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Relationship between Clusterin Gene Expression and *In-vitro* Sperm Characteristics in Caprine Semen

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ABSTRACT

The present experiment was undertaken to study the relationship between clusterin (CLU) gene expression and *in vitro* sperm characteristics in buck semen. Fresh semen samples were collected from 12 bucks maintained in the organized goat farms by artificial vagina. Normalization of initial concentration of spermatozoa was carried out in all buck semen samples before proceeding for RNA isolation. So, initial concentration of each sample was made equal. The spermatozoa were isolated from buck semen samples by swim- up protocol using sperm TALP. Total RNA from the buck spermatozoa were extracted and first strand cDNA was synthesized from 1µg total RNA by using commercial kits. Absolute quantification of CLU gene transcripts in semen samples from 12 bulls was performed by plotting standard curve. Variations in levels of CLU gene transcripts (2500-22546000 copies) were found among 12 different buck semen samples. *In vitro* sperm characteristics were also studied from 12 buck semen samples. Variations in sperm characteristics such as sperm motility (60.0 - 80.0%), sperm viability (72.0 - 93.0%), sperm morphology (73.0 - 91.0%), plasma membrane integrity (50.0 - 82.0%), acrosome integrity (81.0 - 93.0%), DNA integrity (82.0 - 93.0%) and MMP (46.0 - 74.0%) were found among buck semen samples. All *in vitro* sperm characteristics were highly (negatively) correlated ($p<0.01$) with expression levels of CLU gene transcripts in spermatozoa. From this study, it is evident that ejaculated buck semen has variations in transcription pattern of CLU gene in spermatozoa among bucks and expression levels of CLU transcripts have negative correlation with *in vitro* sperm characteristics in buck semen samples.

Keywords: Clusterin gene, i*n vitro* sperm characteristics, buck semen

A number of seminal plasma proteins have been identified as molecular markers of fertility in different species. Proteins such as osteopontin, prostaglandin D synthase, bovine seminal plasma proteins (BSP A1, A2 and A3), heparin binding proteins (HBPs), fertility associated antigen, phospholipase A2, sperm adhesion Z13, clusterin (CLU) and heat shock proteins(HSPs) have been reported as indicators of fertility (Killian *et al.*, 1993; Cancel *et al.,*1999; McCauley *et al.,* 2001and Moura *et al.,* 2006).

CLU is the one of the fertility associated proteins, which was first isolated from ram rete testis fluid and it showed signs of clustering with rat Sertoli cells and erythrocytes. Hence its name, CLU was derived (Fritz *et al.,* 1983). In the male reproductive tract, it is produced by Sertoli cells and principal epididymal epithelial cells and is translocated to abnormal germ cells and spermatozoa (Sylvester *et al.,* 1984).

CLU is involved in various physiological processes such as it binds and agglutinates abnormal spermatozoa, prevent oxidative damage to the sperm and inhibit complement

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induced sperm lysis in male reproductive tract (Moreno *et al.,* 2002). CLU mainly exists on the surfaces of immature, low motile or morphologically abnormal spermatozoa regardless of whether or not the semen is normal (Carlsson *et al.,* 2004). Abnormal increases and decreases in clusterin are often indicative of poor quality semen (Martinez *et al*., 2008).

CLU present in the rete testis fluid is synthesized by Sertoli cells and adheres to the surface of testicular spermatozoa. When spermatozoa move through the rete testis and efferent duct, testis-derived CLU is replaced by CLU from the epididymal or seminal vesicle (Griffiths *et al*., 2009).CLU expression is negatively associated with the percentage of normal sperm morphology (Santos *et al*., 2014) and high expression of CLU promotes the uptake of stress-damaged proteins by dendritic cells in male reproductive tract (Merlotti *et al.,* 2015).

However, expression of CLU and its relationship with fertility potential of semen have not been fully explored. Hence the present experiment was undertaken to study the transcription pattern of CLU gene and its correlation with *in vitro* sperm characteristics in buck semen.

MATERIALS AND METHODS

Plasticware and glassware

All the plasticware used for the present study viz., centrifuge tubes, microcentrifuge tubes, microtips (different graduations) and PCR tubes were procured from Thermo Scientific, USA. Real time PCR strips and master clear cap strips were procured from Biorad, USA. All the glassware used in this study viz., laboratory bottles, microscopic cover slips 18 mm × 18 mm, microscopic slides, conical flasks and beakers were procured from Borosil, India.

Chemicals

Chemicals required for PCR, gel electrophoresis and *in vitro* semen evaluation were procured as detailed below. For real time PCR: RNeasy® mini kit (Qiagen, Germany), high capacity cDNA reverse transcription kit (Thermo Scientific,USA), Taq DNA polymerase 2.0x master mix red (Ampliqon, Denmark), SYBR green I dye (SYBR premix Ex Taq, Takara,Japan), Nuclease free water (Ambion,USA); For gel electrophoresis: Tris acetate gel running buffer (TAE) (50X) (Medox®, India), gel loading dye (6X) (Thermo Scientific, USA), gel red nucleic acid stain (Biotium, Canada), agarose (Sigma-Aldrich, USA), DNA marker 100 bp plus ladder (Thermo Scientific, USA), DNA marker 50 bp plus ladder (Thermo Scientific, USA) and For gel extraction: MinElute® Gel Extraction Kit(Qiagen,Germany).

Chemicals required for *in vitro* evaluation of semen such as eosin stain, nigrosin stain, rose bengal stain, tris buffer, phosphate buffer saline (PBS), thiobarbituric acid and trichloroacetic acid (TBA-TCA) solution, JC-1 stain (5, 5', 6, 6' - tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolylcarbocyanine iodide), carboxy fluorescein diacetate (CFDA), propidium iodide (PI), dimethyl sulphoxide (DMSO), giemsa stain, formaldehyde, sodium citrate, sodium pyruvate, sodium chloride, sodium lactate, potassium chloride, calcium chloride, sodium dihydrogen orthophosphate, magnesium chloride, heparin, fatty acid free bovine serum albumin (BSA), gentamicin, and phenol red were procured from Sigma- Aldrich, USA.

Absolute quantification of CLU gene transcripts

Fresh semen samples were collected from 12 bucks maintained in the organized goat farms by using artificial vagina. The volume of semen immediately after collection was noted and concentration of spermatozoa was assessed by Neubauer's counting chamber from 12 bucks. Normalization of initial concentration of spermatozoa was carried out before proceeding for RNA isolation. So, initial concentration of each sample was made equal. The spermatozoa were isolated from buck semen samples by swim- up protocol using sperm TALP.

Total RNA from the buck spermatozoa were extracted by RNeasy® mini kit (Qiagen, Germany) and first strand cDNA was synthesized from 1µg total RNA by high capacity cDNA reverse transcription kit (Thermo Scientific,USA). Confirmation of CLU gene was performed by conventional PCR with product size 302 bp.

The eluted cDNA was sequenced commercially and was subjected to BLAST analysis. Sequencing of the cloned CLU gene from buck semen showed 98% identity to

the known caprine sequence (EMBL Accession NO: XM_018052776.1).

The specific size of amplicon 302 bp was observed on 2.0 per cent agarose gel electrophoresis. The concentration of cDNA was 25.9 ng/µ,which contained 7.7×10^{10} copies/µl. A series of 10-fold dilutions starting from 7.7×10^{10} to 7.7×10^{1} were prepared. The correlation between the cDNA dilution and the threshold cycle Ct values in real time PCR was analyzed by plotting a standard curve. Quantification of the nucleic acids in the unknown samples was performed by direct comparison with these standards. A linear regression relationship was observed with a coefficient of determination (R^2) of 0.926 and a slope of -2.238.

Assessment of *in vitro* sperm characteristics

In vitro sperm characteristics such as sperm motility, viability, morphology, plasma membrane integrity, acrosome integrity, DNA integrity and MMP were also assessed from 12 different buck semen samples by standard protocol.

Statistical analysis

Statistical analysis was carried out by Karl Pearson's coefficient of correlation described by Snedecor and Cochran (1994).

RESULTS AND DISCUSSION

Expression of CLU gene in buck semen

Absolute quantification of CLU gene transcripts in 12 buck semen samples was performed. Variation in levels of CLU gene transcripts (2500-22546000 copies) were found among 12 different buck semen samples (Table 1).

This is the first study to document expression levels of CLU gene transcripts from ejaculated buck spermatozoa. But, localization of CLU was demonstrated in previous studies on sperm acrosome, neck and end piece of tail and was associated with late spermatids and spermatozoa by indirect immunofluorescence method (Sylvester *et al*., 1984). Northern blot analysis showed that two isoforms of CLU (on testicular and epididymal sperm occur due to tissue-specific post-translational modifications (Sylvester *et al*., 1991). Western blot analysis of sperm membrane extracts from testicular, caput and cauda spermatozoa revealed that testicular CLU was associated with testicular sperm and epididymal CLU was predominantly associated with caput sperm (Mattmueller and Hinton, 1991).

Table 1: Absolute quantification of CLU gene in buck semen samples (n=12)

Buck number	CLU gene transcripts (number of copies)
1	10440
2	1732510
3	33940
4	11377340
5	9460
6	12900
7	22546000
8	1235880
9	13000
10	1277430
11	2500
12	1084130

Evaluation of *in vitro* sperm characteristics of buck semen

In vitro sperm characteristics were also studied from 12 buck semen samples. Variation in sperm characteristics such as sperm motility (60.0 - 80.0%), sperm viability (72.0 - 93.0%), sperm morphology (73.0 - 91.0%), plasma membrane integrity (50.0 - 82.0%), acrosome integrity (81.0 - 93.0%), DNA integrity (82.0 - 93.0%) and MMP (46.0 - 74.0%) were found among buck semen samples (Table 2). All the above *in vitro* sperm characteristics were negatively correlated ($p<0.01$) with expression levels of CLU gene transcripts (2500-22546000 copies) in spermatozoa isolated from 12 different buck semen samples.

In physiological state, CLU prevents oxidative damage by reactive oxygen species (ROS) attack on the spermatozoa (Moreno *et al.,* 2002). It binds and agglutinates abnormal spermatozoa in bulls (Ibrahim *et al.,* 1999) and act like a chaperone, protecting sperm from the toxic effects

Table 2: Evaluation of *in vitro* sperm characteristics of buck semen samples (n=12)

Buck Number	Absolute quantification of CLU gene	*In vitro* sperm characteristics						
		Sperm motility %	Sperm livability	Sperm morphology	Sperm plasma membrane integrity %	Sperm acrosome integrity %	Sperm DNA integrity %	Sperm MMP %
			Live %	Normal %				
1	10440	80	87	91	77	90	93	69
2	1732510	70	79	79	60	84	86	53
3	33940	80	88	90	67	90	91	63
4	11377340	60	75	77	56	83	85	49
5	9460	80	91	90	80	92	91	71
6	12900	80	88	90	72	91	90	66
7	22546000	60	72	73	50	81	82	46
8	1235880	70	83	84	62	87	87	57
9	13000	80	90	90	70	92	93	65
10	1277430	70	81	82	62	88	87	56
11	2500	80	93	90	82	93	91	74
12	1084130	70	84	87	63	89	88	59
Coefficient of correlation		-0.800**	-0.807**	-0.827**	-0.715**	-0.805**	-0.787**	-0.736**

of protein precipitation (Humphreys *et al.,* 1999 and Wilson and Smith, 2000). CLU has the ability to inhibit complement-induced sperm lysis (Jenne and Tchopp, 1989) and exerts protective effect on reproductive tract cells including spermatozoa against apoptosis (Bailey *et al.,* 2002) and assists in removal of damaged sperm from epididymis in human beings (Zalata *et al*., 2012).

Under pathological condition, high level of CLU expression in response to oxidative stress could be the cause for low viability of spermatozoa in buck semen samples and this association probably occurs as a result of ability of CLU to bind damaged portions of hydrophobic regions of sperm membrane (Bailey and Griswold, 1999). Because ROS are highly reactive at pathological levels, they exert significant damage on biomolecules, such as proteins, lipids and nucleic acids (Riffo and Parraga, 1996).

CLU expression was negatively associated with the percentage of normal sperm morphology (Santos *et al*., 2014). But, positive correlation exists between levels of ROS and percentage of spermatozoa with many kinds of abnormalities like, abnormal heads, acrosome abnormalities, mid piece anomalies, cytoplasmic droplets and tail defects (Sabeti *et al.,* 2016).

The sperm plasma membrane is mostly composed of PUFAs which are susceptible to OS due to the presence of double bond (Agarwal and Seleh, 2002). As LPO cascade proceeds in the sperm, almost 60% of the fatty acid is lost from the membrane. LPO affects membrane structure and functions such as fluidity, ion gradients, receptor transduction, transport processes and membrane enzymes (Sikka *et al.,* 1995). Peroxides, products of LPO constitute a potential hazard to the structural and functional integrity of spermatozoa (Srivastava *et al.,* 2006).

The acrosome reaction involves fusion of the plasma and outer acrosomal membranes coupled with the release of hydrolytic enzymes allows the sperm to penetrate the zona pellucida and fertilize an oocyte (Abou and Tulsiani, 2000). Induction of premature acrosomal reaction by ROS altered mitochondrial function and reduced the sperm motility.

ROS induced many kinds of DNA damage, including DNA base modifications, chromatin cross linkage and breakage of DNA strand (de Rosa *et al.,* 2003; Evenson and Wixon, 2006). Failure of chromatin decondensation by ROS in OS reduced the viability and fertility of the sperm cells (Cooter *et al.,* 2005 and Wongtawan *et al.,* 2006). ROS can damage DNA and proteins either through oxidation of

DNA bases (primarily guanine via lipid peroxyl or alkoxyl radicals) or through covalent binding to MDA resulting in strand breaks and cross-linking (Ernster, 1993). ROS can also induce oxidation of critical -SH groups in proteins and DNA, which will alter structure and function of spermatozoa with an increased susceptibility to attack by macrophages (Aitken *et al*., 1994).

ROS may also adversely affect sperm motility via alterations in mitochondrial function. MMP has been used as a measure of mitochondrial function and is linked to ATP synthesis, import of mitochondrial proteins, calcium homeostasis and metabolite transport (Baumber *et al.,* 2000). ROS stimulates proteins of the BCL-2 family, followed by releasing of mitochondrial cytochrome C and concomitant activation of caspase 9 and 3 which result in disruption of MMP (Espinoza *et al.,* 2009 and Kothari *et al.,* 2010).

CONCLUSION

From this experiment, it is evident expression levels of CLU gene have varied among the buck semen samples and expression levels of CLU transcripts have negative correlation with *in vitro* sperm characteristics.

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