International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2017; 5(5): 1997-2000

Received: 03-07-2017
Accepted: 04-08-2017

M Vengatachalam
M.V. Sc, Scholar, Department of Veterinary Physiology, Madras Veterinary College, Chennai, Tamil Nadu, India

K Loganathasamy
Assistant professor, Department of Veterinary Biochemistry, Madras Veterinary College, Chennai, Tamil Nadu, India

V Leela
Professor and Head, Department of Veterinary Physiology, Madras Veterinary College, Chennai, Tamil Nadu, India

J Ramesh
Assistant Professor, Department of Animal Nutrition, Madras Veterinary College, Chennai, Tamil Nadu, India

K Vijayarani
Professor and Head, Department of Animal Biotechnology, Madras Veterinary College, Chennai, Tamil Nadu, India

Bhaskaran Ravi Latha
Professor and Head, Department of Veterinary Parasitology, Madras Veterinary College, Chennai, Tamil Nadu, India

Correspondence
K Loganathasamy
Assistant professor, Department of Veterinary Biochemistry, Madras Veterinary College, Chennai, Tamil Nadu, India

Correlation between Clusterin expression and lipid peroxidation status of Caprine semen

M Vengatachalam, K Loganathasamy, V Leela, J Ramesh, K Vijayarani and Bhaskaran Ravi Latha

Abstract
The present investigation has been undertaken to study the transcription pattern of clusterin (CLU) gene in buck semen and to assess *in vitro* sperm characteristics of buck semen which are screened for CLU gene. 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. Confirmation of CLU and GAPDH gene was performed by conventional PCR with product size 302 and 227 bp, respectively. 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).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. Relative normalized gene expression was analyzed between housekeeping gene (GAPDH) and target gene (CLU) for all the 12 buck semen samples. Expression of GAPDH did not show significant difference. But, expression of CLU was up regulated in buck number 2, 4, 7, 8, 10 and 12 and there was no change in expression of CLU in buck number 1,3,5,6,9 and 11, when compared to threshold regulation of GAPDH. LPO status of semen samples was assessed by estimation of MDA concentration. Variations in MDA (1.12-1.96 µmol/ml) concentration from 12 different buck semen samples were highly (positively) correlated ($p<0.01$) with expression levels of CLU gene transcripts in spermatozoa. *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 positive correlation with malondialdehyde (MDA) concentrations of buck semen samples and negative correlation with *in vitro* sperm characteristics.

Keywords: Clusterin gene, Malondialdehyde, *In vitro* sperm characteristics, buck semen

Introduction
Clusterin (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, clusterin was coined [1, 2]. It is expressed in most mammalian tissues and associated with the clearance of cellular debris and apoptosis. The protein has been implicated in many diverse physiological processes such as programmed cell death, regulation of complement mediated cell lysis, membrane recycling, cell–cell adhesion, lipid transportation, sperm maturation, tissue remodelling, stabilization of stressed proteins in a folding-competent state and src induced transformation. In the male reproductive tracts, it is produced by Sertoli cells and principal epididymal epithelial cells and is translocated to abnormal germ cells and spermatozoa [3]. CLU is involved in various physiological processes such as it binds and agglutinates abnormal spermatozoa in bulls, prevent oxidative damage to the sperm, inhibit complement induced sperm lysis in male reproductive tract [4]. CLU mainly exists on the surfaces of immature, low motile or morphologically abnormal spermatozoa regardless of

whether or not the semen is normal [5]. Abnormal increases and decreases in CLU are often indicative of poor quality semen [6].

CLU 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 clusterin from the epididymal or seminal vesicle [7].

However, expression of CLU and its correlation with fertility potential of semen have not been fully elucidated till date. Hence the present study is undertaken to prospect the role of clusterin in fertility assessment of buck semen to study the transcription pattern of clusterin gene and its correlation with malondialdehyde concentration in buck semen.

Materials and Methods

Materials

Plastic ware and glassware

All the plastic ware used for the present study viz., centrifuge tubes, microcentrifuge tubes, microtips (different graduation) 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 18mm × 18mm, 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 100bp plus ladder (Thermo Scientific, USA), DNA marker 50bp plus ladder (Thermo Scientific, USA) and For gel extraction: MinElute® Gel Extraction Kit (Qiagen, Germany).

Methods

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 and GAPDH gene was performed by conventional PCR with product size 302 and 71 bp, respectively.

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/µl 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.

Statistical analysis

Statistical analysis was carried out by Karl Pearson's co-efficient of correlation described by Snedecor and Cochran (1994) [8].

Results

Expression of CLU gene in buck semen

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

Relative normalized gene expression was analyzed between housekeeping gene (GAPDH) and target gene (CLU) for all the 12 buck semen samples. Expression of GAPDH did not show significant difference. But, expression of CLU was up regulated in buck number 2, 4, 7, 8,10 and 12 and there was no change in expression of CLU in buck number 1,3,5,6,9 and 11, when compared to threshold regulation of GAPDH (Table 2).

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

Table 2: Relative normalised gene expression of CLU and GAPDH in buck semen samples (n=12)

Buck number	Comparison of CLU expression with GAPDH
1	No change
2	Upregulated ↑
3	No change
4	Upregulated ↑
5	No change
6	No change
7	Upregulated ↑
8	Upregulated
9	No change
10	Upregulated ↑
11	No change
12	Upregulated ↑

LPO status of buck semen

LPO status of semen samples was assessed by estimation of MDA concentration from 12 bucks. Variations in MDA concentration among buck semen samples were observed (MDA level 1.12-1.96 µmol/ml). MDA concentrations were highly (positively) correlated ($p<0.01$) with expression levels of CLU gene transcripts in spermatozoa isolated from 12 different buck semen samples (Table 3).

Table 3: Evaluation of LPO status of buck semen samples (n=12)

Buck Number	Concentration of MDA (µMol/mL)
1	1.22
2	1.60
3	1.29
4	1.87
5	1.17
6	1.20
7	1.96
8	1.56
9	1.25
10	1.55
11	1.12
12	1.51
Coefficient of correlation (Between absolute quantification of CLU gene and concentration of MDA	0.819**

Coefficient of correlation with ** indicates high (positive) correlation ($p<0.01$) within the same row

Discussion

Expression of CLU gene in buck semen

In the present study, total RNA were isolated especially from spermatozoa of ejaculated buck semen and were converted into cDNA and qRT-PCR was conducted for analysing the expression level of CLU gene. Absolute quantification of CLU gene in buck semen samples showed variations in levels of CLU gene transcripts (2,500-22,546,000copies). 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 [3]. Northern blot analysis showed that two isoforms of CLU (on testicular and epididymal sperm) occur due to tissue-specific post-translational modifications [9]. Western blot analysis of sperm membrane extracts from testicular, caput and cauda spermatozoa revealed that testicular clusterin was associated with testicular sperm and epididymal CLU was predominantly associated with caput sperm [10].

Correlation between CLU gene expression and LPO status of buck semen

In the present study, expression of CLU gene was positively correlated with MDA level in buck semen samples. MDA is a stable peroxidation product of polyunsaturated fatty acids, usually cross linked to protein. It is a diagnostic tool for assessing the lipid peroxidation (LPO) status [11, 12].

Lipid peroxidation is one of pathological effects from reactive oxygen species (ROS). Reactive oxygen species (ROS), especially superoxide anion (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH) are highly reactive oxidizing agents that belong to the class of free radicals [13, 14] . They have one or more unpaired electron and react with macromolecules foe compensation of their deficit electron. ROS plays a dual role in male fertility. At lower concentration, ROS enhances sperm capacitation, acrosome reaction and fertilization. At higher concentration, ROS inflict severe damage to spermatozoa [15, 16].

ROS is associated with oxidation of membrane polyunsaturated fatty acids (PUFA) [17-19]. Spermatozoa are susceptible to oxidation of their plasma membrane by ROS due to the existence of higher concentration of PUFA in the membrane [20, 21]. PUFA play an important role in ion transport and sperm membrane fluidity. Therefore, oxidation of sperm membrane PUFA by ROS cause sperm membrane dysfunctions and inability of sperm to bind and fuse with oocyte for successful fertilization [22] because the spermatozoa are unable to repair the damage induced by ROS because they lack cytoplasmic enzyme systems which are required to accomplish this repair [20].

CLU is a sensitive cellular biosensor of oxidative stress due to its chaperone activity functions to protect cells from the deleterious effect of oxidative and proteotoxic stress [23]. The presence of both the AP-1 and CLU - specific regulatory elements in the CLU promoter gene specifically make the CLU gene an extremely sensitive biosensor to exogenous or endogenous stress and particularly to free radicals and their derivatives [24].

Conclusions

From this experiment, it is evident expression levels of CLU gene have varied among the bucks and expression levels of CLU transcripts have positive correlation with malondialdehyde (MDA) concentrations of buck semen samples.

Acknowledgements

The authors are grateful to Dean, Madras Veterinary College and higher authorities of Tamil Nadu Veterinary and Animal Sciences University for providing necessary facilities and funds to carry out the experiment.

References

1. Blaschuk O, Burdzy K, andFritz IB. Purification and characterization of a cellaggregating factor (clusterin), the major glycoprotein in ram rete testis fluid. J Biol.Chem.1983; 25:7714-7720.
2. Fritz IB, Burdzy K, Setchell B, Blaschuk O. Ram rete testis fluid contains a protein (clusterin) which influences cell-cell interaction *in vitro*. Biol. Reprod. 1983; 28:1173-1180.
3. Sylvester SR, Skinner MK, Griswold MD. A sulphated glycoprotein synthesized by Sertoli cells and by epididymal cells is a component of the sperm membrane. Bio. Reprod. 1984; 31(5):1087-101.
4. Moreno RC, Boilard M, Sullivan R, Sirard MA. Characterization of secretory proteins from cultured cauda epididymal cells that significantly sustain bovine sperm motility *in vitro*. Mol. Reprod. Dev. 2002; 63:500-509.
5. Carlsson L, Ronquist G, Nilsson BO. Dominant prostasomeimmunogens for sperm-agglutinating autoantibodies of infertile men. J. Androl. 2004; 25:699-705.
6. Martinez HJ, Mateo S, Vidal-Taboada JM. Identification of proteomic differences in asthenozoospermic sperm samples. Hum. Reprod. 2008; 23:783-791.
7. Griffiths GS, Galileo DS, Aravindan RG. Clusterin facilitates exchange of glycosyl phosphatidylinositol-

linked SPAM1 between reproductive luminal fluids and mouse and human sperm membranes. Biol. Reprod. 2009; 81:562-570.
8. Snedecor GW Cochron WG. Statistical Methods. The Iowa State Univ. Press, USA, 1994.
9. Sylvester SR, Morales C, Griswold MD. Localization of sulphated glycoprotein-2 (clusterin) on spermatozoa and in the reproductive tract of the male rat. Bio. Reprod. 1991; 45(1):195-2077.
10. Mattmueller DR, Hinton BT. *In vivo* secretion and association of clusterin (SGP-2) in luminal fluid with spermatozoa in the rat testis and epididymis. Molecular reproduction and development. 1991; 30(1):62-69.
11. Aitken RJ, Clarkson JS, Fishel S. Generation of reactive oxygen species, lipid peroxidation, and human sperm function. Biol.Reprod.1989b; 40:183-97.
12. Laudat A, Lecourbe K, Guechot J, Palluel AM. Values of sperm thiobarbituric acid-reactive substance in fertile men.Clin. Chim. Acta. 2002; 325:113-15.
13. Agarwal A, Prabakaran SA. Mechanism, measurement and prevention of oxidative stress in male reproductive physiology. Ind. J. Exp. Biol. 2005a; 43:963-974.
14. Aitken RL, West KM, Buckingham DW. Leukocyte infiltration into human ejaculate and its association with sperm quantity, oxidative stress, sperm function. J. Androl. 1994; 15:343-352.
15. Agarwal A, Saleh RA, Bedaiwy MA. Role of reactive oxygen species in the pathophysiology of human reproduction. Fert and steril. 2003; 79:829-43.
16. Agarwal A, Prabakaran SA. Oxidative stress and antioxidants in male infertility: a difficult balance. Iran J. Rep. Med. 2005b; 3:1-8.
17. Alvarez JG, Touchstone JC, Blasco L, Storey BT. Spontaneous lipid peroxidation and production of hydrogen peroxide and superoxide in human spermatozoa. J. Androl. 1987; 8:338-48.
18. Alvarez JG, Storey BT. Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa. Mol. Reprod. Dev. 1995; 42:334-346.
19. Fraczek M, Szkutnik D, Sanocka D, Kurpisz M. Peroxidation component of sperm lipid membrane in male fertility. Ginekol Pol. 2001; 72:73-79.
20. Jones R, Mann T, Sherins R. Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxidase, and protective action of seminal plasma. Fertil Steril. 1979; 31:531-537.
21. Aitken RJ, Clarkson JS, Hargreave TB, Irvine DS, WcFC. Analysis of the relationship between defective sperm function and the generation of reactive species in case of oligozoospermia. J. Androl. 1989a; 10:3214-20.
22. Mammoto A, Masumoto N, Tahara M, Ikebuchi Y, Ohmichi M, Tasaka K *et al.* Reactive oxygen species block spermegg fusion via oxidation of sperm sulfhydryl proteins in mice. Biol. Reprod. 1996; 55:1063-68.
23. Trougakos IP, Gonos ES. Oxidative stress in malignant progression; the role of clusterin,a sensitive cellular biosensor of free radicals. Adv. Cancer Res. 2009; 104:171-210.
24. Michel D, Chatelain G, Herault Y, Brun G. The expression of the avian clusterin gene can be driven by two alternative promoters with distinct regulatory elements. Eur. J. Biochem. 1995; 229:215-223.