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Association of Osteopontin Gene Promoter Single Nucleotide Polymorphisms with Bull Semen Quality

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Abstract

Osteopontin (OPN) is a protein found at higher concentrations in the seminal plasma of bulls with above average fertility. Polymorphisms have been reported within the OPN gene promoter that can affect production of this protein and thus, affect fertility. Therefore, Angus (n = 5) and Angus x Gelbvieh (Balancer, n = 14) and Angus x Brahman (n = 15) bulls were evaluated for presence of single nucleotide polymorphisms (SNP) in the *Bos taurus* OPN gene (GenBank: AY878328.1) promoter region, and their possible effects on bull semen quality as evaluated by computer-assisted semen analysis (CASA). Semen was collected by electroejaculation 6 to 9 times from each bull, and each semen collection was evaluated by CASA for motile, progressive and rapid sperm within 5 mins of ejaculation. The bulls were genotyped for reported single nucleotide polymorphisms (SNP) in the promoter region of the OPN gene through amplification of two 700 base pair (bp) DNA fragments and sequencing of the resulting PCR products. Seven SNP sites were identified, at bp 3379, 3490, 3492, 5075, 5205, 5209, and 5263 of the OPN gene. The SNP identified at bp 5205, 5209 and 5263 had not been previously reported. Individual SNP sites were evaluated as the main effect on CASA sperm motility variables in a SAS MIXED model for repeated measures. A thymine to guanine substitution at bp 3379 was associated with increased ($P \leq 0.02$) percentage of motile, progressive and rapid sperm in Angus x Brahman bulls, and tended ($P \leq 0.10$) to increase the same sperm motility parameters in Angus, and Angus x Gelbvieh bulls. The percentages of motile, progressive and rapid sperm were similar ($P \geq 0.05$) among genotypes for the other 6 SNP identified. These results suggest that identification and genotyping of polymorphisms within the promoter region of the bovine OPN gene may be useful for selecting bulls with improved sperm motility parameters.

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Keywords

Osteopontin, Polymorphism, Sperm Motility, Bull

1. Introduction

Osteopontin (OPN) is a ubiquitous, multi-function protein that has been found in higher concentrations in ejaculates of bulls that produce higher conception rates [1]. Cancel *et al.* [2] find that in the bull, OPN is secreted in the ampullae and seminal vesicles, where it is believed to bind to sperm through CD44 and surface integrins. Osteopontin has also been shown to facilitate capacitation and viability of bovine sperm [3]. Ejaculated sperm carries the bound OPN protein to the site of fertilization [4] where it is thought to play a role in the sperm-egg interaction through OPN-integrin complexes present on the surface of the sperm and oocyte [5].

In vitro studies indicate that pre-treatment of either bovine semen or oocytes with purified OPN increases fertilization rate and embryo development [6]. Ejaculated bovine sperm treated with OPN antibodies prior to *in vitro* fertilization results in decreased fertilization rates and increased polyspermy [7]. These findings are in agreement with Hao *et al.* [8], who report OPN reduced polyspermy in a dose dependent manner, while increasing fertilization efficiency during pig in vitro fertilization.

Osteopontin is present in bovine milk at a concentration of about 18 mg/L [9]. While 18 mg/L only represents about 0.05% of the total milk protein, studies have demonstrated that polymorphisms within the OPN gene promoter region show significant associations with milk protein concentrations [10] [11]. Because higher concentrations of OPN in semen are associated with fertility, any polymorphism that occurs in the OPN gene may affect concentration of OPN in seminal plasma and thus, serve as a marker for fertility. This study investigates the polymorphic nature of the bovine OPN gene promoter region to determine any association of polymorphisms with bull semen quality.

2. Materials and Methods

2.1. Bull Management

The University of Arkansas Animal Care and Use Committee approved all practices and techniques utilized in this study. A group of Angus (n = 5) and Angus x Gelbvieh (Balancer, n = 14) bulls ranging in age from 5 to 9 y and weighing between 710 to 955 kg were maintained at the University of Arkansas Beef Research Unit. These bulls were allotted to two separate drylots to reduce social (dominance) stress. The bulls were fed 0.45 kg of high concentrate feed 3 times a wk and *ad libitum* grass hay. The concentrate feed was formulated by University of Arkansas feed mill to contain 10% crude protein, and the grass hay was produced at the University of Arkansas Beef Research Unit and contained 89% dry matter and 11.6% crude protein. A second group of bulls (Angus x Brahman, n = 15) bulls were maintained at the USDA Small Farms Research Center near Booneville, Arkansas. These bulls, averaging 16 mo of age and 478 kg body weight, were maintained on common bermudagrass pastures overseeded with rye grass.

Before inclusion in the study, all bulls received a breeding soundness evaluation to insure they met or exceeded the minimum acceptable standards for semen quality. Semen was collected by electroejaculation weekly from the Angus and Angus x Gelbvieh bulls over a 9 wk period during summer (July 15 through September 19). The Angus x Brahman bulls were collected monthly from May to September. Immediately after collection, semen samples were placed into a water bath at 35˚C to avoid temperature shock.

2.2. Semen Analysis

Each semen collection was analyzed using a computer assisted sperm analysis (CASA; Hamilton Thorne Biosciences, Beverly, MA) within 5 mins of ejaculation. Prior to analysis, each semen sample was diluted with Dulbecco's PBS (#D-5773, Sigma, St. Louis, MO) to achieve a concentration of ~ 25 × 106 sperm/mL before loading onto a 2X-CEL (Hamilton Thorne Biosciences) slide. The CASA system scanned 10 areas along the length of the slide and captured 30 video frames per viewing area to construct a composite of the sperm motility variables. A minimum of 400 spermatozoa was counted on each slide to achieve a representation of the entire se-

men collection sample. The sperm variables measured were percent motile, progressive and rapid sperm. Motile sperm were defined as those with a path velocity ≥ 30 μm/s and progressive velocity ≥ 15 μm/s. Progressive sperm had a path velocity ≥ 50 μm/s and straightness ≥ 70%. Rapid sperm were defined as the percentage of progressive sperm with path velocity > 50 μm/s.

2.3. DNA Extraction

Blood samples were obtained from each test bull via tail vein in 8 mL vacuum tubes containing EDTA (Vacutainer #366643, BD, Franklin Lakes, NJ), and were placed on ice for transport to the lab. Genomic DNA was extracted using a DNeasy Blood and Tissue Kit (QIAGEN, Cat. No. 69504; Valencia, CA) using a mammalian whole blood protocol. Isolated DNA was rehydrated in TRIS-EDTA (TE) buffer (10 mM TRIS, 1 mM EDTA, pH 8.0, Sigma #93283) and quantified using a Qubit fluorometer (Invitrogen, Cat. No. Q32857; Eugene, OR), utilizing a Quant-iT dsDNA high sensitivity assay kit (Invitrogen; Cat. No. Q32854). Following DNA quantitation, samples were frozen and stored at −20˚C until use. Upon thawing, DNA samples were diluted to a concentration of 20 ng/μL in sterile, PCR-quality water and stored at 4˚C.

2.4. Primer Design

Primers for PCR amplification were designed using Primer 3 (v1.1.4) software [12]. The desired area of amplification was copied from the reference sequence of the OPN gene [GenBank: AY878328.1] and inserted into Primer 3 to generate forward and reverse primers flanking the desired area of amplification. The selected primers were then checked for uniqueness within the *Bos taurus* genome using Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, Bethesda, MD).

Two separate sets of primers were used to produce two DNA fragments of interest. Primers OPN3307F: 5'-AGC CCA CCA CCA AAT ACC TA-3' and OPN4006R: 5'-TCT GAA GGA CTG GCT TAG ATT TC-3' were used to amplify a 700 base pair (bp) region between bp 3307 and 4006 of the OPN gene promoter region that had reported polymorphisms [11], including OPN3907 which is believed to be a polymorphism linked to protein concentration in milk. Another set of primers, OPN4816F: 5'-TCC CTC CCT CTA CGT TTT CA-3' and OPN5528R: 5'-CAT CCC AAA AGG GCA TAG AA-3', amplified the region between bp 4816 and 5528 of the OPN gene promoter region that also had reported polymorphisms [11].

2.5. PCR Conditions and Sequencing

Polymerase chain reaction amplification was performed in a 50 μL total reaction volume that included 5 μL of 10 x PCR buffer, 1.5 mM $MgCl_2$, 250 μM dNTP mix, 40 pM of each primer, 100 ng of genomic DNA and 2 units of Biolase DNA polymerase (Bioline USA, Inc; Taunton, MA). Annealing temperature gradients were run to test the uniqueness of the primers and confirm the annealing temperature. The temperature cycles for DNA amplification were as follows: 35 cycles of 94˚C denaturation for 1 m, 59˚C annealing temperature for 45 s and 72˚C for 1 m extension time. Before sequencing, the PCR product was purified using a QIAquick PCR purification kit (QIAGEN; Cat. No. 28104). A sample of each purified product was run through a 1% agarose gel using TBE buffer (#BM-260A, Boston BioProducts, Ashland, MA) to confirm size and the presence of a single PCR product.

An ABI 3130xe (AME Bioscience; Toroed, Norway) analyzer was used for automated DNA sequencing by the DNA Technologies Laboratory at the University of Arkansas. Electropherograms for both forward and reverse complement DNA sequences were evaluated for polymorphisms using BioEdit Sequence Alignment Editor (Ibis Therapeutics, Version 7.0.5.3). Completed DNA sequences were aligned in ClustalW2 (European Molecular Biology Laboratory Outstation-European Bioinformatics Institute, Cambridge, UK; Version 2.0) to identify differences in genetic sequences among bulls and the reported normal reference sequence.

2.6. Statistical Analysis

Data were analyzed using the PROC MIXED model of SAS (SAS Inst., Inc.; Cary, NC) to determine any effect of individual SNP genotypes on CASA sperm motility measures. The percentages of motile, progressive and rapid sperm were described using the MEANS procedure. Differences at $P < 0.05$ were considered to be statistically significant. Within bulls, sperm motility data from each semen collection were analyzed as repeated

measures over time. Because of the differences in location, management, and semen collection interval, data collected from Angus and Angus × Gelbvieh bulls were analyzed separately from data collected from the Angus x Brahman bulls.

3. Results

Through amplification and sequencing of two separate DNA fragments, (bp 3307 to 4006 and bp 4816 to 5528; 700 and 707 bp products, respectively) of the OPN promoter region, 7 SNP sites were identified. Reported SNP sites were confirmed at bp 3379, 3490, 3492, and 5075, while previously unreported SNP were found at bp 5205, 5209, and 5263 of the OPN gene. Genotype and allele frequencies are summarized in **Table 1**. Minor allele frequencies for each sire group are presented in **Table 2**.

The minor allele frequencies reported in Holstein bulls for SNP 3379, 3490 and 3492 are 0.61, 0.80 and 0.61, respectively [11]. Across all beef bulls evaluated, the minor allele frequencies for the same SNP were lower at 0.45, 0.16 and 0.43, respectively. The minor allele frequency for the 7 SNP varied across sire groups (**Table 2**). The SNP located at bp3490, 5075, 5209 and 5263 of the OPN gene promoter occurred the least frequently in the Brahman x Angus bulls evaluated.

Genotypes within each SNP were compared for any effect on the percentage of motile, progressive and rapid sperm, as determined by CASA. No differences ($P > 0.05$) were detected in the percentages of motile, progressive and rapid sperm for bulls represented in genotypes for SNP 3490, 3492, 5075, 5205, 5209 and 5263. Substitution of guanine for thymine at bp 3379 was associated with an increase ($P \leq 0.02$) in the percentage of motile, progressive and rapid sperm collected from Brahman x Angus bulls (**Table 3**). This substitution also resulted in a trend ($P \leq 0.10$) for increased motile, progressive and rapid sperm in Angus and Angus x Gelbvieh bulls.

Table 1. Genotype and allele frequencies of single nucleotide polymorphisms within the promoter region of the OPN gene.

Polymorphism[a]	Homozygous primary allele	Heterozygous	Homozygous minor allele	PAF[b]	MAF[c]
T3379G	0.294	0.500	0.206	0.544	0.456
G3490A	0.735	0.206	0.059	0.838	0.162
A3492G	0.265	0.617	0.118	0.574	0.426
C5075T	0.676	0.265	0.059	0.809	0.191
C5205T	0.765	0.206	0.029	0.868	0.132
G5209A	0.618	0.353	0.029	0.794	0.206
G5263A	0.882	0.118	-----	0.941	0.059

[a]Single nucleotide polymorphism occurred at the base pair number indicated; the first letter represents the primary allele reported for the normal reference sequence and the letter following the number represents the resulting minor allele; [b]Primary Allele Frequency; [c]Minor Allele Frequency.

Table 2. Minor allele frequency of single nucleotide polymorphisms within the promoter region of the OPN gene for different sire groups.

	Angus (n = 5)	Angus x Gelbvieh (n = 14)	Brahman x Angus (n = 15)
Polymorphism[a]	Frequency[b]	Frequency[b]	Frequency[b]
T3379G	0.40	0.43	0.50
G3490A	0.20	0.32	0.00
A3492G	0.50	0.39	0.43
C5075T	0.20	0.32	0.13
C5205T	0.10	0.14	0.13
G5209A	0.20	0.29	0.13
G5263A	0.10	0.14	0.03

[a]The first letter represents the primary allele found in the reported, normal reference sequence and the letter following the base pair number represents the resulting minor allele. [b]Minor allele frequency.

Table 3. Effects of osteopontin polymorphism T3379G on mean percentage (±SE) of motile, rapid progressive and rapid sperm of Angus x Gelbvieh and Angus x Brahman bulls.

	T3379G Genotypes			*P* value
Sperm motility parameter[c]	TT	TG	GG	
Motile sperm (%)				
Angus and Angus-Gelbvieh	58.4 ± 3.2[a]	60.4 ± 3.0 [a]	67.6 ± 4.1[a]	0.096
Angus-Brahman	47.4 ± 5.8[b]	54.7 ± 3.2[b]	72.7 ± 5.8[a]	0.017
Progressive sperm (%)				
Angus and Angus-Gelbvieh	39.8 ± 2.5[a]	44.1 ± 2.3[a]	46.8 ± 3.2[a]	0.104
Angus-Brahman	36.9 ± 5.2[b]	42.2 ± 2.9[b]	57.7 ± 5.2[a]	0.023
Rapid sperm (%)				
Angus and Angus-Gelbvieh	54.4 ± 3.3[a]	57.8 ± 3.0[a]	64.3 ± 4.1[a]	0.080
Angus-Brahman	42.8 ± 5.7[b]	48.3 ± 3.1[b]	66.2 ± 5.7[a]	0.016

[a,b]Means within rows with different superscripts are significantly different ($P < 0.05$). [c]Motile = path velocity ≥ 30 μm/s and progressive velocity ≥ 15 μm/s; Progressive = path velocity ≥ 50 μm/s and straightness ≥ 70%; Rapid = progressive % with path velocity > 50 μm/s.

Numerically, the percentages of motile, progressive and rapid sperm showed consecutive increases for semen collected from bulls with the TT, TG and GG genotypes, respectively.

Genotypes within each SNP were compared for any effect on the percentage of motile, progressive and rapid sperm, as determined by CASA. No differences ($P > 0.05$) were detected in the percentages of motile, progressive and rapid sperm for bulls represented in genotypes for SNP 3490, 3492, 5075, 5205, 5209 and 5263. Substitution of guanine for thymine at bp 3379 was associated with an increase ($P \leq 0.02$) in the percentage of motile, progressive and rapid sperm collected from Brahman x Angus bulls (**Table 3**). This substitution also resulted in a trend ($P \leq 0.10$) for increased motile, progressive and rapid sperm in Angus and Angus x Gelbvieh bulls. Numerically, the percentages of motile, progressive and rapid sperm showed consecutive increases for semen collected from bulls with the TT, TG and GG genotypes, respectively.

4. Discussion

Killian *et al.* [1] evaluated proteins in the seminal plasma of Holstein bulls for potential markers for fertility. The bulls were of known fertility, based on pregnancy date from least 1000 inseminations per bull. A 55 kDa protein was found in higher concentrations in the seminal plasma of high fertility bulls when compared with bulls of average or below average fertility. This 55 kDa protein was later identified as osteopontin [13]. Any SNP within the osteopontin gene could positively or negatively influence expression of this protein in seminal plasma and thus, influence fertility. Therefore, the current study was conducted to identify any such polymorphisms that might influence on bull fertility.

Our results confirmed previously reported SNP sites at bp 3379, 3490, 3492, and 5075, and identified previously unreported SNP at bp 5205, 5209, and 5263 of the OPN gene. A previous study [11] reported a SNP located at bp 3907 influenced milk qualitative traits including protein content. This SNP resulted in deletion of a thymine within a polyT tract. The frequency of this polymorphism was reported to be only 0.05; none of the bulls in the current study were found to have this SNP.

Bull fertility assessment was based on *in vitro* sperm motility variables measured by CASA in the current study. This type of analysis provides objective, repeatable assessment of sperm characteristics [14], which correlate well with fertility in bulls [15] [16]. Farrell *et al.* [15] evaluated the relationship various CASA sperm variables and lifetime fertility of Holstein bulls, based on non-return rates after insemination. Sperm motility was identified as the single most important sperm parameter associated with fertility. Lin *et al.* [17] investigated potential candidate genes for sperm quality and fertility in boars and found that a polymorphism in intron 6 of the porcine OPN gene was associated with sperm motility and litter size. In the present study, a SNP at bp 3379 of the OPN promoter region was associated with an increase in the percentage of motile, progressive and rapid

sperm. The minor allele frequency of this polymorphism ranged from 0.40 to 0.50 among the groups of bulls evaluated. The frequency that the minor allele was detected suggests it occurs relatively frequently and could be used as a basis for selection.

5. Conclusion

Seven polymorphisms were identified within the promoter region of the bovine osteopontin gene. A SNP at bp 3379, resulting in substitution of guanine for thymine, was associated with improved sperm motility, progressive motility and progressive sperm with rapid motility. Although our data was limited, the results of this study suggested that further study was merited to determine associations OPN promoter and gene polymorphisms with bull semen quality.

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List of Abbreviations

Base Pair (bp)
Computer-Assisted Semen Analysis (CASA)
Deoxynucleotide (dNTP)
Dulbecco's Phosphate Buffered Saline (PBS)
Osteopontin (OPN)
Polymerase Chain Reaction (PCR)
Single Nucleotide Polymorphisms (SNP)
TRIS-Borate-EDTA Buffer (TBE)
TRIS-EDTA Buffer (TE)