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Effects of Osteopontin Single Nucleotide Polymorphisms on Bull Semen Quality

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of the requirements for the degree of
Master of Science in Animal Science

By

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Abstract

Two groups of bulls were utilized to evaluate if single nucleotide polymorphisms (SNP) in the *Bos taurus* OPN gene (GenBank accession # AY878328.1) promoter region were related to bull semen quality variables as evaluated by computer assisted semen analysis (CASA). Group 1 consisted of 19 Angus and Balancer bulls, ranging in age from 5 to 8 years, which were collected weekly during a 2-month period (July 15, 2010 through September 19, 2010). Group 2 consisted of 16 Brahman influenced (1/8 to 3/16 Brahman) bulls, mean age = 1.1 ± 0.1 year, that were collected monthly for a 7 month period (February 27, 2007 to August 16, 2007). Each collection was evaluated for motile, progressive, rapid, medium, slow, static, path velocity (VAP), progressive velocity (VSL), track speed (VCL), lateral amplitude (ALH), beat frequency (BCF), straightness (STR), linearity (LIN), elongation and area variables using CASA after semen collection. Each bull was genotyped for reported SNP in the promoter region of the OPN gene through PCR amplification of two 700 base pair fragments and sequencing of the resulting PCR product. Eight SNP sites were identified in Group 1 at base pairs 3379, 3490, 3492, 4967, 5075, 5205, 5209, and 5263 of the OPN gene and additional SNP at base pairs 3541, 3800 and 5262 in Group 2. Individual SNP sites were evaluated as the main effect on CASA measured variables in a SAS MIXED model for repeated measures. The SNP analysis found differences ($P \leq 0.05$) within groups for motile, rapid, progressive, VAP, VCL, ALH, LIN and elongation variables. Haplotypes also were constructed and compared for differences in sperm variables. In Group 1, certain haplotypes appear to be more favorable for percent motile, progressive and rapid sperm, as well as VAP, VSL, VCL, elongation and total abnormalities than bulls identified with

haplotype 1 (reported normal reference sequence). In Group 2, various differences were identified among haplotypes, but were only more favorable than haplotype 1 for BCF and live percentage. These results suggest that polymorphisms within the promoter region of the bovine OPN gene might be useful in certain populations for the selection of bulls with improved sperm quality.

This thesis is approved for recommendation
to the Graduate Council.

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To the farm crew at Savoy for all the help they provided during and after our research.

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All my great friends that I acquired here, thank you for always keeping things fun.

Dedication

I would like to dedicate this thesis to my family who has always pushed me to do my best and pursue my dreams.

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Chapter 1: Review of Literature

Introduction

Livestock producers and the artificial insemination industry depend on sires with high proven fertility. Recently, there have been several reports pointing to the use of seminal plasma protein concentrations and genetic markers as a more useful method for determining potential fertilizing capacity, rather than the evaluation of sperm quality, concentration and motility to predict a bull's fertilizing ability. Even though determining sperm cell concentration, motility and morphology is a simpler, more convenient method, it provides limited information on potential fertility of a sire (Elliot, 1978; Correa et al., 1997; Brahmkshtri et al., 1999). There is obvious evidence that sperm motility is important for fertilization (Mortimer, 1997) but, within groups of bulls that met a certain threshold for sperm motility, the contribution of this variable is limited in determining differences in fertility scores (Flowers, 1997). Artificial insemination centers have found bulls with similar sperm motility variables can differ in their non-return rates by as much as 20 to 25 % (Killian et al. 1993; Larson & Miller, 2000). It also has been shown that breeding soundness evaluations (BSE) can increase the fertility of range bulls as a group (Chenoweth et al., 1992) by removing bulls that do not meet the necessary thresholds (Hawkins et al., 1996) of 30 % motile sperm and 70 % normal morphology. However, a BSE was unable to eliminate subfertile sires with normal sperm parameters or predict fertilizing capacity (Braundmeier & Miller, 2001). Therefore, it is important to determine the subfertile sires that show normal semen quality by other means, leading to other venues of measuring fertility (Braundmeier & Miller, 2001).

Sperm acquires the ability to fertilize oocytes *in vivo* while in the epididymis (Bedford, 1966; Amann & Griel, 1974), but there is also evidence that accessory sex gland secretions may influence sperm physiology and fertilization. During ejaculation, sperm is mixed with secretions from accessory sex glands that contribute the majority of semen volume and components. Some secreted proteins present are known to affect sperm function and properties (Yanagimachi, 1994), and depletion of the accessory sex glands reduces subsequent embryonic development and survival (Chen et al., 2002). These reports suggest that components in the secreted accessory sex gland fluid may have a paternal affect on fertilization and embryonic survival. Henault et al. (1995) demonstrated that fluid collected from accessory sex glands of bulls enhanced the oocyte penetrating ability of sperm from the cauda epididymis, further indicating accessory sex gland secretions affects on post-ejaculation fertilizing ability. The potential influence of seminal plasma proteins on male reproduction has thus lead to the search for functional and molecular markers for fertility.

Killian et al. (1993) reported, based on protein analysis of ejaculates, in normal Holstein bulls with known reproductive history, four fertility-associated proteins present in seminal plasma. Two proteins (26 kDa, pI 6.2; 55 kDa, pI 4.5) were predominantly higher in bulls with known higher non-return rates (NRR), and two proteins (16 kDa, pI 6.7; 16 kDa, pI 4.1) were more prevalent in lower-fertility bulls. Later, the two proteins that were more prominent in higher fertility bulls were identified as prostaglandin D synthase (Gerena et al., 1998) and osteopontin (OPN, Cancel et al., 1997). Based on relative protein density levels, OPN was positively correlated ($r = 0.48$) with fertility (Cancel et al., 1997). Additional reports on proteomic analysis in seminal plasma yielded

other fertility-associated peptides that appear to be positively correlated with fertility such as heparin-binding protein-30, (which is also named fertility-associated antigen; FAA; Bellin et al., 1998) and Type-2 tissue inhibitor of metalloproteinases (TIMP-2; McCauley et al. 2001; Dawson et al., 2002). The presence of these proteins was associated with a 13 to 19 % increase in fertility of bulls (Bellin et al., 1994). Therefore, these studies provide evidence that seminal plasma proteins may have a paternal affect on fertilization.

Structure of Osteopontin

Osteopontin is a multifunctional protein that is typically involved in cell adhesion and tissue remodeling. It has been shown to stimulate cell-to-cell communication (Leibson et al., 1981; Miyauchi et al., 1991) and reduce reactive oxygen species (Hwang et al., 1994; Goldberg & Hunter, 1995). It also of interest in cancer research since it has been associated with malignant transformation and plays a crucial role in determining the oncogenic potential of various cancers (Weber, 2001; Rittling & Chambers, 2004). However, the main focus of this paper is related to the association as a fertility protein.

Osteopontin was first isolated from the mineralized matrix of bovine bone (Franzen & Heinegard, 1985). Subsequent studies detected the ubiquitous protein in numerous tissues and bodily fluids. Besides osteogenic cells, OPN mRNA has been detected in tendon cells, brain, kidney, ovary, and many other tissues (Kerr et al., 1991; Yoon et al., 1987; Young et al., 1990; Craig & Denhardt, 1991). Osteopontin also has been found in multiple fluids including seminal plasma (Killian et al., 1993; Cancel et al., 1997), urine (Shiraga et al., 1989; Khori et al., 1993; 1992) and milk (Sorensen et al.,

1993). Brown et al. (1992) also studied the expression of OPN in normal adult human tissues and found that OPN is present on the luminal surfaces of epithelial cells of the gastrointestinal tract, gall bladder, pancreas, and the urinary and reproductive tracts. Osteopontin expression also has been observed during the peri-implantation period in several species, including humans (Apparao et al., 2001; Von Wolff et al., 2001), goats (Joyce et al., 2005), pigs (Garlow et al., 2002), and rabbits (Apparao et al., 2003), demonstrating its importance in the female reproductive tract. The protein also has been found in uterine fluid and epithelium, with the highest concentration occurring during the luteal phase of the estrous cycle (Gabler et al., 2003).

Osteopontin is an acidic protein, rich in aspartic acid, glutamic acid and serine (Sorensen & Petersen, 1994). Amino acid sequences of OPN have been determined through cDNA sequences for multiple species including bovine (Kerr et al., 1991), swine (Zhang et al., 1990) and human (Khori et al., 1992); all of which show 40 % DNA similarity. These structural studies have shown that OPN is an arginine-glycine-aspartate (RGD) containing glycoprotein that includes a calcium binding domain, fibronectin cell binding site, heparin-binding domains, thrombin cleavage site and a polyaspartic acid domain. It also contains ~30 monosaccharides (Prince et al., 1987), and Sorensen et al. (1995) reported the existence of 28 phosphorylation and 3 O-glycosylation sites in the bovine milk OPN. These binding sites give rise to the variety of reported molecular weights of secreted OPN, which varies from 25 to 80 kDa (Denhardt et al., 2001). Bovine OPN, with the 278 amino acid residue backbone, has a predicted molecular weight of 41 kDa (Kerr et al., 1991) but isoforms of 14 to 55 kDa have been found in seminal plasma and accessory sex glands (Cancel et al., 1997 and 1999).

There are several functional domains on OPN. The arginine, glycine and aspartic acid (RGD) sequence links to β sheets and is a site for integrin binding. Antibodies against this RGD sequence inhibit cell adhesion and the deletion of the sequence renders OPN incapable of activating integrin receptors (Butler, 1995). Thrombin cleaves human OPN between amino acids R168 and S169 (Denhardt et al., 2001), resulting in two peptides of 28 to 35 kDa that increase the ability to induce cell adhesion and chemotaxis (Helluin et al., 2000). Sorensen et al. (1995) reported that bovine milk OPN appears to have a second thrombin cleavage site, found a few amino acids before the RGD sequence. Osteopontin also binds CD44 membrane glycoproteins, which is important for cell-cell interaction and signaling, migration and anti-apoptosis (Weber et al., 1996). Osteopontin also has shown indications of performing as a substrate for matrix metalloproteases MMP-3 and MMP-7, and the cleaved fragments enhance adhesion and migration *in vitro* through ligation to receptors including β1 integrin (Agnihotri et al., 2001).

The calcium binding domain is found between aspartic acid at 216 and serine at 228 amino acid residues in human OPN, and this amino acid fragment is absent in the OPN protein isolated from bovine milk (Kerr et al., 1991). In humans, osteopontin helps avert calcium crystal formation in the kidney (Mazzalli et al., 2002). Osteopontin also has two commonly regarded heparin-binding domains, located close to the SVVYGLR sequence and between D298 and I305 amino acid residues (CD44 binding motif). These domains may be of importance to male reproduction because some heparin-binding proteins (HBP) in seminal plasma have been found to have an association to fertility scores of bulls (Bellin et al., 1994, 1996; McCauley et al., 2001). These HBPs secreted

by accessory sex glands during ejaculation bind to sperm and mediate biochemical events related to capacitation (Miller et al., 1990).

Osteopontin's Role in the Male and Fertilization

Cancel et al. (1999) found, through the use of indwelling catheters in bulls' reproductive tracts and immunohistochemical analyses, that OPN is present on the epithelial surface and lumina of the ampulla and seminal vesicle but undetectable in the testis, epididymis, vas deferens, prostate and bulbourethral glands. This does differ from that found in the rat where OPN mRNA was found in the epididymal epithelium (Siiteri et al., 2005) and immunocytochemistry identified the protein in the cytoplasm of epithelial cells. However, the presence of osteopontin in seminal plasma appears to be relevant to its potential for aiding in fertilization.

In the rat, the Sertoli cells are thought to synthesize OPN, specifically a 60-kD OPN isoform that has been found in spermatogonia and early spermatocytes (Luedtke et al., 2002). However, in bull seminiferous tubules there has been detection of OPN mRNA, but were only associated with certain stages of the seminiferous cycle (Rodriguez et al., 2000). This suggests that OPN synthesis in the seminiferous tubules may be regulated by events that control spermatogenesis. The presence of OPN in the seminiferous tubules of the rat suggests that OPN may function as an anchor protein between germ cells, Sertoli cells and the extracellular matrix. Osteopontin may interact with cell surface integrins and CD44 (Gabler et al., 2003). Integrins are important for the movement of cells through the compartments during spermatogenesis. Thus, OPN may mediate intracellular events in both germ cells and Sertoli cells.

It is thought that OPN and other bovine seminal plasma proteins bind to sperm during ejaculation and therefore are carried to the site of fertilization (Souza et al., 2008). Once sperm reach the oocyte for fertilization, it has been suggested that OPN functions in the sperm-oocyte interaction and fertilization events (Moura et al., 2006). Souza et al. (2008) investigated the localization of fertility-related proteins on ejaculated sperm and the modifications that occur during exposure to oviductal secretions to give insight into their role in male fertility. Three separate incubation conditions were conducted: ejaculated sperm, ejaculated sperm with isthmic oviductal fluid, and ejaculated sperm with isthmic oviductal fluid incubated in ampullary oviductal fluid. An aliquot of semen from each incubation media was subjected to immunocytochemistry and analyzed by laser scanning confocal microscopy. Results showed that OPN binding was primarily found on the acrosomal cap of ejaculated sperm with less fluorescence on the post-equatorial segment and midpiece. Incubation of ejaculated sperm with isthmic oviductal fluid for 4 hours increased fluorescence of the post-equatorial segment, but without alterations to binding on the acrosomal cap or midpiece. Subsequent incubation with ampullary oviductal fluid did not change fluorescence on previous mentioned binding sites. Sperm cells with non-intact acrosomes showed reductions in fluorescence on the acrosomal area when compared to cells with intact acrosomes. However, fluorescence increased on the post-equatorial region of sperm cells with non-intact acrosomes. Results demonstrated the binding patterns of OPN on ejaculated sperm and sperm treated with isthmic and ampullary oviductal fluid. These binding patterns suggested that OPN and other seminal plasma proteins may be involved with the cholesterol and phospholipids removal of the outer acrosomal membrane (Manjunath and Therien, 2002). In this

model, bovine seminal plasma proteins remain bound to sperm through the female reproductive tract. When reaching the oviduct, the bound seminal plasmas interact with the HDL of the cell membrane to trigger a cholesterol influx to initiate capacitation. These findings propose that spermatozoa reach the site of fertilization with OPN bound to the equatorial and post-equatorial area, but have varying degrees of OPN attached to the acrosomal cap. Additionally, the link of OPN to the central region of the spermatozoa may be of importance because it is thought that membrane fusion occurs using the segments close to the equatorial region of the sperm (Gaddum-Rosse, 1985).

Through molecular characteristics of OPN, investigational support and hypotheses of Darribere et al. (2000) and Gabler et al. (2003), an early model of OPN's role in the sperm-egg interaction was depicted. In this model, seminal plasma OPN binds ejaculated sperm via surface integrins creating an OPN-integrin complex. This complex is then able to bind to oocyte integrins during fertilization events. Souza et al. (2008) and Moura et al. (2005) elaborate on these mechanisms by which OPN functions with sperm and the oocyte. It suggests that osteopontin binds to sperm during ejaculation, possibly through the interaction of OPN with surface integrins and/or CD44 receptors, and remains bound through the female reproductive tract to the site of fertilization. Oviductal fluid contains OPN (Gabler et al., 2003) which may attach to the zona prior to sperm reaching the site of fertilization. Upon reaching the oocyte, sperm-bound OPN will interact with the zona pellucida or form cross-links with OPN previously bound to the zona pellucida. Upon entering the perivitelline space, OPN bound to the equatorial area of the acrosome reacted sperm may link with integrin and CD44 receptors on the oocyte membrane. The cross-linking could then trigger intracellular signaling,

possibly affecting post-fertilization events. These proposed mechanics would lend to explain why exogenous OPN has positive effects on fertilization and early embryonic development.

Further research supports the idea that OPN is involved with the oocyte-sperm interaction and fertilization via *in vitro* fertilization studies. Osteopontin antibodies added to fertilization media, caused a reduction in the number of sperm bound to oocytes and the percentage of fertilized oocytes (88.64 % compared to 28.7 %; Goncalves, 2007). The addition of oocytes to non-luteal oviductal fluid and OPN antibodies also decreased the blastocyst percentage found on day 8 (from 22 % to 10.5 %) and hatched blastocysts on day 11 (from 8 % to 3 %; Goncalves et al., 2003). The RGD amino acid sequence in OPN molecules facilitates its linkage with α_5 and α_v integrins (Denhardt et al., 2001; Wai and Kuo, 2004) and the ability of osteopontin to support cell adhesion is prevented when the RGD sequence is mutated (Liaw et al., 1995; Xuan et al. 1995). Treatment of oocytes or sperm with a peptide lacking the RGD sequence or with α_5 and α_v integrin antibodies greatly reduced the number of sperm bound to the zona pellucida and fertilization percentages. Results from the use of antibodies against OPN, the RGD sequence and integrins help support the proposed mechanisms of action in which OPN functions in the sperm-egg interaction and fertilization. Furthermore, the addition of purified OPN (from milk) to sperm and oocytes showed to have a positive outcome of IVF. Goncalves et al., (2003) supported this concept through the incubation of oocytes with purified OPN which increased the cleavage rates at day 4 (from 78.1 % to 85.8 %), blastocyst development at day 8 (from 24.2 % to 33.8 %) and hatched blastocysts at day 11 (from 10.6 % to 18.5 %). Monaco et al. (2009) found similar results, with greater percentages of cleaved

zygotes and compact morulae-blastocysts were documented when incubated with certain concentrations of purified OPN *in vitro*. It also has been found that through the treatment of fertilization media with rat recombinant OPN greatly enhanced fertilization of swine oocytes by as much as 41 %. The same study also demonstrated that OPN reduced the rate of polyspermy in a dose-dependent manner and reduced the number of sperm in oocytes when compared to the untreated control (Hao et al., 2006). However, OPN also shows positive relationships with fertility when added to semen. Bull semen frozen with different concentrations of OPN had greater *in vitro* fertilization rates (85 to 78 % compared to 75 to 69 %) and blastocyst development at day 8 (45 % to 37 % compared with 33 % to 29 %) when compared to a control of untreated semen (Goncalves et al., 2008). The observation that the addition of OPN has an effect on post-fertilization development of embryos suggests that OPN may be associated with the triggering of intra-cellular events.

Osteopontin Gene Polymorphisms

Fertility traits are lowly heritable characteristics; however, some studies have stated that genetics may have a strong effect on fertility (Veerkamp & Beerda, 2007). It is estimated that about one-third of the decline in pregnancy rates of dairy cows can be accounted for by genetics (Shook, 2006). This reproductive decline is most likely due to the push for increased production traits. Recent research has utilized the use of microsatellite markers and quantitative trait loci (QTL) in the region of the OPN gene to determine the effects of single nucleotide polymorphism on fertility, milk protein percentage and other correlated traits (Leonard, 2005).

Osteopontin exists in many tissues and the protein is present in milk (Sorensen et al., 1993), urine (Shiraga et al., 1989; Khori et al., 1993; 1992) and seminal plasma (Killian et al., 1993). The average osteopontin concentration for human milk lies in a range of 3 to 10 µg/ml (Senger et al., 1989), while in cow's milk, a concentration of 8 mg/ml has been detected (Bayless et al., 1997), leading to the investigation of the osteopontin genes effects on milk production traits in dairy cattle.

With the use of whole genome scans, QTL have been identified that affect milk production traits on bovine chromosome 6 (BTA6) close to the osteopontin gene location. Ron et al., (2001) localized a QTL, which appears to have an effect on protein percentage in milk, to a confidence interval of 4 cM in the region of the osteopontin gene. Several other recent reports have identified a QTL near the same region that appears to affect milk yield, fat yield and protein yield. Cohen et al. (2004) were the first to begin sequencing candidate genes in this region. This QTL has now been fine mapped to a 420-kb interval between genes ABCG2 and LAP3. Within this region lie only four known genes: *ABSP*, *MEPE*, *PKD*2, and *OPN*.

Through sequencing and QTL analysis, Schnabel et al. (2005) identified the OPN gene (*OPN*) as the functional candidate for the QTL affecting milk protein percentage in the interstitial BTA6. A total of 12.3 kb of the OPN gene as sequenced and submitted to GenBank (Accession # AY878328.1). Within this submitted sequence, nine single nucleotide polymorphisms (SNP) were identified in eight sires, and of which, four of the sires all share the protein percentage decreasing QTL allele IBD. Therefore, it is likely that a SNP can be responsible for the detected variation in protein percentage. The only concordant SNP was *OPN3907*, located about 1,240 bp upstream from the *OPN*

transcription initiation site. This polymorphism occurs within a multiple thymine (T) sequence which normally produces 9 thymines, but the SNP results in a 10 thymine sequence.

Conclusion

Research has found that a single polymorphism or multiple polymorphisms found within a gene can alter transcription and ultimately, the resulting protein. Polymorphisms found within the coding region of the gene can modify the structure of the subsequent protein while non-coding polymorphisms can also have an effect on the quantity of the protein produced. A coding polymorphism example is a base change in the p53 human gene, causing a change in the amino acid sequence leading to a change in the overall subsequent protein structure (Buchman et al., 1988). An example of a non-coding polymorphism, the tumor necrosis factor -α (TNF- α) gene, located in the major histocompatibility complex (MHC), has demonstrated that a specific genotype could be associated with TNF-α expression (Jacob et al., 1990; Abraham et al., 1993; Pociot et al., 1993; Candore et al., 1994), suggesting that regulatory polymorphisms found in the TNF locus may be responsible for the variations seen. Also in the TNF- α gene, cell lines with differing genetic haplotypes have been shown to cause variations in TNF-α production (Abraham et al., 1993).

Understanding the effects single nucleotide polymorphisms can have on protein production, variations within the OPN gene could have similar outcomes for the fertility specific 55 kDa OPN protein, possibly, ultimately affecting fertilization events.

Osteopontin has been shown to have a significant association with fertility when present in higher concentrations of a bull's ejaculate. It is thought that through OPN-integrin complexes that form on the surface of ejaculated sperm, OPN may play a role in the sperm-egg interaction by aiding in binding through these complexes and also possibly initiating secondary messenger systems that aid in post-fertilization events. Therefore, it could be valuable to producers and the A.I. industry to determine relationships of SNP in the OPN gene with fertility and their use as potential fertility markers.

Chapter 2: Effects of Osteopontin Single Nucleotide Polymorphisms on Bull Semen Quality

Abstract

Two groups of bulls were utilized to evaluate if single nucleotide polymorphisms (SNP) in the *Bos taurus* OPN gene (GenBank accession # AY878328.1) promoter region were related to bull semen quality variables as evaluated by computer assisted semen analysis (CASA). Group 1 consisted of 19 Angus and Balancer bulls, ranging in age from 5 to 8 years, which were collected weekly during a 2-month period (July 15, 2010 through September 19, 2010). Group 2 consisted of 16 Brahman influenced (1/8 to 3/16 Brahman) bulls, mean age = 1.1 ± 0.1 year, that were collected monthly for a 7 month period (February 27, 2007 to August 16, 2007). Each collection was evaluated for motile, progressive, rapid, medium, slow, static, path velocity (VAP), progressive velocity (VSL), track speed (VCL), lateral amplitude (ALH), beat frequency (BCF), straightness (STR), linearity (LIN), elongation and area variables using CASA after semen collection. Each bull was genotyped for reported SNP in the promoter region of the OPN gene through PCR amplification of two 700 base pair fragments and sequencing of the resulting PCR product. Eight SNP sites were identified in Group 1 at base pairs 3379, 3490, 3492, 4967, 5075, 5205, 5209, and 5263 of the OPN gene and additional SNP at base pairs 3541, 3800 and 5262 in Group 2. Individual SNP sites were evaluated as the main effect on CASA measured variables in a SAS MIXED model for repeated measures. The SNP analysis found differences ($P \leq 0.05$) within groups for motile, rapid, progressive, VAP, VCL, ALH, LIN and elongation variables. Haplotypes also were constructed and compared for differences in sperm variables. In

Group 1, certain haplotypes appear to be more favorable for percent motile, progressive and rapid sperm, as well as VAP, VSL, VCL, elongation and total abnormalities than bulls identified with haplotype 1 (reported normal reference sequence). In Group 2, various differences were identified among haplotypes, but were only more favorable than haplotype 1 for BCF and live percentage. These results suggest that polymorphisms within the promoter region of the bovine OPN gene might be useful in certain populations for the selection of bulls with improved sperm quality.

Introduction

Osteopontin (OPN) is a ubiquitous, multi-function protein that has been found in higher concentrations of Holstein bull ejaculates that have known higher conception rates (Killian et al., 1993). Cancel et al. (2005) found that in the bull, OPN is secreted in the ampulla and seminal vesicles of the reproductive tract, where it is believed that OPN binds to sperm through CD44 and surface integrins. Ejaculated sperm then carries the bound OPN protein to the site of fertilization (Souza et al., 2008) where it is thought that OPN plays a role in the sperm-egg interaction through OPN-integrin complexes present on the surface of the sperm and oocyte (Darribere et al., 2000; Gabler et al., 2003). *In vitro* studies also have indicated that pre-treatment of bovine oocytes with purified OPN increased fertilization rate and embryo development (Goncalves et al., 2003 & 2006), as well as the ability to reduce polyspermy in porcine oocytes in a dose dependent manner (Hao et al., 2006). Osteopontin also has been shown to facilitate capacitation and viability of bovine sperm (Erikson et al., 2007a; Monaco et al., 2009), and OPN-treated bovine sperm are associated with increased *in vitro* fertilization and cleavage rates (Goncalves et al., 2008). Furthermore, studies have found that bovine ejaculated sperm

treated with OPN antibodies prior to *in vitro* incubation caused greater rates of binding to the zona pellucida but decreased rates of fertilization (Erikson et al., 2007b). These findings are similar to that in the pig (Hao et al., 2006), indicating that OPN may function to reduce polyspermy and facilitate fertilization events. Recent genetic research has demonstrated that polymorphisms within the OPN gene promoter region may be positively associated with higher protein concentrations and protein percentages in milk (Schnabel et al., 2005; Leonard et al., 2005). Therefore, any SNP that occur in the OPN gene might also affect fertility and thus, serve as a marker for fertility.

The objective of this study was to investigate the polymorphic nature of the *Bos taurus* OPN gene promoter region and determine any association of polymorphisms with bull semen quality variables.

Materials and Methods

Bull Management – Group 1

Methods and procedures involving the use of animals in this study were approved by the University of Arkansas Animal Care and Use Committee (Protocol # 11001). A group of 19 bulls, consisting of Angus and Balancer breeds, ranging in age from 5 to 9 years and weight from 709.8 to 954.8 kg were used for the study. Bulls were allotted into two separate groups prior to and during the project to reduce social (dominance) stress. During this period, bulls were fed 0.45 kg of high concentrate feed 3 times a week as well as *ad libitum* grass hay to help maintain body weight and condition. Prior to the test period, semen was collected from the bulls and evaluated to ensure sperm variables exceeded the minimum acceptable standards for a breeding soundness exam (BSE). The

experimental period ran for two months from July 15, 2010 through September 19, 2010 and bulls were maintained at the University of Arkansas Beef Research Unit near Savoy, AR. Bulls were collected via electro-ejaculation weekly for 9 consecutive weeks during the test period.

Bull Management - Group 2:

The committee of animal welfare at the USDA-ARS Dale Bumpers Small Farms Research Center, Booneville, AR, approved animal procedures used in this experiment. Sixteen Brahman-influenced bulls (1/8 to 3/16 Brahman) with a mean age of 1.1 + 0.1 year and mean body weight (BW) of 478 kg were used for the study. Prior to the test period, bulls were maintained on common bermudagrass pastures overseeded with Elbon rye. Bulls were blocked by BW, scrotal circumference (SC), percentage motile and progressive sperm, and total sperm (millions of cells) across three collection dates (February 27, March 20 and April 17, 2007) before beginning the experiment. For the experiment period, bulls were randomly assigned to graze tall fescue pastures for 121 days (April 17 to August 16, 2007). During this time, bulls were collected once monthly via electroejaculation.

Semen Analysis

Each semen collection was analyzed using a computer assisted sperm analysis (CASA; Hamilton Thorne Biosciences; Beverly, MA) after ejaculation. The sperm variables measured described in Table 1. Prior to analysis, each semen sample was diluted with Dulbecco's PBS to achieve a concentration of ~ 25 $x10^6$ sperm/ml before loading onto a 2X-CEL (Hamilton Thorne Biosciences; Beverly, MA) slide. The CASA

system scanned 8 to 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 sperm cells were counted on each slide to achieve a representation of the entire semen collection sample. A diluted sperm sample was also mounted with Orcein stain on a microscope slide for analysis of percent live.

DNA Extraction

Blood samples were obtained from each test bull via tail vein in EDTA, 8 ml vacuum tubes. Samples were placed on ice for transport to the lab. The samples were centrifuged at 1,200 g for 20 minutes at room temperature then buffy coats were extracted and stored at -20^{o} C. Genomic DNA was extracted using a Puregene DNA Purification kit (Gentra Systems; Cat. No. D-5000; Minneapolis, MN) with a mammalian whole blood protocol for purifying 300 µl of whole blood. However, 100 µl buffy coat was used due to the more concentrated nature of the white blood cells. The protocol included the use of red blood cell (RBC) lysis solution, protein precipitation solution, 100% isopropanol, 70% ethanol and DNA hydration solution for the isolation and preservation of the genomic DNA. All centrifugation steps were carried out at 13,000-16,000 x *g* in a microcentrifuge. Red blood cell lysis solution was added at a volume of 900 µl to the buffy coat volume in a 1.5 ml microcentrifuge tube. Red blood cell lysis solution and buffy coat was incubated for 1 minute and then centrifuged for 20 seconds. Supernatant was removed by pipette, leaving behind the pellet. This step was repeated with 300 µl RBC lysis solution, making sure to resuspend the pellet before incubation. After removing supernatant, the sample was placed on ice to cool for 1 minute. During this time, 100 µl of protein precipitation solution was added to the cell lysate. The

mixture was vortexed vigorously for 20 seconds to resuspend the pellet and thoroughly disperse the cell lysate throughout the solution. The sample was then centrifuged for 1 minute. The supernatant containing the DNA was poured into a clean 1.5 ml microcentrifuge tube containing 300 µl of 100% Isopropanol (2-propanol). The mixture was inverted approximately 50 times and then centrifuged, creating a small, white pellet of DNA. Supernatant was poured off and the tube was drained briefly on an absorbent paper. Following this, 300 µl of 70% ethanol was added to the tube with the DNA pellet and carefully inverted to disperse the DNA. The tube was then centrifuged for 1 minute. The supernatant was poured off, and 100 µl of DNA hydration solution was added to the tube. Contents were inverted to disperse DNA pellet. Isolated DNA samples were stored at 4^{o} C.

Isolated DNA was quantified using a Qubit™ fluorometer (Invitrogen; Cat. No. Q32857; Eugene, OR) and Quant-iT™ dsDNA high sensitivity assay kit (Invitrogen; Cat. No. Q32854; Eugene, OR). For DNA quantitation, the protocol for Quant-iT™ dsDNA HS assay was performed. Qubit™ assay tubes (Invitrogen; Cat. No. Q32856; Eugene, OR) were only used in the flourometer for determining DNA concentration. Prior to quantitation, a working solution was made by diluting Quant-iT dsDNA™ HS reagent 1:200 in Quant-iT™ dsDNA HS buffer. For the two standard solutions, 190 µl of the working solution was added to tubes and 10 µl of Quant-iT™ dsDNA HS standards were added. The standards were placed in the fluorometer in the order of 0 ng/ml then 10 ng/ml for calibration. For DNA samples, clean assay tubes were used and 199 µl of working solution added followed by 1 µl of isolated DNA solution. Duplicate readings were taken of each sample and recorded.

Following DNA quantitation, DNA samples were diluted to a concentration of 20 ng/µl. Sterile PCR quality water was used to dilute the samples in clean microcentrifuge tubes. Using the recorded DNA quantitation readings, a dilution factor was calculated for each sample to reach the desired DNA concentration in a total volume of 100 µl. Diluted DNA samples were stored at 4^{o} C.

Primer Design

Primers for PCR amplification were designed using Primer 3 (v1.1.4) software (Rozen and Skaletsky, 2000; Whitehead Institute for Biomedical Research; Cambridge, MA). The desired area of amplification was copied from the reference sequence of the OPN gene (GenBank accession # 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 bp region between base pairs 3307 and 4006 of the OPN gene promoter region that has reported polymorphisms (Schnabel et al., 2005), including *OPN3907* which is believed to be a polymorphism positively linked to higher 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 base pair 4816

and 5528 of the OPN gene promoter region that also has reported polymorphisms (Schnabel et al., 2005).

PCR Conditions

Polymerase chain reaction amplification was performed in a 50 µl total reaction volume; which included 5 µl of 10x PCR buffer, 1.5 mM $MgCl_2$, 250 µM dNTP mix, 40 pM of each primer, 100 ng of 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^{o} C denaturation for 1 minute, 59^{o} C annealing temperature for 45 seconds, and 72^{o} C for 1 minute extension time.

Gel Electrophoresis

The PCR products were separated using a 1% agarose gel using TBE buffer at 90 volts for a period of 4 hours. Five µl of Hyperladder II (Bioline USA, Inc.; Taunton, MA) was used as the standard to confirm size of the PCR product.

PCR Purification

Before genetic sequencing, the PCR product was purified to remove the primers, nucleotides, polymerases and salts used during the amplification process. Amplified DNA product was purified using QIAquick PCR purification kit (QIAGEN; Cat. No. 28104; Valencia, CA) and PCR purification spin protocol (QIAGEN; Valencia, CA), designed for the isolation of DNA fragments from PCR reactions. All centrifugation

steps were carried out at 10,000 x g in a conventional tabletop microcentrifuge at room temperature for 30-60 seconds. Five volumes of Buffer PBl were added to 1 volume of PCR sample and mixed. QIAquick spin columns were placed in 2 ml centrifuge tubes, and the sample was poured into the column then centrifuged, binding DNA in the column filter. Flow through in the 2 ml centrifuge tube was discarded and QIAquick column placed back in the same tube. To wash the bound DNA, 750 µl of Buffer PE was added to the column and then the sample was centrifuged. Again, flow through was discarded, then tube was centrifuged for an additional 1 minute. QIAquick column was then placed in a clean 1.5 ml microcentrifuge tube and DNA was eluted by adding 30 µl of Buffer EB and centrifuged. Flow through then contained purified PCR product. Purified product was stored at 4^{o} C.

Genetic Sequencing

For DNA sequencing, the DNA template and primers were diluted according to PCR product size. A DNA template ranging from 500-1000 bp was diluted to 5-20 ng and both forward and reverse primers were diluted to 3.4 pmol. DNA template and either forward or reverse primer was placed in a clean 1.5 ml microcentrifuge tube and the total volume of the tube was brought to 13 µl with sterile PCR quality water. 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 (Hall, 2005; Ibis Therapeutics, Version 7.0.5.3).

Completed sequences for Groups 1 and 2 were inserted separately and aligned in ClustalW2 (European Molecular Biology Laboratory Outstation-European Bioinformatics Institute, Hinxton, Cambridge, United Kingdom; Version 2.0) to show differences in genetic sequences among bulls and the reported normal reference sequence.

Statistical Analysis

Data were analyzed using a PROC MIXED model of SAS (SAS Inst., Inc.; Cary, NC). Semen collection readings for motile, progressive, rapid, medium, slow, static, path velocity (VAP), progressive velocity (VSL), track speed (VCL), lateral amplitude (ALH), beat frequency (BCF), straightness (STR), linearity (LIN), elongation and area variables were separated using the LSMEANS procedure. Each individual SNP site was analyzed as the main effect in a PROC MIXED model for repeated measures. The model included the individual SNP site and CASA measured variables. Unique haplotypes also were analyzed as the main effect in the PROC MIXED model for repeated measures with the same model as the SNP analyses.

Results and Discussion

Killian et al (1993) evaluated proteins in the seminal plasma of Holstein bulls for potential markers for fertility. The bulls were of known fertility, based on pregnancy data from at 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. Any polymorphisms 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 verify any such polymorphisms and determine their influence on bull fertility.

Through the amplification and sequencing of two separate DNA fragments, OPN gene base pair 3307 to 4006 and base pair 4816 to 5528 (700 bp and 707 bp products respectively) of the promoter region, 8 SNP sites were identified for bulls in Group 1 and 11 SNP sites for bulls in Group 2. The SNP sites found in Group1 were located at base pairs 3379, 3490, 3492, 4967, 5075, 5205, 5209, and 5263 of the OPN gene. The SNP sites found in Group 2 were located at base pairs 3379, 3490, 3492, 3541, 3800, 4967, 5075, 5205, 5209, 5262 and 5263. Genotype and allele frequencies for Groups 1 and 2 are summarized in Tables 2 and 3 respectively. Individual SNP sites were evaluated as the main effect for motile, progressive, rapid, medium, slow, static, path velocity (VAP), progressive velocity (VSL), track speed (VCL), lateral amplitude (ALH), beat frequency (BCF), straightness (STR), linearity (LIN), elongation and area variables as measured by CASA.

Group 1: Analysis of the individual SNP sites from bulls in Group 1 (Angus and Balancer) are summarized in Tables 4-11. All sperm quality variables were similar (P = 0.096) among genotypes for base pair 3379 (T to G; Table 4). Bulls with the GA genotype at base pair 3490 (G to A; Table 5) were higher (P = 0.013) than bulls with the GG homozygous primary allele genotype for LIN; however, bulls with the GG genotype were greater (P = 0.021) for elongation than bulls with the GA genotype. Bulls with the GG homozygous minor allele genotype at base pair 3492 (A to G; Table 6) were higher (P = 0.036) than bulls with the AA genotype for LIN. The analysis showed that bulls

with the CT genotype at base pair 4967 (C to T; Table 7) were higher ($P = 0.016$) than bulls with the CC homozygous primary allele genotype for percentage motile, progressive and rapid sperm cells. Bulls with the CT genotype at base pair 5075 (C to T; Table 8) were higher ($P = 0.013$) for LIN than bulls with the CC homozygous primary allele genotype; however, bulls with the CC genotype were higher ($P = 0.021$) for elongation than bulls with the CT genotype. Bulls identified with the CC homozygous primary allele genotype at base pair 5205 (C to T; Table 9) were higher ($P = 0.018$) for BCF than bulls with the TT homozygous minor allele genotype; also, bulls with the CT genotype were higher ($P = 0.05$) for elongation than bulls with the CC genotype. Bulls identified with the GA genotype at base pair 5209 (G to A/C; Table 10) were higher ($P = 0.036$) for VAP, VCL and ALH than bulls with the GG homozygous primary allele genotype. Bulls found with the GG and GA genotypes at the same base pair were higher ($P = 0.033$) for BCF than bulls with the AA genotype; also, bulls with the GG genotype were higher ($P = 0.036$) for STR than bulls with the GA, GC and AA genotypes. Bulls with the GG genotype at 5263 (G to A; Table 11) were higher ($P = 0.048$) for BCF than bulls with the GA genotype.

Haplotypes also were constructed based on the 8 SNP sites, producing 11 unique haplotypes for analysis. Haplotype construction and frequencies are summarized in Table 12. Bulls identified with haplotype 1 were the same as the normal reported reference sequence. Sperm motility for bulls identified with haplotypes 8 and 9 (82.3 and 81.3 % respectively; Summarized in Table 13) were significantly higher ($P < 0.05$) than that found of bulls with haplotypes 6, 7, 5 and 1 (57.3, 56.4, 54.8, and 42.3 % respectively). Bulls with haplotypes 8 and 9 were higher ($P < 0.05$) than bulls with

haplotypes 2, 6, 10, 5, 7, 3 and 1 for progressive motility (63.4 and 59.6 % respectively vs. 43.8, 43.6, 41.9, 40.8, 39.8, 37.3 and 31.4 % respectively), and also higher ($P < 0.05$) for rapid motility than bulls with haplotypes 6, 3, 7, 5 and 1 (79.2 and 80.0 % respectively vs. 55.1, 53.7, 53.1, 49.2, and 37.6% respectively). Path velocity (VAP) analysis showed that bulls with haplotypes 3 and 9 (152.3 and 145.7 µm/sec respectively) were greater ($P < 0.05$) than that of bulls with haplotypes 5 and 1 (117.3 and 101.6 µm/sec respectively). Bulls with haplotypes 3, 8, 9, 2 and 7 (117.3, 117.0, 114.3, 106.5 and 103.3 µm/sec respectively) also were greater ($P < 0.05$) for progressive velocity (VSL) than bulls with haplotype 1 (85.2 µm/sec). Track speed was greater ($P < 0.05$) for haplotypes 3, 9, 2 (265.1, 257.1, and 251.4 µm/sec respectively) than bulls with haplotypes 7, 4, 6, 5 and 1 (222.6, 218.8, 218.2, 194.6 and 181.1 µm/sec respectively). Bulls with haplotype 1 had a greater ($P < 0.05$) beat frequency (BCF) than bulls with haplotypes 7, 11 and 4 (26.0 vs. 21.5, 19.6 and 17.7 respectively). Straightness (STR) also was greater ($P < 0.05$) for bulls with haplotypes 1, 5 and 8 (84.3, 82.4 and 81.7 respectively) than bulls with haplotypes 2, 4, 10 and 11 (76.1, 75.7, 75.2 and 74.6 respectively). Linearity (LIN) was greater ($P < 0.05$) for bulls with haplotypes 5 and 8 (54.8 and 52.1 respectively) than bulls with haplotypes 2, 10 and 11 (45.2, 44.9 and 44.0 respectively). Elongation also was greater ($P < 0.05$) for bulls with haplotypes 10, 9, 5 and 11 (47.4, 47.1, 47.0 and 46.2 % respectively) than bulls with haplotypes 6, 8 and 1 (42.6, 42.3 and 40.1 % respectively). Area was greater ($P < 0.05$) for bulls with haplotype 1 (4.9 μ^2) than bulls with haplotypes 7, 10, 5 and 3 (4.6, 4.5, 4.5 and 4.4 μ^2 respectively). A greater occurrence ($P < 0.05$) of total sperm abnormalities were found

for bulls with haplotype 1 (15.7) than bulls with haplotypes 6, 9, 8 and 4 (9.1, 7.8, 7.3 and 7.1 respectively).

Group 2: Analysis of the individual SNP sites for bulls from Group 2 (Brahman influenced) are summarized in Tables 14-24. The analysis showed that bulls with the GG homozygous minor allele genotype at base pair 3379 (T to G; Table 14) were higher (P = 0.033) for percentage motile, progressive, rapid and live sperm than bulls with either the TT or TG genotypes. At the same base pair, bulls with the TG genotype were greater (P = 0.040) for BCF and STR than bulls with the TT genotype. At base pair 5263 (G to A; Table 23) bulls with the GG primary allele genotype were greater (P = 0.027) for ALH than bulls with the GA genotype. Comparison of the various sperm quality variables among the genotypes for the remaining polymorphisms (Tables 15-24) detected for bulls in Group 2, none ($P > 0.05$) were found to be significant.

Haplotypes also were constructed based on the 11 SNP sites, producing 11 unique haplotypes for analysis. Haplotype construction and frequencies are summarized in Table 25. Haplotype numbers are unique to each test group besides bulls identified with haplotype 1, which were the same as the normal reported reference sequence. The effect of the various haplotypes in sperm quality parameters is presented in Table 26. Sperm motility for bulls with haplotypes 7 and 9 (74.3 and 74.7 % respectively) were significantly higher ($P < 0.05$) than that found of bulls with haplotype 2 (36.8 %). Bulls with haplotypes 9 and 7 were higher ($P < 0.05$) for progressive sperm motility than bulls with haplotype 11 (63.0 and 55.7 % vs. 24.3 % respectively). Rapid sperm motility for bulls with haplotype 7 (66.5 %) were also higher ($P < 0.05$) than bulls with haplotype 2 (33.5 %). Path velocity (VAP) was greater ($P < 0.05$) for bulls with haplotype 6 (130.2

µm/sec) than bulls with haplotype 11 (88.4 µm/sec). Progressive velocity (VSL) was greater ($P < 0.05$) for bulls with haplotypes 6 and 1 (113.4 and 103.5 µm/sec respectively) than bulls with haplotype 11 (69.8 µm/sec). Lateral amplitude (ALH) was greater ($P < 0.05$) for bulls with haplotypes 2, 6 and 4 (7.3, 7.2 and 6.9 respectively) than bulls with haplotype 8 (5.2). Bulls with haplotype 4 (29.5) also were greater ($P < 0.05$) for beat frequency (BCF) than bulls with haplotypes 1 and 5 (23.6 and 22.9 respectively). Linearity was greater ($P < 0.05$) for bulls with haplotype 8 (63.3) than bulls with haplotype 11 (48.7). Live percentage was greater ($P < 0.05$) for bulls with haplotype 7 (82 %) than bulls with haplotype 1 (63%).

Through the analysis of the individual SNP sites and their effects on bull semen quality, two particular SNP (Group 1: CT genotype at C4967T and Group 2: GG genotype at T3379G) were found to be the more favorable genotypes. The comparison showed that within each of the individual SNP sites, bulls with the CT genotype at base pair 4967 in Group 1 and bulls with the GG genotype at base pair 3379 in Group 2 both had higher ($P \leq 0.05$) measurements for percent motile, progressive and rapid sperm cells. These measurements, even with the inability to predict fertility scores, are still the most important means of influencing fertility. However, the genotypic frequencies for these genotypes within each group were relatively low (CT: n = 1, 5.3 % and GG: n = 2, 12.4 %), thus, could limit their use as convenient markers of fertility.

Through the analysis of semen quality of bulls with extensive breeding information (minimum 6,346 first services per bull), Farrell et al. (1997) found that CASA measured sperm variables were correlated to lifetime fertility of the bull. In that experiment, Farrell used multiple combinations of sperm variables to correlate to bull

fertility, with the ALH, BCF, LIN, VAP and VSL combination yielding the highest correlation ($r^2 = 0.98$). Their results suggest that certain CASA measured sperm variables and their relationships may be useful for selecting bulls with greater potential fertility. With this information, the OPN haplotype effects on sperm quality may be useful in determining the potential fertility of bulls.

Conclusion

Through comparison of bull haplotypes, based on SNP in the OPN gene promoter region, we found that genotypes may be useful for selecting bulls with improved semen quality. This may give insight to a potential method for developing a genetic approach for predicting bull fertility. Through a genetic approach, producers and AI studs or organizations may be able to identify and possibly eliminate subfertile sires and also determine high fertility sires.

Our data set is limited and additional data would be needed to confirm these findings. Furthermore, because OPN density in ejaculates has been shown to affect bull fertility rates, SNP and haplotype effects on OPN levels would be the next step in determining the usefulness as potential markers of fertility.

Table 1: Description of Sperm Variables Measured by the Hamilton-Thorne Sperm Analyzer

Variable	Description
Motile	Path velocity ≥ 30 µm/sec and progressive velocity ≥ 15 µm/sec
Progressive	Path velocity ≥ 50 µm/sec and straightness ≥ 70%
Rapid	Progressive % with path velocity > 50 µm/sec
Medium	Progressive % with path velocity < 50 µm/sec but > 30 µm/sec
Slow	Path velocity < 30 µm/sec and progressive velocity < 15 µm/sec
Static	Sperm not moving at all.
Path velocity (VAP)	Average velocity of the smoothed cell path in µm/sec
Progressive velocity (VSL)	Average velocity measured in a straight line over entire track
Track speed (VCL)	Average velocity measured over the actual point-to-point track
Lateral amplitude (ALH)	Mean width of the head oscillation as the sperm swims
Beat frequency (BCF),	Frequency of sperm head crossing the sperm average path
Straightness (STR)	Departure of average sperm path from straight line (VSL/VAP)
Linearity (LIN)	Departure of actual sperm track from straight line (VSL/VCL)
Elongation	Ratio (%) of head width to head length
Area	Average size in square microns of all sperm heads

Table 2: Genotype and allele frequencies of single nucleotide polymorphisms found within the promoter region of the OPN gene of Angus and Balancer bulls.

Polymorphism[a]	Homozygous primary allele	Heterozygous	Homozygous minor allele	PAF[b]	MAF[c]
T3379G	0.368	0.421	0.211	0.579	0.421
G3490A	0.526	0.368	0.105	0.711	0.289
A3492G	0.368	0.421	0.211	0.579	0.421
C4967T	0.947	0.053	---------	0.974	0.026
C5075T	0.526	0.368	0.105	0.711	0.289
C5205T	0.789	0.158	0.053	0.868	0.132
G5209A	0.474	0.421	0.053	0.711	0.263
G5209C	0.474	0.053	---------	0.711	0.026
G5263A	0.842	0.158	---------	0.921	0.079

[a] Single nucleotide polymorphism occurred at the base pair number represented, first letter represents primary allele found in the reported normal reference sequence and the letter following the digits represents the resulting minor allele

[b] Primary Allele Frequency

[c] Minor Allele Frequency

Table 3: Genotype and allele frequencies of SNP found in the promoter region of the OPN gene of Brahman influenced bulls.

Polymorphism[a]	Homozygous primary allele	Heterozygous	Homozygous minor allele	PAF[b]	MAF[c]
T3379G	0.188	0.688	0.124	0.532	0.468
G3490A	0.938	0.062	---------	0.969	0.031
A3492G	0.125	0.875	---------	0.563	0.437
G3541A	0.938	0.062	---------	0.969	0.031
T3800C	0.938	0.062	---------	0.969	0.031
C4967T	1.000	---------	---------	1.000	---------
C5075T	0.813	0.187	---------	0.907	0.093
C5205T	0.75	0.250	---------	0.875	0.125
G5209A	0.75	0.250	---------	0.875	0.125
C5262T	0.875	0.125	---------	0.938	0.062
G5263A	0.938	0.062	---------	0.969	0.031

[a] Single nucleotide polymorphism occurred at the base pair number represented, first letter represents primary allele found in the reported normal reference sequence and the letter following the digits represents the resulting minor allele

[b] Primary Allele Frequency

[c] Minor Allele Frequency

Table 4: Effects of osteopontin polymorphism T3379G on sperm quality variables in Angus and Balancer bulls.

	T3379G*			
Variable	**TT**	**TG**	**GG**	**P value**
No. of Bulls	7	8	4	----
Motile	58.4 ± 3.2 [a]	60.4 ± 3.0 [a]	67.6 ± 4.1[a]	0.096
Progressive	39.8 ± 2.5 [a]	44.1 ± 2.3 [a]	46.8 ± 3.2 [a]	0.104
Rapid	54.4 ± 3.3 [a]	57.8 ± 3.0 [a]	64.3 ± 4.1 [a]	0.080
VAP	127.8 ± 3.5 [a]	132.1 ± 3.2 [a]	135.2 ± 4.4 [a]	0.210
VSL	100.5 ± 2.9 [a]	104.6 ± 2.7 [a]	106.2 ± 3.7 [a]	0.246
VCL	224.0 ± 6.1 [a]	227.24 ± 5.6 [a]	235.2 ± 7.7 [a]	0.268
ALH	8.5 ± 0.2 [a]	8.5 ± 0.2 [a]	8.9 ± 0.3 [a]	0.248
BCF	22.3 ± 0.7 [a]	21.7 ± 0.6 [a]	21.9 ± 0.9 [a]	0.544
STR	78.9 ± 0.8 [a]	79.0 ± 0.7 [a]	78.1 ± 1.0 [a]	0.473
LIN	48.0 ± 0.9 [a]	49.0 ± 0.8 [a]	47.7 ± 1.1 [a]	0.383
Elongation	44.1 ± 0.5 [a]	44.3 ± 0.5 [a]	45.4 ± 0.6 [a]	0.147
Area	4.7 ± 0.05 [a]	4.6 ± 0.04 [a]	4.6 ± 0.06 [a]	0.401
Live	73.1 ± 2.1 [a]	73.8 ± 1.9 [a]	77.5 ± 2.7 [a]	0.215

[a, b] Means within rows with different superscripts are significantly different

***** Genotypic frequencies for TT, TG and GG are 0.368, 0.421 and 0.211 respectively

Table 5: Effects of osteopontin polymorphism G3490A on sperm quality variables in Angus and Balancer bulls.

	G3490A*			
Variable	**GG**	**GA**	**AA**	**P value**
No. of Bulls	10	7	2	----
Motile	62.8 ± 2.7 [a]	58.7 ± 3.2 [a]	62.7 ± 5.8 [a]	0.348
Progressive	42.9 ± 2.1 [a]	44.3 ± 2.5 [a]	40.9 ± 4.6 [a]	0.535
Rapid	59.3 ± 2.7 [a]	55.9 ± 3.3 [a]	59.1 ± 5.9 [a]	0.440
VAP	131.5 ± 2.9 [a]	130.9 ± 3.5 [a]	131.4 ± 6.3 [a]	0.902
VSL	102.2 ± 2.4 [a]	105.4 ± 2.9 [a]	103.5 ± 5.3 [a]	0.409
VCL	231.8 ± 5.0 [a]	221.5 ± 6.0 [a]	229.8 ± 10.8 [a]	0.203
ALH	8.7 ± 0.2 [a]	8.3 ± 0.2 [a]	8.9 ± 0.4 [a]	0.150
BCF	22.1 ± 0.6 [a]	21.9 ± 0.7 [a]	21.3 ± 1.2 [a]	0.571
STR	78.0 ± 0.6 [a]	80.1 ± 0.8 [a]	77.8 ± 1.4 [a]	0.053
LIN	47.3 ± 0.7 [b]	50.4 ± 0.9 [a]	46.9 ± 1.5 [ab]	0.013
Elongation	45.0 ± 0.4 [a]	43.3 ± 0.5 [b]	45.9 ± 0.9 [a]	0.021
Area	4.6 ± 0.04 [a]	4.6 ± 0.05 [a]	4.7 ± 0.08 [a]	0.505
Live	75.1 ± 1.7 [a]	72.5 ± 2.1 [a]	77.4 ± 3.8 [a]	0.267

[a, b] Means within rows with different superscripts are significantly different

***** Genotypic frequencies for GG, GA and AA are 0.526, 0.368 and 0.105 respectively

Table 6: Effects of osteopontin polymorphism A3492G on sperm quality variables in Angus and Balancer bulls.

	A3492G*			
Variable	**AA**	**AG**	**GG**	**P Value**
No. of Bulls	7	8	4	----
Motile	62.5 ± 3.2 [a]	58.0 ± 3.0 [a]	65.6 ± 4.1 [a]	0.150
Progressive	42.7 ± 2.5 [a]	41.8 ± 2.4 [a]	46.5 ± 3.2 [a]	0.252
Rapid	59.2 ± 3.2 [a]	55.1 ± 3.0 [a]	61.6 ± 4.2 [a]	0.226
VAP	132.2 ± 3.5 [a]	130.7 ± 3.3 [a]	130.8 ± 4.5 [a]	0.748
VSL	103.3 ± 3.0 [a]	102.8 ± 2.7 [a]	105.1 ± 3.7 [a]	0.620
VCL	233.7 ± 6.0 [a]	225.3 ± 5.6 [a]	223.2 ± 7.7 [a]	0.300
ALH	8.9 ± 0.2 [a]	8.4 ± 0.2 [a]	8.4 ± 0.3 [a]	0.210
BCF	22.3 ± 0.7 [a]	21.7 ± 0.6 [a]	21.8 ± 0.9 [a]	0.523
STR	78.3 ± 0.8 [a]	78.6 ± 0.7 [a]	79.9 ± 1.0 [a]	0.234
LIN	47.0 ± 0.9 [b]	48.6 ± 0.8 [ab]	50.2 ± 1.1 [a]	0.036
Elongation	44.2 ± 0.5 [a]	44.4 ± 0.5 [a]	45.3 ± 0.6 [a]	0.190
Area	4.7 ± 0.05 [a]	4.6 ± 0.04 [a]	4.6 ± 0.06 [a]	0.335
Live	74.6 ± 2.1 [a]	73.3 ± 1.9 [a]	76.1 ± 2.7 [a]	0.400

[a, b] Means within rows with different superscripts are significantly different

***** Genotypic frequencies for AA, AG and GG are 0.368, 0.421 and 0.211 respectively

Table 7: Effects of osteopontin polymorphism C4967T on sperm quality variables in Angus and Balancer bulls.

	C4967T*		
Variable	**CC**	**CT**	**P Value**
No. of Bulls	18	1	----
Motile	60.0 ± 2.0 [b]	82.3 ± 8.0 [a]	0.015
Progressive	42.0 ± 1.5 [b]	63.4 ± 6.2 [a]	0.004
Rapid	56.8 ± 2.0 [b]	79.2 ± 8.2 [a]	0.016
VAP	130.6 ± 2.1 [a]	143.1 ± 8.9 [a]	0.186
VSL	102.7 ± 1.8 [a]	117.0 ± 7.4 [a]	0.076
VCL	227.2 ± 3.7 [a]	238.6 ± 15.3 [a]	0.479
ALH	8.6 ± 0.1 [a]	8.7 ± 0.6 [a]	0.823
BCF	22.0 ± 0.4 [a]	22.0 ± 1.8 [a]	0.978
STR	78.6 ± 0.5 [a]	81.7 ± 2.0 [a]	0.154
LIN	48.1 ± 0.5 [a]	52.1 ± 2.2 [a]	0.100
Elongation	44.6 ± 0.3 [a]	42.3 ± 1.3 [a]	0.103
Area	4.6 ± 0.03 [a]	4.6 ± 0.1 [a]	0.805
Live	74.2 ± 1.3 [a]	78.3 ± 5.3 [a]	0.457

[a, b] Means within rows with different superscripts are significantly different

***** Genotypic frequencies for CC and CT are 0.947 and 0.053 respectively

Table 8: Effects of osteopontin polymorphism C5075T on sperm quality variables in Angus and Balancer bulls.

	C5075T*			
Variable	**CC**	**CT**	**TT**	**P Value**
No. of Bulls	10	7	2	----
Motile	62.8 ± 2.7 [a]	58.7 ± 3.2 [a]	62.7 ± 5.8 [a]	0.348
Progressive	42.9 ± 2.1 [a]	44.3 ± 2.5 [a]	40.9 ± 4.6 [a]	0.535
Rapid	59.3 ± 2.7 [a]	55.9 ± 3.3 [a]	59.1 ± 5.9 [a]	0.440
VAP	131.5 ± 2.9 [a]	130.9 ± 3.5 [a]	131.4 ± 6.3 [a]	0.902
VSL	102.2 ± 2.4 [a]	105.4 ± 2.9 [a]	103.5 ± 5.3 [a]	0.409
VCL	231.8 ± 5.0 [a]	221.5 ± 6.0 [a]	229.8 ± 10.8 [a]	0.203
ALH	8.7 ± 0.2 [a]	8.3 ± 0.2 [a]	8.9 ± 0.4 [a]	0.150
BCF	22.1 ± 0.6 [a]	21.9 ± 0.7 [a]	21.3 ± 1.2 [a]	0.571
STR	78.0 ± 0.6 [a]	80.1 ± 0.8 [a]	77.8 ± 1.4 [a]	0.053
LIN	47.3 ± 0.7 [b]	50.4 ± 0.9 [a]	46.9 ± 1.5 [ab]	0.013
Elongation	45.0 ± 0.4 [a]	43.3 ± 0.5 [b]	45.9 ± 0.9 [a]	0.021
Area	4.6 ± 0.04 [a]	4.6 ± 0.05 [a]	4.7 ± 0.08 [a]	0.505
Live	75.1 ± 1.7 [a]	72.5 ± 2.1 [a]	77.4 ± 3.8 [a]	0.267

[a, b] Means within rows with different superscripts are significantly different

***** Genotypic frequencies for CC, CT and TT are 0.526, 0.368 and 0.105 respectively

Table 9: Effects of osteopontin polymorphism C5205T on sperm quality variables in Angus and Balancer bulls.

	C5205T*			
Variable	**CC**	**CT**	**TT**	**P Value**
No. of Bulls	15	3	1	----
Motile	60.7 ± 2.2 [a]	61.4 ± 4.7 [a]	69.7 ± 8.2 [a]	0.306
Progressive	43.3 ± 1.7 [a]	41.7 ± 3.7 [a]	44.9 ± 6.5 [a]	0.680
Rapid	57.5 ± 2.2 [a]	57.5 ± 4.8 [a]	66.4 ± 8.4 [a]	0.319
VAP	130.6 ± 2.4 [a]	135.7 ± 5.1 [a]	127.0 ± 8.9 [a]	0.388
VSL	103.5 ± 2.0 [a]	105.3 ± 4.3 [a]	97.6 ± 7.5 [a]	0.386
VCL	226.9 ± 4.1 [a]	235.4 ± 8.9 [a]	218.8 ± 15.4 [a]	0.362
ALH	8.5 ± 0.2 [a]	8.7 ± 0.3 [a]	8.8 ± 0.6 [a]	0.725
BCF	22.4 ± 0.5 [a]	21.3 ± 1.0 [ab]	17.7 ± 1.7 [b]	0.018
STR	79.2 ± 0.5 [a]	77.9 ± 1.1 [a]	75.7 ± 2.0 [a]	0.105
LIN	48.5 ± 0.6 [a]	48.5 ± 1.3 [a]	46.0 ± 2.2 [a]	0.298
Elongation	44.2 ± 0.3 [b]	46.0 ± 0.7 [a]	43.7 ± 1.3 [ab]	0.050
Area	4.6 ± 0.03 [a]	4.5 ± 0.07 [a]	4.6 ± 0.1 [a]	0.135
Live	74.2 ± 1.4 [a]	75.7 ± 3.1 [a]	73.1 ± 5.3 [a]	0.668

[a, b] Means within rows with different superscripts are significantly different

***** Genotypic frequencies for CC, CT and TT are 0.789, 0.158 and 0.053 respectively

Table 10: Effects of osteopontin polymorphism G5209A/C on sperm quality variables in Angus and Balancer bulls.

	G5209A/C*				
Variable	**GG**	**GA**	**GC**	**AA**	**P Value**
No. of Bulls	9	8	1	1	----
Motile	57.1 ± 2.9 [a]	64.2 ± 2.9 [a]	62.6 ± 8.2 [a]	69.7 ± 8.2 [a]	0.104
Progressive	41.3 ± 2.3 [a]	44.9 ± 2.3 [a]	41.9 ± 6.5 [a]	44.9 ± 6.5 [a]	0.279
Rapid	53.6 ± 2.9 [a]	61.2 ± 2.9 [a]	59.7 ± 8.3 [a]	66.4 ± 8.3 [a]	0.088
VAP	126.2 ± 3.1 [b]	136.4 ± 3.1 [a]	134.9 ± 8.8 [ab]	127.0 ± 8.8 [ab]	0.036
VSL	101.8 ± 2.6 [a]	106.2 ± 2.6 [a]	100.8 ± 7.5 [a]	97.6 ± 7.5 [a]	0.257
VCL	217.2 ± 5.3 [b]	237.8 ± 5.3 [a]	242.5 ± 15.0 [ab]	218.8 ± 15.0 [ab]	0.015
ALH	8.1 ± 0.2 [b]	9.0 ± 0.2 [a]	9.1 ± 0.6 [ab]	8.8 ± 0.6 [ab]	0.008
BCF	22.3 ± 0.6 [a]	22.0 ± 0.6 [a]	22.7 ± 1.7 [ab]	17.7 ± 1.7 [b]	0.033
STR	80.4 ± 0.7 [a]	78.0 ± 0.7 [b]	75.2 ± 1.9 [b]	75.7 ± 1.9 [b]	0.036
LIN	49.6 ± 0.8 [a]	47.8 ± 0.8 [a]	44.9 ± 2.2 [a]	46.0 ± 2.2 [a]	0.062
Elongation	43.5 ± 0.4 [c]	45.2 ± 0.4 [ab]	47.4 ± 1.3 [a]	43.7 ± 1.3 [bc]	0.050
Area	4.7 ± 0.04 [a]	4.6 ± 0.04 [a]	4.5 ± 0.1 [a]	4.6 ± 0.1 [a]	0.207
Live	73.4 ± 1.9 [a]	75.3 ± 1.9 [a]	76.8 ± 5.3 [a]	73.1 ± 5.3 [a]	0.492

[a, b] Means within rows with different superscripts are significantly different

***** Genotypic frequencies for GG, GA, GC and AA are 0.474, 0.421, 0.053 and 0.053 respectively

Table 11: Effects of osteopontin polymorphism G5263A on sperm quality variables in Angus and Balancer bulls.

	G5263A*		
Variable	**GG**	**GA**	**P Value**
No. of Bulls	16	3	----
Motile	60.4 ± 2.1 [a]	65.7 ± 4.7 [a]	0.316
Progressive	42.9 ± 1.7 [a]	44.3 ± 3.7 [a]	0.750
Rapid	57.3 ± 2.2 [a]	61.8 ± 4.8 [a]	0.406
VAP	132.1 ± 2.3 [a]	127.2 ± 5.1 [a]	0.401
VSL	104.4 ± 1.9 [a]	98.7 ± 4.3 [a]	0.237
VCL	229.4 ± 4.0 [a]	220.0 ± 8.8 [a]	0.343
ALH	8.6 ± 0.2 [a]	8.3 ± 0.3 [a]	0.446
BCF	22.4 ± 0.4 [a]	20.0 ± 1.0 [b]	0.048
STR	79.0 ± 0.5 [a]	77.6 ± 1.2 [a]	0.260
LIN	48.4 ± 0.6 [a]	48.3 ± 1.3 [a]	0.935
Elongation	44.3 ± 0.3 [a]	45.6 ± 0.7 [a]	0.110
Area	4.6 ± 0.03 [a]	4.6 ± 0.07 [a]	0.607
Live	74.3 ± 1.4 [a]	74.8 ± 3.1 [a]	0.886

[a, b] Means within rows with different superscripts are significantly different

***** Genotypic frequencies for GG and GA are 0.842 and 0.158 respectively

Table 12: Haplotype construction and frequency for Angus and Balancers bulls based on the individual SNP sites

	Base pair of OPN gene where polymorphism occurred									
Haplotype	3379	3490	3492	4967	5075	5205	5209	5263	Haplotype Frequency	No. of Bulls
1[a]	TT	GG	AA	CC	CC	CC	GG	GG	0.105	2
2	TT	GG	AA	CC	CC	CC	GA	GG	0.105	2
3	TT	GG	AA	CC	CC	CT	GA	GG	0.053	1
4	TT	GG	AA	CC	CC	TT	AA	GA	0.053	1
5	TT	GG	GG	CC	CC	CT	GA	GA	0.053	1
6	TG	GA	AG	CC	CT	CC	GA	GG	0.105	2
7	TG	GA	AG	CC	CT	CC	GG	GG	0.316	6
8	GG	GA	GG	CT	CT	CC	GG	GG	0.053	1
9	TG	GG	AA	CC	CC	CC	GA	GG	0.053	1
10	GG	GG	AG	CC	CC	CC	GC	GG	0.053	1
11	TG	GG	AG	CC	CC	CT	GA	GA	0.053	1

[a] Bulls with haplotype 1 were the same as the reported normal reference sequence

Table 13: Effects of osteopontin bull haplotypes from Group 1 on sperm quality variables.

	Haplotype										
Variable	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Motile	42.3 ± 6.4^{d}	66.8 ± 5.4^{ab}	56.6 ± 7.7^{bc}	69.7 ± 7.7^{ab}	54.8 ± 7.7^{cd}	57.3 ± 5.4^{cd}	56.4 ± 3.3^{cd}	82.3 ± 7.7^{a}	81.3 ± 7.7^{ab}	62.6 ± 7.7^{abcd}	72.8 ± 7.7^{abc}
Progressive	31.4 ± 5.1^{b}	43.8 ± 4.3^{b}	37.3 ± 6.1^{b}	44.9 ± 6.1^{ab}	40.8 ± 6.1^{b}	43.6 ± 4.3^{b}	39.8 ± 2.6^{b}	63.4 ± 6.1^{a}	59.6 ± 6.1^{a}	41.9 ± 6.1^{b}	47.1 ± 6.1^{ab}
Rapid	37.6 ± 6.5^{c}	63.3 ± 5.5^{ab}	53.7 ± 7.7^{bc}	66.4 ± 7.7^{ab}	49.2 ± 7.7^{bc}	55.1 ± 5.5^{bc}	53.1 ± 3.3^{bc}	79.2 ± 7.7^{a}	80.0 ± 7.7^{a}	59.7 ± 7.7^{abc}	69.7 ± 7.7^{ab}
VAP	101.6 ± 6.8^{d}	140.0 ± 5.8^{abc}	152.3 ± 8.2^{a}	127.0 ± 8.2^{bc}	117.3 ± 8.2^{cd}	129.1 ± 5.8^{bc}	129.5 ± 3.5^{bc}	143.1 ± 8.2^{abc}	145.7 ± 8.2^{ab}	134.9 ± 8.2^{abc}	137.4 ± 8.2^{abc}
VSL	85.2 ± 6.0^{b}	106.5 ± 5.1^{a}	117.3 ± 7.2^{a}	97.6 ± 7.2^{ab}	96.5 ± 7.2^{ab}	103.3 ± 5.1^{ab}	103.3 ± 3.1^{a}	117.0 ± 7.2^{a}	114.3 ± 7.2^{a}	100.8 ± 7.2^{ab}	102.0 ± 7.2^{ab}
VCL	181.1 ± 11.5^{d}	251.4 ± 9.8^{a}	265.1 ± 13.8^{a}	218.8 ± 13.8^{bcd}	194.6 ± 13.8^{cd}	218.2 ± 9.8^{bcd}	222.6 ± 5.9^{bc}	238.6 ± 13.8^{abc}	257.1 ± 13.8^{a}	242.5 ± 13.8^{ab}	246.5 ± 13.8^{ab}
ALH	6.9 ± 0.4^{b}	9.6 ± 0.4^{a}	9.8 ± 0.5^{a}	8.8 ± 0.5^{a}	7.1 ± 0.5^{ab}	8.6 ± 0.4^{a}	8.3 ± 0.2^{a}	8.7 ± 0.5^{a}	9.4 ± 0.5^{a}	9.1 ± 0.5^{a}	9.1 ± 0.5^{a}
BCF	26.0 ± 1.4^{a}	22.1 ± 1.2^{ab}	21.7 ± 1.7^{ab}	17.7 ± 1.7^{b}	22.7 ± 1.7^{ab}	22.6 ± 1.2^{ab}	21.5 ± 0.7^{b}	22.0 ± 1.7^{ab}	22.8 ± 1.7^{ab}	22.7 ± 1.7^{ab}	19.6 ± 1.7^{b}

$^{a, b, c\ d}$ Means within rows with different superscripts are different ($P \leq 0.05$)

Table 13 (cont.): Effects of osteopontin bull haplotypes from Group 1 on sperm quality variables.

	Haplotype										
Variable	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
STR	84.3 ± 1.5^{a}	76.1 ± 1.3^{c}	76.7 ± 1.8^{bc}	75.7 ± 1.8^{c}	82.4 ± 1.8^{ab}	79.7 ± 1.3^{abc}	79.2 ± 0.8^{abc}	81.7 ± 1.8^{ab}	78.8 ± 1.8^{abc}	75.2 ± 1.8^{c}	74.6 ± 1.8^{c}
LIN	49.7 ± 1.8^{abc}	45.2 ± 1.5^{c}	46.7 ± 2.1^{bc}	46.0 ± 2.1^{bc}	54.8 ± 2.1^{a}	49.4 ± 1.5^{abc}	49.2 ± 0.9^{bc}	52.1 ± 2.1^{ab}	47.9 ± 2.1^{abc}	44.9 ± 2.1^{c}	44.0 ± 2.1^{c}
Elongation	40.1 ± 1.0^{d}	45.6 ± 0.8^{abc}	44.7 ± 1.1^{abc}	43.7 ± 1.1^{bc}	47.0 ± 1.1^{ab}	42.6 ± 0.8^{cd}	44.6 ± 0.5^{abc}	42.3 ± 1.1^{cd}	47.1 ± 1.1^{ab}	47.4 ± 1.1^{a}	46.2 ± 1.1^{ab}
Area	4.9 ± 0.1^{a}	4.7 ± 0.08^{ab}	4.4 ± 0.1^{b}	4.6 ± 0.1^{ab}	4.5 ± 0.1^{b}	4.6 ± 0.08^{ab}	4.6 ± 0.05^{b}	4.6 ± 0.1^{ab}	4.6 ± 0.1^{ab}	4.5 ± 0.1^{b}	4.6 ± 0.1^{ab}
Live	69.2 ± 4.5^{a}	75.6 ± 3.8^{a}	75.8 ± 5.4^{a}	73.1 ± 5.4^{a}	71.3 ± 5.4^{a}	71.5 ± 3.8^{a}	73.6 ± 2.3^{a}	78.3 ± 5.4^{a}	81.0 ± 5.4^{a}	76.8 ± 5.4^{a}	80.0 ± 5.4^{a}

$^{a, b, c d}$ Means within rows with different superscripts are different ($P \leq 0.05$)

Table 14: Effects of osteopontin polymorphism T3379G on sperm quality variables in Brahman influenced bulls.

	T3379G*			
Variable	**TT**	**TG**	**GG**	**P Value**
No. of Bulls	3	11	2	----
Motile	47.4 ± 5.8 [b]	54.7 ± 3.2 [b]	72.7 ± 5.8 [a]	0.017
Progressive	36.9 ± 5.2 [b]	42.2 ± 2.9 [b]	57.7 ± 5.2 [a]	0.023
Rapid	42.8 ± 5.7 [b]	48.3 ± 3.1 [b]	66.2 ± 5.7 [a]	0.016
VAP	106.3 ± 6.4 [a]	110.9 ± 3.5 [a]	121.6 ± 6.4 [a]	0.118
VSL	92.2 ± 6.2 [a]	97.0 ± 3.4 [a]	106.5 ± 6.2 [a]	0.127
VCL	164.7 ± 9.8 [a]	176.9 ± 5.3 [a]	188.1 ± 9.8 [a]	0.115
ALH	6.2 ± 0.3 [a]	6.6 ± 0.2 [a]	6.7 ± 0.3 [a]	0.246
BCF	23.8 ± 1.4 [b]	27.3 ± 0.7 [a]	26.8 ± 1.4 [ab]	0.040
STR	80.2 ± 2.3 [b]	86.5 ± 1.2 [a]	86.5 ± 2.3 [ab]	0.030
LIN	54.7 ± 2.3 [a]	58.0 ± 1.2 [a]	58.9 ± 2.3 [a]	0.208
Elongation	44.1 ± 1.6 [a]	46.5 ± 0.9 [a]	46.1 ± 1.6 [a]	0.201
Area	4.6 ± 0.1 [a]	4.8 ± 0.1 [a]	4.8 ± 0.1 [a]	0.355
Live	0.66 ± 0.04 [b]	0.70 ± 0.02 [b]	0.81 ± 0.04 [a]	0.033

[a, b] Means within rows with different superscripts are significantly different

***** Genotypic frequencies for TT, TG and GG are 0.188, 0.688 and 0.124 respectively

Table 15: Effects of osteopontin polymorphism G3490A on sperm quality variables in Brahman influenced bulls.

	G3490A*		
Variable	**GG**	**GA**	**P Value**
No. of Bulls	15	1	----
Motile	56.9 ± 2.7 [a]	53.7 ± 10.5 [a]	0.771
Progressive	44.4 ± 2.4 [a]	39.8 ± 9.5 [a]	0.646
Rapid	51.0 ± 2.7 [a]	45.3 ± 10.4 [a]	0.607
VAP	113.0 ± 2.9 [a]	96.9 ± 11.2 [a]	0.183
VSL	98.7 ± 2.8 [a]	85.8 ± 10.7 [a]	0.263
VCL	178.3 ± 4.4 [a]	152.6 ± 16.9 [a]	0.163
ALH	6.6 ± 0.1 [a]	6.0 ± 0.6 [a]	0.334
BCF	26.5 ± 0.6 [a]	27.3 ± 2.4 [a]	0.771
STR	85.2 ± 1.0 [a]	87.5 ± 4.1 [a]	0.591
LIN	57.4 ± 1.0 [a]	59.0 ± 3.9 [a]	0.707
Elongation	46.0 ± 0.7 [a]	45.3 ± 2.8 [a]	0.815
Area	4.7 ± 0.06 [a]	4.8 ± 0.2 [a]	0.742
Live	0.72 ± 0.02 [a]	0.67 ± 0.07 [a]	0.544

[a, b] Means within rows with different superscripts are significantly different

***** Genotypic frequencies for GG and GA are 0.938 and 0.062 respectively

Table 16: Effects of osteopontin polymorphism A3492G on sperm quality variables in Brahman influenced bulls.

	A3492G*		
Variable	**AA**	**AG**	**P Value**
No. of Bulls	2	14	----
Motile	48.5 ± 7.4 [a]	57.9 ± 2.8 [a]	0.255
Progressive	43.3 ± 6.7 [a]	44.3 ± 2.5 [a]	0.889
Rapid	47.0 ± 7.3 [a]	51.1 ± 2.8 [a]	0.607
VAP	115.3 ± 8.0 [a]	111.5 ± 3.0 [a]	0.670
VSL	103.5 ± 7.6 [a]	97.1 ± 2.9 [a]	0.450
VCL	172.0 ± 12.1 [a]	177.4 ± 4.6 [a]	0.682
ALH	6.0 ± 0.4 [a]	6.6 ± 0.1 [a]	0.182
BCF	23.6 ± 1.7 [a]	27.0 ± 0.6 [a]	0.076
STR	81.8 ± 2.8 [a]	85.8 ± 1.1 [a]	0.208
LIN	57.7 ± 2.8 [a]	57.5 ± 1.0 [a]	0.962
Elongation	43.8 ± 2.0 [a]	46.3 ± 0.7 [a]	0.263
Area	4.4 ± 0.2 [a]	4.8 ± 0.06 [a]	0.056
Live	0.63 ± 0.05 [a]	0.73 ± 0.02 [a]	0.094

[a, b] Means within rows with different superscripts are significantly different

***** Genotypic frequencies for AA and AG are 0.125 and 0.875 respectively

Table 17: Effects of osteopontin polymorphism G3541A on sperm quality variables in Brahman influenced bulls.

	G3541A*		
Variable	**GG**	**GA**	**P Value**
No. of Bulls	15	1	----
Motile	58.0 ± 2.7 [a]	36.8 ± 10.3 [a]	0.066
Progressive	45.1 ± 2.4 [a]	29.2 ± 9.3 [a]	0.120
Rapid	51.7 ± 2.6 [a]	33.5 ± 10.2 [a]	0.105
VAP	111.5 ± 2.9 [a]	119.0 ± 11.3 [a]	0.534
VSL	97.5 ± 2.8 [a]	103.9 ± 10.8 [a]	0.576
VCL	175.4 ± 4.4 [a]	195.8 ± 17.0 [a]	0.265
ALH	6.5 ± 0.1 [a]	7.3 ± 0.6 [a]	0.168
BCF	26.5 ± 0.6 [a]	27.1 ± 2.4 [a]	0.836
STR	85.2 ± 1.0 [a]	87.0 ± 4.1 [a]	0.680
LIN	57.6 ± 1.0 [a]	57.0 ± 3.9 [a]	0.889
Elongation	46.1 ± 0.7 [a]	44.8 ± 2.8 [a]	0.679
Area	4.7 ± 0.06 [a]	4.7 ± 0.23 [a]	0.749
Live	0.72 ± 0.02 [a]	0.62 ± 0.07 [a]	0.223

[a, b] Means within rows with different superscripts are significantly different

***** Genotypic frequencies for GG and GA are 0.938 and 0.062 respectively

Table 18: Effects of osteopontin polymorphism T3800C on sperm quality variables in Brahman influenced bulls.

	T3800C*		
Variable	**TT**	**TC**	**P Value**
No. of Bulls	15	1	----
Motile	56.1 ± 2.7 [a]	65.5 ± 10.5 [a]	0.401
Progressive	43.5 ± 2.4 [a]	53.2 ± 9.4 [a]	0.340
Rapid	50.0 ± 2.7 [a]	60.3 ± 10.3 [a]	0.347
VAP	111.4 ± 2.9 [a]	121.7 ± 11.2 [a]	0.391
VSL	97.3 ± 2.8 [a]	106.5 ± 10.8 [a]	0.423
VCL	175.5 ± 4.4 [a]	194.3 ± 17.0 [a]	0.304
ALH	6.5 ± 0.1 [a]	7.0 ± 0.6 [a]	0.440
BCF	26.6 ± 0.6 [a]	26.9 ± 2.4 [a]	0.908
STR	85.2 ± 1.0 [a]	88.0 ± 4.0 [a]	0.509
LIN	57.3 ± 1.0 [a]	60.7 ± 3.9 [a]	0.423
Elongation	45.8 ± 0.7 [a]	48.3 ± 2.8 [a]	0.399
Area	4.7 ± 0.06 [a]	4.6 ± 0.20 [a]	0.643
Live	0.71 ± 0.02 [a]	0.78 ± 0.07 [a]	0.383

[a, b] Means within rows with different superscripts are significantly different

***** Genotypic frequencies for TT and TC are 0.938 and 0.062 respectively

Table 19: Effects of osteopontin polymorphism C5075T on sperm quality variables in Brahman influenced bulls.

	C5075T*		
Variable	**CC**	**CT**	**P Value**
No. of Bulls	13	3	----
Motile	54.4 ± 2.9 [a]	66.6 ± 6.0 [a]	0.088
Progressive	42.1 ± 2.6 [a]	53.1 ± 5.4 [a]	0.087
Rapid	48.5 ± 2.8 [a]	59.7 ± 5.9 [a]	0.108
VAP	111.6 ± 3.1 [a]	114.0 ± 6.5 [a]	0.744
VSL	97.2 ± 3.0 [a]	100.8 ± 6.2 [a]	0.615
VCL	176.6 ± 4.7 [a]	177.0 ± 9.9 [a]	0.974
ALH	6.5 ± 0.2 [a]	6.4 ± 0.3 [a]	0.779
BCF	26.3 ± 0.7 [a]	27.6 ± 1.4 [a]	0.406
STR	84.8 ± 1.1 [a]	87.6 ± 2.3 [a]	0.298
LIN	57.1 ± 1.1 [a]	59.4 ± 2.3 [a]	0.379
Elongation	45.9 ± 0.8 [a]	46.1 ± 1.6 [a]	0.929
Area	4.7 ± 0.06 [a]	4.8 ± 0.1 [a]	0.765
Live	0.70 ± 0.02 [a]	0.77 ± 0.04 [a]	0.159

[a, b] Means within rows with different superscripts are significantly different

***** Genotypic frequencies for CC and CT are 0.813 and 0.187 respectively

Table 20: Effects of osteopontin polymorphism C5205T on sperm quality variables in Brahman influenced bulls.

	C5205T*		
Variable	**CC**	**CT**	**P Value**
No. of Bulls	12	4	----
Motile	55.3 ± 3.0 [a]	61.03 ± 5.2 [a]	0.364
Progressive	44.5 ± 2.7 [a]	43.1 ± 4.7 [a]	0.800
Rapid	49.9 ± 3.0 [a]	52.7 ± 5.2 [a]	0.646
VAP	114.2 ± 3.2 [a]	105.5 ± 5.6 [a]	0.199
VSL	100.7 ± 3.1 [a]	89.4 ± 5.3 [a]	0.085
VCL	180.3 ± 4.9 [a]	165.9 ± 8.4 [a]	0.161
ALH	6.6 ± 0.2 [a]	6.2 ± 0.3 [a]	0.195
BCF	27.1 ± 0.7 [a]	25.1 ± 1.2 [a]	0.168
STR	86.1 ± 1.2 [a]	83.2 ± 2.0 [a]	0.241
LIN	57.8 ± 1.1 [a]	56.6 ± 2.0 [a]	0.597
Elongation	46.1 ± 0.8 [a]	45.6 ± 1.4 [a]	0.775
Area	4.7 ± 0.1 [a]	4.9 ± 0.1 [a]	0.296
Live	0.70 ± 0.02 [a]	0.74 ± 0.04 [a]	0.388

[a, b] Means within rows with different superscripts are significantly different

***** Genotypic frequencies for CC and CT are 0.750 and 0.250 respectively

Table 21: Effects of osteopontin polymorphism G5209A on sperm quality variables in Brahman influenced bulls.

	G5209A*		
Variable	**GG**	**GA**	**P Value**
No. of Bulls	12	4	----
Motile	55.3 ± 3.0 [a]	61.0 ± 5.2 [a]	0.364
Progressive	44.5 ± 2.7 [a]	43.1 ± 4.7 [a]	0.800
Rapid	49.9 ± 3.0 [a]	52.7 ± 5.2 [a]	0.646
VAP	114.2 ± 3.2 [a]	105.5 ± 5.6 [a]	0.199
VSL	100.7 ± 3.1 [a]	89.4 ± 5.31 [a]	0.085
VCL	180.3 ± 4.9 [a]	165.9 ± 8.4 [a]	0.161
ALH	6.6 ± 0.2 [a]	6.2 ± 0.3 [a]	0.195
BCF	27.1 ± 0.7 [a]	25.1 ± 1.2 [a]	0.168
STR	86.1 ± 1.2 [a]	83.2 ± 2.0 [a]	0.241
LIN	57.8 ± 1.1 [a]	56.6 ± 2.0 [a]	0.597
Elongation	46.1 ± 0.8 [a]	45.6 ± 1.4 [a]	0.775
Area	4.7 ± 0.1 [a]	4.9 ± 0.1 [a]	0.296
Live	0.70 ± 0.02 [a]	0.74 ± 0.04 [a]	0.388

[a, b] Means within rows with different superscripts are significantly different

***** Genotypic frequencies for GG and GA are 0.750 and 0.250 respectively

Table 22: Effects of osteopontin polymorphism C5262T on sperm quality variables in Brahman influenced bulls.

	C5262T*		
Variable	**CC**	**CT**	**P Value**
No. of Bulls	14	2	----
Motile	55.2 ± 2.8 [a]	67.5 ± 7.3 [a]	0.138
Progressive	42.8 ± 2.5 [a]	53.8 ± 6.6 [a]	0.143
Rapid	49.2 ± 2.7 [a]	60.9 ± 7.2 [a]	0.163
VAP	111.8 ± 3.0 [a]	113.7 ± 8.0 [a]	0.824
VSL	97.6 ± 2.9 [a]	100.2 ± 7.7 [a]	0.754
VCL	177.0 ± 4.6 [a]	174.4 ± 12.1 [a]	0.839
ALH	6.6 ± 0.1 [a]	6.3 ± 0.4 [a]	0.500
BCF	26.5 ± 0.6 [a]	26.8 ± 1.7 [a]	0.889
STR	85.1 ± 1.1 [a]	87.1 ± 2.9 [a]	0.527
LIN	57.2 ± 1.0 [a]	59.9 ± 2.8 [a]	0.373
Elongation	46.0 ± 0.7 [a]	45.9 ± 2.0 [a]	0.974
Area	4.7 ± 0.1 [a]	4.8 ± 0.2 [a]	0.707
Live	0.70 ± 0.02 [a]	0.82 ± 0.05 [a]	0.054

[a, b] Means within rows with different superscripts are significantly different

***** Genotypic frequencies for CC and CT are 0.875 and 0.125 respectively

Table 23: Effects of osteopontin polymorphism G5263A on sperm quality variables in Brahman influenced bulls.

	G5263A*		
Variable	**GG**	**GA**	**P Value**
No. of Bulls	15	1	----
Motile	57.1 ± 2.7 [a]	50.2 ± 10.5 [a]	0.531
Progressive	44.6 ± 2.4 [a]	36.7 ± 9.5 [a]	0.428
Rapid	51.1 ± 2.7 [a]	43.5 ± 10.3 [a]	0.490
VAP	112.9 ± 2.9 [a]	98.9 ± 11.2 [a]	0.248
VSL	98.7 ± 2.8 [a]	86.2 ± 10.8 [a]	0.281
VCL	178.6 ± 4.3 [a]	147.8 ± 16.8 [a]	0.097
ALH	6.6 ± 0.1 [a]	5.2 ± 0.5 [b]	0.027
BCF	26.7 ± 0.6 [a]	24.3 ± 2.4 [a]	0.344
STR	85.2 ± 1.0 [a]	87.2 ± 4.1 [a]	0.605
LIN	57.2 ± 1.0 [a]	63.3 ± 3.9 [a]	0.145
Elongation	46.2 ± 0.7 [a]	43.3 ± 2.8 [a]	0.344
Area	4.7 ± 0.1 [a]	4.8 ± 0.2 [a]	0.689
Live	0.72 ± 0.02 [a]	0.60 ± 0.07 [a]	0.135

[a, b] Means within rows with different superscripts are significantly different

***** Genotypic frequencies for GG and GA are 0.938 and 0.062 respectively

Table 24: Haplotype construction and frequency for Group 2 (Brahman influenced) bulls based on individual SNP sites

	Base pair of OPN gene where polymorphism occurred												
Haplotype	3379	3490	3492	3541	3800	4967	5075	5205	5209	5262	5263	Haplotype Frequency	No. of Bulls
1[a]	TT	GG	AA	GG	TT	CC	CC	CC	GG	CC	GG	0.125	2
2	GT	GG	AG	GA	TT	CC	CC	CC	GG	CC	GG	0.063	1
3	TG	GG	AG	GG	TC	CC	CC	CC	GG	CC	GG	0.063	1
4	TG	GG	AG	GG	TT	CC	CC	CC	GG	CC	GG	0.250	4
5	TG	GG	AG	GG	TT	CC	CC	CC	GG	CT	GG	0.063	1
6	GG	GG	AG	GG	TT	CC	CT	CC	GG	CC	GG	0.063	1
7	GG	GG	AG	GG	TT	CC	CC	CT	GA	CC	GG	0.125	2
8	TG	GG	AG	GG	TT	CC	CC	CT	GA	CC	GA	0.063	1
9	GG	GG	AG	GG	TT	CC	CT	CC	GG	CT	GG	0.063	1
10	TG	GA	AG	GG	TT	CC	CT	CC	GG	CC	GG	0.063	1
11	TT	GG	AG	GG	TT	CC	CC	CT	GA	CC	GG	0.063	1

[a] Bulls with haplotype 1 were the same as the reported normal reference sequence

Table 25: Effects of osteopontin bull haplotypes from Group 2 on sperm quality variables.

	Haplotype										
Variable	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Motile	48.5 ± 7.0^{bc}	36.8 ± 9.9^{c}	65.5 ± 9.9^{abc}	51.0 ± 4.9^{abc}	60.3 ± 9.9^{abc}	71.3 ± 9.9^{abc}	74.3 ± 7.0^{a}	50.2 ± 9.9^{abc}	74.7 ± 9.9^{ab}	53.7 ± 9.9^{abc}	45.2 ± 9.9^{abc}
Progressive	43.3 ± 6.3^{abc}	29.2 ± 9.0^{bc}	53.2 ± 9.0^{abc}	40.4 ± 4.5^{abc}	44.5 ± 9.0^{abc}	56.3 ± 9.0^{abc}	55.7 ± 6.3^{ab}	36.7 ± 9.0^{abc}	63.0 ± 9.0^{a}	39.8 ± 9.0^{abc}	24.3 ± 9.0^{c}
Rapid	47.0 ± 6.9^{ab}	33.5 ± 9.8^{b}	60.3 ± 9.8^{ab}	44.8 ± 4.9^{ab}	52.5 ± 9.8^{ab}	65.2 ± 9.8^{ab}	66.5 ± 6.9^{a}	43.5 ± 9.8^{ab}	68.7 ± 9.8^{ab}	45.3 ± 9.8^{ab}	34.3 ± 9.8^{ab}
VAP	115.3 ± 7.8^{ab}	119.0 ± 11.1^{ab}	121.7 ± 11.1^{ab}	111.2 ± 5.5^{ab}	112.5 ± 11.1^{ab}	130.2 ± 11.1^{a}	117.3 ± 7.8^{ab}	98.9 ± 11.1^{ab}	114.9 ± 11.1^{ab}	96.9 ± 11.1^{ab}	88.4 ± 11.1^{b}
VSL	103.5 ± 7.5^{a}	103.9 ± 10.5^{ab}	106.5 ± 10.5^{ab}	98.0 ± 5.3^{ab}	97.2 ± 10.5^{ab}	113.4 ± 10.5^{a}	100.8 ± 7.5^{ab}	86.2 ± 10.5^{ab}	103.2 ± 10.5^{ab}	85.8 ± 10.5^{ab}	69.8 ± 10.5^{b}
VCL	172.0 ± 11.8^{a}	195.8 ± 16.7^{a}	194.3 ± 16.7^{a}	181.1 ± 8.3^{a}	174.2 ± 16.7^{a}	203.9 ± 16.7^{a}	182.7 ± 11.8^{a}	147.8 ± 16.7^{a}	174.5 ± 16.7^{a}	152.6 ± 16.7^{a}	150.2 ± 16.7^{a}
ALH	6.0 ± 0.4^{ab}	7.3 ± 0.5^{a}	7.0 ± 0.5^{ab}	6.9 ± 0.3^{a}	6.4 ± 0.5^{ab}	7.2 ± 0.5^{a}	6.6 ± 0.4^{ab}	5.2 ± 0.5^{b}	6.1 ± 0.5^{ab}	6.0 ± 0.5^{ab}	6.4 ± 0.5^{ab}

$^{a, b, c\ d}$ Means within rows with different superscripts are different ($P \leq 0.05$)

Table 25 (cont.): Effects of osteopontin bull haplotypes from Group 2 on sperm quality variables.

	Haplotype										
Variable	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
BCF	23.6 ± 1.6[b]	27.1 ± 2.3[ab]	26.9 ± 2.3[ab]	29.5 ± 1.1[a]	22.9 ± 2.3[b]	25.0 ± 2.3[ab]	25.9 ± 1.6[ab]	24.3 ± 2.3[ab]	30.7 ± 2.3[ab]	27.3 ± 2.3[ab]	24.3 ± 2.3[ab]
STR	81.8 ± 2.9[a]	87.0 ± 4.1[a]	88.0 ± 4.1[a]	86.5 ± 2.0[a]	85.0 ± 4.1[a]	86.2 ± 4.1[a]	84.3 ± 2.9[a]	87.2 ± 4.1[a]	89.2 ± 4.1[a]	87.5 ± 4.1[a]	77.0 ± 4.1[a]
LIN	57.7 ± 2.8[ab]	57.0 ± 3.9[ab]	60.7 ± 3.9[ab]	56.0 ± 2.0[ab]	58.8 ± 3.9[ab]	58.2 ± 3.9[ab]	57.3 ± 2.8[ab]	63.3 ± 3.9[a]	61.0 ± 3.9[ab]	59.0 ± 3.9[ab]	48.7 ± 3.9[b]
Elongation	43.8 ± 2.0[a]	44.8 ± 2.9[a]	48.3 ± 2.9[a]	47.3 ± 1.4[a]	45.0 ± 2.9[a]	46.2 ± 2.9[a]	47.3 ± 2.0[a]	43.3 ± 2.9[a]	46.8 ± 2.9[a]	45.3 ± 2.9[a]	44.5 ± 2.9[a]
Area	4.4 ± 0.2[a]	4.7 ± 0.2[a]	4.6 ± 0.2[a]	4.8 ± 0.1[a]	4.9 ± 0.2[a]	4.8 ± 0.2[a]	4.8 ± 0.2[a]	4.8 ± 0.2[a]	4.7 ± 0.2[a]	4.8 ± 0.2[a]	5.0 ± 0.2[a]
Live	0.63 ± 0.05[b]	0.62 ± 0.07[ab]	0.78 ± 0.07[ab]	0.67 ± 0.04[ab]	0.80 ± 0.07[ab]	0.81 ± 0.07[ab]	0.82 ± 0.05[a]	0.60 ± 0.07[ab]	0.83 ± 0.07[ab]	0.67 ± 0.07[ab]	0.73 ± 0.07[ab]

[a, b, c d] Means within rows with different superscripts are different ($P \leq 0.05$)

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