STUDIES ON SEQUESTRATION OF PDC-109 PROTEIN ON CRYODAMAGE AND *IN VITRO* FERTILITY OF CROSSBRED BULL SPERMATOZOA

THESIS

Submitted in partial fulfilment of the requirements for the degree

of

Doctor of Philosophy

in

VETERINARY GYNAECOLOGY AND OBSTETRICS

By

Dr. Neeraj Srivastava

Roll No. 1118

To

DEEMED UNIVERSITY

INDIAN VETERINARY RESEARCH INSTITUTE

IZATNAGAR - 243 122 (U.P.)

2011

भारतीय पशु चिकित्सा अनुसंधान संस्थान
(सम विश्वविद्यालय)

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Dated: March, 2011

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Certified that the research work embodied in this thesis entitled **"Studies on sequestration of PDC-109 protein on cryodamage and *in-vitro* fertility of crossbred bull spermatozoa"** submitted by **Dr. Neeraj Srivastava, Roll No. 1118,** for the award of **Doctor of Philosophy Degree** in **Veterinary Gynaecology and Obstetricsat Indian Veterinary Research Institute, Izatnagar,** is the original work carried out by the candidate himself under my supervision and guidance.

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Acknowledgements

I am blessed to have the opportunity to thank the Almighty god, the supreme power for bestowing His grace and guiding me through this chapter of my life.

The short journey has finally come to an end, and here I stand looking for new goals. The journey was made enlighting and comfortable by the numerous known and unknown hands, who showed me right path in the hours of total darkness.

It gives me immense pleasure to express my indebtness towards my advisor ***Dr. S.K. Srivastava,*** *Principal Scientist, Animal Reproduction division. I shall remain ever grateful to him for his constructive counsel, erudite suggestions and painstaking efforts in planning and execution of this study, and timely preparation of this thesis.*

I consider my self fortunate and privileged to have worked under the guidance and supervision of ***Dr. L.P. Singh,*** *Principal Scientist, Animal Reproduction Division. I will ever cherish his faith and confidence in my abilities and ever so whole hearted support I received from him even after his superannuation. I wish I could live up to his expectations.*

I owe my special thanks to ***Dr. S.K. Ghosh****, Senior Scientist, Animal Reproduction division and* ***Dr. S.K. Meur,*** *Principal Scientist, NRL, division of Physiology and Climatology for their exceptional guidance, constructive counsel, patience, constant encouragement and meticulous supervision along the course of study.*

I extend my grateful acknowledgements and veneration to the members of my advisory committee, ***Dr. P. Joshi,*** *Principal Scientist, Animal Biochemistry and Food Sciences,* ***Dr. (Smt.) G. Taru Sharma,*** *Principal Scientist and Head and* ***Dr. Sadhan Baug,*** *Senior Scientist, division of Physiology and Climatology for their caI am blessed to have the opportunity to thank the Almighty god, the supreme power for bestowing His grace and guiding me through this chapter of my life.*

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I owe my special thanks to ***Dr. S.K. Mishra,*** *Principal Scientist and Head, Division of Pharmacology and Toxicology,* ***Dr. R. Somvanshi,*** *Principal Scientist and Head, Division of Pathology,* ***Dr. A.K. Tiwari,*** *Principal Scientist and* ***Dr. P. Dole****, Scientist, division of Animal Biotechnology and* ***Dr. Pallab Choudhury,*** *Senior Scientist, division of Bacteriology and Mycology for their valuable guidance, sparing valuable time and providing the facilities for biochemical analysis.*

I am highly indebted to ***Dr. R. Tiwari,*** *Senior Scientist, ATIC and* ***Dr. Amit Kumar,*** *Scientist, LAR, who's selfless, ever helping attitude and keen interest in my work made even daunting tasks look easy.*

I record my sincere gratitude to ***Dr. M.C. Yadav,*** *Ex-Head and* ***Dr. S.K. Agarwal,*** *Head, Animal Reproduction for their valuable guidance and timely advice.*

I am indebted to my teachers, ***Dr. Umashankar, Dr. Harendra Kumar, Dr. S. Mahmood, Dr. S. Mehrotra, Dr. G.K. Das, Dr. J.K. Prasad, Dr. S.K. Singh*** *of*

Animal Reproduction division, ***Dr. A.C. Majumdar,*** *division of Physiology and Climatology;* ***Dr. Bhaskar Sharma, Dr. Meena Kataria*** *and* ***Dr. M. Saini,*** *division of Animal Biochemistry and Food Sciences for their generous support and co-operation during the entire period of my Ph.D. course and research work.*

I extend my sincere thanks to the ***Director,*** *IVRI for granting permission and study leave to pursue my Ph.D. study,* ***Joint Director*** *(Academic),* ***Joint Director*** *(Research) and* ***Scientific Co-ordinator*** *for providing necessary facilities to complete this work.*

My sincere thanks go to ***Dr.s R.P. Tripathi, P.P. Dubey, Mr.s Yogendra, Ayodhya Prasad, K.K. Khar, K.K. Mishra, Hamid, Subhash, Sircar, Pathak, B.K. Das, Brijlal, Yousuf, Ramsaroop, Gaurav, Sameer, Inder pal, Veerpal*** *and all other staff.*

Mere words are not sufficient to express my indebtness to my friends and colleagues ***Dr.s Tamilarasan, G. Puri, Arvind, Pradeep, Zerome, Gokul*** *and* ***Perumal*** *for their whole hearted support.*

It is my pleasure to record my wholehearted thanks to ***Mr. Dharmendra*** *and* ***Mr. Javed*** *for beautifully typing and preparing this manuscript.*

There are no words to pay tribute to my late parents who had to toil hard to bring me up to this stage. I stand here because of their constant encouragement, self sacrifice and support.

I feel beautiful to express the stupendous weight of gratitude beyond words, to my affectionate and loving family, my spouse and daughter, whose love, dedication and sacrifice made me what I am. I dedicate what ever I have and whatever I do to my family.

List is endless and omissions are part of human error, I thank one and all for their help and best wishes.

Date : ***(Neeraj Srivastava)***

Place : *Izatnagar*

Abbreviations

ATP	:	Adenosine Tri Phosphate
ADPase	:	Adenosine Tri Phosphatase
BSA	:	Bovine Serum Albumin
BSP	:	Bovine Seminal Plasma protein
Ca^{2+}	:	Calcium ion
cAMP	:	Cyclic Adenosine Mono phosphate
Cl^{-}	:	Chloride ion
CTC	:	Chlortetracycline
^{o}C	:	Degree centigrade
DNA	:	Deoxy ribo nucleic acid
DEAE	:	Diethylaminoethyl
EDTA	:	Ethylene Diamino Tetra Acetic acid
FAA	:	Fertility Associated Antigen
FBS	:	Foetal Bovine Serum
FITC-PSA	:	Fluoresentisthiacynate-*Pisum Sativum* Agglutinin
Fn-II	:	Fibronectin type II
FSH	:	Follicle Stimulating Hormone
GAG	:	Glycosaminoglycans
GSP	:	Goat Seminal Plasma protein
GFF	:	Goat Follicular Fluid
HBP	:	Heparin Binding Protein
HDL	:	High Density Lipoproteins
HSP	:	Horse Seminal Plasma protein
h	:	Hour
HCl	:	Hydrochloric acid
HOST	:	Hypo Osmotic Swelling Test
HRPO	:	Horse Radish peroxidase
IgG	:	Immunoglobulin G
kDa	:	Kilo dalton
Kg	:	Kilogram
L	:	Litre
lb	:	Pound

LDF	:	Low Density lipoprotein Fraction
LH	:	Lutenizing hormone
LPC	:	Lyso Phosphatidyl Choline
M	:	Molar
mg	:	Milligram
Mg^{2+}	:	Magnesium ion
min	:	Minutes
ml	:	Millilitre
mm	:	Millimeter
µg	:	Microgram
µl	:	Microlitres
mM	:	Milli Molar
mOsm	:	Milli Osmol
NaCl	:	Sodium chloride
NaOH	:	Sodium Hydroxide
NCM	:	Nitro Cellulose Membrane
ng	:	Nanogram
NMR	:	Nuclear magnetic resonance
PBS	:	Phosphate Buffered Saline
PMSF	:	Phenyl Methyl Sulfonyl Fluoride
PVA	:	Poly Vinyl Alcohol
RNA	:	Ribo Nucleic Acid
RSP	:	Ram Seminal Plasma protein
RVD	:	Regulatory Volume Decrease
SDS-PAGE	:	Sodium dodecyl sulphate- poly acrylamide gel electrophoresis
Sec	:	Second
SFP	:	Seminal Fluid Protein
SVS	:	Seminal Vesicular Protein
TALP	:	Tyrode's albumin-lactate-pyruvate
TCM-199	:	Tissue Culture Media-199
TIMP	:	Tissue Inhibitor of Metallo Protease
Tris	:	Tris (hydroxy methyl) amino methane
TSP	:	Total Seminal Protein
V	:	Volume
W	:	Weight

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1 Introduction

India possesses one fifth of the world total cattle (185.5 million) and more than half of the buffalo population (97.6 million) which contribute significantly to the national wealth and economy in the form of milk, meat, hide, manure and draught power. (Chakrabarti *et al.*, 2005). However, due to poor genetic make up of large nondescript livestock population owned by rural farmers, production of individual cow is very low. The artificial insemination (AI) has played a major role for the past few decades in the genetic improvement of cattle and buffaloes by increasing selection intensities of males and wide dissemination of their valuable germ plasm after cryopreservation, but still the result is far behind than the expectation.

Although, research on semen preservation for AI purposes has a long history over the centuries, the problem still lies with available cryopreservation technique especially in cross bred bull semen. The cryopreservation itself decreases motility and survivability of post thaw spermatozoa to about 50 per cent (Cormier and Bailey, 2003). Moreover, there is also variable degree of morphological, physiological and biochemical alterations in remaining population of live spermatozoa making them unsuitable for optimum fertility. There are factors extrinsic or intrinsic responsible for post thaw semen quality. Systematic studies on various aspects of these factors are very much required for augmentation of fertility and productivity of Indian livestock.

Bovine seminal plasma contains factors, which may have either beneficial and/or detrimental effect to sperm function. Some of these factors are contributed by accessory sex glands. These are proteinous/non proteinous but the nature and characteristics of most of these factors are not well understood (Manjunath *et al.*, 2008). Several accessory sex gland

proteins are known to bind to the spermatozoa membrane and affect its function and properties (Yanagimachi, 1994). Various studies have shown that accessory sex gland secretions participate in key events related to sperm function, fertilization, and embryo development in the female reproductive tract (Henault *et al.,* 1995). However, evidence linking seminal plasma proteins with fertility indices has been a major challenge (Moura *et al.,* 2006) now a days for reproductive biologist.

There are three major classes of proteins namely the spermadhesins (Topfer-Peterson *et al.,* 1998), cysteine-rich secretory proteins (CRISP, Schambony *et al.,* 1998) and Fn-II type proteins in seminal plasma of ungulates, which interact with the sperm surface and participate in sperm function (Topfer-Peterson, 1999). The Spermadhesins family is restricted to ungulates, where as families of cysteine-rich secretory proteins and Fn-II proteins are expressed in male genital tract of many mammalian species, indicating common role in fertilization (Manjunath and Therien, 2002). In other species too, the Fn-II type proteins constitute the major seminal plasma proteins *viz.* horse (Menard *et al.,* 2003), goat (Villemure *et al.,* 2003) and bison (Boisvert *et al*., 2003) that are characterized by their conserved gelatin binding fibronectin-II (Fn-II) type module (Smith et al., 2000). Related proteins have also been identified in rat, mouse, hamster and human seminal plasma (Leblond *et al.,* 1993). These proteins are secretory products of the seminal vesicles (Manjunath *et al.,* 1987).

It is now well established that bovine seminal plasma proteins and their relatives represent a new super family of proteins in mammals. They bind to sperm membrane choline phospholipids (Desnoyers and Manjunath, 1992) and capacitation factors, namely high density lipoproteins, heparin and glycosaminoglycans at ejaculation (Chandonnet *et al.,* 1990). The interaction of bovine seminal plasma proteins with spermatozoa results in the efflux of phosphatidylcholine (PC) and cholesterol (from intracellular to extra cellular) which appears to be an important step in the capacitation process, a necessary event before fertilization can occur (Therien *et al*., 1998; Moreau and Manjunath, 1999). It has been established that PDC-109 (BSP-A1/-A2) in seminal plasma is beneficial during fertilization in female reproductive tract by inducing capacitation, zona binding and acrosome reaction (Therien *et al.,* 1998), hyper-activation (Sanchez-Leungo *et al.,* 2004) and by forming oviduct sperm reservoir (Gwathmey *et al.,* 2003). In contrast, these proteins also induce changes in the sperm plasma

membrane by continuously stimulating cholesterol and phospholipids efflux (Therien *et al.,* 1997), thus have harmful effect on spermatozoa.

Cholesterol has stabilizing effect on sperm membrane (Yeagle, 1985); hence its efflux is expected to provoke reorganization or destabilization of membrane (Manjunath and Therien, 2002). The integrity of the plasma membrane is important for the spermatozoa to withstand harmful effect of cryopreservation process. Exposure of spermatozoa for 4 h to bovine seminal plasma proteins leads to about 25 percent cholesterol efflux (Moreau and Manjunath, 2000). Thus, the continuous exposure of spermatozoa to seminal plasma is detrimental to the sperm membrane, which may render the membrane very sensitive to sperm storage in liquid or frozen states (Viswanath and Shannon, 1997). The epididymal spermatozoa are more resistant to cold shock than ejaculated spermatozoa and this character is cellular and not influenced by fluid environment in which spermatozoa are suspended. Transit of spermatozoa from cauda to ejaculate renders them susceptible to cold shock (Lasley and Bogart, 1944) which may be due to prolong effect of BSP proteins on spermatozoa. Arangasamy (2003) reported that prolonged exposure of spermatozoa to higher doses of HBP had detrimental effect on spermatozoa motility and membrane integrity. The damage was dose dependent with the higher doses showing higher damages. Therefore, BSP proteins in seminal plasma act like a double-edged sword, being both beneficial and detrimental to sperm.

The hypothesis that there is a strong need to undertake work on sequestration of seminal plasma protein PDC-109 from the ejaculate at the time of collection itself was supported by inference from experimental work of Nauc and Manjunath (2000). They quantified the BSP proteins in bovine seminal plasma (Holstein breed) on ejaculated sperm membrane and also on frozen thawed spermatozoa by Radio Immune Assay. The result indicated that 31.4 to 46.7 mg/ml of BSP proteins are present in seminal plasma (total protein fractions 75.5 to 93.0 mg/ml) and the major BSP protein was BSP-A1/-A2 (PDC-109). The average ratio of BSP-A1/-A2, BSP-A3 and BSP-30 kDa was 10:1:1 respectively. In the same experiment, the result showed that all three BSP proteins were present in the sperm membranes even after several washings and represented a ratio of 4:1:1 for BSP-A1/-A2, BSP-A3 and BSP-30 kDa respectively. Similarly, the average ratio of BSP proteins in frozen-thawed sperm was 3:1:1 for BSP-A1/-A2, BSP-A3 and BSP-31 kDa respectively. Above findings clearly indicate

that although egg yolk used in semen extenders binds with BSP proteins and causes its sequestration (Singh *et al.,* 2007), even then the egg yolk mediated sequestration is not complete to prevent cryocapacitation during preservation of semen at ultra low temperature. Also it is possible that the lipoproteins in egg yolk facilitate the non regulated cryocapacitation (Cormier *et al.,* 1997). This necessitates to evolve technique for sequestration of BSP proteins from the ejaculate at the time of collection itself and to assess its effect on fertilizing ability of frozen thawed spermatozoa.

It is, therefore hypothesized that detrimental effect of PDC-109 (major constituent of BSP proteins) on the spermatozoa during cryopreservation process could be minimized to certain extent by sequestration of PDC-109 from the ejaculate at the collection time as well as during processing. Thus the remaining concentration of PDC-109 in seminal plasma will exert minimum detrimental effect during cryopreservation process. The available literatures so far reveal no information on this aspect hence the proposed experiment was designed with following objectives:

1. **Sequestration of PDC-109 at semen collection as well as during processing.**
2. **To investigate the effect of sequestrated semen on minimization of cryo damages and *in vitro* fertilizing ability of spermatozoa.**

2 Review of Literature

Seminal plasma proteins

In bovines, seminal plasma proteins partly originate from the blood plasma by exudation through the lumen of the male genital tract, and partly synthesized and excreted by testis (Kato *et al*., 1985), epididymis (Turner and Reich, 1987), vas deferens (Feng *et al.,* 1995) and seminal vesicles (Manjunath *et al.,* 1994). Aumuller *et al*. (1988) reported that most of the proteins of seminal plasma are secretory products of the seminal vesicle. The seminal vesicle secretion constitutes a portion of seminal plasma on ejaculation and influences the metabolism, motility and surface properties of spermatozoa (Peitz, 1988) thus influencing the fertilizing ability. Henault and Killian (1996) hypothesized that one could improve the fertility of sub fertile spermatozoa by combining them with seminal plasma obtained from a highly fertile bull semen. Further, Amann *et al*. (1999) reported that a peptide derived from the seminal plasma protein (proaposin) can increase the fertility of bovine spermatozoa.

2.1 Protein content in bovine seminal plasma

Electrophoretic profile of seminal plasma of cattle and buffaloes showed 7 to 10 and 9 to 12 different proteins, respectively. Albumin and immunoglobulin were identified in the seminal plasma of both cattle and buffaloes. At least 4 to 6 proteins in the seminal plasma were antigenically similar to those in the blood serum whereas 5 to 6 and 6 to 7 proteins were specific to cattle and buffalo seminal plasma respectively (Kulkarni, 1985).

The average total protein concentration in seminal plasma was 11.6, 8.7, 10.3 and 10.7 gms/100 ml in Sindhi, Jersey, Jersey x Sindhi and Sindhi x Friesian bulls respectively (Seshagiri and Pattabhiraman, 1991). Kulkarni *et al*. (1995) reported mean values of total proteins in seminal plasma 5.63 ± 0.23 (2.8 to 8.7) and 2.43 ± 0.08 (0.6 to 3.7) gms/100 ml

in cattle and buffalo, respectively.

The overall mean values of total protein, heparin-binding (HB) and gelatin-binding proteins were recorded as 29.0 ± 2.7, 2.61 and 0.2 mg/ml respectively in Murrah buffaloes (Arangasamy *et al.,* 2005). Nauc and Manjunath (2000) revealed that in Holstein bull semen BSP proteins represented 40 to 57 per cent of the total seminal plasma proteins (25 to 47 per cent of BSP-A1/-A2, 3 to 5 per cent of BSP-A3 and 3 to 7 per cent of BSP-30 kDa) and 4 to 6 per cent of sperm total protein (2.5 to 4 per cent of BSP-A1/-A2, 0.4 to 0.9 per cent of BSP-A3 and 0.5 to 1 per cent of BSP-30 kDa). The concentration of PDC-109 has been reported to be 15 to 20 mg/ml in bovine seminal plasma (Calvete *et al.*, 1994).

2.2 Heparin-/gelatin-binding proteins in seminal plasma

Several studies suggest that seminal plasma contains factors that modulate the fertilizing ability of spermatozoa. A number of factors in seminal plasma have been investigated like heparin binding protein (Miller *et al*., 1990), clusterins, heat shock proteins, acrosin and many other unidentified proteins as molecular fertility markers (Kulkarni *et al.,* 1996). HBP are abundant on the surface of ejaculated spermatozoa but are less abundant on the plasma membrane of epididymal spermatozoa (Miller *et al.*, 1990). Phelps and Ax (1992) found that distribution of HBP on sperm surface to be 1) over the anterior tip of the head, 2) patches localized to the equatorial region and 3) over the entire head. The phosphorylcholine containing phospholipids, to which the HBP are supposed to bind, constitute more than 73 per cent of total lipids present in the plasma membrane of bovine sperm (Clegg and Foote, 1973).

Miller *et al.* (1990) described three groups of seminal plasma heparin-binding proteins with molecular weight of 14 to 17, 24 and 30 kDa. Proteins of 14 to17 kDa had pl of 4.1 to 6.0 and were associated with sperm membrane and fertility. One of the heparin-binding proteins that bind to spermatozoa had a molecular mass of 30 kDa which is synthesized by the seminal vesicle and prostate glands (McCauley *et al.*, 1999). It was observed that this 30 kDa protein has sequence similarity to DNase -1 and has been termed Fertilization Associated Antigen (FAA). Another HBP with a molecular mass of 24 kDa was supposed to be related with fertility. This protein has an amino acid sequence related to tissue inhibitor of metallo proteinases -2 (McCauley *et al.,* 2001).

Chandonnet *et al.* (1990) reported the ability of these proteins to bind to heparin as

well as gelatin. These proteins are secretory products of seminal vesicles (Manjunath and Sairam, 1987) and were found to modulate sperm properties (Bellin *et al.,* 1988).The presence of HBP on sperm membrane was indicative of the fertility potential of the bulls. Bulls with detectable quantity of HBP on sperm membrane were 11 percentage points more fertile than bulls with undetectable quantity of HBP on sperm membrane (Bellin *et al.,* 1995). The difference in bull fertility may be related to production and secretion of high affinity HBP complex into seminal fluid and/or the ability of sperm to bind and incorporate these complexes in its plasma membrane. HBP were found to modulate capacitation and zona binding ability of buffalo epididymal spermatozoa (Arangasamy *et al.,* 2005).

2.3 Bovine Seminal Plasma (BSP) proteins

The major protein fraction (40 to 57 per cent) of bovine seminal plasma is represented by a family of phospholipids-binding proteins collectively called as BSP proteins (Therien *et al.,* 1995). The three major heparin-binding acidic proteins secreted by seminal vesicles (Gerwig *et al.,* 1996) in bovine seminal plasma are BSP-A1/-A2, BSP-A3 and BSP-30 kDa (Manjunath and Sairam, 1987). BSP-A1 and BSP-A2 are similar but differ only in glycosylation and their mixture is called PDC-109 or gonadostatins (Esch *et al.,* 1983). BSP-A3 does not contain any carbohydrate whereas BSP-30 kDa is also a glycoprotein (Manjunath *et al.,* 1988). The molecular weight of BSP-A1, BSP-A2 and BSP-A3 are between 12 and 15 kDa whereas the mass of BSP-30 kDa protein is 30,000 Da (Manjunath *et al.*1987). BSP-A3 is homologous to BSP-A1/-A2 (PDC-109) with 70 per cent sequence identity including presence of two tandem Fn-II domains (Seidah *et al.,* 1987). The structure of each BSP protein contains two tandemly arranged homologous domains that are similar to the type II structure present in the collagen-binding domain of fibronectin (Calvete *et al.,* 1996,b). This structural property allows BSP proteins to bind heparin, gelatin (Cardin and Weintraub, 1989), high density lipoprotein (HDL), choline phospholipids and low-density lipoprotein fraction (LDF) from hen's egg yolk (Therien *et al.,* 1997).

During normal course of mating in mammals, spermatozoa migrate to the site of fertilization within minutes of ejaculation after serially undergoing process of capacitation, acrosome reaction, hyper activation and zona binding for successful union of gametes. These changes include reorganization of membrane proteins, metabolism of membrane phospholipids

and reduction in membrane cholesterol level (Yanagimachi, 1994). These are thought to aid sperm movement towards oviduct as well as enable spermatozoa to move away from the oviductal epithelium (Demott and Saurez, 1993) and to provide the motive thrust needed for penetration of the zona pellucida (Rathi *et al.*, 2001). A recent report demonstrated that BSP-A1/-A2 (PDC-109) proteins enable spermatozoa to bind to the oviductal epithelium and plays a major role in the formation of the oviductal spermatozoa reservoir (Gwathmey *et al.*, 2003).

Seminal plasma contains BSP-homologous proteins in a variety of mammalian species: such as pB1 (Calvete *et al.*, 1995 b), HSP-1, HSP-2 and HSP-12 kDa, (Calvete *et al.*, 1995 a) and GSP-14 kDa, GSP-15 kDa, GSP-20 kDa and GSP-22 kDa (Villemoure *et al.*, 2003) in boar, stallion and goat respectively. BSP like antigens are also present in rat, hamster and human seminal plasma (Leblond *et al.*, 1993).

2.3.1 BSP-A1/-A2 (PDC-109)

BSP-A1/-A2 or PDC-109 was named following the nomenclature system of Tatemoto and Mutt (1980). The nomenclature is based on their amino (N-) and carboxy (C-) terminal amino acids (one letter system). PDC-109 therefore designates "the peptide (P) having N-terminal aspartic acid (D) and C-terminal cysteine (C) with 109 amino acids".

Purification and primary structure determination of PDC-109 have been reported (Esch *et al.*, 1983). It is a polypeptide of 109 amino acids and contains two tandemly repeating fibronectin type-II (Fn-II) domains, preceded by a 23 residue *N*-terminal domain. This protein is present at about 15-25 mg/mL concentration in the seminal plasma (Scheit *et al.*, 1988).

Binding of PDC-109 with spermatozoa plasma membrane

Manjunath *et al.* (1988) revealed that BSP proteins bind to the plasma membrane of spermatozoa. Around the same time Aumuller *et al*. (1988) reported that the major protein of the bovine seminal plasma (PDC-109) binds to the surface of the spermatozoa. Freshly ejaculated and washed spermatozoa bind approximately 9.3×10^6 molecules of PDC-109 per cell (Calvete *et al.*, 1994). PDC-109 was detected on ejaculated sperm by indirect immunofluorescence and Western blots, but it was not detected on epididymal spermatozoa. Purified PDC-109 when added to the epididymal spermatozoa bind to the plasma membrane overlying the

acrosome (Gwathmey *et al.,* 2003). This was demonstrated by using indirect immunofluorescence and by labeling sperm directly with fluorescein-conjugated PDC-109. Sanchez-Luengo *et al*. (2004) suggested that PDC-109 also binds to the mid piece of sperm once they pass the ampulla of the vas deferens during emission.

As demonstrated by Muller *et al.* (1998), binding of PDC-109 to membrane is a very rapid, biphasic process with half times of less than one second. Maximal binding of PDC-109 to small unilamellar vesicles was achieved with a stoichiometric ratio of 10-11 PC molecule/ PDC-109 molecules. Gasset *et al*. (2000) found maximal binding of PDC-109 to phosphatidylcholine model membranes at a 12:1 phosphatidylcholine to protein molar ratio. The process was independent of the membrane structure and involved a slight conformational change of the protein.

PDC-109 *vis a vis* oviduct spermatozoa reservoir

Experiments conducted by Gwathmey *et al.,* (2003) revealed that significantly fewer epididymal than ejaculated spermatozoa bind to explants of oviduct epithelium. Addition of PDC-109 to epididymal spermatozoa increased epithelial binding to the level observed for ejaculated sperm. In addition, binding of ejaculated sperm to oviduct epithelium was inhibited by addition of excess soluble PDC-109. Ejaculated spermatozoa lost the ability to bind to oviduct epithelium after heparin-induced capacitation, but treatment with PDC-109 restored binding. *In vivo,* loss of PDC-109 from spermatozoa during capacitation could therefore account for release of spermatozoa from the oviduct reservoir. The loss of PDC-109 could be initiated *in vivo* by an increase in heparin like glycosaminoglycans in the oviduct (Parrish *et al*., 1989) or by follicular fluid entering the oviduct with the cumulus mass. These results demonstrated that PDC-109 enables spermatozoa to bind to oviduct epithelium and plays major role in formation of the bovine oviduct sperm reservoir necessary for fertilization. It has been shown in numerous studies that the binding of sperm to the oviduct presents a mechanism for selecting competent sperm population characterized by morphology, motility, membrane integrity, cytosolic calcium levels, sperm protein tyrosine phosphorylation, capacitation and a high fertilizing competence (Gualtieri and Talevi, 2003).

PDC-109 *vis a vis* spermatozoa hyper activation

PDC-109 binds to the choline group of phosphorylcholine-containing phospholipids in a very rapid process (Muller *et al*., 1998). Interaction of PDC-109 with artificial phosphorylcholine vesicles provoked permeability changes and partial disruption of the vesicles (Gasset *et al*., 2000). Thereby, it stimulates cholesterol after binding to the sperm membrane and phospholipids efflux in the early stages of capacitation (Manjunath and Therien, 2002). Furthermore, it binds to choline lipids, thereby increasing the heparin-docking sites on the spermatozoa surface (Therein *et al.*, 1999).

Sanchez-Leungo *et al*., (2004) described a novel biochemical feature of PDC-109 during its interaction with bovine epididymal spermatozoa. Studying the activity of plasma membrane-bound calcium ATPases in bovine spermatozoa, they found a strong stimulatory effect of PDC-109 on spermatozoa motility. This effect was dose-dependent, saturable, and potentially irreversible on sperm plasma membrane ATPases, resulting in increased motility of the spermatozoa. Preincubation of PDC-109 at temperature above 37ºC and pH ranging from below 6.5 and above 8.5 led to loss of the stimulatory effect. Suarez and Ho (2003) indicated that increased free calcium plays a major role in regulating hyper activated motility. They further showed that although hyper activation and capacitation occur simultaneously, both events are regulated by different pathways.

PDC-109 *vis a vis* spermatozoa capacitation and acrosome reaction

The biochemical characteristics of BSP proteins have been well described (Manjunath *et al*., 1988). These bind to spermatozoa membrane choline phospholipids at ejaculation (Desnoyers and Manjunath, 1992) as well as capacitation factors such as HDL and heparin (Manjunath *et al*., 1989), and potentiate spermatozoa capacitation induced by HDL and heparin (Therein *et al*., 1995). The binding of PDC-109 to spermatozoa membrane increase the number of heparin binding sites on the spermatozoa surface and binding of heparin to these sites induce capacitation (Visconti and Kopf, 1998).

Capacitation of bovine spermatozoa by Glycosamino-glycans (GAGs) is stimulated at a maturational step induced by seminal plasma. Ejaculated spermatozoa require 9 h of incubation with GAGs to undergo acrosomal exocytosis without addition of zonae pellucida, whereas

epididymal spermatozoa require 22 h (Lee *et al*., 1985). A 20-min exposure of epididymal spermatozoa to seminal plasma reduces the time required for the acrosome reaction to 9 h (Lee *et al.*, 1985). The exposure of epididymal spermatozoa to seminal plasma *in vitro* enables those spermatozoa to be capacitated by heparin and to respond to zonae pellucida with an increase in acrosome reactions in a manner similar to ejaculated spermatozoa (Florman and First, 1988). These findings suggest that BSP proteins present in seminal plasma may be responsible for capacitation and acrosome reaction of spermatozoa. However, PDC-109 does not actually induce capacitation *per se* but rather enhances capacitation in the presence of high-density lipoproteins, such as those found in bovine follicular fluid (Manjunath *et al*., 1989; Therien *et al*., 2001). Thus, PDC-109 would not initiate capacitation until promoters are available in the oviduct.

PDC-109 *vis a vis* spermatozoa cholesterol efflux

During the transit through the female genital tract, mammalian spermatozoa must undergo the process of capacitation and acrosome reaction before successful fertilization of the ovum (Chang, 1951). Sperm capacitation is a multi step process that is not well understood and involves several biochemical and ultra structural changes in the sperm membrane. Many studies have shown that sperm capacitation is accompanied by a change in the lipid composition of the sperm plasma membrane (involves a decrease in the membrane cholesterol: phospholipids ratio, Davis, 1981). These changes appear to be a reversible phenomenon that influences the fluidity and ionic permeability of the sperm membrane (Langlais and Roberts, 1985). Several studies have demonstrated that cholesterol influx reduces the rate of spontaneous acrosome reacted spermatozoa (Davis, 1980; Fleming and Yanagimachi, 1981) and also inhibit fertilization by either inhibiting or delaying capacitation in bovines (Ehrenwald *et al.*, 1988 a). They have also shown that the efflux of membrane cholesterol leads to bovine sperm capacitation and predisposes the spermatozoa to penetrate ova at a higher rate than those spermatozoa with higher cholesterol levels.

During capacitation cholesterol is lost from the sperm plasma membrane and when sufficient cholesterol is removed the membrane becomes unstable, enhancing its ability to fuse with the outer acrosomal membrane, resulting in the acrosome reaction (Nolan *et al.*, 1992). During a typical freeze-thaw cycle, the sperm membrane must undergo phase transition during

cooling and again at re-warming. Holt *et al*. (1992) obtained some evidence that phase transitions might be involved in the manifestation of cryoinjury during the re warming of cells at thawing. Besides causing physical disruption of the plasma membrane by the induction of lipid packing, lipid phase transition effects cause non-linear kinetic responses in some enzymes, including some of the membrane ATPases whose activity depends upon the physical state of annular lipids (Kimelberg, 1977). It is likely that such effects are partly responsible for the poor control of intracellular calcium concentration, which is evident at temperature below 17°C (Bailey *et al.,* 1994). Moreover, the cryopreserved spermatozoa membrane has to undergo similar fluidity changes to those seen during capacitation. They are permeable to calcium ions which promote both capacitation and the acrosome reaction. These changes in the sperm membrane cause limited survival of capacitated spermatozoa (Watson, 1995).

In non-capacitated spermatozoa, cholesterol is distributed over the entire sperm head, but after induction of the acrosome reaction, cholesterol redistributes from the equatorial to the apical region of the sperm head. Davis (1981) reported that in ejaculated spermatozoa, coating substances and other components of seminal plasma occupy the acrosomal region of the sperm head and have to be removed before capacitation can occur. Rate of capacitation depends on the rate of cholesterol depletion from the sperm plasma membrane (Gadella *et al*., 2001), thus, spermatozoa with high cholesterol content (e.g. spermatozoa from man, bull and stallion) are slow to undergo capacitation whereas spermatozoa with lower cholesterol content (*e.g.* from boar and ram) seem to capacitate much faster (Witte and Schafer-Somi, 2007).

Therefore, if compounds that increase cholesterol removal from sperm membranes such as BSA or cyclodextrins containing no cholesterol are added to the medium, sperm capacitation is enhanced (Visconti *et al.,* 1999). Much of cryopreserved sperm damage depends on the structural stability of the plasma membrane (De Leeuw *et al.,* 1993). The susceptibility of the plasma membrane to undergo lipid phase transitions during cooling is inversely related to the level of cholesterol present. Lower cholesterol levels are present in boar and ram spermatozoa, which are considered to be sensitive to cooling than in rabbit and human spermatozoa that are less susceptible (Gadella *et al*., 2001)

The net cholesterol efflux that is observed during capacitation is affected by the capacity of the medium to bind cholesterol. Many studies have shown that albumin (Davis *et al*., 1980)

and high-density lipoprotein (HDL, Ehrenwald *et al.,* 1990) are acceptor of cholesterol. HDL, the only classes of lipoprotein present in bovine as well as human oviduct and follicular fluid appears to be a more efficient acceptor of cholesterol than albumin (Ehrenwald *et al.,* 1990). Therefore, HDL appears to play an important role in the sperm sterol modification that occurs during at an early stage of capacitation.

Muller *et al*. (2002) studied the interaction of PDC-109 with cholesterol by employing spin-labelled analogues. It was found that PDC-109 does not interact directly with cholesterol molecules. However, in the presence of phospholipids, a strong reduction of cholesterol motion by PDC-109 was observed. The selectivity observed for the phosphocholine-containing lipids, phosphatidylcholine and sphingomyelin, is the highest among other lipids for their interaction with PDC-109 (Thomas *et al.,* 2003). The binding of Lysophosphatidyl choline (LPC, an egg yolk lipid) to PDC-109 is 250-fold stronger than that of phosphorycholine, the smaller negative entropy of binding associated with LPC resulting in its significantly stronger binding to PDC-109 (Anbazhagan and Swamy, 2005).

Cholesterol at a concentration of 1 µg/ml in semen is able to inhibit spontaneous acrosome reaction. It is present in sperm plasma membrane (13 nmol/10^8 cells in human spermatozoa) as well as in human seminal plasma at the rate of 250 µg/ml (Rathi *et al.,* 2001). Wide variation between bulls as well as between ejaculates has been reported by various workers. The cholesterol content of bull spermatozoa varies from 7.87 to 16.75 µg per $50x10^6$ spermatozoa (Sinha *et al.,* 1996). Therien *et al.* (1998) reported cholesterol content of 720 ± 41 µg/ $50x10^8$ spermatozoa in bull. Kadirvel (2006) reported mean cholesterol content as 9.92 ± 1.6 and 5.38 ± 1.85 µg/$50x10^6$ spermatozoa in fresh and frozen thawed semen samples in buffalo bulls respectively. Therien *et al.* (1995) observed that amount of cholesterol did not change up to 48 h of storage at 4°C but phospholipids content of spermatozoa decreases by 30 per cent between 1 h and 24 h of storage and consequently the cholesterol phospholipids ratio increases from 0.19 to 0.28 between 1 h and 48 h of storage.

Harshan (2007) observed that treatment of spermatozoa with PDC-109 led to an increased cryoinjury. The damage was dose dependent with the higher doses showing higher damages. Dhami *et al*. (2004 a) observed that the total cholesterol in seminal plasma was highest in cross-bred in comparison to Gir bulls (45.93 ± 12.58 vs. 31.87 ± 2.39 mg per cent).

Detrimental effect of PDC-109 on spermatozoa

The biochemical characteristics of PDC-109 have been well described by Manjunath *et al.* (1988). This binds to sperm membrane choline phospholipids at ejaculation (Desnoyers and Manjunath, 1992) as well as capacitation factors such as high density lipoprotein (HDL) and heparin (Manjunath *et al*., 1989). This potentiates sperm capacitation induced by HDL and heparin (Therien *et al*., 1997) and is beneficial for spermatozoa function. In contrast, recent studies show that the PDC-109 induces changes in sperm plasma membrane by stimulating cholesterol and phospholipids efflux (Therien *et al*., 1999). This lipid efflux by PDC-109 is time and concentration dependent. At higher concentration of PDC-109 (*i.e*. seminal plasma) and/or longer exposure (as in modulated semen), more cholesterol and phospholipids are removed. The removal of lipids, particularly cholesterol, results in spermatozoa membrane destabilization. Evidence shows that the decrease in cholesterol content in plasma membrane also appears to decrease spermatozoa resistance to cold shock and freezing (Darin-Bennett and White, 1977). It is obvious that prolonged exposure of spermatozoa to seminal plasma that contains PDC-109 has deleterious effect on spermatozoa. Therefore PDC-109 in seminal plasma has the potential to act as both beneficial and detrimental factors to spermatozoa depending on its concentration in seminal plasma and exposure time.

2.3.1.1 Isolation and purification of PDC-109

Seminal plasma protein PDC-109 has been purified and characterized by various workers (Esch *et al.,* 1983; Aumuller *et al.*1988; Calvete *et al.,* 1996 b).

Miller *et al.* (1990) described method of isolation for HBP using heparin-Sepharose affinity chromatography. Activation of Heparin-Sepharose column was done using citrate buffer and equilibrated with phosphate buffer containing 2mM EDTA followed by addition of clear seminal plasma. The unabsorbed proteins were washed with same equilibration buffer. The unbound proteins were eluted as 3 ml fractions with 20 ml of same equilibration buffer containing 1 M NaCl.

Manjunath *et al.* (1987) isolated BSP-A1/-A2, BSP-3, and BSP-30 kDa proteins using Gelatin-Agarose affinity chromatography. The absorbed fractions were resolved on Sephadex G-75 and G-200 columns under the specific conditions and the BSP proteins isolated

by this procedure were lipid-free (Manjunath and Sairam, 1987). The purity of the proteins (yield 90 to 95 per cent) was assessed by SDS-PAGE (Laemmli, 1970). For isolation of BSP proteins Manjunath *et al.* (2002) carried out get filtration chromatography on a Sepharose CL04 column (70x 2.5 cm) equilibrated with PBS at a flow rate of 80 ml/h. After a 40-min interval, fractions of 3 ml were collected and the absorbance was determined at 280 nm.

Other workers have also isolated seminal plasma protein PDC-109 following different protocol with greater degree of purity. Calvete *et al*. (1996 a) used size-exclusion chromatography of seminal plasma for isolation and the peak containing PDC-109 was applied to a DEAE-Sephadex A25 column. At high salt concentration only PDC-109 was retained in the column due to its reported affinity for alkyl groups (Desnoyers and Manjunath, 1993).

A combination of heparin affinity chromatography and DEAE ion exchange chromatography was also used by Gasset *et al*. (1997). Seminal plasma was applied on to a Heparin-Sepharose column and bound proteins were eluted with 10mM o-phosphorylcholine. The eluted proteins were extensively dialyzed against 20 mM Tris-HCL buffer (pH 6.4) containing 1 M NaCl, and loaded on a DEAE-Sephadex column. The column was washed to remove the unbound proteins. PDC-109, which is the only protein retained under this ionic strength was eluted with 10 mM o-phosphorylcholine.

Gwathmey *et al*. (2003) also isolated and purified PDC-109 from bull seminal plasma following above method.

2.3.1.2 Sequestration of PDC-109 from ejaculate

The role of glycerol in cryopreservation is that it contributes to spermatozoa integrity conservation (Anchordoguy *et al*., 1987); however the protection afforded by egg yolk is more complex. The egg yolk has been shown to increase the spermatozoa fertilizing ability when present in extenders for semen storage at ambient temperature (Phillips and Lardy, 1940) and appears to prevent spermatozoa cell damage at cooling and freezing (Lasley and Mayer, 1944). Various components of egg yolk have been investigated to identify the most active component (s) responsible for protective effect (Watson, 1976). Evidence indicates that low-density lipoprotein fraction (LDF), shows the highest protective ability, however the mechanism by which this protection is provided to sperm remains elusive. It is speculated that

LDF associates with sperm membranes and provides protection against membrane damage. Viswanath *et al.* (1992) suggested that EY lipoproteins compete with detrimental seminal plasma cationic peptides in binding to the sperm membrane and thus protect the spermatozoa. The average concentration of BSP proteins in semen is 35 to 50 mg/ml (Nauc and Manjunath, 2000) and of LDF in 20 per cent egg yolk is 10 to 15 mg/ml (Manjunath *et al.*, 2002). Semen is normally diluted 10 times or higher (20 per cent) with egg yolk containing medium (EYTG extender) prior to cryopreservation. At this dilution, most of the BSP proteins may be associated with LDF. However, cholesterol efflux by BSP proteins may start well before extenders containing EY is added, necessitating sequestration of BSP proteins at the time of collection of ejaculate itself.

Although no work has been reported on this aspect, several workers raised hyper immune sera containing polyclonal antibodies against PDC-109 (Gwathmey *et al.*, 2003) for their studies in formation of oviductal reservoir. Similarly, Nauc and Manjunath (2000) raised polyclonal antibodies to develop RIA for quantification of BSP proteins.

2.4 Physico morphological characteristics

2.4.1 Semen volume

Volume of semen ranges from 1 to 15 ml with an average of 4 ml in bulls (Roberts, 1982). Under usual breeding conditions, the volume of semen in males does not change much, but the volume may be influenced by factors like body weight, age, pre-coital stimulation and frequency of semen collection and scrotal size. With the advancement of age of bull and scrotal size and weight, there is a gradual increase in the ejaculate volume (Tomar *et al.*, 1985).

Various workers reported varying amount of ejaculate volume for different breeds of bulls. Mishra *et al.* (1989) reported an average volume of 5.19 ± 0.27 ml in four cross bred bulls with varying levels of inheritance (Sahiwal x Jersey x Holstein x Red Dane). Sagdeo *et al.* (1990) reported an average volume of 3.06 ml and 3.55 ml in Jersey and Holstein crosses, respectively as compared to 2.79 ml in purebred Jersey. An average ejaculate volume of 3.92 ± 0.24 ml in freezable semen samples of crossbred bulls was also observed by (Loyi, 2008).

2.4.2 Mass activity

Mass activity of semen is one of the most common seminal attributes used for assessing its quality due to its simplicity (Mishra and Tyagi, 2006). Kjaestad *et al.* (1993) found significant relationship between motility and field fertility whereas Salisbury *et al.* (1978) reported that the motility of fresh spermatozoa does not predict either the fertility or the freezability of semen. Mass activity is significantly and positively correlated with the spermatozoa concentration, initial motility and live spermatozoa per cent (Patel *et al.*, 1989). Variation in mass activity may also be influenced by the season (Tomar *et al.*, 1985).

Mass activity is also consistently higher in the pure breeds than their crosses (Surya Prakasam and Rao, 1993). Mishra *et al.* (1988) reported average mass motility of 4.06 ± 0.13 in three breed crosses (Jersey x Holstein x Sahiwal). Mohanty (1999) recorded much lower mass activity both in good (2.15 ± 0.09) as well as in poor (1.85 ± 0.06) freezable crossbred bull semen. Loyi (2007) reported mass activity of 3.4 ± 0.10 in freezable semen samples of crossbred bulls.

2.4.3 Concentration

The spermatozoa concentration in a bull semen ranges from 300-2500 million/ml with an average of 1200 million/ml (Roberts, 1982). Similar, mean values have been reported by Agarwal *et al.* (1987) in 3/4th cross bred bulls and Patel *et al.* (1989) in Kankrej cross bred bulls. Mohanty (1999) recorded a spermatozoa concentration of 999.83 to 1114.50 million/ ml in crossbred bulls and found to have a higher spermatozoa concentration in good freezable than non freezable semen samples. Loyi (2008) reported a sperm concentration of 724.09 ± 53.05 million/ml in freezable semen samples of crossbred bulls.

2.4.4 Individual motility

Spermatozoa become essentially mature on reaching to cauda epididymis in that they achieve motility and capability of fertilizing the eggs (Bedford, 1975). The spermatozoa are immotile in the cauda epididymis due to visco-elastic effect of a glycol protein "immobilin". This effect was found to be reversible and non-sperm specific (Usselman *et al.*, 1984). The maturation of spermatozoa during their epididymal transit involves physico-biochemical and morphological alteration resulting into cyto-physiological changes to achieve capacity for motility

and fertility (Orgebin-Crist *et al.*, 1976). The individual motility depends on the factors like age of the bull (Javed *et al.*, 2000), temperature, frequency of collection and sexual excitement before collection (Mishra *et al.*, 1994).

Assessment of individual motility requires screening of individual cells; hence after a semen dilution with suitable extender, individual cells are viewed under 400 magnifications. Motility pattern of individual cells is characterized, counted and expressed as per cent.

The individual motility percentage reported in cross-bred bulls was 83.60 ± 13 (Singh *et al., 1992*), 72.5 ± 0.0 (Mishra et al., 1994) and 77.92 ± 0.33 (Shukla and Mishra, 2005) in fresh semen. Individual motility of 82.99 ± 0.27 (Agarwal *et al*., 1987), 82.98 ± 2.72 (Patel *et al*., 1989), 79.74 ± 0.64 (Singh and Pangawkar, 1990), 55.00 ± 2.02 (Sharma *et al*,. 1991), and 51.0 ± 11.63 per cent (Suryaprakasan and Rao, 1993), was reported for crossbred bulls of BS x HF x H, HF x J x H, HF x S, HF x J and HF x ND inheritance respectively. A reduction of 41 to 53 per cent in spermatozoa motility after cryopreservation was reported by Rasul *et al.*, (2001). Dhami *et al.* (1994 a) reported an average post-thaw motility 55.83 ± 8.28 per cent in crossbred bulls. Loyi (2008) reported per cent individual motility of 75.5 ± 0.84 and 43.61 ± 1.33 in freezable fresh and post-thaw semen samples of crossbred bulls. An average post-thaw motility percentage of 35.48 ± 2.16 (HF x J x H bull; Sharma,1988) 33.98 ± 1.64 (HF x BS x H ; Pathak,1988) and 34.89 ± 1.4 (HF x J x H ; Vyas *et al*. ,1992) were reported by various workers. Prasad *et al*. (2000) categorized semen having 40 per cent or above post-thaw motility as freezable in crossbred bulls.

Addition of heparin-binding proteins at the concentration of 40, 30, and 20 µg/ml performed well to maintain motility when compared to 10, 50 and 60 µg/ml, indicating that high as well as low concentration of heparin-binding protein concentration have detrimental effect on spermatozoa motility (Arangasamy,2003). Harshan *et al*.(2006) reported that spermatozoa of all the PDC-109 protein treated groups had significantly lower motility than the spermatozoa of control group at post-thaw stage but the effects are dose dependent with higher concentrations being deleterious. In frozen-thawed goat semen addition of glutathione (5mM) increased the motility of thawed spermatozoa (Sinha *et al.*, 1996). Addition of catalase, GSH, taurine, and other antioxidants helped to maintain post-thaw motility of bull spermatozoa (Alvarez and storey, 1983).

2.4.5 Per cent live spermatozoa

The estimation of per cent live spermatozoa in a semen sample has direct and positive correlation with fertility of a bull. The live and dead spermatozoa could be differentiated by their reaction to certain stains: dead sperm being colored by the vital dye, and live sperm not being stained. Eosin is the essential cellular stain used whereas the Nigrosin provides a dark background so that observation of the stained and unstained sperm cells can easily be made. All sperm that are even partially stained are considered dead.

Various workers reported live sperm per cent of 79 ± 2.2 in fresh semen (Branton, 1946) and, 70 ± 2.5 to 85 ± 2.1 in freezable samples (Gopal Krishna and Rao, 1978) in crossbred bulls. The per cent viability was reported as 87.38 ± 0.63 (Rao and Rao, 1978), 91.84 ± 1.18 (Patel et al. 1989) and 84.13 ± 2.31 (Srivastava, 2000) in different genotypes of crossbred bulls. The mean live spermatozoa for good and poor quality ejaculate in crossbred bulls were recorded as 82.57 ± 1.33 and 56.78 ± 1.71 by Gebreselassie (2009) in contrast to 78.32 ± 1.36 and 72.15 ± 2.18 per cent respectively observed by Loyi (2008). Harshan (2007) indicated that though lower doses of PDC-109 do not affect viability at pre freeze level, a higher amount of PDC-109 is detrimental for sperm viability (80.25 ±0.89, 83.17 ±0.80, 83.08 ± 0.92 and 83.66 ± 1.05 in groups treated with 120, 40, 20 µg/mL of PDC-109 as compared to control respectively). However at post thaw stage, decrease in viability was noticed in all PDC-109 treated groups (25.67 ± 0.97, 43.92 ± 0.87, 52.08 ± 1.07 and 62.25 ± 1.14 in groups treated with 120, 40, 20 µg/mL of PDC-109 as compared to control respectively). Similar finding was reported by Fiol de Cuneo *et al*. (2004) observing a decrease in sperm motility and viability when PDC-109 (0.5, 1.5 and 3.0 µg) was incubated with cryopreserved bovine spermatozoa for 4 h in the presence of heparin.

2.4.6 Hypo-Osmotic Swelling (HOS) response

The importance of integrity of sperm membrane is unquestionable not only for sperm metabolism but also for desirable changes in the properties required for successful union of male and female gametes. Hence the assessment of membrane function may be a useful indicator for determining the fertilizing ability of spermatozoa (Jeyendran *et al*. 1984).

Panday *et al.* (1986) reported a positive correlation between motility and swollen spermatozoa with that of *in-vitro* fertilizing ability of spermatozoa. However, Chan *et al.* (1992) reported insignificant correlation between sperm swelling and *in-vitro* sperm fertilizing capacity. The HOS response correlates well with the ability of spermatozoa to penetrate denuded hamster oocyte and present a high correlation with the ability of the spermatozoa to fertilize the ovum *in vitro* (Vander ven *et al.,* 1986; Avery *et al.,* 1988). Jeyendran *et al.* (1984) reported that the dead spermatozoa taking the eosin stain may not necessarily be HOS negative and a live spermatozoon preventing the eosin stain uptake may not always swell.

Okada *et al.* (1990) reported positive correlation between the results of HOST and hamster egg penetration assay for specimens with high or low fertility rates. In contrast, McClure and Tom (1991) found no significant correlation for spermatozoa from infertile patients, but only for spermatozoa from fertile donors in humans. Nevertheless, it was shown that combination of HOST with other spermatozoa parameters could improve its predictive potential (Chan *et al*., 1992). Combining HOST results with other parameters (*e.g*. motility and viability) is useful in order to improve correlations with the fertility rate *in vitro* (McClure and Tom, 1991).

Agarwal (1997) reported a significant positive correlation of HOST with sperm motility and sperm viability. Gebreselassie (2009) reported that the mean values of HOST positive spermatozoa for good and poor quality fresh semen were 73.13 ± 1.50 and 54.56 ± 1.99 per cent respectively. A significant positive correlation of HOST with mass motility, initial progressive motility, live count, total intact acrosome and sperm concentration were found by Prasad *et al,* (1999) as well as Srivastava and Kumar, (2006). A HOS response in fresh and frozen-thawed semen of cross bred bull was reported as 74.4 ± 0.80 and 47.8 ± 0.97 per cent respectively, indicating 60 per cent reduction in HOS positive response after cryopreservation (Rasul *et al.,* 2001). Loyi (2007) reported post thaw HOS positive response of 48.21 + 2.18 per cent in good quality freezable semen samples of crossbred bulls. Addition of vitamin E and Ascorbic acid in Egg Yolk Tris Glycerol (EYTG) extender increased the HOS response in frozen thawed semen when compared to control (48.5 ± 1.3 vs. 43 ± 2.1 per cent, Agarwal, 1997). In buffaloes, HOS response after addition of heparin binding proteins at a concentration of 40 and 20 µg differed significantly ($p<0.05$) with control and gave high HOS values (72.95 ± 1.31 and 70.13 ± 0.84 vs. 50.30 ± 2.04 respectively) in fresh semen (Arangasamy, 2003).

Harshan (2007) reported maximum tail swelling in buffalo spermatozoa in the group treated with PDC-109 at the rate of 40 µg/mL (76.33 ± 1.08, 73.43 ± 1.03 and 71.83 ± 0.95 per cent in group treated with 40 and 20 µg/ml of PDC-109 as compared to control respectively) in pre-freeze semen, whereas at post-thaw stage, a significant decrease in viability (36.33 ± 1.02, 44.92 ± 0.81 and 59.33 ± 0.93 per cent in group treated with 40 and 20 µg/mL of PDC-109 as compared to control respectively) of spermatozoa was observed**.** A range of 22.6 ± 3.9 to 58.8 ± 6.3 per cent in post-thaw HOS response in cross-bred bulls was reported by Pant *et al.* (2002). Efforts to minimize the levels of reactive oxygen species (ROS) may improve the HOS response in frozen semen (Dandeker *et al.,* 2002).

2.4.7 Acrosomal integrity

The acrosomal integrity of mammalian spermatozoa is pre-requisite for capacitation, normal acrosome reaction and successful fertilization *in vivo* (Hartree and Srivastava, 1965). The acrosome contains a number of hydrolytic enzymes and several acid hydrolases. The enzymes localized in the acrosome, determine the spermatozoa penetrating and fertilizing capacity. Acrosome can be detached from the spermatozoa head under the influence of different physical and chemical factors (Hartree and Srivastava, 1965). Optimum fertility depends on the acrosome being structurally and functionally intact (Srivastava *et al.,* 1965). Borgohain (1984) observed that acrosomal damage of the spermatozoa of Holstein-Friesian, Jersey and their crosses had significantly negative correlation with conception rates in cows. Saacke (1970) reported a significant positive relationship between the per cent intact acrosome and non return rate.

Stains like Eosin-blue and fast green have been used to assess the acrosome characteristics. It is now being superseded by fluorescent tests (Harrison and Vickers, 1990). The most commonly used staining method to detect acrosomal changes, based on staining intensity and contrast of background is Giemsa stain (Watson, 1975) or fluoresceinated lectins (Batova *et al.,* 1993). Percent intact acrosome is determined by staining spermatozoa with different dye.

Acrosomal integrity by Giemsa stain

Agarwal (1997) found the mean per cent intact acrosome in frozen-thawed bull spermatozoa 61.20 ± 1.44 (range 24 to 78) per cent. Sharma *et al.,* (1992) reported 69.40 per

cent post-thaw intact acrosome in crossbred bull spermatozoa and observed that acrosomal integrity per cent was positively correlated with pre-freeze and post-thaw motility. Prasad, (1997) and Srivastava (2000) recorded a positive correlation of acrosomal integrity with post-thaw livability and motility. Paudel (2002) observed 9 per cent reduction in intact acrosome from fresh to pre-freeze and 28 per cent from pre-freeze to frozen-thawed state in crossbred bull spermatozoa. The per cent intact acrosome was significantly higher in good quality semen than poor quality semen. A significantly positive correlation between intact acrosome, motility and live sperm was also observed (Agarwal, 1997). In two experiment Lambrechts *et al.*, (1999) found intact acrosome percentage to be 89.3 ± 2.3 and 93.5 ± 2.2 in African buffalo epididymal spermatozoa. More than 90 per cent of spermatozoa were observed with intact acrosome in fresh semen of breeding bulls by Giemsa staining (Aguero *et al*., 1994).

b. Acrosome reacted live spermatozoa by FITC-PSA stain

The integrity of spermatozoa membranes can be assessed by using a variety of lectins that bind to glucoconjugates of the outer acrosomal membrane or the outer acrosomal matrix such as fluoresceinated isothiacynate- *pisum sativam* agglutinin (FITC-PSA, Cross and Watson, 1994; Maxwell and Johnson, 1997). FITC- PSA labeling can effectively monitor acrosomal integrity as has been confirmed by electron microscopy assessment (Meyers *et al*., 1995). However only two pattern of staining was observed with stallion spermatozoa, representing sperm cells with intact acrosome and missing acrosome. Graham *et al.* (1990) established that fluorescently labeled PSA could be used in flow cytometry to assess the percentage of cells with or without intact acrosome based on comparisons with naphthol yellow/erythrosin B. An assessment of the percentage of cells with intact acrosome is also done when the acrosome reaction was induced with lysophosphatidylcholine. However, only two patterns of staining were observed with stallion spermatozoa, representing sperm cells with intact acrosomes and missing acrosomes. The AR in progress was not classified (Cheng *et al*., 1996).

Since the intact membrane of live cells excludes a variety of charged dyes, such as trypan blue or Propidium Iodide (PI), incubation with these dyes results in selective labeling of dead cells, while live cells show no or minimal dye uptake. For light microscopic evaluation, a relatively high concentration of dye is required. At these concentrations, eosin, and many other

dyes are toxic, which can lead to underestimation of the proportion of live cells (Woelders *et al.*, 1991). Moreover, eosin dye is used nearly exclusively to stain fresh ejaculated spermatozoa, because glycerol, a most extensively used cryoprotectant, interferes with staining. Therefore now a day fluorescent stains such as PI or similar other dyes in combination with FITC- PSA are most widely used for evaluating integrity of frozen-thawed spermatozoa plasmalemma followed by examination with fluorescence microscopy or flow cytometry (Garner and Johnson, 1995).

In order to detect changes taking place specifically in the live sperm population, Maxwell and Johnson (1997) stained the spermatozoa with PI, and the PI-positive cells were excluded from the estimate of acrosome intact and acrosome reacted. The proportion of an acrosome reacted spermatozoa estimated by FITC-PSA fluorescence tended to be higher than by the CTC method but as yet the acrosome status has not been confirmed in these spermatozoa by electron microscopy (Maxwell and Johnson, 1997).

Gillian *et al*. (1997) carried out an experiment to validate the assumption that F and B pattern would be acrosome intact and the AR pattern would be reacted; cells were stained with both CTC and FITC-PSA. PSA binds to the acrosomal matrix (Cross *et al*., 1986) thus allowing visualization of the acrosome when intact. All F category cells were intact, as were the majority of the B pattern spermatozoa, and most of the AR pattern cells were acrosome-reacted as determined by FITC-PSA fluorescence. Although a minority of the cells displaying the B pattern appeared to be acrosome-reacted (rather than acrosome intact), these cells often exhibited patchy fluorescence in the acrosomal region when assessed by PSA and this may correspond to cells undergoing acrosomal exocytosis (Stock and Fraser, 1987). It is likely that cells in this category have reasonably intact plasma and outer acrosomal membranes for complexed CTC to bind to, with small perforations accounting for the patchy FITC-PSA fluorescence observed. It would be necessary to confirm this observation by visualizing the ram sperm surface with electron microscopy.

2.4.8 *In vitro* capacitation and acrosome reaction

An indirect test of capacitation status using the antibiotic chlortetracycline (CTC) has provided a new insight into the functional status of spermatozoa (Fraser, 1995). With CTC as

a fluorescent probe, spermatozoa show various fluorescent patterns, which have been correlated with predominantly, non capacitated (Pattern F), capacitated or partially capacitated but have not acrosome reacted (Pattern B) and acrosome reacted (AR) sperm population (Ward and Storey, 1984; Collin *et al*., 2000). Indeed, the antibiotic chlortetracycline (CTC, a fluorescent compound) accumulates and fluoresces in membrane compartments in which high concentrations of calcium ions exist next to hydrophobic sites (Tsien, 1998). It has been reported that CTC can be used as a fluorescent probe to visualize the course of capacitation and acrosome reaction in spermatozoa in mouse and bull (Saling and Storey, 1979; Fraser, 1982; Fraser *et al.*, 1995). A flow-cytometric assay for capacitation has also been developed based on CTC-fluorescence (Maxwell and Johnson, 1997). Gillian *et al.*, (1997) reported that for the ram semen, the process of freezing and thawing increased the number of spermatozoa showing the capacited B pattern but had little effect on the number of acrosome reacted spermatozoa. They reported that at 0 h, pattern F and B was observed in 61.3 & 19.5 per cent for fresh and 6.7 & 54.0 per cent for frozen thawed spermatozoa respectively. Curry (2000) observed increase in pattern 'B' spermatozoa from 16 per cent in fresh semen to 39 per cent in frozen thawed spermatozoa. However, after 6 h of incubation, pattern 'B' and 'AR' were 54 and 41 per cent respectively in fresh semen in comparison to 35 and 64 per cent in frozen-thawed spermatozoa. Process of freezing and thawing increased the number of 'AR' spermatozoa. Changes in CTC staining pattern have also been reported for bull spermatozoa (Cormier et al., 1997): after either cryopreservation or cooling to 4° C there is a similar increase in the capacitated B staining pattern. Cormier and Bailey (2003) demonstrated that cryopreservation induces partial or complete capacitation of bull spermatozoa that is revealed by a greater proportion of CTC fluorescent pattern B in bulls. Cooled and frozen-thawed spermatozoa apparently become more "capacitated" as determined by this test. CTC staining is currently assay of choice because it distinguishes three stages of sperm activation: non capacitated acrosome-intact, capacitated and acrosome reacted. A clear understanding of how CTC interacts with the sperm surface at the molecular level is lacking and unfortunately, the evaluation of CTC staining is performed on fixed sperm cells.

In buffaloes, addition of heparin-binding proteins has dose dependent effect on induction of acrosome reaction for 1 to 5 h incubation. At every hour of incubation, there is a gradual

increase in acrosome reacted sperm per cent in a concentration dependent manner (Arangasamy, 2003). Heparin treatment (10µg/ml) enhanced the capacitation rate of buffalo spermatozoa and fertilization rate of oocytes under *in vitro* conditions (Chauhan *et al.*, 1997). They also observed that the percentage of acrosome reacted sperm were increasing during every hour of culture and reached maximum up to 84.33 ± 1.62. Therien *et al.*, (1995) reported a maximum acrosome reacted spermatozoa percentage when BSP proteins were added at the rate of 40µg/ml of semen. Harshan *et al*. (2006) reported an increase in the number of acrosome reacted spermatozoa in PDC-109 treated groups than the control at varying incubation periods at pre-freeze (7.33 ± 0.33, 6.75 ± 0.46 and 3.92 ± 0.39 at 0 h.; 45.25 ± 0.48, 39.33 ± 0.83 and 30.50 ± 0.97 per cent at 4 h. of incubation in group treated with 40, 20 µg/ml of PDC-109 and control respectively) and post-thaw (19.60 ± 0.79, 17.0 ± 0.70 and 5.92 ± 0.31 at 0 h. and 22.58 ± 0.76, 19.08 ± 0.81 and 6.42 ± 0.39 at 4 h. of incubation in group treated with 40, 20 µg/ml of PDC-109 and control respectively). The number of acrosome reacted live spermatozoa were maximum in the group treated with PDC-109 at a rate of 40 µg/ml. At post- thaw stage, per cent acrosome reacted spermatozoa at each time interval of incubation was higher than the corresponding pre-freeze acrosome reacted spermatozoa of same treatment group.

2.5 Cryopreservation of bovine semen

The success of cryopreservation of semen depends on numerous factors, which may be peculiar to each species and therefore optimized protocol is used for each species. When cells are frozen they are subjected to stress resulting from the water-solute interactions that arise through ice crystallization. Exposure of cells to the hyper osmotic, yet unfrozen, solution causes withdrawal of intracellular water, consequent cell shrinkage and possible influx of ions (Mazur, 1984).

The sperm plasma membrane is the primary site of damage induced by cryopreservation (Hammersteds *et al.*, 1997). Noiles *et al.* (1995) suggested that membrane phase transition occurs between 4°C and 0°C in mouse sperm during freezing process which is associated with elevated membrane fragility due to imbrittlement of phase transition and other ultra structural modifications of the plasma membranes during cooling. The rewarming may play a role in the poor fertility of cryopreserved sperm. It seems plausible that reorganization of sperm membrane

lipids disturbs the lipid-lipid and lipid-protein interactions required for normal membrane function (Parks and Graham, 1992).

The damage to bovine spermatozoa during cryopreservation is not well understood. However changes in motility (Kakar and Anand, 1981) and acrosome morphology (Kumar, 1989) of buffalo spermatozoa following freezing and thawing process have been observed. Moreover a considerable loss in motility of sperm occurs after equilibration (Tuli *et al*., 1988). The motility is not affected during dilution, cooling and equilibration but greater damage occurs during freezing and thawing process (Rasul *et al.,* 2000). These hazards can be minimized by optimizing the cooling and freezing rates and using appropriate diluents in which spermatozoa are frozen (Kumar *et al.,* 1992).

Furthermore, despite preservation of adequate motility, cryopreserved human sperm exhibit significant membrane damage as indicated by hypo-osmotic swelling tests (Check and Check, 1991). One interpretation of this data is that as the post-thaw temperature increases, the plasma membrane is subjected to structural rearrangements involving lipids and proteins, the extent and nature of which is governed by interactions of temperature and solute effects during the freezing process.

2.6 Capacitation like changes (cryocapacitation)

Semen cryopreservation is an important tool for assisted reproduction, although the fertility of frozen-thawed spermatozoa is reduced due to sub lethal damage that is not completely understood (Viswanath and Shannon, 1997). However it has been recently recognized that cryopreservation procedures (dilution, cooling, freezing/thawing) induce capacitation-like changes in spermatozoa (Bailey *et al.,* 2000). Watson (1995) suggested that cryopreservation induced modifications to sperm membranes make them more reactive to their environment after thawing such that cryopreserved sperm are in partially capacitated state. Indeed, recent studies have reported similarities between the changes associated with capacitation and cryoinjury, such as plasma membrane reorganization and fluidization, and calcium influx to the spermatozoa (Maxwell and Johnson, 1997). Perez *et al.,* (1996) showed that cryopreserved ram sperm undergo capacitation more quickly than fresh controls as assessed by chlortetracycline (CTC) fluorescent assay. The CTC fluorescence also revealed a greater proportion of pattern

B "capacitated" (Ward and Storey, 1984) spermatozoa due to cooling and immediately after thawing in mice (Fuller and Whittingham, 1996), bull (Cormier *et al.*, 1997), ram (Gillian *et al.* 1997) and boar (Maxwell and Johnson, 1997). Furthermore, spermatozoa were confirmed to be functionally capacitated by cooling and cryopreservation, as reflected by their ability to fertilize homologous oocytes *in vitro* without any pretreatment of cooled sperm, normally required for fresh semen (Cormier *et al*, 1997; Fuller and Whittingham, 1997). Cormier *et al.* (1997) hypothesized that commercial cryocapacitation procedures including equilibration for 4 h predisposed bovine spermatozoa to undergo premature capacitation. Once capacitated, spermatozoa exhibit elevated metabolic rates, increased membrane fluidity and permeability, and if they do not achieve fertilization, they undergo spontaneous acrosome reaction due to uncontrolled influx of Ca^{2+}. This cryocapacitation (Bailey *et al.*, 2000; Watson, 1995) reduces the fertilizing life span of capacitated spermatozoa and is thought to be partially responsible for reduced fertility of frozen-thawed bull semen (Cormier and Bailey, 2003). The mechanism by which this cryocapacitation occurs is not fully understood and hence needs to be elucidated.

2.6.1 Minimization of cryocapacitation

Certain compounds are known to preserve structural and functional integrity of biological membranes during freezing and dehydration (Mazur, 1970). Many of these compounds are found at high concentrations in a wide variety of organisms that survive osmotic stress, severe dehydration, and low temperature stress. Indeed these agents are now being considered to have an important role in the molecular biology of osmo-regulation and the ability of certain organisms to survive low intracellular water activities (Crowe *et al.*, 1984). The spermatozoa plasma membrane lipids respond to temperature changes by alterations in their physical phase state. Although regions of fluid and gel phase lipids coexist at physiological temperatures, reduction of temperature favours fluid to gel transitions; the presence of sterols is to inhibit these phase changes (Holt, 2000). As spermatozoa are not adapted to undergo the temperature changes involved in cryopreservation, they can not modify their lipid content to suit the environmental conditions.

This useful strategy of lipids is widely used in nature to compensate for the change in ionic permeability and enzyme activity, which result from phase transitions (Hazel, 1995). Trehalose is found in a number of plants and animals that can retard dehydration freezing

(Crowe and Clegg, 1987) by forming hydrogen bonds with the polar head groups of phospholipids and helps in preventing fusion events of juxtaposed membranes (Anchordoguy *et al.,* 1987; Crowe *et al.,* 1984).

Improvement in post-thaw motility of spermatozoa and better fertility of sheep inseminated with diluents containing glycine has been reported (Sanchez-Partida, *et al.,* 1992) who had shown that glycine enhances the quality of cryopreserved ram as well as equine spermatozoa (Koshinen *et al.,* 1989), but its effects on bovine spermatozoa have not been studied.

Rudolph *et al.* (1986) reported that the three membrane stabilizing agents, proline betaine, and trehalose interacts with phospholipids by increasing the area of these molecules in phospholipid monolayers, fluidizing bilayers of small unilamellar vesicles, and reducing the co-operativity of the gel to liquid phase transition in bulk solution.

The events associated with cryocapacitation were reversed by chelating the calcium with EGTA and by adding epididymal and seminal plasma (Harkema *et al.,* 1998). Similar reversibility was also observed in spermatozoa agglutination when agglutinated spermatozoa were exposed to EGTA and to epididymal and seminal plasma (Harayama *et al.,* 1994). The concentration of unbound calcium in the cell could be made very low by introducing calcium specific chelators such as EGTA which specifically binds Ca^{2+} with high affinity and lower the free Ca^{2+} level.

Studies on preservations of unfrozen semen revealed that controlling of oxidation by exogenous antioxidants in the extender such as catalase, greatly helped to maintain spermatozoa quality (Foote, 1970). Excessive production of ROS was also associated with a reduction in the motility and fertilizing capacity of stored spermatozoa which could be improved by the addition of antioxidants such as super oxide dismutase and catalase to semen diluents (Stojanov *et al.,* 1994).

Butylated hydroxytoluence (BHT), a free radical scavenger known to interact with biological membranes with their fluidity and phase transition behaviour, has been shown to decrease the permeability of bovine spermatozoa membranes (Hammersted and Andrews, 1997). Suleiman *et al.,* (1996) concluded that vitamin E seems to protect against the loss of

the spermatozoa motility by lipid peroxidation. Supplementation of vitamin E improved spermatozoa motility and increased the possibility of fertilization.

2.7 Zona binding assay

Successful fertilization is known to involve several sequential steps. These are: oviduct spermatozoa reservoir, hyper activation and capacitation in the female reproductive tract, binding of the capacitated spermatozoa to the zona pellucida (ZP), induction of spermatozoa acrosome reaction, penetration of ZP, and fusion of sperm with the vitelline membrane of the oocyte. These vital steps in fertilization form the basis for development of assay for the goal of predicating the fertility of semen. Binding of sperm to ZP is a prerequisite for sperm penetration through the ZP and its fusion with oolemma.

Sperm-ZP recognition and the binding are the earliest stages in the interaction between spermatozoa and oocyte. Fazeli *et al*. (1993) recommended the use of such assay for routine evaluation of bovine semen. Brahmkshri *et al*. (1999) further stressed upon the use of this bioassay as a test of fertilizing potential.

Several variation of fertilization assay has been used. One variation is to use zona-pellucida free hamster oocytes instead of homologous oocytes (Floreman *et al.,* 1989). A homologous oocyte with intact ZP would test the fertilizing ability of spermatozoa under more physiological conditions. Sinowatz *et al*. (2003) investigated zona binding among bovine and porcine oocyte with equine spermatozoa under *in vitro* conditions and compared with homologous zona binding in bovines. Their results indicated insignificant differences in zona binding between heterologous and homologous assays. Cox *et al.,* (1994) compared efficiency of goat spermatozoa to penetrate cattle and sheep intact oocytes. They reported that goat spermatozoa were highly efficient in penetrating cattle and sheep oocytes as compared with homologous fertilization, and that goat spermatozoa penetrated equally well cattle and sheep oocytes.

The relative number of sperm cells bound to the zona pellucida of oocytes in two bulls has correlation with their 56 day non-return of 73.2 and 67.5 per cent respectively (Fazeli *et al*., 1993). Kadirvel (2006) reported zona binding ability of *in vitro* capacitated and cryopreserved buffalo spermatozoa as 115.8 ± 20.8 and 53.8 ± 14.5 in fresh and frozen thawed spermatozoa respectively. Exposure of spermatozoa from cauda epididymis to accessory sex gland fluid resulted in direct increase in zona binding ability, followed by a further increase during capacitation *in*

vitro. Incubation of spermatozoa with heparin or oviductal fluid increased the ability of spermatozoa to bind to ZP (Topper *et al.,* 1999). The outcome of the *in vitro* fertilization system differs among the bulls (Shi *et al*., 1991). In some studies, significant correlations between embryo cleavage and field fertility have been demonstrated (Shamsuddin *et al.,* 1993), not in others (Schneider *et al.,* 1996). Harshan *et al.,* (2006) reported that treatment with PDC-109 increased the number of spermatozoa bound to ova than the control group at the pre-freeze stage. Arangasamy (2003) reported an increase in the number of buffalo cauda epididymal spermatozoa bound to ZP when treated with either HBP or gelatin binding seminal plasma proteins. Fazeli *et al.*,(1993) reported that binding ability of the frozen-thawed bull spermatozoa significantly reduced as compared with that of control ($p<0.05$) or *in vitro* capacitated spermatozoa ($p<0.01$). They pointed out a negative correlation between acrosomal damage and zona binding ability of spermatozoa after freezing-thawing. Similarly reduced binding ability to the zona pellucida after freezing and thawing in dog (Strom *et al.,* 2000), in boar (Fazeli *et al.*,1999) and in human (Cormier and Bailey, 2003) was also reported.

3 Materials and Methods

The proposed study was conducted at the Germ Plasm Centre (GPC), Indian Veterinary Research Institute, Izatnagar, Bareilly (UP). The institute is located at an altitude of 564 feet above the mean sea level, at a latitude of 28° North and a longitude of 79° East. Bareilly has a subtropical climate and experience duel extremes of hot and cold weather with the relative humidity ranging between 15 to 85 per cent in different months of the year.

Chemicals

The chemicals used in this study are listed below with their source in parenthesis.

Acetic acid (glacial), bromophenol blue, EDTA, HCI, methanol, isopropanol, glycine, acrylamide, bisacrylamide, agarose, sodium lauryl sulphate (SDS), Coomassie brilliant blue R-250 and TEMED (Merck, India), protein molecular markers were procured from Bangalore Genei. All other chemicals used in the experiments were procured from Sigma Aldrich, USA.

Sterilization of articles

For all the experimental purposes including semen collection and processing (exceptions if any have been indicated in the body of experiments), all the glass wares were washed thoroughly with soap water, rinsed twice in double distilled water and then dipped in 10 per cent HCl for 12 h. Thereafter; glasswares were washed vigorously with tap water, rinsed twice in double distilled water, dried and sterilized in a hot air oven at 160° C for 1 h.

Autoclaving at 10 Ibs pressure (110° C) was done for buffer solutions (20 min), rubber articles and artificial vagina (10 minutes). The stains, polyvinyl alcohol and other polyethylene articles were exposed to ultraviolet rays for one hour before use.

For protein isolation work, glass column was cleaned thoroughly with soap water, tap water, rinsed with distilled water and dried in oven. Thereafter glass column was filled with chromic acid and allowed to stand for 2 h, chromic acid was removed and the column was cleaned thoroughly with tap water followed by distilled water.

3.1 Isolation of seminal plasma protein PDC-109 from crossbred bull semen

3.1.1 Experimental animals and collection of semen

Crossbred (F x J x H) bulls maintained at the Germ Plasm Centre of I.V.R.I. were used for the collection of the semen. These bulls were reared under the identical feeding and managemental conditions during the entire duration of the study.

Semen was collected during morning h using artificial vagina as per the standard practice. The sample was immediately supplemented with a protease inhibitor cocktail used at the manufacturer's recommended concentrations (P 8340, Sigma Aldrich, USA). For the sake of uniformity only those ejaculates that had a mass activity of +3 and above (on a scale of 0 to +5) and individual motility of 70 per cent or above were selected for further processing.

3.1.2 Selection of ejaculates

Ejaculates were selected based on the basis of mass activity as well as individual motility.

The mass activity of the semen sample was determined by assessing the motility of the spermatozoa just after semen collection. It was observed under the low power of microscope without cover slip and was graded on the scale of 0 to +5. The semen samples showing mass activity of +3 or above were only utilized for experimental work.

Individual motility was recorded as percentage of progressively motile spermatozoa in a semen sample (Salisbury *et al*. 1985). This was assessed by placing a drop of the diluted semen on a clean, grease free glass slide mounted on a stage maintained at 37^{o}C and observed under high power magnification (400X). The semen sample was extended so that approximately 15 to 20 spermatozoa were visible under the visual field of microscope. Samples having individual motility of 70 per cent or above were selected for the study (Salisbury *et al.*, 1985).

3.1.3 Processing of seminal plasma and storage

Each ejaculate was centrifuged at 4000 g for 20 min to remove the suspended spermatozoa and particulate debris. The cell pellet was discarded and supernatant plasma was collected. It was further centrifuged at 10000 g for 60 min at 5°C to clear the seminal plasma and was stored at -20°C until analysis.

3.1.4 Total protein estimation

Total protein content of seminal plasma was estimated as per the standard procedure described by Lowry *et al.* (1951).

Method

Standard Bmvine Serum Albumin (BSA) containing 10 to 50 µg of solution was pipetted in five different test tubes (in duplicate). Volume of each tube was made up to 0.5 ml with double distilled water (DDW). In another test tube, 0.5 ml of DDW was taken as blank solution. 0.5 ml of diluted sample containing approximately 20 to 40 µg protein was taken in a separate tube as test solution. To this 2.5 ml of reagent C (Annexure) was added and mixed well. This was allowed to stand for 10 min. at room temperature. Thereafter 0.25 ml of reagent D (Annexure) was added to each test tube and mixed immediately. 30 min. after development of bluish colour, absorbance was read at 750 nm in spectrophotometer. A standard curve was drawn by plotting protein concentration against absorbance and values of protein in unknown samples were calculated from standard curve.

3.1.5 Isolation of PDC-109

PDC-109 was isolated as per the method described by Gasset *et al.* (1997) with minor modification. The method consists of progressive purification of seminal plasma proteins by using Heparin-Sepharose affinity chromatography followed by DEAE-Sephadex ion-exchange chromatography.

Method

Clean glass column (height/circumference, 11/7 cm; Vensil, India) was marked at 5 ml level and Heparin-Sepharose (HS) media was loaded into the glass column upto the mark. Activation or regeneration of HS column was carried out by passing 10 ml each of citrate

buffer solution containing100 mM citrate buffer, (pH 5.5) 1 M NaCl, 0.025% sodium azide and Tris-HCl solution containing 100 mM Tris-HCl (pH 8.8), 1 M NaCl and 0.025% sodium azide. At least 100 ml of each buffer was passed alternately. Thereafter column was equilibrated with 100ml of solution containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 2mM PMSF and 0.025% sodium azide (equilibration buffer). Clear seminal plasma (0.5 ml) was diluted three times with the equilibration buffer and loaded into the equilibrated column. Once the sample entered the HS media, the flow was stopped for 15 min to allow proteins to bind. The column was extensively (7 to 8 times) washed with equilibration buffer to remove unabsorbed proteins. The adsorbed heparin binding proteins (HBP) were eluted in 3 ml fractions with elution buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 100 mM choline chloride and 0.025% sodium azide. The column was washed thoroughly with equilibration buffer for storage of used HS media. Finally storage buffer (0.5 M NaCl and 0.05% sodium azide) was passed through the column and it was stored in storage buffer at 5^{o} C.

The optical density (OD) of eluted HBP fractions was observed in spectrophotometer at 280 nm. Selected fractions were analyzed for protein content by the standard procedure given by Lowry *et al.* (1951). The fractions containing proteins were pooled and dialyzed against solution containing 50 mM Tris-HCl (pH 7.4), 150mM NaCl, 5 mM EDTA, and 0.025% sodium azide and stored at -20^{o} C till further purification and isolation of PDC-109.

The fraction used for further purification to obtain PDC-109 was concentrated by dialyzing against solution containing 20mM Tris-HCl (pH 6.5) and 1M NaCl. Thereafter previously cleaned glass column was marked at 5ml level and DEAE-Sephadex (DS) media was loaded up to the mark. Activation of DS was done by washing the column successively with 100 ml each of 0.4 M Tris-HCl (pH 6.5) with 4M NaCl ; 0.2 M Tris-HCl (pH 6.5) with 4M NaCl and 0.1M Tris-HCl (pH 6.5) with 2 M NaCl. The column was equilibrated by passing 100 ml of 20 mM Tris-HCl (pH 6.5) with 1M NaCl. The dialyzed protein sample was then loaded (sample volume was less than 5 per cent of the bed volume) into the DS column. Once the sample entered the DS media, the flow was stopped for 15 min to allow desired proteins to bind. The column was washed extensively with equilibration buffer to remove the unadsorbed proteins. The elution of bound PDC-109 was carried out using 20mM Tris-HCl (pH 6.5), 1M NaCl with 100 mM choline chloride in 3 ml fractions. The OD of eluted protein

fractions (3 ml) were observed in spectrophotometer at 280 nm. Pooled fractions were analyzed for protein content by the standard procedure given by Lowry *et al*. (1951) and thereafter dialyzed against Tris-Citrate buffer (Annexure) fortified with antibiotics to remove NaCl and to balance the pH. The protein thus isolated was identified against protein molecular weight markers by SDS-PAGE. The dialyzed protein was used to raise antisera in male rabbit.

3.1.6 Identification of purified protein

The isolated and purified protein was analyzed by discontinuous Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) as described by Laemmli (1970).

Method

a. Gel preparation

For discontinuous SDS-PAGE, glass plates with 1.5 mm spacers were assembled in gel casting mould. The comb was inserted between the plates. The notched plate was marked 1cm below the comb teeth for stacking gel. Before removing comb, each well is marked serially. The 15 per cent resolving gel solution (Annexure) was poured between glass plates. The gel surface was immediately layered with a small amount of water or isopropanol and allowed to polymerize for about half an hour. After the polymerization of resolving gel was complete, the water above the gel was decanted and the gel surface was rinsed twice with distilled water. The stacking gel solutions (Annexure) were prepared and poured on to the separating gel. Comb was inserted between the plates immediately and the gel was allowed to polymerize for an hour. After the polymerization of the stacking gel, the comb was removed carefully and the wells were rinsed with water. The plates with the gel were then carefully taken out of the mould and put in electrophoresis apparatus. Electrode buffer (Annexure) was poured in upper and lower tanks of the apparatus and it was connected to the power supply pack (Pharmacia, Model EPS 500/400).

b. Sample preparation and electrophoretic run

Protein samples were prepared by mixing protein solution (at least 150 to 200 µg) with sample buffer (Annexure, 4:1) and 5 per cent mercaptoethanol. The processed protein samples were kept in water bath at 60 to 70^{o} C for about 5 min and loaded into the wells using Hamilton syringe. Protein molecular weight marker (Fermentas, India) was also loaded into first well.

The electrophoresis was carried out at room temperature in constant voltage mode at 50 volts till the dye entered the resolving gel, thereafter electrophoresis was carried out at 100 volts. The power supply was disconnected after the dye front reached the bottom of the gel. The gel plates were prised apart and the gel was carefully removed by repeated soaking in distilled water. The gel was put in fixing solution (Annexure) for 1 to 2 h. After removing the gels from fixing solution the gel was stained with Coomassie Brilliant Blue R-250 for 1 h. It was then destained using the destaining solution (Annexure) and the gels were stored in 7 per cent acetic acid.

3.2 Raising of hyper immune sera

Four adult (aged 1 to 1.5 years, weighing 1.5 to 2.0 kg) NewZealand White male rabbits were used for raising anti-sera against purified PDC-109. Pre immune blood was collected from all the four rabbits just before immunization, sera was separated and stored at -20^{o} C for further use. For primary immunization 100 µg of PDC-109 was mixed with an equal volume of Freund's complete adjuvant (FCA) to form an emulsion. Emulsification was checked by placing a drop of the mixture on the surface of water. Each rabbits were injected subcutaneously with 100 µg of PDC-109 in FCA immediately after blood was collected to obtain pre immune serum. First booster dose, containing 100 µg of PDC-109 emulsified with equal amount of Freund's incomplete adjuvant (FIA) was given subcutaneously at 2 weeks interval whereas second booster was given on day 21 followed by third booster on 28th day. Rabbits were bled five days after third booster to assess the level of antibody produced in the serum. For this about 5.0 ml blood was collected, serum separated and immuno-diffusion test was performed to assess the presence of 'antibodies against PDC-109'. When the precipitating line turned dark then about 15 ml rabbit blood was collected by puncturing heart with the help of 20 ml syringe and hypodermic needle. Blood was kept undisturbed in a wide mouth test tube to allow clotting. The clotted blood was brought to laboratory under ice and kept at 4^{o}C for 1 h. The serum was separated by centrifugation of the clotted blood at 1500 g for 10 min. Sera were mixed with 5 ppm sodium azide and 0.5ml aliquots were made. These aliquots were stored at -20^{o}C till IgG rich fractions (antibodies against PDC-109) were isolated from it.

3.2.1 Immuno diffusion test

To assess the presence of antibodies (IgG) against PDC-109 in raised hyper immune sera, immune-diffusion test was carried out as described by Colligon *et al*. (1992).

Method

The procedure consisted of taking cleaned (wiped with absolute alcohol) glass slide and placing them on a horizontal surface. A solution of 1 per cent agar in PBS (Annexure) was dissolved by boiling it in conical flask. About 4.5 ml of melted agar in PBS was placed over each glass slide (forming a thickness of 1.5 mm) very carefully so that it does not flow out of slide and allowed to solidify. Once it was solidified, holes were punched out in a concentric manner using hub of a needle and excess agar was removed from wells using needle. Thereafter central well was filled with PDC-109 solution (100 µg in 1 ml of PBS) and surrounding wells were filled with serially diluted hyper immune sera (1:2 to 1:16 in PBS). This procedure was repeated for each rabbit (and each collection) separately. These plates were then incubated in humid boxes at 37^{o} C for 48 to 72 h. The precipitation lines developed at varying time interval for each rabbit which indicated presence of hyper immune sera (IgG) against protein (PDC-109). These gels were dried on the glass plates after staining with Coomassie brilliant blue R-250 solution (Annexure) and stored as permanent record.

3.2.2 Checkerboard assay (ELISA)

Checkerboard assay was carried out to determine titre of the antibody raised against PDC-109.

Antigen

Purified PDC-109 was taken in concentrations ranging from 50 to 400 ng in coating buffer (Annexure) for determining the most suitable antigen concentration for ELISA.

Antisera

The antisera raised in rabbit against PDC-109 were used in two-fold dilutions ranging from 1:50 to 1: 25600. Two columns of well were used as control, which consisted of pre-immune sera. The serum dilutions were made in PBS (Annexure, pH 7.4) containing 0.05 per cent Tween-20 (PBST).

Method

The antigen (PDC-109) was dissolved in coating buffer (Annexure). To each well of a microtitre assay plate about 100 µl optimally diluted antigen was coated and incubated in a humid chamber overnight at 4°C. The plates were washed thrice to remove unbound antigen using PBST (Annexure) and the wells were filled with 3 per cent BSA fraction V in washing buffer (Annexure) to block any remaining protein binding sites by incubating at 37°C for 2 h. The plates were washed thrice with washing buffer. Antisera raised against PDC-109 (primary antibody) were diluted and each well was filled with 100 µl of antibody solution in the aforesaid dilutions. The plates were incubated in humid chamber at 37°C for 1 h. The plates were again washed thrice with washing buffer. To each well 100 µl of 1:10,000 diluted goat anti-rabbit IgG HRPO conjugate was added and plates were further incubated at 37°C for 1 h in humid chamber. After incubation plates were washed thrice with washing buffer. 100 µl of freshly prepared substrate solution (Annexure) was added in each well and plates were kept in dark at room temperature for 20 to 30 min for the color development. The reaction was stopped by adding 100 µl of sulphuric acid (1N) and the absorbance was measured at 492 nm using Multiscan EX, Labsystems ELISA reader apparatus.

3.2.3 Western blot of seminal plasma protein PDC-109

Enzyme linked immuno transfer blot was done as per Towbin *et al.* (1979) to detect the specificity of the raised antisera. The proteins were resolved on 15 per cent SDS-PAGE. Proteins on the gel were transferred electrophoretically onto nitrocellulose membrane (NCM, Sigma Aldrich, USA) using blotting apparatus (ATTO, Japan) as per protocol described below.

Method

After electrophoresis, the gel was taken off from the plates and kept in transfer buffer (Annexure) to remove excess SDS from the gels. Eight to Ten Whatman No. 3 filter papers of a size little larger than gel were stacked one by one on the anode plate after soaking in the transfer buffer. Care was taken to avoid air bubbles in between stacks. Membrane kept pre wet in transfer buffer and was placed over the paper stacks. Then the gel was placed, making sure to avoid air bubbles. Orientation of the membrane was marked before stacking filter

papers (Whatman filter paper no. 3) exactly of gel size over the gel after soaking them in transfer buffer. The complete stack was saturated with ice-cold transfer buffer before the cathode plate was placed in position over the stack and a current of 3 to 5 mA/cm^2 was applied for 3 h. After transfer, the gel was stained to check the efficiency of transfer and the membrane was subjected to immunological detection.

Development of blot

The membrane, after transfer, was incubated overnight in 5 per cent (w/v) skim milk in PBS-T (PBS containing 0.2 per cent Tween 20), pH 7.4 for blocking the nonspecific binding sites. After blocking, the membrane was washed thrice with PBS-T (5 min each) and incubated with 1:400 diluted rabbit anti PDC-109 serum at 37^{o}C for 2 h.

Thereafter, the membrane was washed thrice with PBS-T as described above and incubated for 1 h at 37^{o}C with 1:5000 diluted goat anti rabbit HRPO conjugate (Sigma, USA).After washing, the protein antibody reaction was detected by incubating the membrane with substrate (Annexure). The colour reaction was terminated by washing the membrane with distilled water to prevent background coloration.

3.3 Isolation of 'antibody against PDC-109'

The antisera collected from immunized male rabbits showing dark precipitation lines at immunodiffusion test were selected for isolation of antibody (IgG rich fraction) against PDC-109. The ion exchange chromatography procedure as described by Colligon *et al*. (1992) was employed to purify the 'antibody against PDC-109'.

Method

10 ml of hyper-immune sera was added in 20 ml of saturated ammonium sulphate solution (34 per cent, SAS) slowly by continuously stirring at 4° C for 2 h. The solution was centrifuged at 9000 g for 10 min to collect precipitate. This precipitate was dialyzed overnight against 0.01M phosphate buffer (pH 8.0) at 4^{o}C. The pre cleaned glass columns were mounted on the stand and were marked at 20 cm height. The DEAE-Sephadex media (Sigma Aldrich, USA) was poured till the mark. The packed column was equilibrated with 0.01M phosphate buffer (pH 8.0) at room temperature. The previously dialyzed solution was applied on to the

top of equilibrated column. When flow rate of 2 ml/5min. decreased, top of the column bed (DS media) was stirred lightly. The column was washed in two steps. Column containing DS media was washed with 3 bed volumes (35 ml, first washing) of 0.01M phosphate buffer (pH 8.0) until all the unretained proteins comes out which was monitored by measuring absorbance of the fraction (5 ml each) at 280 nm. Afterwards fractionation on a column was done using a 0.03M phosphate buffer containing 100 mM NaCl (second washing). The OD of the eluted 5 ml fractions were then observed in spectrophotometer at 280 nm and selected peaks were analyzed by 15 per cent SDS-PAGE. The protein content was estimated as described earlier.

3.3.1 Lyophilization and addition of 'antibodies against PDC-109'

Five bulls used in the present study had an average concentration of 21.7 mg/ml of PDC-109 with average ejaculate volume of 3.14 ml. IgG rich fraction (antibodies against PDC-109) was added in the ejaculates at the ratio of 1:1 with PDC-109 to bind and sequester the protein from the ejaculates.

After estimation of concentration of 'antibodies against PDC-109' (IgG-rich) in pooled eluted fractions, 3.14 ml elute containing 21.7 mg/ml of 'antibodies against PDC-109' was transferred in graduated test tubes. These tubes were then subjected to vacuum lyophilization to reduce the volume. This resulted in each test tube containing 68.14 mg of 'antibodies against PDC-109' in lyophilized form. These test tubes were kept at -20º C until used for collection of ejaculates in treatment groups (gr.3 and 4).

3.4 Sequestration of PDC-109 from the ejaculate

Proposed experiment was conducted in four groups as mentioned below.

Group 1 : Semen collected and diluted in Egg Yolk Tris Glycerol (EYTG) extender as per routine procedure.

Group 2 : Direct collection of ejaculate in EYTG extender.

Group 3 : Direct collection of ejaculate in test tubes coated with, 'antibodies against PDC-109'.

Group 4 : Semen collection as per routine procedure and diluted in extender without egg yolk but containing 'antibodies against PDC-109'.

After collection of ejaculates in each group, it was processed further for cryopreservation following routine procedure.

3.4.1 Physico-morphological characteristics

In addition to volume, mass activity and individual motility, semen samples from all four groups were subjected to following physico-morphological assessment.

a. Spermatozoa concentration

The concentration of spermatozoa (millions/ml) in semen samples was determined by using haemocytometer (Salisbury *et al.,* 1985).

b. Individual Motility

Individual motility was assessed as described earlier for fresh semen sample.

c. Per cent live Spermatozoa

The per cent live spermatozoa was determined by adopting differential staining technique using Eosin-Nigrosin stain as described by Lasley and Bogart, (1942). A drop of semen was taken on a clean, grease free pre warmed glass slide and 4 to 5 drops of Eosin-Nigrosin stain was added. This was mixed gently using a blunt fine glass rod. After 30 sec to 1 min a thin smear was made on another clean, grease free glass slide. The smear was dried in air and examined under oil immersion objective. A total of 200 spermatozoa were counted in each slide. The stained and partially stained spermatozoa were considered as dead.

d. Hypo-osmotic swelling (HOS) response

HOS response was assessed as described by Jeyendran *et al*. (1984) for human spermatozoa.

Method

Briefly, solution A & solution B were prepared. Solution A contained 0.734 g sodium citrate where as solution B contained 1.351 g fructose in 100 ml of distilled water. 0.5 ml of each solution was taken in a clean, pre warmed (37^{o} C) test tube. In this tube (containing hypo-osmotic solution of 150 mOsm/L) 0.1 ml of semen was added and mixed well. The sperm suspension was incubated in water bath at 37^{o}C for 45 min. After incubation a drop of

eosin-Y solution was added. A small drop of the suspension from the bottom of the tube was placed on a clean, grease free glass slide and covered with cover slip. The slides were examined under the high power magnification (400 X) of a phase contrast microscope. A minimum of 200 spermatozoa were counted and the different types of tail swelling pattern were recorded. Similar procedure was followed for the semen samples at pre-freeze and frozen-thawed stage of both control and treatment groups.

Spermatozoa were classified as per cent HOS responsive according to the presence of the tail swelling patterns (Prasad *et al*., 1999) as mentioned below.

Pattern A: No swelling-complete loss of membrane integrity.

Pattern B: Swelling at the tip of the tail

Pattern C: Different types of hair-pin like swelling

Pattern D: Complete tail swelling.

The sperm cells displaying the swelling pattern B, C and D were considered positive for the HOST (HOST reactive), where as pattern A was considered as HOST negative (HOST non-reactive).

e. Acrosomal integrity by Giemsa stain

Acrosomal integrity of fresh, pre-freeze and post-thaw spermatozoa was assessed using Giemsa stain (Watson, 1975).

Method

A smear of diluted semen sample was prepared on a clean, grease free glass slide and air-dried. This was dipped in Hancock's fixative (Annexure) for 15 min for fixation of smear. Fixed smear was washed in slow running water (10 minutes), rinsed with distilled water and air-dried. The smear was stained in Giemsa working solution (Annexure) for 3 to 4 h. Slide was then removed from the stain solution and rinsed quickly in distilled water, air dried and mounted in DPX mountant. The slide was examined under oil immersion objective of the microscope to assess acrosomal integrity. At least 200 spermatozoa were counted and categorized as acrosome intact or acrosome reacted in each sample and percentage calculated.

f. Acrosome reacted live spermatozoa by FITC-PSA stain

The acrosomal status of spermatozoa in semen samples was also assessed using fluorescein labeled lectin from the peanut plant, *Arachis hypogaea* (FITC-PSA) using a slightly modified version of the procedure described by Sukardi *et al*. (1997).

Method

Modification included use of PBS instead of HEPES buffer and removal of excess PI by diluting the contents several fold and centrifugation instead of filtration of the solution.

Preparation of reagents

1. PI solution 500 µg/ml: 20 mg of PI dissolved in 40.0 ml of PBS.
2. FITC-PSA 100 µg/ml: 1mg of FITC-PSA dissolved in 10.0 ml of PBS.

100 µl of semen sample was taken in a microcentrifuge tube and volume made up to 1.0 ml with PBS (Annexure). The sample was washed twice by centrifugation at 170 g for 10 min Supernatant was removed and the final volume was made up to 100 µl with PBS to which 2.0 µl of PI solution was added to get a final concentration of 10 µg/ml PI in the sperm suspension. The spermatozoa were allowed to interact with PI exactly for 2 min. The excess PI was removed by adding 10 fold volume of PBS and centrifuged gently at 800 g for 5 min. The supernatant was removed and final volume was again adjusted to 100 µl with PBS. From this 20 µL of diluted semen suspension was smeared on clean grease free glass slide in duplicate and dried. Spermatozoa were permeabilized by flooding the slide with 100% methanol for 5 min. Excess methanol was removed by washing the slides with PBS. Permeabilized slides were then flooded with FITC-PSA working solution (40 µg FITC PSA/ml) in PBS. The slides were kept in dark chamber at 37^{o} C for half an hour. Excess FITC-PSA was removed by rinsing the slides with PBS. A drop of anti fade solution of 0.22M 1, 4-diazo-bicyclo (2, 2, 2) octane was placed on the stained smears in order to preserve fluorescence. A cover slip was placed, pressed and edges sealed with color less nail varnish. Slides were examined within two hours under the fluorescence (Nikon Microphot FXA EPI-FL3; Japan) with FITC filter set at 40 x magnification. A total of 200 spermatozoa were counted and categorized as follows:

PSA positive and PI negative : Acrosome intact live (AIL)

PSA positive and PI positive : Acrosome intact dead (AID)

PSA negative and PI negative: Acrosome reacted live (ARL)

PSA negative and PI positive : Acrosome reacted dead (ARD)

PSA positive sperm showed green to yellowish green fluorescence. PI positive spermatozoa showed red colored nuclear material, indicating damaged membrane, as intact membrane is impermeable to PI. Cells which retained staining of the equatorial segment were considered fully acrosome reacted as these cells were considered totally devoid of PSA staining. The PI-positive cells were excluded from the estimate of acrosome intact and acrosome reacted live spermatozoa.

g. Washing of spermatozoa

Washing of spermatozoa was necessitated for estimation of cholesterol content of spermatozoa and *in vitro* capacitation and acrosome reaction.

Immediately after evaluation, fresh and frozen thawed spermatozoa were washed using Percoll density gradient (Strom *et al*. 2000) to remove egg yolk particles, dead cells and debris.

Method

One ml layer of 40 per cent Percoll (V/V, Sigma Aldrich, USA) in non capacitating medium (NCM , Annexure)) was pipetted carefully over a 1 ml layer of 80 per cent Percoll (V/V in NCM) in a disposable 15 ml centrifuge tube. One ml fresh or thawed semen was gently layered on top of the two steps Percoll column. This test tube was centrifuged at 400 g for 30 min. After centrifugation, the pellets were washed once again with NCM and resuspended in NCM to make 100 million spermatozoa per ml immediately after collection, at pre-freeze and after freezing-thawing (post-thaw). This procedure was followed to make an aliquot of 1 ml (in duplicate) of all the four groups in cryovials and stored at -20^{o}C till used for *in vitro* assays *viz*. cholesterol estimation and CTC assay.

h. Cholesterol assay

The cholesterol content in spermatozoa was estimated as per the method of Bligh and Dyer (1959) described below:

Method

A total of 100 million washed spermatozoa were taken in a 10 ml vial. The sperm pellet was extracted with 20 volume of chloroform: Methanol (1:1, V/V) solution and vertexed for 20 sec. There after it was centrifuged at 800 g for 5 min. The spermatozoa was evaporated to dryness under liquid Nitrogen gas and kept at -20°C. At the time of estimation, 0.5 ml of chloroform was added to each vial and cholesterol was estimated by cholesterol assay kit (Span Diagnostics Ltd, India).

In a test tube marked 'blank' 1000 µl of reagent 1 (Annexure) whereas in another tube marked 'standard' 1000 µl of reagent 1 and 10 µl of reagent 2 (Annexure) was added. In third tube marked 'test' 1000 µl of reagent 1 was mixed with 10 µl of washed spermatozoa sample. All three test tubes were incubated at 37° C for 10 min and O.D. was observed for blank, standard and test at 505 nm in spectrophotometer. Cholesterol content of spermatozoa was calculated by application of formula as below:

Cholesterol concentration = O.D. (Test)/O.D. (Standard) x 200

i. *In vitro* capacitation and acrosome reaction

The CTC method was used to determine spermatozoa capacitation was slightly modified from Fraser *et al.* (1995). The procedure adopted was as follows

Method

The CTC solution (Annexure) for determination of capacitation and acrosome reaction was prepared daily and pH adjusted to 7.8. The solution was protected from light and maintained at 4° C till use. 10 µl of spermatozoa suspension was mixed with equal volume of CTC solution on a previously cleaned grease free slide at room temperature. After five seconds, 1.5 ml of gluteraldehyde (12.5 per cent v/v in 20 mM Tris-HCl, pH 7.4) was added to the samples. Finally a drop of 0.22 M, 1, 4- diaza-bicyclo (2, 2, 2) octane dissolved in glycerol: Phosphate-buffered saline (9:1) was added to retard the fading of CTC fluorescence. The slides were covered with cover slips and examined on the same day. The CTC fluorescence was observed with a Nikon microscope equipped with phase contrast and epifluorescent optics. All samples were processed in duplicate and the experiment was replicated six times. At least 200

spermatozoa per slide were examined and classified according to one of the three staining pattern as described by Fraser *et al.* (1995).

Pattern F : Uniform bright fluorescence over the whole head, characteristic of non capacitated cells

Pattern B : Fluorescence-free band in post-acrosomal region (capacitated cells)-.

Pattern AR : Dull fluorescence over the whole head except for a thin punctate band of fluorescence along the equatorial segment (acrosome reacted cells)

No fluorescence was observed when CTC was omitted from the preparation.

3.4.2 Pre freeze and post-thaw evaluation

Immediately at the end of equilibration period (at pre-freeze stage) and at post-thaw stage, semen straws were thawed at 37° C for 40 sec. in water bath and viability (live per cent), individual motility, HOS response, acrosomal integrity, cholesterol efflux, and *in vitro* capacitation and acrosome reaction was assessed. Similarly, above parameters as well as heterologous zona binding were assessed at post-thaw stage.

Zona binding assay

Spermatozoa of all the four groups were subjected to heterologous (buffalo oocyte) zona binding assay after freezing-thawing (post-thaw). The method of Fazeli *et al*. (1993) was followed.

Method

Collection of ovaries

Ovaries from sexually mature buffaloes were collected in sterile and chilled normal saline solution (Annexure) supplemented with antibiotics from local abattoir just after slaughter and transferred to the laboratory. The ovaries were washed 6 to 8 times with NSS fortified with antibiotics.

Oocyte collection

Follicular fluid from surface follicle (>2 mm) of buffalo ovaries were collected by aspiration method with 18 gauge needle attached to a 5 ml syringe containing oocyte collection

medium (OCM, Annexure). The collected cumulus oocyte complexes (COC) were poured in a 50 ml centrifuge tube and kept undisturbed for half an hour in a CO_2 incubator at 39°C, 5% CO_2 and 95% humidity to allow the COC to settle. The supernatant was decanted and the sediments containing the COC were poured into large square searching Petri dish containing OCM.

Oocytes having compact, multilayered, cumulus oocyte complex (COCs) and evenly granulated cytoplasm were selected under a stereo-zoom microscope and transferred to another petri dish containing OCM. The selected COCs were washed 6 to 7 times with OCM and finally 4 to 5 washings in oocyte maturation media (OMM, Annexure) were carried out.

***In vitro* maturation of oocytes**

The COCs were cultured in 50-100 µl (10-12 oocytes/drop) of maturation media under mineral oil in petri dishes. They were incubated at 39°C, 5% CO_2 and 95% humidity for 24 h. The ova were assessed for maturation after 24 h under stereo zoom microscope based on their expanded cumulus cell mass (Liu *et al*., 1991).

Sperm capacitation and *in vitro* insemination of matured oocytes

Capacitation of spermatozoa was carried out by placing them in eppendorf tube, covered with mineral oil in 5% CO_2 and 95% humidity using modified TALP (Annexure) as capacitation medium. Thawed semen was washed to remove the dead cells using non capacitating medium (NCM). The spermatozoa were then washed twice with the sperm TALP (Annexure) by centrifugation at 170 g for 10 minutes. The spermatozoa suspension was resuspended in fertilization TALP (Annexure) and concentration was adjusted at 5 x 10^7 sperm / ml. The aliquots were placed under mineral oil in eppendorf tube in 5% CO_2 incubator with 95% humidity at 37°C for up to 6 h.

After maturation, oocytes were removed by gentle pipetting with capillary tube and transferred in disc containing fertilization TALP followed by washing 6 to 8 times in fertilization TALP. After washing, 10 to 12 oocytes were placed in 50 µl of fertilization TALP and 20 to30 µl of capacitated sperm was added to it and co-incubated at 39°C, 5% CO_2 and 95% humidity for 18 to 20 h in CO_2 incubator. To remove free and loosely attached spermatozoa to zona pellucida, fresh fertilization TALP was added to droplets 6 to 7 h later for washing and

process was repeated 2 to 3 times. Thereafter droplets containing oocytes and spermatozoa were again transferred back to CO_2 incubator for remaining period of incubation.

Evaluation of sperm zona binding attachment

After 18 to 20 h of incubation, the penetrated oocytes were washed and processed further for counting the number of spermatozoa bound to zona pellucida (binding index) and number of oocyte bound with spermatozoa (binding per cent).

At least 60 oocytes were used to estimate the zona pellucida binding of each group of thawed spermatozoa.

3.5 Statistical analysis

ANOVA was carried with PROC GLM of SAS 9.2 available in Genetics division of Indian Veterinary Research Institute.

4 Results

4.1 Isolation of seminal plasma protein PDC-109 from crossbred bull semen

For isolation of PDC-109 ejaculates showing mass activity +3 or more and individual motility 70 per cent and above were only selected from cross-bred (F x J x H) bulls. A total of 24 ejaculates were collected. The mean volume, mass activity and individual motility were 3.14 ± 0.5 ml, 3.1 ± 0.4 and 78.25 ± 1.79 per cent respectively.

4.2 Protein content

The protein content in seminal plasma was estimated by standard procedure of Lowry *et al.* (1951, Fig. 1).

The average concentration of total protein (TP) was 90.1 ± 5.6 mg/ml whereas concentration of isolated Heparin Binding Protein (HBP) was 27.9 ± 0.6 mg/ml representing about 30.9 per cent of total protein. The average concentration of isolated purified PDC-109 was 21.7 ± 0.42 mg/ml constituting 24.1 per cent of the total protein content of seminal plasma.

4.3 Elution profile of the purified proteins

The elution profile of HBP and PDC-109 has been presented in Fig. 2 and 3**.** It was evident from the results that maximum elution of HBP occurred in fraction 3 and 4, trailing off to minimum by fraction 6, whereas maximum elution of PDC-109 was observed in fraction 2 and 3. Choline chloride at a concentration of 100 mM was found suitable for the elution of PDC-109 from DEAE-Sephadex column.

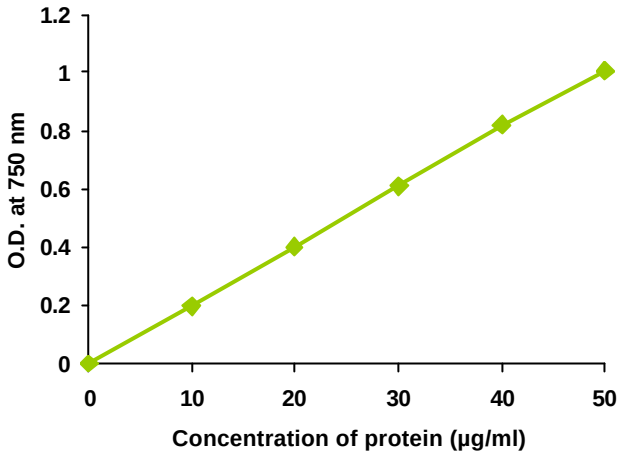


Fig. 1 : Standard curve for protein estimation

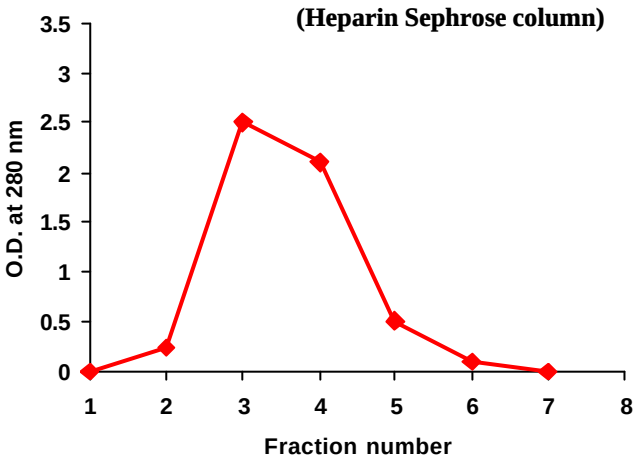


Fig. 2 : Elution curve of HBP of crossbred bull semen using 100 mM choline chloride on affinity chromatography, equilibrated with 150 mM NaCl

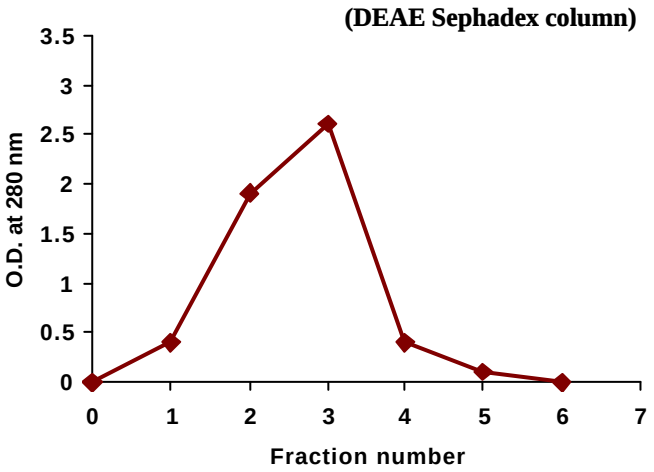


Fig. 3 : Elution curve of PDC-109 from HBP fraction of crossbred bull semen using 100 mM choline chloride on ion exchange chromatography, equilibrated with 1 M NaCl

4.4 Electrophoretic profile of seminal plasma proteins

SDS-PAGE analysis of HBP and purified PDC-109 of seminal plasma was carried out to assess the purity and to characterize the purified proteins.

Heparin Binding Proteins

The protein bands representing BSP-A1/-A2, BSP-A3 and BSP-30 kDa were observed in the SDS-PAGE (i.e. 14.7, 16.5, and 30 kDa). The bands representing BSP-A1 and BSP-A2 were too close to visualize them as individual bands (Fig. 4).

PDC-109

Purified PDC-109 appeared as doublets on the SDS-PAGE representing the two glycosylated forms of protein BSP-A and BSP-A2. They were observed around 14.7 kDa range. (Fig. 5).

4.5 Assessment of 'antibodies against PDC-109'

Purified PDC-109 was injected in four New Zealand White male rabbits for raising antisera. The production of 'antibodies against PDC-109' (IgG-rich fraction) in all four male rabbits was assessed by immunodiffusion asssay. Three out of four male rabbits responded to antigen (PDC-109) by producing antisera indicated by the presence of clear bands in immunodiffusion assay (Fig.6). Serial dilution of hyperimmune sera with PBS (1:16 in rabbit A, 1:4 in rabbit B and C reacted with antigen (100 µg in 1 ml PBS) by producing dark bands.

Evaluation of antibody/antigen titre using ELISA

Checker-board assay was carried out with progressively diluted antibody (raised in rabbits against PDC-109) and antigen samples.

On plotting the graph of obtained ELISA reading (O.D. at 492 nm) against antibody titre or antigen concentration, an antibody titre of 1 in 400 and an antigen concentration of 225 ng were found optimum.

Western blot of seminal plasma proteins

Purified PDC-109 were resolved on SDS-PAGE gel and transferred electrophoretically to Nitro Cellulose Membrane (NCM). The NCM were then probed with antibody raised

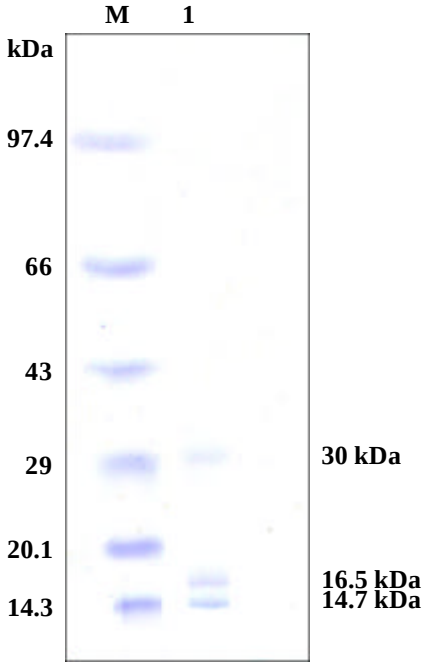


Fig. 4 : **SDS-PAGE of HBP from crossbred bull semen purified by affinity chromatography in 15% gel under reduced condition and stained with Coomassie Brilliant blue G-250**

Lane M : Standard protein marker

Lane 1 : HBP

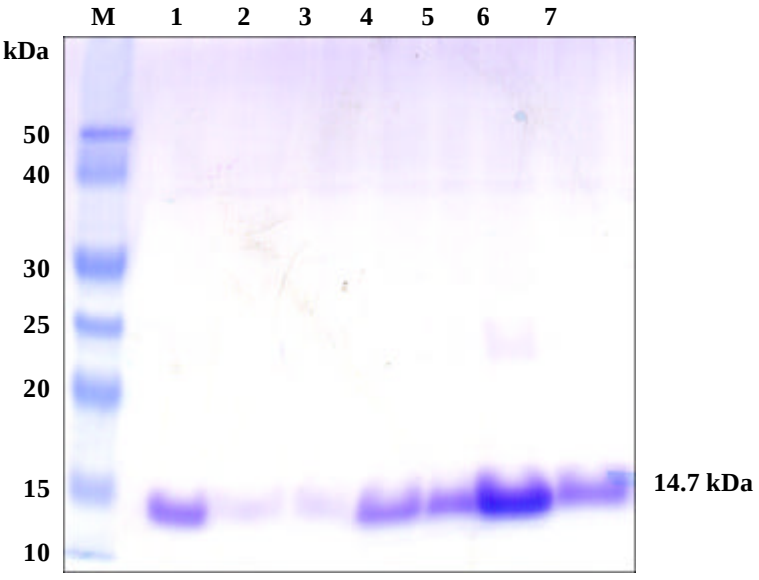
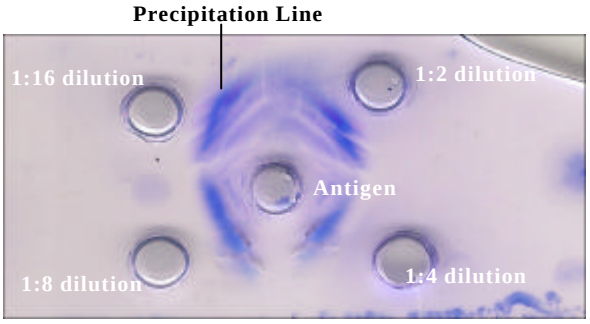


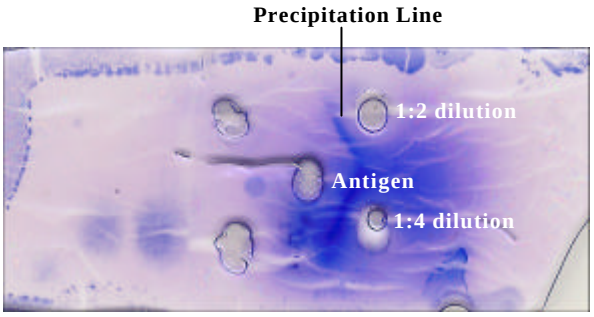
Fig. 5 : **SDS-PAGE of PDC-109 from HBP fraction of crossbred bull semen purified by ion exchange chromatography in 15% gel under reduced condition and stained with Coomassie Brilliant G-250**

Lane M : Standard protein marker

Lane 1-7 : PDC-109 from different bulls



(a) Antisera from rabbit A



(B) Antisera from rabbit B & C

Fig. 6 : Immunodiffusion assay in 1% agar in PBS, precipitation line showing interaction of PDC-109 with its antisera at 1:2 to 1:16 dilutions. Hyperimmune sera diulted 1:2 to 1:16 in PBS.
Antigen 100 µg diluted in 1 ml of PBS.

against PDC-109. The development of a single brown band in the lane examined indicated the specificity of the antisera raised (Fig. 7).

Purification of 'antibodies against PDC-109' from antisera

Isolation of IgG rich fraction from immunized rabbit sera by ammonium sulphate precipitation followed by DEAE ion-exchange chromatography resulted in a pure fraction (first Peak, Fig. 8). Chromatographic pattern of purified rabbit 'antibodies against PDC-109' with phosphate buffer pH 8.0 (first peak) and phosphate buffer + 100 mM NaCl (second peak) is shown in Fig 8.

Concentration of 'antibodies against PDC-109' in eluted fractions

The concentration of 'antibodies against PDC-109' in two pooled eluted fractions (first and second peak) was 15.7 ± 0.3 and 10.5 ± 0.4 mg/ml on protein basis.

SDS-PAGE analysis of 'antibodies against PDC-109'

Figure 9 shows the results of SDS-PAGE for determining the purity of IgG by ammonium sulphate precipitation followed by DEAE ion-exchange chromatography in which a distinct polypeptide band with molecular weight about 50 kDa corresponding to rabbit IgG heavy chains and diffused band, molecular weights of 25 kDa corresponding to rabbit IgG light chains is evident. The SDS-PAGE analysis showed that purification of IgG by ion-exchange chromatography could yield an antibody rich fraction from rabbit hyper immune sera in two peaks.

4.6 Physico-morphological characteristics

Effect of sequestration of PDC-109 on various physico-morphological characteristics in fresh, pre-freeze (at the end of equilibration period) and post-thaw semen has been presented below.

a. Individual motility

The mean value of individual motile spermatozoa in fresh, pre-freeze and post-thaw semen has been presented in Table 1, Fig. 10.

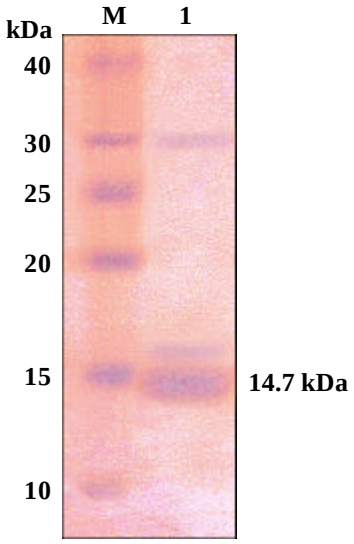


Fig. 7 : Western blot of PDC-109 probed with anti-PDC-109 antisera

Lane M : Standard protein marker

Lane 1 : PDC-109 probed with antisera

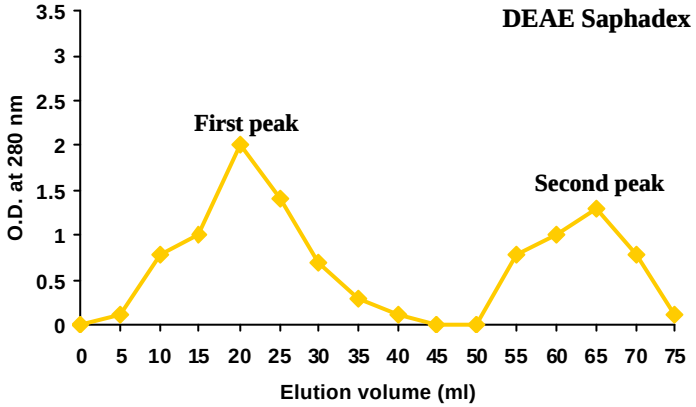


Fig. 8 : Elution curve of purified antibodies against PDC-109' by ion exchange chromatography with phsphate buffer pH 8.0 (first peak) and phosphate buffer + 100 mM NaCl (second peak)

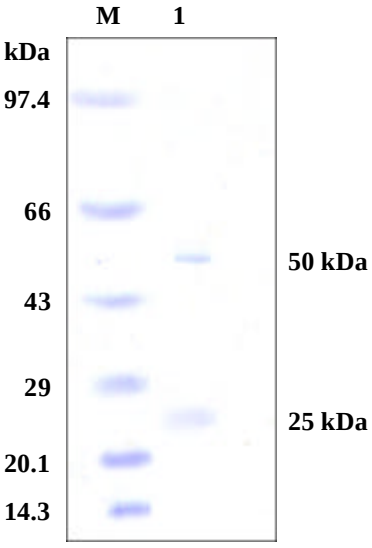


Fig. 9 : SDS-PAGE of IgG rich fraction, purified by ion exchange chromatography in 15% gel under reduced condition and stained with Coomassie Brilliant blue G-250 showing heavy and light chains

Lane M : Standard protein marker

Lane 1 : Antibodies against PDC-109

Table 1 : Mean(±SE) effect of sequestration of PDC-109 on individual motility

Stage of processing	No. of ejaculates (n)	Per cent individual motility			
		Group 1	Group 2	Group 3	Group 4
Fresh	6	78.50±1.83	78.83±1.79	78.17±1.83	77.87±1.80
Pre freeze	6	72.17±1.82[a]	71.0±1.80[a]	75.33±1.80[a]	59.67±1.81[b!]
Post-thaw	6	47.00±1.86[a]	48.67±1.85[a]	56.17±1.83[c!]	40.33±2.93[b*]

Means bearing different superscripts in a row differ significantly (* : $p<0.05$, ! : $p<0.01$)
Per cent decrease from mean value (fresh) 31.5, 30.6, 22.0 and 37.5

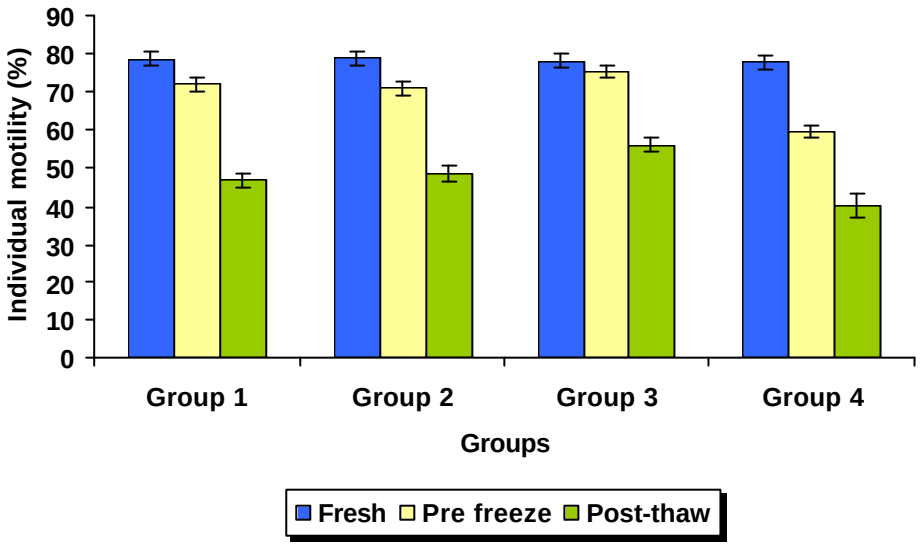


Fig. 10 : Effect of sequestration of PDC-109 on individual motility. The vertical column represent mean±SE

Fresh semen

The mean values were 78.50 ± 1.83, 78.83 ± 1.79, 78.17 ± 1.83 and 77.87 ± 1.80 per cent in group 1, 2, 3 and 4 respectively.

There was no significant difference in per cent individual motility of spermatozoa in different treatment and control groups.

Pre-freeze

The pre-freeze per cent individual motility of spermatozoa was 72.17 ± 1.82, 71.0 ± 1.80, 75.33 ± 1.80 and 59.67±1.81 in group 1, 2, 3 and 4 respectively.

The per cent individual motility of spermatozoa differed significantly ($p<0.01$) in group 4 as compared to control and other treatment groups.

Post-thaw

The mean value of post-thaw individual motility was 47.00 ± 1.86, 48.67 ± 1.85, 56.67 ± 1.83, and 40.33 ± 2.93 per cent in group 1, 2, 3 and 4 respectively.

There was significant difference in group 3 ($p<0.01$) and group 4 ($p<0.05$) compared to group 1 (control) and group 2.

In these groups per cent decrease in motility observed at pre-freeze stage from mean values in fresh semen was 6.33, 7.83, 2.74 and 18.2 in group 1, 2, 3 and 4 respectively, indicating highest decrease in group 4 than other three groups.

Per cent decrease in motility observed in post-thaw semen from pre-freeze values was 25.17, 22.83, 9.16 and 19.34 in groups 1, 2, 3 and 4 respectively, indicating lowest decrease in group 3.

b. Per cent live spermatozoa

The results observed in fresh, pre-freeze and post-thaw semen samples have been presented in Table 2 (Fig. 11 and 12)**.**

Fresh semen

The mean per cent live spermatozoa observed in fresh semen samples were 83.83 ± 2.18, 82.50 ± 2.17, 86.33 ± 2.18 and 81.83 ± 2.16 in group 1, 2, 3 and 4 respectively.

Table 2 : Mean(±SE) effect of sequestration of PDC-109 on live spermatozoa

Stage of processing	No. of ejaculates (n)	Per cent live spermatozoa			
		Group 1	Group 2	Group 3	Group 4
Fresh	6	83.83±2.18	82.50±2.17	86.33±2.18	81.83±2.16
Pre freeze	6	75.17±2.42[a]	75.33±2.18[a]	83.83±2.18[b*]	70.33±2.18[a]
Post-thaw	6	60.33±2.19[a]	61.50±2.19[a]	69.50±2.16[b*]	49.67±2.16[c!]

Means bearing different superscripts in a row differ significantly (* : p<0.05, ! : p<0.01)
Per cent decrease from mean value (fresh) 23.5, 21.0, 16.8 and 32.2

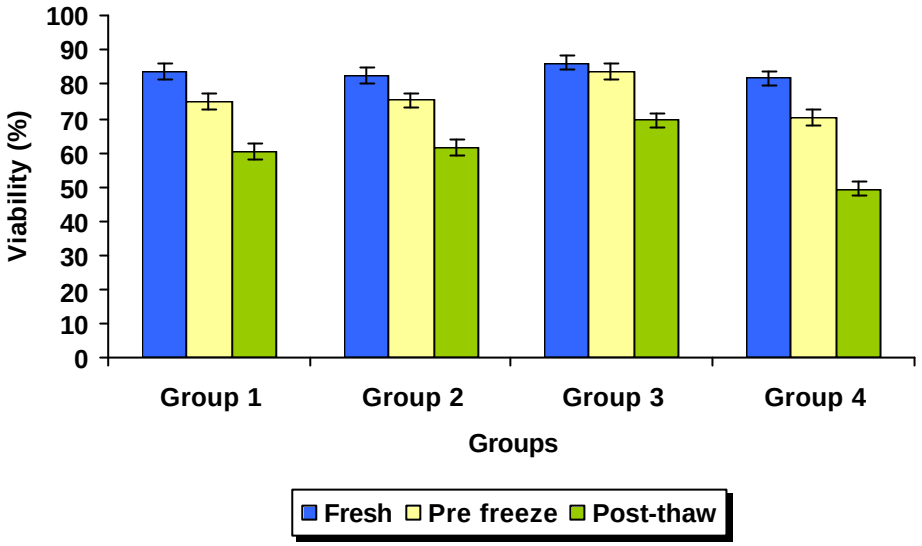


Fig. 11 : Effect of sequestration of PDC-109 on live spermatozoa. The vertical column represent mean±SE

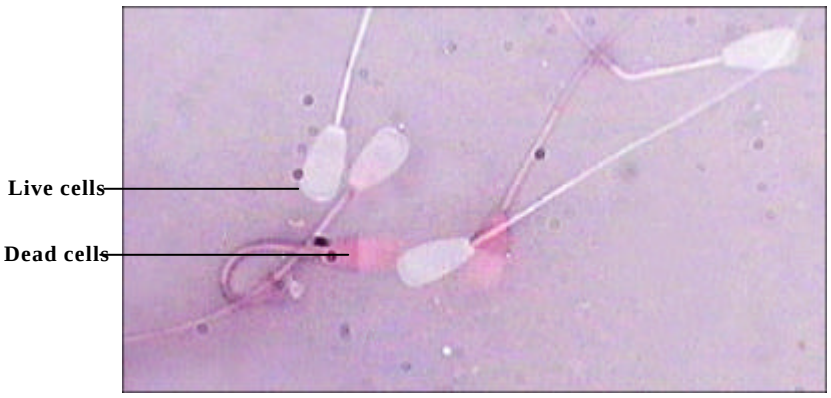


Fig. 12 : Live and dead spermatozoa after staining with eosin-nigrosin

The above results showed higher mean values in group 3 whereas it was comparable in group 2 and 4 with group 1 (control).

Pre-freeze

The mean per cent live spermatozoa at pre-freeze stage were 75.17 ± 2.42, 75.33 ± 2.18, 83.83 ± 2.18 and 70.33 ± 2.18 in group 1, 2, 3 and 4 respectively.

The data presented above showed a significant ($p<0.05$) difference in per cent viable spermatozoa in group 3 as compared to control as well as other two treatment groups whereas no difference was observed in group 2 and 4 than control.

Post-thaw

The mean post-thaw per cent live spermatozoa were 60.33 ± 2.19, 61.50 ± 2.19, 69.50 ± 2.16 and 49.67 ± 2.16 in group 1, 2, 3 and 4 respectively.

The values differed significantly in group 3 ($p<0.05$) as well as in group 4 and ($p<0.01$) compared to control. However it was similar in group 2 and group 1.

In four groups per cent decrease in viability observed at pre-freeze stage from mean values in fresh semen was 8.66, 7.17, 2.5 and 11.5 in group 1, 2, 3 and 4 respectively, indicating lowest decrease in group 3 and highest in group 4.

Per cent decrease in viability observed in post-thaw semen from pre-freeze value was 14.84, 13.83, 14.33 and 20.66 in groups 1, 2, 3 and 4 respectively, indicating highest decrease in group 4.

c. Hypo osmotic swelling response

The mean values of HOS responsive spermatozoa in fresh, pre-freeze and post-thaw semen samples has been shown in Table 3 and depicted in Fig. 13 and 14.

Fresh semen

The mean per cent HOS responsive spermatozoa was 77.17 ± 1.75, 77.5 ± 1.62, 83.33 ± 1.71, and 76.83 ± 1.69 in group 1, 2, 3 and 4 respectively.

There was no significant difference in HOS responsive spermatozoa in different groups.

Table 3 : Mean(±SE) effect of sequestration of PDC-109 on HOS response of spermatozoa

Stage of processing	No. of ejaculates (n)	Per cent HOS response			
		Group 1	Group 2	Group 3	Group 4
Fresh	6	77.17±1.75	77.5±1.62	83.33±1.71	76.83±1.69
Pre freeze	6	67.5±1.75	68.17±1.75	78.83±1.69	63.33±1.72
Post-thaw	6	58.50±1.32[a]	60.5±1.75[a]	68.67±1.62[b*]	47.50±1.92[c!]

Means bearing different superscripts in a row differ significantly (* : $p<0.05$, ! : $p<0.01$)
Per cent decrease from mean value (fresh) 18.68, 17.0, 14.7 and 29.3

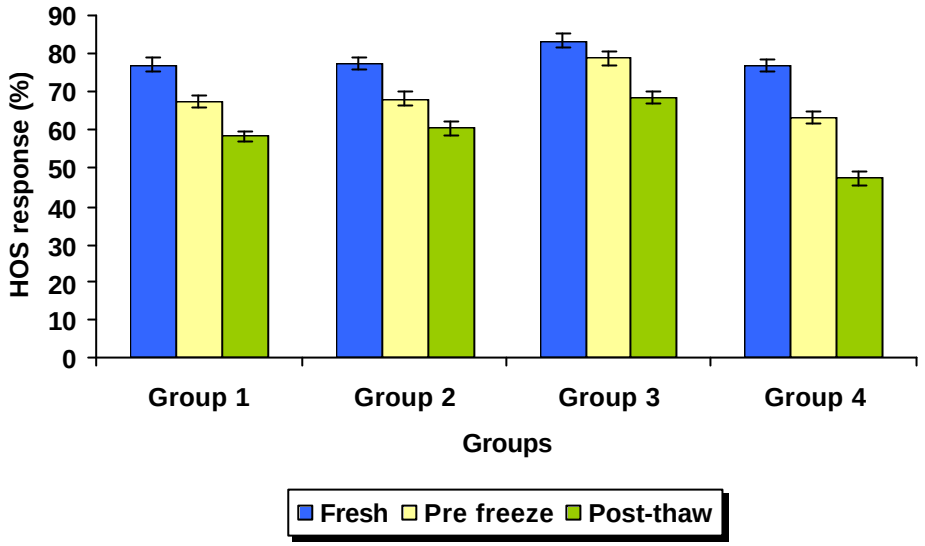


Fig. 13 : Effect of sequestration of PDC-109 on HOS response. The vertical column represent mean±SE

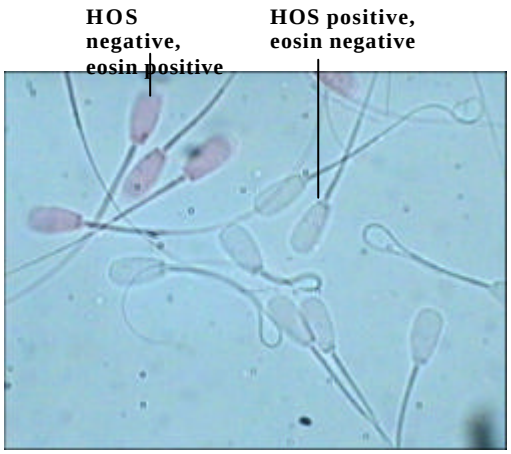


Fig. 14a : Spermatozoa showing HOS response

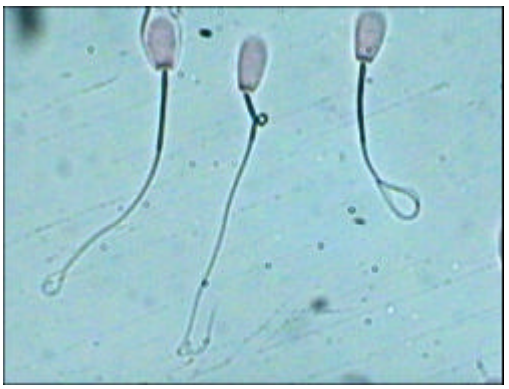


Fig. 14b : Spermatozoa showing various pattern of tail coiling

Pre-freeze

The mean pre-freeze per cent HOS responsive spermatozoa was 67.5 ± 1.75, 68.17 ± 1.75, 78.83 ± 1.69, and 63.33 ± 1.72 in group 1, 2, 3 and 4 respectively.

There was non significant difference in different groups.

Post-thaw

The mean post-thaw per cent HOS responsive spermatozoa was 58.50 ±1.32, 60.5 ± 1.75, 68.67 ± 1.62 and 47.53 ±1.92 in group 1, 2, 3 and 4 respectively.

The HOS response was significantly different in group 3 ($p<0.05$) and group 4 ($p<0.01$) compared to control and group 2.

In these groups per cent decrease in HOS responsive spermatozoa observed at pre-freeze stage from mean values in fresh semen was 9.67, 9.33, 4.5 and 13.5 in groups 1, 2, 3 and 4 respectively, indicating lowest decrease in group 3 than other three groups.

Per cent decrease in HOS response observed in post-thaw semen from pre-freeze values was 9.0, 7.67, 10.67 and 15.83 in groups 1, 2, 3 and 4 respectively, indicating maximum decrease in group 4.

d. Acrosomal integrity by Giemsa stain

The mean values of spermatozoa with intact acrosome in all groups as determined by Giemsa staining in fresh, pre-freeze and post-thaw semen samples (Table 4**,** Fig. 15 and 16) were assessed.

Fresh semen

The per cent spermatozoa with intact acrosome were 84.50 ± 2.38, 78.67 ± 2.31, 85.00 ± 2.40, and 79.00 ± 2.40 in group 1, 2, 3 and 4 respectively.

No significant difference in mean values was observed in different groups.

Pre-freeze

The mean pre-freeze values were 72.83 ± 2.39, 71.67 ± 2.30, 81.33 ± 2.38, and 65.83 ± 2.29 in group 1, 2, 3 and 4 respectively.

Table 4 : Mean(±SE) effect of sequestration of PDC-109 on acrosomal integrity of spermatozoa by Giemsa stain

Stage of processing	No. of ejaculates (n)	Per cent acrosomal integrity			
		Group 1	Group 2	Group 3	Group 4
Fresh	6	84.50±2.38	78.67±2.31	85.00±2.40	79.00±2.40
Pre freeze	6	72.83±2.39^{a}	71.67±2.30^{a}	81.33±2.38^{b*}	65.83±2.29^{a}
Post-thaw	6	61.83±2.1^{a}	60.67±2.38^{a}	75.17±2.38$^{b!}$	42.67±2.31$^{c!}$

Means bearing different superscripts in a row differ significantly (* : $p<0.05$, ! : $p<0.01$)
Per cent decrease from mean value (fresh) 22.7, 18.0, 8.83 and 36.3

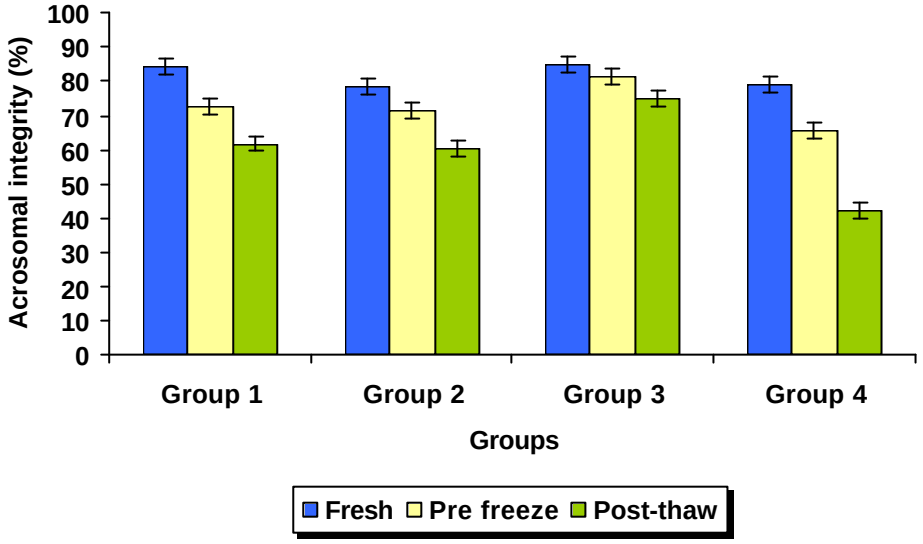


Fig. 15 : Effect of sequestration of PDC-109 on acrosomal integirty of spermatozoa by giemsa stain. The vertical column represent mean±SE

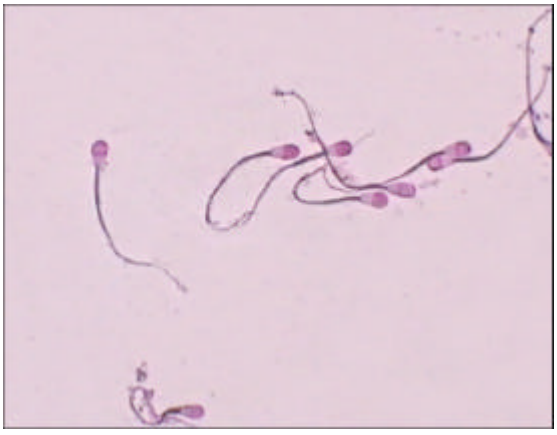


Fig. 16a : Giemsa stained spermatozoa showing acrosome intact and abnormal

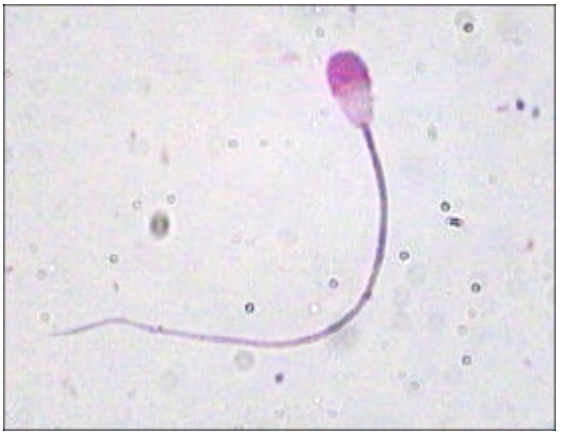


Fig. 16b : Giemsa stained normal spermatozoa

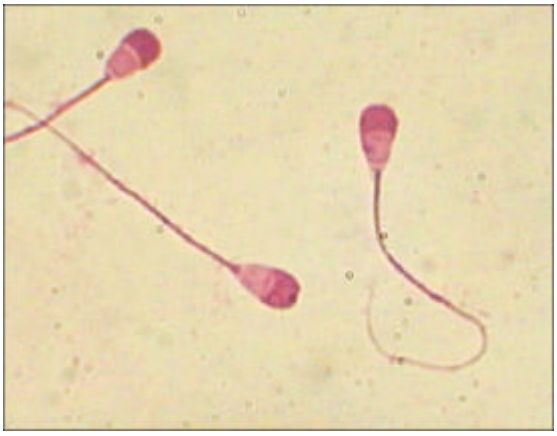


Fig. 16c : Giemsa stained spermatozoa showing pear shape

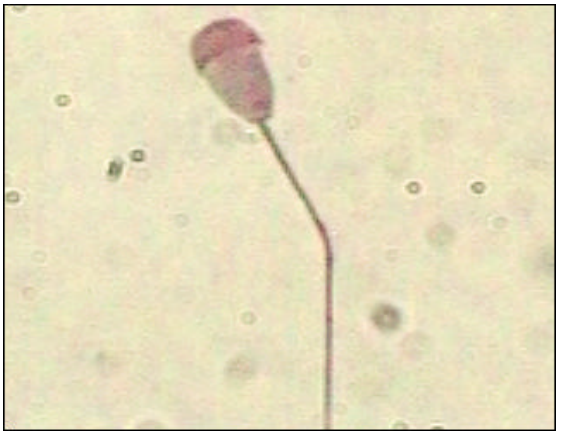


Fig. 16d : Giemsa stained swollen and detached acrosome

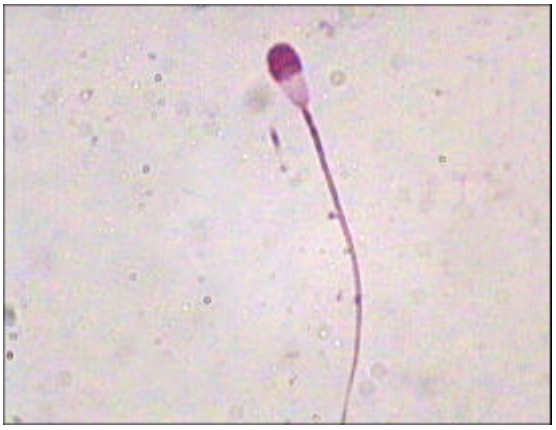


Fig. 16e : Giemsa stained spermatozoa showing elongated head

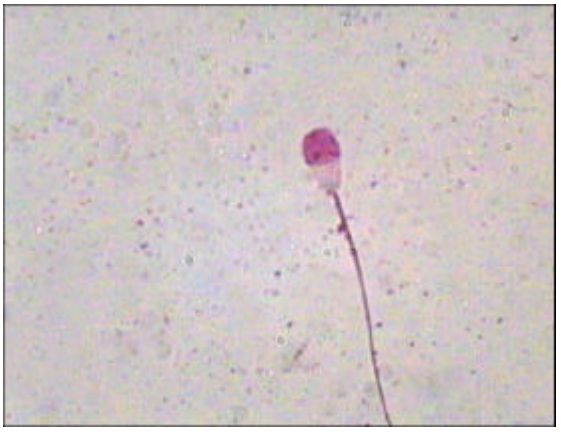


Fig. 16f : Giemsa stained spermatozoa showing swollen head

The per cent spermatozoa with intact acrosome was significantly ($p<0.05$) different in group 3 when compared to group 1 (control) and 2.

Post-thaw

The mean post-thaw value for spermatozoa with intact acrosome was 61.83 ± 2.1, 60.67 ± 2.38, 75.17 ± 2.38, and 42.67 ± 2.31 in group 1, 2, 3 and 4 respectively.

The values were significantly different ($p<0.01$) in group 3 as well as in group 4 than group 1 (control).

In four groups decrease in per cent intact acrosome observed at pre-freeze stage from that of fresh semen was 11.67, 7.0, 3.67 and 13.17 in groups 1, 2, 3 and 4 respectively, indicating lowest decrease in group 3 and highest in group 4.

Per cent decrease observed in post-thaw spermatozoa with intact acrosome from pre-freeze values was 11.0, 11.0, 6.16 and 23.16 in groups 1, 2, 3 and 4 respectively, indicating minimum decrease in group 3 and maximum in group 4.

e. Acrosome reacted live spermatozoa by FITC-PSA stain

The mean value of live spermatozoa showing acrosome reaction as assessed by FITC-PSA and PI in fresh, pre-freeze and post-thaw semen samples has been presented in Table 5 and depicted in Fig. 17 and 18.

Fresh semen

The mean per cent live spermatozoa in fresh semen samples were 5.33 ± 2.14, 6.67 ± 2.11, 5.43 ± 2.09 and 5.33 ± 2.11 in group 1, 2, 3 and 4 respectively. No significant difference in mean values was observed in different groups.

Pre-freeze

The mean pre-freeze values were 30.83 ± 2.10, 31.38 ± 2.10, 20.33 ± 2.14 and 27.33 ± 2.20 in group 1,2, 3and 4 respectively.

The mean value of acrosome reacted live spermatozoa was significantly ($p<0.05$) different in group 3 and 4 than control.

Table 5: Mean(±SE) effect of sequestration of PDC-109 on acrosome reacted live spermatozoa by FITC-PSA stain

Stage of processing	No. of ejaculates (n)	Per cent acrosome reacted spermatozoa			
		Group 1	Group 2	Group 3	Group 4
Fresh	6	05.33±2.14	6.67±2.11	5.43±2.09	5.33±2.11
Pre freeze	6	30.83±2.10^{a}	31.38±2.10^{a}	20.33±2.14^{b*}	27.33±2.20^{c*}
Post-thaw	6	43.67±2.11^{a}	44.33±2.11^{a}	30.83±2.10$^{b!}$	36.67±2.30$^{c!}$

Means bearing different superscripts in a row differ significantly (* : $p<0.05$, ! : $p<0.01$)
Per cent increase from mean value (fresh) 36.8, 37.6, 25.4 and 31.4

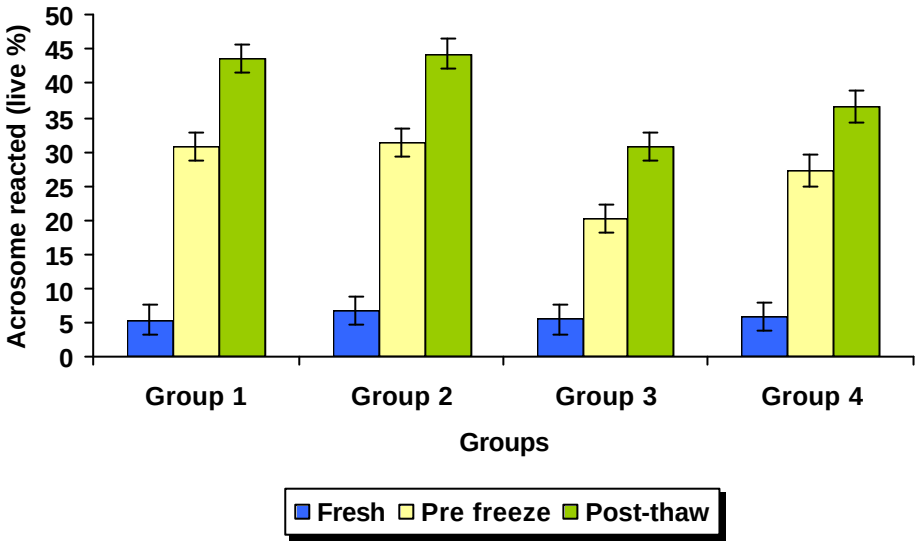


Fig. 17 : Effect of sequestration of PDC-109 on percent acrosome reacted live spermatozoa stained by FITC-PSA + PI. The vertical column represent mean±SE

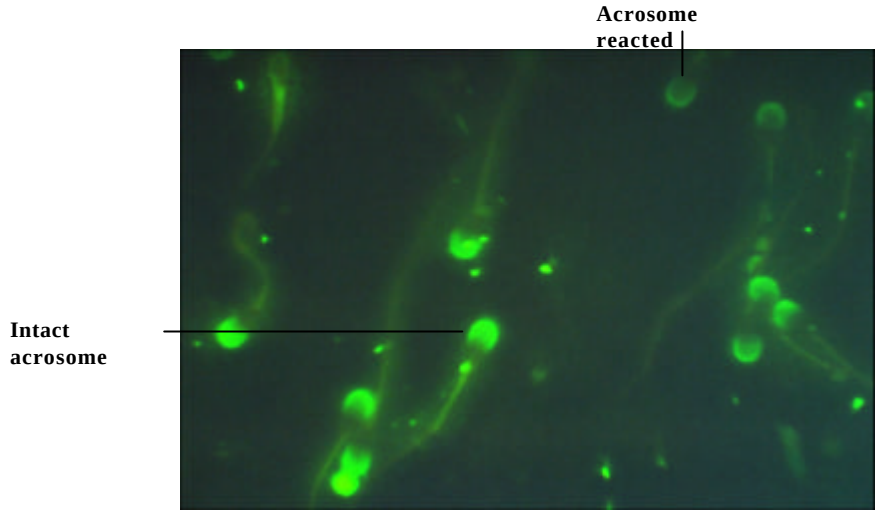


Fig. 18a : Spermatozoa acrosome stained with FITC PSA

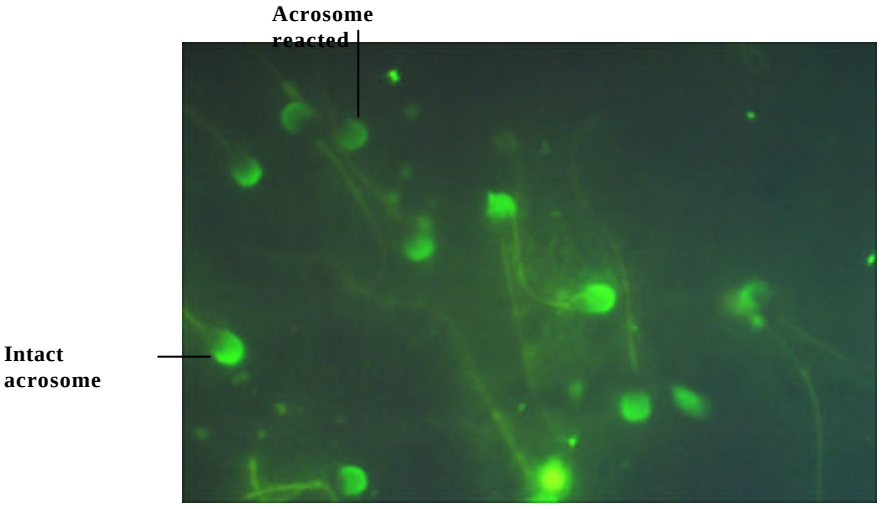


Fig. 18b : Spermatozoa acrosome stained with FITC PSA

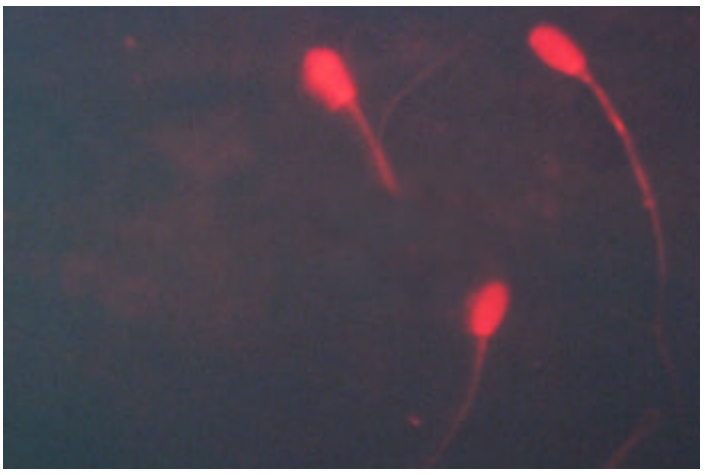


Fig. 18c : Spermatozoa acrosome stained with PI showing dead spermatozoa

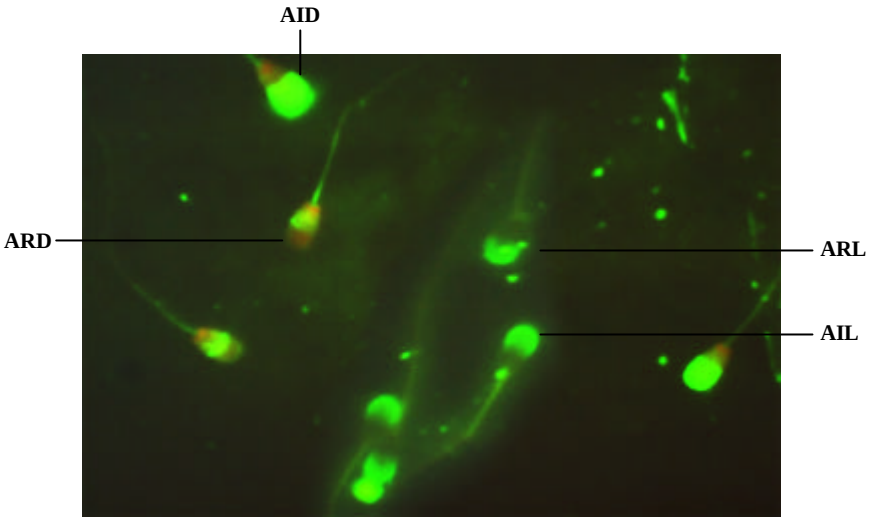


Fig. 18d : Spermatozoa acrosome stained with FITC PSA + PI

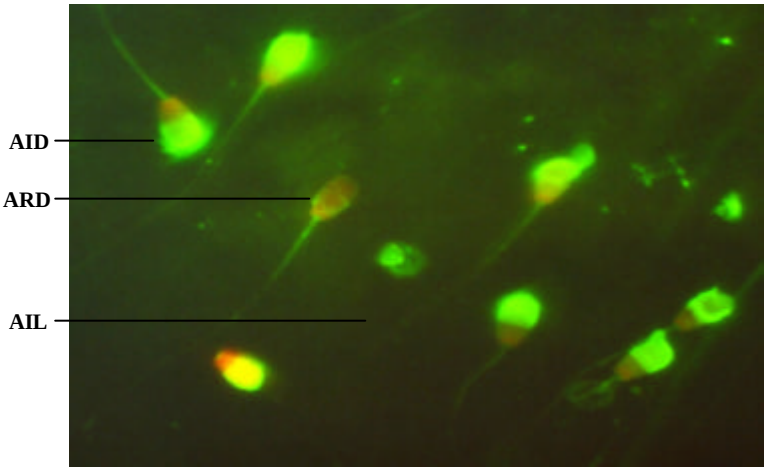


Fig. 18e : Spermatozoa acrosome stained with FITC PSA + PI

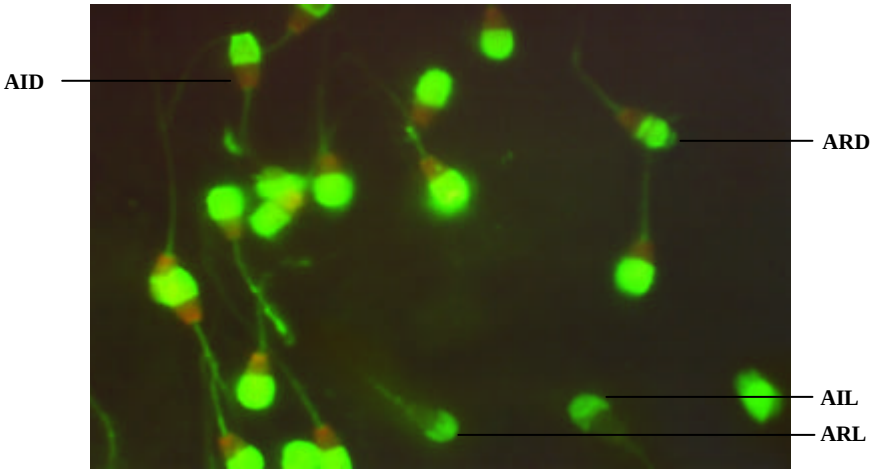


Fig. 18f : Spermatozoa acrosome stained with FITC PSA + PI

Post-thaw

The mean post-thaw values were 43.67 ± 2.11, 44.33 ± 2.11, 30.83 ± 2.10 and 36.67 ± 2.30 in group 1, 2, 3 and 4 respectively.

The mean values differed significantly in group 3 ($p<0.01$) and group 4 ($p<0.05$) than control.

In these groups, per cent increase in mean value of acrosome reacted live spermatozoa at pre-freeze stage from fresh was 25.5, 24.7, 14.9 and 22.0 in group 1, 2, 3 and 4 respectively.

Per cent increase in mean value at post-thaw stage from pre-freeze value 12.8, 12.9, 10.5 and 9.3 in group 1, 2, 3 and 4 respectively. The results indicated minimum increase in acrosome reacted spermatozoa in group 3 than other groups.

f. Cholesterol content of spermatozoa

The mean cholesterol content of spermatozoa (µg/100 million) in fresh, pre-freeze and post-thaw semen samples has been presented in Table 6 and Fig. 19.

Fresh semen

The mean cholesterol content of spermatozoa (µg/100 million) in fresh semen was 23.35 ± 0.93, 20.85 ± 0.91, 23.35 ± 0.93 and 20.85 ± 0.93 in group 1, 2, 3 and 4 respectively.

The mean values in group 2, 3 and 4 showed no significant difference with that of group 1.

Pre-freeze .

The mean pre-freeze cholesterol content was 9.72 ± 0.93, 9.43 ± 0.82, 14.95 ± 0.96, and 8.50 ± 0.82 (µg/100 million spermatozoa) in group 1, group 2, group 3 and group 4 respectively.

A significant ($p<0.05$) difference in cholesterol content of spermatozoa was observed in group 3 compared to group 1 (control), 2 and 4.

Post-thaw

The mean post-thaw cholesterol content of spermatozoa was 7.26 ± 0.75, 7.61 ± 0.93, 11.60 ± 0.89, and 6.93 ± 0.93 (µg/100 million spermatozoa) in group 1, 2, 3 and 4 respectively.

Table 6 : Mean(±SE) effect of sequestration of PDC-109 on cholesterol content of spermatozoa

Stage of processing	No. of ejaculates (n)	Cholesterol content of spermatozoa (µg/100 million)			
		Group 1	Group 2	Group 3	Group 4
Fresh	6	23.35±0.93	20.85±0.91	23.35±0.93	20.85±0.93
Pre freeze	6	9.72±0.93[a]	9.43±0.82[a]	14.95±0.96[b*]	8.50±0.82[a]
Post-thaw	6	7.26±0.75[a]	7.61±0.93[a]	11.60±0.89[b*]	6.93±0.93[a]

Means bearing different superscripts in a row differ significantly (* : $p<0.05$)

Per cent decrease from mean value (fresh) 16.1, 13.2, 11.7 and 13.92

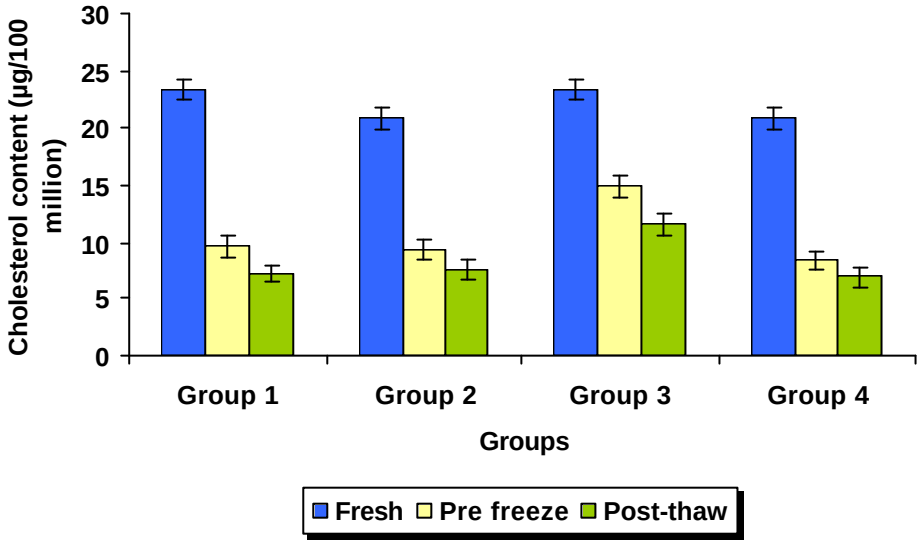


Fig. 19 : Effect of sequestration of PDC-109 on cholesterol content of spermatozoa. The vertical column represent mean±SE

The values were significantly ($p<0.05$) different in group 3 compared to group 1 (control).

Decrease in per cent cholesterol content of spermatozoa at pre-freeze stage from that of fresh semen was 13.63, 11.42, 8.4 and 12.35 in groups 1, 2, 3 and 4 respectively, indicating lowest decrease in group 3.

Post-thaw decrease in per cent cholesterol content of spermatozoa from that of mean value in pre-freeze semen was 2.46, 1.82, 3.35 and 1.57 in groups 1, 2, 3 and 4 respectively.

g. *In vitro* capacitation and acrosome reaction

Pattern F (non capacitated or acrosome intact)

The per cent spermatozoa showing pattern F in fresh, pre-freeze and post-thaw semen samples (Table 7, Fig. 20 and 21) were assessed.

Fresh semen

The mean value was 79.83 ± 1.36, 80.81 ± 1.30, 80.67 ± 1.36, and 80.17 ± 1.29 in group 1, 2, 3 and 4 respectively in fresh semen.

Pre-freeze

The pre-freeze per cent spermatozoa exhibiting pattern F were 28.83 ± 1.31, 29.50 ± 1.36, 59.67 ± 1.31 and 19.00 ± 1.36 in group 1, 2, 3 and 4 respectively.

The results showed a significant difference of mean values in group 3 ($p<0.01$) and group 4 ($p< 0.05$) whereas it was non significant in group 2 than group 1.

Post-thaw

The post-thaw mean value for spermatozoa showing pattern F was 14.83 ± 1.29, 16.83 ± 1.30, 44.50 ± 1.36 and 17.33 ± 1.31 per cent in group 1, 2, 3 and 4 respectively.

The above values showed a significant ($p<0.01$) difference in group 3 compared to group 1 (control).

Spermatozoa showing pattern F in pre-freeze semen revealed a decrease of 51.0, 51.33, 21.0 and 61.17 per cent from mean values in fresh semen in groups 1, 2, 3 and 4 respectively, indicating minimum decrease in group 3 whereas it was highest in group 4.

Table 7 : Mean(±SE) effect of sequestration of PDC-109 on pattern F in CTC stain

Stage of processing	No. of ejaculates (n)	Per cent non capacitated spermatozoa			
		Group 1	Group 2	Group 3	Group 4
Fresh	6	79.83±1.36	80.83±1.30	80.67±1.36	80.17±1.29
Pre freeze	6	28.83±1.31[a]	29.50±1.36[a]	59.67±1.31[b!]	19.00±1.36[c*]
Post-thaw	6	14.83±1.29[a]	16.83±1.30[a]	34.50±1.36[b!]	17.33±1.31[a]

Means bearing different superscripts in a row differ significantly (* : $p<0.05$, ! : $p<0.01$)
Per cent decrease from mean value (fresh) 65.0, 64.0, 36.2 and 62.8

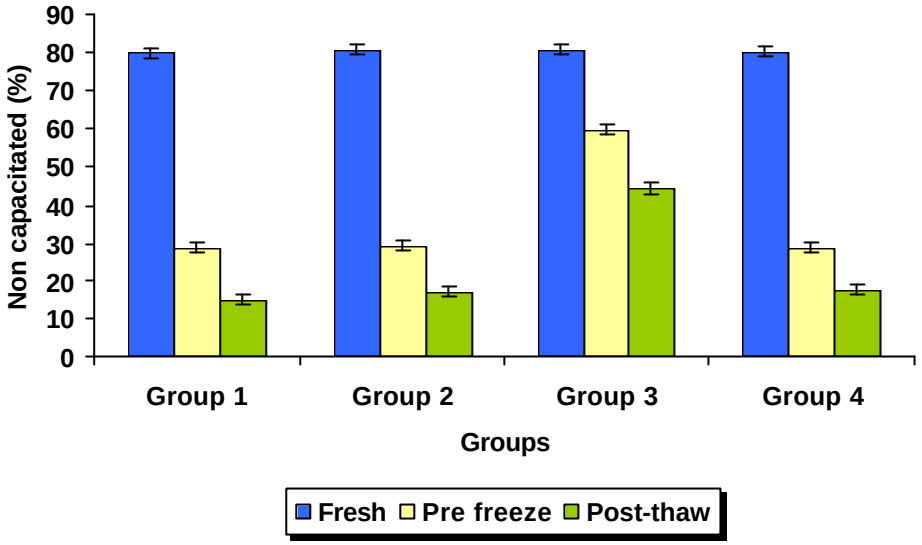


Fig. 20 : Effect of sequestration of PDC-109 on percent non capacitated spermatozoa in CTC stain. The vertical column represent mean±SE

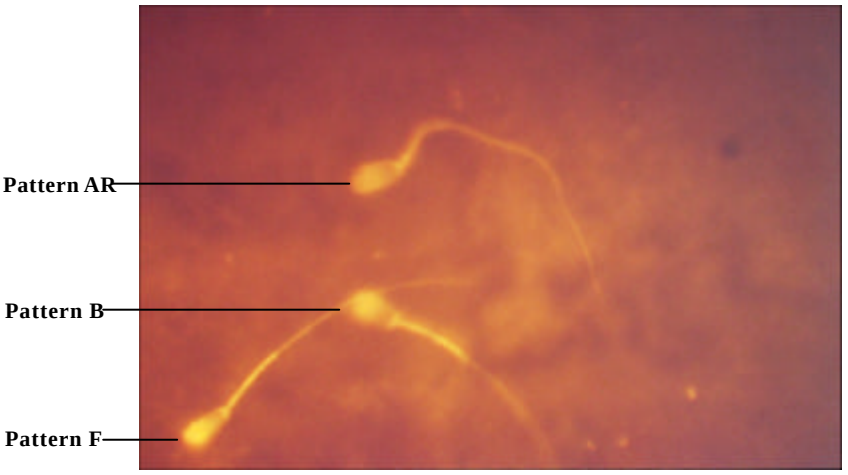


Fig. 21a : Spermatozoa stained with CTC showing non capacitated, capacitated and acrosome reacted head

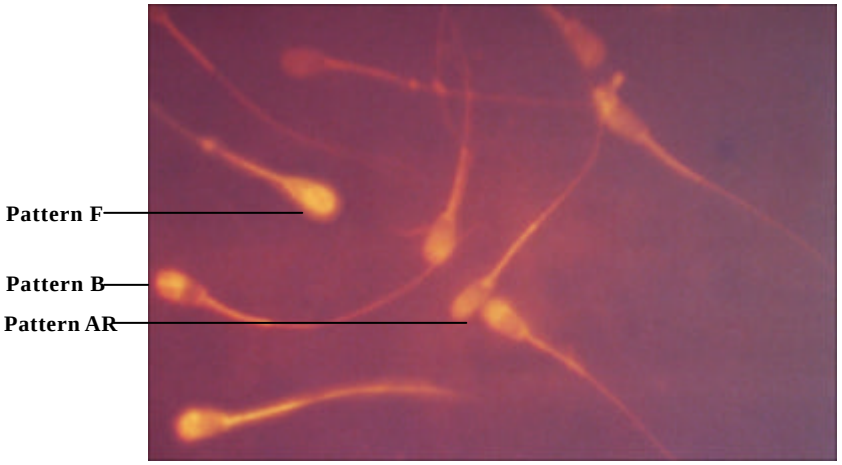


Fig. 21b : Spermatozoa stained with CTC showing non capacitated, capacitated and acrosome reacted head

The mean post-thaw value for pattern F spermatozoa at post-thaw stage showed a decrease of 14.0, 12.67, 15.17 and 1.67 per cent from those of pre-freeze mean values in groups 1, 2, 3 and 4 respectively.

Pattern B (capacitated)

The mean per cent spermatozoa showing pattern B in fresh, pre-freeze and post-thaw semen samples has been presented in Table 8 and Fig. 21 and 22.

Fresh semen

The mean value of spermatozoa exhibiting pattern B was 15.00 ± 1.57, 13.83 ± 1.50, 13.50 ± 1.48 and 14.67 ± 1.42 per cent in group 1, 2, 3 and 4 respectively.

Pre-freeze

The mean pre-freeze value of pattern B spermatozoa was 46.83 ± 1.37, 51.83 ± 1.57, 29.33 ± 1.32 and 50.67 ± 1.67 per cent in group 1, 2, 3 and 4 respectively.

The mean values were significantly ($p<0.01$) different in group 3 than group 1 (control).

Post-thaw

The mean per cent post-thaw spermatozoa showing pattern B was 52.00 ± 1.42, 55.17 ± 1.59, 41.50 ± 2.1 and 58.33 ± 1.9 in group 1, 2, 3 and 4 respectively.

The results above indicated a significant ($p<0.05$) difference in group 3 than group 1 (control).

Pattern B spermatozoa in pre-freeze semen showed a increase of 31.83, 38.0, 15.83 and 36.0 per cent from mean values in fresh semen in groups 1, 2, 3 and 4 respectively, indicating minimum increase in group 3.

The mean value at post-thaw stage showed a increase of 5.17, 3.34, 12.17 and 7.66 per cent from mean values in pre-freeze semen in groups 1, 2, 3 and 4 respectively.

Pattern AR (acrosome reacted)

The mean per cent spermatozoa exhibiting pattern AR in fresh, pre-freeze and post-thaw semen samples have been presented in Table 9 and Fig. 21 and 23.

Table 8 : Mean(±SE) effect of sequestration of PDC-109 on pattern B in CTC stain

Stage of processing	No. of ejaculates (n)	Per cent capacitated spermatozoa			
		Group 1	Group 2	Group 3	Group 4
Fresh	6	15.00±1.57	13.83±1.50	13.50±1.48	14.67±1.42
Pre freeze	6	46.83±1.37[a]	51.83±1.57[a]	29.33±1.32[b!]	50.67±1.67[a]
Post-thaw	6	52.00±1.42[a]	55.17±1.59[a]	41.50±2.1[b*]	58.33±1.9[a]

Means bearing different superscripts in a row differ significantly (* : $p<0.05$, ! : $p<0.01$)
Per cent increase from mean value (fresh) 37.0, 41.3, 28.0 and 43.66

Table 9 : Mean(±SE) effect of sequestration of PDC-109 on pattern AR in CTC stain

Stage of processing	No. of ejaculates (n)	Per cent acrosome reacted spermatozoa			
		Group 1	Group 2	Group 3	Group 4
Fresh	6	5.17±1.74	5.33±1.52	5.83±1.68	5.16±1.70
Pre freeze	6	24.33±1.36^{a}	18.67±1.90^{a}	11.00±1.74$^{b!}$	30.33±1.79^{a*}
Post-thaw	6	33.17±1.84^{a}	28.00±1.72^{a}	24.00±1.74^{b*}	24.33±1.80^{a}

Means bearing different superscripts in a row differ significantly (* : $p<0.05$, ! : $p<0.01$)
Per cent increase from mean value (fresh) 28.0, 22.7, 8.17 and 19.2

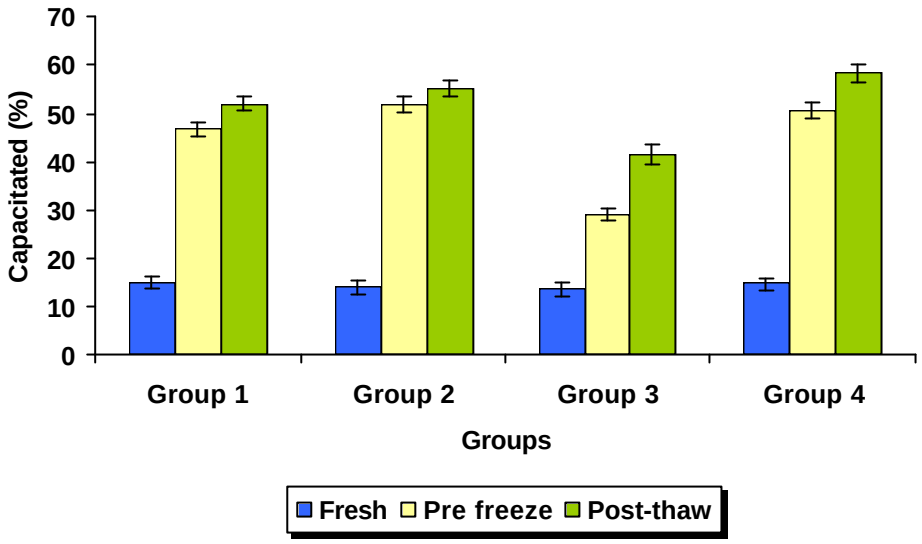


Fig. 22 : Effect of sequestration of PDC-109 on percent capacitated spermatozoa in CTC stain. The vertical column represent mean±SE

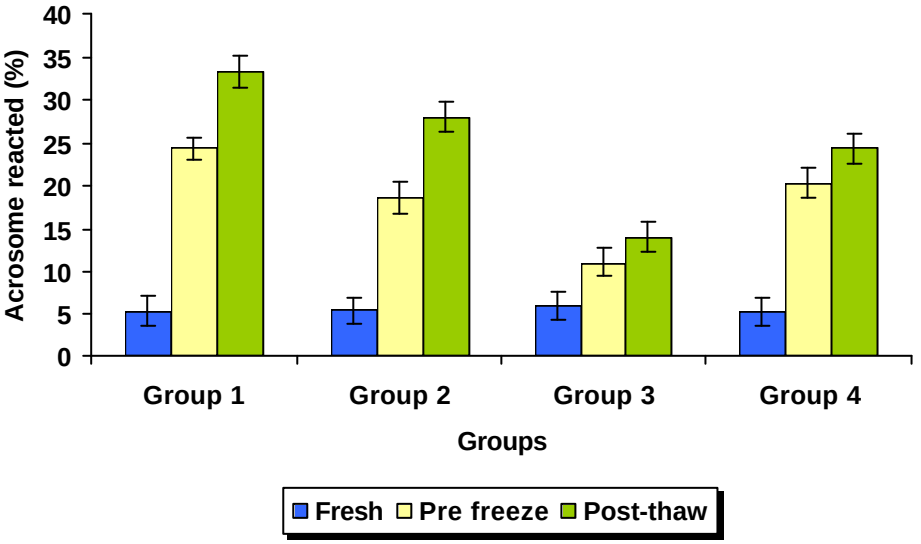


Fig. 23 : Effect of sequestration of PDC-109 on percent acrosome reacted spermatozoa in CTC stain. The vertical column represent mean±SE

Fresh semen

The per cent mean value of pattern AR spermatozoa was 5.17 ± 1.74, 5.33 ± 1.52, 5.83 ± 1.68, and 5.16 ± 1.70 in group 1, 2, 3 and 4 respectively.

Pre-freeze

The mean per cent spermatozoa exhibiting pattern AR was 24.33 ± 1.36, 18.67 ± 1.90, 11.00 ± 1.74 and 30.33 ± 1.79 in group 1, 2, 3 and 4 respectively in pre-freeze condition.

The mean values were significantly different in group 3 ($p<0.01$) and group 4 ($p<0.05$) compared to group 1 (control).

Post-thaw

The per cent post-thaw value of pattern AR spermatozoa was 33.17 ± 1.84, 28.00 ± 1.72, 24.00 ± 1.74 and 24.33 ± 1.80 in group 1, 2, 3 and 4 respectively.

The results above indicated a significant ($p<0.05$) difference in mean values in group 3 than group 1 (control).

Spermatozoa showing pattern AR in pre-freeze semen revealed a increase of 19.16, 13.34, 5.17 and 25.17 per cent from mean values in fresh semen in groups 1, 2, 3 and 4 respectively, indicating minimum increase in group 3.

Pattern AR Spermatozoa at post-thaw stage showed a increase of 8.84, 9.33, 3.0 and 6.0 per cent from mean values in pre-freeze semen in groups 1, 2, 3 and 4 respectively.

h. Zona binding ability of post-thaw spermatozoa

The mean values of zona binding assay (binding per cent and binding index, BP and BI, respectively) for semen samples of all the four groups after freezing-thawing (Table 10, Fig. 24 and 25) were evaluated.

The BP and BI for semen samples were 58.06 ± 4.7 and 47.16 ± 7.5; 56.92 ± 3.8; and 43.26 ± 6.2; 65.63 ± 4.7 and 79.96 ± 7.6, and 50.76 ± 4.7 and 31.77 ± 8.6 in group 1, 2, 3 and 4 respectively.

Table 10 : Mean(±SE) effect of sequestration of PDC-109 on heterologous sperm zona binding

Parameters	No. of ejaculates	Group 1	Group 2	Group 3	Group 4
No. of oocytes examined	6	62	65	64	65
No. of sperm bound oocytes	6	36	37	42	33
No. of zona bound sperm.	6	2924	2812	5118	2065
Binding per cent	6	58.06±4.7	56.92±3.8	65.63±4.7	50.76±4.7
Binding index	6	47.16±7.5^{a}	43.26±6.2^{a}	79.96±7.4$^{b!}$	31.77±8.6^{c*}

Means bearing different superscripts in a row differ significantly (* : $p<0.05$, ! : $p<0.01$)

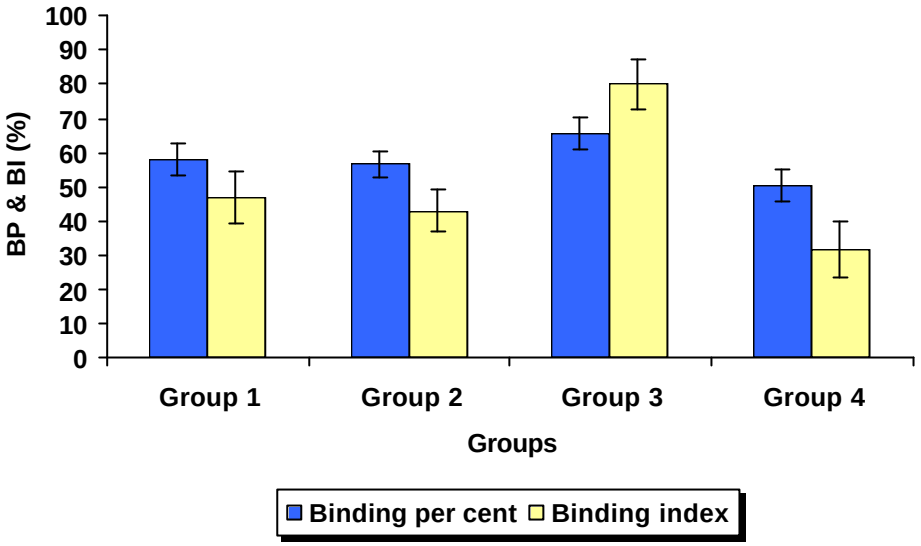


Fig. 24 : Effect of sequestration of PDC-109 on heterologous zona binding. The vertical column represent mean±SE

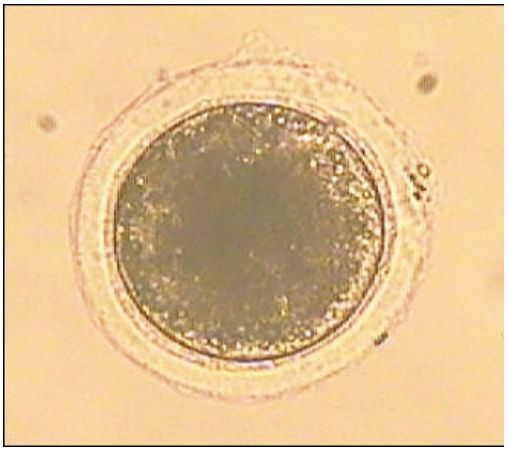


Fig. 25a : Denuded buffalo oocyte after removal of surrounding cumulus cells

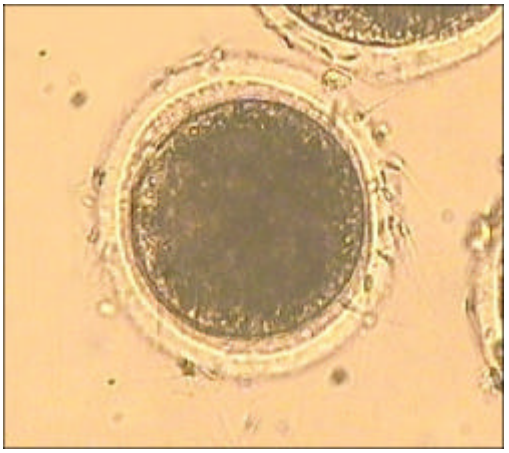


Fig. 25b : Capacitated spermatozoa bound to zona pellucida of a matured oocyte

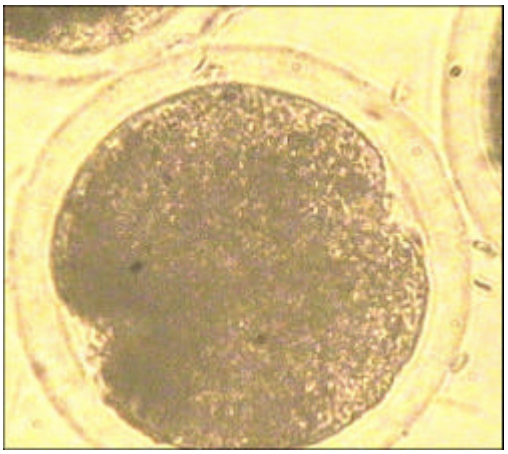


Fig. 25c : Penetrated oocyte showing first cellular division

The above results indicated significant difference in group 3 ($p<0.01$) and group 4 ($p<0.05$) compared with group 1 (control) for BI in post-thaw spermatozoa. No significant difference in values for BP in three groups in comparison to group 1 was observed.

5 Discussion

It has been established that PDC-109 in seminal plasma is beneficial during fertilization in female reproductive tract by inducing capacitation, acrosome reaction and zona binding, hyper-activation as well as by helping in formation of oviduct-spermatozoa reservoir. In contrast, bovine seminal plasma proteins also induce changes in the sperm plasma membrane by continuously stimulating cholesterol and phospholipids efflux in a time and concentration dependent manner. It was therefore postulated that detrimental effect of excess PDC-109 (majority constituent of BSP proteins) on the spermatozoa during cryopreservation could be minimized to certain extent by sequestration of PDC-109 from the ejaculate at the time of collection as well as during processing.

Thus the present study was designed to investigate the effect of sequestration of PDC-109 at collection as well as during processing and the effect of sequestered semen on minimization of cryodamages and *in vitro* fertilizing ability of spermatozoa.

Briefly, PDC-109 was isolated, purified and characterized, antisera raised against it in rabbits and then the 'antibodies against PDC-109' was added in extender to cause sequestration of PDC-109 from the ejaculates of various groups. Ejaculates thus collected were then evaluated for various seminal parameters and results were compared with control. For the purpose of investigation four groups formed were group 1: control; group 2: Direct collection of ejaculates in EYTG; group 3: Collection of ejaculates in test tubes coated with 'antibodies against PDC-109', later processed in EYTG and group 4: Extender containing 'antibodies against PDC-109' but no EY.

5.1 Isolation of PDC-109

5.1.1 Total protein concentration

Protein content was estimated by Lowry's protocol (Lowry, 1951, Fig. 1) in seminal plasma before isolation of PDC-109. The value of total proteins/ml (90.1 ± 5.6) present in seminal plasma is in agreement with the findings of Kulkarni *et al*. (1995), Harshan (2007) and Loyi (2008) but was lower than those reported by Seshagiri and Pattabhiraman (1991). The difference might be due to difference in breed and age.

Isolated Heparin Binding Proteins (HBP) of crossbred bull semen in terms of quantity (mg/ml) and per cent of total proteins in present investigation were 27.9 ± 0.6 and 30.9, respectively. The HBP fraction isolated in this study does not represent the whole of the heparin binding class of proteins in crossbred bull seminal plasma. As the proteins were eluted from heparin-Sepharose column using choline chloride, thus they represent only that fraction of HBP which bind to choline chloride. The findings reported here are in agreement with results of Harshan (2007). Nauc and Manjunath (2000) evaluated the concentration of each BSP proteins by respective radio immune assays in the seminal plasma of 25 individual bull ejaculates and indicated that BSP proteins represent 31.4 to 46.7 mg/ml in seminal plasma whereas the total protein was 73.5 to 93.0 mg/ml. Nauc and Manjunath (2000) also observed variations in the concentration of total proteins and BSP proteins in split ejaculates from the same bull. They reported that BSP proteins in seminal plasma constituted an average of 47 per cent of the total protein fraction. In a study conducted by Bellin *et al*. (1994) the average concentration of total HBP represented 19.2 and 0.14 mg/ml of ejaculate in bovine seminal plasma and sperm membrane, respectively.

In the present investigation, PDC-109 constituted 21.7 ± 0.42 mg/ml, representing an average of 24.1 per cent of total protein fraction of crossbred bull seminal plasma. Above findings are in agreement with results reported by Harshan (2007) but lower than Nauc and Manjunath (2000). In the study conducted by Nauc and Manjunath (2000) concentration of PDC-109 was much higher when compared with other seminal plasma proteins, and this protein alone represented an average of 38 per cent of the total protein fraction. While Nauc and Manjunath (2000) had utilized radio immune assay of whole seminal plasma to quantitate PDC-109, the present study employed progressive purification of the protein using two stage

chromatographic procedures, each of these steps leading to a loss of certain amount of protein. This might be the reason for the low recovery rate of PDC-109 and other proteins observed in this study. Calvete *et al.* (1994) quantitated PDC-109 (15-20 mg/ml) which is lower than the value in present investigation. Difference in value might be due to difference in purification procedure.

5.1.2 Elution profile of the purified proteins

The elution profile of all the HBP proteins purified by affinity chromatography resembled the typical graph of affinity chromatography with a sharp rise in the O.D. and a gradual tailing. Elution of HBP from heparin-Sepharose column using choline chloride revealed (sharp rise in fraction 3 and 4, trailing in 5 and 6) similar elution profiles (Fig.2). Similar observations (sharp rise in fraction 2 and 3) were made for the elution of PDC-109 from DEAE-Sephadex column using choline chloride (Fig.3). Elution pattern for isolation of HBP and PDC-109 observed in present investigation was also reported by Harshan (2007).

5.1.3 Electrophoresis of seminal plasma proteins

Eluted seminal plasma protein *viz*. HBP and PDC-109 after affinity and ion-exchange chromatography were subjected to discontinuous SDS-PAGE to assess the purity of the protein and to characterize the proteins for their molecular weight.

In the present study the analysis of HBP proteins on SDS-PAGE revealed three bands in the region of 14 to 17 kDa and 28 to 30 kDa (Fig.4). The result is in accordance with the findings of Manjunath *et al.* (1989), who reported that BSP-A1, BSP-A2 and BSP-A3 had apparent molecular masses ranging from 15 to 17 kDa and the BSP-30 kDa protein had a molecular mass of 28 to 30 kDa. BSP-A1 and -A2 also designated as PDC-109 which has an identical amino acid sequence but their difference resides in their degree of glycosylation. The two proteins essentially represent the same protein groups hence the similarity in the SDS-PAGE profile. BSP-30kDa is also a glycoprotein, whereas BSP-A3 does not contain any carbohydrate (Salois *et al.*, 1999).

In this study after electrophoresis of the PDC-109, purified protein appeared as doublets, representing the two proteins (BSP-A1 and -A2) constituting PDC-109. Gwathmey *et al*. (2003) also observed purified PDC-109 as doublets. The bands represent the glycosylated and non-glycosylated forms (Manjunath and Sairam, 1987; Gerwig *et al.*, 1996).

5.1.4. Production and evaluation of antisera

a. Immunodiffusion assay

Hyper immune sera was raised in four male New Zealand White rabbits. The production of antisera ('antibodies against PDC-109' or IgG rich fractions) was subjected to immunodiffusion test. Figure 6 shows that three out of four male rabbits responded to antigenic stimulation with PDC-109 by producing antisera as evident from appearance of clear band in immunodiffusion assay. Absence of antigenic response in fourth rabbit could be attributed to non specific factors such as sub clinical disease or individual variation in responding to antigenic stimulation.

b. Enzyme Linked Immuno Sorbent Assay

The antisera collected from rabbits were evaluated for its titre by checker board assay employing serial dilutions of antigens and antibodies. An antibody titre of 1:400 was found suitable and an antigen concentration of 225 ng was found optimum. The above findings were in accordance with those reported by Harshan (2007).

5.1.5 Western blot of PDC-109

Isolated and purified PDC-109 was probed with the raised antisera. The development of a single brown band in the lane indicated the specificity of the antisera (Fig.7). Nauc and Manjunath (2000) reported that antibody raised against PDC-109 did not cross react with any other antigen verifying the specificity of the antigen-antibody interaction. A doublet was detected by anti-PDC-109 in the alcohol precipitate of crude and purified PDC-109 (Harshan, 2007).

5.1.6 Purification and SDS-PAGE analysis of 'antibodies against PDC-109'

The hyper immune sera collected from antigenic responsive male rabbits were pooled and subjected to ammonium sulphate precipitation followed by DEAE ion-exchange chromatography which resulted in a pure fraction as seen by first peak in Figure 8. Chromatographic pattern of purified rabbit 'antibodies against PDC-109' with phosphate buffer pH 8.0 (first peak) and phosphate buffer + 100 mM NaCl (second peak) is shown in Figure 8. The concentration of 'antibodies against PDC-109' in two pooled eluted fractions was 15.7 ± 0.3 and 10.5 ± 0.4 mg/ml on protein basis for first and second peak respectively.

Figure 9 shows the result of SDS-PAGE for determining the purity of IgG by ammonium sulphate precipitation followed by DEAE ion-exchange chromatography in which a distinct polypeptide band with molecular weight of about 50 kDa corresponding to rabbit IgG heavy chains and diffused band, molecular weight of 25 kDa, corresponding to rabbit IgG light chains is visible. The SDS-PAGE analysis showed that purification of IgG rich fractions could be achieved using phosphate buffer and phosphate buffer + NaCl on ion-exchange chromatography.

5.1.7 Evaluation of seminal attributes at initial stage

For isolation and purification of PDC-109, semen samples were evaluated for seminal attributes before separation of seminal plasma.

a. Volume

The mean ejaculate volume of semen sample recorded in present study was 3.14 ± 0.5 ml. The finding is in agreement with other workers (Mishra *et al.,* 1989; Patel *et al.,* 1989). Loyi (2007), while working on freezable and non freezable semen samples of crossbreds, observed that ejaculate volume had no influence on semen quality. However, ejaculate volume has an important bearing on total number of frozen semen straws harvested from each bull, affecting economics of semen production. The variation in the semen volume could be influenced by the age (Raja and Rao, 1983), individual bull factor, month of collection (Rao and Rao, 1978) and season (Bhoite *et al.,* 2008).

b. Mass activity

In present study, the average mass activity of semen samples was 3.1 ± 0.4 on a scale of 0 to 5. The result is in agreement with findings of other workers (Mishra *et al.,* 1989; Patel *et al.,* 1989). Higher mass activity is associated with freezability (Sethi *et al.,* 1989). A consistently high mass activity is indicative of fertility of a bull (Agarwal *et al.,* 1987).

c. Concentration

The average spermatozoa concentration in present study was found to be 840.55 ± 35.42 million/ml. The finding is comparable with those reported by Agarwal *et al*. (1987) and Patel *et al*. (1989). The concentration of spermatozoa is important for the production of

maximum number of frozen semen straws. Loyi (2007) observed that concentration of spermatozoa in the semen was significantly ($p<0.05$) correlated with the individual motility. Concentration of spermatozoa is greatly influenced by individual bull factor (Agarwal *et al.,* 1987), season (Patel *et al.,* 1989), breed, environmental factors (Tomar *et al.,* 1985) and age (Rao and Rao, 1978).

5.1.8 Physico morphological characteristics of treatment groups

Before discussing the effect of sequestration of PDC-109 on the various spermatozoa parameters evaluated in the study, a discussion about the possible mode of action of PDC-109 on spermatozoa would be helpful.

At ejaculation spermatozoa are exposed to the seminal fluid, which contains the PDC-109 contributed by seminal vesicles. During this brief exposure the proteins remove a significant amount of cholesterol, which is also accompanied by the release of some phospholipids. Manjunath and Therien (2002) found that this cholesterol efflux from epididymal spermatozoa is concentration and time dependent. Studies with BSP proteins by the same authors have shown that an exposure of 15 to 30 min leads to 7 to 15 per cent cholesterol efflux while an exposure of 4 h leads to 25 per cent cholesterol efflux. Though the BSP proteins induce a significant cholesterol efflux, this is insufficient to complete capacitation. A second cholesterol efflux induced by HDL (discussed later) in the female genital tract may be essential for completion of capacitation and acrosome reaction. Studies on goat spermatozoa by Iborra *et al*. (2000) indicated that removal of about 30% cholesterol from sperm membranes did not induce any capacitation and acrosome reaction. A release of about 50-65 per cent cholesterol was required to induce capacitation and acrosome reaction. Ehrenwald *et al*. (1988a) did not detect any acrosome reacted bovine spermatozoa even after 31 per cent cholesterol efflux. This lipid efflux resulting in decreased cholesterol to phospholipids ratio, may slightly destabilize the sperm membrane, thus priming for spermatozoa capacitation. Davis (1980) observed that capacitation involves a decrease in membrane cholesterol to phospholipids ratio. During capacitation, cholesterol is lost from the sperm plasma membrane, and when sufficient cholesterol is removed, the membrane becomes unstable, enhancing its ability to fuse with the outer acrosomal membrane, resulting in the acrosome reaction (Nolan *et al.,* 1992). These initial changes appear to be a reversible phenomenon that influences the fluidity and ionic permeability

of the sperm membrane (Langlais and Roberts, 1985). Davis (1981) based on several studies, proposed that the loss of cholesterol from the sperm membrane and the resulting decrease in the cholesterol/phospholipids (C/P) molar ratio is an important step in the process of spermatozoa capacitation. This concept is supported by other studies (Fleming and Yanagimachi, 1981; Go and Wolf, 1985; Ehrenwald *et al.,* 1988a; Cross, 1996).

The PDC-109 and 30 kDa proteins were found to stimulate specific cholesterol and phospholipids efflux of up to 29.5 ± 4.7 per cent (Therien *et al.,* 1999). This cholesterol and phospholipid efflux induces plasma membrane lipid reorganization, ultimately increasing, membrane permeability to Ca^{2+}, HCO_3^- and K^+ (Visconti and Kopf, 1998). High intracellular concentrations of these ions are required for a spermatozoon to undergo the acrosome reaction (Parrish *et al.,* 1999) as well as to fuse with the oocyte (Brewis and Moore, 1997).

In order to enable the spermatozoa to undergo heparin-mediated capacitation, heparin must interact with sperm membrane bound BSP proteins. Therien *et al.* (1997) observed that ejaculated spermatozoa are coated with all three BSP proteins, all of which promote heparin-induced capacitation. Binding of antibodies to any one type of BSP proteins prevents the interaction of heparin with the other BSP proteins possibly by steric hindrance (Lane *et al.,* 1999).

PDC-109 can not stimulate cholesterol efflux or acrosome reaction of the epididymal spermatozoa in the absence of heparin (Therien *et al.,* 1998). Factors present in the follicular fluid (heparin, heparin like proteoglycans or other glycosaminoglycans) are necessary for the PDC-109 to act at the right time in the right place (Therien *et al.,* 1995). Because of this requirement the sperm begin their capacitation only within the female genital tract remaining incapacitated until they reach the appropriate time and place for capacitation.

The destabilization of the sperm plasma membrane is also contributed by prolonged exposure of spermatozoa to PDC-109 during cryopreservation process (Therien *et al.,* 1995). The increase in permeability of sperm plasma membrane could be due to the direct effect of cholesterol efflux induced by the presence of PDC-109 in higher concentration in seminal plasma. The increase in permeability of plasma membrane produces capacitation like changes in cryopreserved spermatozoa.

The value of seminal attributes of the semen samples of all four groups were also studied in fresh semen with the view to make effective comparison of values obtained at later stages (pre-freeze and after freezing-thawing) of experiments. The mean values of seminal attributes in fresh semen were important for discussion of similar values especially for HOST, cholesterol estimation and, *in vitro* capacitation as well as acrosome reaction. The pre-freeze evaluation of various seminal physico-morphological charactestics, *in vitro* capacitation, acrosome reaction and cholesterol content of spermatozoa in different groups were carried out with the aim of evaluating the effect of sequestration of PDC-109 on the spermatozoa before they were subjected to the stress of cryopreservation. The data obtained would also help to appreciate the degree of change or protection provided by the sequestration of PDC-109 from the seminal plasma to the spermatozoa during subsequent cryopreservation process. In addition to above, heterologous zona binding assay was carried out using post-thaw spermatozoa of four groups.

a. Effect of sequestration of PDC-109 on individual motility

The mean value of pre-freeze individual motility of spermatozoa was significantly ($p<0.01$) different in group 4 than group 1 (control). In these groups per cent decrease in motility observed at pre-freeze stage from mean values in fresh semen was 6.33, 7.83, 2.74 and 18.2 in group 1, 2, 3 and 4 respectively, indicating highest decrease in group 4 than other three groups (Table 1, Fig. 10).

The mean value of individual motile spermatozoa after freezing-thawing showed (Table 1) a significant difference in group 3 ($p<0.01$) and group 4 ($p<0.05$) than group1 (control). Per cent decrease in motility observed in post-thaw semen from pre-freeze values was 25.17, 22.83, 9.16 and 19.34 in groups 1, 2, 3 and 4 respectively, indicating lowest decrease in group 3.

The result obtained for individual motility in fresh semen is in agreement with those reported by Singh *et al.* (1972) and Loyi (2007). Ejaculates having individual motility of 70 per cent or more were considered freezable, (Loyi, 2007). Significant correlation between individual motility and fertility had been observed by Singh *et al.* (1972) and with freezability by Rana and Dhami (2004).

Harshan (2007) while using epididymal spermatozoa, observed that the group treated with 40 µg PDC-109/ml had significantly higher ($p<0.05$) pre-freeze motility than the spermatozoa in control. In the same study, it was observed that spermatozoa of the group treated with high concentration of PDC-109 (120 µg/ml) had significantly lower pre-freeze motility than the other groups. The results indicated that PDC-109 stimulates motility of spermatozoa but the effects were dose dependent with higher concentrations being deleterious. Similar results were obtained in a study by Kumar (2005) and Harshan *et al.* (2006), where the buffalo cauda epididymal spermatozoa were treated with heparin-binding protein.

PDC-109 changes biophysical membrane properties, eventually resulting in increased spermatozoa motility. Sanchez-Luengo *et al.* (2004) purified PDC-109 from bull seminal vesicle extracts and studied its effect on spermatozoa motility. The experiments demonstrated that PDC-109 significantly increases spermatozoa motility. The authors studied calcium-pumping mechanisms by monitoring the effect of PDC-109 on various parameters of enzyme activity of $Ca^{(2+)}$-ATPase in epididymal sperm plasma membranes and compared them with $Ca^{(2+)}$-ATPase activities from other organs and from epididymal spermatozoa of different species. They observed that enzyme activities of both $Mg^{(2+)}$ - dependent and $Mg^{(2+)}$-independent $Ca^{(2+)}$-ATPases increased in a dose-dependent manner following the addition of the PDC-109 (range 5-20 mg/ml). Preincubation of PDC-109 at temperatures above 37°C and pH ranging from below 6.5 and above 8.5 led to the loss of the stimulatory effect. An analysis of enzyme kinetics pointed to irreversible, cooperative interaction of PDC-109 with the enzyme. The effect was organ-specific, that is, restricted to spermatozoa ATPases, but it was not species-specific, as it could be elicited also in rat spermatozoa. Sanchez-Luengo *et al.* (2004) found that on addition of 2 µM of PDC-109, the straight line velocity increased from a basal value of 9.6 ± 0.14 to 42.4 ± 0.10 µm/s. Average path velocity (µm/s) increased from a basal value of 19.2 ± 0.07 to 52.2 ± 0.11 after adding 2 µM proteins. Curvilinear velocity (µm/s) ranged from 44.5 ± 0.13 without protein to 100.9 ± 0.23 after addition of 2 µM of PDC-109. Increasing the amount of PDC-109 to 4 µM did not further enhance the velocity. The relative number of spermatozoa with enhanced motility was not increased after further addition of PDC-109.

The change in membrane phospholipids concentration in mid-piece may be involved in alterations of other plasma membrane components of the sperm tail, resulting in an increase

in the intracellular Ca^{2+} and cAMP levels that support increased motility. The role of cAMP in regulating mammalian spermatozoa motility is well established (Yanagimachi, 1994).The removal of cholesterol with a resultant change in sperm plasma membrane fluidity could modulate Ca^{2+} and HCO_3 ion fluxes, leading to activation of adenyl cyclase resulting in increased cAMP, it would in turn induce protein tyrosine phosphorylation (Visconti *et al.,* 1998). Lin and Kan (1996) reported that the changes in cholesterol level in the plasma membrane over the middle piece and principal piece may increase the lateral mobility of membrane components.

Therien *et al.* (1995) observed that PDC-109 stimulated the efflux of spermatozoa cholesterol in a dose dependent manner observing that motility of bovine spermatozoa decreased from about 80 to 90 to 10 to 20 per cent in the beginning of the incubation period (6 to 8 h) when 120 µg/ml of PDC-109 was present. The effect could be due to a higher efflux of cholesterol than the level, which provides optimum membrane fluidity for motility. Therefore, sequestration of PDC-109 from the semen samples by the combined effect of antibodies against and LPC from egg yolk in group 3 resulted in higher pre-freeze motility percentage. It seems that presence of antibodies against PDC-109 alone (in absence of LPC from egg yolk) in premeditated ratio of 1:1 with protein in group 4 was not sufficient to cause sufficient sequestration of protein leading to loss of motility per cent, a decrease of 18.2 per cent (Table 1) from that of fresh semen. Alternately, a continuous and steady stimulation of spermatozoa metabolism by remaining PDC-109 in seminal plasma due to incomplete sequestration of protein in group 4 might lead to loss of energy, leading to spermatozoa wasting and decreased motility.

At post-thaw stage spermatozoa of all the protein treated groups had significantly lower motility than the spermatozoa of control group in a dose dependent manner with the highest dose showing the greatest reduction in spermatozoa motility Harshan (2007). Similar findings were also reported by Kumar (2005).

It was evident from the findings of Harshan (2007) that prolonged exposure of spermatozoa with higher concentration of PDC-109 as during cryopreservation process leads to damages to sperm plasma membrane caused by excessive cholesterol efflux. The stability of sperm plasma membrane required for viability and regulating calcium uptake, is disturbed after prolonged exposure to PDC-109 resulting in inhibitory effect on individual motility of

post thaw spermatozoa. Therefore, sequestration of PDC-109 by antibodies against it and lysophosphatidylcholine from egg yolk in group 3 was the reason for spermatozoa showing higher post-thaw individual motility. It is probable that sequestration of PDC-109 from seminal plasma in group 4 was not sufficient and remaining amount of protein exerted its deleterious effect on spermatozoa motility. Also, it was observed that no added benefit could be derived from direct collection of ejaculates in EYTG extender (group 2) when compared to routine processing procedure as far as individual motility is concerned. This might be due to similar level of sequestration of PDC-109 from ejaculates from both groups thus showing identical post-thaw individual motility.

b. Effect of sequestration of PDC-109 on per cent live spermatozoa

The per cent live spermatozoa in pre-freeze semen (Table 2, Fig. 11) showed a significant ($p<0.05$) difference only in group 3 compared to group 1 (control). In four groups per cent decrease in viability observed at pre-freeze stage from mean values in fresh semen was 8.66, 7.17, 2.5 and 11.5 in group 1, 2, 3 and 4, respectively, indicating lowest decrease in group 3 and highest in group 4.

It is evident from Table 2 that mean post-thaw live spermatozoa was significantly different in group 3 ($p<0.05$) and group 4 ($p<0.01$) than group 1 and 2. Per cent decrease in viability observed in post-thaw semen from pre-freeze value was 14.84, 13.83, 14.33 and 20.66 in groups 1, 2, 3 and 4 respectively, indicating highest decrease in group 4 (Table 3).

The mean value in fresh semen is in accordance with the report of Mishra *et al.* (1989), Bhoite *et al.* (2008) and Loyi (2008). The per cent live spermatozoa is an important parameter while selecting ejaculates for further processing in cryopreservation. Loyi (2007) reported significantly ($p<0.05$) higher percentage of live spermatozoa in freezable ejaculates than the non-freezable ejaculates.

Harshan (2007) had observed decrease in viable spermatozoa per cent in group containing higher amount of PDC-109 (120 *vs*. 40 µg PDC-109/ml). His experiment indicated that though lower doses of PDC-109 did not affect viability, a higher amount of PDC-109 in seminal plasma is detrimental for spermatozoa viability.

Fiol de Cuneo *et al.* (2004) observed a decrease in spermatozoa motility and viability when cryopreserved bovine spermatozoa was incubated with PDC-109 (0.5, 1.5 or 3.0 mg/ ml) for 4 h in the presence of heparin. In accordance with these results, Way *et al.* (2000) reported that exposure of bovine spermatozoa to accessory sex gland fluid accelerated cell death and that rapid removal of spermatozoa from seminal plasma was critical for maximal viability. Similar results were obtained with respect to motility and viability when PDC-109 was tested in the absence of heparin. Therefore, it appeared that these effects of the PDC-109 were not dependent on the interaction of PDC-109 with heparin.

Manjunath *et al.* (2002) reported that although PDC-109 is beneficial for spermatozoa function, they might be detrimental to the spermatozoa if exposed for longer periods. In earlier studies, the toxic effect of seminal plasma or accessory sex gland fluid on spermatozoa was found to be concentration and time dependent (Shannon and Curson, 1972). Dott *et al.* (1979) suggested that the toxic effect of seminal plasma is immediate and persists even after washing spermatozoa. Thus the toxic effect of seminal plasma or accessory gland fluid could be attributed to the PDC-109 which are present in very high concentration in the seminal plasma of bull and are not removed from spermatozoa surface even by several washing.

Thus it could be assumed that sequestration of PDC-109 from the semen samples by antibodies against it and by lysophosphatidylcholine (LPC) from egg yolk in group 3 could have led to decrease in detrimental effect of the protein vis-à-vis spermatozoa viability. With lower concentration of PDC-109 remaining in the semen samples, there was reduction in protein induced metabolic activity and damaging efflux of cholesterol and phospholipids from sperm plasma membrane was also decreased. Consequently, there was reduced sperm membrane destabilization, resulting in higher viability percentage in group 3. However, absence of similar beneficial effect in group 4 might be due to presence of insufficient quantity of 'antibodies against PDC-109' in extender in premeditated ratio of 1:1 with PDC-109 to cause desired sequestration of protein leading to loss of viability.

Harshan (2007) reported that at post-thaw stage addition of PDC-109 in the semen sample led to decrease in the per cent viability of spermatozoa after freezing-thawing, the reduction in viability per cent was observed to be dose dependent with the group treated with highest dose of protein showing the greatest reduction in viability per cent.

PDC-109 was shown to initiate cholesterol and phospholipids efflux from spermatozoa membrane (Therien *et al.,* 1998) and this could trigger a decreased stability of the spermatozoa to a series of stress factors to which spermatozoa are exposed during cryopreservation and thawing. Exposure of spermatozoa for 4 h to BSP proteins leads to about 25 per cent cholesterol efflux (Moreau and Manjunath, 2000). Hence, the loss of cholesterol causes the spermatozoa cells to become more susceptible to stress. Altering the lipid composition of sperm plasma membranes not only affects the ability of spermatozoa to capacitate and to acrosome react, it also affects the way spermatozoa respond to cryopreservation (Purdy and Graham, 2004).

It has been reported that loss of PDC-109 bound to sperm plasma membrane occurs during cryopreservation. This loss has been reported to be more than 70 per cent (Nauc and Manjunath, 2000). They carried out RIA aided quantification of BSP in the freeze-thawed spermatozoa and found a decrease in spermatozoa bound protein on an average of 84.0, 79.9 and 74.0 per cent for BSP-A1/-A2, BSP-A3 and BSP-30 kDa, respectively. This reduction in protein itself could either be the cause of increased susceptibility to membrane damage as a result of associated lipid efflux leading to cryodamages which is mirrored by decreased spermatozoa viability in present study.

Other workers have also reported that spermatozoa, following exposure to seminal plasma, become more sensitive to cold shock and freeze-thaw. Way *et al.* (2000) reported that exposure of spermatozoa to accessory sex gland fluid for longer period, is toxic and removal of spermatozoa from seminal plasma or accessory sex gland fluid is critical for maximum viability. The decrease in cholesterol content of spermatozoa appears to decrease spermatozoa resistance to cold shock and freezing. It could be assumed therefore, that as the unbound PDC-109 content of semen sample was sequestered by antibodies against it and also by LPC from egg yolk, it led to significant ($p<0.05$) increase in viability percent in group 3. The beneficial effect of sequestration of PDC-109 had manifested itself at pre-freeze stage. However, it appears that presence of 'antibodies against PDC-109' in extender in premeditated ratio of 1:1 with protein was not enough to cause sufficient sequestration of protein in group 4 leading to loss of viability. It was also observed that direct collection of ejaculates in EYTG extender (group 2) did not offer any added benefit with respect to spermatozoa viability in comparison to routine processing where ejaculates were collected and extender was added later on. This

might be due to similar level of sequestration of PDC-109 by presence of egg yolk LPC in both groups manifesting in identical viability per cent.

c. Effect of sequestration of PDC-109 on hypo osmotic swelling response

The mean values of hypo-osmotic swollen spermatozoa in pre-freeze sample were higher in group 3, and lower in group 4 than group 1 and 2. In these groups (Table 3, Fig. 13) per cent decrease in HOS responsive spermatozoa observed at pre-freeze stage from mean values in fresh semen was 9.67, 9.33, 4.5 and 13.5 in groups 1, 2, 3 and 4 respectively, indicating lowest decrease in group 3 than other three groups.

The HOS response in post-thaw semen was significantly different in group 3 ($p<0.05$) and group 4 ($p<0.01$) than group 1 and 2. Per cent decrease in HOS response observed in post-thaw semen from pre-freeze values as evident from Table 3 was 9.0, 7.67, 10.67 and 15.83 in groups 1, 2, 3 and 4 respectively, indicating maximum decrease in group 4.

HOST is an important indicator of spermatozoa membrane integrity which is required very much for spermatozoa metabolism as well as for successful fertilization. Additives like vitamin E and ascorbic acid in semen samples increased HOS response in spermatozoa at post-thaw (Agarwal *et al.,* 1987).

While working with cauda epididymal spermatozoa of buffaloes, Harshan (2007) observed maximum pre-freeze HOS response in the group treated with PDC-109 at the rate of 40 µg/ml which was attributed to beneficial effect of PDC-109 in stabilizing the sperm membrane in a first step (Greube *et al.*, 2001). The decrease in HOS response of spermatozoa with the increase in the concentration of PDC-109 was reported by Arangasamy (2003), Kumar (2005) and Harshan *et al.* (2006).

The plasma membrane is a key organelle with respect to spermatozoa fertilizing ability. A sensitive way of testing plasma membrane functionality is to examine the ability of spermatozoa to moderate it's swelling in response to hypo-osmotic stress (volume regulatory ability). Khalil *et al.* (2006) found significant correlations between volumetric parameters and spermatozoa-oviduct binding capacity. Spermatozoa of bulls with high non return rates responded to hypo tonicity as 'perfect osmometers'. Sub fertile bulls had lower binding indices and deficiencies in volume recovery after hypotonic challenge, indicating, that intact volume regulatory ability was a necessary prerequisite for binding to oviduct epithelium and was related to fertility.

The influence of PDC-109 on spermatozoa HOS response could also be due to the efflux of cholesterol by it during the initial exposure of the spermatozoa to the protein. Davis (1981), has shown that cholesterol alters the bulk biophysical properties of biological membranes. This steroid can increase the orientation order of the membrane lipid hydrocarbon chains and as a consequence can reduce the ability of membrane proteins to undergo conformational changes this may control their functions due to the fact that membrane is less fluid. Therefore, high concentration of cholesterol in the membrane might inhibit membrane protein functions. Studies of several membrane associated ion transporter (*e.g.* Na^+, K^+, K^+-ATPase and GABA transporter) by cholesterol, support the idea that both direct and indirect effects of this steroid on regulation of enzyme/ion channel activity could be involved (Vermuri and Philipson, 1989).

In the present study, higher pre-freeze HOS response observed in group 3 could be due to the removal of deleterious effect of long time exposure of spermatozoa to PDC-109 by its sequestration through antibodies and also by the presence of LPC from egg yolk. At the same time decrease in HOS response in spermatozoa of group 4 could be due to insufficient sequestration of PDC-109 by antibodies in extender used. Probably more quantity of 'antibodies against PDC-109' in extender could have offered desired protection level to spermatozoa against deleterious effect of protein.

While working on buffalo cauda epididymal spermatozoa, Harshan (2007) observed that HOS response in the entire PDC-109 treated group was significantly lower than the spermatozoa of control group at post-thaw stage. The reduction in HOS response was observed to be dose dependent with the highest dose showing the greatest reduction in hypo osmotic swollen spermatozoa. Similar findings were also reported by Kumar (2005).

In the present study, the observation on post-thaw HOS response was similar to those observed in viability percentage and individual motility. This is along the expected lines as it was observed that sequestration of PDC-109 from semen samples brought about a significant increase in viability and motility after freezing-thawing. HOST is a good mirror of the integrity of sperm membrane; therefore, any change in sperm membrane integrity is reflected in change in HOS response. Higher HOS response in spermatozoa of group 3 compared to control could be attributed to reduction in sperm membrane damage due to sequestration of

PDC-109 by the antibodies against it and LPC from egg yolk. Similar protection of sperm plasma membrane was not observed in group 4 probably due to insufficient sequestration of protein by the low dose of antibodies as well as absence of egg yolk LPC. It was also observed that direct collection of ejaculates in EYTG extender (group 2) did not offer any added benefit with respect to HOS response in comparison to routine processing where ejaculates were collected and extender was added later on. This might be due to similar level of sequestration of PDC-109 by egg yolk in both groups.

d. Effect of sequestration of PDC-109 on acrosomal integrity

The acrosomal intactness of spermatozoa was determined using giemsa stain.

The pre-freeze mean value (Table 4, Fig. 15) was significantly ($p<0.05$) different in group 3 than group 1 (control). In four groups decrease in per cent intact acrosome observed at pre-freeze stage from that of fresh semen was 11.67, 7.0, 3.67 and 13.17 in groups 1, 2, 3 and 4 respectively, indicating lowest decrease in group 3 and highest in group 4.

The mean value of spermatozoa with intact acrosome after freezing-thawing was significantly different ($p<0.01$) in group 3 and 4 than group 1 (control). Per cent decrease observed in post-thaw spermatozoa with intact acrosome from pre-freeze values was 11.0, 11.0, 6.16 and 23.16 in groups 1, 2, 3 and 4 respectively, indicating minimum decrease in group 3 and maximum in group 4 (Table 4, Fig. 15)..

Harshan (2007) observed that addition of PDC-109 in semen samples containing cauda epididymal spermatozoa of buffalo had no significant difference in pre-freeze acrosomal integrity than control whereas in post-thaw semen samples negative dose dependent effect on post-thaw acrosome integrity with control group showing significantly ($p<0.05$) higher values than the protein treated groups was reported. Various studies had shown that PDC-109 exposed spermatozoa had a greater susceptibility to acrosome damage (Therien *et al.*, 1998).

In a study by Harshan (2007) the cauda epididymal spermatozoa from buffalo was used .The semen samples from buffalo cauda epididymis had apparently no seminal plasma protein and hence there was no detrimental effect of added dose of PDC-109 on sperm plasma membrane. Therefore when measured quantity of PDC-109 was added to such semen samples a protective effect of stabilizing the sperm plasma membrane in a first step was observed.

However in the normal course of cryopreservation process, semen samples contain ample quantity of PDC-109 which causes detrimental effect on sperm plasma membrane stability. This was the reason that in present study when excess quantity of PDC-109 was sequestered from seminal plasma by addition of antibodies against it as in group 3, a higher response of pre-freeze and post-thaw acrosome integrity was observed. However, it was observed that beneficial effect of addition of 'antibodies against PDC-109' (at 1:1 ratio with protein) in group 4 was not observed due to absence of added benefit of protection offered by LPC from egg yolk in extender. It could be safely assumed that addition of higher dose of 'antibodies against PDC-109' in extender would lead to desired protection of spermatozoa vis-à-vis acrosome integrity. It was also observed that direct collection of ejaculates in EYTG extender (group 2) offered no added benefit for spermatozoa acrosome integrity as compared to routine cryopreservation procedure which might be due to adverse effect of remaining PDC-109 in both groups where its sequestration was not complete.

e. Effect of sequestration of PDC-109 on acrosome reacted live spermatozoa by FITC-PSA stain

The mean per cent acrosome reacted live spermatozoa was assessed by FITC-PSA and PI. Number of spermatozoa with intact acrosome is positively correlated with capacitation and acrosome reaction leading to successful fertilization. Borgohain (1984) observed that acrosomal damages of the spermatozoa of Holstein-Friesian and their crosses had significantly negative correlation with conception rates in cows.

The mean value of acrosome reacted live spermatozoa at pre-freeze stage in present study was significantly ($p<0.05$) different in group 3 and 4 than control. In four groups, per cent increase in mean value of acrosome reacted live spermatozoa at pre-freeze stage from fresh was 25.5, 24.7, 14.9 and 22.0 in group 1, 2, 3 and 4 respectively.

At post-thaw stage the mean values differed significantly in group 3 ($p<0.01$) and group 4 ($p<0.05$) than control. Per cent increase in mean value at post-thaw stage from pre-freeze value was 12.8, 12.9, 10.5 and 9.3 in group 1, 2, 3 and 4 respectively. The results indicated minimum increase in acrosome reacted spermatozoa in group 3 than other groups (Table 5, Fig. 17).

Harshan (2007) observed that addition of PDC-109 in semen samples containing cauda epididymal spermatozoa of buffalo had no significant difference in acrosome integrity at pre-freeze level however at post-thaw stage, addition of PDC-109 in semen samples of cauda epididymal buffalo spermatozoa had negative effect on post thaw acrosome integrity, with value in the control group being significantly ($p<0.05$) higher than the protein treated groups. The number of acrosome reacted live spermatozoa were maximum in the group treated with PDC-109 at rate of 40 µg/ml. At post thaw stage, the level of acrosome reacted spermatozoa at each time interval of incubation was higher than the corresponding pre-freeze acrosome reacted spermatozoa of same treatment group. On the other hand, Gillian *et al*. (1997) reported that the process of freezing-thawing increased the number of spermatozoa showing capacitated B pattern in ram semen but it had little effect on the number of acrosome reacted spermatozoa. They observed that at 0 h, pattern F and B as 61.3/ 19.5 and 6.7/54 per cent for fresh and frozen thawed spermatozoa, respectively. The freezing process and changes in sperm plasma membrane brought about by prolonged action of PDC-109 alters the influx of Ca^{2+} in bovine spermatozoa. Both fresh and frozen thawed spermatozoa in Ca^{2+} supplemented medium accumulated calcium rapidly but thawed spermatozoa did so faster than fresh spermatozoa (Bailey *et al*., 1994). The influx of Ca^{2+} is a critical step in the process of capacitation, hence an increased calcium influx is supposed to bring about capacitation and acrosome reaction more rapidly.

In present study a minimum increase in spermatozoa showing pattern AR at pre-freeze stage in group 3 might be a result of sequestration of PDC-109 by addition of antibodies against it and also by presence of LPC from egg yolk in extender. Sequestration of PDC-109 from the semen samples in group 3 resulted in lower cholesterol and phospholipids efflux, reduced damages to sperm plasma membrane, reduced Ca^{2+} influx and hence reduction in percentage of acrosome reacted spermatozoa. As added protection offered by LPC was not available to spermatozoa of group 4, an increase in percentage of acrosome reacted spermatozoa was observed. It was also evident from the study that no added benefit could be observed in direct collection of ejaculates in EYTG extender (group 2) than routine cryopreservation procedure which might be due to similar level of sequestration of PDC-109 by LPC present in egg yolk in both groups.

f. Effect of sequestration of PDC-109 on cholesterol content of spermatozoa

The mean cholesterol content of spermatozoa in fresh semen in the group 1 (control) was 23.35 ± 0.93 µg/100 million with comparable values in group 2, 3 and 4.

At pre-freeze stage a significant ($p<0.05$) difference (Table 6, Fig. 19) in cholesterol content of spermatozoa in Group 3 compared to group 1 (control) was observed. Decrease in per cent cholesterol content of spermatozoa observed at pre-freeze stage from that of fresh semen was 13.63, 11.42, 8.4 and 12.35 in groups 1, 2, 3 and 4 respectively, indicating lowest decrease in group 3.

The mean post-thaw value (Fig. 22) was significantly ($p<0.05$) different in group 3 than group 1 (control). Decrease in per cent cholesterol content of spermatozoa observed at post-thaw stage from that of mean value in pre-freeze semen was 2.46, 1.82, 3.35 and 1.57 in group 1, 2, 3 and 4 respectively.

The cholesterol content of spermatozoa varies widely between bulls as well as different ejaculates of the same bull. Sinha *et al.* (1996) reported cholesterol content of spermatozoa varying from 7.87 to 16.75 µg/50 x 10^6 spermatozoa in crossbred bulls. On the other hand Therien *et al.* (1998) reported cholesterol content of 720 ± 41 µg/50 x 10^8 spermatozoa in Holstein bull. The previous report also showed wide variation between and within the species. The cholesterol content of spermatozoa in different species were reported as 1.13 ± 0.12 nmol/10^7 spermatozoa in human (Zarintash and Cross 1996), 720 ± 41 µg/5 x 10^9 spermatozoa in bull (Therien *et al.*, 1998), 143 ± 29.9 µg (free cholesterol)/10^9 spermatozoa in boar (Cerolini *et al.*, 2001), 16.2±0.3 µg (free cholesterol) /10^9 spermatozoa in turkey (Douard *et al.*, 2000).

Gadella *et al.* (2001) suggested that spermatozoa with high cholesterol content (*e.g.* spermatozoa from man, bull and stallion) are slow to undergo capacitation whereas spermatozoa with lower cholesterol content (*e.g.* from boar and ram) seem to capacitate much faster.

The net cholesterol efflux that observed during capacitation was affected by the capacity of the medium to bind cholesterol e.g. albumin (Davis *et al.*, 1980) and high density lipoproteins (Ehrenwald *et al.*, 1990) are acceptor of cholesterol. Muller *et al.* (2002) suggested that

PDC-109 in the presence of phospholipids causes strong efflux of cholesterol from sperm plasma membrane. However, in the present study, the PDC-109 was sequestered from seminal plasma by addition of 'antibodies against PDC-109'. Therefore sequestration of PDC-109 from seminal plasma led to reduced cholesterol efflux and consequently higher presence of cholesterol content in spermatozoa of group 3. Anbazhagan and Swamy (2005) reported that LPC binds with PDC-109, making it unavailable for cholesterol efflux. As the egg yolk in extender used in group 4 was replaced by 'antibodies against PDC-109', the beneficial effect of LPC of egg yolk was not available to spermatozoa. Probably addition of 'antibodies against PDC-109' was not in sufficient concentration for desirable sequestration of protein.

Kadirvel (2006) reported mean cholesterol content as 5.38 ± 1.85 µg/50 million spermatozoa in frozen-thawed semen of buffalo bulls. A significant reduction of cholesterol content after cryopreservation recorded in the study is in agreement with the reports of Cerolini *et al*. (2001). They found that free cholesterol content decreased where as phospholipid and triglycerol content increased in boar semen after freezing-thawing. Douard *et al*. (2000) however, observed that the amount of cholesterol did not change throughout the 48 h storage at 4^{o}C but the phospholipids content of spermatozoa decreased by 30 per cent between 1 h and 24 h of storage as consequently the cholesterol phospholipids ratio increased from 0.19 to 0.28 between 1 h and 48 h of storage.

Therien *et al*. (1998) observed that PDC-109 causes cholesterol and phospholipids efflux in a time and concentration dependent manner, resulting in changes in sperm plasma membrane. At higher concentration of PDC-109 (i.e. seminal plasma) and /or longer exposure (as in modulated semen), more cholesterol and phospholipids are removed, which may decrease spermatozoa resistance to cold shock and freezing (Darin-Bennett and White, 1977). Harshan (2007) also observed that treatment of spermatozoa with PDC-109 led to an increased cryoinjury.

In group 3, the excess quantity of PDC-109 in seminal plasma was sequestered by addition of antibodies against it. Thus lower efflux of cholesterol was the reason behind higher cholesterol content of spermatozoa in group 3. Similar beneficial effect of addition of 'antibodies against PDC-109' in semen ejaculates of group 4 was less pronounced. Even then the cholesterol content of spermatozoa in group 4 was comparable to that of group 1 (control). No significant

difference in cholesterol content of spermatozoa of group 2 was observed from that of group 1 (control), indicating that no clear benefit could be obtained by direct collection of ejaculates in EYTG extender in comparison to routine processing as for as cholesterol efflux is concerned. This might be due to adverse effect of identical concentration of PDC-109.

g. Effect of sequestration of PDC-109 on *in vitro* capacitation and acrosome reaction

In present experiment, CTC-fluorescence assay of spermatozoa was performed allowing classification of spermatozoa in non-capacitated (pattern F), capacitated (pattern B) and acrosome-reacted (AR) categories, as described originally by Ward and Storey (1984).

Pattern F, B and AR

Fresh semen

The mean values of spermatozoa exhibiting *in vitro* capacitation and acrosome reaction in fresh semen as evident by pattern F, B and AR (Fig. 19) in Group 2, Group 3 and Group 4 were comparable to that of group 1 (control).

Pattern F (Non capacitated or acrosome intact spermatozoa)

The mean per cent spermatozoa showing pattern F at pre-freeze stage revealed (Table 7) a significant difference in group 3 ($p<0.01$) and group 4 ($p<0.05$) than group 1 (control). Spermatozoa showing pattern F (Fig. 20) in pre-freeze semen revealed a decrease of 51.0, 51.33, 21.0 and 61.17 per cent from mean values in fresh semen in groups 1, 2, 3 and 4 respectively, indicating minimum decrease in group 3 whereas it was highest in group 4.

The mean post-thaw value (Fig. 18) for spermatozoa showed a significant ($p<0.01$) difference in group 3 than group 1 (control). Pattern F spermatozoa at post-thaw stage exhibited a decrease of 14.0, 12.67, 15.17 and 1.67 per cent from mean values in pre-freeze semen in group 1, 2, 3 and 4 respectively.

In the present study a significant ($p<0.01$) increase in pattern F (non capacitated or acrosome intact) at pre-freeze stage in group 3 than group 1 (control) was observed. Harshan *et al.* (2006) reported a decrease in number of non capacitated spermatozoa in PDC-109 treated groups than the control at varying incubation period. Lusignan *et al.* (2007) reported that the PDC-109 increased epididymal spermatozoa capacitation in boar as compared with

control. This effect reached a maximum level at 20 µg/ml of PDC-109 (2.2 fold higher than control). It was observed that increase in number of capacitated spermatozoa at pre-freeze stage has negative effect on conception rate (Roncoletta *et al.,* 2006).

Arangasamy (2003) reported that addition of HBP has dose dependent effect on induction of acrosome reaction and decrease in spermatozoa showing pattern F. At every hour of incubation, there was a gradual increase in acrosome reacted spermatozoa and consequent decrease in acrosome intact (pattern F) spermatozoa after addition of protein in a concentration dependent manner. Chauhan *et al*. (1997) reported that heparin treatment (10 µg/mL) enhanced the capacitation rate of buffalo spermatozoa and decreased the percentage of acrosome intact, non capacitated (pattern F) spermatozoa. Florman and First (1988) observed that exposure of spermatozoa to seminal plasma *in vitro* increased the percentage of capacitated spermatozoa. These findings suggest that increased per cent of spermatozoa showing capacitation and acrosome reaction might be due to PDC-109 present in seminal plasma causing cholesterol and phospholipids efflux after prolonged exposure (Harshan, 2007). It therefore follows that sequestration of PDC-109 by antibodies against it from semen samples would lead to lowered cholesterol and phospholipids efflux and greater sperm plasma membrane stability. Thus in group 3 significantly ($p<0.01$) increased number of spermatozoa exhibited pattern F (acrosome intact or non capacitated) at pre-freeze and post-thaw stage. At the same time the similar beneficial effect was lower in group 4. This might probably be due to sequestration of excess harmful PDC-109 was not sufficient by antibodies against it in group 4 where protective effect of LPC from egg yolk was also not there resulting in decreased number of non capacitated spermatozoa. Addition of higher dose of antibodies against PDC-109 could have increased acrosome intact/non capacitated spermatozoa in the group 4 which has to be elucidated further. It was evident from the study that no added benefit could be derived from direct collection of ejaculates in EYTG extender (group 2) than routine cryopreservation procedure as the level of sequestration of PDC-109 by LPC present in egg yolk was identical in both groups.

Pattern B (capacitated)

The mean value of spermatozoa showing pattern B was significantly ($p<0.01$) different in group 3 than group 1 (control). Pattern B spermatozoa in pre-freeze semen showed (Table

8, Fig. 22) a increase of 31.83, 38.0, 15.83 and 36.0 per cent from mean values in fresh semen in group 1, 2, 3 and 4 respectively, indicating minimum increase in group 3.

The mean value for post-thaw spermatozoa showing pattern B indicated a significant ($p<0.05$) difference in group 3 than group 1 (control). The mean value at post-thaw stage showed an increase of 5.17, 3.34, 12.17 and 7.66 per cent from mean values in pre-freeze semen in group 1, 2, 3 and 4 respectively. The protective effect of sequestration of PDC-109 from semen samples of group 3 was evident at pre-freeze stage itself whereas detrimental effect of protein was more evident in other groups at same stage. This was the reason for resulted difference in mean values in post-thaw stage (Fig. 22).

Changes in CTC staining pattern have been reported for bull spermatozoa by Cormier *et al.* (1997). They observed that after either cryopreservation or cooling to 4°C there was a similar increase in the capacitated B staining pattern. Chauhan *et al.* (1997) observed that heparin treatment of spermatozoa led to increase in percentage of capacitated and acrosome reacted spermatozoa during every hour of culture and reached maximum up to 84.33 ± 1.62 per cent. Therien *et al.* (1995) reported maximum capacitated and acrosome reacted spermatozoa when PDC-109 was added at the rate of 40 µg/ml of semen. These findings suggest that PDC-109 present in seminal plasma might be responsible for capacitation and acrosome reaction of spermatozoa. Ehrenwald *et al.* (1988a) had shown that efflux of membrane cholesterol leads to bovine spermatozoa capacitation. Harshan *et al.* (2006) reported an increase in the number of capacitated and acrosome reacted post-thaw spermatozoa in PDC-109 treated group than the control at varying incubation periods. Similar findings were also reported by Arangasamy (2003).

In the present investigation, a significant ($p<0.01$) difference in spermatozoa showing pattern B in group 3 from that of control was observed. Efflux of cholesterol and phospholipids by PDC-109 leading to capacitation has been discussed earlier. In present investigation, PDC-109 was sequestered from semen samples by addition of antibodies against it in group 3. Thus the deleterious effect of protein on cholesterol and phospholipids efflux was avoided and might be the reason for significant decrease in spermatozoa showing pattern B in this group. It was also evident from the study that no additional benefit could be derived from direct collection of ejaculates in EYTG extender (group 2) than routine cryopreservation procedure. This might be due to similar level of sequestration of PDC-109 in both groups.

Pattern AR (acrosome reacted)

The mean per cent of pre-freeze acrosome reacted spermatozoa (Fig. 21) were significantly ($p<0.01$) lower in group 3 and ($p<0.05$) higher in group 4 and comparable in group 2 with group 1. Spermatozoa showing pattern AR in pre-freeze semen (Table 9, Fig. 23) revealed a increase of 19.16, 13.34, 5.17 and 25.17 per cent from mean values in fresh semen in group 1, 2, 3 and 4 respectively, indicating minimum increase in group 3.

The results indicated a significant difference ($p<0.05$) in post-thaw mean values (Table 9) in group 3 and 4 than group 1 (control). At post-thaw stage pattern AR spermatozoa showed an increase of 8.84, 9.33, 3.0 and 6.0 per cent from mean values in pre-freeze semen in group 1, 2, 3 and 4 respectively.

Gillian *et al.* (1997) reported that the process of freezing-thawing increased the number of spermatozoa showing capacitated B pattern in ram semen but it had little effect on the number of acrosome reacted spermatozoa. They observed that at 0 h, pattern F and B as 61.3, 19.5 and 6.7, 54 per cent for fresh and frozen thawed spermatozoa, respectively. On the contrary, Harshan *et al.* (2006) reported an increase in the number of acrosome reacted spermatozoa in PDC-109 treated groups than control at varying incubation periods at pre-freeze stage. The number of acrosome reacted live spermatozoa were maximum in the group treated with PDC-109 at rate of 40 µg/ml. At post thaw stage, the level of acrosome reacted spermatozoa at each time interval of incubation was higher than the corresponding pre-freeze acrosome reacted spermatozoa of same treatment group. Similar findings were reported by Perez *et al.* (1996), who found that freezing-thawing of ram spermatozoa results in an increased proneness to capacitation and acrosome reaction. The freezing process and changes in sperm plasma membrane brought about by prolonged action of PDC-109 alters the flux of Ca^{2+} in bovine spermatozoa. Both fresh and frozen thawed spermatozoa in Ca^{2+} supplemented medium accumulated calcium rapidly but thawed spermatozoa did so faster than fresh spermatozoa (Bailey and Buhr, 19953). The influx of Ca^{2+} is a critical step in the process of capacitation, hence an increased calcium influx is supposed to bring about capacitation more rapidly.

In present study a significant ($p<0.01$) decrease in spermatozoa showing pattern AR at pre-freeze stage in group 3 might be a result of sequestration of PDC-109 by addition of antibodies against it and also by presence of LPC from egg yolk in extender. Sequestration of

PDC-109 from the semen samples in group 3 resulted in lower cholesterol and phospholipids efflux, reduced damages to sperm plasma membrane, reduced Ca^{2+} influx and hence reduction in percentage of acrosome reacted spermatozoa. As added protection offered by LPC was not available to spermatozoa of group 4, an increase in percentage of acrosome reacted spermatozoa was observed. Also, it was evident from the study that no added benefit could be observed in direct collection of ejaculates in EYTG extender (group 2) than routine cryopreservation procedure which might be due to similar level of sequestration of PDC-109 by LPC present in egg yolk in both groups.

5.1.9 Effect of sequestration of PDC-109 on zona binding assay

Zona binding assay was carried out to investigate the effect of sequestration of PDC-109 from the semen samples on the ability of frozen-thawed crossbred bull spermatozoa to bind with ova from buffaloes (heterologous). Zona binding assay consisted of determination of binding per cent (BP) and binding index (BI) of post-thaw spermatozoa.

The results indicated a positive and significant difference in BI in group 3 ($p<0.01$) and group 4 ($p<0.05$) compared to group 1 (control). No significant difference in mean values (Table 9, Fig. 22) for BP in three groups compared to control was observed (Table 10, Fig. 24).

Harshan (2007) observed that addition of PDC-109 at the rate of 40 µg/ml in semen samples did not increase the number of frozen-thawed spermatozoa bound with *in vitro* matured oocyte (256.09 ± 14.85 and 250.13 ± 11.56 spermatozoa in control and treatment group, respectively). Arangasamy (2003) reported an increase in the number of buffalo cauda epididymal spermatozoa binding to zona pellucida when treated with HBP at pre-freeze stage.

Topper *et al.* (1999) showed *in vitro* capacitation leads to an increased capacity of fresh bovine spermatozoa to bind to zona pellucida. Since the binding between spermatozoa and the zona pellucida involves hydrophobic as well as ionic interactions (Urch and Patel, 1991), the decreased net negative charge that takes place during capacitation may facilitate the binding of bovine zona pellucida proteins to spermatozoa.

Experiment on boar spermatozoa binding with zona pellucida have shown that PDC-109 competed effectively with zona pellucida glycoproteins for boar spermatozoa spermadhesin AWN binding (Dostalova *et al*., 1995). Since AWN is actively involved in spermatozoa zona

binding, presence of PDC-109 in the media surrounding the ova could have reduced the number of spermatozoa bound to ova. Spermadhesin, which has lectin like properties in relation to the oligosaccharides residues of the zona pellucida glycoproteins, act as glycoprotein receptor of the zona (Calvete *et al.,* 1996 b). In the present investigation, the PDC-109 was sequestered from semen samples of group 3 by addition of antibodies against it. Additionally LPC present in egg yolk also caused sequestration of PDC-109. Therefore PDC-109 might not be available in sufficient quantity in post-thaw semen samples to compete effectively with zona pellucida glycoprotein for binding with spermatozoa spermadhesin AWN. This might be the reason for significantly ($p<0.01$) higher response of spermatozoa of group 3 with respect to BI and non significant increase in BP as compared to spermatozoa of group 1 (control).The extender used for group 4 contained 'antibodies against PDC-109' in place of egg yolk. Addition of antibodies alone might have caused incomplete sequestration of PDC-109 which led to higher cholesterol efflux and damages to sperm membrane. Thus a lower BP and BI, as an indicator of reduced zona binding ability of spermatozoa, were observed in group 4 than group 1 (control). It is evident from the study that no added benefit could be derived from direct collection of ejaculates in EYTG extender than routine cryopreservation procedure which indicates negative effect on spermatozoa zona binding in these groups.

6 Summary and Conclusions

The present work entitled **'studies on sequestration of PDC-109 protein on cryodamage and *in vitro* fertility of crossbred bull spermatozoa'** was conducted at the Germ Plasm Centre, Indian Veterinary Research Institute, Izatnagar, Bareilly (UP). The study was designed to investigate the effect of sequestration of PDC-109 from ejaculates of crossbred bulls at collection as well as during processing on minimization of its detrimental effect. This was revealed by differences in various physico-morphological characterstics of spermatozoa during pre-freeze and post-thaw stages of cryopreservation.

For present investigation, PDC-109 was isolated, purified, characterized and antisera was raised against it in rabbits. The 'antibodies against PDC-109' was used for sequestration of PDC-109 from the ejaculates of treatment groups at collection time. Ejaculates thus collected were then evaluated for various physico-morphological characterstics and result was compared with control.

Heparin binding proteins (HBP) and PDC-109 were isolated from seminal plasma using 100 mM choline chloride on heparin-Sepharose and DEAE-Sephadex columns. The concentration of HBP proteins and PDC-109 in seminal plasma was found to be 27.9 ± 0.6 and 21.7 ± 0.42 mg/ml constituting 30.9 and 24.1 per cent of the total protein content (90.1 ± 5.6 mg/ml) of seminal plasma respectively. Purified PDC-109 was observed in SDS-PAGE gels as doublets representing the two glycosylated forms of protein. These BSP proteins were observed in the HBP fraction of the seminal plasma as revealed in SDS-PAGE. The protein

bands representing BSP-AI/-A2, BSP-A3 and BSP-30 kDa were observed in the SDS-PAGE having molecular weight of approximately 14.7, 16.5 and 30 kDa, respectively.

Isolated and purified PDC-109 was used to raise antisera in rabbits. Three out of four male rabbits responded to antigen by producing antisera as indicated by the presence of clear bands in immunodiffusion assay. The titre of the antisera was established using a checker board ELISA and a titre of 1:400 for antibody and an antigen concentration of 225 ng were found optimum. Western blot of PDC-109 established the specificity of the raised antisera. The concentration of 'antibodies against PDC-109' in two pooled eluted fractions was 10.5 ± 0.4 and 15.7 ± 0.3 mg/ml on protein basis. Elutes were dialyzed to concentrate the protein.

For the purpose of investigation four groups formed were group 1: control; group 2: Direct collection of ejaculates in EYTG; group 3: collection of ejaculates in tubes coated with 'antibodies against PDC-109', processed in EYTG later and group 4: Extender containing antibodies against PDC-109 but no EY.

In the treatment group 3 and 4, 3.14 ml of elute containing a total of 68.14 mg of 'antibodies against PDC-109' was transferred, lyophilized and later on used for collection of ejaculates. Semen samples of all the four groups were processed for cryopreservation. Pre-freeze and post-thaw evaluation of various physico-morphological characterstics *viz*. individual motility, viability, HOS response, acrosomal integrity, cholesterol content of spermatozoa, *in vitro* capacitation and acrosome reaction were carried out with the aim of evaluating the effect of sequestration of PDC-109 on the spermatozoa. In addition to above, heterologous sperm-zona binding assay was also carried out using post-thaw spermatozoa of four groups. The data thus obtained helped to appreciate the degree of change or protection provided by the sequestration of PDC-109 from the seminal plasma to the spermatozoa during subsequent cryopreservation process.

Evaluation of various physico-morphological characteristics revealed that at pre freeze stage, the mean per cent value was significantly different ($p<0.05$) for viability and acrosomal intactness in group 3 and individual motility in group 4 as compared to other groups. At the same time a significant difference in per cent mean value was observed in post-thaw spermatozoa

for individual motility ($p<0.01$ and $p<0.05$), viability ($p<0.05$ and $p<0.01$), HOS response ($p<0.01$ and $p<0.01$), acrosomal intactness ($p<0.05$ and $p<0.01$) and per cent acrosome reacted live spermatozoa ($p<0.01$ and $p<0.05$) in group 3 and 4 respectively. The effect observed might be due to protective effect of sequestration of PDC-109 by antibodies against it at pre-freeze and post-thaw stage. However, it appears that presence of 'antibodies against PDC-109' in extender in premeditated ratio of 1:1 with protein was probably inadequate to cause sufficient sequestration of protein in group 4 leading to lower mean values in these parameters.

Protective effect of sequestration of PDC-109 from ejaculates was observed by reduced efflux of spermatozoa cholesterol in group 3. The mean value for cholesterol content of spermatozoa was significantly different ($p<0.05$) in group 3 than other groups at pre-freeze and post-thaw stage. A decrease of cholesterol content 8.4 and 3.35 µg/100 million spermatozoa was observed at pre-freeze (from fresh) and post-thaw (from pre-freeze) stage in group 3 as compared to other three groups. Thus lower efflux of cholesterol was the reason behind higher cholesterol content of spermatozoa at post-thaw stage in group 3 **(**7.26 ± 0.75, 7.61 ± 0.93, 11.60 ± 0.89, and 6.93 ± 0.93 µg/100 million spermatozoa in group 1, 2, 3 and 4 respectively). However, similar beneficial effect of sequestration of PDC-109 in group 4 was less pronounced which might be due to absence of LPC from egg yolk in extender. Probably addition of higher doses of 'antibodies against PDC-109' in this group might have resulted in desirable beneficial effect.

In vitro capacitation and acrosome reaction status was evaluated in spermatozoa of all four groups at pre-freeze and post-thaw stage using CTC fluorescent assay, which allows simultaneous classification of heterogenous spermatozoa population into non-capacitated, capacitated and acrosome reacted groups. It was observed that sequestration of PDC-109 from semen samples led to increased number of non capacitated spermatozoa in group 3 as compared to control (28.83 ± 1.31, 29.50 ± 1.36, 59.67 ± 1.31 and 19.00 ± 1.36 per cent in group 1,control; 2, 3 and 4 respectively) at pre-freeze stage. Similar increase in mean value was observed at post-thaw stage (14.83 ± 1.29, 16.83 ± 1.30, 44.50 ± 1.36 and 17.33 ± 1.31 per cent in group 1, control; 2, 3 and 4 respectively) also. At the same time there was a

decrease in mean per cent AR spermatozoa in pre-freeze (24.33 ± 1.36, 18.67 ± 1.90, 11.00 ± 1.74 and 30.33 ± 1.79 in group 1, control; 2, 3 and 4, respectively) and post-thaw samples (33.17 ± 1.84, 28.00 ± 1.72, 24.00 ± 1.74 and 24.33 ± 1.80 in group 1, control; 2, 3 and 4 respectively) in group 3 as compared to other groups. Consequently, a significant decrease in mean value of capacitated spermatozoa was observed at pre-freeze ($p<0.01$; 46.83 ± 1.37, 51.83 ± 1.57, 29.33 ± 1.32 and 50.67 ± 1.67 per cent in group 1, control; 2, 3 and 4 respectively) and post-thaw ($p<0.05$; 52.00 ± 1.42, 55.17 ± 1.59, 41.50 ± 2.1 and 58.33 ± 1.9 in group 1, control; 2, 3 and 4 respectively) stage in group 3 compared to control. An increase in number of non capacitated spermatozoa and decrease in acrosome reacted spermatozoa was shown to have a positive effect on conception rate. The results in present study indicated that sequestration of PDC-109 from semen samples had protective effect on spermatozoa and even after prolonged exposure of spermatozoa to PDC-109 the protective beneficial effect continued.

Zona binding assay was carried out to investigate the effect of sequestration of PDC-109 from the semen samples on the ability of frozen-thawed bull spermatozoa to bind with ova from buffaloes (heterologous). It was evident from the present work that sequestration of PDC-109 from semen samples has significant and positive effect by increasing the Binding index in group 3 than control (79.96 ± 7.6 *vs*. 47.16 ± 7.5). However, no significant difference was found in four groups for binding per cent. The results above indicated that sequestration of PDC-109 from semen samples reduced competence of available free PDC-109 with zona pellucida glycoprotein resulting in higher value in BI.

Conclusions

From the present study, it is concluded that

- PDC-109, a seminal plasma protein is present in crossbred bull which has adverse effect on cryopreservation and fertilizing ability of spermatozoa.
- Sequestration of PDC-109 using antibodies against it, minimized the cryodamage as evidenced by lower cholesterol efflux as well as improvement in post-thaw physico-morphological characteristics of spermatozoa

- Comparative increase in binding index in PDC-109 sequestered group revealed improvement in fertilizing ability of spermatozoa

Future research directions

It is reflected from present study that adverse effect of PDC-109 on cryopreservation can be minimized by adding the antibodies against it in the semen, however further study is warranted on following points before making any recommendation.

- Study on large sample number to confirm the exact quantity of antibodies against PDC-109 for its sequestration as well as a substitute for egg yolk in extender being used routinely.
- *In vivo* study using PDC-109 sequestered semen is required to assess the fertilizing ability of spermatozoa.

7 Mini Abstract

Seminal plasma not only facilitates the transport of spermatozoa in the female reproductive tract it also contains factors that influence spermatozoa motility and fertility. Recent evidence suggests that certain proteins in the seminal plasma are detrimental for cryopreservation of semen. The major protein fraction of bovine seminal plasma is represented by a family of related proteins designated as Bovine Seminal Plasma proteins *viz.* BSP-A1/-A2 (PDC-109), BSP-A3, and BSP-30 kDa. PDC-109 is a major constituent of BSP proteins. They bind to sperm membrane choline phospholipids and high density lipoproteins (HDL) at ejaculation and thereby potentiate the sperm capacitation induced by HDL. Also BSP proteins induce changes in the sperm plasma membrane by stimulating cholesterol and phospholipids efflux in a concentration and time dependent manner thus making it sensitive to liquid or frozen state. This cholesterol and phospholipids efflux causes spermatozoa acrosome damages. It was, thus postulated that sequestration of majority constituent BSP protein especially PDC-109 could lead to minimization of its detrimental effect on spermatozoa. The present investigation was designed to study the effect of sequestration of PDC-109 on physico-morphological characterstics of spermatozoa at pre-freeze and post-thaw stages of cryopreservation as well as zona binding ability of post-thaw spermatozoa with heterologous (buffalo) oocyte. Semen samples from crossbred bulls were collected, seminal plasma separated for isolation and purification of PDC-109 by affinity followed by ion-exchange chromatography. The purity of purified PDC-109 was assessed by SDS PAGE and then used for raising antisera against it in rabbits. 'Antibodies against PDC-109' was isolated from raised antisera by ion-exchange chromatography and used for addition in semen samples at 1:1 ratio with protein. For the purpose of investigation four groups formed were group 1: control; group 2: Direct collection of ejaculates in EYTG; group 3: Collection of ejaculates in test tubes coated with 'antibodies against PDC-109', later processed in EYTG and group 4: Extender containing 'antibodies against PDC-109' but no EY. Result from the study indicated higher per cent mean value for individual motility, viability, HOS response, acrosomal intactness and per cent acrosome reacted live spermatozoa in treatment groups where PDC-109 was sequestered by addition of antibodies against it. Sequestration of PDC-109 in group 3 also led to reduction in spermatozoa cholesterol efflux, thus showing beneficial effect of such procedure. *In vitro* capacitation and acrosome reaction status was evaluated in spermatozoa of all four groups at pre-freeze and post-thaw stage using CTC fluorescent assay. It was observed that sequestration of PDC-109 from semen samples led to increased number of non capacitated spermatozoa in group 3 as compared to control (59.67 ± 1.31 *vs*. 28.83 ± 1.21 per cent respectively) at pre-freeze stage. Similar increase in mean value was observed at post-thaw stage also. At the same time there was a decrease in mean per cent AR spermatozoa in pre-freeze and post-thaw samples in group 3 as compared to other groups. The result in present study indicated that sequestration of PDC-109 from semen samples had protective effect on spermatozoa and even after stress of cryopreservation process the protective beneficial effect continued. Result of zona binding (heterologous) assay revealed that sequestration of PDC-109 from semen samples has positive effect by increasing the binding index in group 3 than control. However, no effect was observed in four groups with respect to binding per cent. It was thus evident that sequestration of PDC-109 from semen samples reduced competence of available free PDC-109 with zona pellucida glycoprotein resulting in higher value in BI. The study indicated that PDC-109 is partly responsible for destabilization of sperm plasma membrane during cryopreservation and therefore its sequestration from semen samples resulted in protective effect on spermatozoa. Addition of 'antibodies against PDC-109' in extenders used for semen processing is an option worth considering minimizing damages during semen cryopreservation.

8 लघु सारांश

वीर्य प्लाज्मा न केवलइ मादा जननागों में शुक्राणु का परिवहन बढ़ाता है, वरन शुक्राणु की गतिशीलता व जननशीलता भी अपने कारकों से बनाये रखता है। शोध परिणाम दर्शाते हैं, कि वीर्य प्लाज्मा में उपस्थित कुछ प्रोटीन वीर्य हिमीकरण हेतु हानिकारक है। वीर्य प्लाज्मा में उपस्थित मुख्य प्रोटीन बोवाइन वीर्य प्लाज्मा प्रोटीन समुदाय के होते हैं जैसे बीएसपी–ए1/ए2 (पीडीसी–109), बीएसपी–ए3 एवं बीएसपी–30 किलो डाल्टन। पीडीसी–109, बीएसपी प्रोटीनों का मुख्य घटक है। ये वीर्य–स्खलन के समय शुक्राणु झिल्ली कोलीन फास्फोलिपिड व उच्च घनत्व लाइपोप्रोटीनों से युक्त हो एचडीएल द्वारा कराये गये शुक्राणु अग्रपिडल अभिक्रिया को बढ़ा देते हैं। बी.एस.पी. प्रोटीन कोलेस्टेरॉल व फास्फोलिपिड को सान्द्रण व समय आधारित तरीके से वाह्य निर्गम कर शुक्राणु प्लाज्मा झिल्ली में परिवर्तन कर देती है जिससे तरल अथवा हिमीकृत अवस्था में संवेदनशील हो जाती है। कोलेस्टेराल व फास्फोलिपिड का बाहर निकलना शुक्राणु के एक्रोसोम को क्षतिग्रस्त करता है। अतः यह सोचा गया, कि बीएसपी प्रोटीन के मुख्य अवयव पीडीसी–109 का पृथक्करण शुक्राणु को होने वाले हानिकारक प्रभाव को न्यूनतम कर सकता है। वर्तमान अध्ययन पीडीसी–109 के पृथककरण का प्रभाव शुक्राणु के भौतिक व आकास्की संबंधी हिमन पूर्व व पश्चात गुणों पर ज्ञात करने हेतु व तरलीकरण पश्चात शुक्राणु की विजातीय अण्डाणु से युक्त होने की क्षमता पर प्रभाव ज्ञात करने हेतु किया गया। संकर साड़ों से वीर्य संग्रतीत कर प्लाज्मा से एफिनिटी व आयन एंक्सचेज क्रोमेटोग्राफी द्वारा पीडीसी–109 का पृथक्कीकरण किया गया। एसडीएस पेज द्वारा पीडीसी–109 की शुद्धता आंकी गई व इसको खरगोशों में एन्टीसीरम तैयार करने हेतु प्रयुक्त किया गया। आयन एक्सचेंज क्रोमोटोग्राफी द्वारा एन्टी सीरा से पीडीसी–109 के विरूद्ध एन्टीबाडीज प्रथक्कृत की गई तथा वीर्य नमूनों में प्रोटीन के साथ 1:1 अनुपात में प्रयुक्त की गई। प्रयोग के लिए 4 समूह बनाये गये1. नियत्रंण 2. ईवाईटीजी में सीधे सग्रंहण 3. पीडीसी109 विरूद्ध एन्टीबॉडी युक्त परखनली में संग्रहण तत्पश्चात ईवाईटीजी उपचार 4. पीडीसी–109 विरूद्ध एन्टीबॉडी युक्त किन्तु ईवाई रहित तनुकारक। परिणामों से ज्ञात हुआ कि एकल गतिशीलता, जीवितता, एचओएस प्रभाव व एक्रोसोम की अक्षुण्णता का प्रतिशत उन उपचार समूहों में अधिक था जहाँ पीडीसी–109 का पृथक्करण एन्टीबॉडी मिलाकर किया गया था। समूह 3 में पीडीसी109 पृथक्करण का प्रभाव शुक्राणु से कोलेस्टेराल निर्गत करने में कमी के रूप में पाया गया, जो लाभदायक है। चारों समूहों के शुक्राणुओं में सीटीसी फ्लूरोसेन्ट पैसे द्वारा हिमन पूर्व व तरलीकरण पश्चात अन्तः पात्ते कैपेसिटेशन व एक्रोसोम क्रिया मापी गई। यह देखा गया कि पीडीसी–109 द्वारा समूह 3 में नियंत्रण को अधिक सख्या में अधपिडंक अभिक्रिया रहित शुक्राणु पाये गये (59.67±1.31 vs 28.83±1.21प्रतिशत) ऐसी ही वृद्धि तरलीकरण पश्चात भी ज्ञात हुई। समूह 3 में हिमन पूर्व व तरलीकरण पश्चात एक्रोसोम प्रतिक्रिया युक्त शुक्राणुओं का प्रतिशत अन्य समूहों की अपेक्षा कम ज्ञात हुआ। अध्ययन के परिणाम दर्शाते हैं कि वीर्य नमूनों में पीडीसी–109 प्रथक्करण का शुक्राणु पर प्रभाव रक्षात्मक है व हिमीकरण की क्रिया के पश्चात भी यह बना रहता है। विजातीय अन्तः पात्रे निषेचन के परिणाम दर्शाते हैं कि वीर्य पीडीसी–109 पृथक्करण का समूह 3 में नियत्रंण की अपेक्षा बांइडिग इन्डेक्स बढ़ाने में घनात्मक प्रभाव रहा। यद्यपि चारों समूहो में बाइड़िग प्रतिशत पर कोई प्रभाव ज्ञात नहीं हुआ। अतः यह स्पष्ट हुआ कि वीर्य नमूनों से पीडीसी–109 पृथक्करण द्वारा जोना पैलुसिडा ग्लाइकोप्रोटीन से युक्त होने की उपलब्ध मुक्त पीडीसी–109 की क्षमता घटी व उच्च पेनिट्रेशन इन्डेक्स दर प्राप्त हुई।

अध्ययन द्वारा ज्ञात हुआ, कि पीडीसी–109 हिमीकरण क्रिया में शुक्राणु प्लाज्मा झिल्ली का स्थायित्व नष्ट करने हेतु उत्तरदायी है व इसका पृथक्कीरण शुक्राणु को सुरक्षा प्रदान करता है। पीडीसी–109 विरूद्ध एन्टीबाडीज वीर्य तनुकारकों में मिलाकर वीर्य हिमन द्वारा उत्पन्न शुक्राणु क्षतियों को न्यूनतम किया जा सकता है।

9 References

Agarwal, R.K. (1997). Studies on the effect of certain biological fluids on *in vitro* fertilizing ability of buffalo spermatozoa. M.V. Sc. Thesis submitted to IVRI, Izatnagar, U.P.

Agarwal, S. K. Shanker, U. and Nautiyal, L. P. (1987). Physical characteristics of 3/4th crossbred bull semen. *Indian Vet. Med. J.***11**: 59-60.

Agarwal, Y. and Vanha-Pertulla, T. (1987). Effects of secretory particles in bovine seminal vesicles secretions on sperm motility and acrosome reaction. *J. Reprod. Fertil.,* **29:** 389-399.

Aguero, A., Miragaya, M.H., Mora, N.G., Chaves, M.G., Nield, D.M. and Beconi, M.T. (1994). Effect of vitamin E addition on equine sperm preservation. *Communications Biologicas,* **13:** 343-356.

Ahzar, M., Liweilte, Mary, M., Buhr, Thomas, G., Kroetsch and Peuls, P. (2002). Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. *Biol. Reprod.,* **66:** 354-360.

Alvarej, J.G. and Storey, B.T. (1983). Role of super Oxide dismutase in protecting rabbit spermatozoa from O_2 toxicity due to lipid peroxidation**.** *Biol. Reprod*. **28**: 1129: 1136.

Alvarez, J.G. and Storey, B.T. (1992). Evidence for increased lipid peroxidative damage and loss of superoxide in human spermatozoa. Superoxide dismutase as major enzyme protectant against oxygen toxicity. *J. Androl.,* **8:** 338-348.

Amann, R.P., Seidel, G.E. and Brink, Z.A. (1999). Exposure of thawed frozen bull sperm to a synthetic peptide before artificial insemination increases fertility. *J. Androl*. **20:** 42-46.

Anbazhagan, V. and Swamy, M.J. (2005). Thermodynamics of phosphorycholine and lysophosphatidycholine binding to the major protein of bovine seminal plasma, PDC-109. *FEBS lett.*, **579**: 2933-2938.

Anchrodoguy, T.J., Rudolph, A.S., Carpenter, J.F. and Crowe, J.H. (1987). Modes of interaction of cryoprotectants with membrane phospholipids during freezing. *Cryobiology*, **24:** 324-331.

Aranasamy, A. (2003). Isolation of buffalo seminal plasma proteins and their effect on *in vitro* capacitation, acrosome reaction and fertilizing potential of spermatozoa. Ph. D. Thesis submitted to IVRI.Izatnagar.

Arangasamy, A., Singh, L.P., Ahmed, N., Ansari, M.R. and Ram, G.C. (2005). Isolation and characterization of heparin and gelatin binding buffalo seminal plasma proteins and their effect on cauda epididymal spermatozoa. *Anim. Reprod.Sci.*, **90**: 243-254.

Aumuller, G. and Scheit, K. (1987) Immunohistochemistry of secretory proteins in the bull seminal vesicle. *J. Anat.*, **150**: 43-48.

Aumuller, G. Vesper, M., Seitz, J., Kemme, M. and Scheit, K-H. (1988). Binding of a major secretory protein from bull seminal vesicles to bovine spermatozoa *Cell Tissue Res.*, **252**: 377-384.

Austin, C.R. (1951). Observation on the penetration of the sperm into the mammalian egg. *Aust. J. Sci. Res.*, **B-4**: 581-596.

Avery, S., Bolton, V., Mason, B.A. and Mills, C. (1988). The use of hypo-osmotic sperm swelling test as an indicator of IVF outcome. *Human Reprod.*, **3**:94.

Avery, S., Bolton, V.N. and Mason, B.A.(1990). An evaluation of the hypo osmotic swelling test as a predictor of fertilizing capacity *in vitro* .*Int. J.Androl.*, **13**: 93-99.

Bailey, J.L. and Buhr, M.M. (1993). The calcium control of fresh and cryopreserved bovine spermatozoa in response to A23187. *Cryobiology*, **30:** 470-481.

Bailey, J.L., Bilodeau, J.F. Cormier, N. (2000). Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. *J. Androl.* **21**: 1-7.

Bailey, J.L., Robertson, L. and Buhr, M.M. (1994). Caclium regulation, computerized motility parameters and fertility of bovine spermatozoa. *Can. J. Anim. Sci.*, **74:** 53-58.

Batova, I., Mollova, M. and Ivanova, M. (1993). Involvement of human seminal plasma glycoprotein defined by a peptide specific monoclonal antibody in the process of

sperm capacitation and sperm zona interaction. *Human Reprod. Suppl.* **1:** 88, Abstract, 255.

Baumber, J., Ball, B.A., Gravance, C.G., Medina, V. and Davies-Morel, M.C.G. (2000). The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential and membrane lipid peroxidation. *J. Andrology*, **21:** 895-902.

Bedford, J.M. (1975). Maturation, transport and fate of spermatozoa in the epididymis. In: Handbook of physiology, Sec. 7, Vol. 5, Male Reproduction (D.W. Hamilton and R.O. Greep, eds), Amer. Physiol. Soc., Bethesda, M.D. pp.303-317.

Bellin, M.E. Hawkins, H.E., Oyarzo, J.N., Vanderboom, R.J. an Ax, R.L. (1995). Monoclonal antibody detection of heparin-binding proteins on sperm corresponds to increased fertility of bulls. *J. Anim. Sci.*, **74**: 173-182.

Bellin, M.E. Oyarzo, J.N., Hawkins, H.E., Zhang, H.M., Smith, R.G., Forest, D.W., Sprott, L.R. and Ax, R.L. (1988). Fertility-associated antigen on bull sperm indicates fertility potential. *J. Androl. Sci.*, **76**: 2032-2039.

Bellin, M.E., Hawkins, H.E. and Ax, R.L. (1994). Fertility of range beef bulls grouped according to presence or absence of heparin binding proteins in sperm membranes and seminal fluid. *J. Androl. Sci.*, **72**:2441-2448.

Bergeron, A., Crete, M.H., Brindle, Y. and Manjunath, P. (2004). Low-density lipoprotein fraction from hen's egg yolk decreases the binding of the major proteins of bovine seminal plasma to sperm and prevents lipid efflux from the sperm membrane. *Biol.Reprod.* **70**: 708-717.

Bhoite, U.Y., Sutar, D.A. and Ulmek, B.R. (2008). Studies on semen quality of crossbred bulls. *Indian Vet. J.*, **85:** 395-397.

Bhosrekar, M.R., Char, S.N., Patil, B.R. and Ranganekar, D.V. (1984). Effect of thawing temperature and time on the forward motility, live count and acrosome maintenance of bovine spermatozoa. *Ind. J. Anim. Sci.*, **54:** 1126.

Blighs, E.G. and Dyer, W.J. (1949). A rigid method of total lipid extraction and purification. *Can J. Biochem. Physiol.*. **37**: 911-917.

Boisvert, M., Bergeron, A., Lazure, C. and Manjunath, P. (2003). Isolation and characterization of gelatin binding bison seminal vesicle secretory proteins. *Biol. Reprod.,* **70:** 651-661.

Borgohain, B.N. (1984). Studies on the reproductive soundness of breeding bulls. Ph.D. Thesis, IVRI, Izatnagar, (U.P.).

Brahmkshri, B.P., Edwin, M.J., John, M.C., Nainar, A.M. and Krishnan, A.R. (1999). Relative efficacy of conventional sperm parameters and sperm penetration bioassay to assess bull fertility *in vitro*. *Anim. Reprod. Sci.*, **54:** 159-168.

Branton, C. (1946). Physiology and morphology of spermatozoa from different levels of the reproductive tract of the bull. M.S. Thesis, Cornell Univ. In: Physiology, of Reproduction and Artificial Insemination of Cattle. Second Edn. 1978. Eds G.W. Salisbury, N.L. Van-Demark and J.R. Lodge, W.H. Freeman and Co.San Francisco. Pp. 224-225.

Brewis, I.A. and Moore, H.D.M. (1997). Molecular mechanisms of gamete recognition and fusion at fertilization. *Hum. Reprod. Natl. Suppl. J.*, BFS, **2:** 156-165.

Buhr, M.M., Canvin, A.T. and Bailey, J.L. (1989). Effects of semen preservation on boar spermatozoa head membranes. *Gamete Res.*, **23:** 441-449.

Calvete, J.J., Mann, K., Sanz, L., Raida, M. and Topfer-Petersen, E. (1996a). The primary structure of BSP-30 kDa, a major lipid-, gelatin-, and heparin-binding glycoprotein of bovine seminal plasma. FEBS Lett., **399:** 147-152.

Calvete, J.J., Mann, K., Schafer, W., Sanz, L., Reinert, M., Nessau, S., Raida, M. and Topfer-Petersen, E. (1995a). Amino acid sequence of HSP-1, a major protein of stallion seminal plasma: effect of glycosylation on its heparin- and gelatin-binding capabilities. *Biochem. J.*, **310:** 616-622.

Calvete, J.J., Raida, M., Gentzel, M., Urbanke, C., Sanz, L., Topfer-Petersen, E. (1997). Isolation and characterization of heparin- and phosphorylcholine-binding proteins of boar and stallion seminal plasma: primary structure of porcine pB1. FEBS Lett., **407:** 201-206.

Calvete, J.J., Raida, M., Sanz, L., Wempe, F., Scheit, K.H., Romero, A. and Topfer-Petersen, E. (1994). Localization and structural characterization of an oligosaccharide O-linked to bovine PDC-109. Quantitation of the glycoprotein in seminal plasma and on the surface of ejaculated and capacitated spermatozoa. *FEBS Lett.*, **350:** 203-206.

Calvete, J.J., Sanz, L., Reinert, M., Dostalova, Z., Topfer-Petersen, E. (1995 b). Heparin-binding proteins on bull, boar, stallion, and human spermatozoa. In: Jamieson BGM, Ausio, J. and Justine J.L. (Eds), Advances in Spermatozoal Phylogeny and Taxonomy, 166. Paris : Memoires du Museum National D'Histoire Naturelle, 515-524.

Calvete, J.J., Topfer-Petersen E. And Sanz, L. (1996 b). Spermadhesin-A novel class of mammalian lectins? In Lectins: Biology, Biochemistry, Clinical Biochemistry. (E. van Driessche, P. Rouge, C. Beeckmans, T.C. Bog-Hansen, Eds). Textop, Helkrup, 185-192.

Cardin, and Weintraub, H.J. (1989). Molecular modeling of protein-glycosaminoglycan interactions. *Arteriosclerosis,* **9:** 21-32.

Cerolini, S., Maldjan, A., Pizzi, F. and Ghozzi, T.M. (2001). Changes in sperm quality and lipid composition during cryopreservation of goat semen. *Reproduction,* **121:** 395-401.

Chakrabarti, S.K., Hakeem, P.M.A. and Arun, S. (2005). 17th livestock census reports. Govt. of India (www.dahd.nic.in).

Chan, J.P., Tredway, D.R., Pang, S.C., Coresill, J.U. and Su, B.C. (1992). Assessment of sperm for cryopreservation using the hypo-osmotic viability test. *Fert. Steril.* **58(4):** 841-844.

Chandonnet, L., Roberts, K.D., Chapdelaine, A. and Manjunath, P. (1990). Identification of heparin-binding proteins in bovine seminal plasma. *Mol. Reprod. Dev.,* **26**: 313-318.

Chang, M.C. (1951), Fertilizing capacity of spermatozoa deposited into fallopian tubes *Nature*. **168**: 607-608.

Chauhan, M.S., Singla, S.K., Manik, R.S. and Madan, M.L. (1997). Increased capacitation of buffalo sperm by heparin as confirmed by electron microscopy and *in vitro* fertilization. *Indian J. Exp. Biol.,* **35:** 1038-1043.

Check, M.L. and Check, J.H. (1991). Poor hypo-osmotic swelling test results from cryopreserved sperm despite preservation of sperm motility. *Arch. Androl.*, **26:** 37-41.

Cheng, Feng-Pang, Fazeli, Alireza, Voorhout, W.F., Marks,,A., Bevers, M.M. and Colenbrander, Ben (1996). Use of peanut agglutinin to assess the acrosomal status and the zona pellucida-induced acrosome reaction in stallion spermatozoa. *J. of Androl.*, **17(6)** :674-682.

Clegg, E.D. and Foote, (1973). *J. Reprod. Fertil.*, **34:** 379-388.

Colligan, J.E., Kruisbeck, A.M., Mangulies, D.H., shevach, E.M. and strobe, W. (1992).Production of antibodies. In: Current protocols in Immunology. Vol.I. pp.201-253.

Collin, S., Sirard, M.A., Dufour, M., Bailey, J.L. (2000). Sperm calcium levels and chlortetracycline fluorescence patterns are related to the *in vivo* fertility of cryopreserved bovine semen. *J. Androl.,* **21:** 938-943.

Combes, G.B., Varner, D.D., Schroeder, F., Burghardt, R.C. and Blanchart, T.L. (1998). Effects of supplemental cholesterol on post thaw motility and plasma membrane integrity of equine spermatozoa. In Proc. of the 7th Intt. Symp. on Equine Reprod. Pretoria, South Africa, pp. 35-36.

Cormier, N. and Bailey, J.L. (2003). A differential mechanism involved during heparin and cryopreservation induced capacitation of bovine spermatozoa. *Biol. Reprod.,* **69:** 177-185.

Cormier, N., Sirard, M.A. and Bailey, J.L. (1997). Premature capacitation of bovine spermatozoa is initiated by cryopreservation. *J. Androl.,* **18(4):** 461-468.

Cox, J.F., Catalan, A., Saravia, F., Avila, J. And Maria, A.S. (1994). *In vitro* fertilization of cattle and sheep follicular oocytes by goat spermatozoa. *Small Rum. Res.,* **15:** 55-58..

Cross, N.L. (1996). Effect of cholesterol and other sterols on human sperm acrosomal responsiveness. *Mol. Reprod. Dev.,* **45:** 212-217.

Cross, N.L. and Watson, S.K. (1994). Assessing acrosomal status of bovine sperm using fluoresceinated lectins. *Theriogenology*. **42**; 89-98.

Cross, N.L., Morales, P., Overstreet, J.W. and Hanson, F.W. (1986). Two simple methods for detecting acrosome-reacted human sperm. *Gamete Res.,* **15**: 213-226.

Crowe, J.H. and Clegg, J.S. (1987). Dry biological systems. Academic Press, New York, **88:** 287-288.

Crowe, J.H., Crowe, L.M., Chapman, D. (1984). Preservation of membrane in hydro biotic organisms: the role of trehalose. *Science* **223:** 701-703.

Curry, M.R. (2000). Cryopreservation of semen from domestic livestock *Rev. Reprod.* **5:**46-52.

Dandekar, S.P., Nadkarni, G.D., Kulkarni, V.S. and Punekar, S. (2002). Lipid peroxidation and antioxidant enzymes in male infertility. *J. Post Graudate. Med.* **48:** 186-189.

Darin-Bennett, A. and White, I.G. (1977). Influence of the cholesterol content of mammalian spermatozoa on susceptibility to cold shock. *Cryobiology,* **14:** 466-477.

Darin-Bennett, A., Poulos, A. and White, I.G. (1973). The effects of cold shock and freeze-thawing in release of phospholipids by ram, bull and boar spermatozoa. *Aust. J. Biol. Sci.,* **26:** 1409-1420.

Davis, A.P., Graham, J.K. and Foote, R.H. (1987). Homospermic versus heterospermic insemination of zona-free haemster eggs to assess fertility of fluorochromelabelled acrosome-reacted bull spermatozoa. *Gamete Res.,* **17:** 343-354.

Davis, B.K. (1975). Inhibition of fertilizing capacity in mammalian spermatozoa by natural and synthetic vasicles. *Symp. Pharmacol. Effect Lipids,* **42:** 145-157.

Davis, B.K. (1980). Interaction of lipids with the plasma membrane of sperm cells. I. The antifertilization action of cholesterol. *Arch. Androl.,* **5:** 249-254.

Davis, B.K. (1981). Timing of fertilization in mammals: sperm cholesterol/phospholipids ratio as a determinant of the capacitation interval. *Proc. Natl. Acad. Sci.,* USA, **78:** 7560-7564.

Davis, B.K., Byrne, R. and Bedigian, K. (1980). Studies on the mechanism of capacitation: albumin-mediated changes in plasma membrane lipids during *in vitro* incubation of rat sperm cells. *Proc. Natl. Acad. Sci.,* USA **77:** 1546-1550.

De Leeuw, F.E., De Leeuw, A.M., Den Dass, J.H., Colenbraader, B. and Verkleij, A.J. (1993). Effects of various cryoprotective agents and membrane stabilizing compounds on bull sperm membrane integrity after cooling and freezing. *Cryobiology,* **30:** 32-44.

De mott, R.P. and Suarez, S.S. (1993). Hyper activated sperm progression in the mouse oviduct. *Biol. Reprod.* **46**: 779-785.

Del Sorbo, C., Fasano, G., Fabbrocini, A., Lubrano, L.S. and Sansone, G. (1995). Piruvato quale substrato energetico in extenders crioprotettivi. Effetti sulla motilia allo scongelamento di spermatozoi bufalini *Bubalis bubalis.* Proc. 7th Meeting Nazionale "Studio sulla efficienza riproducttiva degli animalia di interesse zootecnico". Bergamo, Italy vol. 1, pp. 585-588.

De-Leeuw, F.E., Colenbrander, B. and Verkleji, A.J. (1991). The role membrane damage plays in cold shock and freezing injury. In Johnson, I.A., Rath, D., eds., *Boar Semen Preservation II. Berlin*: Paul Parey Scientific Publishers **95**: 104.

Desnoyers, L. and Manjunath, P. (1992). Major proteins of bovine seminal plasma exhibit novel interaction with phospholipids. *J. Biol. Chem.,* **267**: 10149-10155.

Desnoyers, L., and Manjunath, P. (1993). Interaction of a novel class of phospholipids binding proteins of bovine seminal fluid with different affinity matrices. *Arch. Biochem. Biophys.,* **305**: 341-349.

Dhami, A.J. and Sahni, K.L. (1994a). Effects of various cooling (from 30°C to 5°C), equilibrium and diluent treatments on freezability, post-thaw thermo resistance, enzyme, leakage and fertility of bubaline spermatozoa. *Buffalo J.,* **2**: 147-159.

Dhami, A.J. Jani, V.R., Mohan, G. and Sahni, K.L. (1994b). Effect of extenders and additives on freezability, post thaw thermo resistance and fertility of frozen Murrah buffalo semen under tropical climate. *Buffalo.J.,* **1:**35-45.

Dostalova, Z., Calvete, J.J., Sanz, L., Topfer-Petersen, E. (1995). Boar spermadhesin AWN-1. Oligosaccharide and zona pellucida binding characteristics. *Eur. J. Biochem.,* **230:** 329-336.

Dott, H.M., Harrison, R.A.P. and Foster, G.C.A. (1979). The maintenance of motility and surface protperties of epididymal spermatozoa from bull, rabbit and ram in homologous seminal and epididymal plasma. *J. Reprod. Fertil.,* **55**: 113-124.

Douard, V., Hermier, D. and Blesbois, E. (2000). Changes in turkey semen during liquid *in vitro* storage. *Biol. Reprod..,* **63:** 1450-1456.

Drevius, L.O. (1971). Permeability coefficients of bull spermatozoa for water and polyhydric alcohols. *Exp. Cell Res.,* **69**:212-216.

Drevius, L.O. and Eriksson, H. (1996). Osmotic swelling of mammalian spermatozoa. *Ezp. Cell Res.,* **42**: 136-156.

Duru, N.K., Morshedi, M.S., Schuffner, A. and Oehninger, S. (2001). Cryopreservation thawing of fractionated human spermatozoa is associated with membrane phosphatidyl serine externalization and not DNA fragmentation. *J. Androl.,* **22:** 646-651.

Ehrenwald, E., Foote R.H. and Parks, J.E. (1990). Bovine oviduct fluid components and their potential role in sperm cholesterol efflux. *Mol. Reprod. Dev.,* **25:**195-204.

Ehrenwald, E., Parks, J.E. and Foote, R.H. (1988b). Cholesterol efflux from bovine sperm : II. Effect of reducing sperm cholesterol on penetration of zona free hamster and *in vitro* matured bovine ova. *Gamete Res.,* **20:** 413-420.

Ehrenwald, E., Parks, J.E. and Foote, R.H. (1988a). Cholesterol efflux from bovine sperm I. Induction of acrosome reaction with lysophosphatidylcholine after reducing sperm cholesterol. *Gamete Res.,* **20**: 145-157.

Ephen, J.K. (1961). Effect of egg yolk levels and rate of freezing on survival of bovine spermatozoa after storage. *Ind. Vet. J.*, **38:**508-511.

Esch, F.S., Ling, N.C. Bohlen, P., Ying, S.Y. and Guillemin, R. (1983). Primary structure of PDC-109, a major protein constituent of bovine seminal plasma. *Biochem. Biophys. Res. Commun.*, **113**:861-867.

Fazeli, A., Duncan, A.E., Watson, P.F. and Holt, W.V. (1999). Sperm-oviduct interaction: Induction of capacitation and preferential binding of uncapacitated spermatozoa to oviduct epithelial cells in porcine species. *Biol. Reprod.*, **60**:879-86.

Fazeli, A.R., Steenweg, W., Bevers, M.M., de Loos, F.A.M., Van den Broek, J. and Colenbrander, B. (1993). Development of sperm zona pellucida binding assay for bull semen. *Vet. Rec.*, **132:**14-16.

Feng, H.M., Wen, R.Q., Wang, Q.H., Jiang, Y.J. and Li, O.K. (1995). Proteins in fluid from the proximal vas deferens of normal fertile and vasectomised men. *International. J. Androl.*, **18**:63-66.

Fiol de Cuneo, M., Vincenti, L.M., Martini, A.C., Ponce, A.A. and Ruiz, R.D. (2004). Effects of PDC-109 on bovine sperm functional activity in presence or absence of heparin. *Theriogenology*, **62(1-2**): 207-216.

Fleming, A.D. and Yanagimachi, R. (1981). Effects of various lipids on the acrosome reaction and fertilizing capacity of guinea pig spermatozoa with special reference to the possible involvement of lysophospholipids in the acrosome reaction. *Gamete. Res.*, **4:** 253-273.

Florman, H.M., and First, N.L. (1988). The regulation of acrosomal exocytosis. II. The zona pellucida-induced acrosome reaction of bovine spermatozoa is controlled by extrinsic positive regulatory elements. *Dev. Biol.*, **128:**464-73.

Florman, H.M., Tombes, R.M., First, N.L. and Babcock, D.F. (1989). An adhesion associated agonist from the zona pellucida activates G protein promoted elevations of internal Ca^{2+} and pH that mediate mammalian sperm acrosomal exocytosis. *Dev. Biol.*, **135**: 33-146.

Foote, R.H. (1970). Influence of extender, extension, and rate and glycerolating technique on fertility of frozen bull semen. *J. Dairy Sci.*, **53:**1478.

Fraser, L.R. (1982). Ca^{2+} is required for mouse sperm capacitation and fertilization *In vitro*. *J. Androl.*, **3:**412-419.

Fraser, L.R. (1995).Ionic control of sperm function. *Reprod. Fertil. Dev*. **7**: 905-925.

Fraser, L.R., Abeydeera, LR. and Niwa, K. (1995). Regulating mechanisms that modulate bull sperm capacitation and acrosomal exocytosis as determined by chlortetracycline analysis. *Mol.Reprod. Dev.* **40**:233-241.

Fuller, S.J. and Whittingham, D.G. (1996). Effect of cooling mouse spermatozoa to 4^{o}C on fertilization and embryonic development. *J. Reprod. Fertil.,* **108:** 139-145.

Fuller, S.J. and Whittingham, D.G. (1997). Capacitation-like changes occur in mouse spermatozoa cooled to low temperature. *Mol. Reprod. Dev.,* **46:** 318-324.

Gadella, B.M., Rathi, R., Brouwers, J.F.H.M., Stout, T.A.E. and Colenbrander, B. (2001). Capacitation and acrosome reaction in equine spermatozoa. *Anim. Reprod.Sci*. **68**: 249-265.

Galantino-Homer, H.L., Visconti, P.E. and Kopf, G.S. (1997). Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3959-monophosphate-dependent pathway. *Biol. Rerprod.,* **56:** 707-719.

Galli, A., Basetti, D., Martigoni, M., Bornaghi, V. and Mofill, M. (1991). Frozen bovine semen quality and bovine cervical mucus penetration test. *Theriogenology*, **35(4)**:837-844.

Garner, D.L. and Johnson, L.A. (1995). Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. *Biology of Reproduction*. **53**:276-84.

Gasset, M, Magdaleno, L. and Calvete J. J. (2000). Biophysical study of the perturbation of model membrane structure caused by seminal plasma protein PDC-109. *Arch. Biochem. Biophys.,* **374(2):**241-7.

Gasset, M., Saiz, J.I., Laynez, J., Sanz, L., Gentzel, M., Peterson, E.T. and Calvete, J.J. (1997). Conformational features and thermal stability of bovine seminal plasma protein PDC-109 oligomers and phosphorycholine-bound complexes. *Eur. J. Biochem.,* **250:** 735-744.

Gebreselassie, G.A. (2009). Studies on physico-morphological characteristics and certain enzymes in freezable and non-freezable semen ejaculates in cross bred bulls. M.V.Sc. thesis, submitted to Deemed University, IVRI, Izatnagar, India.

Gerwig, G.L., Calvete, J.J., Topfer-Peterson, E. and Vliegenthart, J.F. (1996). The structure of the O-linked carbohydrate chain of bovine seminal plasma protein PDC-109 revised by H-NMR spectroscopy: a correction. *FEBS Lett.*,**387:**99-100.

Gillan, L., Evans, G. and Maxwell, W.M.C. (1997). Capacitation status and fertility of fresh and frozen-thawed ram spermatozoa. *Reprod. Fertill. Dev.,* **9:**481-487.

Go, K.J. and Wolf, D.P. (1985). Albumin-mediated changes in sperm sterol content during capacitation. *Biol. Reprod.,* **32:** 145-153.

Gopal Krishna, T. and Rao, A.R. (1978). Semen characteristics in Murrah buffalo bulls. *Indian. Vet.J.,* **55**:216.

Graham, J.K., Kune, E and Hamersledt, R.H. (1990). Analysis of germ cell viability, acrosomal integrity and mitochondrial function using flow cytometry. *Biol. Reprod.* **43:**55-64.

Greube, A., Muller, K., Topfer-Peterson, E., Herrmann, A. and Muller, P. (2001). Influence of the bovine seminal plasma protein PDC-109 on the physical state of membranes. *Biochemistry,* **40:** 8326-8334.

Griffin, J.F., Nunn, W.R. and Hartigan, P.J. (1971). An immune response to egg yolk semen diluent in dairy cows. *J. Reprod. Fertil.,* **25:** 193-199.

Gualtieri, R. and Talevi, R. (2003). Selection of highly fertilizable component bovine spermatozoa through adhesion to the fallopian tube epithelium *in vitro*. *Reproduction,* **125:**251-258.

Gwathmey, T.M. Ignotz, G.G. and Suarez, S.S. (2003). PDC-109 (BSP-A1/-A2) promotes bull sperm binding to oviductal epithelium *in vitro* and may be involved in forming the oviductal sperm reservoir, *Biol. Reprod.,* **69:**809-815.

Hammerstedt, R.H. and Andrews, J.C. (1997). Metabolic support of normothermia. In Karow, A.M., Critser, J.K. (Eds), Reproductive Tissue Banking. San Diego Academic Press. Pp, 139-166.

Hancock, J.L. (1952). The spermatozoa of sterile bulls. *J. of Exptl. Biol.,* **30**: 50-56.

Harkema, W., Harison, R.A.P., Miller, N.G.A., Topper, E.K. and Woolders, H. (1998). Enhanced binding of zona pellucida proteins to the acrosomal region of intact spermatozoa in response to fertilizing conditions: a flow cytometric study. *Biol. Reprod.* **58**:421-430.

Harrison, R. A.P. and Vickers, S.E. (1990) Use of fluorescent probes to asses membrane integrity in mammalian spermatozoa *J. Reprod. Fert.,* **88**:343-352.

Harrison, R.A.P. ((1996). Capacitation mechanisms and the role of capacitation as seen in entherian mammals. *Reprod. Fertil. Dev.,* **8:** 581-594.

Harshan, H.M. (2007). Modulation of structural and functional properties of buffalo cauda epididymal spermatozoa by sperm binding seminal plasma protein (PDC-109). Ph.D. Thesis submitted to IVRI, Izatnagar.

Harshan, H.M., Singh, L.P., Arangasamy, A., Ansari, M.R. and Kumar, S. (2006). Effect of buffalo seminal plasma heparin binding protein (HBP) on freezability and *in vitro* fertility of buffalo cauda spermatozoa. *Ani. Reprod. Sci.,* **93 :**(1-2): 124-133.

Hartree, F. and Srivastava, P.N. (1965). Chemical composition of ram spermatozoa. *J. Reprod. Fertil.,* **9:**47-60.

Haryama, H., Miyano, T., Miyake M, Kusunoki, H. and Kato, S. (1994). Identification of anti-agglutinin for spermatozoa in epididymal boar plasma. *Mol. Reprod. Dev.* **37:**436-445.

Hazel, J.R. (1995). Thermal adaptation in biological membranes: is homeoviscous adaptation the explanation. *Ann. Rev. Physiol*. **11:** 73-88.

He, L., Bailey, J.L. and Buhr, M.M. (2001). Incorporating lipids into boar sperm decreases chilling sensitivity but not capacitation potential. *Biol. Reprod.,* **64:** 69-74.

Heeres, A.A., Merton, J.S., Hazerlegar, W. Van, Wagtendonk-de Leeuw, A.M., Komp, B. (1996). Optimization of sperm-oocyte ratio during IVF of bovine cumulus-oocytes-complexes. *Theriogenology* **45:**266.

Henault, M.A. and Killian, G.J. (1996). Effect of homologous and hererologous seminal plasma on the fertilizing ability of ejaculated bull spermatozoa assessed by penetration of zona free bovine oocytes. *J. Reprod. Fertil.,* **108:**199-204.

Henault, M.A., killian, G.J., Kavanaugh, J.F. and Griel, L.C. (1995). Effect of accessory sex gland fluid from bulls of different fertilities on the ability of cauda epididymal sperm to penetrate zones-free ovine oocytes. *Biol. Reprod.* **52:**390-397.

Holt, W.V. (2000). Basic aspects of frozen storage of semen. *Ani.Reprod. Sci*. **62:** 3-22.

Holt, W.V. and North, R.D. (1994). Effects of temperature and restoration of osmotic equilibrium during thawing on the induction of the plasma membrane damage in cryopreserved ram spermatozoa. *Biol. Reprod.,* **51:** 414-424.

Holt, W.W., Head, M.F. and North, R.D. (1992). Freeze-induced membrane damage in ram spermatozoa is manifested after thawing-observation with experimental cryomicroscopy. *Biol. Reprod.,* **46:** 1086-1094.

Iborra, A., Companyo, M., Martinez, P. and Morros, A. (2000). Cholesterol efflux promotes acrosome reaction in goat spermatozoa. *Biol. Reprod.,* **62:** 378-383.

Ignotz, G.G., Lo, M.C., Perez, C.L., Gwathmay, T.M. and Suarez, S.S.(2001). Characterization of Fucose-binding protein from bull sperm and seminal plasma that may be responsible for formation of the oviductal sperm reservoir. *Biology of Reprod.* **64**: 1806-1811.

Iqbal, N. and Hunte, A.G. (1995). Comparison of various bovine sperm capacitation systems for their ability to alter the net negative surface charge of spermatozoa. *J. Dairy Science,* **78:** 84-90.

Javed, M.T., Khan, A. and Kauser, R. (2000). Effect of age and season on some semen parameters of Nili-Ravi buffalo bulls. *Veterinarski-Archiv.,* **70:** 83-94.

Jeyendran, R.S., Holmgren, W.J. Bilefeld, P. and Wentz, A.C. (1992). Fertilizing capacity of various populations of spermatozoa within an ejaculate. *J. Assist. Reprod. Genet.,* **9:32**-35.

Jeyendran, R.S., Vander Ven, H.H., Parez-Pelaez, M., Crabo, B.G. and Zaneweld, L.J.D.(1984). Development of an assay to assess the functional integrity of the human membrane and its relationship to other semen characteristics. *J. Reprod. Fertil.,* **70**: 219-228.

Juan, M.F., Gallego, M., Barrios, B., Osada, J., Perez, J.C. and Blanco, T.M. (2006). Immunohistochemical localization of sperm preserving proteins in the ram reproductive tract. *J. Androl.,* **15:** 255-265.

Kadirvel, G. (2006). Studies on cryocapacitation of buffalo spermatozoa and its minimization. Ph.D. thesis submitted to deemed University IVRI, Izatnagar, U.P.

Kakar, S.S. and Anand, S.R. (1981). Changes in ATP, Adenhylate energy charge and AMP during the freezing of buffalo semen. *J. Reprod. Fertil.* **62:**543-548.

Kato, M., Sung, W.K., Kato, K. and Goodman, D.S. (1985). Immunohistochemical studies on the localization of cellular retinol binding protein in rat testes and epididymis. *Biol. Reprod.,* **32:** 172-189.

Katz, D.F., Overstreet, J.W. and Hanson, F.W. (1980). A new quantitative test for sperm penetration into cervical mucus. *Fertil. Steril.,* **33**: 179-186.

Khalil, A.A.Y., Petrunkina, A.M., Sahin, E., Waberski, D. and Topfer-Peterson, E. (2006). Enhanced binding of sperm with superior volume regulation to oviductal epithelium. *J. Androl..,* **27**(6): 754.

Khorasami, A.M., Cheung, A.P. and Lee, C.Y.G. (2000). Cholesterol inhibitory effects on human sperm-induced acrosome reaction. *J. Androl.,* **21:** 586-594.

Killian, G.J., Chapman, D.A. and Rogowski, L.A. (1993). Fertility associated proteins in Holstein bull seminal plasma. *Biol. Reprod.,* **49:**1202-1207.

Kimelberg, H.K. (1977). The influence of membrane fluidity on the activity of membrane-bound enzymes. IN: Poste, G., Niclolson, G.L. (eds), dynamic aspects of cell surface organization Vol. 3 Elsevier/North-Holland, Amsterdarm, pp. 205-293.

Kjaestad, H., Ropstad, E. and Andersen, B.K. (1993). Evaluation of spermatological parameters used to predict the fertility of frozen bull semen. *Acta Veterinariascandinavica,* **34:** 299-303.

Klein, V., Gimpl, G. and Fahrenholz, F. (1995). Alteration of the myometrial plasma membrane cholesterol content with β-cyclodextrin modulates the binding affinity of the oxytocin receptor. *Biochem.,* **34:** 13784-13793.

Koshinen, E., Junnila, M., Katila, T. and Saini, S. (1989). A preliminary study on the use of betaine as a cryoprotective agent in deep-freezing of semen. *Zentralbl. Veterinarmed. Reihe A.* **36:**110-114.

Kulkarni, B.A. (1985). Immunological studies on Indian buffalo and cattle. *Arch. Androl.,* **14**:231-238.

Kulkarni, B.A., Dhande, S.G. and Patnakar, D.D. (1995). A comparative study on seminal plasma protein profiles of the buffalo and cattle bulls. *Buffalo J.,* **1**: 125-129.

Kulkarni, B.A., Dhande, S.G. and Patnakar, D.D. (1996). Seminal plasma protein profiles of Holstein Friesian, Jersey and cross bred bulls. *Ind. J. Anim. Sci.,* **11**:133-137.

Kumar, A. (2005). Effect of seminal plasma heparin binding and non-heparin binding proteins on the freezability and *in vitro* fertility of buffalo cauda spermatozoa M.V.Sc. thesis submitted to deemed university, IVRI, Izatnagar.

Kumar, S. (1989). Studies on the effect of various levels of certain cryoprotectants on deep freezing of buffalo semen. Ph.D. Thesis Deemed Univ. IVRI, Izatnagar, pp.

Kumar, S., Sahni, K.L. and Mohan, G. (1992). Effect of different levels of glycerol and yolk on freezing and storage of buffalo semen in milk, tris and sodium citrate buffers. *Buffalo J.,* **2**:151-156.

Ladbroke, B.D., Williams, R.M. and Chapman, D. (1968). Studies on lecithin-cholesterol-water interactions by differential scanning calorimetry and X-ray diffraction. *Biochem. Biophys. Acta,* **150:** 333-340.

Laemmli, V.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London),* **227:**660-685.

Lambrechts, H., Van Nicherk, F.F., Coetzer, W.A., Clotete, S.W.D. and Van der Horst, G. (1999). The effects of cryopreservation on the survivability, viability and motility of epididymal African buffalo (*Syncercus caffer*) spermatozoa. *Theriogenology.*, **52(7):** 1241-1249.

Lane, M., Therien, I., Moreau, R., Manjunath, P. (1999). Haparin and high density lipoprotein mediate bovine sperm capacitation by different mechanisms. *Biol. Reprod.,* **60:** 169-175.

Langlais, j. and Roberts, K.A. (1985). A molecular membrane model of sperm capacitation and acrosome reaction of mammalian spermatozoa. *Gamete Res.,* **12:**183-224.

Lasley, J.F. and Bogart, R. (1942). A comparative study of epididymal and ejaculated spermatozoa of boar. *J. Anim. Sci.,* **3:**360-370.

Lasley, J.F. and Bogart, R. (1944). Some factors affecting the resistance of ejaculated spermatozoa of the boar to different environmental conditions. *Am. J. Physiol.* **141**: 619.

Lasley, J.F. and Mayer, D.T. (1944). A variable physiological factor necessary for the survival of bull spermatozoa. *J.Anim. Sci*.**3**:129-135.

Lasso, J.L., Noiles, E.E., Alvare, J.G. and Storey, B.T. (1994). Mechanism of superoxide dismutase loss from human sperm cells during cryopreservation. *J. Androl.,* **15:** 255-265.

Leblond, E., Desnoyers, L. and Manjunath, P. (1993). Phosphorycholine-binding proteins form the seminal fluids of different species, share antigenic determinants with the major proteins of bovine seminal plasma. *Mol. Reprod. Dev.,* **34:** 443-449.

Lee, C.N. and Ax, R.L. (1984). Concentration and composition of glycosaminoglycans in the female bovine reproductive tract. *J. Dairy Science,* **67:** 2006-2009.

Lee, C.N., Handrow, R.R., Lenz, R.W. and Ax, R.L. (1985). Interactions of seminal plasma and glycosaminoglycans on acrosome reactions in bovine spermatozoa *in vitro*. *Gamete Res.,* **12:** 345-55.

Lenz, R.W., Ax, R.L., Grimek, H.J. and First, N.L. (1982). Proteoglycan from bovine follicular fluid enhances an acrosome reaction in bovine spermatozoa. *Biochem. Biophys. Res. Commun.,* **106:** 1092-98.

Lin, Y. and Kan, F.W. (1996). Regionalization and redistribution of membrane phospholipids and cholesterol in mouse spermatozoa during in vitro capacitation. *Biol. Reprod.,* **55:** 1139-1146.

Lindhomer, C. (1974). The importance of seminal plasma for human sperm motility. *Biol. Reprod.,* **10:** 533-542.

Liu, J.M., Jin, Z.Q., Zhao, X.X. and Zhu, Y.D. (1991). The development of bovine follicular oocytes matured in different culture media. *Vet. Res. Commun.,* **15(4):** 270-280.

Lowry, O.H., Rosenberg, N.J. Farr, A.L. and Randall, R.J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.,* **193:** 265-275.

Loyi, T. (2008). Comparative studies on electrolytes and total seminal plasma protein between freezable and non freezable crossbred bull semen. M. V. Sc thesis submitted to the Deemed University, Indian Veterinary Research Institute, Izatnagar.

Lusignan, M.F., Bergeron, A., Crete, M.H., Lazure, C. and Manjunath, P. (2007). Induction of epididymal boar sperm capacitation by Pb1 and BSP-A1/-A2 proteins, members of the bsp protein family. *Biiol. Reprod.,* **76:** 424-432.

Majidi, J., Andolalizadeh, J., Amirkhiz, M.B. and Majidi, S. (2007). Production and purification of polyclonal antibody against bovine immunoglobulins in rabbits. *Afr. J. biotechnol*. **6 (12)**: 1369-1372.

Manjunath, P. (1984). Gonadotropin release and stimulatory and inhibitory proteins in bull seminal plasma. In: Sairam M.R., Atkinson, L.E. (Eds). Gonadal Proteins and Peptides and Their Biological Significance. *Singapore: World Scientific Publishing Company*, pp. 49-61.

Manjunath, P. and Sairam, M.R. (1987). Purification and biochemical characterization of major acidic proteins (BSP-A1, BSP-A2 and BSP-A3) from bovine seminal plasma. *Biochem. J.,* **241:** 685-692.

Manjunath, P. and Therien, I. (2002). Role of seminal plasma phospholipids binding proteins in sperm membrane lipid modifications that occurs during capacitation. *J. Reprod. Immunol.,* **53:** 109-119.

Manjunath, P., Baillargeon, L., Marcel, Y., Seidah, N., Chrétien, M., Chapdelaine, A. (1988). Diversity of novel proteins in gonadal fluids. In: Chrétien MMK (ed)., Molecular biology of brain and endocrine peptidergic systems. New York: Plenum Press, pp. 259-273.

Manjunath, P., Bergeron, A., Lefebvre, J. and Ton, J. (2008). Seminal plasma proteins : function and interaction with protective agents during semen preservation. *Biochemica et Biophysica Acta.,* **1784:** 891-899.

Manjunath, P., Chandonnet, E., Leblond, E. and Desnoyers, S.L. (1994). Major proteins of bovine seminal vesicles bind to spermatozoa. *Biol. Reprod.,* **50:** 27-37.

Manjunath, P., Chandonnet, E., Leblond, E. and Desnoyers, S.L. (1993). Major proteins of bovine seminal plasma bind to spermatozoa. *Biol. Reprod.,* **49:** 27-37.

Manjunath, P., Marcel, Y.L., Uma, J., Seidah, N.G., Chrétien, M. and Chapdelaine, A. (1989). Apolylipopotein A-I binds to a family of bovine seminal plasma proteins. *Biochem. J.,* **264:** 16853-16857.

Manjunath, P., Nauc, V., Bergeron, A., Menard, M. (2002). Major proteins of bovine seminal plasma bind to the low density lipoprotein fraction of hen's egg yolk. *Biol. Reprod.,* **67:** 1250-1258.

Manjunath, P., Sairam, M.R. and Uma, J. (1987). Purification of four gelatin binding proteins from bovine seminal plasma by affinity chromatography. *Biosci. Rep.,* **7:** 231-237.

Manjunath, P., Soubeyrand, S., Chandonnet, L. and Roberts, K.D. (1994). Major proteins of bovine seminal plasma inhibit phospholipase A_2. *Biochem. J.* **303:** 121-128.

Martinez, P. and Moros, A. (1996). Membrane lipid dynamics during human sperm capacitation. *Frontiers in Bioscience*, **1:** 103-117.

Maxwell, W.M. and Johnson, L.A. (1997). Chlortetracycline analysis of boar spermatozoa after incubation; flow cytometric sorting, cooling, or cryopreservation. *Mol. Reprod. Dev.,* **46:** 408-418.

Mayer, D.T. and Lasley, J.F. (1945). The factor in egg yolk affecting resistance, storage potentialities and fertilizing capacity of mammalian spermatozoa. *J.Animal Sci.* **4**:261-269.

Mazur, P. (1970). Cryobiology: the freezing of biological systems. *Science*, **168:** 939-949.

Mazur, P. (1984). Freezing of living cell: mechanisms and inplications. *Am. J. Physiol.*, **247:** C125-C142.

McCauley, T.C., Zhang, H.M., Bellin, M.E. and Ax, R.L. (1999). Purification and characterization of fertility-associated antigen (FAA) in bovine seminal fluid. *Mol. Reprod. Dev.,* **54:** 145-153.

McCauley, T.C., Zhang, H.M., Bellin, M.E. and Ax, R.L. (2001). Identification of a heparin binding protein in bovine seminal fluid as tissue inhibitor of metalloproteinases-2. *Mol. Reprod. Dev.,* **38:** 336-341.

McClure, R.D. and Tom, R. (1991). Human sperm hypo-osmotic swelling test: relationship to sperm fertilizing ability. *Int. J. Fertil.,* **36:** 360-366.

Medeiros, C.M., Forell, F., Oliveira, A.T. and Rodrigues, J.L. (2002). Current status of cryopreservation : why isn't it better. *Theriogenology,* **57:** 327-344.

Meizel, S. and Tuner, K.O. (1983). Stimulation of an exotic event, the hamster sperm acrosome reaction by cis-unsaturated fatty acids. *FEBS Lett.,* **161:** 315-318.

Menard, M., Nauc, V., Lazure, C., Vaillancourt, D. and Manjunath, P. (2003). Novel purification method for mammalian seminal plasma phospholipid proteins reveals the presence of novel membrane of this family of protein in stallion seminal fluid. *Mol. Repord. Dev.,* **66:** 349-357.

Meyers, S.A., Overstreet, J.W., Liu, K.L.M. and Drobnis, E.Z. (1995). Capacitation *in vitro* of stallion spermatozoa: comparison of progesterone-induced acrosome reaction in fertile and sub fertile males.*J. Androl*.**16:**47-54.

Miller, D.J., Winer, M.A. and Ax, R.L. (1990). Heparin binding proteins from seminal plasma bind to bovine spermatozoa and modulate capacitation by heparin. *Biol. Reprod.,* **42:** 899-915.

Miller, K., Pomorski, T., Miller, P. and Hermann, A. (1998). Stability of transbilayer phospholipid asymmetry in viable ram sperm cells after cryotreatment. *J. Cell. Sci.,* **112:** 11-20.

Mishra, A. K. and Tyagi, S. (2006) Recent trends in the semen collection, evaluation and cryopreservation. National seminar on artificial insemination: Acceptability, impact, constraints and solution. 12-13th Oct. 95-105, Izatnagar.

Mishra, A.K., Patel, S.H., Joshi, B.V., Jaiswal, R.S. and Trivedi, K.R. (1994). Characteristics and freezability of cross-bred bull semen under Indian conditions. Proc. 4th Intl. Buffalo Congr. Sao Paolo, Brazil, **3:** 495-497.

Mishra, T. P., Saxena, V. B. and Tripathi, S. S. (1988). Cryopreservation of semen of crossbred bulls of two different genotypes. *Indian J. Anim. Reprod.* **58**: 1424-1426.

Mishra, T.P., Saxena, V.B. and Tripathi, S.S. (1989). Physico-morphological characters and trace elements in semen of Murrah buffalo bulls. *Indian Vet. J.*, **66:** 1031-1034.

Mohanty, D. N. (1999). Studies on reproductive soundness, freezability and fertility in crossbred bulls. Ph.D Thesis submitted to Deemed University, Indian Veterinary Research Institute, Izatnagar.UP, India.

Morales, P., Cross, N.L., Overstreet, J.W. and Hanson, F.W. (1989). Acrosome intact and acrosome reacted human sperm can initiate binding to the zona pellucida. *Dev. Biol.,* **133:** 385-392.

Moreau, R. and Manjunath, P. (1999). Characterization of the lipid efflux particles generated by seminal phospholipids binding proteins. *Biochem. Biophys Aus.,* **1438:** 175-184.

Moreau, R. and Manjunath, P. (2000). Characteristics of the cholesterol efflux induced by novel seminal phospholipids-binding proteins. *Biochem. Biophys. Acta,* **1457:** 24-32.

Moreau, R., Therien, L., Lazure, C. and Manjunath, P. (1998). Type II domains of BSP-A1/-A2 proteins: binding properties, lipid efflux, and sperm capacitation potential. *Biochem. Biophys. Res. Comm.,* **246:** 148-154.

Moura, A.A., Koc, H., Chapmon, D.A. and Killian, G.J. (2006). Identification of proteins in the accessory sex gland fluid associated with fertility indexes of dairy bulls: A proteomic approach. *Androl. J.,* **27(2):** 201-211.

Muller, P., Erlemann, K.R., Muller,K., Calvette,J.J., Topfer-Peterses, E. Marienfeld, K. and Herrmann, A.(1998). Biophysical characterization of the interaction of bovine seminal plasma protein PDC-109 with phospholipids vesicles. *Eur. Biophys. J.,* **27:** 438-447.

Muller, P., Greube, A., Topfer-Petersen, E. and Herrmann, A. (2002). Influence of the bovine seminal plasma protein PDC-109 on cholesterol in the presence of phospholipids. *Eur. Biophys. J.,* **31:** 438-447.

Nauc, V. and Manjunath, P. (2000). Radio immunoassays for bull seminal plasma proteins (BSP-A1/A2, BSP-A3, and BSP-30- kilo Daltons) and their quantification in seminal plasma and sperm. *Biol. Reprod.,* **63:** 1058-1066.

Noiles, E.E., Balley, J. and Storey, B.T. (1995). Temperature dependence of the water permeability of murine sperm shows a discontinuity between 4 and O°C. *Cryobiology,* **32:** 220-238.

Nolan, J.P., Graham, J.K. and Hammerstedt, R.H. (1992). Artificial induction of exocytosis in bull sperm. *Arch. Biochem. Biophys,* **292:** 311-322.

Okada, A., Inowata, K., Matsuhashi, M., Fusio, K., Miura, K., Shina, K. and Shirai, M. (1990). Correlation of the hypo-osmotic swelling test, semen score and the zona free hamster egg penetration assay in humans. *Int. J. Androl.*, **13:** 337-343.

Orgebin-Crist, M.C., Danzo, G.J. and Cooper, T.J. (1976). Re-examination of the dependence of epididymal sperm viability on the epididymal environment. *J. Reprod. Fert.* (Suppl.), **24:** 115-120.

Pace, M.N., Graham, E.F.C. (1974). Components in egg yolk which protect bovine spermatozoa during freezing. *J. Anim. Sci.*, **39:** 1144-1149.

Panday, J.J., Mortimer, D. and Sawers, R.S. (1986). A standardized approach for evaluating the penetration of human spermatozoa into cervical mucus *in vitro*. *Fertil. Steril.*, **45(3):** 357-365.

Pant, H.C., Mittal, A.K., Patel, S.H., Shukla, H.R., Kasiraj, R. and Prabhakar, J.H. (2002). The hypo osmotic swelling test : an assay of cell membrane integrity and quality of frozen thawed buffalo semen. *Int. J. Anim. Reprod.*, **23(1):** 8-11.

Paridya, I.J., Mortimer, D. and Sawers, R.S. (1986). A standardized approach for evaluating the penetration of human spermatozoa into cervical mucus *in vitro*. *Fertil. Steril.*, **45(3):** 357-367.

Parks, J.E. and Graham, J.K. (1992). Effects of cryopreservation procedures on sperm membranes. *Theriogenology*, **38:** 209-222.

Parrish, J.J., Susko-Parrish, J.L. and First, N.L. (1985). Effect of heparin and chondroitin sulfate on the acrosome reaction and fertility of bovine sperm in vitro. *Theriogenology*, **24:** 537-549.

Parrish, J.J., Susko-Parrish, J.L., Crister, E.S., Leibfried-Rutledge, L., Barnes, F., Eyestone, W. and First, N.L. (1988). Bovine *in vitro* fertilization. 11th Tech. Conf. A.I. and Reprod., pp. 120.

Parrish, J.J., Susko-Parrish, J.L., First, N.L. (1989). Capacitation of bovine spermatozoa by heparin: inhibitory effect of glucose and role of intracellular pH. *Biol. Reprod.*, **41:** 683-699.

Parrish, J.J., Susko-Parrish, J.L., Graham, J.K. (1999). *In vitro* capacitation of bovine spermatozoa: role of intra cellular calcium. *Theriogenology* **51:** 461-472.

Patel K. V. Dhami A.J. Kodagali S.B. (1989) Seminal characteristics and their interrelationships in Kankrej x Jersey and Kankrej x Holstein Friesian crossbred bulls. *Indian Vet. J.* **66:** 737-743.

Patel K. V. Dhami A.J. Kodagali S.B. (1989) Seminal characteristics and their interrelationships in Kankrej x Jersey and Kankrej x Holstein Friesian crossbred bulls. *Indian Vet. J.* **69:** 437-443.

Pathak, N (1988). Studies on the semen of crossbred (Holstein-Friesian x Brown Swiss x Hariana) bulls with special reference to cryopreservation. M V Sc. Thesis, IVRI, Izatnagar, UP.India.

Paudel, P. K, (2002). Studies on cryopreservation-induced damages and effect of chlorpromazine and some antioxidants on frozen thawed spermatozoa of crossbred bulls. PhD. Thesis submitted to deemed university, Indian Veterinary Research Institute. Izatnagar.

Peitz, B. (1988). Effects of seminal vesicle fluid components on sperm motility in the house mouse. *J. Reprod. Fertil.,* **83:** 169-176.

Perez, L.J., Valcarcel, A., delas Heras, M.A., Moses, D.F. and Baldassare, H. (1996). Evidence that frozen-thawed ram spermatozoa show accelerated capacitation *in vitro* as assessed by the chlortetracycline assay. *Theriogenology,* **46:** 131-140.

Petrunkina, A.M., Gropper, B., Topfer-Petersen, E. and Gunzel-Apel, A.R. (2005). Volume regulatory function and sperm membrane dynamics as parameters for evaluating cryoprotective efficiency of a freezing extender. *Theriogenology* **63:**1390-1406.

Petrunkina, A.M., Harrison, R.A.P., Tsolova, M., Jebe, E. and Topfer-Petersen (2007). Signalling pathways involved in the control of sperm cell volume. *Reproduction,* **133:** 61-73.

Phelps, B. and Ax, R.L. (1992). Effect of cold shock on boar sperm treated with butylated hydroxyl toluene. *Biol. Reprod.,* **21:**319-325.

Philips, P.H. and Lardy H.A. (1940). Yolk-buffer pabulum for the cryopreservation of bull semen. *J.Dairy Sci*.**23**:399-404.

Polge, C. and Rowsen, LE.A. (1952). Fertilizing capacity of bull spermatozoa after freezing at (-) 79^{o}C, *Nature*, **169:**626.

Prasad, J. K (1997). Studies on enzymatic changes and *in vitro* fertility test in relation to cryopreservation of crossbred bull semen M V Sc. Thesis submitted to deemed university, Indian Veterinary Research Institute. Izatnagar.

Prasad, J. K., Kumar, S., Mohan, G., Shankar, U. and Agarwal, S. K. (1999). Hyposmotic swelling test (HOST) and its response in fresh freeze-thawed semen. *Indian.J. Anim. Sci.* **69**: 766-769.

Prasad, J. K., Kumar, S., Mohan, G., Shankar, U. and Agarwal, S. K. (2000). Biochemical studies pertaining to freezability of cross bred bull semen. *Indian. J. Vet.Res*. **8:** 37-40.

Purdy, P.H. and Graham, J.K. (2004). Effect of adding cholesterol to bull sperm membranes on sperm capacitation, the acrosome reaction, and fertility. *Gamete Biol.***71**:522-527.

Raja, C.K.S.V. and Rao, A.R. (1983). Semen characteristic of Brown-Swiss crossbred bulls in relation to age. *Indian Vet. J.* **60**: 451-454.

Rana, C.M. and Dhami, A.J. (2004). Physical attributes, intact acrosome, HOS test and freezability of semen of Gir and Jafarabadi bulls. *Indian Vet. J.,* **81:** 406-410.

Rao, A.V. N. and Rao, Y.V. R. (1996). Semen characteristic and freezability of crossbred bulls. *Indian Vet. J.* **73**: 1086-1088.

Rao, B. K. and Rao, A. R. (1978). Semen characteristic of crossbred bulls. *Indian Vet. J.* **55**: 692-700.

Rasul, Z., Ahmad, N. and Anzal, M. (2000). Changes in motion characteristics, plasma membrane integrity and acrosome morphology during cryopreservation of buffalo spermatozoa. *J. Androl.* **22**:278-283.

Rathi, R., Colebrander, B., Bevers, M.N. and Gadella, B.M. (2001). Evaluation of *in vitro* capacitation of stallion spermatozoa. *Biol. Reprod.* **65:**462-470.

Roberts, S. J. (1982). Veterinary obstetrics and genital diseases. Second Ed. CBS Publishers and Distributors, p.622.

Roncoletta, M., Morani, E.S.C., Esper, C.R., Barnabe, V.H. and Franceschini, P.H. (2006). Fertility-associated proteins in Nelore bull sperm membranes. *Ani. Reprod. Sci.,* **91(1-2):** 77-87.

Rudolph, A.S., Crowe, J.H. and Crowe, L.M. (1986). Effects of three stabilizing agents-proline, betaine, and trehalose-on membrane phospholipids. *Archieves of Biochem. and Biophys.*, pp. 134-143.

Saacke, R.G. (1970). Semen quality tests and their relationship to fertility. Proc. 4th Tech. Confr. NAAB. p.22.

Sagdeo, L. R., Chitnis, A. B., Deshmukh, S. N and Kaikini, A. S. (1990). Studies on semen freezability of pure Jersey and Crossbred bulls with varying levels of exotic inheritance. *Indian J.Anim.Reprod.* **11:** 79-84

Saling, P.M. and Storey, B.T.(1979). Mouse gamete interactions during fertilization in vitro: chlortetracycline as fluorescent probe for the mouse sperm acrosome sperm acrosome reaction. *J. cell. Biol.* **83**:544-555.

Salisbury, G. W. VanDemark, N. L. and Lodge, J. R. (1978). *Physiology of reproduction and artificial insemination of cattle* 2nd ed. by W.H Freeman and Company. p: 268—274.

Salois, D., Menard, M., Paquette, Y. and Manjunath, P. (1999). Complementary deoxyribonucleic acid cloning and tissue expression of BSP-A3 and BSP-30 kDa: phosphatidylcholine and heparin-binding proteins of bovine seminal plasma. *Biol. Reprod.,* **61:** 288-297.

Sanchez-Luengo, S., Aumuller, G., Albrecht, M., Sen, P.C. Rohm, K.and Wilhelm, B. (2004). Interaction of PDC-109, the major secretory protein from bull seminal vesicles, with bovine sperm membrane Ca(2+)-ATPase. *J. Androl.,* **25**(2):234-44.

Sanchez-Partida, L.G., Maxwell, W.M., Paleg, L.G. and Satchell, B.P. (1992). Proline and glycine betaine in cryoprotective diluents for ram spermatozoa. *Reprod. Fertil. Dev*. **4:** 113-118.

Sanz, L., Calvete, J.J., Mann, K., Gabius, H.J., Topfer-Peterson, E. (1993). Isolation and biochemical characterization of heparin-binding proteins form boar seminal plasma: a dual role for spermadhesins in fertilization. *Mol. Reprod. Dev.,* **35:**37-43.

Saxena, V.B. and Tripathi, S.S. (1979). Semen characterstic and preservability in red dane bulls. *Indian J. of Anim. Sci.,* **49:** 878-883.

Schambony, A., Hess, O., Gentzel, M. and Topfer-Peterson, E. (1998). Expression of CRISP proteins in the male equine genital tract. *J.Reprod. Fertil.,* Suppl. **53:**67-72.

Scheit, K.H. Kemme, M., Aumuller, G., Seitz, J., Hagendroff, G. and Zimmer, M. (1988). The major protein bull seminal plasma: Biosynthesis and biological function. *Biosci. Rep.,* **8**:589-608.

Schneider, C.S., Elington, J.E., Wright, R.W. (1996). Effects of bulls with different field fertility on in vitro embryo cleavage and development using sperm co-culture systems. *Theriogenology*. **145**: 262.

Seidah, N.G., Manjunath, P., Rochemont, J., Sairam, M.R. and Chrétien, M. (1987). Complete amino acid sequence of BSP-A3 from bovine seminal plasma. Homology to PDC-109 and to the collagen-binding domain of fibronectin. *Biochem. J.,* **243**:195-203.

Seshagiri, V.N. and Pattabhiraman, S.R. (1991). Characterization of seminal plasma proteins of Sindhi, Jersey and crossbred bulls. *Ind. Vet. J.*, **68**:1143-1146.

Sethi, R.K., Raina, V.S., Joshi, B.K. and Gurnani, M. (1989). Multistage selection of crossbred males, and effect of their age and body weight on semen quality and freezability. *Indian J. Anim. Sci.,* **59:** 171-174.

Shannon P, Vishwanath R. (1995). The effect of optimal concentration of sperm on the fertility of fresh and frozen bovine semen and a theoretical model to explain the fertility differences. *Anim. Reprod. Sci*. **39:** 1-10

Shannon, P. and Curson, B. (1972). The effect of egg yolk levels on bovine seminal plasma toxins. In: Proceedings of the 7th ICAR and AI; 1972; Munich Germany, **4**:1363-1367.

Sharma, M. L (1988). Studies on the semen quality of triple crossbred bulls with reference to cryopreservation. M V Sc. Thesis, IVRI. Izatnagar, UP. India.

Sharma, M. L. Mohan, Greesh. And Sahni, K. L. (1991). Physical characteristics of semen of triple cross-bred bulls. *Indian Vet. Med. J.* **15**: 302-305.

Sharma, M. L. Mohan, Greesh. And Sahni, K. L. (1992). A study on acrosomal damage on cryopreservation of crossbred bull semen. *Indian Vet. J.* **69**: 962-964.

Shi, D.S., Lu, K.H., Gordon, I., Polge, C. (1991). Variation in cleavage and embryonic development of bovine oocytes in vitro fertilized with different bull ejaculates. *Theriogenology*. **35**: 271.

Shivaji, S., Scheit, K.H. and Bhargava, P.M. (1990). Proteins of seminal plasma. *J. Willey and Sons,* Inc, New York., pp.145-160.

Shamsuddin, M., Larsson, B. (1993). *In vitro* development of bovine embryos after fertilization using semen from different donors. Reprod. *In Domestic. Anim.* **28**:77-84.

Shukla, M.K. and Mishra, A.K. (2005). Correlations between seminal characteristics in cross breds. *Indian. J. Anim. Sci.* **75**: 263-266.

Singh D.M and Pangawkar G. R (1990). Studies on some characteristics exotic and crossbred bull spermatozoa. *J. Anim. Reprod*. **11**:92-95.

Singh, B., Singh, B.P. and Tomar, N.S. (1972). Fertility index in Hariana bull. *Indian J. Anim. Hlth.,* **6:** 57-63.

Singh, L.P., Harshan, H.M. and Ansari, M.R. (2007). Effect of egg yolk and seminal plasma heparin binding protein interaction on the freezability of buffalo cauda epididymal spermatozoa. *Anim. Reprod. Sci.,* **99:** 395-400.

Singh, M.P., Sinha, S.N. and Singh, B. (1992). Some characteristics of Murrah bulls. *Indian J.Anim. Sci.***52**:594-598.

Sinha, M.P., Sinha, A.K., Singh, B.K. and Prasad, R.L. (1996). The effect of glutathione on the motility, enzyme leakage and fertility of frozen goat semen. *Anim.Reprod. Sci.* **41:**237-243.

Sinowatz, F., Wessa, E., New Miller, C. and Palma, B. (2003). On the species specificity of sperm binding and sperm penetration of the zona pellucida. *Reprod. In Domest. Anim*. **38(2):** 141-146.

Smith, S.P., Hashimoto, Y., Pickford, A.R., Campbell, I.D. and Werner, J.M. (2000). Interface characterisation of the type II module pair from fibronectin. *Biochemistry,* **39:** 8374-8381.

Sofikitis, N.V., Miragawa, I. and Zavos, P.M. (1992). Capacitation and acrosome reaction and outcome of hypo osmotic swelling test in human sperm. *Mol. Androl.,* **4:** 363-368.

Soubeyrand, S. and Manjunath, P. (1997). Novel seminal phospholipase A_2 is inhibited by the major proteins of bovine seminal plasma. *Biochem. Biophys. Acta,* **1341:** 183-188.

Srivastava, P.N., Adam, C.E. and Hartree, E.P. (1965). Enzymic action of acrosomal preparation on the rabbit ovum *in vitro. J. Reprod. Fertil.,* **10**:61-67.

Srivastava, S. (2000). Effect of ascorbic acid, caffeine and chloroquine on the freezability of bovine semen. M.V.Sc. thesis submitted to IVRI (Deemed University), Izatnagar, U.P.

Srivastava, S. and Kumar, S. (2006). Effects of certain additives on the freezability of crossbred bull semen. *Indian. J.Anim. Reprod.* **27:** 1-5.

Stock, C.E. and Fraser, L.R. (1987). The acrosome reaction in human sperm from men of proven fertility. *Hum. Reprod. (Old)* **2**: 109-119.

Stoganov, T., Rhodes, S.C., Maxwell, W.M.C. and Evans, G. (1994). The effect of antioxidants on the pregnancy rate after insemination with liquid stored ram spermatozoa (abstract). *Proc. Soc.Reprod. Biol.* 26:116.

Strom H, B., Larsson, B., Linde-Forsberg, C. and Rodriguez-Martinez, H. (2000). Evaluation of chilled and frozen-thawed canine spermatozoa using a zona pellucida binding assay. *J. Reprod. Fert.,* **119**: 201-206.

Strzezek, J., Saiz, Cidoncha, F., Wysocki, P., Tyszkiewicz, A. and Jastrzebski, M. (2002). Seminal plasma proteins as markers of biological value of boar semen. *Anim. Sci. Papers and Rep.,* **20(4):** 255-266.

Suarez, S.S, and Ho, H.C. (2003). Hyperactivated motility in sperm. *Reprod. Domest. Anim.,* **38:**119-124.

Sukardi, S., Curry, M.R. and Watson, P.F. (1997). Simultaneous detection of the acrosomal status and viability of incubated ram spermatozoa using fluorescent markers, *Anim. Reprod. Sci.,* **46:89**-96.

Suleiman, S.A., Ali, M.E., Zaki, Z.M.S., El-Malik, E.M.A. and Nasr, M.A. (1996). Lipid peroxidation and human sperm motility : protective role of vitamin E. *J. Androl.,* **17:** 530-537.

Suryaprakasam, T. B and Narasimha Rao, A. V. (1993). Studies on seminal characteristics of multiple breed A.I. bulls. *Indian. Vet. J.* **70:** 629-632.

Tatemoto, K., and Mutt, V. (1980). Isolation of two novel candidate hormones using a chemical method for finding naturally occurring polypeptides. *Nature,* **285**(5):417-418.

Thakkar, J.K., East, J., Seyler, D. and Franson, R.C. (1983). Surface active phospholipase A_2 in mouse spermatozoa. *Biochem. Biophys. Acta.,* **754:** 44-50.

Therien, I. and Manjunath, P. (1993). Effect of progesterone on bovine sperm capacitation and acrosome reaction. *Biol. Reprod.* **69:** 1408-1415**.**

Therien, I., Bleau, G. and Manjunath, P. (1995). Phosphatidylcholine binding proteins of bovine seminal plasma modulates capacitation of spermatozoa by heparin. *Biol. Reprod.,* **52:**1372-1379.

Therien, I., Moreau, R. and Manjunath, P. (1998). Major proteins of bovine seminal plasma and high-density lipoprotein induce cholesterol efflux from epididymal sperm. *Biol. Reprod.* **59:**768-776.

Therien, I., Moreau, R. and Manjunath, P. (1999). Bovine seminal plasma phospholipids binding proteins stimulate phospholipids efflux from epididymal sperm. *Biol. Reprod.,* **61**:590-598.

Therien, I., Souberyrand, S. and Manjunath, P. (1997). Major proteins of bovine seminal plasma modulate sperm capacitation by high-density lipoprotein. *Biol. Reprod. ,* **57**:1080-1088.

Therien, I., Bousquet, D. and Manjunath, P. (2001). Effect of seminal phospholipid-binding proteins and follicular fluid on bovine sperm capacitation. *Biol. Reprod.,* **65:** 41-51.

Thomas, C.J., Anbazhagan, V., Ramakrishnan, M., Sultan, N., Surolia, I. and Swamy, M.J. (2003). Mechanism of membrane binding by the bovine seminal plasma protein, PDC-109: A surface Plasma resonance study. *Biophys. J.,* **84:**3037-3044.

Tomar, N.S. and Misra, B.S. (1985). Comparative analysis of non-motile and progressively motile semen of buffalo bulls. *Indian Vet. J.,* **48:** 702-705.

Topfer-Peterson, E. (1999). Carbohydrate based interactions on the route of a spermatozoon to fertilization. *Hum. Reprod. Update,* **5**:314-329.

Topfer-Peterson, E., Romero, A., Valera, P.F., Ekhlasi-hundrieser, M., Dostalova, Z., Sanz, L. and Calvete, J.J. (1998). Spermadhesins: a new protein family. Facts, hypothesis and perspectives. *Andrologia,* **30:**217-224.

Topper, E.K., Killian, G.J., Way, A., Enyel, B. and Woelders, H. (1999). Influence of capacitation and fluids from the male and female genital tract on the zona binding ability of bull spermatozoa. *J. Rep.Fertil.,* **115:**175-183.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of protein from polyacrylaminde gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl.Acad. Sci. USA:* **76:** 4350-4354.

Travis, A. and Kopp, G.S. (2002). The role of cholesterol efflux in regulating the fertilization potential of mammalian spermatozoa. *The J. Clin. Invest.,* **110:** 731-736.

Travis, A. and Kopp, G.S. (2002). The role of cholesterol efflux in regulating the fertilization potential of mammalian spermatozoa. *The J. Clin. Invest.,* **110:** 731-736.

Tsien, R.Y.(1998). Fluorescent indicators of ion concentrations. In: D.L: Taylor and Y.L. Wang (Editors), Fluorescence microscopy of living cells in culture. Part B> Quantitative Fluorescence Microscopy-imagine and spectroscopy Methods in cell Biology. *Vol.* 30. Chapter S. Academic Press. New York, pp. 127-156.

Tuli, R.K. Batabyal, A.K. Singal, S.P. and Razdan, M.N. (1988). Seminal characteristics and deep freezing of cross bred bull semen using Tris yolk glycerol extender. *Ind. J. Anim. Prodn.,* **9(1):** 16-18.

Turner, T.T. and Reich, G.W. (1987). Influence of proteins in rat cauda epididymal lumen fluid on cauda epididymal sperm motility. *Gamete Res.,* **18:**267-278.

Unpublshed report (2010). Germ Plasm Centre, IVRI, Izatnagar.

Urch, U.A. and Patel, H. (1991). The interaction of boar sperm acrosin with its natural substrate, the zona pellucida and polysulphated polysaccharides. *Development*, **III**: 1165-1172.

Usselman, M.C., Carr, D.W. and Acott, T.S. (1984). Rat and bull sperm immobilization in the cauda epididymal, a comparative mechanism. *Annals of New York Academy of Science*, **438**:530-531.

Vander-Ven, H.H., Jeyendran, R.S., Alhasani, S., Parez-Pelaez, M., Diedrich, K. and Zaneweld, L.J.D. (1986). Correlation between human sperm swelling in hypo-osmotic medium and *in-vitro* fertilization. *J. Androl.*, **7:** 190-196.

Vemuri, R. and Philipson, K.D. (1989). Influence of sterols and phospholipids on sarcolemma and sarcoplasmic reticular cation transporters. *J. Biol. Chem.*, **264(15):** 8680-8685.

Villemure, M., Lazure, C. and Manjunath, P. (2003). Isolation and characterization of gelatin-binding proteins from goat seminal plasma. *Reprod. Biol. Endocrinol.* **1**:39.

Visconti, P.E. and Kopf, G.S. (1998). Regulation of protein phosphorylation during sperm capacitation *Biol. Reprod.*, **59:**1-6.

Visconti, P.E., Galantino-Homer, H., Ning, X.P., Moore, G.D, Valenzuela, J.P., Jorgez, C.J., Alvarez, J.G. and Kopf, G.S. (1999). Cholesterol efflux-mediated signal transduction in mammalian sperm. *J. Biol. Chem.*, **274**:3235-3242.

Visconti, P.E., Glantino, Homer H., Moore, G.D., Bailey, I., L., Ning, X., Fenner, M and Kopf, G.S. (1998). The molecular basis of sperm capacitation. *J. Androl.* **18**(2):242-248.

Vishwanath, R. and Shannon, P. (1997). Do sperm cells age? A review of the physiological changes in sperm during storage at ambient temperature. *Reprod. Fertil. Dev*.**9:**321-331.

Vishwanath, R., Shannon, P. and Curson, B. (1992). Cationic extracts of egg yolk and their effect on motility, survival and fertilizing ability of bull sperm. *Anim.Reprod.Sci*.**29**: 185-194.

Vyas, S. Mohan, G. Dhami, A.J. and Sahni, K.L (1992). Studies on the norms and correlations of initial and post-thaw seminal attributes of triple crossbred bulls. *Int. J.Anim. Sci.* **7:** 73-76.

Ward, C.R. and storey, B.T. (1984). Determination of the time course of capacitation in mouse spermatozoa using a chlortetracycline fluorescence assay. *Dev.Biol.*, **104:**287-296.

Watson, P.F. (1975). Use of Giemsa stain to detect changes in the acrosome of frozen ram spermatozoa. *Vet. Rec.,* **97**:12-15.

Watson, P.F. (1976). The protection of ram and bull spermatozoa by low density lipoprotein fraction of egg yolk during storage at 5°C and deep freezing. *J. Therm. Biol.* **1:**137-141.

Watson, P.F. (1994). Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post thawing function. *Reprod. Fertil. Dev*. **7**: 871-891.

Way, A.L., Griel, L.C. Jr. and Killian, G. (2000). Effects of accessory sex gland fluid on viability, capacitation and acrosome reaction of cauda epididymal spermatozoa. *J. Androl.,* **21:** 213-219.

Witte, T.S. and Sachafer-somi, S. (2007). Involvement of cholesterol, calcium and progesterone in the induction of capacitation and acrosome reaction of mammalian spermatozoa. *Ani. Reprod. Sci.* **102**: 181-193.

Woelders, H., Matthijs, A. and Engel, B. (1991). Effects of trehlose and sucrose, osmolarity of the freezing medium, and cooling rate on viability and intactness of bull sperm after freezing and thawing. *Cryobiology,* **35**:93-105.

Wolf, D.E., Hagopian, S.S. and Isogima, S. (1986). Changes in sperm plasma membrane diffusability after hyperactivation during *in vitro* capacitation in the mouse. *J. Cell. Biol.,* **102:** 1372-1377.

Yanagimachi, R. (1994). Mammalian fertilization, In: Physiology of Reproduction. 2nd Ed. E. Knobil. and J. D. Neill, eds. Raven press, New York, pp. A189-A317. Yeagle, D.L. (1985) Cholesterol and the cell membrane *Biochem. Biophys. Acta.* **822**:267-287.

Yeagle, D.L. (1985). Cholesterol and the cell membrane. *Biochem. Biophys. Acta*. **822**:267-287.

Yeagle, P.L. (1989). Lipid regulation of cell membrane structure and function. *FASEB J.*, **3:** 1833-1842.

Zarintash, J., Renee and Cross, L.N. (1996). Unesterified cholesterol content of human sperm regulates the response of the acrosome to the agonist, progesterone. *Biol. Reprod.,* **55:** 19-24.

Zhang, B.R., Larsson, B., Lundeheim, N., Rodriguez-Martinez, H. (1997). Relationship between embryo development in vitro and 56-day non return rates of cows inseminated with frozen-thawed semen from dairy bulls. *Theriogenology*. **48:**221-231.

Zhao, Xian-Mian, Song, Xue-Xiang, Kawai, Y. and Niwa, K. (2002). Penetration *In vitro* of zona free pig oocytes by homologous and heterologous spermatozoa. *Theriogenology*. **58**:995-1006.

Annexure

PROTEOMICS

I. Reagents and solutions for Lowry's method

1. Reagent A: 2% sodium carbonate in 0.1 N Sodium hydroxide (NaOH)
2. Reagent B: 0.5% Copper sulphate ($CuSO_4.5H_2O$) in 1% Potassium sodium tartrate
3. Reagent C: 50 ml of Reagent A was mixed with 1 ml of Reagent B (Alkaline copper solution). Only freshly prepared solutions were used.
4. Reagent D: Dilute the Folin ciocalteu's reagent to make it 1N in water (50 percent reagent + 50 ml Distilled water).

II. Reagents and solutions for SDS-PAGE

i) Separating gel buffer (8x)

(3.022 M Tris-HCl buffer, pH 8.4, containing 0.8% (w/v) SDS and 0.232% (v/v) TEMED)

Tris	36.6 g
SDS	0.8 g
TEMED	0.232 ml

pH was adjusted with HCl and volume was made up to 100 ml.

ii) Stacking gel buffer (8x)

(1M Tris-HCl, pH 6.8, containing 3.2% (w/v) SDS and 0.232% (v/v) TEMED)

Tris	12.11 g
SDS	3.2 g
TEMED	0.232 ml

pH was adjusted with HCl and volume was made up to 100 ml.

iii) Acrylamide solution (30%)

30g of acrylamide and 0.8 g of bis-acrylamide were dissolved in distilled water by stirring and volume was made to 100 ml.

iv) Sample buffer (5x)

(0.312 M Tris-HCl buffer, pH 6.8, containing 0.125% (w/v) bromophenol blue 10% (w/v) SDS and 50% (v/v) glycerol)

1.8 g of Tris was dissolved in 15 ml of water and pH was adjusted with HCl. To this solution SDS (5 g), bromophenol blue (0.0625 g) and glycerol (25 ml) were added and volume was made to 50 ml with water.

v) Ammonium pesulphate solution (APS, 20% w/v)

vi) Electrode buffer

(25 mM Tris, 192 mM glycine and 1% (w/v) SDS, pH 8.3)

The following ingredients were dissolved in distilled water to make 500 ml.

Tris	1.515 g
Glycine	7.2 g
SDS	0.50 g

Solution i-iv were filtered through Whatman filter No. 1 and stored at 4°C. Electrode buffer was freshly prepared.

vii) Separating (Resolving) gel recipe (for 32 ml)

Acrylamide (%)	4	5	10	15	18
Separating gel buffer (ml)	4	4	4	4	4
Acrylamide stock solution (ml)	4.26	5.34	10.68	16.02	19.20
D.water (ml)	23.71	22.66	17.32	11.98	8.80
APS (ml)	0.16	0.16	0.16	0.16	0.16

viii) Stacking gel recipe (3.30% acrylamide, for 8.0 ml)

Stacking gel buffer	1 ml
Acrylamide stock solution	0.88 ml
Water	6.08 ml
APS	0.04 ml

ix) Fixing solution

20% (v/v) isopropanol and 10% (v/v) acetic acid

x) Staining solution for SDS-PAGE and Immunodiffusion

0.875 g of Coomassie Brilliant Blue R-250 was dissolved in 225 ml of methanol and 50 ml of acetic acid by stirring overnight. The volume was made to 500 ml with water and the solution was filtered through Whatman filter paper. The staining solution was stored at room temperature in a brown bottle.

xi) Destanining solution

20% (v/v) methanol and 10% (v/v) acetic acid (for 24 hours with 3-4 changes) followed by 10% (v/v) acetic acid (for 12 hours).

xii) Gel storage solution

7% (v/v) acetic acid

III Reagents for western blotting

i) Transfer buffer

Tris base	18.2 g
Glycine	86.5 g

	Methanol	1200 ml
	Distilled water up to	6000 ml
ii)	**PBST**	
	PBST	100 ml
	Skim milk powder	5 g

III. Reagents for ELISA

i)	**Phosphate buffered saline (PBS, pH 7.2)**	
	NaCl	8.0 g
	KCl	0.2 g
	KH_2PO_4	0.2 g
	$NaH_2 PO_4$	1.15 g
	H_2O	1000 ml
ii)	**Washing buffer (PBST)**	
	0.05% Tween-20 in PBS	
iii)	**Coating buffer (0.05 M carbonate buffer, pH 9.5)**	
	Sodium carbonate	1.5 g
	Sodium bicarbonate	2.93 g
	Sodium azide	0.20 g
	Distilled water	1000 ml
iv)	**Blocking solution**	
	BSA	3.0 g
	PBST	100 ml
v)	**Conjugate diluted in PBS (pH 7.4)**	
vi)	**Substrate buffer (Citrate buffer, 0.1 M, pH 4.6)**	
vii)	**Substrate solution**	
	Ortho-phenylene diamine	25 mg
	Citric acid phosphate buffer, pH 5.0	25 ml
	Mixed and 25 µl H_2O_2 added. Protected from light.	
viii)	**Stopping solution (1 M H_2SO_4)**	
	Conc. H_2SO_4	5.4 ml
	Distilled water	94.6 ml

IV. SEMENOLOGY

i)	**Semen diluting fluid**	
	Sodium chloride	1 g
	Eosin	0.05 g

	Formalin	1 ml
	Distilled water	100 ml
ii)	**Nigrosin-Eosin stain**	
	Eosin-γ	1.67 g
	Nigrosin	10 g
	Sodium cirate buffer (2.9%)	100 ml
iii)	**Solutions for giemsa staining**	
	Giemsa stain solution (stock)	
	Giemsa stain	1 g
	Methanol	98 ml
	Glycerol	32 ml

Giemsa stain powder was ground with absolute methanol in a glass pestle and mortar. Glycerol was added and stored at 37°C for 1 week. During this period the stain mixture was mixed well for few min every day.

Sorenson's 0.1 M phosphate buffer (pH 7.0)

i)	**Solution A:** 0.1 M Potasium dihydrogen phosphate soution	
	Potassium dihydrogen phosphate (Anhydrous)	13.609 g
	Double glass distilled water	1000 ml
ii)	**Solution B:** 0.1 M Disodium hydrogen orthophosphate solution	
	Disodium hydrogen phosphate	14.198 g
	Double glass distilled water	1000 ml

Sorenson's 0.1 M phosphate buffer was prepared by mixing 17 ml solution 'A' and 33 ml solution 'B' and the pH was adjusted to 7.0

Hancock's fixative

Sodium chloride	10 g
Sodium bicarbonate	0.5 g
Formulain	125 ml
Distilled water upto	1000 ml

Giemsa working solution

Stock Giemsa solution	3.0 ml
Sorenson's 0.1 M phosphate buffer	2.0 ml
Double glass distilled water	45.0 ml

iv)	**Chlortetra cycline (CTC) stain**	
	CTC stain 750 µM	1.54 mg
	Cystine 5 mM	3.5 mg
	NaCl 130 mM	30 mg

	Tris 20 mM	9.72 mg
	Double glass distilled water to make up volume to 4 ml	
v)	**Tris dilutor**	
	Tris (Hydroxyl methyl) amino methane	3.028 g
	Citric acid	1.675 g
	Penicillin G sodium (IU/ml)	500-1000
	Streptomycin sulphate (µg/ml)	500-1000
	Double glass distilled water	100 ml
vi)	**Tris-egg yolk glycerol dilutor**	
	Tris (Hydroxyl methyl) amino methane	3.028 g
	Citric acid	1.675 g
	Fructose	1.25 g
	Penicillin G sodium (IU/ml)	500-1000
	Streptomycin sulphate (µg/ml)	500-1000
	Double glass distilled water	100 ml
	Glycerol-7%	
	Egg yolk-10%	
vii)	**PBS (pH 7.4)**	
	Sodium chloride (150 mM)	9 g
	Disodium hydrogen phosphate	5.75 g
	Sodium dihydrogen phosphate	1.47 g
	Double distilled water	1000 ml
viii)	**Normal saline**	
	Sodium chloride	9.0 g
	Double distilled water	1000 ml

V. ZONA BINDING ASSAY

i) Oocyte collection medium (OCM), pH 7.3-7.4, 280-290 m Osm/kg

Stock OCM solution

Ingredients	**Concentration (mM)**
NaCl	136.89
KCl	2.68
KH_2PO_4	1.46
NaH_2PO_4	8.09
$CaCl_2PO_4$	0.90
$MgCl_2$ (Anhydrous)	1.00

D-glucose	5.54
Sodium pyruvate	0.32
Gentamicin (50 µg/ml)	500 µl
Phenol red	10 µg/ml

Working OCM [3mg/ml BSA (Fraction V), 10% EBS]

OCM stock solution	45 ml
Estrous buffalo serum (EBS)	5 ml
BSA (Fraction V)	150 mg

ii) Tissue culture medium-199 (TCM-199)

Stock TCM-199 media: Cat No. M7528 Lot 73K244492, with Earle's salt and 25 mM HEPES.

0.01 g L-glutamine and 0.1 ml gentamicin were added in 100 ml TCM-199 and stored at 4^{o}C. This prepared TCM-199 was always used for making different media in the present study.

iii) Oocyte Maturation Medium (0MM) pH 7.2-7.4, 280 mOsm/kg

TCM-199	5 ml
FBS	500 µl
GFF	250 µl
FSH-P	0.5 µg/ml
BSA Fraction-V	15 mg
LH	10 IU/ml

iv) Fertilization TALP, pH 7.6-7.8, 280-300 mOsm

Fertilization stock solution

Ingredients	**Concentration (mM)**
NaCl	114
KCl	3.20
NaH_2PO_4	0.34
C_2Cl_2 $2H_2O$	2.00
$MgCl_2$ (Anhydrous)	0.50
$NaHCO_2$	25.00
Sodium lactate	1.86 µg/ml
Gentamicin (50 µg/ml)	50.0 µg/ml
Phenol red	10 µg/ml

Heparin stock solution

Heparin	12 mg
Fertilization stock solution	2000 ml

Pyruvate stock solution

Sodium pyruvate	33 mg
Fertilization stock solution	2000 ml

Sperm TALP [10 µg/ml heparin, 0.25 mM Pyruvate, 3 mg/ml BSA (V)]

Fertilization stock solution	25 ml
Heparin stock solution	41.66 µl
Pyruvate stock solution	41.66 µl
BSA (Fraction-V)	75 mg

Fertilization TALP [10 µg/ml heparin, 0.25 mM Pyruvate, 6 mg/ml BSA (V)]

Fertilization stock solution	5 ml
Heparin stock solution	8.34 µl
Pyruvate stock solution	8.34 µL
BSA (Fraction-V)	30 mg

v) Complete Tyrode's Medium (TALP, Parrish *et al.*, 1988) pH 7.4

NaCl	100 mM	5.844 g
KCl	3.1 mM	0.231 g
$NaHCO_3$	25 mM	2.100 g
NaH_2PO_4	0.3 mM	0.03597 g
Na Lactate	21.6 mM	1.846 ml
$CaCl_2$	2 mM	0.2208 g
$MgCl_2$	0.4 mM	0.0986 g
HEPES	10 mM	2.383 g
Na Pyruvate	1 mM	0.110 g
Glucose	5 mM	0.900 g

vi) Non Capacitating Medium (NCM) pH 7.4

KCl	2.7 mM	0.201 g
KH_2PO_4	1.5 mM	0.04 g
Na_2HPO_4	8.1 mM	1.150 g
NaCl	137 mM	8.00 g
Glucose	5.55 mM	0.999 g
Pyruvate	1 mM	0.11 g

vii) Modified complete Tyrods's medium (Sp TALP, pH 7.4)

NaCl	100 mM	5.844 g
KCl	3.1 mM	0.231 g

$NaH\ CO_3$	25 mm	2.100 g
NaH_2PO_4	0.3 mM	0.03597 g
Na lactate	21.6 mM	1.846 ml
$CaCl_2$	2 mM	0.2208 g
$MgCl_2$	0.4 mM	0.986 g
HEPES	10 mM	2.383 g
Na Pyruvate	1 mM	0.110 g
BSA		6 mg/ml

VI. CHOLESTEROL ESTIMATION

Reagent 1: Cholesterol reagent

Reagent 2: Working cholesterol standard, 200 mg%

Reagent 3: Precipitating reagent

Vitae

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