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Study on correlation between expression levels of osteopontin gene and *in vitro* sperm characteristics in bovine semen

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Abstract

The present investigation has been undertaken to study the expression level of osteopontin (OPN) gene and its relationship with *in vitro* sperm characteristics in bovine semen. Fresh semen samples from 12 Jersey cross breed bulls were collected from organized breeding center by using artificial vagina. The initial concentration of semen sample was checked immediately before proceeding for RNA isolation. Motile sperms were isolated by the swim-up procedure using sperm-TALP medium. Total RNA from bull spermatozoa was extracted and first strand cDNA was synthesized by using commercial kits. Confirmation of OPN and GAPDH genes by conventional PCR product size 100 and 198 bp was performed. Fragment of 267 bp OPN gene containing real time PCR primers was cloned in to a plasmid vector and was used to construct standard curves for the absolute quantification of OPN gene. The initial copy numbers of OPN gene for all the 12 bull semen was calculated. Variations in levels of OPN gene transcripts (12,000 - 650, 00,000 copies) were found among 12 different bull semen samples. The results of absolute quantification assays were correlated ($p < 0.01$) with the *in vitro* sperm characteristics such as sperm motility, viability, acrosomal integrity, plasma membrane integrity and mitochondrial membrane potential except sperm morphology. From this study, it is evident that ejaculated bull semen has variations in expression level of OPN gene among bull spermatozoa and expression levels of OPN gene have correlation with *in vitro* sperm characteristics.

Keywords: Osteopontin, gene expression, sperm characteristics, bull semen

Introduction

Semen analysis is essential to determine fertility potential of male animals in livestock industry (AI) for dissemination of superior germplasm. Bulls from artificial insemination centers show differences in non-return rates of about 20 to 25 percentage, but these results are not explained by routine semen analysis. The existence of sub fertile sires appears to show normal semen quality but poor fertility rate and it has stimulated the study of other markers of fertility, such as molecular components of the seminal plasma [1].

A number of seminal plasma proteins have been identified as molecular markers of fertility in different species. Proteins such as osteopontin (OPN), prostaglandin D synthase, bovine seminal plasma proteins (BSP A1, A2 and A3), heparin binding proteins (HBPs), fertility associated antigen (FAA), phospholipase A2, sperm adhesion Z13, clusterin (CLU) and heat shock proteins (HSPs) have been reported as indicators of fertility [2,3,4,5]. Interestingly, proteomics has identified large number of proteins that are differentially expressed in sperm membrane, seminal plasma, accessory sex gland fluid and epididymal fluid in case of both fertile and sub fertile bulls [6].

Osteopontin (OPN) is a multifunctional glycoprophosphoprotein, belongs to a family of small integrin-binding ligand N-linked glycoprotein (SIBLING) [7]. It binds to cells through integrin and nonintegrin receptors [8].

OPN is secreted by ampulla and vesicular glands in bulls. OPN mRNA was found in rat epididymal epithelium and its presence in the cytoplasm of epididymal epithelial cells was confirmed by immunocytochemistry [9]. OPN enhances sperm capacitation, acrosome reaction and prevent polyspermy in porcine and bovine species [10]. It also affects the rate of embryo development in the early stages. It increases sperm viability by blocking apoptotic pathways [11]. Expression of OPN is more in the accessory sex gland fluids of high fertile bulls than in low fertile bulls [5]. OPN was detected at greater concentrations in the seminal plasma than in sperm cells of buffalo semen [12].

But, study on expression level of OPN and its association with fertilizing capacity of semen is scarce. Hence, the present investigation was undertaken to study the expression level of osteopontin (OPN) gene and its relationship with *in vitro* sperm characteristics in bovine semen.

Materials and Methods

Collection of semen sample and separation of spermatozoa from semen

Fresh semen samples from 12 Jersey cross breed bulls were collected by using artificial vagina. The volume and concentration of the individual samples were recorded. The spermatozoa were isolated from bull semen samples by swim up protocol using sperm TALP.

The initial concentration of semen samples were checked immediately by standard Neubauer hem cytometer microscopic visual method before proceeding for RNA isolation. Thus normalization of initial concentration was done so that initial amount of every sample was made equal.

Polymerized chain reaction (PCR), cloning and expression of OPN gene

Total RNA from the bull spermatozoa were extracted by the RNeasy® Mini Kit, Qiagen as per manufacturer's protocol. The purity and concentration of the samples were measured at A280/260 by using NanoDrop™ 1000 spectrophotometer. The quantity and integrity of the sperm RNA was verified by checking 28S and 18S ribosomal RNA bands after electrophoresis on formaldehyde containing 1% (w/v) agarose gel. The first strand cDNA was synthesized from 1µg total RNA using M-MuLV Reverse Transcriptase Revert Aid™ H minus first strand cDNA synthesis kit (Thermo Scientific) as per manufacturer's protocol. The following commercially synthesized primers (Life Technologies) were used to amplify specific bovine transcripts. Osteopontin (100 bp corresponding to bases of the bovine sequence, EMBL accession no. AY878328): forward 5' ATG CAT GAC GCA CCT AAG AAG 3', reverse 5' TAT CCT TGG CCT TTG GCG T 3'; Osteopontin (267 bp corresponding to bases of the bovine sequence, EMBL accession no. AY878328): forward 5' ATG CAT GAC GCA CCT AAG AAG 3', reverse 5' TCA ATT GAC CTC AGA AGA GGC 3' and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [13]. forward 5' GTC TTC ACC ACC ATG GAG 3', reverse 5' GTC ATG GAT GAC CTT GGC 3'. The size of each specific RT-PCR product is assigned in parentheses. Each PCR was performed in a 20 µl volume containing 10 µl of amplicon master mix, 1.0 µl of forward primer (10pm/ µl), 1.0 µl of reverse primer (10pm/ µl), 1.0 µl of cDNA template, 7.0 µl of Nuclease free water. The following individual amplification programmes were run in a thermo cycler (Eppendorf Master cycler®, Germany) for each factor. OPN: 30 cycles 94 °C for 15 seconds 52 °C for 15 seconds 72 °C for 10 seconds; GAPDH: 94 °C for 20 seconds 52 °C for 20 seconds 72 °C for 25 seconds. All PCR programmes started with an initial denaturation step at 94 °C for 2 min and ended with an elongation phase at 72 °C for 1 min.

Ten µl of each reaction mixture was subjected to electrophoresis on a 2 per cent agarose gel in a tris acetate EDTA (TAE) buffer containing gel red at a concentration of 0.35ml/ml of the gel and resultant bands were visualized

under UV light gel documentation system (Bio Rad, USA). PCR-amplified fragments of about 100 µl were purified with GenElute™ PCR clean-up kit (sigma-Aldrich, USA, Cat. No. NA1020) they were then ligated into the PTZ57R/T vector using TA cloning kit transformed into *Escherichia coli* DH5α cells and plated on appropriate indicator plates following the manufacturer's protocol. Plasmids carrying the target fragments were isolated with the Hi Yield™ plasmid mini kit (RBC Cat. NO. YPD100).

Absolute quantification of OPN gene

Plasmid DNA was recovered from the transformed *E. coli* using Hi Yield™ plasmid mini kit (RBC Cat. NO. YPD100) as described earlier. The OD value of the plasmid DNA standard concentration was measured at 260 nm/280 nm on Thermo Scientific Nano Drop™ 1000 spectrophotometer (Nano Drop Technologies, LLC, Wilmington, DE, USA) as 67ng/µl and purity of 1.6.

Plasmid copy numbers were calculated using the formula described by Whelan *et al.* (2003) [14].

$$\begin{aligned} \text{DNA (copy number)} &= \frac{6.02214199 \times 10^{23} \text{ (copy/mol)} \times \text{DNA amount (g)}}{\text{DNA length (DP)} \times 660 \text{ (g/mol/dp)}} \\ &= \frac{6.02214199 \times 10^{23} \times 6.7 \times 10^{-8}}{267 \times 660} \\ &= 2.28 \times 10^{11} \end{aligned}$$

Master stock containing 2.28 x 10¹¹ copies/µl were used for preparation of 10-fold dilution series to generate standard curve (Figure 1-a, b,c,d).

The following mixture was added into the qRT- PCR plate (Quantitative real time polymerase chain reaction) and kept for qRT-PCR reaction as duplicates in a real time thermal cycler (Bio Rad CFX 96™ Real time machine).

Reagents	Volume
SYBR master mix	10.0 µl
Forward primer (10pm/ µl)	0.5 µl
Reverse primer (10pm/ µl)	0.5 µl
Template cDNA	2.0 µl
Nuclease free water	7.0 µl
Total reaction mixture	20 µl

The fluorescence intensities of the standards were plotted against the respective cycle number. In the fit-point method which was employed for calculation, a crossing line of fixed fluorescence intensity was defined within the log-linear phase of amplification.

Intersection of the crossing line with the respective amplification curve of a sample was defined as the crossing point, which was directly proportional to the starting amount of target in the sample. Plotting of crossing points of the external standards versus the concentration, yielded a linear calibration curve from which the concentration of the unknown sample could be calculated. The data were accepted only if the NTC had no amplification.

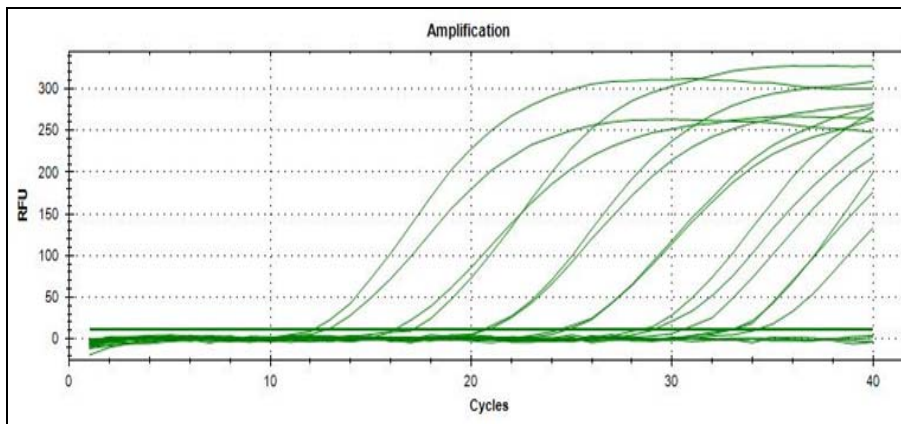


Fig A: Amplification plot for 10-fold serial dilutions ranging from 2.28×10^{11} to 2.28×10^5 copies/ μ l plasmid DNA

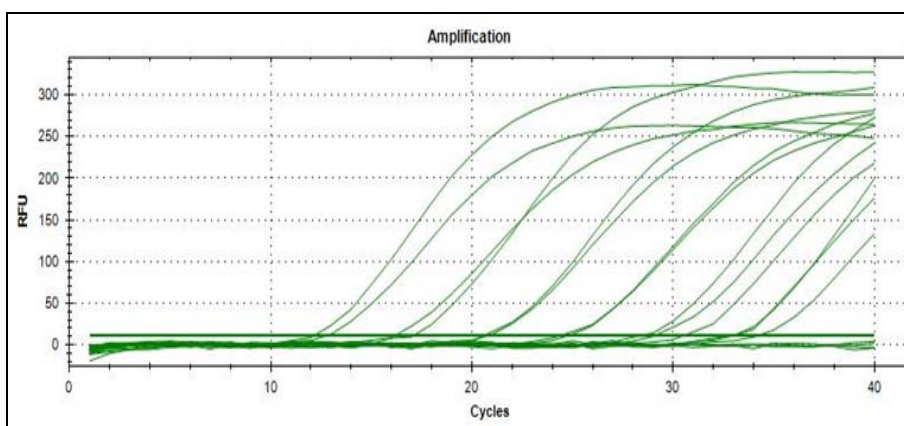


Fig B: Amplification plot for OPN gene along with plasmid DNA

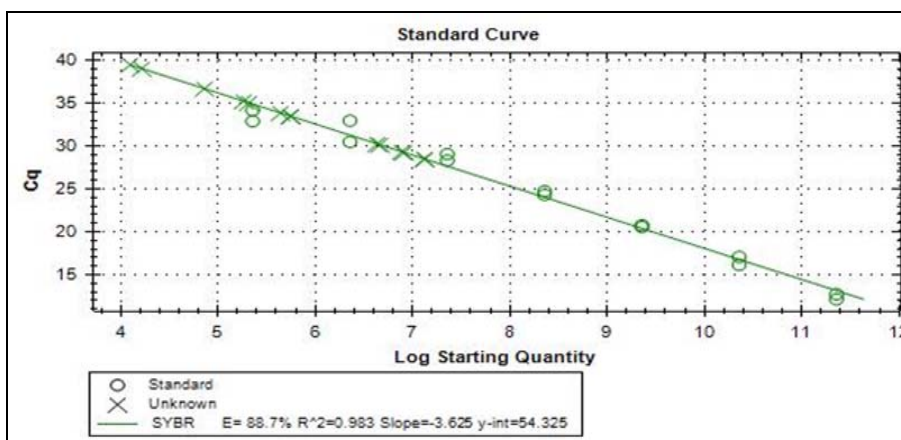


Fig C: Standard curve

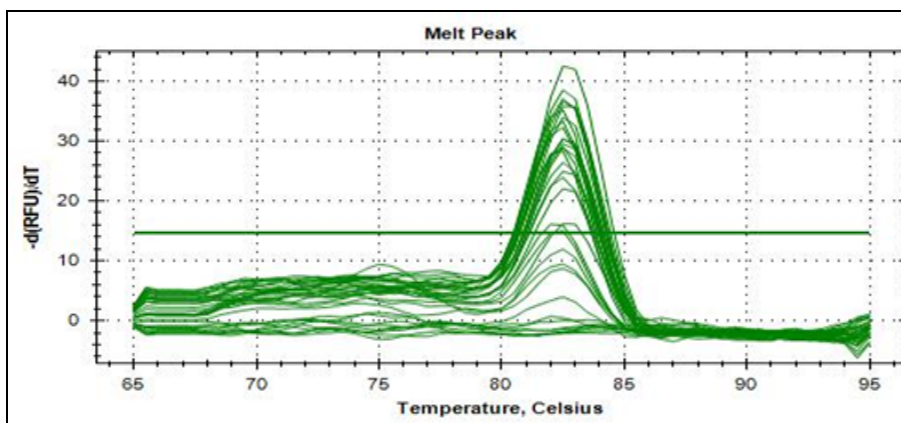


Fig D: Melting curve analysis

Evaluation of *in vitro* sperm characteristics

The sperm motility was assessed by placing a drop of fresh semen on clean grease free glass slide and covered with a cover slip. Minimum of three fields were scanned under bright field microscopy to assess the mass motility of spermatozoa and graded in terms of percent ranging from 0-100 multiples of 10 [15]. The sperm viability was determined by the supravital eosin and nigrosin stain technique [16]. Sperm morphology was assessed by rose bengal stain [17]. Functional membrane integrity was assessed using osmotic resistance test (hypo-osmotic swelling test - HOST) [18]. Acrosomal integrity was assessed by Giemsa stain. Acrosomal intact spermatozoa showed acrosomal cap and acrosomal nonintact spermatozoa lost acrosomal cap [19]. Mitochondrial membrane potential was assessed by using JC-1 (5, 5', 6, 6' - tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide). Spermatozoa with high MMP exhibited red-orange fluorescence and those with medium to low MMP exhibited green fluorescence. Spermatozoa without MMP did not exhibit fluorescence [20].

Statistical analysis

The data collected were analyzed using SPSS® 20.0 software package. Statistical analysis was carried out by Karl Pearson's coefficient of correlation [21].

Results

Absolute quantification of OPN gene

Absolute quantification of OPN gene transcripts in semen samples from 12 bulls was performed. Variations in levels of OPN gene transcripts (12,000 - 650,000 copies) were found among 12 different bull semen samples.

Evaluation of *in vitro* sperm characteristics

Analysis of *in vitro* sperm characteristics from 12 different bull semen samples showed sperm motility (70.0 - 90.0%), viability (79.0 - 92.0%), morphology (76.0 - 88.0%), plasma membrane integrity (60.0 - 70.0%), acrosome integrity (78.0 - 89.0%) and MMP (55.0 - 68.0%). All *in vitro* sperm parameters except sperm morphology were highly correlated ($p < 0.01$) with expression levels of OPN gene transcripts (12,000 - 65,000,000 copies) in spermatozoa isolated from 12 different bull semen samples (Table 1).

Table 1: Correlation between expression of OPN gene and *in vitro* sperm characteristics in bull semen samples (n=12)

Bull No.	Starting Quantity of OPN gene (No. of copies)	Sperm Motility%	Sperm Viability	Sperm morphology Normal%	Plasma membrane integrity%	Sperm Acrosome Integrity%	Sperm MMP%
			Live%				
1	7800000	80	87	85	65	82	63
2	13000000	90	88	85	67	84	63
3	180000	70	80	84	62	80	57
4	16524	70	80	76	60	78	55
5	560000	70	81	81	63	80	59
6	400000	70	81	84	62	80	59
7	12000	70	79	78	57	78	56
8	4600000	80	84	84	64	82	60
9	12800000	80	88	86	68	84	64
10	65000000	90	92	88	70	89	68
11	140000	70	80	82	60	80	58
12	60000000	90	90	85	68	87	66
Coefficient of correlation		0.800*	0.828*	0.574	0.752*	0.911*	0.839*

Coefficient of correlation with * indicates high correlation ($p < 0.01$) within the same row

Discussion

Absolute quantification of OPN gene transcripts in semen samples from 12 bulls showed variations in levels of OPN gene transcripts (12,000 - 650,000 copies). This is the first study to document expression levels of OPN gene transcripts from ejaculated bull spermatozoa.

The results of *in vitro* sperm characteristics revealed that sperm motility, viability, plasma membrane integrity, acrosome integrity, MMP and LPO from 12 different bull semen have higher correlation with OPN transcripts except sperm morphology. Our study corroborated with previous studies where they demonstrated that sperm motility was higher in high fertility bulls with higher expression of OPN gene [5]. Variation in sperm motility among the bulls may be due regulation of nitric oxide (NO) synthesis by OPN because OPN is a general modulator of NO synthesis [22]. OPN is a cell survival factor and protect cells from undergoing apoptosis of epithelial, endothelial and smooth muscle cells [23]. And reported that OPN binds to integrin receptors located on the epithelial surfaces of accessory sex glands and protect them from bacterial infections and influence host defense mechanism in inflammatory conditions. In this way, a

protective effect for spermatozoa travelling in the male reproductive tract is ensured. Differences in NO synthesis level in male reproductive tract might be the reason for the spermatozoa having variations in viability among the bulls in our study. Though more number of spermatozoa was found morphologically normal from bull semen having high amount of OPN gene in our study, no direct relationship was established with respect to OPN level and sperm morphology. But another protein called ubiquitin is a suitable marker of sperm abnormalities because it covalently links to the surface of defective spermatozoa [24]. Hence its association with OPN needs to be established for better correlation.

OPN is localized in acrosomal cap of spermatozoa. Integrin and CD44 receptors are also found in acrosomal cap of spermatozoa and OPN secreted from accessory sex glands binds with receptors [25]. But in case of acrosomal non intact spermatozoa, either OPN or receptors might be lacking [26]. Hence low amount of OPN was detected in spermatozoa of bull semen possessing low acrosomal integrity in this study. Spermatozoa that exhibit high MMP have intact acrosome, normal motility, normal morphology and high fertilizing capacity [27, 28]. NO stimulated proteins of Bcl-2 family

followed by releasing mitochondrial cytochrome C and concomitant activation of caspase 9 and 3 which causes disruption in MMP. Upregulation of OPN synthesis down regulates NO production thereby preventing disruption in MMP of spermatozoa from bull semen displaying high amount of OPN in this study.

Conclusions

From this experiment, it is obvious that OPN gene is expressed in spermatozoa of ejaculated bull semen. Expression levels of OPN transcripts have varied among the bulls tested. Correlation is found between levels of OPN gene transcripts and *in vitro* sperm characteristics.

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