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Protamine 3 expressions in crossbred bull spermatozoa may not be a prognostic marker for differentiating good and poor quality semen

Sushil Kumar, Umesh Singh, Indrajit Ganguly#, Rajib Deb*, Rani Singh, Sandeep Mann, G. Sengar, D. K. Mandal, Mahesh Kumar and Arjava Sharma

Molecular Genetics Laboratory, Project Directorate on Cattle (ICAR), Meerut, Uttar Pradesh-250001, India.

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Protamines are short and highly basic sperm-specific nuclear proteins. Though the expression profile of protamines 1 and 2 is well known, but there is little knowledge of protamine 3 expression in bovine semen. In this study, Frieswal (HF x Sahiwal) crossbred bulls were categorized into two groups (good and poor quality) based on the initial progressive motility of semen and other seminal parameters. The mRNA expression of PRM3 gene among two groups was evaluated by real time quantitative polymerase chain reaction (PCR) using TaqMan chemistry, where peptidylprolyl isomerase A (PPIA) was used as an internal control. Our finding revealed that expression of PRM3 was down regulated in poor quality semen producers as compared to good quality semen producing group, but unfortunately no significant difference of transcript abundance was observed between the groups. To shed light on present findings, it can thus assume that PRM3 may not be a prognostic marker to differentiate good and poor quality bull semen in Frieswal cattle.

Key words: Protamine 3, Frieswal, semen, expression.

INTRODUCTION

To differentiate finally into spermatozoa, haploid spermatids undergo complex morphological and physiological changes during spermatogenesis. These changes include chromatin remodeling and condensation mediated through the replacement of somatic histones by transition proteins and protamines (Wykes and Krawetz, 2003). The first step in this process occurs in haploid round spermatids and involves replacement of somatic histones with the transition proteins (TNP1 and TNP2). Subsequently, in elongating spermatids, the protamines (PRM1 and PRM2) replace TNP1 and TNP2. The resulting chromatin is highly condensed and transcriptionally silent. In bovine, PRM1, PRM2, and TNP2 genes encode basic chromosomal proteins and are located in a compact gene cluster as observed in mouse, rat and human (Balhorn et al., 2000; Engel et al., 1992; Ferraz et al., 2010; Schluter et al., 1992; Singh and Rao, 1988; Le Lannic et al., 1993). The protamine gene cluster contains a fourth gene, designed protamine 3 (PRM3), located between the PRM2 and TNP2 genes in rat, human and mouse

*Corresponding author. E-mail: drrajibdeb@gmail.com. Tel: +911212645598. Fax: +911212656021.

Table 1. Seminal qualities (least square mean values) of good vs poor quality semen producing Frieswal bulls for the present study.

Particular	Good	Poor
Volume (ml)	$4.39 \pm 0.27_a$	$3.11 \pm 0.26_b$
Concentration (millions/ml)	$923.433 \pm 106.21_a$	$692.23 \pm 99.65_b$
Number of sperm/ejaculate	$3905.66 \pm 94.62_a$	$2039.24 \pm 198.01_b$
Motility (%)	$57.61 \pm 1.41_a$	$18.45 \pm 1.61_b$
PTM (%)	$40.24 \pm 6.24_a$	$26.88 \pm 5.92_b$

a,b Row wise means with different subscripts differ significantly at $p < 0.05$. PTM: Post thaw motility.

(Singh and Rao, 1988; Schluter and Engel, 1995; Kramer and Krawetz, 1998; Cho et al., 2001; Schluter et al., 1996). The timing of PRM3 mRNA translation is developmentally regulated in spermiogenesis and it codes for a small intronless protein localized in the cytoplasm of elongated spermatids (Kleene, 1989; Schluter et al., 1996). It has also been established that the PRM3 gene is conserved in diverse mammalian species including bovine and PRM3 –/– knockout experiment showed a reductions in sperm motility of male mice, lacking PRM3 protein, without compromising their fertility (Kleene, 1989). To date, the PRM3 gene has not been studied adequately and there is a paucity of published literature describing its function, expression profile, regulation pattern and association with sperm motility and fertility in bull. Recently, we have observed higher PRM1 transcripts in normal crossbred Frieswal bulls compared to motility impaired group ($p<0.05$) as well as a non-significant difference of PRM2 transcripts between the groups (Grzmil et al., 2008). The present research was undertaken to elucidate the PRM3 transcript abundance in ejaculated spermatozoa of normal and impaired crossbred Frieswal (HF X Sahiwal) bull semen using Taqman-real time PCR based quantification.

MATERIALS AND METHODS

Fresh semen samples were collected from categorized crossbred Frieswal bulls into normal (good) and impaired groups (poor) according to their seminal quality parameters viz. volume, concentration, number of sperm/ejaculates, progressive motility (>40% considered as good) and post thaw motility (PTM). The concentration of sperm was estimated by using a photometer (Accucell, IMV- France). The testis tissues of Buffalo (*Bubalus bubalis)* were collected in RNA later and stored at -80°C before use. Assessment of membrane integrity (HOST test) of spermatozoa was performed as per procedure described elsewhere (Grzmil et al., 2008). To rule out the possibility of spermatozoa and contaminating somatic cells, the semen samples were purified through a discontinuous Percoll (Sigma-Aldrich) gradient (40:80) centrifugation (20 min at 300 *g*, 25°C) as described earlier (Grzmil et al., 2008). The motile spermatozoa were kept at -80°C in RNA later (Ambion, Austin, TX, USA) until RNA extraction. The quantity and purity of total RNA isolated from purified spermatozoa and buffalo testis were measured by using ND-1000 spectrophotometer (Nano-Drop, Fisher Thermo, and Wilmington, DE, USA).

Total RNA isolated from crossbred bull spermatozoa and buffalo (*B. bubalis)* testicular tissues were reverse transcribed to complementary DNA using Random primers and M-MuLV reverse transcriptase (Protoscript, NEB) according to the manufacturer's instructions. The cDNA product was stored at -20°C. Genomic DNA contamination was checked by PCR, using intron spanning primer specific to bovine PRM1 (Forward 5'AGATACCGATGCTGCCTCAC3', Reverse 5'GTGGCATGTTCAAGATGTGG3) gene. Bovine genomic DNA isolated from blood by GenElute™ Blood Genomic DNA Kit (Sigma–Aldrich) was used as a positive control. A diluted 1:10 solution of the cDNA was used to elucidate the differential expression of PRM3 in mature spermatozoa of good and poor quality crossbred Frieswal (HF X Sahiwal) bull. The expression of PRM3 mRNA was quantified by real-time PCR (Step One, Applied Biosystems, Foster City, CA, USA) using TaqMan probe chemistry. The PRM3 (FAM, PN4351372) and PPIA (VIC, PN4448489) gene probes were obtained from Applied Biosystems (Foster City, CA, USA). PPIA gene was used as an endogenous control. All the PCR reactions were performed in optical 48-well reaction plates in triplicate. The amplification was carried out in 10 µl volume containing 5 µl TaqMan 2X Universal PCR Master mix, 0.5 µl Probe (20X), 2 µl of cDNA template and 2 µl DNase/RNase free sterile water. PCR cycling conditions were: initial denaturation of 95°C for 10 min followed by 40 cycles of denaturation 95°C for 30 s; annealing for 60°C for 60 s and extension 60°C; 55 s. For gene of interest negative and positive controls were included. Samples were quantified by the ΔΔCt method (Martin-Coello et al., 2011).

Data are presented as mean ± SEM and analyzed by using SPSS statistical program (SPSS 10.0 for Windows; SPSS, Inc., Chicago, IL, USA). The experiment was reproduced thrice. Significant differences were determined by one-way ANOVA using the SPSS program. Gene expression pattern of the PRM3 gene between good and poor quality semen producers were compared using Student *t*-test.

RESULTS AND DISCUSSION

Bulls were categorized into normal (good) and impaired (poor) groups based on basic semen parameters like volume, sperm concentration, number of sperm/ejaculates, initial progressive motility and post thaw motility (PTM). The overall seminal attributes of bull spermatozoa categorized as good and poor are shown in Table 1. Presence of genomic DNA, the intron-spanning primers of PRM1 gene produced an amplicon of 334 bp, whereas a single band corresponding to the size of 234 bp observed in case of pure cDNA without genomic DNA contamination (Figure 1). Sperm cDNA samples, devoid

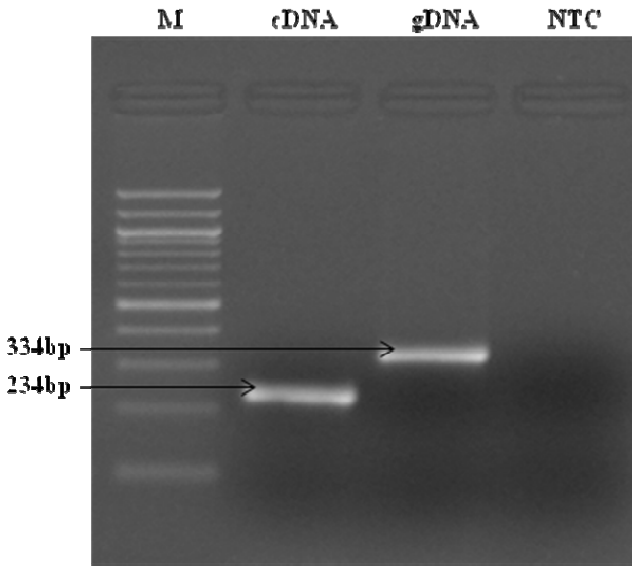

Figure 1. The intron-spannig primer of PRM1 was used to detect the genomic DNA contamination. Lane 1 = M, 100 bp DNA ladder, Lane 2 = PRM1 produced 234 bp amplicon size with cDNA sample, Lane 3 = PRM1 produced 334 bp amplicon size with gDNA sample and Lane 4 = NTC, No template control.

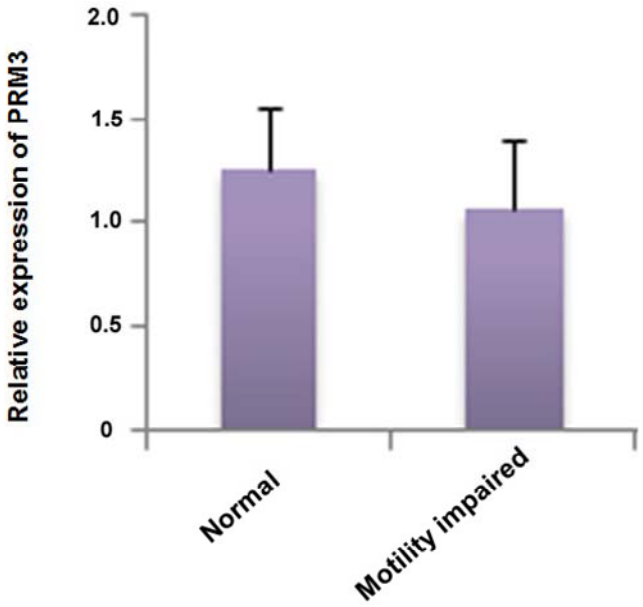

Figure 2. PRM3 mRNA expression in normal (good) (n=10) and motility impaired (poor quality) (n= 9) semen producing Frieswal bulls. Expression of mRNA was normalized using PPIA (Peptidyl Prolylisomerise A) as housekeeping gene. Data of two independent experiments indicating mean mRNA expression ± standard error for triplicates.

of genomic DNA contamination, were used for down-stream qPCR. The mRNA expression of PRM3 was monitored in good (normal) and poor (motility impaired) quality semen producing crossbred Frieswal bulls. In the present investigation, we observed the relative transcript abundance of PRM3 was down regulated in poor quality crossbred Frieswal bulls, however, the difference was not significant ($p>0.05$) between the groups (Figure 2). Similarly, we also analyzed the different relative mRNA expression profile of PRM3 in Frieswal bull semen categorized according to semen volume, concentration, number of sperm/ejaculates and post thaw motility (PTM). The results are shown in Table 2.

The sperm cell is a highly differentiated cell type that results from a specialized genetic and morphological process termed spermatogenesis. During spermatogenesis, various modifications occur which is associated with the DNA condensation of the chromatin. Concomitant with these visible changes in chromatin organization, the histone and non-histone proteins are removed from the DNA and replaced for a period of time by several transition proteins. These proteins are subsequently replaced by protamines during the final stages of spermatid maturation, chromatin reorganization and condensation. In spermatozoa formation, the histone-to-protamine transition plays an important role. It is well known that the protamines are the most abundant basic sperm-specific proteins present in sperm nuclei. During spermatogenesis, protamines are replaced by somatic histones that are essential for sperm formation and function (Domenjoud et al., 1990). The PRM1 and PRM2 genes contain a single intron whereas; PRM3 is an intron-less gene that is conserved in mammals. The expression of the PRM3 gene sequence was first reported in rat testis (Singh and Rao, 1988), located in the cytoplasm instead of the nucleus (Kleene, 1989). The genomic sequences of the PRM1 and PRM2 genes are organized in the form of a loop domain together with the transition protein 2 gene (TNP2) and a sequence called PRM3 (Choudhary et al., 1995; Schluter et al., 1996; Singh and Rao, 1988; Krawetz and Dixon, 1988; Wykes and Krawetz, 2003; Nelson and Krawetz, 1993). Due to their spatial organization, they may allow a coordinated expression of these genes during spermiogenesis. In humans, while the protamine (PRM1 and PRM2) and transition protein (TNP2) genes are expressed at high levels, the potential role of PRM3 is expressed at very low levels (Schluter et al., 1996; Singh and Rao, 1988; Krawetz and Dixon, 1988). The predicted amino-acid sequence of PRM3 is not at all related to other protamines, as it lacks arginine clusters and, instead, is rich in glutamic acid. Therefore, PRM3 is not likely to bind DNA. In mouse, PRM3 exhibits severe clear difference from the other protamines, that is, PRM1 and PRM2 (Martins et al., 2004). PRM3 –/– knock-out male mice, lacking PRM3 protein, showed a reduction in sperm motility without compromising their fertility (Kleene, 1989). The role of PRM3 related to bovine fertility and sperm motility is not yet clear and attempted till today. In the present investigation, we could observe

Table 2. Least square mean values for mRNA relative expression of PRM3 gene in Frieswal bulls categorized according to semen volume, concentration, number of sperm/ejaculate and post thaw motility percentage.

Parameter	Good	Poor	p Value
Volume (ml)	1.34±0.48 (n=8)	1.16±0.45 (n=9)	ns
Concentration (millions/ml)	1.54±0.39 (n=8)	1.34±0.36 (n=9)	ns
Number of sperm/ejaculate	1.50±0.36 (n=7)	1.43±0.41(n=6)	ns
PTM (%)	1.29±0.47 (n=9)	1.13±0.44 (n=9)	ns

ns: Non-significant; PTM: post thaw motility.

non-significant difference of PRM3 transcript abundance between normal and motility impaired groups. Our previous study showed a significantly decreased level of PRM1 mRNAs in spermatozoa of motility impaired group compared to normal crossbred Frieswal bulls. On the other hand, the expression of PRM 2 transcript level was not significantly different between the groups (Grzmil et al., 2008). The lowest mRNA expression of PRM3 was found in bovine testis as compared to other protamines (Ganguly et al., 2013). Together with these findings, our results indicate that protamine 3 expression does not vary significantly among good and poor quality matured bull spermatozoa of Frieswal crossbred cattle. The present preliminary claim must be verified by further experiments to find out the exact role of Protamine 3 to differentiate good and impaired spermatozoa among bulls of different cattle breeds need to be imitated.

Conclusions

The present study although revealed a down regulation of PRM3 transcripts in impaired semen producers as compared to normal crossbred bulls, however, significant difference of transcripts abundance between good and poor quality semen producers could not be observed. The present results thus suggest very little probable role of PRM3 gene in sperm motility as well as other seminal parameters among Frieswal crossbred bulls. However, further studies to understand detailed molecular function, expression profile, regulation pattern of PRM3 gene and its association with sperm motility and fertility in bulls need to be undertaken.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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