Haryana Vet. (June, 2014) **53 (1)**, 50-54

Research Article

LEPTIN GENE TRANSCRIPTS STUDY IN *IN VITRO* CAPACITATED SPERMATOZOA OF BUFFALO BULL SEMEN

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Received: 11.11.2013; Accepted: 19.03.2014

ABSTRACT

Leptin, known as a potential satiety factor, plays an important role in homeostasis of both metabolism and reproduction. The present study was carried out on 10 buffalo bull semen ejaculates. After collection, the semen was evaluated by physical and microscopic methods and by Computer assisted semen analysis (CASA). *In vitro* capacitation was done in sperm Talp (sp-TALP) media containing heparin and was confirmed by head to head agglutination and acrosomal reaction after staining with dual stain. Head to head attached acrosome reacted sperm cells were also high at the end of incubation. Fresh and *in vitro* capacitated spermatozoa were investigated for the presence of leptin gene transcript using reverse transcriptase-PCR (RT-PCR) analysis. After RT-PCR, the amplified PCR product was purified and cloned. The cloned product was further sequenced and analyzed to study the phylogenetic relationship of leptin gene transcript of buffalo with other species. RT-PCR analysis revealed that leptin gene transcripts were expressed in fresh as well as *in vitro* capacitated spermatozoa of buffalo bull semen. Based on band intensity it was found that expression of gene was slightly higher in *in vitro* capacitated spermatozoa as compared to fresh ejaculated spermatozoa. Presence of higher expression of leptin gene transcripts in *in vitro* capacitated spermatozoa may suggest its role in regulating process of capacitation in this species.

Key words: *In vitro* capacitation, acrosomal reaction, RT-PCR, cloning

Leptin,with molecular weight of 16 kDa and 167 amino acids, is a pleiotropic cytokine like peptide hormone, produced by obese gene and identified in 1994 by positional cloning in mouse and humans (Zhang *et al*., 1994). The leptin gene has been mapped to chromosome 4 in bovine (Stone *et al*., 1996; Pomp *et al*., 1997) and chromosome 8 in buffalo (Vallinato *et al*., 2004). The bovine leptin gene consists of three exons and two introns (about 18.9 kb) are spans, equivalent to that of human or mouse gene (Taniguchi *et al.*, 2008). Leptin functions as a satiety factor in the regulation of body weight (Ahima *et al*., 2000), and as a permissive regulator of human reproductive maturity serving as mediator in a wide range of neuroendocrine functions (Tena-Sempere *et al*., 2007). Leptin profoundly effects reproduction by exerting its biological effects via interaction with the leptin receptor. Studies have pointed to a direct role of leptin in male reproductive function (Tena-Sempere *et al*., 2002) and in the female fertility (Brannian *et al.*, 2001). However, the role of this hormone in the regulatory network controlling male reproductive function is still a matter of debate (Aquila *et al*., 2005). Recently, leptin secretion by sperm suggested that the sperm has ability to modulate its metabolism, according to its energy needs, independently by systemic leptin expression. This may represent a protective mechanism in male reproduction to guarantee the accumulation of energy substrates to maintain fertilizing capability of the gamete (Consiglio *et al*., 2009).

There is little information about role of leptin in reproductive performance of farm animals, especially in buffalo bull. Hence, the present study was conducted to analyse the expression of leptin gene transcript in the fresh and in *in vitro* capacitated buffalo bull semen.

MATERIALS AND METHODS

Collection and Examination of Semen: Semen was collected from Murrah buffalo bulls (*Bubalus bubalis*) maintained at the Semen Bank, Hisar using artificial vagina (IMV, France) maintained at 42^0C. Semen having mass activity of +3.0 and above on a subjective scale of 0 to 5 were used in this study. Semen samples were evaluated for various macroscopic examinations and microscopic parameters. Percentage of live

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spermatozoa was estimated by differential staining technique using Eosin-Nigrosin stain (Campbell *et al*., 1953). Minimum of hundred spermatozoa were counted under the oil immersion of a phase contrast microscope for estimating the percentage of live (unstained) spermatozoa. The pinkish (eosinophilic) and partially stained spermatozoa were classified as dead (Fig. 1). Hypo-osmotic swelling test was performed to determine the membrane integrity. The appearance of curls in tail of a sperm signifies that water has been transported in a physiological manner into the cell to reach osmotic equilibrium. This is indicated by an intact flagellar membrane (Fig. 1). The samples were then processed in culture medium, modified Tyrodes's bicarbonate-buffered medium (sp-TALP) as described by Parrish *et al*. (1988). Working sp-TALP medium was prepared by adding Ca^{2+} (2mM), pyruvate (1mM), $NaHCO_3$ (25mM) to 2X stock diluted in the ration of 1:1 with distilled water.

Sperm Capacitation and Acrosome Reaction: *In vitro* capacitation using sp-TALP supplemented with heparin and acrosomal reaction induction using lysophosphotidyl choline (LPC) was done following the protocol of Roy and Atreja (2008). After assessing the motility of sperms at different time intervals, the extent of sperm agglutination was determined as a qualitative test of sperm capacitation. LPC is known to induce acrosome reaction only in capacitated cells, hence the percentage of acrosome reacted cells represents the percentage of capacitated cells. After the stipulated incubation time, the samples were subjected to dual staining procedure as described by Sidhu *et al*. (1992) to differentiate the physiological as well as degenerative acrosome loss. The smears were viewed under oil immersion. All types of cells were counted randomly in different fields. At least 100 cells were screened for the assessment for capacitation.

Evaluation of Semen: Evaluation of semen was done by computer assisted semen analyser (CASA) HTM-Version-12 IVos analyser. Motility of spermatozoa was judged by CASA by keeping 5µl semen over Leja^{R}standard count four chamber slide as per the manufacturer's instructions. The individual motility of spermatozoa was recorded as percentage of progressively motile spermatozoa. Viability of spermatozoa was determined using Ident dye as per the manufacturer's instructions.

RNA Isolation: Total RNA was isolated from fresh as well as capacitated spermatozoa using Trizol (Life technologies) reagent as per the manufacturer's instructions. The cDNA was synthesized from the total RNA with random decamer primers using cDNA kit (Ambion™).

Polymerase Chain Reaction (PCR): The cDNA synthesized was amplified by PCR using leptin gene specific primers (Table 1). Primer blast was used to design the primers for exon-2 of leptin gene and β-actin (positive control for PCR reaction). Cyclic conditions for amplification of leptin gene consisted of initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation at 95°C for 45 sec, annealing at 57^0C for 45 sec, primer extension at 72°C for 45 sec and a step of final extension at 72°C for 10 min. A product size of 397 bp and 115 bp was observed for exon-2 and β-actin, respectively on gel electrophoresis.

Purification of PCR Product and Cloning: PCR products were purified using QIAquick gel extraction kit (QIAGEN) before custom sequencing. The pJET1.2/ blunt cloning vector (2974 bp; Fermentas) was used for cloning the PCR product of leptin gene exon 2.

Nucleotide Sequencing and Phylogenetic Analysis: Purified PCR products of leptin gene exon-2 were sequenced using BigDye terminator chemistry and on automated DNA sequencer (ABI PREISM ™3130XL Version 3.0) in the department. The partial length leptin gene sequences of buffalo and leptin gene sequences of different species available in the GenBank database were used for phylogenetic analysis. Evolutionary relationships among different species were also determined using MEGA 4.0 software.

Table 1
Primers used for the amplification of leptin gene and β-actin

Gene	Forward	Reverse	Product length
Exon-2	5'-CCAGGGAGTGCCTTTCATTA-3'	5'-ATGGCCACGGTTCTACCTC-3'	397 bp
β-actin	5'-CAATTCCATCATGAAGTGT-3'	5'-GATCTCTTTCTGCATCCT-3'	115 bp

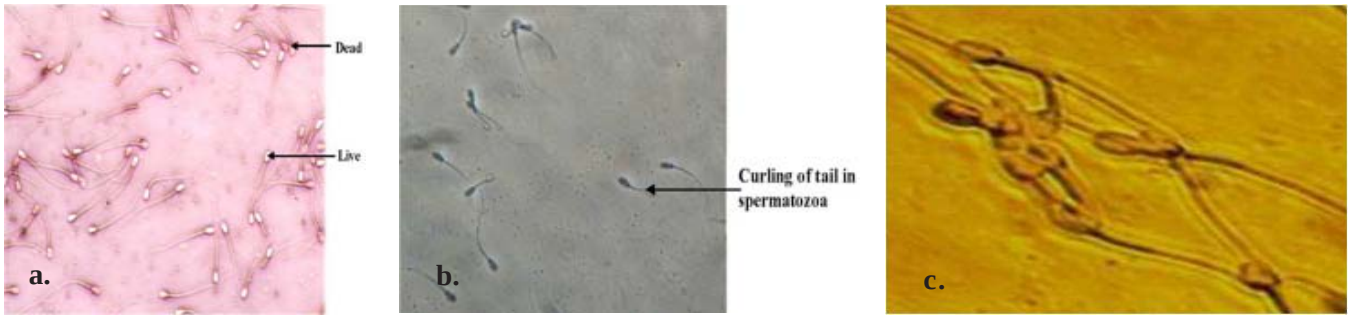

Fig 1. Microscopic examination of semen (a) Live and dead spermatozoa (b) Hypo-osmotic swelling test showing curling of tail in spermatozoa (c) Head to head agglutination of spermatozoa after *in vitro* capacitation.

RESULTS AND DISCUSSION

Physical and Physiological Attributes: Average volume of 10 buffalo bull semen ejaculate under experiment was 5.5±0.01ml. The semen characteristics viz. ejaculate volume, colour consistency, pH, mass activity, individual motility, sperm concentration viability and abnormal sperm count were evaluated. Colour and consistency of semen of all bull ejaculate was milky white and medium thick, respectively. The pH of semen varied from 6.8 to 7.3. Concentration of sperms varied from 942 to 1500 million/ml (average 1200 million/ml). Viability (%) of sperms varied from 86% to 93% (mean 89%) and average morphological abnormality was less than 4%. The finding of average volume of semen ejaculates was in close conformity with the earlier report of Dhami (1992). Average mass activity was higher than that reported by Gill *et al.* (1974).

Evaluation of Semen by CASA: Total motility (%) of spermatozoa varied from 86 to 96% (average 91%) while the progressive motility varied from 14 to 21% (mean 17%). Viability varied from 86 to 96% (average 91%). Evaluation by CASA was more accurate than manual handling because CASA value was recorded by screening different fields under fluorescent microscope and thus providedthe highest level of accuracy for measuring sperm velocities and motion parameter.

***In vitro* Capacitation of Buffalo Spermatozoa:** *In vitro* capacitation was carried out in different media supplement and the spermatozoa changed their motility patterns. During incubation period, capacitated spermatozoa showed characteristic behaviour with head to head attachment wandering around and many sticking with each other (Fig. 1).

Assessment of Acrosomal Reaction: Assessment of acrosomal reaction by dual staining (Trypan blue and Giemsa) method revealed four types of staining patterns (Fig. 2). The staining patterns were: Live spermatozoa with acrosome intact (LAI; pink acrosome and unstained post-acrosomal region), live spermatozoa with acrosome reacted or lost (LAR; unstained acrosomal region and unstained post-acrosomal region), dead spermatozoa with acrosome intact (DAI; pink acrosome and light-blue or light brown post-acrosomal region) and dead spermatozoa with acrosome reacted or lost (DAR; unstained or light-blue acrosomal region and light blue or light-brown post-acrosomal region).

In vitro capacitation with heparin showed that the percentage of LAI, DAI, LAR and DAR was 87%, 6%, 4% and 3%, respectively at 0 h of incubation. During half an hour to 2 h of incubation, LAI, DAI, LAR and DAR varied from 75 to 70%, 5 to 8%, 10 to 15% and 4 to 7%, respectively. After 3 hours of incubation, LAI, DAI, LAR and DAR were 58, 10, 20, 12%, respectively. Addition of lysophosphotidyl choline (100µg/ml) after 4 h increased the LAR cells percentage from 20 to 80

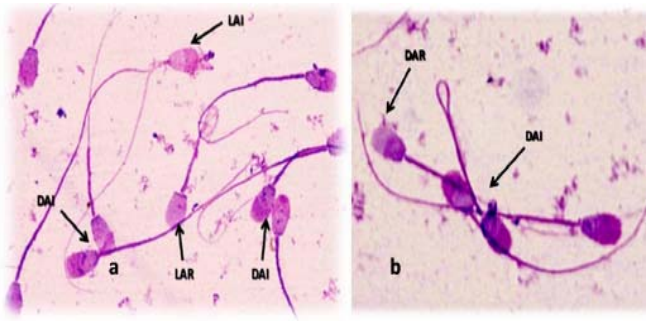

Fig 2. Buffalo spermatozoa stained with dual staining technique (a & b). LAR (live acrosome reacted); DAR (dead acrosome reacted); LAI (live acrosome intact); DAI (dead acrosome intact).

(Table 2). The present findings of evaluation of acrosomal reaction were similar to that of observed by Sidhu *et al*. (1992).

Leptin Receptor Gene Transcripts in Fresh and *in vitro* Capacitated Spermatozoa : The cDNA amplified by PCR using leptin gene exon-2 primers yielded a product of 397 bpin fresh and capacitated spermatozoa (Fig. 3). A house keeping gene, β-actin, was also amplified using β-actin gene specific primers which yielded a product size of about 115 bp (Fig. 3). The comparison of the intensity of band of the exon-2 gene leptin in fresh and *in vitro* capacitated sperms revealed that the leptin gene was expressed more in the *in vitro* capacitated sperms.

Sequence Analysis of Leptin Gene Exon-2: The nucleotide sequences of leptin gene exon-2 showed 97.9% similarity to that of *Bubalus bubalis* (AY495587.1). Phylogenetic analysis also revealed that the *B. bubalis* was closer to *Bos* species than any other species (Fig. 4). The *B. bubalis* was least identical to pig (*Sus scrofa*).

In the present study, we observed that the expression of leptin gene transcript was higher in *in vitro* capacitated sperms than in fresh sperms. Aquila *et al*. (2005) reported that treating washed pooled human sperms from normal samples with leptin and incubating them under uncapacitating condition increased both the cholesterol efflux and protein tyrosine phosphorylation. They hypothesized that the leptin may have a role in modulating sperm energetic substrate availability during capacitation. Additionally, these authors demonstrated that sperms can modulate its metabolism and could secrete leptin based on its energy needs independent of systemic leptin expression. This may represent a protective mechanism in male reproduction to guarantee the accumulation of energy substrates to

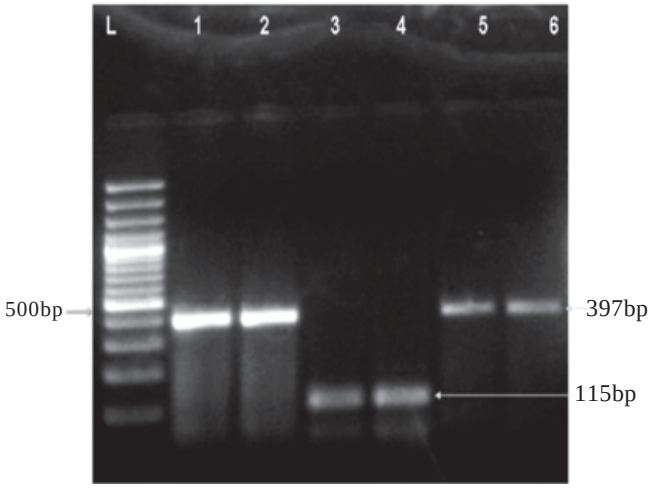

Fig 3. Agarose gel electrophoresis of RT-PCR product. Lane L: 100bp ladder; Lanes 1-2: PCR product of exon-2 (397bp) after capacitation; Lanes 3-4: PCR product of â-actin (115bp); Lanes 5-6: PCR product of exon-2 before capacitation.

maintain the gamete fertilizing capability. The increased expression of the leptin gene in the *in vitro* capacitated sperm might have happened to modulate the energy requirement by increasing the leptin receptors. Lampiao and du Plessis (2008) showed that *in vitro* leptin had beneficial effects on human sperm function and concluded that the hormone could play a role in enhancing the fertilization capacity of human spermatozoa via increasing motility and acrosome reaction. Therefore, leptin, through its receptor, appears to affect both capacitation and acrosome reaction suggesting its possible role in the acquisition of fertilizing ability of sperm.

The present study revealed that the results analyzed through CASA was more accurate than that observed by manual method. The mRNA expression of leptin gene was slightly higher in *in vitro* capacitated spermatozoa as compared to fresh ejaculated spermatozoa. Higher presence of leptin gene transcripts in *in vitro* capacitated

Table 2

Acrosomal reaction after incubation in media containing heparin and lysophosphotidyl choline

Sperm incubation time (hr)		LAI %	DAI %	LAR %	DAR %
Heparin (10µg/ml)	0 hr	87	6	4	3
	1/2 hr	75	5	10	4
	1 hr	70	8	15	7
	2 hr	70	8	15	7
	3 hr	58	10	20	12
Lysophosphotidylcholine (100µg/ml)	4 hr	5	3	80	14

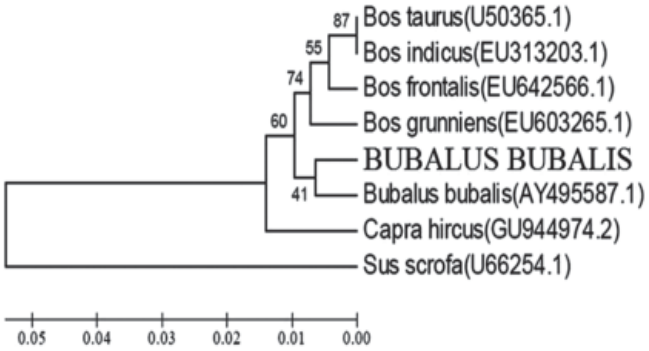

Fig 4. Phylogram of buffalo leptin gene exon-2 depicting the evolutionary relationship with other species.

spermatozoa was suggestive of its possible role in regulating process of capacitation in this species.

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