. At least two ejaculates from each bull were analyzed; each sample consisted of 0.5 ml diluted semen.

Semen ejaculates were obtained from two artificial insemination companies (Lagoa da Serra Inseminação Artificial and Pecplan), where samples were collected and analyzed according to international standards (5% maximum abnormal acrosomal morphology), in a diluent consisting of 0.2 mol Tris-base/l, 0.065 mol Citrate acide/l, 0.026 mol fructose/l, 7% glycerol, 20% egg yolk and antibiotics. The cryopreservation procedure was as follows: 4°C to -10°C, decreasing 4°C per minute: -10°C to -130°C, at a 40°C per minute rate with liquid nitrogen (-196°C).

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Extraction and detection of semen proteins

The method used for extraction of total spermatozoon proteins was acid extraction as described by Dyck and Buhr (1993). Each 0.5-ml diluted semen sample was thawed at 37°C for 30 s. Seminal plasma and spermatozoon separation was done by centrifugation at 30,000 *g* for 30 min. The spermatozoon pellet was washed twice with 0.05 M Tris/l, pH 7, and 0.025 M sucrose/l by centrifugation at 30,000 *g* for 10 min, resuspended in 100 µl 0.18 N H_2SO_4, incubated at room temperature for 30 min, and centrifuged at 30,000 *g* for 30 min. Total protein content in the supernatant was quantified (Bradford, 1976).

The method used for membrane protein extraction was that described by Jean *et al*. (1995). Spermatozoon separation was similar to the previous procedure; however, pellet washes were done using a phosphate-buffered solution, followed by 30,000 *g* centrifugation for 10 min. The pellet was resuspended in 400 µl 0.6 M KCl/l and 0.002 M phenylmethylsulfonylfluoride/l (PMSF), incubated on ice for 30 min, and centrifuged at 30,000 *g* for 5 min. The supernatant membrane protein content was quantified, according to Bradford (1976).

Seminal plasma protein extraction followed seminal plasma and spermatozoon separation by centrifugation at 30,000 *g* for 30 min. Proteins were precipitated with 3.6 ml analytical acetone followed by another centrifugation at 12,000 *g* for 5 min. The pellet was resuspended in 1 ml 0.1 mol Tris/l, pH 7.5. Samples were quantified (Bradford,1976), and 5 µg total proteins was loaded in each gel lane. Separation was done in 14% SDS-PAGE and detected by silver staining, according to Blum *et al*. (1987).

Phospholipase A_2 activity tests

Total semen protein was acid extracted from two frozen-thawed samples of each bull. Thirty-one animals were used to determine phospholipase A_2 activity, including: Caracu (1), Gir (6), Nelore (4), Guzera (2), Danish (1), Holstein (4), Aberdeen Angus (1), Limousin (2), Simmental (4), Girolandesa (5), and the bulked semen. In each sample, an average of 30 to 80 µg total proteins was extracted, by detecting fatty acids released after enzymatic cleavage by phospholipase A_2 into a phospholipid and dying the product using Rhodamine 6G, followed by quantification in a spectrophotometer at 530 nm (Alonso Marques, 1998).

Calcium concentrations in bovine seminal plasma

Nitroperchloric digestion (2 ml nitric acid and 1 ml perchloric acid) for 2 h in a digestor block with an average temperature of 200°C was carried out in 0.5 ml thawed seminal plasma from 37 animals, obtained after semen centrifugation at 30,000 *g* for 30 min. After that, seminal plasma was resuspended in 50 ml 0.5% lanthanun oxide, according to Hommonnai (1978), modified. After digestion reading was done over a 12- to 24-h period in an atomic absorption spectrophotometer (GBC-932AA), at 422.7 nm.

Acrosome reaction

Acrosome reaction was executed in accordance with Parrish *et al.* (1988). Based on protein analysis considering all variations detected frozen-thawed semen from 14 selected bulls of different breeds were used, including the following: Simmental (3), Nelore (2), Holstein (1), Gir (6), Caracu (1), and Aberdeen Angus (1). Spermatozoa were washed once in modified TALP Tyrode buffer containing BGM-1 and BSA (Parrish *et al*., 1988). Analysis of spontaneous acrosome reaction due to the freeze-thaw process was made with a negative control, which consisted of counting stained spermatozoon in air-dried smears before incubation (0 h). After resuspension in the same solution, heparin was added. Heparin is a glycosaminoglycan not directly leading to acrosome reaction, but rather predisposing sperm to changes typical of acrosome reaction (Ax and Lenz, 1987). Incubation for 4 h at 38°C followed. Lysophosphatidylcholine, acting as a fusogen component which stimulates the acrosome reaction (Llanos *et al.*, 1995), was added and incubation continued for 15 min. To determine acrosome reaction, air-dried smears were stained with naphtol yellow and eritrosin B (Lenz *et al*., 1983). Two hundred spermatozoa were examined at 1000X. Spermatozoa that had not undergone the acrosome reaction appeared dark, with a distinctly apical edge while the others were much lighter in the acrosomal area. Acrosome reaction index was calculated by dividing acrosome reaction rate at 4 h by that at 0 h.

Analysis of gels

Protein molecular mass and respective densitometry analysis were determined by the ImageMaster Videodocumentation System (1996).

Statistical analysis

Statistical analysis for standard curves and correlations was carried out by linear regression (Statistica for Windows, 1993). For calcium concentration and phospholipase A_2 activity in different bull semen samples, variance analysis and Duncan's multiple range test were done by the SAS program (SAS Institute Inc., 1989).

RESULTS

Electrophoretic profile of semen proteins

An analysis of total protein electrophoretic profiles and respective densitometry in the 37-bull samples of several breeds (Aberdeen, Caracu, Danish, Gir, Girolandesa, Guzera, Holtein, Limousin, Nelore and Simmental) are represented in Figure 1. Figure 1 shows quantitative differ-

ences in proteins with molecular masses of 31.8, 29.7, 25.4, 20.9, 20.2, 18.4, 16.2 and 16 kDa. Qualitative differences (presence or absence of bands) were observed in proteins of low molecular mass (27.5, 21.4, 16.8 and 16.2 kDa). Spermatozoon membrane protein analysis showed differences in proteins with molecular masses estimated at 31.1 and 16.7 kDa (Figure 1). Quantitative differences were found in proteins of 66, 60, 45, 36, 29, 27.3, 20.9, 20.2, 18.8, 17.3, 16.3 and 15.7 kDa. Eight samples with some proteinaceous differences found among the 37 animals analyzed are represented in Figure 1. When analyzing the electrophoretic profile of seminal plasma proteins, quantitative differences were found in 94.5-, 29.9-, 24.9-, 18.1-, 17.4- and 16.2-kDa protein bands, while qualitative differences were found in 24.9- and 21.5-kDa bands. The diluent was also analyzed and many proteins were disregarded.

Phospholipase A_2 activity test

Thirty-one semen samples were analyzed. In five of them, enzymatic activity could not be detected by the colorimetric method, which has a lower limit of detection of 0.001 units min^{-1} μl^{-1}. The mean enzymatic activity of the 31 bulls was 0.573 units min^{-1} μl^{-1}. PLA_2 variance analysis was significant among animals ($P < 0.01$), with enzyme activity that varied from 0.08 to 1.8 units min^{-1} μl^{-1} (Table I).

Analysis of calcium concentration in the seminal plasma

Calcium concentration was calculated for 37 animals. The mean calcium concentration in the seminal plasma revealed significant differences among bulls ($P < 0.05$). The mean among bulls was 265 µg/ml (Table I).

Acrosome reaction analysis

Fourteen of the 37 samples were analyzed for their capacity to undergo *in vitro* acrosome reaction. The 14 samples had an acrosome reaction index varying from 1.5 to 10, with an average of 3.9, agreeing with results of Feliciano Silva *et al*. (1996).

Semen protein, phospholipase A_2 activity and calcium concentration correlation between acrosome reaction

All proteins showing polymorphism in the electrophoretic profiles and therefore of possible importance in the bovine fertilization process were analyzed. Correlation between proteins detected through electrophoresis and phospholipase A_2 activity was found to be nonsignificant. However, significant correlation between 10 proteins and seminal plasma calcium concentration was found, with 7 proteins occurring in spermatozoa and the other 3 in seminal plasma (Table II).

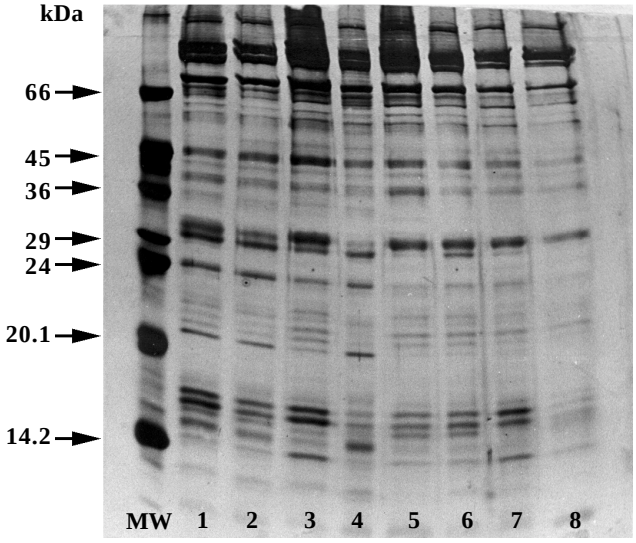

Figure 1 - Electrophoresis in 14% SDS-PAGE of membrane proteins of bovine spermatozoa. (MW) Standard of molecular mass SDS-6H. (1) Nelore, (2) Nelore, (3) Limousin, (4) Simmental, (5) Nelore, (6) Gir, (7) Holstein, and (8) Limousin.

The acrosome reaction presented a significant positive correlation coefficient ($r = 0.71$) only for the spermatozoa membrane protein of 15.7 kDa, and a negative correlation coefficient ($r = 0.61$) for the spermatozoa membrane protein of 31.1 kDa. Acrosome reaction did not present relevant correlation with phospholipase A_2 ($r = 0.06$) or with seminal plasma calcium concentration ($r = 0$).

DISCUSSION

Analyses of the electrophoretic profiles of 37-bull samples showed polymorphism among individuals, with no specificity attributed to breeds, where differences were both qualitative (DNA variations resulting in mutation or deletion possibly preventing protein transcription and/or translation) and quantitative (protein expression alterations, affecting transcription as well as translation) perhaps important in the spermatozoon-ovum-binding process and also in acrosome reaction induction.

Due to limited phospolipase A_2 activity in the thawed bull semen, it escaped detection by conventional methods, e.g., the potentiometric (De Haas *et al.*, 1968). However, a more sensitive colorimetric method has been developed (Alonso Marques, 1998), which allows detection of phospholipase A_2 activity exceeding 0.001 but under 3 units min^{-1} μl^{-1}. Quantitative diversity of phospholipase A_2 activity among bulls, according to Chang *et al*. (1987) may be explained by several factors, possibly interfering in phospholipase A_2 activity even after its transcription and translation. These would be kinase C protein, guanidine nucleotide regulating proteins, IP3, DAG and cAMP. The cAMP can inhibit phospholipase A_2 activity by bind-

Table I - Analysis of variance of phospholipase A_2 (PLA_2) activity and calcium concentration of frozen bovine semen.

Animal	Means of PLA_2 activity[a]	Means of calcium concentration[a] (µg/ml)
Pool [b]	0.005 F	130 FGH
Aberdeen	0 F	194.7 EFGH
Caracu	0.003 F	156.3 EFGH
Danish	0.096 EF	133.7 FGH
Gir 1	-	403 B
Gir 2	0 F	204.8 EFGH
Gir 3	1.108 BCDEF	114.7 GH
Gir 4	0.004 F	110.7 GH
Gir 5	2.814 A	186 EFGH
Gir 6	0.295 CDEF	105.7 GH
Gir 7	0 F	170.3 EFGH
Girolandesa 1	0.006 F	265 CDEF
Girolandesa 2	0.001 F	585 A
Girolandesa 3	0.804 BCDEF	131 FGH
Girolandesa 4	0.375 CDEF	295 BCDE
Girolandesa 5	0.434 CDEF	533 A
Girolandesa 6	-	188 EFGH
Girolandesa 7	-	250 DEFG
Girolandesa 8	-	204.3 EFGH
Guzera 1	0.087 EF	106.7 GH
Guzera 2	1.862 AB	383 BC
Holstein 1	-	401 B
Holstein 2	-	388.8 BC
Holstein 3	0.576 BCDEF	357.8 BCD
Holstein 4	0.188 DEF	215.3 EFGH
Holstein 5	1.446 BCD	92.7 H
Holstein 6	0.188 DEF	193.3 EFGH
Limousin 1	0 F	108 GH
Limousin 2	0.002 F	159 EFGH
Nelore 1	0.001 F	199 EFGH
Nelore 2	1.187 BCDEF	160.3 EFGH
Nelore 3	1.352 BCDE	194.3 EFGH
Nelore 4	0.752 BCDEF	92 H
Simmental 1	1.568 BC	107 GH
Simmental 2	0.456 CDEF	173.7 EFGH
Simmental 3	1.569 BC	171.3 EFGH
Simmental 4	0 F	223.3 EFGH

[a]Means followed by the same letter are not significantly different. [b]Pool of five animals with morphological alterations of sperm. - Samples that were not analyzed.

Table II - Correlation coefficient between semen proteins and calcium concentration in bovine seminal plasma.

Protein origin	Correlation coefficient of calcium with proteins
Total proteins	
31.8 kDa	-0.50
20.9 kDa	0.71
16.2 kDa	0.41
Membrane protein	
31.1 kDa	0.38
27.3 kDa	0.46
20.8 kDa	0.40
17.9 kDa	0.54
Seminal plasma protein	
29.9 kDa	0.61
18.1 kDa	0.34
16.2 kDa	-0.34

ing phospholipase A_2 or replacing the free stored calcium essential for phospholipase A_2 activity (Van Den Bosh, 1980). The semen freezing and thawing process also decreases phospholipase A_2 activity approximately 50% in swine samples when compared with the activity in fresh semen (Dyck and Buhr, 1993).

Calcium concentration differences in bovine seminal plasma suggest a possible genetic control for releasing extracellular calcium; however, these differences stem from individual variability, unattributable to different breeds. The high correlation coefficient between seminal plasma calcium concentration and semen protein suggests that spermatozoon proteins with molecular masses of approximately 31, 27, 20, 18 and 16 kDa, as well as those of seminal plasma with molecular masses of 29, 18 and 16 kDa, participate in extracellular calcium flow activation. Spermatozoon protein of 31 kDa and seminal plasma protein of 16 kDa gave a negative correlation coefficient, associated with lower levels of extracellular calcium.

According to Llanos *et al*. (1995) calcium is essential to acrosome reaction while, according to Rönkkö *et al*. (1994), phospholipase A_2 is also of great importance. However, this research showed no correlation between calcium ions, phospholipase A_2 activity and acrosome reaction. Calcium and phospholipase A_2 activity present in bovine spermatozoa, although varying significantly in bulls, may be sufficient to produce acrosome reaction, and could influence the bovine semen fertilization process. These results differ from those arrived at Florman *et al*. (1989). According to Florman, the calcium can stimulate the acrosome reaction in bovine.

The protein of approximately 15.7 kDa, which presented positive correlation with acrosome reaction may be a ZRK protein, important in the spermatozoon-ovum-binding process while the 31.1 kDa protein is probably ZRK in its dimeric form, which may not stimulate the binding process (as happens in human spermatozoa) essential for regulating cascade in the acrosome reaction (Leyton *et al*., 1992). This 15.7-kDa protein, which presented a significant correlation (0.71) with the acrosome reaction, is a candidate protein marker for fertility.

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RESUMO

Várias proteínas que constituem o espermatozóide têm sido relatadas como sendo proteínas que se ligam ao óvulo no momento da fertilização, bem como íons cálcio têm um papel importante na capacitação e reação acrossômica. Baseado nisto, este estudo teve como objetivo analisar e correlacionar proteínas do sêmen

congelado bovino de diferentes raças, concentração de íons cálcio no plasma seminal e atividade da fosfolipase A_2 do espermatozóide com a reação acrossômica, visando encontrar fatores que influenciem no processo de fertilização bovina. Análises do perfil eletroforético das proteínas (totais e de membrana) do espermatozóide e do plasma seminal bovino revelaram variabilidade protéica entre indivíduos na qual diferenças qualitativas e quantitativas foram identificadas. A quantificação da fosfolipase A_2, bem como da concentração de cálcio no plasma seminal revelaram diversidade genética entre touros. Uma proteína de 15,7 kDa apresentou correlação significativa (0.71) com a reação acrossômica, que pode estar diretamente relacionada com a fertilização *in vivo* e deste modo outros experimentos podem ser realizados a fim de investigar a utilidade deste marcador protéico na verdadeira fertilidade.

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