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Identifying molecular and spermatological markers to detect sperm chromatin fragmentation

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ABSTRACT

Transition nuclear proteins (*TNP*s) are the primary basic proteins produced from the histone replacement. Protamines (*PRMs*) and transition nuclear proteins are important for maintaining the sperm chromatin integrity, resistance to cryopreservational damage, potentiating fertilizing ability of sperm, preventing embryo deaths and abortions. Hence this histone to protamine replacement should be highly conserved among the males of different species. In the present study, blood and semen samples from 43 cattle and 17 buffalo bulls were collected to isolate genomic DNA and to study sperm chromatin fragmentation following varying period of cryopreservation. Single stranded conformational polymorphism (SSCP) was done to detect any single nucleotide polymorphisms (SNPs) in the coding exonic regions of *TNP* 1 and 2 as well as *PRM* 1 and 2 genes. The SSCP analysis did not reveal any SNP in these genes. This may be due to conserved pattern of these genes. The sperm chromatin fragmentation determined by comet assay was significantly different between the acceptable semen donating cattle as well as the buffalo bulls.

Key word: Comet assay, Histone, Protamines (*PRMs*), Single stranded conformational polymorphism (SSCP), Transition nuclear proteins (*TNPs*)

Selection of superior breeding bulls based on progeny performance has got some limitations. Amalgamation of molecular markers with phenotypic selection increases the accuracy of selection, but highly prized bulls with compromised reproductive efficiency will not help in dissemination of good germplasms. The sperm chromatin integrity is very crucial in effecting a full-term, successful pregnancy in mammals (Balhorn 1982). Replacement of histone by transition nuclear proteins (*TNP 1&2*) and finally by protamines (PRM) is necessary for the integrity of sperm chromatin. Protamines (*PRM 1&2*) are pivotal in spermatid differentiation, aberrations in protamine expression can cause infertility in males (Iguchi *et al.* 2006). The mammalian sperm chromatin is packaged into tight, almost crystalline status that is at least 6 times more condensed than in mitotic chromosomes (Fuentes-Mascorro *et al.* 2000). Identifying the molecular markers contributing to sperm chromatin integrity in cattle and buffalo bulls will improve the fertilizing ability and selection of better bulls for use in improving the artificial insemination success rates. Currently sufficient evidences are lacking on the molecular markers contributing to sperm chromatin integrity in cattle and buffalo bulls. Therefore, the present study was undertaken to find out the SNPs present in cattle and buffalo bulls for TNP and PRM gene pairs and assess the effect of cryopreservation on sperm chromatin integrity following variable time periods.

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MATERIALS AND METHODS

Selection of experimental animals

Peripheral blood for genomic DNA extraction was taken from 43 cattle bulls out of which 38 bulls were having normal sperm parameters and 5 bulls were poor semen donors. Similarly out of total 17 buffalo bulls 9 bulls were acceptable semen donors and 8 were poor donors. The semen samples were collected from 3 farms in Punjab, namely, Dairy farm, GADVASU (20 samples), Ludhiana; State Animal Husbandry and Semen Bank, Nabha (13 samples); State Semen Bank Ropar, Punjab (27 samples).

Polymerase chain reaction: PCR was carried out in a 25 µl reaction volume using 4 sets of primers, 1 published primer-pair for *TNP*1 (Panigrahi and Yadav 2010) having a product size of 484 bp (5'-CTGCAAAGCCCCTCATCTT-3' and 5'-CCCTTCTGTTCGGTTGCT -3'). Three pairs of other primers were designed using Primer3 online software (http: //bioinfo.ut.ee/primer3-0.4.0/), viz. *TNP*2 (amplicon length 414 bp; 5'- CTTTCACAGGACTCCCCCAG -3' and 5'-TCCCAATGCATCAGCCCAAT -3'), *PRM*1 (amplicon

209 bp; 5¢- TGCTGAAGTGTCCTCGTGTC -3¢ and 5'-AGGTGGCATTGTTCGTTAGC -3') and *PRM*2 (amplicon 291 bp; 5¹-CACGTTTTACGGGGTGAGGA -3¹ and 5¹-TGGACTCTTCACGTGGCATC -3¹). All PCR products were visualized by agarose gel electrophoresis and photographed using Gel Doc.

SSCP of PCR products: All PCR products were run in 10% polyacrylamide gel (49: 1 acrylamide and bis-acrylamide. 0.5 X TBE) for SSCP analysis in the vertical electrophoretic unit and it was run at 25° C (instead 4° C), 200 Volt for 7–8 h (Mukhopadhyay *et al.* 2011a).

Assessment of sperm chromatin fragmentation: Neutral Comet assay was done to assess the sperm chromatin fragmentation levels in cattle and buffalo bulls with little modification of the protocol given by Singh *et al.* (1988). The experimental animals were grouped into 2. One group containing 7 cattle and 6 buffalo semen donating bulls; samples were used for standardization of protocol at 3 different time intervals: fresh sample (0 day), seventh day post-cryopreserved and 30th day post-cryopreservation. Second group had 30 cattle and 10 buffalo semen donating bulls; samples were used for studying the effect of cryopreservation of sperm chromatin integrity at 90 days (3 months) and 113 days (3 months and 23 days) post-cryopreservation.

RESULTS AND DISCUSSION

Analysis of TNP 1 & 2 and PRM 1 & 2 gene for SNPs: The band pattern obtained by SSCP analysis did not reveal any difference among the different samples (Fig. 1) for *TNP 1* and *TNP 2* genes. The absence of SNP sites in these genes may be due to highly conserved nature of these 2 genes, which are in accordance with Kremling *et al*. (1989). Panigrahi and Yadav (2010) identified 3 novel SNPs in the intronic region of *TNP 2* gene at 205, 341 and 347 bp positions, however, not in the *TNP1* gene. Given the limited literature on the protamine genes in domestic animals, the studies done on human subjects furnished the only comparison. Schlicker *et al.* (1994) did not detect any variation in *TNP* 1 in 36 infertile men. Miyagawa *et al.* (2005) carried out direct sequencing of the PCR-amplified DNA from blood samples in human subjects and observed 7 SNPs in the 1547-bp region of the *TNP2* gene; 2 were in the intron and 5 were in the exon. Absence of SNPs in the target regions in the present study could also be due to the small sample size. Aoki *et al.* (2006) found a total of 3 SNPs in the PRM1 gene. Each polymorphism occurred with similar frequencies between protamine-deficient patients, severely infertile patients, and fertile controls. *Protamine 2* gene is transcribed and translated on low levels in bull spermatids, can be the contributing factor for absence of polymorphism (Maier *et al.* 1990). Aoki *et al.* (2006) identified 7 SNPs in the PRM2 gene; 5 of the 7 SNPs are novel (T66C, C201T, C281T, C290T, and C406T), whereas the remaining 2 have already been reported by Tanaka *et al.* (2003).

Assessment of sperm chromatin fragmentation: In the present study, the per cent intact sperm-chromatin differed significantly among the different days of cryopreservation (Table 1; Fig. 2). Fresh (0 day) semen samples obtained from acceptable cattle bulls had intact sperm chromatin 70.833±0.739 as against acceptable buffalo bulls with 83.333±0.926 intact sperm-chromatin differing significantly (P<0.05).

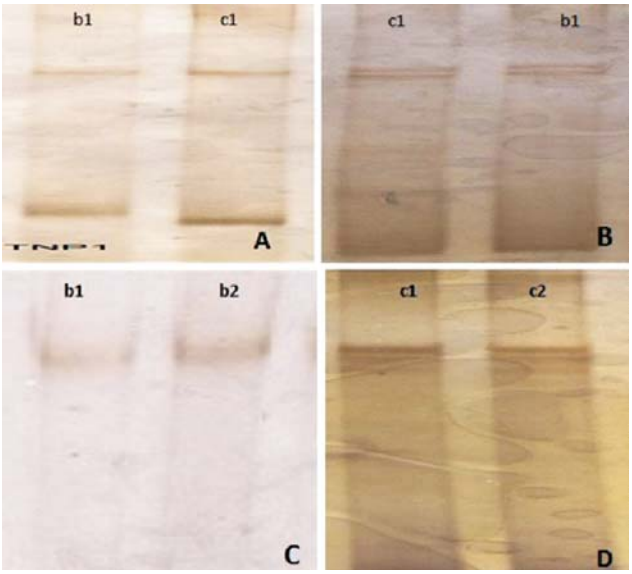

Fig. 1. Band patterns revealed by single strand conformation polymorphism (SSCP) study of the (A) buffalo (b1) and crossbred cattle (c1) for *Transition Protein 1* (*TNP1*) gene, (B) crossbred cattle (c1) and buffalo (b1) for *Transition Protein 2* (*TNP2*) gene, (C) buffalo (b1 and b2) for Protamine 1 (PRM1) gene and (D) crossbred cattle (c1 and c2) for *Protamine 2* (*PRM2*) gene.

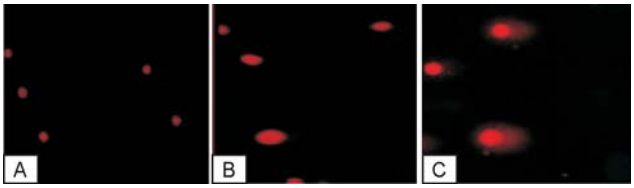

Fig. 2. Single cell gel electrophoresis representing (A) sperm-cells with no fragmented chromatin (Fresh semen), (B) sperm nuclear chromatin with mild fragmentation evident as hallow around the cells (7th Day cryopreservation), (C) highly fragmented sperm nuclear DNA emerging as comet tail (30th day cryopreservation).

Table 1. Per cent high fragmented spermatozoa of acceptable semen donating cattle and buffalo bulls at different time intervals

Species	Fresh (0 day) sample	7 day post cryopreservation	30 day post cryopreservation
Cattle	1.905^{a}±0.294	4.167^{b}±0.359	12.619^{c}±0.781
Buffalo	0.972^{a}±0.296	2.361^{d}±0.139	7.639^{e}±0.427

$^{a, b,c.}$: mean-values with different superscripts differ significantly at 5% (P<5%).

Table 2. Percent highly fragmented sperm chromatin obtained after cryopreservation of bovine (buffalo and crossbred cattle) bull semen for 90 days and 3 months and 23 days

Category	Species	3 Months	3 Month and 23 days
Acceptable semen	Cattle	$4.568^{a}\pm0.195$	$11.142^{e}\pm0.278$
	Buffalo	$1.667^{b}\pm0.417$	$5.278^{a}\pm0.773$
Poor semen	Cattle	$8.500^{c}\pm0.802$	$13.667^{f}\pm0.875$
	Buffalo	$4.479^{d}\pm0.260$	$8.854^{c}\pm0.563$

[a, b,c]: mean-values with different superscripts differ significantly at 5% (P<5%).

Animals belonging to the second group comprised 30 cattle bulls divided into 2 categories. First category was of acceptable semen donating and another category was of poor semen donating. Among 30 cattle bulls 27 were of acceptable semen donating standard and 3 bulls were poor semen donators. Among 10 buffalo bulls 7 bulls were poor semen donating and 3 bulls had semen of acceptable semen donating quality. On performing comet assay of these bull semen after 3 months and again after 3 month and 23 days showed significant differences (P<0.05) for the sperm DNA fragmentation (Table 2).

DNA fragmentation level on different time intervals was high in cattle bulls as compared to buffalo bulls. Also the fragmentation level showed an ascendency on the increase of cryopreservation time in both cattle and buffalo bulls irrespective of acceptable and poor quality. But the percent fragmentation of sperms in poor bulls was much higher as compared to acceptable quality bulls. Mukhopadhyay *et al.* (2011b) revealed that freezing–thawing of bovine semen significantly increased the sperm chromatin breakage. Similarly, Linfor and Meyers (2002) detected the increased DNA damage in response to cooling injury in equine spermatozoa using single cell electrophoresis. Correlation was found between poor semen quality and higher levels of damaged DNA detected as DNA fragmentation using the comet assay by Irvine *et al.* (2000). The above results are further strengthened by the findings of Bell *et al.* (1993) who concluded that cryopreservation and thawing, alone or in combination, are likely to induce membrane damage to sperms. Sparse reports are available in this regard in livestock species associating sperm DNA fragmentation level with seminal attributes (Fraser and Strzezek 2004, 2007 and S³owinska *et al.*2008). Sperm DNA of infertile and subfertile individuals (poor donors) suffer more DNA fragmentation, especially following freezing of samples (Kalthur *et al.* 2007).

The single stranded conformational polymorphism (SSCP) done on transition nuclear proteins and protamine genes in divergent animals indicated that these genes are highly conserved among cattle and buffalo. The sperm chromatin fragmentation determined by comet assay differed significantly (P<0.05) between the acceptable semen donating cattle and buffalo bulls. Cryopreservation of spermatozoa irrespective from acceptable semen donating cattle/ buffalo bulls for longer period induces sperm chromatin fragmentation.

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