Detection of osteopontin transcript in seminal plasma and its association with post-freeze-thaw quality of cryopreserved spermatozoa in mithun (Bos frontalis)

K.K. Baruah1*, A. Dhali2, B. Bora, A. Mech2 and M. Mondal3

ICAR-National Research Centre on Mithun, Jharnapani, Medziphema - 797 106, Nagaland, India.

Received: 07-02-2016 Accepted: 20-08-2016 DOI: 10.18805/ijar.v0iOF.4553

ABSTRACT

The study aimed to detect osteopontin (OPN) transcript in seminal plasma and to assess the relationship between the presence of the transcript and post-freeze-thaw quality of cryopreserved spermatozoa in mithun (Bos frontalis). Semen samples were collected from five adult bulls through rectal massage method and cryopreserved in tris-egg yolk-glycerol extender. OPN transcript was detected by RT-PCR in the RNA purified from seminal plasma. OPN transcript was found to be present consistently in three animals (OPN+) and absent in two animals (OPN-). Although, sperm viability and acrosomal integrity were found similar at different stages of cryopreservation in both the groups, tail abnormality after final dilution and, head and tail abnormalities after equilibration were found significantly (P<0.05) lesser in the OPN+ compared to OPN- animals. The results indicated that OPN is probably important in stabilizing sperm membrane that resulted in better resistance of sperm to cryopreservation process in OPN+ animals.

Key words: Cryopreservation, Mithun, Seminal plasma, Spermatozoa, Osteopontin, Transcript.

INTRODUCTION

Mithun (Bos frontalis) is the domesticated form of wild gaur, which is primarily found in the North-Eastern hilly states of India at an elevation of 300 to 3000 m above sea level. It is also available in the hilly tracts of Bhutan, Myanmar, China and Bangladesh. Mithun is mainly reared as a meat animal and mithun×cattle hybrids are popular milch animal as well (Baruah et al., 2013). The conservation and genetic improvements of this unique species can be achieved proficiently through effective breeding involving artificial insemination (AI) with preserved semen of superior fertilizing capability and genetic merits. Therefore, identifying the mithun bulls with favourable OPN status is expected to augment the outcome of breeding programme in this species.

Seminal plasma is important for maintaining sperm motility and viability (Muino-Blanco et al., 2008). The seminal plasma proteins are adsorbed onto the spermatozoa and topographically reorganized into specific regions of the sperm surface (Metz et al., 1990; Desnoyers and Manjunath, 1992; Souza et al., 2008; Wolf and Voglmayr, 1984). These proteins may change the properties of the sperm membrane in several ways, particularly by binding to it and or modifying the structure or the arrangement of the existing membrane molecules. It is suggested that they maintain the stability of the membrane up to the process of capacitation (Ollero et al., 1994; Fraser et al., 1996; Manjunath and Therien, 2002). The addition of seminal proteins has shown to revert the cryodamage on sperm membrane (Rebolledo et al., 2007).

Osteopontin (OPN) is a secreted extracellular matrix phospho-protein identified in various tissues and fluids including those of the male and female reproductive tracts (Erikson et al., 2007). OPN may be glycosylated, phosphorylated and sulfated, and its expression and post-translational modifications are tissue-specific and regulated by hormones and growth factors (Denhardt and Guo, 1993). In female reproductive tract, OPN is detected in the oviduct, uterus, and placenta (Craig and Denhardt, 1991). In male, OPN is believed to be produced by the ampullae and seminal vesicles of the male reproductive tract in bovine (Boccia et al., 2013). In cattle, OPN is one of the seminal plasma proteins associated with fertility (Killian et al., 1993; Cancel et al., 1997) and high-fertility Holstein bulls have greater concentrations of OPN in their accessory sex gland fluids compared to low-fertility bulls (Moura et al., 2006). The addition of OPN into the IVF system enhances sperm capacitation process and blastocyst yield in buffalo (Boccia et al., 2013).

Previously, we reported the method for cryopreserving mithun spermatozoa (Dhali et al., 2008; Baruah et al., 2013), but no report is available currently on the status of osteopontin in mithun seminal plasma either at protein or...
transcript level. Moreover, no information is available at present on the detection of OPN transcript in seminal plasma in any livestock species. The current study aimed to detect osteopontin transcript in mithun seminal plasma and to establish relationship between the presence of the transcript and post-freeze-thaw quality of spermatozoa in mithun.

MATERIALS AND METHODS

Animals and management: Five healthy adult mithun bulls (4 to 6 years of age) were used for semen collection during the experiments. The animals were maintained in individual bull pens at the research farm of ICAR-NRC Mithun, Jharnapani, Nagaland, India. The animals were fed indoors and daily offered 4 kg concentrates (88% dry matter, 67% total digestible nutrient and 15% crude protein) fortified with mineral mixture (2%) and salt (1%) and, ad libitum mixed green forages (grasses, shrubs, herbs and tree leaves collected from the grazing area on hilly terrain; 19% dry matter and 11% crude protein) and drinking water. Health status of the animals was regularly monitored by a veterinarian. All experimental protocols and animal care met regulations of the “Institute Animal Ethics and Utilization Committee, ICAR-NRC Mithun, Jharnapani, Nagaland, India”.

Semen collection, dilution and preservation: Semen samples (N=4 for each animal) were collected from the bulls through rectal massage method as described previously (Dhali et al., 2008). Briefly, seminal vesicles were massaged for 5 min followed by the gentle milking of ampullae for 3 to 5 min, which resulted in erection and ejaculation. Following ejaculation, neat semen drops were collected into a graduated test tube with the help of a funnel. The samples exhibiting a fresh-sample-motility score of 3 or more were only processed further for freezing and detecting OPN transcript (Dhali et al., 2008).

The samples were diluted and frozen according to the previously described method (Baruah et al., 2013). Briefly, after collection, the samples were partially diluted by adding 1 ml of pre-warmed (37°C) tris-egg yolk extender. The partially diluted samples were maintained at 37°C for further processing. Final dilution of the samples was done with pre-warmed (37°C) tris-egg yolk extender in such a way that after final dilution and glycerol addition each ml of the sample contained 60×10^6 sperm. After final dilution, the total amount of required glycerol was added into the extender containing spermatozoa in a single dose, mixed with extender by swirling action and kept at 4°C for equilibration (4-h). After equilibration, the samples were loaded into pre-cooled (4°C) 0.50 ml straws leaving a small air space at the end and heat sealed. The straws were then frozen in liquid nitrogen vapour for 10 min and immediately plunged into liquid nitrogen for storage.

Assessment of semen and sperm qualities: The volume, mass activity and spermatozoal concentration of semen were determined according to the previously described methods (Baruah et al., 2013). The sperm quality traits (progressive motility, livability, acrosomal integrity and morphological abnormalities) were determined after final dilution, 4-h equilibration (4°C) and warming (37°C) and, freeze-thawing and warming (37°C) according to the previously described methods (Baruah et al., 2013). In brief, progressive motility (total percentage of progressively mobile spermatozoa) was subjectively determined to the nearest of 10% by analyzing 4 to 5 fields of view of the sample placed on a pre-warmed slide (37°C) under a cover slip by two persons. Liveability, morphological abnormalities and acrosomal status were evaluated simultaneously using the trypan blue-Giemsa staining technique. Four different classes of spermatozoa (live with intact acrosome, live with damaged acrosome, dead with intact acrosome and dead with damaged acrosome) were distinguished during the evaluation (Kovacs and Foote, 1992). Total morphological abnormality was determined by adding the proportion of head (abnormal shape or loose), mid-piece (degenerated or bent) and tail (bent or coiled) abnormalities. The frozen samples were evaluated after minimum 7 days of storage in liquid nitrogen.

RNA isolation, Reverse transcription (RT) and polymerase chain reaction (PCR) analysis: During the rectal palpation for semen collection, the initial transparent secretion avoiding neat semen drops was collected separately for each animal (N=4) for RNA purification. The sample was kept on ice immediately after collection and processed within 30 min for total RNA purification from 100 μl sample using Qiagen RNeasy Mini Kit (Qiagen, USA) according to the manufacturer’s instructions. Briefly, RLT buffer was added to the sample and homogenized by passing the content 10 times through a 20-gauge needle fitted to a disposable 2 ml syringe. The lysate was centrifuged for 3 min at 10,000×g; supernatant was removed carefully and one volume of 70% ethanol was added to the cleared lysate and mixed immediately by pipetting. Ethanol added sample was then loaded to an RNeasy spin column and centrifuge for 15 s at 10,000×g. The spin column membrane was washed with RQ1 buffer by centrifugation for 15 s at 10,000×g. RNase-free DNase I (Qiagen, USA) was then added directly (27.3 Kunitz units in 80 μl buffer) onto the spin column membrane and incubated at 30°C for 15 min. The column was subsequently washed with RQ1 and RPE buffers and the total RNA was eluted in 30 μl nuclease free water by centrifugation for 15 s at 10,000×g. Purified RNA was reverse transcribed into cDNA immediately using the High-Capacity cDNA Archive Kit (Applied Biosystems, USA) following the manufacturer’s instructions. Briefly 10 μl of purified RNA was mixed with 10 μl RT mix (containing RT buffer, random primer, dNTPs and multiScribe reverse transcriptase) and incubated at 25°C for 10 min, 37°C for 120 min, 85°C for 5 min and 4°C for 10 min. The synthesized cDNA was stored at 4°C and used within 7 days for PCR analysis. PCR amplification of β-
actin and OPN transcripts was performed using GoTaq Flexi DNA Polymerase kit (Promega, USA). Briefly, 1μl of cDNA was added into 24μl of PCR mix containing 0.2μM of each primer, 1.5mM of MgCl2, 0.2mM of dNTPs and 0.025U GoTaq DNA Polymerase. The PCR protocol involved 40 cycles (95°C for 15 s, 55°C for 15 s and 72°C for 30s) of amplification. The primers used were sense 5'-ACTGGGACGACATGGGAAAGAT-3' and antisense 5'-TGCTCGAAGTCCAAGGGCAGCTG-3' for β-actin (441bp amplicon) and sense 5'-GACGCTGAAACCACTGATGACC-3' and antisense 5'-AGGCTATGGAATCTTGGCTGAGTTT-3' for OPN (425bp amplicon). Following the PCR amplification, 10μl reaction mix was analysed by electrophoresis in ethidium bromide stained agarose gels to detect the amplified products.

Statistical analysis: Statistical analyses were performed using the PASW 18.0.0 software package (SPSS/IBM, Chicago, IL, USA). For the analyses, arcsine transformation was done for the results expressed in percentages. The variations in volume, mass activity and spermatozoa concentration of semen between the groups with different OPN category (present or absent in seminal plasma) were analyzed by ANOVA. The variations in sperm traits (progressive motility, liveability, acrosomal integrity and morphological abnormality) between the groups at each stage of cryopreservation (final dilution, 4-h equilibration or post-freeze-thaw) were analyzed by ANOVA. Additionally, the variations in sperm traits among the stages of cryopreservation for each OPN category were analyzed by ANOVA. Pair wise multiple-comparison procedures between means were conducted using the Student-Newman-Keuls (SNK) test. A probability value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Detection of OPN transcript in seminal plasma: The RT-PCR amplification of β-actin (441 bp amplicon) and OPN (425 bp amplicon) transcripts in mithun seminal plasma is depicted in Figure 1. Similar β-actin amplification could be detected consistently in all the experimental animals in all collections, which indicated that RNA purification and RT procedures were satisfactory. The amplification of OPN was detected consistently in three animals (OPN+) in all collections. In contrast, in two animals, OPN amplification could not be detected in all collections (OPN-).

Quality of semen and sperm in the animals with different OPN status: Volume (ml) of semen was found significantly (p<0.05) greater in the OPN+ (2.1±0.1) compared to OPN- (1.1±0.1) animals. In contrast, the spermatozoa concentration (×10^6/ml) and mass activity (5-point scale) of semen did not differ significantly between the OPN+ and OPN- animals (361±29 vs. 340±10 and 3.8±0.1 vs. 3.7±0.1 respectively). Although progressive motility of the extended semen did not vary significantly at different stages of cryopreservation between the groups, the post-freeze-thaw motility (%) was found marginally greater in OPN+ (38.9±2.3) compared to that of OPN- (35.1±1.5) animals (Figure 2). Progressive motility (Figure 2) and the proportion of live spermatozoa with intact acrosome (Table 1) were found to be significantly (p<0.05) greater after final dilution compared to that after 4-h equilibration and freeze-thawing in both the groups. In contrast, in both the groups, the proportions of live spermatozoa with damaged acrosome, dead spermatozoa with intact and damaged acrosome, and head, tail and total abnormalities were found to be significantly (p<0.05) lesser after final dilution compared to that after equilibration and freeze-thawing (Table 1 and 2). Effect of cryopreservation stage was not significant on mid piece abnormality in both the groups (Table 2).

<table>
<thead>
<tr>
<th>Cryopreservation stage</th>
<th>Category</th>
<th>Live sperm with intact acrosome (%)</th>
<th>Live sperm with damaged acrosome (%)</th>
<th>Dead sperm with intact acrosome (%)</th>
<th>Dead sperm with damaged acrosome (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>After final dilution</td>
<td>OPN-</td>
<td>65.7±2.5</td>
<td>2.5±0.8</td>
<td>8.2±1.2</td>
<td>23.5±2.0</td>
</tr>
<tr>
<td>After final dilution</td>
<td>OPN+</td>
<td>67.9±2.4</td>
<td>2.2±0.5</td>
<td>8.0±0.7</td>
<td>21.9±2.2</td>
</tr>
<tr>
<td>After 4-h equilibration</td>
<td>OPN-</td>
<td>50.2±1.9</td>
<td>4.9±0.7</td>
<td>12.9±0.8</td>
<td>32.4±2.5</td>
</tr>
<tr>
<td>After 4-h equilibration</td>
<td>OPN+</td>
<td>50.2±2.1</td>
<td>5.2±0.6</td>
<td>12.0±0.9</td>
<td>33.1±2.3</td>
</tr>
<tr>
<td>After 4-h equilibration</td>
<td>OPN-</td>
<td>38.6±1.3</td>
<td>6.4±1.1</td>
<td>13.7±1.2</td>
<td>41.3±1.8</td>
</tr>
<tr>
<td>After 4-h equilibration</td>
<td>OPN+</td>
<td>41.3±2.3</td>
<td>5.0±1.1</td>
<td>11.8±1.7</td>
<td>40.7±2.5</td>
</tr>
</tbody>
</table>

Effect of cryopreservation stage was significant (P<0.05) on all parameters within each OPN category
In the current study, we could successfully detect OPN transcript and post-freeze-thaw spermatozoa quality in mithun. The results indicated that the presence of OPN transcript in seminal plasma was associated with the superior spermatozoa quality after freezing-thawing.

Fig 2: Progressive motility (%) of extended mithun semen during different stages of freezing; OPN- and OPN+ indicate absence and presence of osteopontin transcript respectively in seminal plasma; Effect of freezing stage was significant (P<0.05) on all parameters within each OPN category

When various quality traits of spermatozoa were compared between the groups at each stage of cryopreservation, the proportions of live and dead sperms with intact or damaged acrosome were found similar after final dilution, 4-h equilibration or freeze-thawing (Table 1). Although head and mid piece abnormalities did not differ significantly between the groups, tail and total abnormalities were found to be significantly (P<0.05) greater in OPN- animals after final dilution compared to OPN+ animals after final dilution (Table 2).

<table>
<thead>
<tr>
<th>Cryopreservation stage</th>
<th>Category</th>
<th>Head abnormality (%)</th>
<th>Mid piece abnormality (%)</th>
<th>Tail abnormality (%)</th>
<th>Total abnormality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>After final dilution</td>
<td>OPN-</td>
<td>1.2±0.4</td>
<td>0.4±0.2</td>
<td>5.0±0.3*</td>
<td>6.6±0.3*</td>
</tr>
<tr>
<td></td>
<td>OPN+</td>
<td>0.7±0.2</td>
<td>0.4±0.1</td>
<td>3.9±0.3</td>
<td>5.1±0.2</td>
</tr>
<tr>
<td>After equilibration</td>
<td>OPN-</td>
<td>3.1±0.4*</td>
<td>0.6±0.3</td>
<td>7.7±0.6*</td>
<td>11.4±0.5*</td>
</tr>
<tr>
<td></td>
<td>OPN+</td>
<td>1.7±0.3</td>
<td>0.7±0.2</td>
<td>6.2±0.2</td>
<td>8.5±0.4</td>
</tr>
<tr>
<td>After 7 days of freezing</td>
<td>OPN-</td>
<td>2.9±0.5</td>
<td>1.6±0.3</td>
<td>9.2±1.3</td>
<td>13.7±1.9</td>
</tr>
<tr>
<td></td>
<td>OPN+</td>
<td>2.0±0.5</td>
<td>1.0±0.2</td>
<td>6.4±1.2</td>
<td>9.4±1.7</td>
</tr>
</tbody>
</table>

* indicates values were significantly (P<0.05) different between the groups at specific cryopreservation stage

Effect of cryopreservation stage was significant (P<0.05) on all parameters within each OPN category.

Table 2: Proportions of head, mid piece, tail and total abnormalities in extended mithun semen at different stages of cryopreservation. OPN- and OPN+ indicate absence and presence of osteopontin transcript respectively in seminal plasma respectively.

OPN is a fertility associated protein and its abundance in bovine seminal plasma is linked with male fertility (Cancel et al., 1997; Moura et al., 2006). Ampullae and seminal vesicles have been shown as the source of OPN in bull seminal plasma and majority of the protein is synthesized by the epithelial cells of the ampullae (Rodriguez et al., 2000; Cancel et al., 1999). OPN facilitates bovine sperm capacitation and viability (Erikson et al., 2007b; Monaco et al., 2009) and OPN-treated sperm are associated with increased rates of in vitro fertilization, cleavage, and proportion of embryos developed into advanced blastocysts (Goncalves et al., 2008; Monaco et al., 2009). In this study, we detected OPN transcript in seminal plasma and assessed the relationship between the presence/absence of the transcript and post-freeze-thaw spermatozoa quality in mithun. The results indicated that the presence of OPN transcript in seminal plasma was associated with the superior spermatozoa quality after freezing-thawing.
detect OPN transcript in mithun seminal plasma. The presence or absence OPN transcript in seminal plasma for a particular animal was consistent in all the four ejaculates evaluated. The results indicated that the detection of OPN transcript in seminal plasma could serve as a valuable technique for identifying bulls of superior fertility if a positive relation between OPN status and fertility performances is established.

It has been demonstrated previously in different livestock species that seminal plasma proteins have the ability to increase resistance of spermatozoa to cryo-injury (Muino-Blanco et al., 2008). Proteins from the epididymal and seminal fluid are adsorbed onto the spermatozoa and are topographically reorganized into specific regions of the sperm surface (Muino-Blanco et al., 2008; Wolf and Voglmayr, 1984). These proteins may change the properties of the sperm membrane in several ways, particularly by binding to it and modifying the structure or the arrangement of the existing membrane molecules (Muino-Blanco et al., 2008). Even these proteins have the capability to revert cryodamage of the sperm membrane (Muino-Blanco et al., 2008; Rebolledo et al., 2007). Previously, a significant negative correlation is reported between morphological abnormalities and membrane integrity in buffalo sperm following freeze-thawing (Mahmoud et al., 2013). A possible relation between the presence of OPN transcript in seminal plasma and post-freeze-thaw sperm quality was investigated in the current study. Although, sperm viability and acrosomal integrity were found similar at different cryopreservation stages between the groups, tail abnormality after final dilution and, head and tail abnormalities after 4-h equilibration were found significantly lesser in the OPN+ compared to OPN- animals. Even though not statistically different, these abnormalities were found much lesser after freezing in OPN+ animals. The results indicated that OPN is probably important in stabilizing sperm membrane that resulted in better resistance of sperm to cryopreservation process in OPN+ compared to OPN- animals.

CONCLUSIONS

In conclusion, OPN transcript could be detected in the seminal plasma of mithun bulls and presence of the transcript was associated with sperm morphological abnormalities during cryopreservation process. The results indicate that OPN may be important in stabilizing sperm membrane and offer better resistance to sperm to withstand cryopreservation process. Consistent presence of OPN transcript in the seminal plasma indicates that detection of the transcript can be a valuable technique for identifying bulls of superior fertility. In future, it will be fascinating to study if a positive relationship exists between the OPN transcript status in seminal plasma and fertility performances of male.

ACKNOWLEDGEMENT

We thankfully acknowledge the research grant provided for the study by the Department of Biotechnology, Ministry of Science and Technology, Government of India.

REFERENCES


