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Identification of Clusterin in Buffalo (*Bubalus Bubalis*) Semen and its Relation to Semen Quality

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ABSTRACT

The study aimed to identify the clusterin in buffalo seminal plasma and compare the semen quality of high and low clusterin-positive sperm (CPS) bearing buffalo bulls. Semen was collected with artificial vagina technique from buffalo bulls. The anti-clusterin antibody recognized one protein band of ~40 kDa in the western blot. Based on the results of the immunofluorescence assay, bulls were divided into two groups: high CPS and low CPS bulls. The expression of clusterin on sperm cells was further reflected in terms of their quality. The high CPS bulls showed significantly ($P<0.05$) low values for total, progressive, and rapid sperm motility compared to low CPS bulls. The curve linear velocity (VCL), straight linear velocity (VSL), straightness (STR), and linearity (LIN) of sperm were found to be significantly higher in low CPS bulls than in high CPS bulls. Further, high CPS bulls showed significantly more sperm abnormality than that of low CPS bulls. The plasma membrane integrity of sperm was found more in low CPS bulls compared to high CPS bulls. In conclusion, an anti-clusterin antibody recognized ~40 kDa clusterin in buffalo seminal plasma. The high percentage of CPCs in buffalo semen is a negative marker for semen quality.

***Key words*:** CASA, Immunofluorescence assay, Biomarker

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INTRODUCTION

Buffalo is an integral part of rural agriculture in India, and Southeast Asia and spread to many other countries outside Asia, towards the Middle East, Europe, Latin America, North America, and Africa. Despite its incontestable contribution to the animal industry, now this species has started receiving well-deserved recognition from research workers. Out of approximately 55 million breedable buffaloes in India, hardly 15% are bred through artificial insem-

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ination (AI) because of the poor freezability and fertility of buffalo spermatozoa when compared with cattle spermatozoa (Andrabi, 2009). For successful AI in buffalo, bull fertility is essential to ensure optimal reproductive efficiency (Kumar *et al.,* 2012). Generally, bulls are selected for semen collection based on breeding soundness evaluation, but among those selected bulls 20 to 25% difference in conception rates was reported (Larson et al. 2000). Routinely used traditional semen quality tests provide limited information about the semen quality of bulls.

Several proteins are secreted into seminal plasma which plays an important role during sperm capacitation and fertilization; therefore, they can be a potential biomarker for semen quality and bull fertility. Clusterin is one of the seminal plasma proteins that was first identified in ram rete testis fluid, where it showed signs of clustering with rat Sertoli cells and erythrocytes (Blaschuk *et al.,* 1983). It is a ubiquitously expressed amphipathic glycoprotein consisting of two non-identical subunits designated α and β. Several isoforms with varying molecular weights of clusterin occur in the bull reproductive tract and variation in carbohydrate content among these isoforms affects the biological or functional activity of the protein (Ibrahim *et al.,* 1999). In the male reproductive tracts, it is produced by Sertoli cells and principal epididymal epithelial cells (Sylvester *et al.,* 1984) and is translocated to abnormal germ cells and spermatozoa (Ibrahim *et al.,* 2001). However, Ibrahim *et al.* (1999) found that 40 and 70 kDa isoforms of clusterin in the accessory gland fluid are similar to those reported in the cauda epididymis fluid of bulls. Clusterin in male reproductive tracts is involved in various physiological processes such as binding and agglutinates abnormal spermatozoa in bulls, prevent oxidative damage to the sperm and inhibit complement-induced sperm lysis (O'Bryan *et al.,* 1990; Ibrahim *et al.,* 1999; Reyes- Moreno *et al.,* 2002). The presence of clusterin on ejaculated sperm may indicate improper spermatogenesis or irregular epididymal maturation (Ibrahim *et al.,* 2000). Thus, clusterin-positive spermatozoa (CPS) in bull semen is a potential marker for poor semen quality (Ibrahim et al. 2001). Despite significant research works done on the identification of clusterin in the secretion of various parts of the male reproductive tract and the presence of clusterin on ejaculated sperm, no reports have been found in buffaloes. Therefore, the present study has been conducted to identify the clusterin in buffalo seminal plasma and subsequently compare the semen quality of high and low CPS buffalo bulls.

MATERIALS AND METHODS

Animal ethics: Animal experiments were conducted after following the guidelines laid down by the Institute Animal Ethics Committee, ICAR-CIRB, Hisar.

Semen collection and cryopreservation: Ten breeding Murrah buffalo bulls were used for semen collection under the progeny testing program of the institute and maintained under an identical milieu. Semen was collected (twice a week for four weeks) with an artificial vagina technique from the buffalo bulls. The collected semen was conventionally assessed for volume, color, and sperm concentration by using an Accucell bovine photometer (IMV, France) and mass activity and percentage of motile spermatozoa. Sperm motility was subjectively assessed under a phase contrast microscope equipped with a warm stage (37°C) at 40 X magnification. The fresh semen was extended in Andromed (Minitube India Pvt. Ltd, Delhi, India) to make a final concentration of 80 x 10^6 spermatozoa/mL. Thereafter, the extended semen was slowly cooled to 4°C and kept for a period of 3-4 h for equilibration. The equilibrated semen was loaded into 0.25 mL plastic straws (IMV, L'Aigle, France) and frozen into a programmable biological freezer (Mini Digi-cool, IMV Technologies, L'Aigle, France) as described earlier by Kumar et al. (2015a).

SDS PAGE and Western blot: For identification of clusterin in seminal plasma, ejaculates of bulls were pooled and then centrifuged at 600 x g for 10 min at room temperature. The supernatant seminal plasma was recovered and re-centrifuged at 10,000 x g for 1 h at 4°C and then the supernatant was kept at -20°C till further evaluation. The concentration of protein in seminal plasma was determined by Lowry et al. (1951). The proteins were separated by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) by using 10% resolving/separating gel as per the procedure described by Laemmli (1970). After electrophoresis, the gel was destained in a destaining solution (50% (v/v) methanol in water with 10% (v/v) acetic acid.) until protein bands were visualized. Western blot analysis was done as per the method described by Towbin et al. (1979) to identify clusterin in buffalo seminal plasma. Briefly, following electrophoresis, the unstained gel was equilibrated in a transfer buffer for 15 minutes. The polyvinylidene fluoride (PVDF) membrane was cut to the dimensions of the gel and kept wet with methanol for a few seconds and then with transfer buffer for 15–30 minutes. The equilibrated gel was carefully placed on top of the transfer membrane, aligning the gel on the center of the membrane, and another sheet of pre-soaked filter paper was placed on top of the gel. After the transfer of protein on the PVDF membrane, the unsaturated sites were blocked by incubating in 5% skimmed milk powder dissolved in Phosphate-buffered saline with Tween 20 (PBST, 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, and 0.1% (w/v) Tween 20) for 2 h at room temperature with shaking on the rotator shaker. The primary antibody (rabbit anti-clusterin,

Sigma # SAB3500199) diluted 1: 500 in PBS was allowed to bind specific proteins on the membrane for overnight at 4^0C followed by 2-3 washing of 5 minutes each with the washing buffer. The membrane was incubated in 1: 2000 dilution of goat anti-rabbit IgG-AP conjugate (Santacruz Biotechnology, Santacruz, CA, U.S.A) *i.e.* secondary antibody for 2 h at room temperature followed by 2-3 washing of 5 minutes each with the washing buffer. The membrane was visualized by adding freshly prepared substrate solution.

Immunofluorescence Assay: Frozen-thawed semen was centrifuged at 1000 x g for 10 minutes, and the supernatant was discarded. The sperm pellets were resuspended in phosphate-buffered saline (PBS), smear prepared on a clean slide, and air-dried, then fixed with 4 % paraformaldehyde in PBS for 1 h at 4°C. After three washes in PBS, slides were blocked with 1% BSA (fraction V, Sigma #A-9647) for 2 h. The slides were incubated with anti-clusterin antibody (1:100, rabbit anti-clusterin, Sigma # SAB3500199) and left overnight at 4°C. The solution was decanted and the cells were washed with PBS three times for 5 minutes each. After incubation with goat anti-rabbit IgG labeled with FITC (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a dark room for 45 min at room temperature, slides were washed with PBS and covered with a cover slip. Slides were viewed with a fluorescent microscope. The proportion of clusterin-positive spermatozoa was determined by differential counting of 200 sperm cells under a fluorescence microscope (excitation-495 nm and emission-519 nm). The bulls were divided into high and low CPS groups on the basis of clusterin-positive sperm cells, greater than 7% CPS were considered high CPS bulls, and lesser or equal to 7% CPS, the bulls were considered low CPS bulls. The classification based on the finding of Ibrahim et al. (2000). Among 10 bulls, six were low CPS bulls and four were kept in high CPS bulls.

Assessment of plasma membrane integrity: Plasma membrane integrity of frozen-thawed semen was evaluated using a hypo-osmotic swelling test (HOST) as described by Kumar *et al.* (2015a). Briefly, the test was performed by mixing 100 µL semen with 1 mL hypo-osmotic solution (0.735 g sodium citrate $2H_2O$ and 1.351 g fructose in 100 mL distilled water) and for the control group, 1 mL PBS mixed with 100µL frozen-thawed semen and incubated for 60 min at 37°C. For easy visibility one drop of eosin solution was added in the suspension, just before the examination of the slides. After incubation, sperm tail bending/coiling was assessed by placing 15 µL of well-mixed sample on a warm slide (37°C) under light microscopy at 400× magnifications. A total of 200 spermatozoa were counted in different fields and the percentage of spermatozoa positive for the HOS test (having coiled tails) was calculated. The percentage of curled tail spermatozoa in PBS was deducted from that of the HOST to get the true HOST-reactive sperm. The sperm with a coiled tail after incubation were considered to have an intact plasma membrane.

Assessment of sperm abnormalities: The sperm morphological abnormalities were evaluated by the eosin–nigrosin staining method described earlier (Singh *et al.,* 2013). Briefly, after washing sperm samples in PBS at 37 °C, one drop of the suspension containing 2–3 x 10^5 sperm/mL was placed in one corner of a pre-warmed glass slide. Then a thin film smear was prepared using a spreader slide at a 30° angle to disperse the semen suspension over the slide's length and fixed with air drying. The smear was stained with eosin–nigrosin solution (1.67 g eosin, 10 g nigrosin dissolved in 100 mL distilled water) for 3–5 min, excess stain was removed by rinsing the slide in distilled water, dried, and evaluated under a phase contrast microscope at 1,000x magnification. A minimum of 200 spermatozoa were analyzed for each sample.

Assessment of acrosomal integrity: The acrosomal integrity was evaluated using fluorescein-conjugated lectin *Pisum sativum* agglutinin (FITC-PSA) staining method as reported by Kumar et al. (2014) with modification. In brief, the semen sample was diluted in PBS and centrifuged at 1500 rpm for 10 min, and then supernatant was discarded. Finally, a pellet of spermatozoa was re-suspended in 250 µL PBS and then one drop was taken for smear onto a pre-cleaned microscope slide. The smear was dried and fixed with ice-cold methanol for a few seconds. Air-dried slides were incubated with FITC-PSA (50 µg/mL in PBS solution) in the dark for 20 min at room temperature. Subsequently, the excess stain was removed by washing with ultra-pure water, then the slide was air dried, and a cover slip was applied with glycerol and examined under a fluorescent microscope fitted with an excitation filter of 365 nm and a barrier filter of 397 nm. Sperm possessing an intact acrosome showed more intense fluorescence in the acrosome region with a distinct ring, whereas damaged sperm heads had less or no intense fluorescence.

Objective assessment of sperm kinetics and motility: Sperm kinetics and motility were assessed using CASA system CASA (computer-assisted sperm analyzer, Hamilton Thorne, IVOS II) as described earlier (Kumar *et al.,* 2015b). Before analysis under CASA, the semen sample was diluted with pre-warmed Tris buffer to give a sperm concentration of about 40 × 10^6 spermatozoa/mL. One µL prepared semen sample was loaded in a pre-warmed (38°C) 8-chamber Leja slide (depth 20 µm) and analyzed for sperm motility characteristics. For each sample, 5 optical fields around the central reticulum of the chamber were used to count spermatozoa.

The CASA software settings were as follows: temperature =38°C, frame rate= 60Hz, frames acquired=30, minimum contrast=35, minimum cell size=5 pixels, cell size= 9 pixels, cell intensity= 110 pixels, average path velocity (VAP) =50µm/s, straightness (STR) =70%, VAP cut-off= 30 µ/s and VSL cut-off=15 µ/s for recording sperm kinetics and motility. The following motion characteristics were recorded in frozen-thawed samples: total motility (TM, %), progressive motility (PM, %), rapid motility (RM %), straight linear velocity (VSL, µm/s), average path velocity (VAP, (µm/s), curvilinear velocity (VCL, µm/s), average lateral head displacement (ALH, µm/s), beat cross frequency (BCF, Hz), straightness (STR, %) and linearity (LIN, %) of the spermatozoa.

Statistical Analysis: All data were analyzed using SPSS software. The data were expressed as mean ± SE and the differences were compared by unpaired t-test. Relationships between values were analyzed by the Spearman correlation test. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The average concentration of protein in buffalo seminal plasma was found to be 25mg/mL. Western blot analysis of unreduced samples showed that anti-clusterin antibody recognized protein bands of ~40 kDa (Fig. 1).

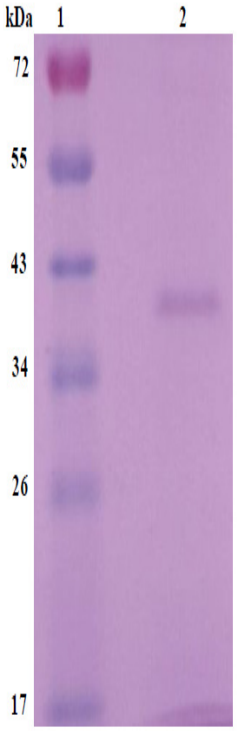

Fig. 1: Western blot of seminal plasma proteins separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE) and probed with the monoclonal antibody MAC393. Lane 1 pre-stained marker, lane 2 seminal plasma clusterin (~40 kDa).

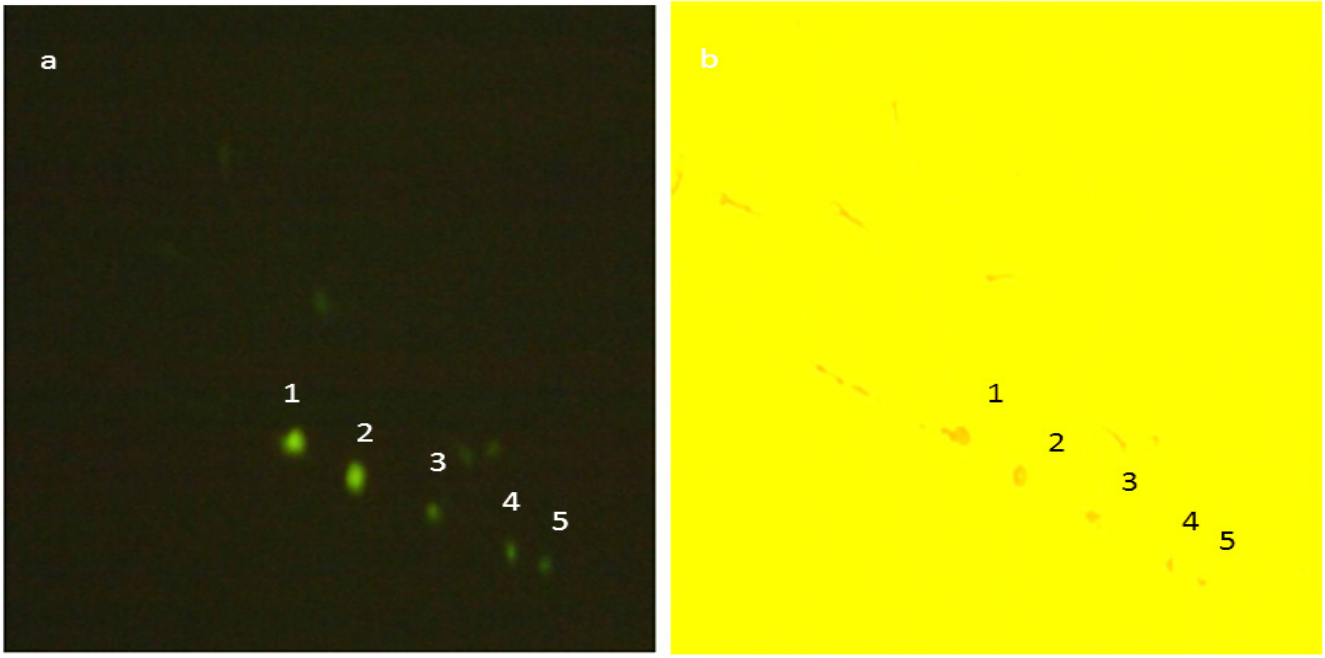

Fig. 2: Immunofluorescence assay of clusterin on frozen-thawed buffalo bull spermatozoa. (a) Spermatozoa stained with anti-clusterin antibody conjugated with FITC anti-rabbit IgG showed green fluorescence indicating clusterin-positive sperm cells. (b) Phase contrast photographs of 'a' showing abnormal morphology. Sperm (1) and (2) exhibiting an extensive staining pattern are teratoids, sperm cells 3, 4, and 5 are decapitated spermatozoa. Magnifications 40X.

After the immunofluorescence assay, we observed that bull no. 1 and 4 showed significantly higher (P<0.05) CPS than bull no. 2 and 3. Therefore, for this study, bull no. 1 and 4 were considered high CPS bulls, and bulls no. 2 and 3 were considered low CPS bulls.

Table 1: Mean (± SE) of the percentage of clusterin positive sperms (CPS), sperm abnormality, HOST, and acrosomal damage of buffalo bull sperm (n=10).

Variable	High CPS bulls	Low CPS bulls
CPS (%)	11.34^{a} ±0.24	6.82^{b} ±0.62
Sperm abnormality (%)	24.09^{a} ±0.94	11.33^{b} ±0.86
HOST (%)	24.14^{b} ±2.27	40.86^{a} ±1.65
Acrosome damages (%)	14.42^{a} ±1.09	13.67^{a} ±1.16

Values with different superscripts within the column differ significantly (P<0.05).

The intensity of fluorescence varied with abnormalities like teratospermatozoa was extensively stained while head and midpiece abnormal spermatozoa appeared weak stained (Fig. 2). The expression of clusterin on sperm cells was further reflected in terms of their quality and we observed that high CPS bulls showed significantly (P<0.05) more morphological abnormality than that of low CPS bulls. The intact plasma membrane integrity of sperm (Table 1) was found more in low CPS bulls compared to high CPS bulls. Further, no significant difference (P<0.05) was observed in acrosomal damage among high and low CPS bulls (Table 1, Fig. 3).

Sperm kinetics and motility of frozen-thawed semen of respective bulls were monitored using the computer-assisted sperm analyzer and results are presented in Table 2. The bulls having higher value for CPS showed significantly (P<0.05) low values for total, progressive and rapid motility compared to low CPS bulls. The VSL and VCL were found significantly higher in low CPS bulls than high CPS bulls. The STR and LIN were found significantly higher (P<0.05) in bull no 2 of low CPS bulls. No significant difference was observed in VAP, ALH and BCF between high and low CPS bulls.

Sperm abnormality is positively correlated while HOST, VSL, VCL, STR, LIN, TM, PM, and RM are negatively correlated with CPS. No significant correlation was observed between acrosomal damage, VAP, ALH, and BCF with CPS (Table 3).

Table 3: Correlation of sperm parameters with clusterin positive sperm

Variable	r	*P*-value
Sperm abnormality (%)	0.614	< 0.001
HOST (%)	-0.486	0.005
Acrosome damages (%)	0.125	NS
VAP (µm/s)	-0.121	NS
VSL (µm/s)	-0.517	0.001
VCL(µm/s)	-0.446	0.012
ALH(µm)	0.013	NS
BCF(Hz)	0.025	NS
STR (%)	-0.625	<0.001
LIN (%)	-0.576	<0.001
TM (%)	-0.725	<0.001
PM (%)	-0.756	<0.001
RM (%)	-0.425	0.075

NS is not significant.

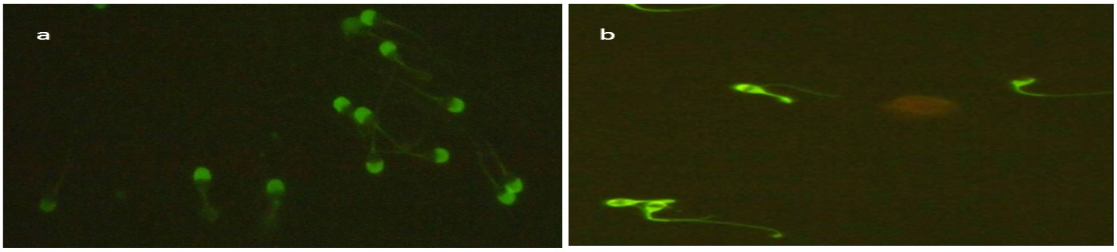

Fig. 3: Evaluation of acrosome damages through FITC-PSA staining. (a) In an intact acrosome, the acrosomal region of the sperm head exhibited a uniform green fluorescence. (b) In an acrosomal damaged sperm, completely or partially acrosomal region do not fluorescence green. Magnifications 40X.

Table 2: Mean (±SE) sperm kinetics and motility parameters of buffalo bull semen (n = 10) evaluated under CASA.

Bulls	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	ALH (µm)	BCF (beats/sec)	STR (%)	LIN (%)	TM (%)	PM (%)	RM (%)
High CPS	106.48^{a} ±1.25	69.84^{b} ±1.62	201.84^{b} ±2.80	8.03^{a} ±0.08	29.10^{a} ±0.17	61.11^{a} ±0.84	37.00^{a} ±0.83	36.44^{b} ±3.76	16.11^{b} ±1.74	20.89^{b} ±2.65
Low CPS	114.09^{a} ±1.64	79.60^{a} ±1.47	230.60^{a} ±2.55	8.08^{a} ±0.09	30.90^{a} ±0.15	73.44^{b} ±0.34	42.33^{b} ±0.33	60.56^{a} ±1.88	30.00^{a} ±2.35	38.00^{a} ±3.53

Values with different superscripts within column differ significantly (P<0.05).

To the best knowledge, we are documenting the first report on the identification of clusterin in buffalo seminal plasma. The results of the present study showed that anti-clusterin antibody recognized one protein band of ~40 kDa in Western blot in buffalo seminal plasma. Similarly, Howes et al. (1998) separated proteins from rete testis fluid (RTF), cauda epididymal fluid (CEF) and seminal plasma of bulls by non-reducing SDS- PAGE and Western blots probed with monoclonal anti-clusterin (MAC393 mAb) showed no reaction in RTF whereas detected ~35 and ~33 kDa protein bands in CEP and only the ~35 kDa protein in seminal plasma. Further, in bull, Western blot of unreduced samples showed that the anticlusterin antibody reacted with a protein of 94 to 100 KDa in RTF and 57 to 76 KDa proteins in CEF (Ibrahim *et al.,* 2001). Collard and Griswold, (1987) showed that rat testis secretes clusterin 73 kDa disulphide-linked heterodimeric proteins. Recently, McReynolds *et al.* (2014) also identified 40 kDa isoform of clusterin on the surface of human sperm. Mature clusterin is secreted as a protein of 76–80 kDa (Burkey *et al.,* 1991; Wong *et al.,* 1993), depending on the degree of glycosylation, which appears like a 40 kDa α and β protein smear by SDS–PAGE under reducing conditions (Leskov *et al.,* 2003). In various studies, different anti-clusterin antibodies recognized different isoforms of clusterin in the male reproductive tract and the variation in carbohydrate content among these isoforms may affect the biological or functional activity of the protein (Ibrahim *et al.,* 1999). This discrepancy in molecular weights of clusterin is attributed to differences in post-translational modification, tissues from which secreted, and the type of antibodies used to recognize the clusterin.

In the present study, our results indicated that the expression of clusterin on sperm cells is having direct relationship with the morphological abnormalities and an inverse relationship with its total, progressive, and rapid motility. In a previous report, an evaluation of semen quality was made on the expression of clusterin, and obtained results were similar to that of our results (Ibrahim *et al.,* 2000). Furthermore, Ibrahim *et al.* (2001) reported that the increase in CPS coincided with the presence of a high percentage of sperm abnormalities in scrotal-insulated bulls. High levels of clusterin were also strongly associated with morphological alterations in hypertensive men (Muciaccia *et al.,* 2012). Recently, Kumar *et al.* (2015) also found that the expression of the clusterin gene was significantly higher among motility-impaired crossbred bull semen compared to the good quality one. Abnormal spermatocytes and morphologically abnormal spermatozoa exhibited intense reactivity with anti-clusterin antibodies, suggesting that clusterin is synthesized by somatic cells in the testis and epididymis and is translocated to abnormal germ cells and spermatozoa (O'Bryan *et al.,* 1994; Clark *et al.,* 1997; Ibrahim *et al.,* 2000). The expression of clusterin in rat prostate showed that castration increases clusterin mRNA in epithelial cells of the prostate before and during the appearance of initial stages of apoptosis (Buttyan *et al.,* 1989). Since, clusterin has been suggested as a possible marker for apoptosis in vivo and also recognized its higher expression in adjacent viable cells than in dying cells in tissues undergoing degeneration in vivo (Walton *et al.,* 1996; Clark *et al.,* 1997) or in vitro (Akakura *et al.,* 1996; Humphreys *et al.,* 1997; Calvo *et al.,* 1998; Viard *et al.,* 1999). Further, we observed that VCL was found higher in low CPS bulls in comparison to high CPS bulls indicating that abnormal spermatozoa have an irregular path while normal spermatozoa travelled along its actual curvilinear path resulting in higher VCL. The importance of VCL (swimming speed) of ejaculated spermatozoa was strongly correlated with IVF rates (Holt et al. 1985; Chan et al. 1989).

Several reports suggest that clusterin is a survival molecule rather than an apoptosis marker because it is up-regulated and transferred from neighboring healthy cells to more stressed cells in tissues undergoing degeneration, thereby exerting a protective function on the bystander cells (Rosenberg and Silkensin, 1995; Koch-Brandt and Morgans, 1996). It is involved in fertility-related processes such as the prevention of oxidative damage (Trougakos and Gonos, 2002) and complement-mediated sperm lysis (Jenne and Tschopp, 1989), agglutination of spermatozoa (Carlsson *et al.,* 2004), membrane protection and membrane remodeling during sperm maturation (Griffiths *et al.,* 2009). The exact reason why this protein with different important functions in the male reproductive tract accumulates on abnormal spermatozoa is still unknown. It is possible that clusterin production is amplified and its accumulation on degenerating germ cells was enhanced for the deterrence of denaturation and stabilization of cell membrane proteins (Ibrahim *et al.,* 2000).

CONCLUSIONS

In conclusion, an anti-clusterin antibody recognized ~40 kDa clusterin in buffalo seminal plasma. The sperm clusterin in buffalo semen is positively correlated with morphologically abnormal spermatozoa and negatively correlated with sperm kinetics and motility parameters. Thus, the high percentage of CPCs in buffalo semen is a negative marker for semen quality.

CONFLICT OF INTEREST

The authors declare that no conflict of interest would prejudice the impartiality of this experiment.

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