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Expression of pregnancy-specific beta-1-glycoprotein, aromatase and nitric oxide synthase in bovine sperm[1]

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ABSTRACT

In search of alternative indicators of fertility in bulls, this research evaluated the effects of breed, type of cattle and age on the relative expression of the genes aromatase, pregnancy-specific beta-1-glycoprotein (PSG1), and nitric oxide synthase (NOS) in bovine semen. Sperm from Jersey, Holstein, Brahman, Brangus, Charolais, Charbray, Senepol, and Simmental bulls was collected and the extracted mRNA was analyzed for expression of the genes using real time polimerase chain reaction (RT-PCR). The relative expression of aromatase was lower ($P < 0.07$) in sperm from Jersey bulls (0.89) than in bulls of all other breeds (average 1.01). The relative expression of PGS1 was greater ($P > 0.02$) in sperm from dairy (1.00) than from beef bulls (0.96). Age did not affect relative expression of the genes under study. Additionally, in data from 31 bulls, expression of NOS was positively correlated with scrotal circumference ($r = 0.31$; $P < 0.08$). As far as we know, these are the first results showing that the genes aromatase, PSG1 and NOS can be detected in bull sperm and may have implications as molecular markers for bull fertility.

Key words: bull fertility, RT-PCR, PSG1, NOS, aromatase

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RESUMEN

Expresión de glicoproteína específica de la preñez, aromatasa y óxido nítrico sintasa en espermatozoides bovinos

En la búsqueda de marcadores alternos para la fertilidad en los toros, se evaluó el efecto de la raza, el tipo de ganado y la edad, sobre la expresión relativa de los genes aromatasa, glicoproteína beta-1-específica de la preñez (GSP1) y óxido nítrico sintasa (NOS) en espermatozoides bovinos. Se recolectó semen de toros Jersey, Holstein, Brahman, Brangus, Charolais, Charbray, Senepol y Simmental para extraer el ARN mensajero y evaluar la expresión de los genes por la reacción en cadena de la polimerasa en tiempo real. La expresión relativa de la aromatasa fue menor ($P < 0.07$) en el semen de toros Jersey (0.89) en comparación a las otras razas (promedio 1.01). La expresión relativa de GSP1 fue mayor ($P < 0.02$) en el semen de toros lecheros (1.00) comparado a los de toros de carne (0.96). La edad no tuvo efecto ($P > 0.05$) sobre la expresión relativa de los genes evaluados. Además, datos obtenidos de 31 toros revelaron que la expresión de NOS correlacionó directamente con la circunferencia escrotal ($r = 0.31$, $P < 0.08$). Hasta donde conocemos, estos resultados muestran por primera vez que la expresión de los genes aromatasa, GSP1 y NOS puede ser detectada en semen bovino, lo que podría tener implicaciones para su uso como marcadores moleculares para la fertilidad de los toros.

Palabras clave: semen, bovinos, GSP1, NOS, aromatasa, fertilidad

INTRODUCTION

Effective reproduction is fundamental in efforts to improve animal production. Research to improve reproductive performance using genetic selection in bovines has been focused on optimizing fertility traits directly in the female (Cammack et al., 2009). However, the results of some studies suggest that cow fertility could be improved by selecting for fertility traits in the bull (Mackinnon et al., 1990).

The sperm cell is a unique feature of the male reproductive system, which was typically thought to serve only by delivering its DNA for the fertilization process. Recently, various studies have found that sperm metabolites may affect other reproductive functions; for example, the sperm can carry ribonucleic acid (RNA) into the oocyte, and it has been suggested that messenger ribonucleic acid (mRNA) might have a role before or after fertilization (Ostermeier et al., 2004; Krawetz, 2005). In addition, sperm RNAs may contribute to subsequent processes such as early embryonic development and implantation (Hamatani, 2012).

In men, certain genes have been involved in fertility: pregnancy-specific beta-1-glycoprotein (PSG1), aromatase and nitric oxide synthase (NOS). For example, sperm from fertile humans expresses a greater quantity of PSG1 compared to non-fertile individuals (Avendaño et al., 2009). It has also been suggested that PSG1 may play a role in embryogenesis and/or implantation (Ha et al., 2010). Further-

more, greater expression of aromatase resulted in higher sperm motility of men (Ostermeier et al., 2004) and buffalos (Tiwari et al., 2008). Thus, the degree of aromatase expression in sperm was suggested as a possible marker for sperm motility (Lambard, 2004; Tiwari et al., 2008). Moreover, NOS is involved in the regulation of spermatogenesis (Ishikawa et al., 2005) and could be involved in the processes of sperm maturation and capacitation (Rodríguez et al., 2005).

Collectively, these genes may play important roles in sperm fertility of men and other species. As far as we know, gene expression from bull sperm has not been studied; therefore, the current research aims to study the expression of the genes PGS1, aromatase and NOS in bovine semen.

MATERIALS AND METHODS

Geographical location

This study was conducted on Puerto Rican dairy and beef cattle herds, located in the following municipalities: Lajas, Cabo Rojo, Humacao, Guánica, Vega Alta, Camuy, Juana Díaz, Santa Isabel, San Germán, Moca, Hatillo, Arecibo, Morovis, Corozal, Toa Alta, Jayuya, Utuado, Ciales, and at the Beef Cattle Farm "Finca Montaña" of the University of Puerto Rico Mayagüez Campus. The semen collection took place from late May 2011 until mid June 2012.

Semen collection

At all the participating farms, a semen sample was collected from each bull of the following breeds: Brahman (n = 21), Brangus (n = 6), Charbray (n = 7), Charolais (n = 16), Simmental (n = 4), Senepol (n = 13), Holstein (n = 30) and Jersey (n = 5). Bulls had to be 18 months of age or older to participate. Before each collection, information on the animal was gathered, including: identification number, breed, scrotal circumference and age. Prior to the collection, a visual and physical examination of reproductive organs was performed. Only bulls considered normal and healthy had their semen collected by electro-ejaculation. The fresh semen was visually evaluated for quality and blood, urine, dirt or pus contamination. Subsequently, the semen sample was observed under a microscope for the presence of microorganisms. A minimum volume of 5.0 mL of semen per animal was then placed in sterilized tubes.

Total RNA isolation from sperm samples

The freshly ejaculated semen was transferred into 15 mL centrifuge tubes. For the isolation of total RNA, RNAzol® RT reagent (Molecular

Research Center, Inc., Cincinnati, OH)[5] was used. For each 1.0 mL of sample in the tube, 1.0 mL of RNAzol® was added, for a 1:1 ratio, within a total volume of 10.0 mL. The samples were homogenized for one minute in the Polytron® Homogenizer and further procedures recommended by the manufacturer were followed. The final RNA pellet was diluted with 50 µL of molecular grade water and placed in 1.7 uL microcentrifuge tubes, which were immediately stored at -80 °C. An Eppendorf BioPhotometer Plus® with Hellma® TrayCell adapter was used to measure the concentration of total RNA. To optimize the quality of the samples and eliminate DNA activity, the RNA was treated with Amplification Grade Deoxyribonuclease-1 (DNase1) (Sigma-Aldrich, Inc., 2010) according to the manufacturer's instructions.

cDNA synthesis and real-time PCR

The reverse transcriptase reaction was performed with Quanta Biosciences qScript cDNA SuperMix® (Gaithersburg, MD) following the manufacturer's procedures for a total volume of 20 µL and using 300 ng of total RNA. The cDNA synthesis was performed in Eppendorff 6321 AG thermocycler as follows: five minutes at 25° C, 40 minutes at 42° C, five minutes at 85° C, and then kept at 4° C. The real time polymerase chain reaction (RT-PCR) was performed in Eppendorf Realplex 22331 with Quanta Biosciences PerfeCta SYBR Green Fast Mix® (Gaithersburg, MD) according to manufacturer's procedures. The following conditions were imposed for the reaction: 95° C for two minutes, followed by 45 cycles at 95° C for 15 seconds, 60° C for one minute and 95° C for 15 seconds. Specific primers for bovine transcripts were obtained from previous publications (Table 1). Final concentrations for the forward and reverse primers were 900 µM.

Statistical analysis

Relative expression of the genes in semen was related to age, breed, and type of cattle (dairy or beef). In addition, scrotal circumference was correlated with expression of the genes. The statistical analysis was performed using Statistical Analysis System (SAS) software (Version 9.1, SAS Institute Inc., Cary, NC, USA).

[5]Company or trade names in this publication are used only to provide specific information. Mention of a company or trade name does not constitute an endorsement by the Agricultural Experiment Station of the University of Puerto Rico, nor is this mention a statement of preference over other equipment or materials.

TABLE 1.—*Primer sequence for amplification of pregnancy specific beta-1-glycoprotein, aromatase, nitric oxide synthase and ribosomal protein S9 (RSP9).*

Gene	Accession No.	Forward Primer	Reverse Primer
Pregnancy specific beta-1 glycoprotein	NM-174411	5'TGG CCT TCT CAG AGT GCA TAG TCA3	5'ATC CTT GAT GTT TCT CAG CGG GTG3'
Aromatase	NM-174305	5'CGA AGT TGT GCC TAT TGC CAG CAT3'	5'AGA GGA ACC TGC AGT GGG AAA TGA3'
Nitric oxide synthase	NM-181037	5'AAA GCA ACC ATC CTG TAC GC3'	5'ATT CCC AAA GGT GCT GGT CA3'
Ribosomal protein S9 (Housekeeping gene)	DT860044	5'CCT CGA CCA AGA GCT GAAG'3	5'CCTCCAGACCTCACGTTTGTTC3'

Bull age and relative gene expression

For evaluation, the data on animal age were categorized in four groups as follows: 18 to 28, 29 to 36, 37 to 60 and 61 to 120 months, and analyzed using the statistical model:

$$Y_{ij} = \mu + \alpha_i + \beta x_{ij} + \varepsilon_{ij}$$

Y_{ij} = relative gene expression (PGS-1, Aromatase, NOS)
μ = overall mean
α_i = effect of the age [(18 to 28), (29 to 36), (37 to 60) and (61 to 120) months]
βx_{ij} = covariate effect, level of expression of RPS9
εi_j = experimental error associated with total or relative expression of RPS9

Bull breed and relative gene expression

$$Y_{ij} = \mu + \alpha_i + \beta x_{ij} + \varepsilon_{ij}$$

Y_{ij} = relative expression (PGS-1, Aromatase, NOS)
μ = overall mean
α_i = effect of breeds (Jersey, Holstein, Brahman, Brangus, Charolaise, Charbray, Senepol and Simmental)
βx_{ij} = covariate effect, level of expression of RPS9
ε_{ij} = experimental error associated with total or relative expression of RPS9

Animal type and relative gene expression

$$Y_{ij} = \mu + \alpha_i + \beta x_{ij} + \varepsilon_{ij}$$

Y_{ij} = relative expression (PGS-1, Aromatase, NOS)
μ = overall mean
α_i = effect of type (dairy or beef cattle)
βx_{ij} = covariate effect, level of expression of RPS9
εi_j = experimental error associated with total or relative expression of RPS9

Scrotal circumference

Thirty-one bulls were restrained and scrotal circumference was measured with a scrotal wand. A simple linear regression between relative gene expression and the housekeeping gene RSP9 was calculated. The Proc Reg model was used to determine the residual values. These residual values were then used to determine the Pearson correlation coefficient between scrotal circumference and relative gene expression.

RESULTS

In this study, the mRNA for the genes PGS1, aromatase, and NOS in bovine semen was detected. The effects of bull breed (Figure 1), type of cattle and a correlation with scrotal circumference (Table 2) were established. The effect of age groups was not significant.

Bull breeds and relative gene expression

The relative expression of aromatase in sperm samples evaluated was lesser ($P < 0.07$, Figure 1) for Jersey bulls (0.89) compared to the other breeds (1.0). However, the relative expression of PGS1 ($P = 0.45$) and NOS ($P = 0.11$) was similar among all breeds tested. Relative expression of PSG1 was 1.00, 1.03, 0.993, 1.15, 0.977, 1.015, 0.97, and 0.920 for Brahman, Brangus, Charbray, Charolaise, Simmental, Holstein, and Jersey, respectively. Corresponding values for NOS were 1.00, 1.02, 0.96, 1.00, 0.88, 0.93, 0.95, and 0.83.

Type of cattle and relative gene expression

The relative expression of PSG1 in sperm was lesser ($P < 0.02$) for beef (0.96) than for dairy cattle (1.00), whereas the relative expression of aromatase (1.00 beef vs. 0.98 dairy) did not differ significantly between the two types of cattle ($P = 0.18$). The NOS showed the same relative expression of 1.00 in both beef and dairy cattle ($P = 0.90$).

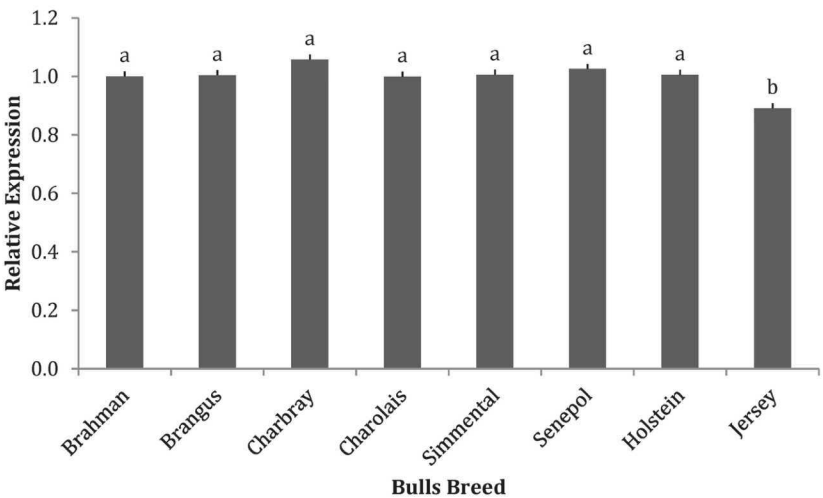

FIGURE 1. Relative aromatase expression in eight of the bull breeds. Different letter represents a breed difference ($P < 0.07$).

TABLE 2.—*Pearson correlations between the gene expression and scrotal circumference in dairy and beef bulls.*[1]

Gene	Correlation	P-value
Pregnancy-Specific Beta-1-Glycoprotein	-0.1966	0.2892
Aromatase	0.1716	0.3559
Nitric Oxide Synthase	0.3149	0.0844

[1]A total of 31 dairy and beef bulls were studied. P-values were calculated using Pearson correlation coefficient.

Bull age and relative gene expression

Significant differences among age groups were not found in expression of aromatase (P = 0.23), PGS1 (P = 0.39), and NOS (P = 0.89), for age categories 18 to 24, 25 to 36, 37 to 60, 61 to 120 months, the respective values were: aromatase, 1.00, 1.05, 1.04, 1.03; PGS1, 1.00, 0.97, 0.97, 0.96; and NOS, 1.00, 1.00, 1.01, 1.03.

Scrotal circumference

The correlation analysis (n = 31) showed that NOS had a positive correlation (P < 0.08) with scrotal circumference but the relative expression of aromatase and PGS1 were not significantly correlated with this anatomical variable (Table 2).

DISCUSSION

Nussbaumer et al. (2006) and Sakurada et al. (2009) stated that the RT-PCR technique is a reliable tool for identifying semen mRNA expression. However, the mRNA is unstable and can deteriorate rapidly (Sakurada et al., 2009). Nonetheless, sperm mRNA has been isolated in mice (Gyllensten et al., 1991), humans (Avendaño et al., 2009; Lambard et al., 2003; Zhao et al., 2006), buffalos (Tiwari et al., 2008), pigs (Hwang et al., 2013) and chickens (Shafeeque et al., 2014). Through RT-PCR assays, semen mRNA can be used for many purposes; in the present research the RT-PCR assay was used to study the presence of sperm mRNA for the genes aromatase, PSG1 and NOS in bulls.

Aromatase is an important key to the male reproductive system and "normal" male reproduction, and sexual development is related to the balance of its activity (Saez, 1994; Carreau et al., 2003). In fact, aromatase expression has been positively related to higher sperm motility in men (Ostermeier et al., 2004), mice (Robertson et al., 1999; 2001) and buffalo (Tiwari et al., 2008). Earlier research suggested that aromatase expression could be used as a possible marker for sperm motility (Lambard, 2004; Tiwari et al., 2008; Carreau et al., 2009). The Jersey

bulls of the present study exhibited lesser aromatase expression than those of the other breeds sampled, which differs from the results of previous research (Tiwari et al., 2008). Aromatase deficiency is related to inability to complete the fertilization process and lesser expression of this gene has been negatively correlated with greater proportions of abnormal and immotile sperm (Lambard, 2004; Tiwari et al., 2008; Carreau et al., 2009).

The presence of PSG1 was reported for the first time in human sperm by Avendaño et al. (2009) and Hamatani (2012). They concluded that sperm from fertile men show greater PSG1 expression than sperm from non-fertile men. Our results demonstrated an effect of animal type on the expression of PSG1 in bull semen being greater for beef ($P = 0.02$) compared to dairy cattle. Based on this result and previous research, we can postulate a possible relation between the PSG1 expression and greater fertility in beef than in dairy cattle. Results obtained in humans and mice support this hypothesis. It has been suggested that sperm could retain some PSG1 mRNA (Carreau et al., 2009), and play a possible role after fertilization, such as in early embryogenesis and implantation (Hamatani, 2012; Martínez et al., 2013), but the physiological pathways involved are not yet clear. Avendaño et al. (2009) reported an age-related pattern of aromatase relative gene expression. However, the present results revealed no such effect in the age groups studied.

The expression of NOS is also related to male sexual and reproductive functions and sperm motility. Tiwari et al. (2008) even suggested that sperm contain NOS transcripts, located in the head and mid-piece. Lewis (1996) obtained high correlation between NOS expression and testicular function. The NOS expression was detected in bull semen in the present study, and a positive correlation with scrotal circumference was revealed ($r = 0.31$, $P < 0.08$). The present and previous studies provide evidence of a possible relation between NOS expression, testicular function and sperm motility. Some experimental results support a NOS role in sperm motility (Hellstrom et al., 1994; Ishikawa et al., 2005), whereas others differ (Rosselli et al., 1995; Weinberg et al., 1995). On the other hand, the overexpression of NOS can cause oxidation of sperm membrane lipids and proteins (Stamler et al., 1992), reduced motility (Balercia et al., 2004), and other negative effects causing infertility in men. Thus, the role of NOS will depend on the level of expression and redox state (Balercia et al., 2004).

These transcripts may have important roles in sperm fertility of men and other species. To our knowledge, the expression of these genes had not been previously studied in bull semen. Therefore, these results document for the first time the presence of aromatase, PGS1 and NOS

transcripts in the semen of the bovine species, with implications for possible functions in sperm motility, fertility, early embryogenesis, implantation and testicular function.

A possible future application of bovine sperm mRNA is as a diagnostic tool for evaluation of fertility. The expression of these genes in bull semen could potentially become a molecular marker for fertility as it has become in humans.

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