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CICLO XXIII

"PROTEIN PROFILES AND BIOCHEMICAL CHARACTERISTICS OF SEMEN: INFLUENCE ON FROZEN-THAWED SPERMATOZOAL QUALITY IN RAMS (*OVIS ARIES*) AND ALPACAS (*VICUGNA PACOS*)"

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RIASSUNTO

Il plasma seminale è un fluido biologico complesso ed è conosciuto come una base nutritiva e protettiva degli spermatozoi. Alcuni componenti del plasma seminale sono di particolare importanza per il metabolismo spermatico, per la funzione, sopravvivenza e trasporto di queste cellule all'interno dell'apparato riproduttore femminile. Si conosce ancora poco come migliorare da un punto di vista biochimico e biofisico il plasma seminale. Nei camelidi, in particolare, l'origine non è conosciuta. Quindi, una serie di esperimenti sono stati condotti per studiare la composizione biochimica del plasma seminale negli arieti e negli alpaca e la relazione tra le caratteristiche biochimiche e la qualità e congelabilità spermatica. Nel primo esperimento, come studio di base, si sono usati due successivi eiaculati raccolti da arieti riproduttori di razza Sarda per osservare l'influenza del tipo di eiaculato sulla congelabilità degli spermatozoi. Si è osservato una più alta % di motilità spermatica nel post-scongelamento dei campioni raggruppati di primo e secondo eiaculato. Nel secondo esperimento, sono stati considerati eiaculati raccolti da arieti di razze venete a numerosità ridottissima per studiare il profilo biochimico, compreso quello proteico. Gli eiaculati sono stati suddivisi in 8 gruppi dividendo il campione in base al diverso trattamento di diluizione e di composizione del medium (TRIS o latte scremato). Sia i campioni freschi che quelli post-scongelamento sono stati valutati considerando l'integrità di membrana plasmatica utilizzando la tecnica in fluorescenza che considera la traslocazione della fosfotidilserina (Test dell'Annessina V) capace di rilevare il grado di apoptosi cellulare. I risultati di questo esperimento indicano una variabilità della composizione del plasma seminale, anche su base individuale, e che l'eterogeneità degli eiaculati (1° vs 2°) è meglio mantenuta quando si è usato il TRIS nel medium di diluizione. Inoltre , è stato osservato una correlazione negativa significativa tra proteine totali, Mg, P e spermatozoi con assenza di risposta al test di apoptosi. Un'altra interessante relazione positiva è tra la risposta negativa al test di apoptosi e proteine con peso molecolare di 75 kDa.

Nel terzo esperimento, seme epididimale, raccolto da soggetti di razze veneto a numerosità ridotta, è stato trattato con TRIS o latte scremato per osservare le caratteristiche post-scongelamento degli spermatozoi. I risultati dimostrano come le qualità spermatiche sono inferiori rispetto agli spermatozoi di eiaculato. Nel quarto esperimento, si è osservato come la dieta può influenzare i componenti del plasma seminale ed in particolare il profilo energetico ela qualità seminale (volume, concentrazione, motilità) in alpaca. Nel quinto esperimento seme di alpaca è stato trattato con 4 differenti tipi di medium di diluizione ed il miglior risultato si è ottenuto utilizzando TRIS con lo 0,8% di fruttosio. Si sono messe in evidenza numerose interessanti correlazioni tra viscosità, % di cellule negative al test dell'apoptosi, Mg, P, proteine totali. In generale si può affermare che la composizione biochimica e la funzione del plasma seminale variano con la dieta e l'eiaculato anche per lo stesso soggetto e tra specie animali. La composizione è un fattore che determina l'utilizzo del singolo soggetto per la conservazione di materiale genetico è dovrebbe esser preso in considerazione per migliorare le tecniche di preparazione di medium di mantenimento o diluizione pre-congelamento per ottimizzare le tecniche di riproduzione assistita negli animali da reddito, per migliorare i programmi di conservazione di razze in pericolo e per migliorare la capacità fertilizzante post-scongelamento.

ABSTRACT

Seminal plasma (SP) is a complex biological fluid and is known to act as a nutritive-protective medium for spermatozoa suspended in it. Some of SP components are very important for sperm metabolism, as well as sperm function, survival and transport in the female reproductive tract. Remarkably little is known about biophysical and biochemical makeup of SP. Specially in camelids, the origin, composition and function of SP remain a mystery. Therefore, a series of experiments was conducted to study the biochemical composition of SP in ram and alpaca, and their possible relations with fresh semen quality and freezability.

In the first experiment, as a basic study, we used two successive ejaculates collected from Sarda rams to observe the effect of ejaculate types (single and pool) on the freezability of spermatozoa. All samples cryopreserved using TRIS-citrate-egg yolk extender with 7% glycerol. We observed higher percentage of motile spermatozoa ($P<0.05$) with a highest motility score in post-thaw spermatozoa of pool of first and second ejaculates. In experiment two, ejaculates collected from Veneto endangered sheep breeds to study biochemical composition including Protein Profiles (PP) of SP in ejaculates and individual ram. Ejaculates were cryopreserved dividing into eight treatment groups using TRIS-based and skim milk-based extenders, and both fresh and post-thaw semen were evaluated for plasma membrane integrity with double fluorescence staining and plasma membrane architecture (phosphatidylserine translocation) with Annexin V assay along with routine semen parameters. Correlation between seminal parameters of fresh and cryopreserved semen with biochemical components of SP were statistically examined. Results of this experiment revealed ejaculate variation, more markedly, individual variation in SP composition and demonstrated that pooled (first and second) ejaculates could maintain sperm heterogeneity better when preserved with TRIS-based extender. Moreover, we observed negative but significant ($P<0.01$) correlation of total protein, Mg, P, and positive correlation ($P<0.05$) of protein bands of 75 kDa with apoptosis marker free viable sperm. In third experiment, caudal epididymal sperm collected from Veneto endangered sheep breed, were cryopreserved with TRIS- based and milk based extender to observe post-thaw sperm characteristics, and results demonstrated that post-thaw sperm quality of was inferior to that of ejaculated spermatozoa. In fourth experiment, we observed that diet could affect some SP components, especially, enzymes and energy profiles, and semen quality in terms of, semen volume, sperm concentration and sperm motility in alpaca. In experiment five, alpaca semen was cryopreserved with four types of extenders and both fresh and post-thaw semen were evaluated for plasma membrane integrity with double fluorescence staining and plasma membrane architecture (phosphatidylserine-PS translocation) with Annexin V. Results

showed that TRIS based extender with 0.8% fructose could promote better post-thaw survivability of alpaca semen. we also demonstrated that there was correlation between biochemical composition of SP and both fresh and post-thaw semen quality. We found significant ($P<0.01$) correlation between semen viscosity, and percentage of apoptosis free sperm, Mg, P, total protein of SP. To our knowledge, this is the first report in the presence of apoptosis marker in alpaca viable sperm. However, our results confirm that biochemical composition and SP function varies with diet, ejaculate within male and between males and species. The composition of SP, therefore, is one factor that determines the suitability of individual male for semen cryopreservation and should also be taken into account in the preparation of medium during assisted reproductive techniques (ART) in order to preserve and possibly enhance post-thaw sperm fertilizing ability.

CHAPTER 1. INTRODUCTION

Semen consists of spermatozoa suspended in the fluid medium, called seminal plasma (SP). Its composition depends firstly, on the proportion of sperm and SP, and is further determined by the size, storage capacity, and secretary output of several different organs which comprise the male reproductive tract (Mann, 1964). After discover of spermatozoa and its motility association with SP by Anton van Leeuwenhoek with the aid of a crude microscope in 1677, main research on male reproduction was mainly fascinated by the structural detail and function of spermatozoa. Since, two compartments of semen (sperm and SP) differ in their origin, composition and function, they must be considered separately. Biochemistry of SP is a relatively modern, but rapidly expanding field of research, particularly the biological significance of various bio-chemical constituents of SP.

The widening practice of artificial insemination (AI) with cryopreserved semen for breeding purposes on a large scale reveals the need for improved standards of sperm evaluation and provides a powerful stimulus for morphological as well as bio-chemical investigations on SP. Functional competence of spermatozoa depend on several factors such as normal spermatogenesis, proper maturation during epididymal transit and composition SP. The availability of better quality semen results from the coordinated action of all parts of the male reproductive tract along with endocrine control (Goeritz et al., 2003). The consideration of these interrelationship could allow the understanding of physiological changes of frozen-thawed spermatozoa.

1.1. PHYSIOLOGY OF SPERMATOZOA

Mammalian spermatozoa consist of the head with a nucleus containing the highly compacted male haploid genome, and the flagellum. In the nucleus, chromosomes are highly condensed and thus imped e any transcriptional activity to replace proteins. The spermatozoa acrosome is a modified secretary granule whose membrane and matrix are spatially segregated into head regions of distinct molecular composition, structure, and function and it allows the sperm to interact with and penetrate the oocyte at fertilization. (Kopf and Gerton, 1991; Eddy and O'Brien, 1993). Flagellum is divided into two components:(i) the midpiece containing the mitochondria, which are believed to generate energy (by oxidative phosphorylation) needed for sperm motility (Eddy and O'Brien, 1994), and (ii) the principal and terminal pieces, which beat propelling the spermatozoon forward (Turner, 2003). In the midpiece of the flagellum, the axonemes are surrounded by outer dense fibers (ODF) and mitochondria, while a fibrous sheath (FS) surrounds the axoneme in the principal piece (Baccetti and Afzelius, 1976).

Sperm plasma membrane (PM) is a dynamic, stable and metabolically inert structure, consists of five specific domains involved in different physiological functions: the acrosome, equatorial segment, basal, midpiece region and tail (Ladha, 1998). The sperm head plasma membrane is separated from the midpiece plasma membrane by the posterior ring and this latter domain is separated from the flagellum plasma membrane by the annular ring. Furthermore, the acrosome-overlying plasma membrane is separated from the post-acrosomal membrane by the equatorial segment. In mature sperm its surface membrane is not in contact with intracellular membranes because vesicle mediated membrane transport is blocked (Flesch and Gadella,2000).

Sperm membranes are composed of lipids and proteins. Lipids are preferentially clustered around integral membrane proteins (Hammerstedt et al. 1990). Some of the integral proteins function as pores or channels and others as receptors for other molecules (Mocè et al., 2010). The lipid matrix of the sperm

PM is a heterogeneous mix of phospholipids, glycolipids, and sterols, which are distributed asymmetrically between the inner and outer leaflets of the membrane (Apel-Paz et al., 2003); where the hydrophilic lipid groups oriented to the external and the hydrophobic fatty acyl chains in the internal membrane.

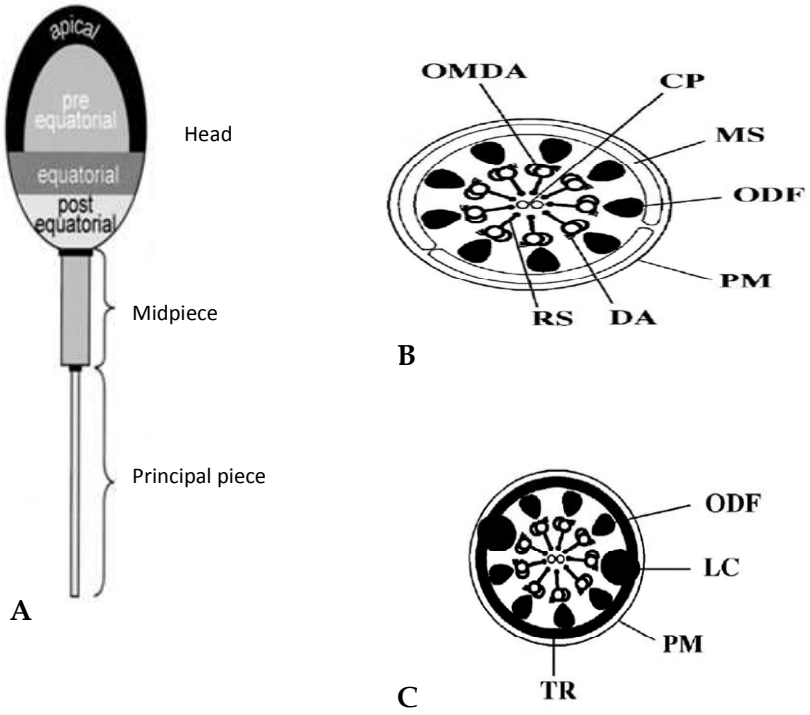


Figure 1.1: Schematic presentation of spermatozoa(A), cross sectin of midpiece(B) and Pricipal piece (C). PM- plasma membrane, MS-mitochondrial sheath, ODFs- outer dense fibers, OMDA- Outer microtubule doublets of the axoneme, DA- Dynein arms, RS- Radial spokes, CP- central pair of microtubule doublets, LC- longitudinal columns of the fibrous sheath, TR- Ttransverse ribs.

Phospholipids are the most representative lipid fraction of the sperm cell membranes and, of these, phosphatidylcholine and phosphatidylethanolamine are the major components (Mann,1964; Mann and Lutwak-Mann, 1981). Different regions of the plasma membrane differ in their cholesterol/phospholipid ratio. The acrosomal membrane of bull spermatozoa has a lower cholesterol/phospholipid ratio than the isolated sperm plasma membrane (Parks et al., 1987). The content of PM cholesterol does not only differ between species,

but also between individual males within a species and between individual ejaculates of a single male (Gadella et al., 2001).

1.1.1. Epididymal Maturation

Testicular spermatozoa are immotile and they must undergo a post-gonadal maturation in the epididymes . The maturation event enables the spermatozoa to exhibit two key functions fundamental to fertilization: 1) the activation of sperm movement and 2) the initiation of sperm capacitation. Sperm have very little endoplasmic reticulum or Golgi apparatus with which to maintain membrane integrity. Therefore, as a 'terminal cells'' to maintain functional status (motility and fertilizing ability) sperm rely on absorption of molecules from the surrounding environment (Amann et al., 1993; Yanagimachi, 1994). During their transit through the epididymis, spermatozoa are subjected to a continually changing luminal environment modified by the secretory and endocytic activities of the cells lining epithelium. Moreover, considerable changes also occur in specific membrane domains.

Principal changes in the sperm PM during epididymal maturation (reviewed by Sostaric et al, 2004):

- **Alteration of topographical configuration of the sperm plasma membrane**
    - Reduction in size and a change in shape of the apical segment
    - Changes in the appearance of the acrosomal contents
    - Changes in the density and distribution of membrane particles
    - Structural remodelling of acrosome by compartmentalization of protein
- **Modification in PM bio-chemical composition**

- Disappearance or redistribution of sperm surface glycoproteins mediated by glycosidases, phosphatases, proteases, glycosyltransferase etc.
- Extensive cross-linking of nuclear protamines by disulphide bonds.
- Increase in total surface negative charge.
- Relocation of surface antigens, and addition, elimination or modification of surface proteins related with acrosome reaction (AR).
- Incorporation with receptors for zona Pellucida(ZP).
- Adsorption and/or integration of several glycoproteins and peptides in the plasma.
- Acquisition of antiagglutinous proteins and proteins related with sperm motility.
- Selective and progressive loss of phospholipids and an elevated proportion of cholesterol [phosphatidylcholine (PC) by 37%, phosphatidylethanolamine (PE) by 45%, phosphatidylinositol (PI) by 79%, phosphatidylserine (PS) by 78%, cardiolipin (C) by 35%, sphingomyelin (Sph) by 17%, ethanolamine plasmalogen (EP) by 61%, and choline plasmalogen (CP) by 9%.]

Sperm are stored in a quiescent state in the cauda epididymes before ejaculation. This gamete reserve permits ejaculation of larger numbers of sperm than the daily testicular production (Copper, 1999). The number of sperm stored varies according to the reproductive pattern, social and mating behavior of a given species (Amann, 1981). Epididymal epithelium maintains the internal environment of lumen through different functions such as, reduction of luminal sodium ion concentration, secretion of viscous mucoprotein that restrict sperm movement, steady production of acids keeping intracellular sperm pH low, protection of sperm membranes by the secretion of decapacitation factor (Copper, 1999). Moreover, epididymal epithelium also involves in prevention of oxidative stress and protection of sperm from the immune system (Hinton et al.,

1995). A series of enzymes produced by the epididymal epithelium, such as glutathiones- transferase, superoxide dismutase, glutathione peroxidase and catalase are important for the elimination of reactive oxygen species and for the removal of toxicants (Cooper, 1999; Hinton et al., 1995).

1.1.2. Sperm activation

In spite of having potentiality to be motile, spermatozoa stay in a dormant state in the cauda epididymides and are activated only after or during ejaculation. Spermatozoa display two types of physiological motility: i) activated motility, observed in freshly ejaculated sperm. This kind of motility is essential for transport through the female reproductive tract to the oviduct, whereas, ii) hyperactivated motility, is seen mostly in capacitated sperm essential for sperm detachment from the oviductal epithelium and to reach the site of fertilization, and to penetrate the cumulus and ZP of the oocyte (Katz and Yanagimachi, 1980; Ho and Suarez, 2001). Flagellum of an activated sperm generates a symmetrical, lower amplitude waveform that drives the sperm in a relatively straight line. In contrast, in hyperactivated sperm, the flagellar beat becomes asymmetrical and higher amplitude, which results in circular or figure-eight trajectories (Yanagimachi, 1994; Ishijima et al, 2002). A variety of mechanisms have been proposed to explain the activation of mammalian sperm motility at ejaculation (Jones and Murdoch, 1996).

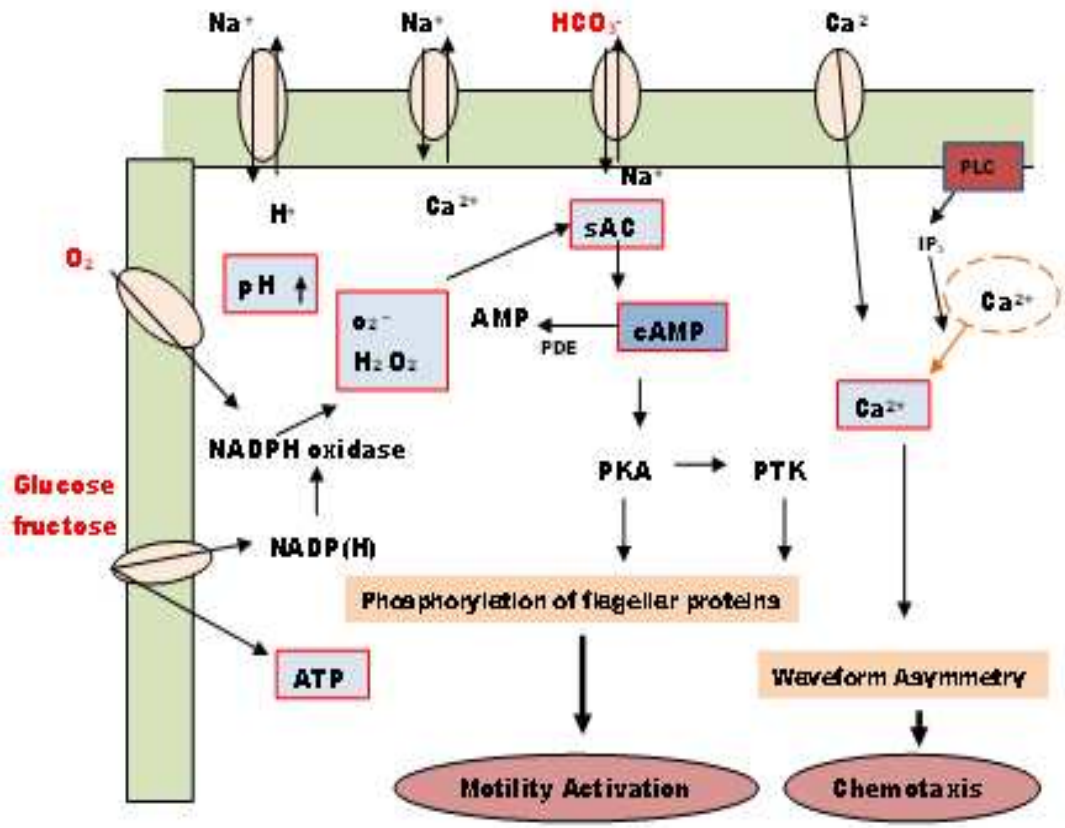


Figure 1.2. A model for sperm motility activation (modified from Aitken, 2000; Inaba, 2003). Three primary stimuli such as exposure to glycolysable sugars, oxygen, and bicarbonate are thought to be responsible for sperm activation. Along with these, several kinds of ionic channels and receptors and enzymes for cyclic nucleotide synthesis have been identified as molecules involved in motility activation. HCO_3^- -bicarbonate, PDEs-phosphodiesterases sAC -Soluble adenylyl cyclase, PKA- cAMPdependent protein kinase, PTK-protein tyrosine kinase, PLC–phospholipase.

Ejaculated spermatozoa differ from epididymal spermatozoa in pH (Hammerstedt et al, 1979), respiration (Lardy and Ghosh, 1952), adenosine triphosphate synthesis and consumption (Cascieri et al, 1976), heparin-binding sites (Nass et al, 1990), and the profile of proteins bound to the PM (Dostalova et al, 1994; Calvete et al, 1996). Ejaculated spermatozoa, while motile, are unable to fertilize and they must undergo complex sequences of capacitation, egg recognition, acrosome reaction, and sperm-oocyte fusion. Since mature spermatozoa are transcriptionally inactive and unable to synthesize new proteins, they rely on protein phosphorylation as a means of altering their functions. All these precisely timed events are regulated by the activation of different intracellular signaling pathways, which often depend on the assembly

of multiprotein signaling complexes (Heydecke et al., 2006). ATP produced by glycolysis or oxidative respiration is absolutely required for phosphorylation itself and cAMP synthesis (Urner and Sakkas, 2003).

1.1.3. Capacitation

Capacitation of spermatozoa is a complex process occurring after sperm ejaculation and is required to produce fertilization of the oocyte *in vivo* and *in vitro*. Visconti et al (2009) have described the possible phenomenon and multfactorial evants involved in capacitation process.

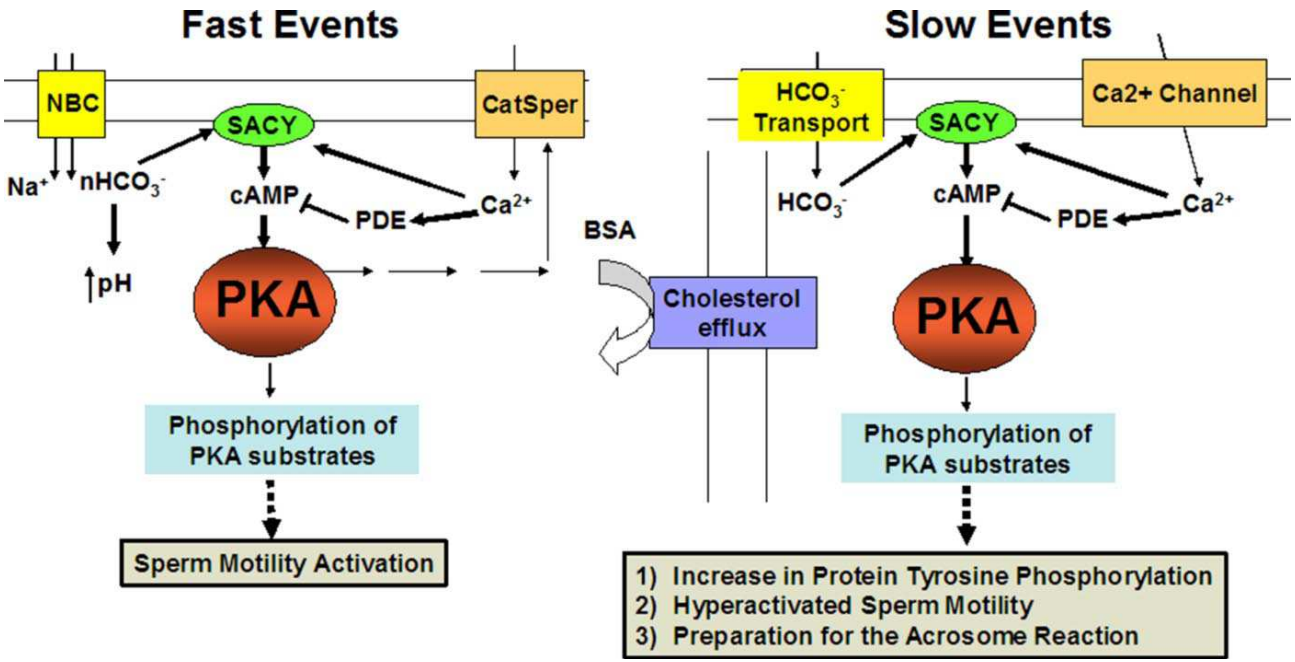


Figure 1.3. Molecular basis of fast and slow events associated with sperm capacitation. (*Fast Events*) As soon as sperm are in contact with an isotonic solution containing HCO^{3-} and Ca^{2-}, a vigorous flagellar movement is observed. At the molecular level, this process depends on the increase in PKA activity and is mediated by a HCO^{3-} and Ca^{2-}, coordinated stimulation of the atypical adenylyl cyclase (SACY). At these instances, it is believed that HCO^{3-} and Ca^{2-}, are transported by a Na^{+}/ HCO^{3-} cotransporter (NBC) and a sperm-specific Ca_{2}^{-}, channel (CatSper). (*Slow Events*) After an extended period of incubation in vivo or in vitro, sperm acquire the ability to fertilize. The fertilization capacity is preceded by the preparation to undergo the exocytotic acrosome reaction and by changes in the motility pattern known as hyperactivation. At the molecular level, these changes are correlated with an increase in tyrosine phosphorylation. This increase is downstream of PKA stimulation; however, opposite to the fast processes, the increase in tyrosine phosphorylation also depends on the presence of cholesterol acceptors in the capacitation medium (Vasconti, 2009).

The reaction occurs during capacitation, are confined mainly at molecular level and cannot be detected at the structural level. The mechanisms of sperm capacitation are associated with PM and intracellular ionic modifications (Yanagimachi, 1994; de Lamirande et al., 1997; Visconti et al., 1998). Major biochemical changes involve :

- removal or inactivation of decapacitation factors on the sperm surface;
- changes in localization of molecular structure, and lateral mobility of integral proteins;
- adsorption onto the sperm of proteins from the female tract;
- alterations in membrane lipid composition, in particular in the cholesterol/phospholipid ratio (Cross, 1998);
- ionic deregulation manifested as increases in internal Ca2+,Na+, and pH (Frase, 1995); generation of reactive oxygen species (de Lamirande et al., 1997);
- an increase in CAMP and protein tyrosine phosphorylation (Visconti et al., 1998).

Like a long term process, this process starts when sperm leave the epididymis, should not be completed until ovulation occurs. Heparin-like Glycosaminoglycans (GAGs) and High density lipoprotreins (HDL), components of the oviductal and follicular fluid are considered to induce and enhance sperm capacitation (Ehrewald et al., 1990; Therien et al., 1997). Because of the long residency of spermatozoa in the isthmus prior to ovulation, the oviduct is considered the principal site for completion of sperm capacitation (Iborra et al., 2000). Harrison (1996) described capacitation as a ''window of destabilization'', as this process does not produce stable 'activated' population, but make spermatozoa susceptible to membrane degeneration and lead to death gradually. The timing for completion of the capacitation process is dependent on the sperm intrinsic 'readiness' or level of maturity at the time of ejaculation, and capacitated sperm has a limited life span (Medeiro, et al., 2002).

1.1.4. Acrosome reaction (AR)

The acrosome reaction (AR) is an exocytotic process that spermatozoa undergo for acquisition of fusogenicity by the equatorial segment of the plasma membrane; required to bind and to fuse with the oolemma (Yanagimachi, 1994). Multiple fusions between the outer acrosomal membrane and overlaying plasma membrane leading to the release of acrosomal enzymes and exposure of the molecules present on the inner acrosomal membrane surface that mediate fusion with oolema (Margalit *et al.,* 1997; Baldi *et al.,* 2000). AR is a well-defined structural and biochemical event, when acrosin, a serine protease, is liberated and play a major role in the attachment to, and penetration of the ZP by spermatozoa (Margalit *et al.,* 1997; Honda *et al.,* 2002). Periacrosomal plasma membrane domain of the spermatozoon contain specific receptors for zona pellucida (Wassarman, 1995) and the binding of these receptors to the egg ligand activates both G protein and tyrosine kinase signaling pathways (Ward and Kopf, 1993) leading to some sequential changes, such as influx of calcium, an elevation of intracellular pH, fusion occurs between the periacrosomal plasma membrane and the outer acrosomal membrane, dispersion of acrosomal contents and finally, release of enzymes (acrosine and hyaluronidase)to facilitate sperm penetration through oocyte investment (Kopf and Gerton, 1991; Meizel, 1984). Cross (1998) proposed that a decrease in the cholesterol content of sperm plasma membrane during capacitation leads to increased membrane fluidity with subsequent increase in membrane permeability and fusion capacity, so that spermatozoa can undergo AR. A number of soluble N-ethylmaleimide-sensitive factor-attachment protein receptor (SNARE) proteins located on the apical side of the sperm acrosome region may couple calcium entry to exocytosis (Evans and Florman,2002). Following penetration of the ZP, sperm adhere to and fuse with PM of the egg mediated by adhesion proteins (Evans and Florman, 2002; Henkel *et al*, 2005b). Egg activation occurs after fertilisation and the process of embryonic development is initiated.

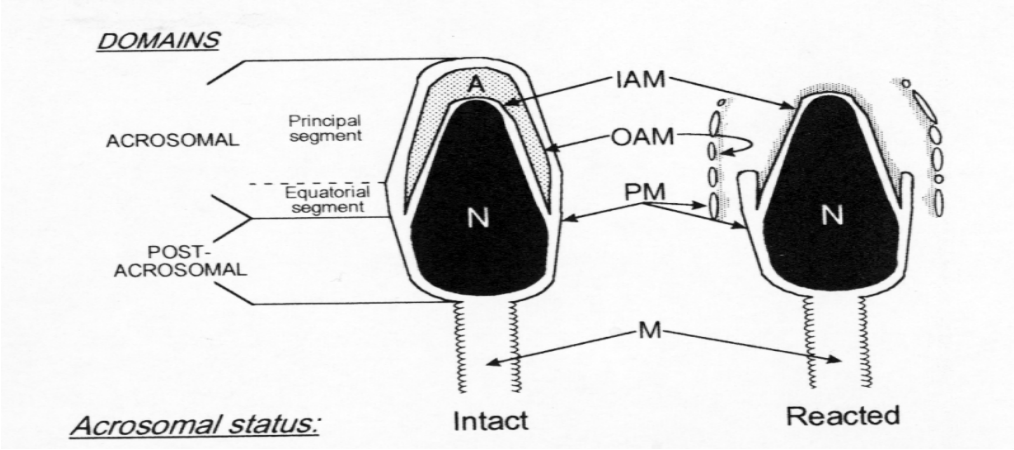


Figure 1.4. Schematic presentation of Acrosome reaction in mammalian spermatozoa. IAM-Internal acrosomal membrane; OAM-Outer acrosome membrane; PM-Plasma membrane; N-Nucleus; M- Mid piece (McLeskey et al., 1998).

1.2. BIOCHEMICAL COMPOSITION AND FUNCTION OF SEMINAL PLASMA

Seminal plasma (SP) is a complex fluid portion of semen in which spermatozoa are suspended at the time of ejaculation. SP contributes to the majority of semen volume (Moura et al., 2007), and its biochemical components are secreted from rete testis, epididymis and accessory sex glands (AGs) of the male reproductive tract (Mann and Lutwak-Mann, 1981). Its pH varies with species, slightly acidic in bulls and rams, and slightly alkaline in camelids. SP mediates the chemical function of the ejaculate. Previously, it was believed that all AGs could be anatomically and functionally "homogenous" due to their similar embryological origin or related morphological structures. Discovery and identification of several substances in AGs secretion, such as, citric acid, prostatic phosphatase, fructose and phosphorylcholine (Mann, 1964) opened the way to think and study different chemical secretory activities of these glands, and their possible roles for spermatozoa. Conventional role for SP is survival medium that facilitates transport of spermatozoa. This view has been contradicted by the advent of reproductive technologies, where it is possible to fertilize ova with washed spermatozoa and produce viable embryos resulting in live offspring without exposure of the female reproductive tract to SP. On the other hand, it has also been observed that the use of preserved semen for artificial insemination (AI) in livestock species, which often involves extensive dilution or removal of SP, results in lower fertility rates than those of natural mating (Tummaruk et al., 2000). This evidence suggests that SP components participate in key events related to sperm function, fertilization and embryo development in the female reproductive tract. However, the knowledge on effects of SP components on spermatozoa and fertility is scattered, and somehow conflicting in ruminant and camelid. However, the identification of active factors in SP and mechanisms by which they act, are not sufficiently understood in different species. Specially in camelids, the origin, composition and function of the viscous component of SP remain a mystery and only some biochemical morphological characteristics of SP

have been described (El-Manna et al., 1986; Garnica et al., 1993, Mosaferi et al., 2005).

SP is made up of *ions:* Na^+, K^+, $Zn^{+,}$ Ca^{++}, Mg^{++}, Cl^- ; *energy substrates*: fructose, sorbitol, glycerylphosphocholine; *organic compounds*: citric acid, amino acids, peptides, low- and high molecular weight proteins, lipid, hormones, cytokines etc. Nitrogenous components such as ammonia, urea, uric acid and creatinine, and reducing substances such as ascorbic acids, hypotaurine also exist in SP of ruminants. The composition of SP is determined by the size, storage capacity and secretory output of different organs of the male reproductive tract. AGs known as seminal vesicle, prostate and bulbourethral gland, contribute most of the volume of the ejaculate.

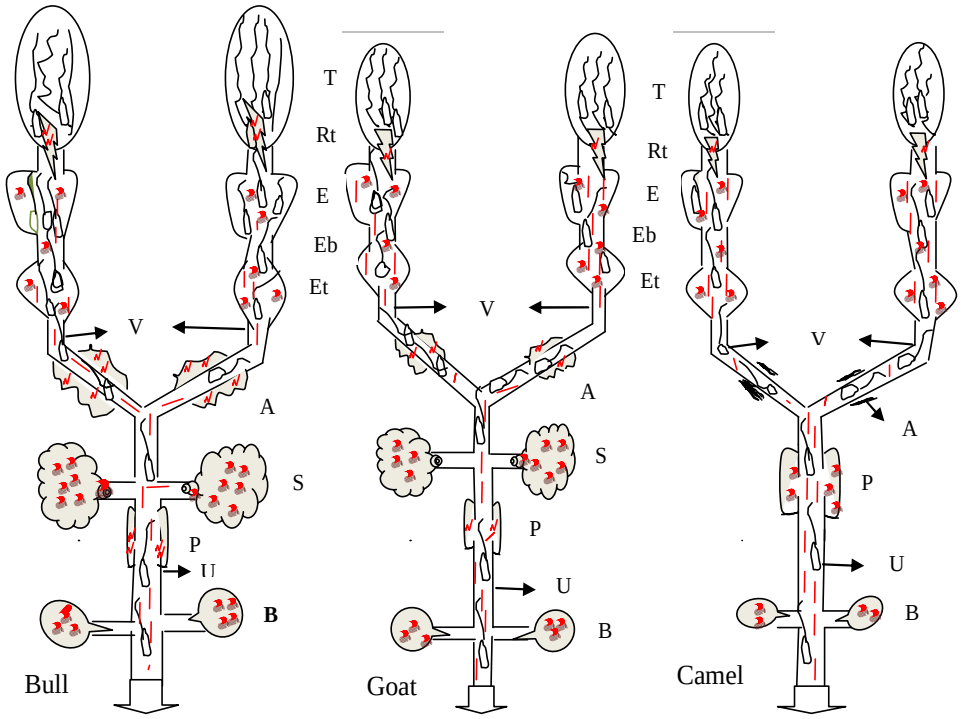


Figure 1.5. Schematic presentation of male reproductive systems of bull, goat, and camel. The drawing illustrates sources and flow of SP (red color). A- Ampullary glands B-Bulbo-urethral gland, E-Head of epididymes, Eb-Body of epididymes, Et-Tail of epididymes, P- Prostate, Rt-Rete testes, S-Seminal vesicle, U-Urethra, V-Vas deference

Seminal vesicle's secretion constitutes major portion of SP (in most of the ruminants except camelids where it is absent) at ejaculation and influences metabolism, motility and surface properties of spermatozoa (Metafora et al., 1989). The protein and biochemical composition of SP varies with breed, animal and ejaculate, due to numerous sources of intra- and inter-animal variation including climate, plane of nutrition, sexual maturity, health status, frequency of collection and ejaculate number (Evans and Maxwell, 1987).

A summary of the most important constituents of SP of ruminants and camelids is given in Figure 1.6.

Differences in concentrations of some elements in SP may be due to variations in exposure from feeding, management and different detection methods. Apart from this variability, there are also changes brought about in SP composition following ejaculation through the action of enzymes present in SP and metabolic activity of spermatozoa suspended in SP.

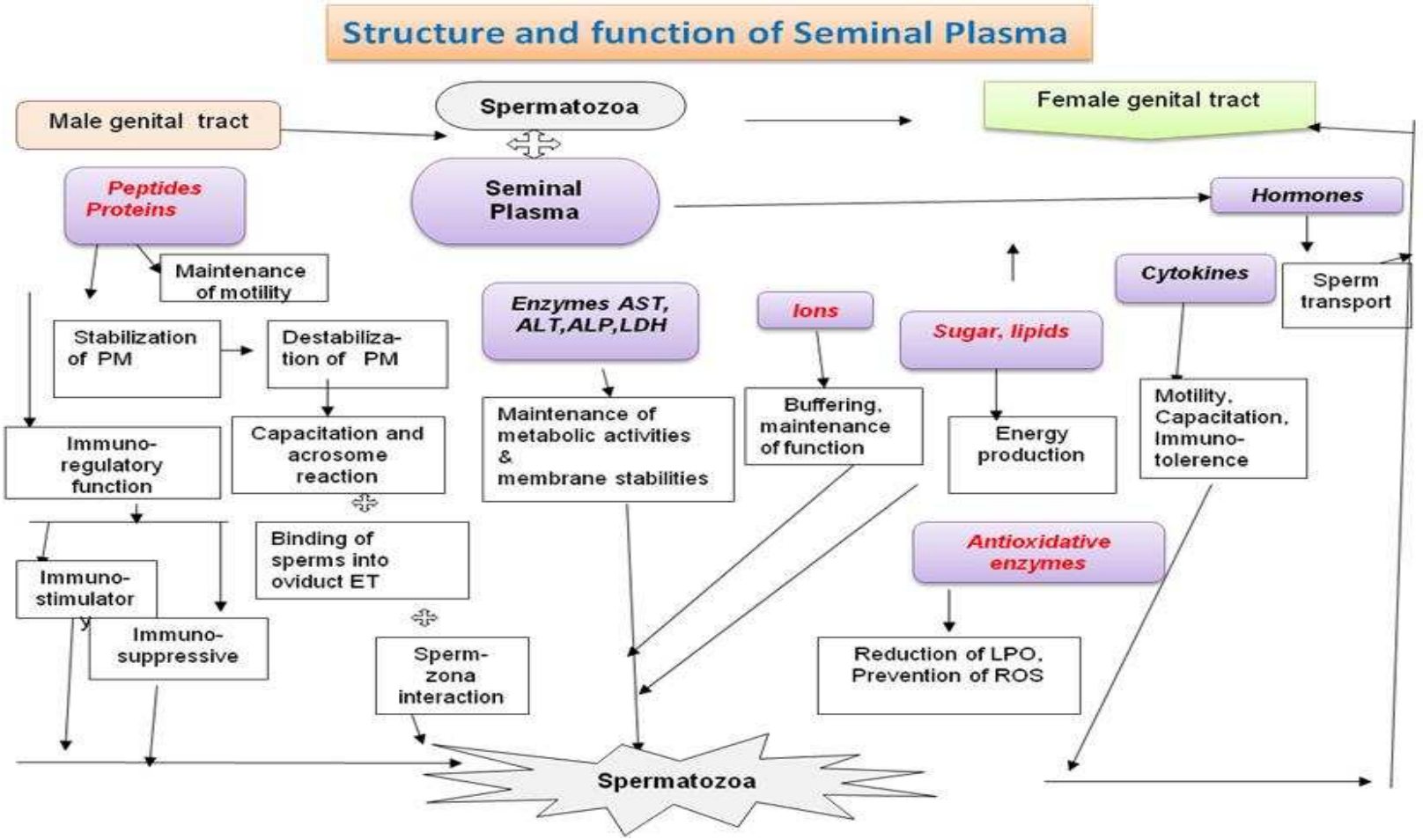
Along with chemical composition, roles of SP on mature spermatozoa have been widely studied with contrasting results. Functions of the component of SP are not fully understood or appreciated. Researches in several species highlight varying roles for SP including: (i). Activation and augmentation of the motility of spermatozoa (ii). Serves as buffer, optimal osmotic and nutrient medium (iii). Prevention of premature activation during physiological transport of spermatozoa and stabilization of the plasma membrane with capacitation inhibitors (Desnoyers and Manjunath, 1992; Villemure et al., 2003) (iv). protection of spermatozoa from being phagocytosed and destroyed in an inflammatory environment (Troedsson et al., 2000; Alghamdi et al., 2004) (v). Regulation of sperm transport and elimination (Troedsson et al., 2005) (vi). hastens ovulation (Marion, 1950) and induces ovulation in pig and camelids (O'Leary et al., 2004; Ratto et al., 2005) (viii). helps in sperm-ovum interaction (Gonçalves et al., 2006; Souza et al., 2008) (ix). activates the expression of embryotrophic cytokines and helps in the preparation of the maternal tract for the developing embryo, particularly by facilitating immune changes required to accommodate pregnancy

(Robertson, 2005) and finally, (x) influences fertility (Rozeboom et al., 2000). Conversely, detrimental effects of SP on motility and viability of spermatozoa after freezing-thawing have also been reported (Schmehl et al., 1986; Garcia and Graham, 1987). In this section, potential components of SP and their specific functions are briefly discussed.

Table 1.1. Composition of SP in ruminants and camelids (Values are mg/dl unless otherwise stated).

Content	Bull	Ram	Goat	Buffalo	Old world camelids	New world camelids
Fructose	150-900	150-600	875	368-815	23.5	3-7
Glucose	300	0.9-1.6	4.8-8.8	13-52	29-42	4-8
Citric acid	340-1150	110-260	-	440-444	9.8	3.1-6.0
Total proteins(g/dl)	3.8	2.30-2.50	0.77- 1.48	-	1.6-2.6	3-4
Total lipids	29	254-396	-	150-175	87	51-115
Phospholipids	149.1	-	57	6.9-59.4	26-48	27-31
Cholesterol	312.16	-	-	117.83	15.3-25.9	0-8
Glutamic acid	1.0-8.0	4.5-5.2	-	4.28	-	-
Na	140-280	120-258	60-183	260-278	-	-
K	80-210	50-140	76-255	192-205	-	-
Ca	35-60	6-15	5-15	30	7.7-8.8	13-31
P	9	4.8-12.0	-	8-9	1.7-4.6	7-17
Cl	110-290	86	82-215	303-347	84-120	263-491
Mg	7-12	2-13	1-4	4.3-5.7	-	2.1-4.85
Zn	2.6-3.7	56-179	-	o.80-1.17	-	-
Testosterone (pg/ml)	210-1310	25-375	-	970	-	-
Oestrogen (pg/ml)	20-166	-	-	43.67	-	-
Prostaglandins (ng/ml)	5-10	500-20,000	-	-	-	-
ALP	246BU/dl	14895-40818 mU/ml	-	315BU/dl	-	50-3143 UI/l
AST	345-623 SFU/ml	190-256 mU/ml	-	166 units/ml	-	-
ALT	15.0-18.3SFU/ml	39-148mU/ml	-	34units/ml	-	0-115 UI/l
LDH	1909 units/ml	968-1697 mU/ml	-	1621 BBU/ml	-	-

Figure 1.6. A model of SP structures and functions. The model focuses the main content of SP and their functions on spermatozoa and the female genital tract.



1.2.1. Proteins

SP contains several proteins, many of which are secretory products of epididymes and seminal vesicles (Chandonnet et al., 1990). There has been intense research on the identification and functions of SP proteins in different animals. A comprehensive review on biochemical characteristics and functions of SP proteins has recently been published by Muiño-Blanco et al. (2008), where the authors have discussed elaborately the protective capability of some low molecular SP proteins against cold shock of spermatozoa and antioxidant properties. The protein component of SP varies among species and has important effects on sperm function (Thomas et al., 2003). The addition and removal of a variety of SP proteins during epididymal maturation and at ejaculation play an important role in the maintenance of plasma membrane stability (Desnoyers and Manjunath,1992), motility (Henricks et al., 1998; Sánchez-Luengo et al., 2004), capacitation (Therien et al., 1998), sperm-egg interaction and fertilization (Yanagimachi, 1994). SP proteins also promote phagocytosis and binding of dead spermatozoa, and polymorphonuclear cells via protease activity (Dacheux et al., 2003). Mammalian spermatozoa acquire their motility and ability to recognize and fertilize oocytes by sequential interactions with proteins present in the epididymal fluid. Major epididymal proteins include lactoferrin, clusterin, Procathepsin D, cholesterol transfer protein etc. It has been suggested that various epididymal proteins fulfill different roles, some by directly modifying the sperm membrane surface or composition and others by contributing to the preservation of sperm integrity (Dacheux et al., 2003).

In bull SP, a group of heparin-binding proteins (HBPs) called bovine seminal plasma proteins (BSPs), are present, which are secreted by seminal vesicles. Four proteins are identified as members of BSP family referred as BSP-A1, BSP-A2, BSP-A3 and BSP-30-kDa. They represent 70-86% of the total protein content of bovine SP (Manjunath and Sairam, 1987; Moura et al., 2007). BSP-A1 and BSP-A2 share the same primary structure and are called together PDC-109

(Esch et al., 1983). At ejaculation, spermatozoa mix with BSPs and these proteins remove some (5–8%) cholesterol (first cholesterol efflux) from the plasma membrane and bind to choline phospholipids preventing free movement of phospholipids, thus stabilizing the plasma membrane (Villemure et al., 2003). Binding sites of BSPs appear to be lipids present on the plasma membrane of spermatozoa. BSP-A1, BSP-A2, and BSP-A3 bind specifically to phospholipids, which contain the phosphorylcholine (PC) groups, whereas BSP-30Kd displays a much broader binding specificity by preferentially binding to choline phospholipids but also interacting with phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol, phosphatidic acid and cardiolipin (Desnoyers and Manjunath 1992). BSPs bind to the sperm surface at acrosome, postacrosome and midpiece region after ejaculation, which are important for initiating motility (Scheit et al., 1988; Manjunath et al., 1994). PDC-109 is known to increase sperm motility and the pumping efficiency of plasma membrane Ca^{2+}-ATPase in an irreversible, cooperative manner (Sánchez-Luengo et al., 2004). When spermatozoa reach oviduct after deposition in the female reproductive tract, they bind to the oviductal epithelium to form a reservoir to prolong their motile life span. BSPs mediate sperm binding to the oviduct epithelium and maintain sperm motility in that region of the female reproductive tract (Ignotz et al., 2001; Gwathmey et al., 2006). BSPs mainly promote capacitation of bovine spermatozoa by inducing phospholipid and cholesterol efflux from the plasma membrane (Thérien et al., 1998; Manjunath and Thérien, 2002). When spermatozoa reach the oviduct, sperm-bound BSPs interact with high density lipoproteins (HDL) present in the oviduct and/or follicular fluids resulting in removal of cholesterol (second cholesterol efflux) as well as removal of BSP proteins from the plasma membrane (Thérien et al., 2001). This reaction destabilizes the plasma membrane and induces some intracellular signal transduction pathways, among which a rise in the plasma membrane permeability to Ca2+ and in its intracellular pH. Sperm-bound BSPs may be inserted into the plasma membrane to establish the communication with calmodulin, which is bound to the inner surface of the plasma membrane and

thus participates in the regulation of events involving Ca2+ transport during capacitation and acrosome reaction (Manjunath et al, 1993). Yu et al. (2003) have proposed that PDC-109 also prevents premature acrosome reaction of spermatozoa in the female reproductive tract by inhibiting protein kinase C activity. All these functions are important in maintaining spermatozoa in an appropriate state to facilitate fertilization in the female reproductive tract. Indeed, the stimulation of BSPs for capacitation is dependent on exposure time and presence of heparin or high density lipoprotein or glycosaminoglycan (GAG) present in the oviductal fluid (Manjunath and Therien, 2002).

SP proteins of other ruminants, like rams (RSP-14 kDa, RSP-16 kDa, RSP-20 kDa and RSP-24 kDa) (Jobim et al., 2005; Bergeron et al., 2005) and goats (GSP-14 kDa, GSP-15 kDa, GSP-20kDa and GSP-22kDa) (Villemure et al., 2003) also share heparin and phosphorylcholine-binding properties similar to BSPs. Very recently, Harshan et al. (2009) have confirmed the presence of PDC-109 in buffalo SP. In goat, GSP-20 and GSP-22 show a strong heparin affinity, whereas GSP-14 and GSP-15 interact weakly (Villemure et al., 2003). RSP-20 and RSP-24, both appear to have higher affinity for heparin compared to RSP-14 and RSP-16 (Bergeron et al., 2005). In small ruminants, specific functions of these proteins are yet to be determined. It seems that they could be involved in sperm capacitation and gamete interaction, stabilizing the sperm membrane in a first step as decapacitating factors and latter participating in the membrane modification during capacitation, as previously suggested for BSPs. Barrios et al. (2005) have postulated a role of RSP-14 in stabilizing membrane phospholipid and cytoskeleton. Furthermore, RSP-14 and RSP-20 have protective effects to recover membrane integrity of cold-shocked spermatozoa (Perez-Pe et al., 2001b). However, more studies are still required to understand clearly the function(s) of these proteins in the sequence of complex processes of capacitation, acrosome reaction and fertilization.

Another protein family known as spermadhesines, has been identified in SP of bulls (Einspanier et al., 1994), rams (Bergeron et al., 2005) and bucks (Melo et

al., 2008). These proteines show a molecular mass of 12- kDa to 16-kDa and was initially designated as aSFP (acidic seminal fluid protein) (Teixeira et al., 2002). These proteins are associated with the sperm surface and are secreted from seminal vesicles (Teixeira et al., 2006). They are multifunctional proteins exhibiting ligand-binding affinities to several oligosaccharides, sulfated polysaccharides, serine protease inhibitor, glycosaminoglycans and phospholipids (Töpfer-Petersen et al., 1998). This kind of binding property may hypothesize aSFPs involvement in sperm capacitation and sperm-egg interactions, which yet to be investigated in ruminants. Besides these, aSFPs restrict lipid peroxidation and regulate mitochondrial activity. They exert an energy-preserving effect by restricting motility and their abilities for the reversible suppression of motility and metabolic activity as well as the protective action against lipid peroxidation may serve to maintain the viability of spermatozoa (Schoneck et al., 1996). It has been proposed that aSFPs do not bind extensively to spermatozoa and is diluted out easily in the female reproductive tract, allowing for rapid restoration of motility of spermatozoa (Dostalova et al., 1994). Calsemin, another heat-stable, low molecular weight acidic protein fraction has been identified in ram SP (Bradley and Forrester, 1982). This protein fraction acts as a Ca_2 -dependent regulator of two enzymes: Ca_2-dependent Mg_2-ATPase and 3′:5′-cyclic nucleotide phosphodiesterase. Very little is known about this protein fraction. It was proposed that calsemin could act like as calmodulin and Ca2-dependent stimulation of flagellar beat activity might occur in the presence of calsemin (Bradley and Forrester, 1982). A calmodulin-like protein (CLP) had also been identified and partially characterized in buffalo SP, where its concentration is relatively high and it might be responsible for some of the physiological changes involved in capacitation and acrosome reaction (Sidhu and Guraya, 1989).

A cell adhesion molecule known as osteopontin (OPN) of 55 kDa is present in SP of bull, derived from ampulla and seminal vesicle (Cancel et al., 1997). OPN is believed to bind to spermatozoa at ejaculation and is assumed to remain bound until sperm reach the site of fertilization (Souza et al., 2008). It participates in

sperm–oocyte interaction affecting fertilization and early embryonic development (Gonçalves et al., 2006; Souza et al., 2008).

A peptide of 47-residues known as Seminalplasmin (SPNL), isolated from bovine SP (Reddy and Bhargava, 1979), had been shown to be a transcription-inhibitory protein (Scheit et al, 1985). The biosynthesis of SPNL occurs in seminal vesicles of bull (Wempe et al., 1990). SPLN, also known as caltrin, acts as a calcium transport regulator on bull spermatozoa (Sitaram et al., 1997) and increases the fluidity of the plasma membrane and acrosomal membrane (Shivaji et al., 1989). Although SPLN inhibits spontaneous acrosomal exocytosis by inhibiting the Na/Ca_2 antiporter pathway, it has been proposed that its presence on the plasma membrane is essential for bringing about the physiologically relevant zona pellucida agonist-induced acrosomal reaction in bull spermatozoa (Clark et al., 1993).

In camelids, research for identification and quantification of protein has mainly been concentrated on a specific protein fraction of high molecular weight, which has GnRH like activities (Paolicchi et al., 1999; Pan et al., 2001), termed as ovulation inducing factor (OIF). The property and nature of OIF Will be dicussed in later section. In our preliminary study, we have observed low molecular protein fractions (like 10 kDa or more) that have good relationship with freezability of alpaca semen. Research works for identification of these proteins fraction are in progress.

It is worthy to point out that the quantity and quality of SP proteins vary with individual animal and some environmental factors like, season of collection, temperature, nutrition and stress (Perez-Pe et al., 2001a). Cardozo et al. (2006) observed (by SDS-PAGE) that several protein bands (molecular weight 20–70 kDa) were absent in SP of certain rams during the non-breeding season. Also in buck, HBPs present a different pattern during breeding and non breeding seasons (La Falci et al., 2002). Though some SP proteins are present in both breeding and non-breeding season, higher concentration is observed in the breeding season (Smith et al., 1999). These seasonal changes in Protein Profiles

could be due to seasonal variation in gonadotrophin levels and their receptors in the testes (Xu et al., 1991) that affect endocrine gonadal function and secretions of epididymes and seminal vesicles. Seasonal variation may occur due to differential protective effect of SP, which may be related with antioxidant enzyme activity (Marti et al., 2007).

1.2.2. Amino acids and Enzymes

There is a broad range of amino acids in SP and most of these amino acids are originated in the testes or epididymides. Their concentrations increase after ejaculation due to extensive proteolytic activity that take place in semen. Amino acids serve as a readily oxidizable substrate for energy yielding reactions in semen (Neumark and Schindler, 1967). The amino acid found in greatest concentration in SP is glutamic acid, which is accompanied by a high level of glutamic oxaloacetic transaminase (GOT) activity (Flipse, 1960). L-Arginine, (a non-essential amino acid) also plays an important role in sperm metabolism and motility. It acts as a source of energy for normal sperm motility in the form of arginine phosphoric acid (Patel et al., 1998). The addition of amino acids has been reported to overcome to some extent the deleterious effects of excessive dilution on semen.

Several enzymes of SP such as,Glutamic oxaloacetic transaminase (GOT)/ Aspartate amino transferase (AST)/(AAT), Glutamic piruvate transaminase (GPT)/ Alanine aminotransferase (ALT), Alkaline phosphatase (ALP) and Lactate dehydrogenase (LDH) are essential for metabolic processes which provide energy for viability, motility and fertility of spermatozoa. These enzymes are used as good indicators of semen quality as they measure the plasma membrane stability of spermatozoa (Corteel, 1980). A positive correlation between membrane damage and AST, ALT and LDH release was reported (Borque and Ayllón, 1996; Sirat et al., 1996). Transaminases are located primarily in the midpiece of spermatozoa (Mann and Lutwak-Mann, 1981). The measurement of these enzymes from SP is considered to be a sensitive indicator

of the plasma membrane damage (Sirat et al., 1996), and altered membrane functions which may occur due to inadequate epididymal maturation associated with increased semen collection frequency. LDH is localized in cytosol and mitochondria of spermatozoa (Burgos et al., 1995) and is loosely bound to the cell wall that facilitates its easy release into SP (Rao et al., 1984). In ruminants, the specific action of this enzyme has not been reported. Research in boar SP revealed that LDH could be responsible for driving glycolysis when O_2 is limited, by carrying NADH-mediated reduction of pyruvate to lactate (Jones, 1997) and reduced LDH activity in SP might result from reduced systhesis of LDH in testicular tissue indicating disturbed sperm function in the testicular parenchyma and changed sperm metabolism. Another important enzyme ALP in SP is derived primarily from the secretion of AGs. ALP activity is observed on sperm head, midpiece and tail fragments. Sperm motility is a complex phenomenon which, involves a variety of biochemical functions such as phosphorylation of proteins by cyclic- AMP dependent protein kinase. ALP is known to regulate this step by dephosphorylation (Tang and Hoskins, 1975). Differences in composition, biophysical characteristics and function of extra and intra cellular membranes of spermatozoa may be important factors for releasing these enzymes in seminal fluids of different animals. Moreover, the presence of enzymes activities follows reproductive seasonality (Gündoğun, 2006).

An ubiquitous enzyme known as Phospholipases A_2 (PLA_2), capable of hydrolyzing the *sn*-2-position of phospholipids, has been detected in spermatozoa and SP of several species (Upreti et al., 1999). It is believed that this enzyme plays a major role in the late maturational events of spermatozoa, particularly in the acrosomal reaction and in sperm-egg fusion (Yuan et al., 2003). In bucks, egg yolk coagulating enzyme (EYCE) or Bulbo-urethral secretion III (SBUIII) presents phospholipase activity. This enzyme is claimed to hydrolyze phospholipids from egg-yolk (present in diluents used for preservation) into unsaturated fatty acids and lysophospholipids, which cause coagulation and toxicity for spermatozoa. SBUIII has been purified, characterized and identified as a monomeric 55–60 kDa *N*-glycosyl-protein (BUS60) that exhibits heparin

affinity. It has a very close homology with pancreatic-lipase-related proteins 2 (PLRP2) (Pellicer-Rubio et al., 1997). In a recent study, Sias et al. (2005) have confirmed (by DNA sequencing) that bulbo-urethral secretion 60 (BUS60) is PLRP2, which has phospholipase A_1 activity. The physiological role of GoPLRP2 is still unknown, but this enzyme might be associated with the reproductive activity of bucks. Generally, bulbo-urethral glands produce a mucoid secretion that cleans the urethra prior to semen passage upon ejaculation. GoPLRP2 might be involved in this cleaning process of the buck genital tract (Sias et al., 2005), which demands intensive research for confirmation.

SP also contain enzymes that protect spermatozoa in the female reproductive tract like platelets activating factor acetylhydrolase (PAFAHs) and its activity has been detected in bovine SP (Parks and Hough, 1993). Soubeyrand et al. (1998) have specified that the enzyme responsible for most of PLA_2 activities in bull SP is a PAFAHs of 60 kDa, which is expressed exclusively in the seminal vesicles and the ampulla. Although it is capable of hydrolysing long-chained phosphatidylcholine, it displays a highly preferential activity towards platelets activating factors (PAF). It is very important to note that mammalian spermatozoa contain a relatively high proportion of ether-linked-phospholipids, which can serve as precursors for PAF synthesis involved in sperm capacitation (Hough and Parks, 1994). Release of sperm-derived PAF within the female reproductive tract could evoke an inflammatory response by triggering neutrophil activation generating superoxide metabolites, causing extensive cellular damage with irreversible arrest of sperm motility (Kovalski et al., 1992). PAFAHs may be responsible to inhibit or delay actions of sperm-derived PAF prior to sperm transport into the oviduct and also to hydrolyze oxidatively damaged phospholipids in spermatozoa and seminal fluids preventing cellular damage (Hough and Parks, 1994). However, the role of PAF-AH in other ruminant reproductive biology still requires further attention.

Arylsulfatase enzymes (AS-A, -B, and -C) have been identified in bull SP, known to remove sulfate from various glycoconjugates and steroid molecules

that are involved in the stability and permeability of the plasma membrane (Gadella et al., 1991). Thus, they could function as *in vivo* capacitating factors prior to spermatozoa swimming out of SP in the female reproductive tract. Arylsulfatase C (a microsomal steroid sulphatase) has been proposed as a promoter of sperm capacitation (Langlais and Roberts, 1985). Very little information is available on the function of these enzymes in ruminants SP. There are also some enzymes in SP whose functions are still unknown. Among them, α-1,4-glucosidase and Prostaglandin-H2 D-isomerase (PGDS) are common. α-1,4-glucosidase has been identified in ram SP and its activity is established as a useful marker of epididymal function. Because epididymides of adult rams are the primary source of α -glucosidase in SP (Tremblay et al., 1990). α-1,4-glucosidase is also related to semen quality specially correlated positively with sperm concentration in ejaculates (Guerin et al., 1981). Tremblay et al. (1990) revealed that the highest percentage of sperm motility was associated with a low α-glucosidase content of SP and seasonal variations of testosterone in adult rams might have negative control on the presence of α-glucosidase in semen. PGDS is the only one of the glutathione-independent lipocalins that is associated with enzyme activity. It has been detected in bull semen (Gerena et al.,1998) and ram (Fouchécourt, et al., 1999). It might be originated from epididymes, as this enzyme could not be detected in the seminal vesicle secretion or in SP of vas deferens-ligated rams. The relationship between PGDS and the physiology of spermatozoa is unknown. It is thought that it might have a role on fertility (Gerena et al., 1998).

1.2.3. Ions

Sperm function is highly dependent on ionic environment (Hamamah and Gatti, 1998). Difference in the dietary mineral level may have a positive effect on the ion concentrations of SP. Cations such as Na, K, Ca, and Mg in SP exert osmotic balance and are components of many important enzymes (Cevik et al., 2007). Na is the principle cation in SP, with an exception in bull semen where Ca

concentration is very high (Setchell and Brooks, 1988). Na ion is an important element for spermatozoa function (Mosaferi et al., 2005). K is a natural metabolic inhibitor and higher K concentration in SP decreases sperm metabolism thereby, decreasing sperm motility (Massányi et al., 2003). Ca triggers the acrosome reaction in mammalian spermatozoa and is also involved with sperm motility (Kaya et al., 2002). Meseguer et al., (2006) have reported that Ca_{2}^{+} concentrations, in seminal plasma, are good predictors of post-thaw semen quality.

Mg, found in nearly all enzymatic systems, is regarded as a marker of seminal vesicle secretions (Wong et al., 2001) and may play an important role in sperm motility (Jobim et al., 2004). Assumpção et al. (2005) observed a negative correlation of abnormal sperm morphology with P, Ca and Na concentrations and a positive correlation with K concentration. Cu is necessary for many enzymes like the Cu-Zn-superoxide-dismutase (SOD), which is involved in cell protection against free radicals. More recently, several investigators have observed a positive correlation of Cu content of buffalo SP with sperm motility (Eghbali et al., 2008).

Zn exerts a protective anti-oxidant activity (Gavella and Lipovac, 1998) and is thought to be the primary factor responsible for the antibacterial activity of SP. It stabilizes the final DNA-nucleoprotein assembly and the plasma membrane of spermatozoa (Lewis-Jones et al., 1996). Metabolic regulation of spermatozoa appears to be mediated through Zn as a regulator of enzymatic activity in the semen and Zn plays an important role in control of motility by controlling energy utilization through adenosine triphosphate systems and through regulation of phospholipid energy reserves (Hidiroglou and Knipfel, 1984). Testosterone is thought to be involved intimately in zinc turnover in accessory sex glands of the ram (Hidiroglou et al., 1979). An inverse relationship between Zn content in bovine SP and fertility of the semen was stated by Swarup and Sekhon (1976). Increased concentration of Cl in SP may play a role in infertility (Cevik et al., 2007). The distribution of major ions between sperm and SP might be a basis for

variation of semen quality of different successive ejaculates and should be considered in the interpretation of fertility evaluation of semen.

1.2.4. Reducing Sugar

Fructose is a major saccharide that is present in ruminant SP. Fructose is synthesized from blood glucose by AGs specially seminal vesicle stimulated by testosterone (Kumar and Farooq, 1994), and its concentration in SP tends to increase during the breeding season (Matsuoka et al., 2006). Fructose plays an important role in metabolism as spermatozoa utilize fructose to produce ATP (Sanchez-Partida, et al., 1999). In both old and new world camelids, SP fructose concentration is very low, which is probably due to the absence of seminal vesicles. The corpus prostate seems to be the principal source of fructose, smaller amounts are contributed by the bulbourethral glands, ampulla ductus deferentis and pars disseminata of the prostate (El-Manna et al., 1986). In camelids SP, glucose is the principle sugar, which is converted into fructose either by phosphorylated pathway or by monophosphorylated pathway through sorbitol dehydrogenase and aldose reductase (Agarwal et al., 2004). The concentration of fructose in SP has often been used as an indicator of the androgen status of animal as its secretion is stricly governed by the level of androgens in the blood (Mann and Lutwak-Mann, 1981). Information on total fructose concentration in SP of different species could help in the determination of appropriate concentration of fructose to be added into semen diluents. Another reducing sugar sorbitol is also present in ruminant SP. The ram spermatozoa posses enzyme sorbitol dehydrogenase that enables sorbitol to be converted to fructose and used as a metabolic substrate (Setchell and Brooks, 1988).

1.2.5. Lipids

A key feature in the function of spermatozoa is the lipid composition of sperm membrane and SP. Lipid profiles consist of cholesterol, phospholipids, diglycerides, tryglycerides and wax esters. The possible source of lipid in SP may

be the epididymes as well as spermatozoa. Pickett and Komarek (1966) observed more lipid content in SP obtained from normal ejaculates than SP collected after vasectomy. Lipid content also varies with ejaculates and higher lipid concentration is observed in first as opposed to the second ejaculates (Iqbal et al., 1984). Seminal lipids may play a significant roles in the sperm structure, metabolism, sperm capacitation and fertilization of female gametes (Hafez, 1987) and its presence in SP is also important because of the ability of sperm cells to take up lipid components or fatty acids from the surrounding environment under determined circumstances (Cerolini et al., 2001).

Among different lipid profiles, the most predominant lipids are phospholipids and cholesterol that have special relevance in the structure and function of the plasma membrane of spermatozoa (Cross, 1998). SP also contains triacylglycerols, their oxidation serving to meet the sperm energy requirements. The intake and use of triacylglycerols is enabled by very-low-density lipoprotein (VLDL) receptor expression on the sperm Membrane (Gundogan and Elitok, 2004). There are different types of phospholipids (Table-2) present in SP, among which the choline plasmalogens are the major phospholipids of ruminant SP .

Table 1.2. Phospholipids composition (% of total phospholipids) of ruminant SP (Andrabi, 2009; Jain and Anad, 1976).

Phospholipids	**Bull**	**Buffalo**	**Goat**
Phosphotidal choline (Choline Plasmogen) (CP)	17.6-32.9	17.3	15.7-22.4
Phosphotidyl choline (PC)	24.5-30.0	21.7-34.1	15.2-22.0
Phosphatidyl ethanolamine (PE)	5.4-10.5	10.8-11.7	1.1-15.6
Phosphatidal ethanolamine (EP)	5.0-16.3	4.1-4.9	1.6-4.3
Sphingomylin (SPH)	11.6-16.3	13.-13.8	10.6-19.8
Phosphatidyl serine (PS)	1.3	2.8	1.1-5.9
Phosphatidyl inositol (PI)	0.8	2.9	1.5-6.0
Lysophosphatidyl ethanolamine (LPE)	1.2-2.2	5.6-6.6	4.3-14.2
Lysophosphatidyl choline (LPC)	1.2-2.2	3.1-3.9	3.2-9.8
Diphosphatidyl glycerol (DOP)	5.0-8.8	3.5-7.4	0.3-0.5
Phosphatidic acid (PA)	0.4	0.5	0.2-1.4

The synthetic capability of spermatozoa is very limited and its behaviour is essentially controlled by the external effectors on which they are suspended and acting through cell surface and plasma membrane (Harrison et al., 1992). Although fructose is the main energy producing substrate, spermatozoa may use phospholipids for energy production in absence of oxidizable soluble carbohydrates (Scott and Dawson, 1968). Phospholipids utilization by spermatozoa may have an effect on the structural integrity of the cell membrane, which might be important in understanding the reduced fertilizing capacity of frozen-thawed spermatozoa. Dawson et al. (1956) identified a glycerol complex namely, glyceryl-phosphorylcholine (GPC). GPC is present almost in all mammals SP but, ram SP contains a concentration of more than one percent than other animals. It is mainly found in the epididymal fluid. Although it is always in intimate contact with spermatozoa, it is unable to be metabolized. Though small amounts of GPC are also produced in the accessory sex organs, the concentration of this substance in the epididymal fluid is an important indicator of the epididymal function (Pardiwalla et al., 1980). While little is known about the functional significance of this component, it was hypothesized that GPC might be an important source of energy for spermatozoa in the female reproductive tract where an enzyme of the female reproductive tract might be responsible for breakdown of GPC (White and Wallace, 1961). Results from some researches have indicated that reduced sperm concentration and motility are associated with a decrease SP lipid content (Taha et al., 2000). Investigation of the actual functions of lipids of SP on spermatozoa could be an interest area of research that might help to improve cryopreservation process. Because, the transfer of proteins through sperm surface is modulated by the distribution of lipids along the membrane (Parks and Graham 1992). Perhaps an exchange of lipids may occur between spermatozoa and SP. This hypothesis needs a comprehensive study. Total lipids and phospholipids have been quantified in alpaca SP (Garnica et al., 1993). Their functions have not yet been studied in camelids, but they may play a role in maturation and protection of the plasma membrane integrity.

1.2.6. Anti-oxidants

The plasma membrane of spermatozoa is rich in phospholipid-bound polyunsaturated fatty acid (PUFA). Therefore, spermatozoa are highly susceptible to lipid peroxidation (LPO) by free radicals or reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide anion (O^{2-}) and hydroxyl radical(-OH). Physiologically, ROS are important mediators of normal sperm function, such as signal transduction mechanisms and play an important role in normal physiological processes such as, sperm capacitation, acrosome reaction, maintenance of fertilizing ability, and stabilization of the mitochondrial capsule in the mid-piece in bovine (Desa et al., 2009; Goncalves et al., 2010) and their beneficial effects on sperm functions depend on the nature and the concentration of the particular ROS involved (de Lamirande and Gagnon, 1993)

It is important to point out that epididymal spermatozoa exhibit a spontaneous capacity for superoxide anion production, mediated by a membrane-bound NADPH oxidase, lead to the generation of peroxides that serve as hydrogen acceptors for phospholipid hydroperoxide glutathione peroxidase in the induction of sperm chromatin condensation (Aitken and Vernet, 1998). In order to maintain proper physiological activities a fine balance between ROS production and recycling around sperm cells is essential (Drevet, 2006). Otherwise, any imbalance leads to the impairment of sperm function through oxidative stress resulting in decreased motility of spermatozoa (presumably by a rapid loss of intracellular ATP), axonemal damage (de Lamirande and Gagnon, 1992), decreased viability and increased morphological defects specially in midpiece, with deleterious effects on the capacitation and the acrosomal reaction of spermatozoa (Kim and Parthasarathy, 1998). Superoxide anion and nitric oxide participate in heparin-induced capacitation in bovine cryopreserved sperm, whereas hydrogen peroxides induce capacitation in ejaculated bovine sperm(O'Flaherty et al., 1999; Breininger et al., 2010).

In physiological condition, there are both intra and extra cellular enzymatic and non-enzymatic antioxidant scavenger system for prevention of ROS to protect spermatozoa from oxidative stress. SP is very important in determining the antioxidant defense system for spermatozoa. Both spermatozoa and SP contain antioxidant enzymes known as superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidases (GPx) and its substrates (GSH and GSSG), and catalase (CAT) (Storey et al., 1998). Indeed, mature spermatozoa have little capacity for repairing oxidative damage because their cytoplasm contains low concentrations of scavenging enzymes (Alvarez and Storey 1989). Surprisingly, SP has higher concentration of antioxidants than any other biological fluids including blood serum (Mann, 1964), and antioxidants that are present in SP mainly compensate for the deficiency in cytoplasmic enzymes. Epididymal enzyme like catalase protects spermatozoa from oxidative damage in the epididymal lumen whereas, SOD and GP secreted from seminal vesicle protect sperm after ejaculation (Zubkova and Robaire, 2004). The localization of SOD on the acrosome, post-acrosome and tail, of GPx on the post-acrosome and apical head, and of GR on the tail have been demonstrated (Marti et al., 2008). There are three SOD isoenzymes (CuZn-SOD, Mn-SOD and extracellular EC-SOD), of which, CuZn-SOD activity is high and Mn-SOD and EC-SOD activities are very low in SP (Eghbali et al., 2008). SOD spontaneously dismutates O^{2-} to form O_2 and H_2O_2, whereas catalase converts H_2O_2 to O_2 and H_2O (Alvarez et al., 1987).

$$2O_2^- + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

$$H_2O_2 \xrightarrow{\text{Catalase}} H_2 + 1/2\,O_2$$

Another important attribute of SP antioxidant scavenger system is the relatively high concentration of reduced glutathione (GSH). Glutathione is the substrate of the selenium containing enzyme glutathione peroxidase (the main enzyme involved in removing hydrogen peroxides) and of glutathione

transferase (an enzyme which catalyses covalent reactions of glutathione with electrophilic substances). Glutathione acts as electron donor, and it can react directly (through its thiolic group) with hydrogen peroxide, superoxide anion and hydroxyl radicals, and its sulphydryl group can react with alkoxyl radicals and hydroperoxides [(Lenzi et al., 1996) for review]. Glutathione reductase regenerates reduced glutathione (GSH) from oxidized glutathione (GSSG) . The equilibrium equation involving GSH and GSSG is:

$$2\,GSH + H_2O_2 \xrightarrow[\text{Peroxidase}]{\text{Glutathione}} GSSG + 2\,H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow[\text{Reductase}]{\text{Glutathione}} 2\,GSH + NADP^+$$

(Calvin et al. 1981)

Along with antioxidative function, glutathione also involves in fructolysis of spermatozoa (Slaweta and Laskowaska, 1987). It is a coenzyme of 1,3-diphosphoglyceric aldehyde dehydrogenase which leads to oxidation of triose phosphate to phosphoglyceric acid, which is reduced to pyruvic acid and then lactic acid, improving metabolic activity and motility of spermatozoa (Sinha et al., 1996). It is obvious that antioxidant activity is dependent on the presence of antiperoxidant factors, which may attach to spermatozoa at ejaculation and can protect them from lipid peroxidative attacks during their passage through the female reproductive tract. In this respect, it should be pointed out that GPx and SOD are found with RSP -14 and 20, which are mainly involved in prevention of cold shock (Marti et al., 2007). Apart from these antioxidants enzymes, albumin (present in SP) is also known to absorb lipid peroxides, and contribute to the protective effect on both the plasma membrane and motility (Alvarez and Storey 1995) . It is noteworthy to point out that levels of antioxidant defensive systems vary with species, season and type of ejaculation (Ollero et al., 1996). Higher activities of GR, GPx SOD and CAT have been observed in semen of poor quality in non-breeding season (Colas et al., 1990) and higher antioxidant protective

system of SP is claimed to ensure fertilizing potential of spermatozoa in adverse condition (Cardozo et al., 2006).

1.2.7. Hormones and Cytokines

Hormones like estrogens, progesterone, testosterone, luteinising hormone (LH), prolactin and prostaglandins have been reported to be present in SP. Steroid hormones and prostaglandin content of SP are the results of the activity of Leydig cells, epididymis, seminal vesicle, prostate and spermatozoa themselves, since sperm contain both aromatase and cyclo-oxygenase II (Hess et al., 2001). PLA_2 anchored to the plasma membrane releases arachidonic acid from phospholipids, which is converted to prostaglandin E_2. . The hormonal profile of SP varies among species. Ram SP contains lower estrogen and testosterone concentrations than that of bull SP. Ram semen contain a fair amount of prostaglandin (PGE), which is known to stimulate the contractile activity of the cervix and uterus *in vitro* and *in vivo* improving transport of spermatozoa in ewes (Gustafsson et al., 1977). Level of PG is shown to be dependent on season (Shore et al., 2003). The presence of immunoreactive prolactin, LH and progesterone in buffaloes (Sidhu and Gill,, 1992) and bulls SP (Razdan et al., 1977) has been reported. The specific functions of these enzymes in SP are unknown. Sidhu and Gill (1992) observed a negative correlations between the levels of immunoreactive prolactin in semen, and motility and viability of spermatozoa. Concentrations of prostaglandins, testosterone and estrogen in the semen have been suggested as indicative of the reproductive status of the bull and ram (Dimov and Georgiev, 1977). Insulin-like growth factor I (IGF-I) deserves increasing interest as it is an important regulator of reproductive functions (Jones and Clemmons, 1995). IGF-I is synthesized in Sertoli cells and Leydig cells under the control of FSH and LH. This potent mitogenic, metabolic and differentiating polypeptide is believed to function as a chemokinetic factor necessary for development of normal germ cells and for maintainance of normal sperm morphology (Glander et al., 1996). IGF-I is present in bovine SP and IGF-I

receptor is present on the acrosomal region of uncapacitated ejaculated bovine spermatozoa (Henricks et al., 1998). Since the acrosomal region is involved in capacitation and acrosome reaction, one could hypothesize their possible regulatory role in sperm pre-fertilization events. IGFs may have an important role in increasing the straight-line velocity and decreasing the amplitude of lateral head displacement (Miao et al., 1998), important for sperm transport through the female reproductive tract. Selvaraju et al. (2009) have studied the effect of IGF-I of Buffalo SP on semen quality and suggested that addition of IGF-I can improve sperm functional parameters by reducing lipid peroxidation levels and maintaining sperm motility by increasing fructose uptake. Such an interesting hypothesis deserves further attention. However, the action of IGFs on semen quality or fertility is related to the action of IGF-binding proteins (IGFBPs) in SP, which probably modulate IGF actions on sperm function (Zhou and Bondy, 1993). Likewise, IGF-II binds to four major BSPs (BSPA1, -A2, -A3, and -30kDa) to exert its function (Desnoyers and Manjunath, 1994). Literaure on the presence and function IGFs and IGFBPs in SP of small ruminants species is very limited.

Cytokines are important factors for anti-inflammatory function of SP. Recently, Vera et al. (2003) have confirmed the presence of cytokines, namely, the interleukins 6 and 10 (IL-6 and IL-10) and tumor necrosis factor alpha (TNFα) in bull semen. IL-10 is known as an anti-inflammatory cytokine and its function in the male reproductive tract is to maintain immunological balance and avoid rejection of spermatozoa. TNFα possesses not only pro-inflammatory activity but also plays an important role in modulating the inflammatory response (Hodge-Dufour et al., 1998). TNFα synthesized from seminal vesicles is testosterone-dependent and its action in the female reproductive tract following insemination may be initiated by plasmin and other enzymes (Robertson et al., 2002).

1.2.8. Vesicular Fractions

Vesicular fractions or membrane vesicles being characterized and localized in the whole ram ejaculate (El-Hajj Ghaoui et al., 2004) have drawn current attention, and are thought to be secreted from testis and epididymes. The precise origin of membrane vesicles or the mechanism by which they are discharged into the semen have not been clearly identified yet. This component of SP could have a positive effect on progressive motility of spermatozoa. Therefore, attachment of membrane vesicles to the sperm surface could delay capacitation and acrosome reaction similar to the action of prostasomes in human SP (Fabiani et al., 1994). Such hypothesis relating to the function of these microscopic organelles requires further investigation. In addition, further work is needed to determine its beneficial effect and influence on the fertilizing ability of spermatozoa of different animals.

1.2.9. Ovulation inducing factor of SP

SP exerts a distinct stimulation in the process of reproduction. From time to time doubts have been expressed as to whether the individual accessory gland secretions or even the entire SP, have any essential role to fulfill the process of reproduction. Evidence demonstrating an effect of SP in hastening or inducing ovulation in ruminants is limited. In some early studies, it was reported that sterile copulation with vasoctomized male was associated with enhanced LH secretion and a higher ovulation rate in spontaneous ovulators like cattle and sheep (Marion, 1950; Jöchle, 1975). Several authors demonstrated that ovulation occurred after intravaginal (Chen et al., 1985) or intramuscular/ intrauterine administration (Pan et al., 1992) of Bactrian SP to female Bactrian camels. Recently, Adams et al. (2005) have reported the existence of ovulation inducing factor (OIF) in SP of alpacas and llamas that could elicite a surge in circulating concentrations of LH and induce ovulation in more than 90% of treated females. OIF is thought not to be species specific, as bull SP can induce ovulation in

females bactrian camels (Chen et al., 1985), lama and alpaca (Ratto et al., 2006). Though ovulation rate was lower with bull SP than alpaca or llama SP, this study proposed the hypothesis that OIF might be a conserved molecule in SP among all mammals. OIF in SP induces ovulation via a systemic rather than a local route (Ratto et al., 2005), though controversy remains in one study where investigators observed ovulation after intravaginal deposition of alpaca semen in female alpacas and llamas (Sumar, 1994). Interestingly, the putative ovulation-inducing factor in SP is completely different from the native LH, HCG, PMSG and PGF-2α and posseses GnRH-like activity but, OIF is not GnRH. The biological activity of OIF on rat pituitary cells was not suppressed when anti-GnRH antibodies were added to the culture media (Paolicchi et al., 1999). The complex composition of OIF in Bactrian camel has been thoroughly studied by (Pan et al., 2001) with ion-exchange chromatography on DEAE-cellulose, HPLC and reverse-phase HPLC. OIF is formed with a multi-layered structure, of which, the outermost layer comprises glycoprotein of 19.431 kDa, the second layer consists of alkaline protein components 19 kDa, and the third layer consists of acid protein components of 13.18752 kDa. These layers with different chemical properties are proposed to be very important in the maintenance of OIF stability in SP matrix. In an earlier study, Pan et al. (1986) proposed that OIF might be synthesized from hypothalamus or pituitary gland as they could not observe any ovulation in female camels after vaginal deposition of accessory gland fluids. But this result demands a further confirmation. More studies should be conducted for proper identification of functional properties of OIF in ruminants semen, which could be an evolutionary asset for diagnosis and treatment of ovulatory problems in female animals

1.2.10.Immunoregulatory and antimicrobial effects of components of SP

SP function in the normal physiology is associated with the ejaculation of spermatozoa and their subsequent survival in the female reproductive tract. Semen is potentially antigenic to the female and during maturation in the epididymis the spermatozoa acquire their antigenic properties (Valentovičová et al., 2005). Antibodies that recognize surface antigens of mature spermatozoa are present in sera of both sexes. Spermatozoa may be exposed to antisperm antibodies in semen and in cervical, ovarian follicular and fallopian fluid (D'Cruz et al., 1990). Additionally, spermatozoa can readily be ingested by vaginal leucocytes and epithelial cells (Phillips and Mahler, 1977). SP plays an immunoregulatory role that is beneficial to survival of spermatozoa in the female reproductive tract. Spermatozoa are coated with some proteins of SP having the immunosuppressive activity in immune reactions and these proteins accompany the migration of spermatozoa to the female reproductive tract (Matoušek, 1985). SP has been shown to interfere, either directly or indirectly, with the function of different types of immunocompetent cells, including Tand B lymphocytes and macrophages (Mukherjee et al., 1983). Immunoglobulin G, an predominant immunoglobulin, is present in SP of bull and buffalo. Bovine seminal Ribonuclease (BS-RNase) appears to be the major immunosuppressive agent of SP. It exerts action through its ability to catalyze the cleavage of RNA and with its dimeric structure plays a key role presumbly in binding to specific cell receptor(s). Its immunosuppressive action is accomplished by interfering strongly with the expression of the interleukin-2 receptor on T cells (Tamburrini et al., 1990). Beside RNase, IL-10 and TNFα also posses local immunosuppressive action and negative control on inflammatory reaction in the female reproductive tract (Hodge-Dufour et al., 1998). Their local action might be responsible for the modulation of the immune response to sperm antigens or even protection of spermatozoa from certain facets of the female immune system (Kelly,1995). Platelet-activating factors acetylhydrolases (PAFAHs) also present

immunosuppression activities by inhibiting actions of sperm derived PAF and its concentration in SP is so high that it could conceivably result in the immunosuppression of the whole uterus (Soubeyrand et al., 1998). Along with the beneficiary effect, the temporary local immunosuppression provided by SP can facilitate infection by viruses and bacteria that might be present in the semen and establish SP as an medium for sexually transmitted disease (Vera et al., 2003).

Interestingly, a very intriguing scenario is also suggested by Teixeira et al. (2002) who have stated that spermadhesine of SP shows immunostimulatory properties within the female reproductive tract of animals. There may be two bases for immunoregulatory functions of SP: i. supporting the elimination of the vast majority of waste spermatozoa that fail to fertilize ii. preservation of the tiny minority of surviving spermatozoa that are able to fertilize oocytes. Immunostimulatory molecules may have a temporally retarded mode of action and they may play a role in the competition between spermatozoa, as the female reproductive tract will become immunologically active only after spermatozoa have reached the ovum or a safe sperm storage site [(Poiani, 2006) for review].

Insemination induces a rapid and transient inflammatory reaction in the female reproductive tract. Detailed studies are conducted on this aspect in rodents, human, and boar (Robertson, 2005). It is not clear exactly which component of semen induces this reaction in ruminant, even information regarding leucocyte populations in the ruminant reproductive tract is limited. Insemination induced inflammatory reaction is regarded as a normal physiological phenomenon that play an important role in the establishment of a state of immune-tolerance during normal pregnancy (Robertson, 2002). Ram semen can enhance the expression of proinflammatory cytokine interleukin-8 in the ovine cervix (Mitchell et al., 2002). Scott et al. (2006) have reported that post inflammatory response increases neutrophils and macrophages and reduces mast cells in vagina, cervix and uterus of ewes, which might be a preparatory phenomenon for the implantation of ovine conceptus. Because, macrophages are believed to have a role in inducing immunotolerance to paternal antigens

(Robertson and Sharkey, 2001). Presence of IL-8 after insemination is thought to enhance cervical dilation, which in turn, facilitates transcervical transport of spermatozoa. Cytokines and prostaglandins synthesized in the male accessory glands are transferred to the female reproductive tract at insemination where they bind to receptors on target cells in cervix and uterus, activating changes in gene expression, leading to modifications in structure and function of tissues of the female reproductive tract (Robertson, 2005). Factors in SP including proteins, cytokines, sex hormones and prostaglandins posses potential biological capabilities to protect spermatozoa from different pathogens both in the male and female reproductive tract (Maegawa et al., 2002). Antimicrobial protein seminoplasmin present in bovine SP, has a potent antibacterial activity and lyses Gram-positive and Gram-negative bacteria (Murti et al., 1994). Seminalplasmin is shown to be a potent inhibitor of reverse transcriptases RNA–dependent DNA nucleotydaltransferases and appears to act by binding strongly to the enzyme (Reddy et al., 1983). It possesses antimicrobial property by inhibiting the growth, as well as nucleic acid and protein synthesis of several bacteria (Scheit et al., 1985) and by increasing bacterial membrane permeability (Sitaram et al., 1992). PLA_2 also exerts a potent antimicrobial action in the SP (Bourgeon et al., 2004). It could be stated that exploration of the nature and interaction between active components of SP and the female reproductive tract in immunoregulatory system may be of particular value to AI in providing increased sperm survival in allowing a larger number of inseminations from an ejaculate

1.3. ASSESSMENT OF SPERMATOZOA

The ultimate goal of semen assessment is to predict the fertilizing ability of the spermatozoa, as well as, to estimate the potential capability of a male as a breeder . Classical sperm parameters, including sperm concentration, motility, morphology and viability estimated by light microscope, are the most used parameters for semen quality assessment in AI laboratories. But physical semen characteristics (both macroscopic and microscopic) and sperm morphology measurements are not always indicative of fertility and reproductive performance (Zhang et al., 1998:Novak et al., 2009). These tests are considered somehow inefficient to correlate properly with some unexamined factors such as repeatability of sperm quality tests, relationships between sperm qualitative and quantitative characteristics in the insemination dose, sperm manipulation and possible injury during insemination, sperm viability in relation to survival in the female reproductive tract, fertilization and early embryonic development (Correa et al.,1997). In truely, the ability to predict the fertility of semen with laboratory tests is still limited, mainly due to the complexity of the spermatozoon and its associated environment. Spermatozoon is multifunctional cell that must possess a large number of attributes that make it potentially fertile. Any spermatozoon lacking any of these attributes or not possessing enough of an attribute will be infertile (Januškauskas and Žilinskas, 2002). In order to increase the predictive power of the test, simultaneous analysis of multiple sperm attributes (Graham et al., 1990) and\or outcomes of several laboratory assessments can be combined statistically to look for the overall effect of several independent sperm parameters (Zhang et al., 1998), which can explain variation among individual.

Therefore, new technologies are being used for the assessment of sperm quality over the last decades and improved diagnostic tools have been used to examine kinetic parameters of motility and to understand complex structural and molecular changes that spermatozoa undergo during its lifespan-ie. complete screening of sperm function. Nowadays, more sophisticated evaluations of sperm quality are performed with multicolor flow cytometry. In this system,

spermatozoa travel in a sunpension one by one through the beam of a laser that causes any fluorescent stain associated with the sperm cell to fluoresce (Graham, 2001). Photomultiplier tubes permit the determination of specific wavelengths emitted by individual compartments in each sperm cell that have been previously stained with a fluorescent dye (Graham and Moce, 2005). Variuos kinds of fluorochromes and compounds conjugated to fluorescent probes have made possible to observe sperm physical characteristics including cell size, shape and internal complexity and to understand their biochemical functional status. Moreover, flow cytometric study increases the repeatability of assessment, removes the subjectivity of assessment and allows simultaneous assessment of multiple Fluorochromes (Gillan et al., 2005). Thus, flowcytometry can provide information on cell viability, acrosomal integrity, mitochondrial function, capacitation status, membrane fluidity apoptotic mechanism and DNA status (Evanson, 1984; Garner et al., 1997; Nagy et al., 2003). In spite of having many advantages for semen evaluation with flow cytrometry, its use is often limited to research by the expense and the requirement of a skilled operator.

1.3.1. Motility

Sperm motility is the most used criterion for routine semen evaluation, where subjective evaluation of quantitative motility (percentage of motile and progressively motile sperm) is mainly performed. Percentage of motile sperm is considered as an indication of cell viability and an indirect method of assessing metabolic activity. Along with subjective evaluation, Computer Automated Sperm Analysis (CASA) has been introduced to evaluate qualitative motility or the kinetic property of the ejaculated individual sperm cells. CASA study helps to improve the accuracy of data collection and to reduce time in the exam performance (Amman Hammerstedt, 1993). Video images for computerized sperm motion analysis are obtained from viewing fields of motile sperm using a microscope. A set number of successive video frames is analyzed at a constant rate and each sperm is classified as either motile or nonmotile. Various kinds of motion characteristics have been reported in many species including bovine

(Anzar et al., 997), goat (Cox et al., 2006), ram (Robayo et al.,2008) using the CASA system. Motility data is characterized as follows:

Table-1.3. Kinematic parameters used for CASA system.

Parameters	**Abbreviation**	**Unit**	**Description**
Average path velocity	VAP	µm/s	measures the mean trajectory of spermatozoa per unit of time.
Curvilinear velocity	VCL	µm/s	Total distance traveled by the sperm head per unit time
Straight line velocity	VSL	µm/s	measures the straight liner trajectory of spermatozoa per unit of time
Amplitude of lateral head displacement	ALH	µm	measure of the side-to-side movement of sperm head
Linearity	LIN	%	(VSL/VCL) X100
Straightness	STR	%	(VSL/VAP)X100
Wobble	WOB	%	(VAP/VCL)X100
Beat/cross frequency	BCF	Hz	Number of times the curvilinear path acrosses the average path per unit time:approximates to the flagellar beat frequency for ejaculate spermatozoa

A high correlation coefficient between motility or velocity of bull spermatozoa and competitive fertility index was reported. CASA has some limitations. Its study is limited in a small population. In the analysis of frozen semen, this system is less capable of identifying non-spermatozoa, such as egg yolk, misleading to high concentration of spermatozoa (Comhaire et al. 1992).

The validity of the results depends on careful sample preparation and the settings used on the instrument (Anzar et al., 1991) to identify correctly moving sperm, nonmoving sperm, and particles (usually static) other than sperm.

1.3.2. Plasma membrane integrity

Generally, eosine\nigrosine dyes are used as supravital stain in laboratory to asses viability. Only live and dead spermatozoa can be identified. Recently, fluorescent staining have been developed to identify a third population known as "moribund or dying cells"(can be misjudged as a live cell with general staining) along with live and dead spermatozoa population using fluorescent microscope or flow cytometer to get a precise information of viable spermatozoa. Various membrane permeable and non-permeable fluorescent dyes are used to evaluate physical intactness of sperm PM (Table 1.4.).

Table 1.4. Common fluorescence dyes used for PM integrity.

Non permeable DNA dyes	**Permeable dye**
Bisbenzimidazoles	Enzyme based dye
Hoechst 33258 (H258),Hoechst 33342 (H342) wavelength-358-488nm	Fluorescein diacetate (FDA)
	6-carboxylfluorescein diacetate (CFDA
Phenanthridines	6-carboxylmethylfluorescein diacetate (CMFDA)
Propidiumiodide (PI),	
Ethidiumhomodimer-1	Calcein acetoxy methyl ester (CAM)
Wavelength-488-568	Wave length- 510-550 nm
YoPro-1	DNA fluorochrome
Wavelength-488-568 nm	SYBR-14
ToPro-3:	Wavelength- 515

Upon entering into living cell, permeable acylated dyes are immediately deacylated by intracellular esterases leaving the probe impermeable. Living cell loaded with this probe fluorescence green. Onthe other hane, the entered probes easily leak out of deteriorated cells with damaged membranes and impermeable DNA dye enter into cell and bind with DNA fluorescenc red.

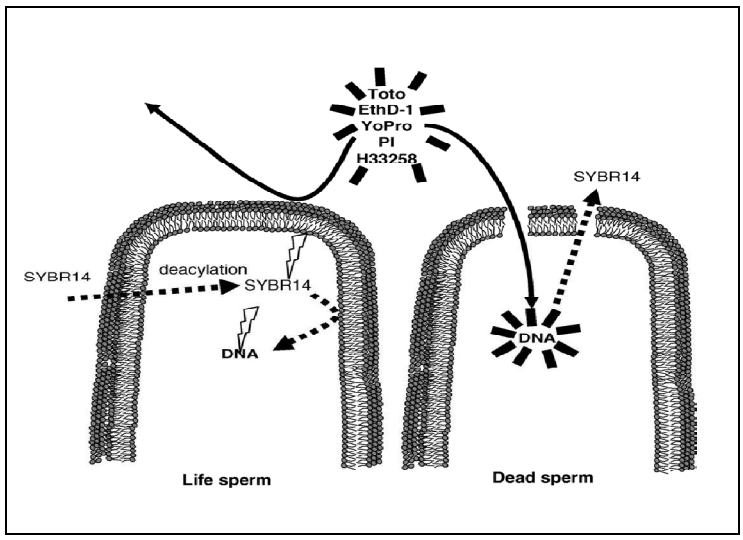


Figure-1.7. Discrimination between intact and plasma membrane damaged sperm using DNA affinity probes (Silva and Gadella, 2006).

Carboxifluorescein acetate combined with propidium iodide(CFDA\PI), Hoescht 33258, or calcein acetate in combination with ethidium homodimer, SYBR-14 with either propidium iodide or ethidum homodimer is the combination of probes most frequently used (Garner and Johnson, 1995). When CFDA\PI is used, population contained green fluorescence over head confirmed as live spermatozoa. Two populations of damaged sperm can be identified; one consisted of sperm with red staining (PI+) over the head (termed as dead), and a second population which consisted of sperm, with an intact acrosome that retained green fluorescence (CFDA+) and a red postacrosomal region (PI+) (termaed as moribund or dying cells). The combination of SYBR-14 and PI or dyes is more used and enables flow cytometric staining immediately after freeze-

thawing without any laborious processing step to remove egg yolk or lipid particles from milk. As these particle don't contain DNA, they remained unlabeled and therefore, don,t interfere in sperm counting (Nagy et al., 2003).

The sperm plasma membrane functional status is of particular importance since transportation of compounds across the sperm membrane is an important biochemical process for sperm viability and maintenance of fertilizing capability (Jeyendran et al., 1984). For this reason, Hypo-osmotic swelling test (HOST) is performed to evaluate the membrane biochemical activity along with evaluation of physical intactness of plasma membrane with vital stains. HOST provides a simple, effective and convenient means of evaluating viability of spermatozoa (Esteves et al., 2007) and is considered as a better indicator of cell membrane integrity than supravital stains (Neild et al., 1999). In HOST solution, spermatozoa with intact and functionally active plasma membranes will undergo swelling (due to the influx of water) to establish an equilibrium between the extra- and intracellular compartments (Jeyendran et al., 1984).

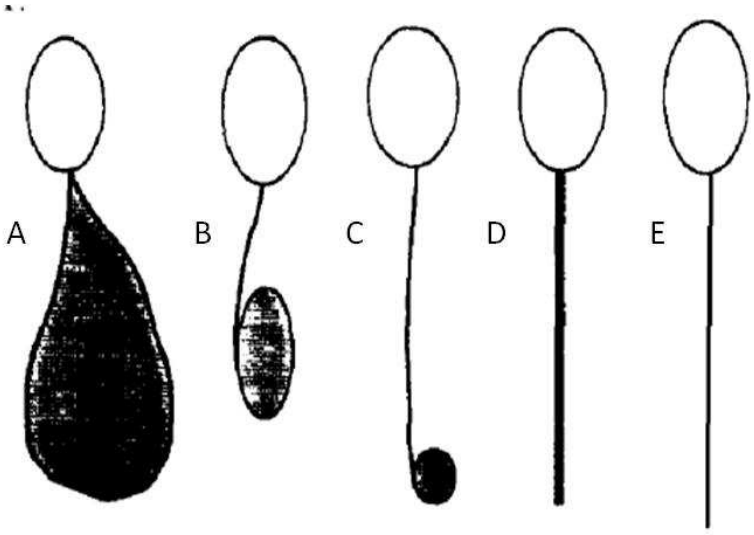


Figure-1.8. Diagrammatic illustration of different sperm swelling patterns as measured by HOST. Type A represents maximal sperm swelling; Types B and C represent intermediate sperm swelling stages; and Type D represents the initial swelling response in the HOS test. Non-swollen (E) spermatozoa are considered to have a functionally inactive or damaged sperm membrane. (Correa et al., 1997).

Though solutions with different osmolalities have been tested both for fresh and frozen-thawed samples from different animals, a solution of fructose and

sodium citrate in distilled water with 100 mOsm gives best results for ruminant spermatozoa (Correa and Zavos, 1994). Mandan et al., (2003) observed a significant positive correlations among HOST and MOT, VSL, VCL, ALH and VAP in buffalo sperm and revealed that of sperm kinematics might depend on plasma membrane integrity. Vidament et al. (1998) observed HOST as the the best predictive test of the freezability of stallion semen. Brito et al. (2003) reported a significant relationship between Post-thaw HOST and *in vitro* fertilization rate. Several studies have evaluated HOST as a better predictor of the fertilizing capacity of spermatozoa than motility in ram, (Vazquez et al., 1988) bull (Correa and Zavos, 1994) and humans (Zaneveld et al., 1990).

1.3.3. Capacitation

Fluorescent antibiotic chlortetracycline (CTC) is routinely used to assess the incidence of sperm capacitation and acrosome reactions. This membrane probe binds to the sperm plasma membrane in a Ca2+/Mg2+ dependent manner and viable sperm cells become labeled with CTC at different parts of the surface membranes where Ca2+ is present above a certain threshold concentration to allow CTC immobilization (reviewed by Silva and Gadella, 2006). Three pattern of CTC fluorescence can be identified- F-pattern (intact non-capacitated sperm), an overall staining of the sperm head, B-pattern for capacitated sperm, a more prominent staining of the apical area of the sperm head and decreased staining at the posterior area of the sperm head (Ward and Storey, 1984); AR pattern (acrosome reacted sperm) with no fluorescence on head.

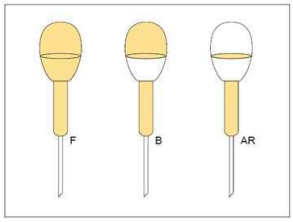


Figure-1.9. Chlortetracycline fluorescent staining patterns for uncapacitated (F), capacitated (B) and acrosome-reacted (AR) spermatozoa. (Curry, 2000).

Thundathil et al. (1999) found a positive correlation between the proportion of F-pattern frozen-thawed bull spermatozoa and the day-56 non-return rate and suggested that fertility increased with the proportion of viable, Fpattern spermatozoa. CTC staining is not amenable to flow cytometric evaluation (Rathi et al., 2001), as flowcytometer can only discriminate different amounts of fluorescence to a lower degree (Silva and Gadella, 2005). For these reasons, CTC staining has largely been superseded by a hydrophobic dye Merocyanine 540 (M540). M540 monitors a decreased packing order of phospholipids in the outer leaflet of the plasma membrane lipid bilayer (Williamsonet al., 1983, Langner and Hui, 1993) which allows its intercalation into the hydrophobic core of the membrane, present in capacitated spermatozoa (Rathi et al., 2001). M540 can also be useful for assessing membrane fluidity (Pen˜ a et al. 2004; Ferna´ndez-Santos et al. 2007; Caballero et al. 2009), which is increased during acrosomal reaction. Inaddition, M540 staining can be combined with the membrane impermeable DNA binding probe Yo-Pro-1 to allow simultaneous analysis of membrane lipid status and cell viability (Rathi et al., 2001) Beside M140, Indo-1 acetoxymethylester (Indo-1 AM) has been used for measuring intracellular Ca2+ in spermatozoa by flow cytometry (Brewis et al. 2000).

Capacitation involves cytosolic pH changes and increasing calcium levels. On this basis, pH indicator 2-,7--bis-(2-carboxyethyl)- 5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF AM, emission at 535 nm) for measuring the capacitation-related pH increase in bovine spermatozoa has also been used (Pons-Rejraji et al.,2009).

1.3.4. Acrosome integrity

Acrosome must remain intact before and during the transit of the sperm to the isthmus until zona binding has been accomplished. Early acrosome reactions render sperm infertile, and therefore, it makes sense to assess acrosome integrity . Two types of fluorescent probes are used to detect acrosomal status. One is chlortetracycline with antibody that bind to externally exposed antigens, and

another includes- lectins with antibodies that bind to intracellular, acrosomal antigens (Januškauskas and Žilinskas, 2002). The lectin conjugates bind to specific carbohydrate moieties of glycoproteins that are exclusively localized in the acrosome. Pisum sativum (green pea; PSA), or of Arachis hypogaea (peanut;PNA) are commonly used lectin conjugates. PNA labeling is specific for the outer acrosomal membrane, whereas, PSA is used for labeling acrosomal matrix glycoproteins. Fluorescein isothyocyanate (FITC)-conjugated PSA or PNA (FITC_PSA or FITC-PNA) is commonly used to assess acrosomal status of spermatozoa from domestic animals (Cross and. Watson, 1994; Sukardi et al., 1997). For cryopreserved spermatozoa PNA is preferred because it exhibits less nonspecific binding to other areas of the sperm and has less affinity to egg yolk particles (Dalimata and Graham, 1997; Thomas et al., 1997). PI in combination with FITC- PSA (Sukardi et al., 1997) or FITC-PNA (Rathi et al., 2001) has been widely used to discriminate between nonviable acrosome reacted and viable acrosome reacted sperm.

1.3.5. Mitochondrial function

Mitochondria produces ATP by oxidative phosphorylation necessary for sperm function and therefore, the functional integrity of mitochondria may thus be important for sperm survival in the female genital tract or during assisted reproductive technology (ART). Changes in mitochondrial membrane potential has been proposed to be a good indicator of sperm functional impairment (Pena et al., 2003). Rhodamine 123 (R123), potentiometric membrane dye, has been used to evaluate sperm mitochondrial function (Evenson et al., 1982; Auger et al., 1989; Graham et al., 1990). It only fluoresces red when the proton gradient over the inner mitochondrial membrane (IMM), is built up. Unstained sperm do not contain functional mitochondria, whereas, positive stained cells are aerobically functional (Garner et al., 1997). R123 is considered not to be very reliable due to its low sensitivity, variability within samples and background staining (Garner et al., 1997). There are several Mitotraker with different color such MitoTracker

Green FM [bovine: Garner et al. (1997)], MitoTracker_ Red FM [boar: (Gadella and Harrison 2002)], MitoTracker_ Red CM-H2TMRos [bovine: Fatehi et al. (2006)] and MitoTracker_ Deep Red FM [stallion: Hallap et al. (2005); ram: García-Á lvarez et al. (2009)] also selectively label the respirating mitochondria and can be used for multi-parametric sperm assessments.

Another mitochondrial stain JC-1 (5,50,6,60-tetrachloro-1,10,3,30-tetraethyl benzimidazolyl-carbocyanine iodide) now commonly used with spermatozoa from different animal species; bull (Garner et al., 1997); ram(Martínez-Pastor et al. 2004); stallion (Ortega-Ferrusola et al.,2009), to distinguish cells having highly functional mitochondria from cells having weakly functional mitochondria. JC-1 is transported into the interior of functioning mitochondria. When the concentration of JC-1 inside the mitochondria increases, the stain forms aggregates which fluoresce orange (Thomas et al., 1998). JC-1 switches from orange fluorescence in the aerobic functional mid-piece towards green fluorescent after IMM depolarization (Garner et al., 1999; Gravance et al., 2000) which indicate weakly functional mitochondria. JC-1 appears to be not suitable for multi-parameter assays due to its emission in both the green and red wavelengths and it does not work effectively on frozen-thawed sperm, probably due to interactions with egg yolk particles from the freezing extender (Garner et al.,1999). Mitochondrial activity has been related to cell motility (Garner et al., 2001; Januskauskas et al., 2005), viability (Garner et al., 1997; Januskauskas et al., 2005) and fertility (Kasai et al., 2002).

1.3.6. Apoptosis marker

Apoptosis or programmed cell death, is an active gene-driven process of self-destruction expressed through signal transduction pathways and it provides a critical means for regulation of the numbers of male gametes during spermatogenesis (Guthrie and Welch, 2005). Many studies have revealed the process of apoptosis and described presence of apoptotic cells in ejaculate from

different animals including human (Jesenberger and Jentsch, 2002; Martin et al., 2004, Martí et al., 2008

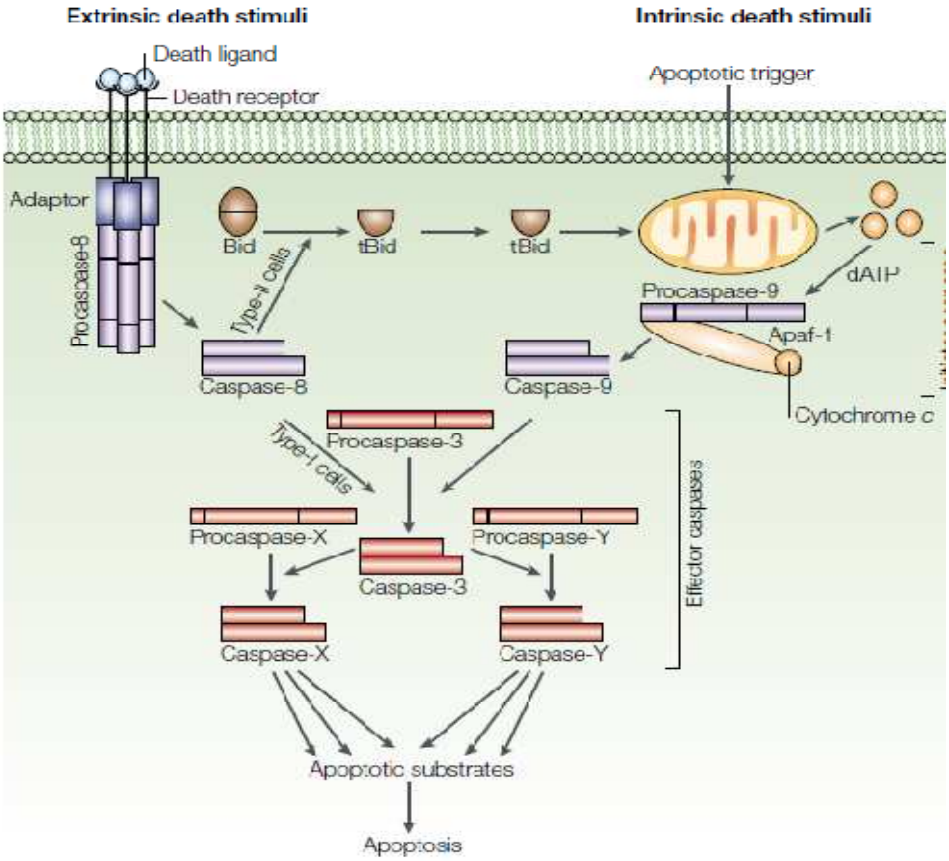


Figure-1. 10. Schematic presentation of apoptotic process. Apoptotic signaling pathways can be divided into two main routes, the death receptor pathway and the mitochondrial pathway, each of which involves the activation of specific initiator caspases such as caspase-8 and caspase-9. Both routes eventually converge at the level of caspase-3 activation. Downstream of the effector caspase-3, several signals serve to mediate apoptosis (Jesenberger and Jentsch, 2002).

After induction of apoptosis, mitochondrial pores are opened and leads to the release of proapoptotic factors from the mitochondria (Ravagnan et al., 2002). Proapoptotic factors of different proteases related to the caspases family (cysteine

proteases with aspartate specificity) are subsequently activated. It has been shown that ejaculated spermatozoa exhibit certain characteristics of apoptotic somatic cells: DNA fragmentation, plasma membrane blebbing, chromatin condensation, caspase activation, loss of mitochondrial membrane potential, decrease in plasma membrane integrity, and externalization of phosphatidylserine on the plasma membrane (Martin et al., 2004;Anzar et al., 2002, Blanc-Layrac et al., 2000, Donnelly et al., 2000). When the cell membrane is disturbed, the phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane (Bratton et al., 1997), and this is one of the earliest changes in the sperm membrane. Markers of terminal apoptosis include activated caspase-3, loss of the mitochondrial membrane potential integrity, and DNA fragmentation, which are expressed by varying proportions of ejaculated sperm (Evenson et al., 2002; Henkel et al., 2004). Since ejaculated mature spermatozoa are unable to undergo apoptosis and don't retain the necessary caspase-mediated mechanism (Weil et al, 1998), presence of apoptotic cells in fresh ejaculate may be the remainders of an earlier abortive apoptotic population (Martin et al., 2004). Moreover, the translocation of PS is considered by some authors to be a physiological event during the capacitation process (Gadella and Harrison, 2002; de Vries et al., 2003), while for others it represents a sign of cellular damage, a feature of the apoptotic phenomenon (Muratori et al., 2004; Kurz et al., 2005).

Externalization of PS can be monitored by the calcium-dependent binding of Annexin-V, appears to be more sensitive indetecting live spermatozoa dysfunctional plasma membrane besides those dead or moribund cells (Guthrie and Welch, 2005). FITC-conjugated Annexin-V and propidium Iodide (Thomas et al., 2006) or 6-CFDA and Annexin-V (Marti et al., 2008) can be used and annexin-V binds to negatively charged phospholipids on the outer leaflet of the plasma membrane in a Ca2+-dependent manner, and displays high affinity for phosphatidylserine.

Although many studies have examined the effects of cryopreservation on the outcomes of routine spermatological evaluations, few studies have focused on the association of cryopreservation with apoptotic induction (Hammadeh et al., 1999; Martin et al., 2004) specially with induction of membrane PS translocation in human (Duru et al., 2001), boar (Pena et al. 2003), and bull (Anzar et al., 2002) sperm cells.

1.3.7. DNA integrity

In mammals, the sperm nucleus is transcriptionally inactive (Imschenetzky et al., 2003) and its chromatin is compacted at a degree of condensation 6-fold higher compared with a somatic cell nucleus (Ward and Coffey, 1991). DNA fragmentation can be caused during spermatogenesis by apoptosis caused by activation of endonucleases or during ejaculation by reactive oxygen species (Evenson, 1999). The degree of DNA damage is clearly correlated with the impairment of embryo development and severe DNA damage cause male infertility (Seli et al., 2004). Therefore, DNA integrity is assessed for proper control of male fertilizing ability. There are several techniques to assess sperm DNA fragmentation such as sperm chromatin structure assay (SCSA), in situ nick translation, the terminal deoxyribonucleotidyl transferase mediated dUTP nick end labelling (TUNEL) and comet assay (Evenson et al., 1980, 2002; Evenson and Tritle, 2004).

The DNA integrity of sperm can be determined by the sperm chromatin structure assay (SCSA) (Evenson et al., 1980). The SCSA is a flow cytometric assay that is the most widespread test for assessing sperm chromatin. This test is relatively simple to perform: sperm samples are submitted to a DNA denaturation step, mixed with an acridine orange (AO) solution and analysed by flow cytometry (Evenson and Jost 2000). AO is a metachromatic fluorochrome that readily intercalates in the DNA. When associated to double-stranded DNA (dsDNA) it fluoresces green, whereas it fluoresces red when associated to single-stranded DNA (ssDNA). DNA fragmentation index (DFI) is calculated by

analysis flourometric data and when DFI in thawed semen is higher than 30%, it decreases in vivo and in vitro fertility (Gandini et al. 2006). Sperm chromatin can be evaluated under a fluorescence microscope after Acridine Orange (AO) staining on slide (Gandini et al. 2006).

The SCSA has been used in almost all domestic animals [bull (Evenson et al., 1980; Januskauskas et al., 2003); boar (Evenson et al., 1994; Boe-Hansen et al., 2005), stallion (Love and Kenney, 1998; Dias et al., 2006), ram (Peris et al., 2004) and dog (Nunez-Martinez et al., 2005)]. SCSA has shown a good correlation with fertility in bulls (Evenson et al., 1980;Sailer et al., 1996), boars (Evenson et al., 1994), stallions (Love and Kenney, 1998) and humans (Evenson et al., 1999; Virro et al., 2004) and considered a useful predictor of fertility, either in vivo (Evenson et al. 1999) or in vitro (Larson-Cook et al. 2003; Gandini et al. 2006).

Another method to detect DNA damage is to allow enzymatic incorporation of fluorescent nucleotide analogs by a terminal nucleotide transferease into single stranded DNA areas (silva and Gadella, 2005). The terminal deoxynucleotidyl transferase (TdT) mediated digoxigenin-dUTP nick-end labeling (TUNEL) assay is commonly used to assess DNA fragmentation in a variety of cells, including spermatozoa (Shen et al., 2002; et al., 2004). The TUNEL assay quantifies the incorporation of TdT mediated fluorescently labeled dUTP at breaks in double-stranded DNA, utilizing a reaction catalyzed by terminal deoxynucleotidyl transferase, the fluorescently labeled fragmented ends of DNA are detectable by flow cytometry and fluorescence microscope. The proportion of TUNELpositive cells appears to correlate well with decreased pregnancy rates using ART (Benchaib et al., 2003).

1.4. EFFECT OF CRYOPRESERVATION

Cryopreservation of spermatozoa has become a powerful technology for the preservation of the valuable genetic patrimony and distribution of males of high fertility and to preserve genome from endangered breed. In order to obtain faster diffusion of individuals of higher genetic merits, AI with cryopreserved semen has been used commercially in dairy cattle for decades. Nowadays, fair to good fertility rates are obtained with AI in comparison to natural mating. Unfortunately, in other ruminant species like sheep, goats, buffaloes and also in camelids, cryopreservation for AI is commercially limited. Cryopreservation is a non-physiological method that involves a high level of adaptation of biological cells to a series of functions like dilution, incubation, cooling, freezing or thawing, which cause ultrastructural, biochemical and functional alterations of spermatozoa (Watson et al.,1992; Holt 2000). Extender used for cryopreservation is made of various compounds, which have energy source (glucose, fructose and lactose etc), buffering capacity (TRIS, TES to main pH of extender), protection against cold shock, cryoprotectant (glycerol), appreciable quantities.) and antibiotics and present proper osmotic pressure and electrolyte balance. The goal of a cryopreservation protocol to preserve biologically functional spermatozoa that must survive in the uterine environment following a in vivo insemination, then to be transported to the oviduct, and maintained there until the oocyte arrives, and must be able to produce a viable embryo.

1.4.1. Changes in spermatozoa

Inspite of extensive development in cryopreservation protocol, fertility with frozen-thawed semen are not comparable to those obtained with fresh semen. Reduction in fertility after cryopreservation arises from both a diminished viability of spermatozoa and a sublethal dysfunction of the surviving population (Watson, 2000), due to increased freeze-thaw-induced morphological and

genomic abnormalities, and reduced rate of sperm motility (Pèrèz. Pe et al., 2002; Martin et al., 2004).

Ejaculate consists of a heterogeneous population of cells, which achieve their full fertility potential at different rates within the female tract and thus maximize the chances of a fertile spermatozoon successfully combining with an egg. It is becoming apparent that the freeze–thaw process results in a more homogeneous cell population, which may be functionally compromised (Leahy et al., 2010). Generally, about 40-50 % of the spermatic population does not survive after freezing-thawing (Watson, 2000). In ram, frozen-thawed semen may have a high proportion (40–60%) of motile cells, although only 20–30% remains biologically functional (Medeiros et al., 2002). After both slow and fast freezing of ram semen, motility is better preserved than the morphological integrity of spermatozoa. The plasma and acrosome membranes are more sensitive than the nucleus and locomotor and mid-piece part of the sperm cell. Moreover, the mitochondrial architecture is altered by freeze–thawing, but the tail filament and fibrils showed no detectable change after freeze–thawing (Salamon amd Maxwell, 2000).

During cryopreservation process, the spermatozoa undergo tremendous chemical and physical insults such as reduced temperature, lethal intracellular ice crystal formation and dissolution, cellular dehydration, osmotic injury during the addition and removal of cryoprotectants, and spermatozoa show marked responses to all of these steps (Gao and Crister, 2000; Gilmore et al., 2000; Watson, 2000).

During cooling, a thermotropic phase transition in the membrane phospholipids from a liquid-crystalline to a gel phase (a functional alteration of the plasma membrane condition), results in more rigid (ordered) membrane structure leading clusters of integral membrane proteins, altering function of ion channel protein, leading to increased membrane permeability (Robertson et al., 1988). Spermatozoa subjected to cryopreservation are very sensitive to a rapid reduction in temperature from 25 to 5°C (Watson, 1981). This produces cold

shock, a membrane transition phase behavior exhibited by most biological membranes (Morris et al., 1987). In addition, changes in temperature affects the asymmetry of membrane phospholipids very markedly. Aminophospholipid transfarase is thought to be responsible for maintenance of this asymmetry (Frog et al., 1993), and changes of temperature produce an alteration of the enzyme and a break of this asymmetry. It associates with an increase in the fluidity of the external plasma membrane, which provokes a major permeability, fusogenisity and instability, shorten the life (Watson, 1995) o spermatozoa. Loss of selective permeability and integrity of the plasma membrane leads to exposure and reorganization of actin binding sites (Holt and North, 1991), redistribution of ions, and changes in the membranes of the acrosome and mitochondria, reduction in pH and cellular metabolism and loss of motility (Quinn and White, 1966; Pickett and Komarek, 1967; Watson, 1995; Gadea, 2003).). In this stage, increased Ca+ uptake contributes to capacitative changes, collectively known as cryo-capacitation, which, latter lead to fusion events between the plasma membrane and underlying outer acrosomal membrane (Watson, 2000). Cryo-capacitated spermatozoa show hyperactivation (HA), which manifested by a vigorous pattern of sperm motility marked by high velocity, wide amplitude and whiplash movement of the flagellum (Mortimer and Maxwell, 1999). Cryo-capacitation lead to a spontaneous acrosomal reaction prior to arrival at the ampulla of the oviduct when prematurely capacitated spermatozoa are used for AI (Bailey et al., 2000) and make spermatozoa unable to form an adequate reservoir and to reach the site of ovulation. These inabilities might account for reduced fertility with cryopreserved spermatozoa in small ruminants.

The damaging effects of cooling and freezing upon sperm membrane varies among domestic species and is influenced by several elements namely, cholesterol/phospholipids ratio, content of lipids in the bilayer, degree of hydrocarbon chain saturation and protein/ phospholipid ratio (Medeiros et al. 2002). It has been observed that, sperm membrane with higher ratio of cholesterol\phopholipd and lower ratio of polyunsaturated\saturated, fatty acid presents more resistance to the changes of temperature (Pringle and Chapman,

1981). Bull, ram, and boar sperm (sensitive), have a higher ratio of unsaturated to saturated fatty acids (2. 5), whereas more resistant sperm from rabbits, dogs, and human have lower ratios (:1). On the other hand, phopspholipid:cholesterol ratio is very high (0.88–0.99) in sperm from species whose sperm are resistant to cold shock (rabbit and human), and low (0.38 and 0.45) in sperm from species whose sperm are susceptible to cold shock (bull and ram; Darin- Bennett and White, 1977).

When cells are frozen, they are subjected to stresses resulting from a water–solute interaction that arise through ice crystallization. Exposure to the hyperosmotic, unfrozen solution causes an efflux of intracellular water, cell shrinkage, and potentially an influx of ions (Mazur, 1984). Thawing reverses the freezing process, forcing the cell to retrace its path through the various environments encountered (Quinn and White, 1966) and the consequent influx of water may cause membrane disruption.

Cryopreserved spermatozoa are very susceptible to changing osmotic conditions encountered during freezing, thawing and processing (Fiser and Fairfall, 1984; Zavos, 1991). Spermatozoa encounter hypertonic conditions during freezing-thawing and an abrupt change in osmotic pressure results in occurrence of osmotic shock, reduced sperm viability and sperm membrane damage (Hammerstedt and Graham, 1990; Correa and Zavos, 1994). Osmotic stress to spermatozoa influences the fragility of sperm plasma membrane leading to rupture of plasma membrane and deachemnt of tail (Silvia et al., 2003). Ram sperm have narrow osmotic tolerance limits, similar to those of boar and bull sperm (Guthrie et al., 2002).

Post-thawed sperm become more sensitive to lipid peroxidation than those from fresh semen (Trinchero et al.,1990), due to the membrane modifications that are produced during the cryopreservation process. Specifically, the activation of an aromatic amino acid oxidase following the death of ram and bull sperm has been identified as a major source of ROS production in the semen of these animals (Upreti et al, 1998). ROS facilitates the acrosome reaction through a

stimulatory effect on the phospholipase A2 (PLA,) activity that is present in spermatozoa and that is stimulated both by the calcium and by the formation of lipid peroxides within the plasma membrane (Goldman et al., 1992). ROS can also induce oxidation of critical -SH groups in proteins and DNA, which will alter structure and function of spermatozoa with an increased susceptibility to attack by macrophages (Aitken et al., 1994). H_2O_2 is the primary ROS responsible for the loss of sperm functions (de Lamirande and Gagnon, 1992; Aitken et al., 1993).

Ultrastructural damage during freeze–thawing is accompanied by biochemical changes, which include: the release of glutamic oxaloacetic transaminase (GOT), losses of lipoproteins and amino acids, decrease in phosphatase activity, decrease in loosely bound cholesterol protein, increase in sodium and decrease in potassium content, inactivation of hyaluronidase and acrosin enzymes, loss of prostaglandins, reduction of ATP and ADP synthesis, decrease in acrosomal proteolytic activity (Salamon and Maxwell, 1995), and also denaturation of DNA (Gillan and Maxwell, 1999). Losses of proteins in the intermediate piece, causes the decrease in ATP's levels without possibility of resynthesis (Watson, 1981). In sheep, it has been observed that, though the majority of sperms lose ATP as a onsequence of damage in membrane membrane, the concentration intracelular ATP in sperms resistant to the cold-shock is kept to levels similar to those of fresh semen for several hours (Moses et al.,1996).

Very recently, Martin et al. (2006) have proposed that cell death and increased membrane permeability observed after cryopreservation might not be solely due to ice formation but also due to a hypothetical apoptosis-like process in spermatozoa. They have observed that apoptotic characteristics appeared as ordered events in bull sperm during the cryopreservation process, as a decrease of the mitochondrial membrane potectial (MMP) observed immediately after dilution in the cryopreservation medium, caspase activation after equilibration and changes in membrane permeability after the complete freezing/thawing

process. Elevated ROS, Glycerol or Ca++ influx may also have a direct toxic effect on the mitochondria, leading to decreased MMP, opening of the mitochondrial pores and release of cytochrome c and other pro-apoptotic factors, such as Bax, which could trigger caspase activation and ultimately lead to an apoptotic-like death (Wündrich et al., 2006) in cryopreserved sperm. White (1993) reported a decrease in the membrane rigidifying phospholipids PE and SM and in a relative increase in the membrane fluidizing phospholipid PC resulted from cryopreservation. The redistribution of phospholipids probably affects membrane function and charge, which can lead to severe membrane dysfunction (Watson,2000). During the early phases of disturbed membrane function, asymmetry of the membrane phospholipids occurs, before the integrity of the plasma membrane is progressively damaged (Martin et al., 1995). On this basis, it can be proposed that apoptotic positive cells are not functionally completely competent. Although many studies have examined the effects of cryopreservation on the outcomes of routine spermatological evaluations, few studies have focused on the association of cryopreservation with apoptotic induction (Hammadeh et al., 1999; Martin et al., 2004) specially with induction of membrane PS translocation in human (Duru et al., 2001), boar (Pena et al. 2003), and bull (Anzar et al., 2002) sperm cells.

Two factors are supposed to be related with overall effect of cryopreservation on sperm cells. Intrinsic factors includes the inherent sperm cell characteristics, such as, the geometry of the cell (diameter, volume, relation surface volume) (Curry et al., 1996), sperm hydration condition, permeability of the plasma membrane to the water and croprotectors, and age or maturation state of spermatozoa (Wolf, 1995). Differences in fatty acid composition and lipid class ratios in spermatozoa among species are also important factors in the freezability of the male gametes (Parks and Lynch, 1992). Among the external factors, cooling and freezing rates, type of cryoprotective agents and their concentration, extender composition, dilution rates, temperature at which glycerol is added to the semen, equilibration time, and thawing rate are considered very important for successful cryopreservation (Mazur, 1994;

Salamon and Maxwell, 1995; Aisen et al., 2000; Paulenz et al., 2004; Anel et al., 2005). Variation in freezability is well documented between animals and in ejaculates of single animal (Watson, 1995) and individual genetic variation might have an impact on to differentiate good and bed freezers (Thurston et al., 2002).

1.4.2. Changes in proteins and enzymes of SP.

Along with osmotic imbalance, the dilution effect of semen presumably removes adsorbed proteins, natural antioxidant and other beneficial components of SP. The removal of these beneficial components by dilution can also create charged differences between spermatozoa causing head to head agglutination (Mann et al,1980). Ashworth et al. (1994) showed that ram spermatozoa died rapidly after extensive dilution of SP. Decreasing temperature during cryopreservation can alter protein function in two ways- directly decreasing the catalytic activity of enzymes (Hammerstedt and Hay, 1980) or indirectly by changing pH of aquous buffer system, altering the surface charge of protein, leading to an impairment of protein-lipid interaction and irreversible denaturation of proteins (Oehninger et al, 2000).

A significant decrease (70–80%) in sperm-bound BSPs was reported after cryopreservation (Nauc and Manjunath, 2000). The induced modifications may further reduce membrane barrier properties and lead to cryocapacitation of frozen-thawed spermatozoa. Theses changes in BSPs might be responsible for deleterious effect on bovine spermatozoa during semen cooling and freezing (Pruitt et al, 1993). In a recent study, Bergeron and Manjunath, (2006) have reported that milk proteins such as casein micelles interact with BSP proteins and block their action, and hypothesized that biding pattern may be similar for homologous protein eg RSPs, GSPs. It is not clear that this binding affinity is beneficial for sperm protection from capacitation or a side effect of milk casein.

In ram, inhibitory factors present in SP are not well characterized. These factors are thought to be proteins of low molecular weight (less than 10kDa), or

to reside in the protein-free fraction have a direct detrimental effect on sperm function (Garcia-Lopez et al., 1996), or may disturb the binding of beneficial proteins to the sperm plasma membrane (Dominguez et al.,2008)

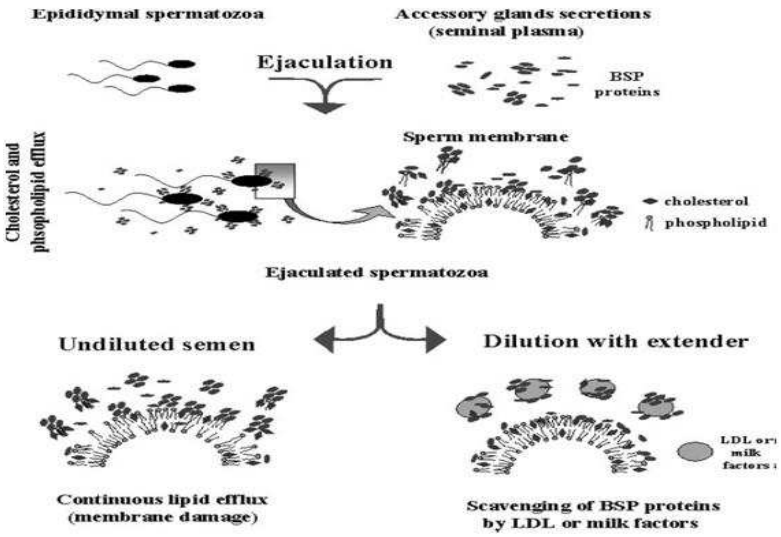


Figure-1.10: Mechanism of sperm protection by egg yolk (EY) and milk (Bergeron and Manjunath, 2006).

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In bull, a heat-labile protein known as aromatic L- aminoacid oxidase, located in the tail of spermatozoa, is responsibe for the toxic effct of SP in ambient temperature (Shannon and Curson, 1981). Aromatic L-amino acid oxidase is a latent enzyme, which becomes active only after death of bovine spermatozoa (Shannon and Curson, 1972). In bucks, SBUIII in SP secreted from the buck bulbo-urethral gland interact with skim milk triglycerides and release sperm-toxic fatty acids, such as oleic acid, appears to be responsible for the deterioration of buck spermatozoa diluted in skim milk. Buck SP produced in the nonbreeding season is more detrimental on motility and survival rate of epididymal spermatozoa in skim milk extender than SP from the breeding season (Pellicer, 1995).

There are few studies evaluating the association of SP proteins and enzymes with semen freezability. Jobim et al. (2004) reported a differences in SP Protein Profile from bulls with low and high semen freezability. On the basis of their

study, the acidic protein of (13-16 kDa) could be used as markers of high semen freezability and protein of 25–26 kDa could be a marker of low semen freezability. A positive correlation between SP total protein content and the freezability of ram semen has been reported by Barrios et al. (2000). Conversely, low protein content in SP reduces its buffering capacity and in turn, semen quality (Paz and Lewin, 1992). In alpaca, we have observed that SP collected from male with high freezability shows some specific protein fractions, which are absent in SP collected from male with low freezing quality (Marion et al., 2010).

Measurement of the enzyme activities leaked into extracellular medium through the injured plasma membrane could be used as a marker to estimate cellular damage occurring during cryopreservation. Dhami and Kodagali (1990) stated that ALP and AST could be used as markers for freezability and fertility in buffalo, as they observed a negative correlation between ALP leakage and motility of crypreserved buffalo spermatozoa. Similarly, Uperti et al. (1996) proposed ALP leakage in SP as a marker for optimisation of cooling and freeze-thaw steps of cryopreservation for ram semen.

1.4.3. Changes in antioxidants of SP

During cryopreservation, the semen is exposed to cold shock and atmospheric oxygen, which in turn, increases sperm susceptibility to lipid peroxidation (LPO) due to higher production of ROS (Salamon and Maxwell, 1995). Dilution of semen is claimed to reduce protective enzyme capacity increasing susceptiblity of the plasma membrane to lipid peroxidation, thus resulting in reduction of sperm motility and damage of the genomic integrity (Kasimanickam et al, 2007). In bovine and ram semen, ROS are generated primarily by dead spermatozoa via an aromatic amino acid oxidase catalyzed reaction (Upreti et al, 1994). Several studies have been conducted on semen viability and enzyme leakage from spermatozoa due to ROS production in frozen bull semen (Dhami and Kodagali, 1990; Upreti et al, 1996) and ram (Marti et al, 2007). Cryopreservation causes reduction in bull sperm gluthathione reductase

levels by 78%, SOD activity by 50% and the level of GPx activity significantly (Bilodeau et al, 2000). In ram, SOD is very much affected by cryoinjury, with a decrease of 65% after freezing-thawing, whereas GPx and GR activities are less affected (Marti et al, 2008). The lower enzymes activities found after the freezing-thawing process could be a result of the loss of the enzyme and/or enzyme inactivation due to low temperature. The study regarding antioxidant property in diluted semen for cryopreservation is very limited in goats, buffaloes and camelids. Natural antioxidants of SP exert a protective effect on the plasma membrane preserving both metabolic activity and viability of cryopreserved spermatozoa (O' Flaherty et al, 1997) and semen samples with highest viability after freezing and thawing have a higher activity of enzymatic antioxidant substances (Nair et al, 2006).

1.5. EFFECTS OF ADDITION OF SP OR ITS COMPONENTS ON FROZEN-THAWED SPERMATOZOA

At ejaculation, macromolecules including glycoproteins in seminal vesicular fluid, become adhered quickly and firmly onto the sperm surface. During the actual freezing of semen, the coating of macromolecules from vesicular secretion may act as a protective shield and minimizes membrane damage. Therefore, resuspension and incubation of post-thawed spermatozoa with whole SP have been used. A lot of studies have been conducted on the effect of SP on the survival of cryopreserved spermatozoa using different plasma removal techniques, such as semen filtration, dialysis and centrifugation. The addition of SP or its component into post-thawed semen increases the oxygen uptake and motility of spermatozoa (White et al, 1987), helps to recover some surface proteins (Dominguez et al, 2008), reverts the cryodamage on the plasma membrane of ram spermatozoa (Rebolledo et al. 2007) and increases overall sperm quality parameters (Maxwell et al, 2007). Catt et al. (1997) observed that

sperm maintained in their own seminal plasma prior to its removal for extension and then cryopreservation showed an increased resistance to cold shock. Addition of seminal plasma proteins to the medium prior to cold-shock, prevents membrane damage by inhibition of protein tyrosine phosphorylation (Pèrez-Pè et al, 2002), and maintains antioxidant enzymes activity and their distribution on the sperm surface (Marti et al, 2008), leading to viable sperm population (Pèrez-Pè et al. 2001b). Even during the sorting process, high dilution negatively affects the percentage of live and motile spermatozoa by lowering concentrations of protective seminal lipids and proteins (Maxwell et al, 1996). Reintroducing SP into the collection medium can compensate these deleterious effects (de Graaf et al, 2009). When spermatozoa (either uncapacitated or capacitated) are exposed to seminal plasma, they are unable to undergo the acrosome reaction and complete fertilization (Cross, 1993; Watson, 1995). The addition of SP prevents premature sperm capacitation and can convert capacitated spermatozoa back to an uncapacitated state, which may allow cryopreserved spermatozoa to remain fertile for a longer interval (Moore et al.,2005).

Soluble protein fractions obtained from membrane vesicle can improve sperm function and fertility when added into post- thawed medium (El-Hajj Ghaoui et al, 2007). The ability of ram SP proteins to repair and protect spermatozoa against cold-shock is somehow dependent on the season of the year (Pèrez-Pe et al. 2001a). The addition of SP collected during winter or autumn (breeding season) significantly improves total and progressive motility of frozen-thawed ram spermatozoa (Barrios et al, 2000; Dominguez et al, 2008). But controversy remains on the beneficial effect of addition of SP into post-thwed semen. Morrier et al. (2003) did not observe any beneficial effect of post- thaw SP treatment on motility, and proposed that the egg protein present in the diluent media could mask the effect of SP. Though there are several reports on beneficial effects of addition of heterogenous SP collected from bull (Gunay et al, 2006) or boar (Fischer et al, 1992) on freezability and fertility of frozen-thawed ram semen, García-López et al. (1996) has stated that both protective and repairing effects SP proteins are highly species specific because neither SP proteins from bull nor BSA

could abile to restore ram sperm membrane integrity. To get a better result with addition of SP, removal of diluents media containing glycerol by centrifugation or percoll density gradient separation procedure prior to addition of SP in frozen-thawed sperm may be a necessary step as it may allow spermatozoa to interact with SP component directly before exposure to the female reproductive tract (Maxwell et al, 1999). In camelids, nature (high viscosity) and component of SP are the major constraint to semen cryopreservation and impede the handling of sperm, reduce sperm activity, hasten sperm death, hinder proper mixing of sperm with extenders or react adversely with extenders. In order to overcome these problems, different liquefying enzymes such as, collagenase, trypsin (Bravo et al, 2000) and/or mechanical techniques involving alternately aspirating and expelling ejaculate through a needle (Apaza et al, 2001) have been practised. Despite the improvement in semen processing, and motility and viability of spermatozoa, fertility of camelids remains low with cryopreserved semen. Therefore, the precise nature of active constituents of SP that can help to improve post-thawed semen quality and fertility remains as a promising area of investigation. Proper understanding of beneficial effects of SP treatment for post-thawed spermatozoa could explore new approaches for cryopreservation, such as, widespread utilization of SP from vasectomized males, which can reduce extensive dilution of SP with commercial diluent or can increase utilization of specific SP proteins to improve the function of semen extenders.

1.5.1. Effect of whole SP or its components on fertility of cryopreserved spermatozoa

Naturally, a vast portion of SP does not accompany spermatozoa during their ascent to the site of fertilization in the female reproductive tract and its removal is necessary for the acquisition of fertilizing capacity. Therefore, the contribution of SP to fertility is a matter of continuing debate. Some components act as fertility factors, while other acts as antifertility factors when evaluated *in vitro*. Many attempts have been made to predict fertilizing capacity of cryopreserved spermatozoa, and a number of studies has been conducted to

relate SP components to fertility both *in vitro* and *in vivo.* When comparing AI with frozen vs fresh semen, it appears that reproductive success and quality of the outcome can be compromised if females are not exposed to SP. It has been shown that spermatozoa exposed to beneficial SP proteins, even at low concentrations, are able to penetrate oocytes earlier than those not exposed to SP proteins (El-Hajj Ghaoui et al, 2007). Addition of SP or component of SP to frozen-thawed ram semen has been shown to increase motility and and to influence sperm penetration through cervical mucus, results in increased fertility after cervical insemination (Gillan and Maxwell, 1999; Maxwell et al, 1999). While, this evidence suggests that impaired function of cryopreserved spermatozoa could be overcome by the addition of whole SP or its specific component, antifertility activity is also related to SP components in a variety of animals including bulls (Fraser et al, 1996). A lot of studies have been conducted to find out the relation between different SP components and fertility. Killian et al. (1993) identified two seminal plasma proteins with high fertility in bulls (26 kDa and 55 kDa) and two proteins that were correlated with low fertility (16 kDa and 16 kDa). The 55kDa fertility-associated protein has been identified as osteopontin (Cancel et al., 1999) and the 26kDa fertility-associated protein as Lipocalin-Type Prostaglandin D synthase (Gerena et al., 1998).

The sugar composition of SP has also been correlated with fertility, mainly due to its importance for sperm energy production (Garner et al, 2001). The concentration of PGE and testosterone in SP of bulls of good fertility is significantly higher than in bulls of low fertility (Shore et al, 2003). Sauerweina et al. (2000) observed that IGF-I concentration in SP of healthy breeding bulls was correlated positively with fertilization or pregnancy rates. Bulls with lower peroxidation in frozen semen have been proven to have a higher fertility rate (Kasimanickam et al, 2007). A positive correlation of AST activities in post-thawed semen with acrosomal damage in ruminant spermatozoa and a negative correlation with fertility was also reported (Zhao-Qi et al,1982). In order to achieve satisfactory fertility rate, importance should be given to learn the effect of SP on sperm fertilizing capacity separately from the activation on motility. From

the economical point of view, further research should be directed to establish the specific role of SP or components on fertilizing capacity of cryopreserved spermatozoa. Moreover, biochemical evaluation of SP could be an important criterion for assessing fertility and diagnosing male reproductive disorders.

Aim of the study

The current status of alpaca and ram semen cryopreservation is still considered poor to fair, respectively. Conclusively, the reasons for these sub-optimal results are still unknown compared with fresh semen. Cryopreserved semen have a short life span. This is presumable due to damage in the plasma membrane, water effux, osmotic imbalance during freezing and reverse rehydrating process during thawing (Eriksson and Rodríguez-Martínez, 2000). Several studies have demonstrated that beneficial effect of addition of SP in post-thawing sperm. Addition of SP modulates sperm motility, resistance to cold shock and stability of the plasma membrane. Generally, most of these studies attempted to disclose the effect of SP proteins. SP protein profiles has been the most studied (Cardezoa et al, Garicia et al 2006; Manjunath and Theirien, 2002) despite SP having other components (Mann & Lutwak-Mann, 1981). Generally, SP contains to macroelements, proteins, enzymes, hormone, cytokine which have specific action on spermatozoa. This is suitable to consider especially ions, enzymes, which also play important role in osmolarity balance in semen plasma membrane stabilization. Moreover, differences in SP content of a semen could well be linked to the different abilities of spermatozoa suspended in SP. Whether these ions, enzymes are differentially represented in ejaculate, whether they can influence to variable indicating sperm resilience to cryopreservation, such as, sperm motility, plasma membrane integrity, essential for fertilization is unresolved. Therefore, a series of experiment was designated to study SP composition in ejaculates (ram) and in individual male (ram, alpaca) and their relation with quality parameters in both fresh and frozen thawed spermatozoa (heterogeneity) in an ejaculate.

The experiment specifically aimed to study :

- Effect of ejaculate types on semen freezability.
- SP bio-chemical composition and protein profiles and their relation with fresh and post thaw semen quality frozen with two extenders.
- Characters of post thaw epididymal sperm frozen with two extenders.
- Effect of diets on biochemical composition of SP and semen quality in alpaca.

Relation of SP biochemical components and protein profiles with post thaw semen quality of alpacas.

CHAPTER 2. MATERIALS AND METHODS

Overall Experimental Design

Experiments, conducted throughout the research period, consisted of the following:

Study of bio-chemical composition of seminal plasma and their influences on freezability of ram semen with the following experiments:

- Comparison of the effects of different ejaculate types on semen freezability in Sarda ram;
- Effects of ejaculates on the freezability of spermatozoa and the relationship with biochemical composition and Protein Profiles (PP) of seminal plasma with semen quality and freezability
    - Effect of ejaculate on freezability of spermatozoa frozen with two diluents
    - Biochemical analysis and measurement of Protein Profiles (PP) with gel electrophoresis);
- Characteristics of post-thawed epididymal spermatozoa collected from endangered sheep breeds in Veneto region

Study of bio-chemical composition of seminal plasma and their influences on quality and freezability of alpaca semen.

- Diet effect on semen quality, biochemical composition and Protein Profiles (PP) of seminal plasma in alpaca.
- Effect of diluents on freezability of semen and their relationship with biochemical composition and Protein Profiles (PP) of SP.
    - Freezing with TRIS-citrate-egg yolk diluents with fructose and glucose

- Freezing with TRIS-citrate-egg yolk and skim milk diluents

Reagents

All chemicals were of 'analytical' grade where commercially available, and unless otherwise stated, were purchased from Applichem, GmBH, Germany. Fructose, 6-carboxifluorescence diacetate (6-CFDA) were purchased from Sigma-Aldrich, GmBH, Germany. AnnexinV-CY3 detection kit was purchased from Abcam, UK.

2.1 COMPARISON OF THE EFFECTS OF DIFFERENT EJACULATE TYPES ON SEMEN FREEZABILITY IN SARDA RAM

This study was conducted to compare effects of ejaculates (inter-individual and intra-indivdual) on post-thaw semen quality in term of freezability.

2.1.1. Animals

Four adult fertile Sarda rams (3-4 years of age) were used in this study. They were maintained in the Department of Animal Reproduction and Biotechnology, Agris- Sardegna, Italy and were housed separately from ewes. All rams were maintained with proper balanced diet and had a free access to water.

2.1.2. Semen collection, evaluation and processing

Semen was obtained from using artificial vagina (40-42°C) fitted with a graduated test tube. Ejaculates were obtained from two consecutive semen collections per ram, once a week in September-November, 2007. Immediately

after collections all ejaculates were placed in a water-bath at 37°C. Sperm concentration was evaluated using a calibrated spectrophotometer. The mass movement, motility score, and percent of motile (motility%) were estimated under phase-contrast microscope with a thermostatically controlled stage at 37°C. T o evaluate sperm motility, semen sample (5µl) was placed under a cover slip on a pre-warmed (37 ◦C) slide and subjectively assessed using phase (400× magnification) and motility score was given, as described by Evans and Maxwell (1987). Sample with a spermatozoa concentration higher than $3X10^9$/ml and motility score higher 3 (on a scale 0-5, where a score of 5 indicates rapid swirling motion and 0 denotes absence of motion) were acceptable. For this study semen samples were grouped as:

F1- First ejaculate from single ram;

S1- Second ejaculate from single ram;

P- Pool of first and second ejaculates collected from single ram;

P1- Pool of first ejaculates collected from different rams.

Pool samples ejaculates were mixed properly to diminish individual variations.

All samples were frozen using two steps dilution methods based on the use of Tris-fructose- egg yolk extender. The base semen extender, consisted of TRIS (hydroxymethyl- aminoethane) (2. 42% w/v), citric acid (1. 36%w/v), fructose (1%w/v), 20% (v/v) egg yolk, penicillin (100,000 IU) and streptomycin (100mg) were divided into two parts- one part without glycerol (A kept at 30°C) and other part with 7% glycerol (B kept at 4°C).

All ejaculates were diluted with extender A and were cooled to 4°C over 2hrs in a refrigerator. Then extender B was added slowly over 15 minutes and diluted semen was loaded into 0.25ml straws with a final sperm concentration of $80X10^6$/straw, and allowed to equilibrate at 4°C for 2hrs. Straws were exposed to liquid nitrogen vapor (-75°C) for 5 minutes and then plunged into liquid nitrogen (-196°C) for storage.

Frozen sample was thawed in a water bath at 37°C for 30 seconds . Post-thawed semen quality were assessed subjectively by a single observer. All samples were presented in a random order unknown to the observer.

2.1.3. Statistical analysis

To evaluate the effect of the ejaculates on sperm quality two ways repeated ANOVA was performed using the statistical software SIGMASTAT 2. 03. The sperm parameters are expressed as mean±SEM per treatment group. $P<0.05$ value was considered significant in this study.

2.2. EFFECTS OF EJACULATES ON THE FREEZABILITY OF SPERMATOZOA AND THE RELATIONSHIP BETWEEN BIOCHEMICAL COMPOSITION, PROTEIN PROFILES (PP) OF SEMINAL PLASMA AND SEMEN QUALITY AND FREEZABILITY (EFFECT OF EJACULATE ON FREEZABILITY OF SPERMATOZOA FROZEN WITH TWO DILUENTS)

This experiment was performed to study **e**ffects of ejaculates on the freezability of semen collected from endangered sheep breeds in Veneto region and relationship of biochemical composition and Protein Profiles of seminal plasma with semen quality and freezability.

2.2.1. Animals

Five adult rams of endangered Veneto breeds (one Foza, two Alpagota, one Lamon and one Padovana) were used in this study. They were maintained in the Agricultural farm TONIOLO of the University of Padova, Italy and were housed separately from ewes. Rams were 2-3 years old and were free from any disease during the study period. All rams were maintained with proper balanced diet and had a free access to water. Before starting the experiment, rams were trained for collecting semen with artificial vagina over 6 months.

2.2.2. Semen collection and evaluation

Semen was collected with artificial vagina (40-42°C) fitted with a graduated test tube. Ejaculates were obtained from two consecutive semen collections per ram within ten minutes, two days in a week with at least three days abstinces period in September-November, 2010.Immediately after collection, semen was transported to the laboratory, and incubated in a water bath at 37°C. Ejaculate volume was recorded directly from calibrated test tube. Sperm concentration

(×10^9 sperm/ml) was measured using Bürker cell chamber after 1:200 dilution of semen in distilled water and sperm motility(%) were assessed subjectively with a phase contrast microscope.

2.2.2.a. Cell viability assessment

Cell viability, defined as physical intactness of plasma and acrosomal membranes, was assessed by fluorescent staining with 6-carboxifluorescein diacetate (6-CFDA) and propidium iodide (PI) (Harrison and Vickers, 1990). Fresh semen was diluted into 1:500 with a saline media, composed of 200 mM sucrose, 50 mM NaCl, 18.6 mM sodium lactate, 21 mM HEPES, 10 mM KCl, 4 mM $NaHCO_3$, 2.8 mM glucose, 0.4 mM $MgSO_4$, 0.3 mM sodium pyruvate, 0.3 mM K2HPO_4, 1.5 IU/mL penicillin, and 1.5 mg/mL streptomycin (pH 6.5). 10µl CFDA (1mM dissolved in water), 10µl PI (0.75 mM dissolved in DMSO) and 10µl formaldehyde (12%w/v) were added into diluted sample and incubated at 37°C for 15 min in dark. The cells were then examined under an Ultraviolet fluorescence microscope (at least 200 cells) and with flow cytometer.

Intact plasma membrane contains esterase which hydrolyze CFDA into carboxyflourescin, resulting in green fluorescence. Whereas, non-intact plasma membrane let PI to enter inside the cell staining DNA, fluorescence red.

Three groups of cells were identified with both Ultraviolet fluorescence microscope(Diaplan, Leitz) and flow cytometer. CF+PI-: spermatozoa with green fluorescence; CF+PI+: spermatozoa with green acrosom and red postacrosomal region; CF-PI+: spermatozoa with red head.

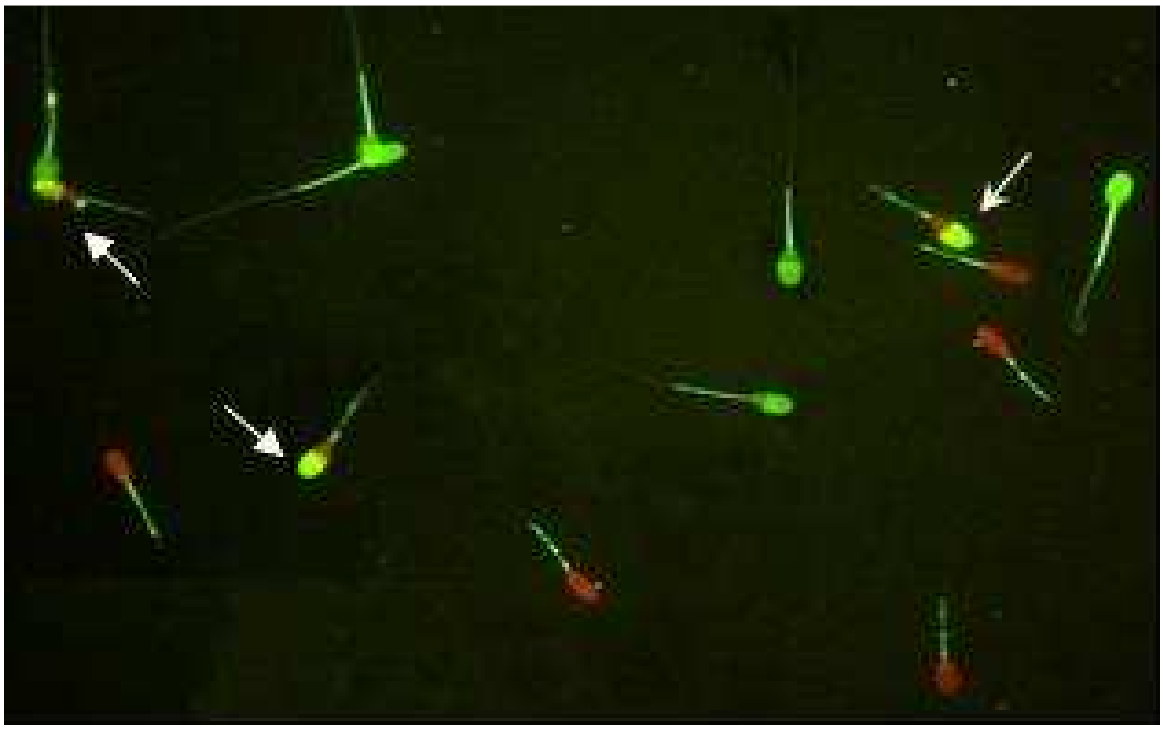


Figure 2.1. Ram spermatozoa stained with CFDA/PI. Viable sperm(CF+PI-, green), dead sperm (CF-PI+,red) and damaged sperm (CF+PI-, green acrosome and red postacrosome, marked with white arrow) was identified.

2.2.2.b. Flow cytometry

All flow cytometry measurements were performed with the use of a flow cytometry (FACScalibur; Becton Dickinson®, San Jose, CA, USA). Plasma membrane ruptured cells were PI positive, and their red fluorescent signal was detected on the FL2-photodetector. Membrane intact cells were CF+positive, and its green fluorescent signal was detected on the FL1- photodetector. Data on at least 20000 spermatozoa per sample were obtained and analyzed.

2.2.2.c. Hypo-osmotic swelling test (HOST)

HOST was used to evaluate the functional intrigrity of the sperm plasma membrane. This was performed by incubating 20µl semen with 200µl of a

100mOsm hypo-osmotic solution (made of 9g fructose and 4. 9g sodium citrate/1 distilled water) at 37 ◦C, for 60 min. After incubation, 5µl of the mixture was spread with a cover slip on a warm slide. A total of 200 sperms were evaluated in different microscopic fields at 40X magnification. The percentage of sperm with swollen and curled tails was recorded as HOST+ve% cells (Revell andMrode, 1994).

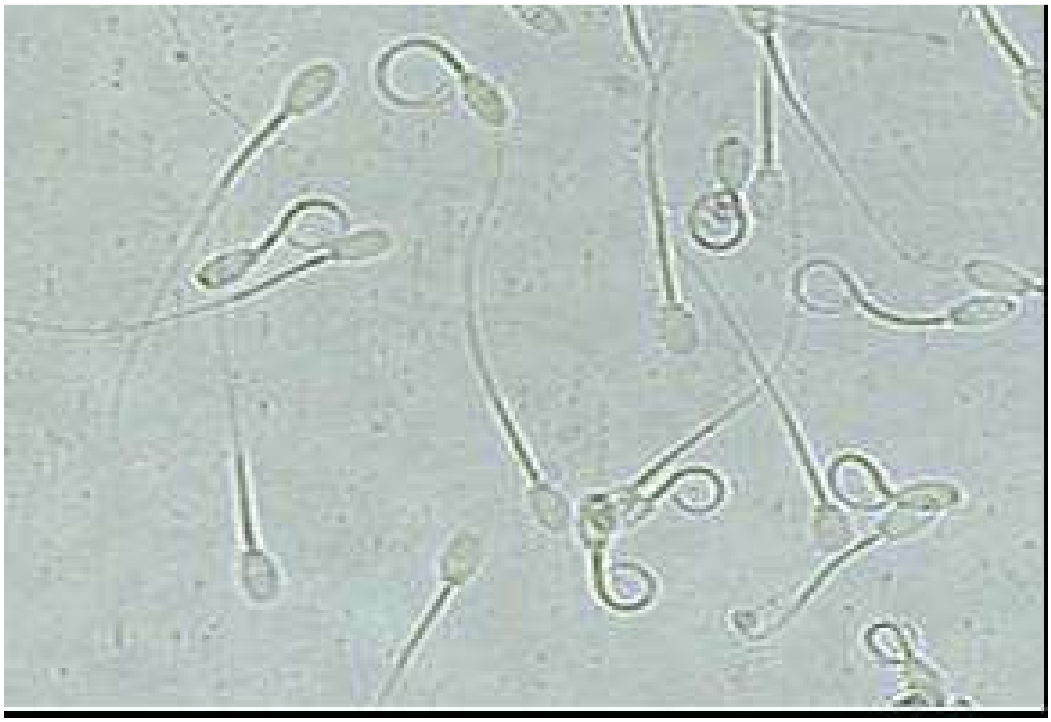


Figure-2.2. Ram spermatozoa in hypo-osmotic solution. Spermatozoa with coiled tail were HOST+ve and spermatozoa with straight tail was considered as non-viable.

2.2.2.d. Apoptosis marker or detection of membrane phosphatidylserine (PS) translocation

Annexin V is a calcium-dependent phospholipid-binding protein with high affinity for PS. The translocation of PS residues to the outer layer of the plasma membrane was detected with the Annexin V-Cy3. 6-CFDA was used to differentiate between viable cells with or without PS translocation and non-viable cells. 50 µl of fresh semen was diluted with 450µl of 1X binding buffer (Apoptosis detection kit, Abcam, UK) and stained with 5µl 6-CFDA (1mM dissolved in

water) and 2µl of Annexin Vcy3. Stained samples were incubated at room temperature for 10 min in dark, later 5µl formaddehyde (12%, w/v) was added and sample were evaluated within short time after preparation (Martí et al.,2008).

3-5µl of sample was placed on a slide and covered with cover slip, and was controlled with ultraviolet fluorescence microscope at 50X magnification. Before starting this experiment, we had justified the fluorescence color of AnnexinV under ultraviolet fluorescence microscope. Generally, AnnexinV positive cells are identified by red fluorescence with a rhodamine (Nikon G-2A) filter, whereas, AnnexinV+ve cells give a dark color under ultraviolet fluoroscope. Three populations were identified and 200 cells were counted from different microscopic fields.

CF+AN- : sperm with green fluorescence indicate viable sperm without PS translocation;

CF+AN+: green fluorescence over the acrosome, mid piece, and dark color on post-acrosome, tail indicating viable sperm but apoptotic positive;

CF-AN+: spermatozoa with dark fluorescence indicated dead sperm either by apoptosis or necrosis.

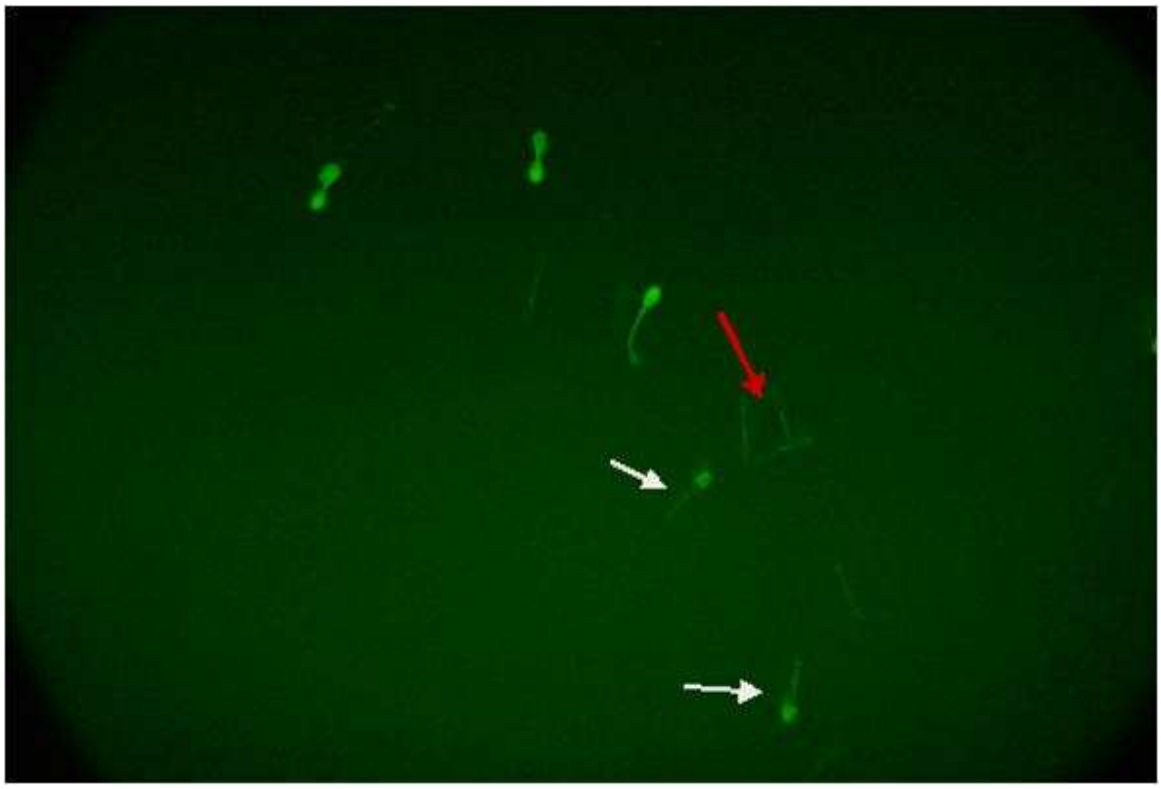


Figure 2.3. Ram spermatozoa with Annexin V staining. Viable sperm without PS translocation (CF+AN-, green), apoptotic sperm (CF+AN+, green acrosome and dark postacrosome- white arrow), dead sperm (CF-AN+, dark- red arrow). were identified with ultraviolet florescent microscope at 50X magnification.

Flow cytometry measurements were performed with a FACSCalibur flow cytometer (Beckton Dickinson Immunocytometry Systems, San Jose, CA). Forward and side scatter values were recorded on a linear scale, while fluorescent values were recorded on a logarithmic scale. Plasma membrane with translocated PS cells were AN+ positive, and their red fluorescent signal was detected on the FL2-photoditector. Membrane intact cells were CF+positive, and its green fluorescent signal was detected on the FL1- photodetector. Data on at least 20000 spermatozoa per sample were obtained and analyzed.

2.2.3. Experimental design and semen processing

Each sample was divided into three parts- part-1 and 2 were used as single and pooled samples, respectively for freezing and part-3 was used to centrifuge (2500 rpm for 10 min) to separate SP and spermatozoa. SP was preserved at -20°C for bio-chemical analysis . On the other hand, centrifuged spermatozoa were used to freeze.

Two diluents, TRIS-citrate- egg yolk and skim milk-egg yolk were used for this experiment. Therefore, all semen samples were divided into 8 treatment groups.

Four groups were frozen with TRIS-based extender and named as-

1T- first ejaculate; **2T-** second ejaculate**; 3T-** pool of first and second ejaculate;

4T- pool of first and second ejaculate without seminal plasma

four groups frozen with milk based extender- named as-

1S- first ejaculate; **2S-** second ejaculate**; 3S-** pool of first and second ejaculate;

4S- pool of first and second ejaculate without seminal plasma

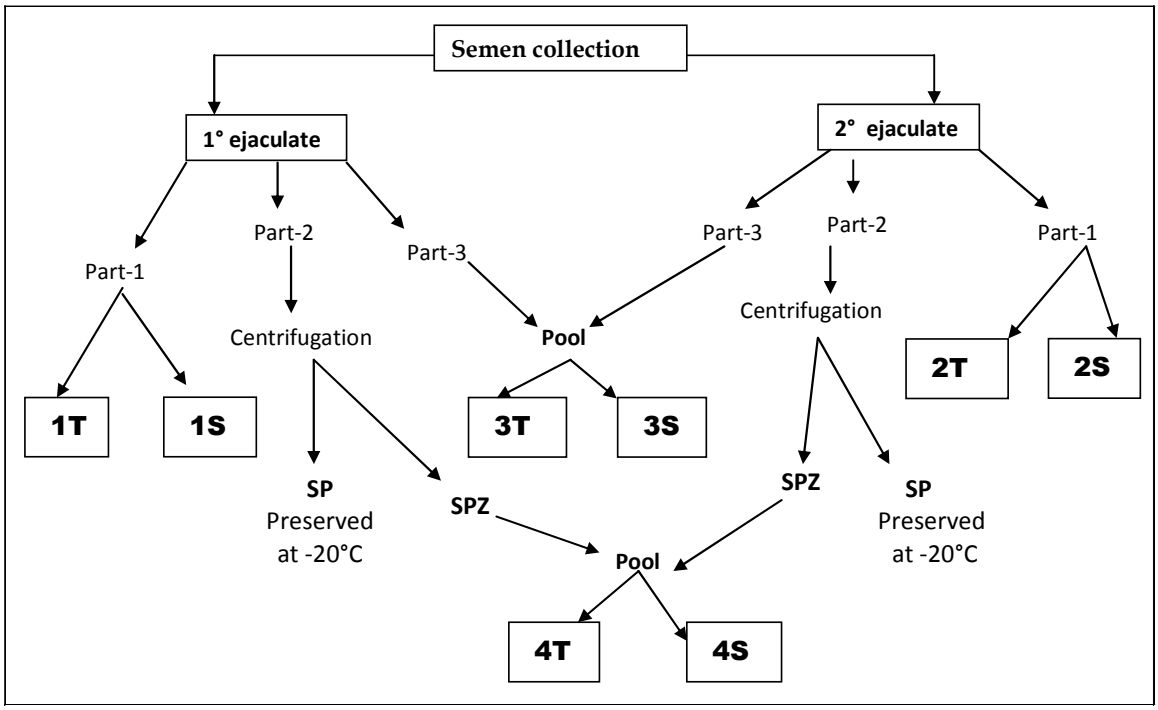


Figure 2.4. Experimental design used for this study. 1- first ejaculate, 2-second ejaculate, 3-pool of first & second ejaculates, 4- pool of first and second ejaculate centrifuges spermatozoa, T- TRIS based extender, S-skim milk extender, SPZ- spermatozoa, SP- seminal plasma

TRIS-based extender (T), consisted of TRIS (hydroxymethyl- aminoethane) (2. 42% w/v), citric acid (1. 36%w/v), fructose (1%w/v), 20% (v/v) egg yolk, penicillin (100,000 IU) and streptomycin (100mg), were divided into two parts- A without glycerol (kept at 30°C) and B with 7% glycerol (kept at 4°C). Samples from T group were diluted with extender A and were cooled to 4°C over 2hrs. Then extender B was added slowly over 15 minutes.

Milk-based extender (S) was prepared from Skim milk powder (11%, w/v) and distilled water, heated to 95°C for 10 min, and after cooling to room temperature (5%, v/v), penicillin (100,000 IU) and streptomycin (100mg) were added. This extender was divided into two parts: E1- without glycerol and kept at 30°C before added into semen, and E2- with 14%(v/v)(final concentration is 7%) glycerol (Paulenz et al., 2004). Samples were diluted with E1 for a final sperm concentration of $800X10^6$/ml. Tubes containing S group diluted samples were wrapped with tissue paper to protect against cold shock and then cooled to 4°C

for 30 min. The cooled semen was then diluted with equal volume of E2 previously maintained at 4°C. Addition of E2 was performed stepwise during a couple of minutes.

Diluted semen (both with T and S) transferred into 0.25ml straws with a final sperm concentration of $200X10^6$/straw, and allowed to equilibrate at 4°C for 2hrs. Before freezing, one straw from all treatment groups were controlled and pre-freezing motility (%) and HOST+ve% sperm were recorded. Straws were exposed to liquid nitrogen vapor (2-3 cm above the liquid nitrogen surface) for 5 min and then plunged into liquid nitrogen (-196°C) for storage.

When required, frozen sample was thawed in a water bath at 37°C for 30s .

2.2.4. Post-thawed sperm assessment

All tests used for fresh semen were applied for thawed samples including sperm motility analysis using a computer assisted semen analysis (CASA) system.

2.2.4.a. Sperm motility analysis

5µl of frozen-thawed sperm sample were placed onto a prewarmed microscope slide covered with a prewarmed coverslip and loaded into the analyzer.

The instrument settings were as follows: number of frames acquired-25; frames rate- 30 Hz; minimum cell cise- 9 pixels, VAP 25 µ/s, STR- 80%,VAP-cutoff -10µ/s. ; VSL cutoff- 15µ/s, minimum contrast-9; cell intensity- 15.

The following motion characteristics were compared: VAP, VCL, VSL, ALH, LIN, STR.

2.2.4.b. Cell viability

5µl of thawed sample were diluted with 195µl of saline extender (described for fresh semen) and 2µl of 6-CFDA, 2 µl of PI and 2 µl of formaldehyde were added into sample (modified from Martí et al.,2008). Stained samples were incubated at 37°C for 15 min in dark. Both fluorescence microscopic and flow cytometric evaluation were performed. Viable sperm% (CF+PI-,green), damaged sperm% (CF+PI+, green acrosome and red postacrosome) and dead% (CF-PI+, red) (Figure-2.1) were recorded.

2.2.4.c. Hypo-osmotic swelling test (HOST)

5 µl of thawed semen was added into 50 µl of fructose-sodium citrate solution (100 mOsm) and incubated for one hour at 37°C. 200 cells were counted where spermatozoa with curled tail was considered as HOST+ve cell (Figure-2.2).

2.2.4.d. Apoptosis marker

50 µl thawed semen sample was mixed with 150 µl 1X buffer (apoptosis detection kit, Abcam, UK) and then stained with 2 µl 6-CFDA (1mM dissolved in water), 1µl Annexine V-Cy3. Sample was incubated for 15 min in dark and then mixed with formaldehyde (12%, w/v) (modified from Martí et al., 2008). All samples were controlled with ultraviolet fluorescence microscope and flow cytometer as described for fresh sample. Viable cell% (CF+AN-, without PS translocation, green), apoptotic positive cells% (CF+AN+, green acrosome and dark/red postacrosome) and dead cells% (CF-A+, dark) (Figure 2.3.) were recorded.

2.3. EFFECTS OF EJACULATES ON THE FREEZABILITY OF SPERMATOZOA AND THE RELATIONSHIP BETWEEN BIOCHEMICAL COMPOSITION, PP OF SEMINAL PLASMA AND SEMEN QUALITY AND FREEZABILITY (BIOCHEMICAL ANALYSIS AND GEL ELECTROPHORESIS OF SP PROTEIN).

Biochemical analysis was performed with an automatic analyzer (Hitachi Boehering Mannheim, 912 Automatic Analyzer) using specific kits. SP enzymes such as alanine amino transfarase (ALT), alkaline phosphatase, glucose (Glu), total protein (TP), triglycerides (TG), cholesterol (Chol), calcium (Ca), sodium (Na), potassium (K), magnesium (Mg), phosphorus (P) and chlorine (Cl) were measured.

One dimensional Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to identify and quantify Protein Profiles (PP) in ram SP.

2.3.1. Preparation of gel

PAGE was performed on 14% polyacrylamide gels according to Laemmli (1970) using a vertical slab gel apparatus (Mini-Protean Tetra system, Bio-red). Resolving gel was prepared with 14% acrylamide\bisacrylamide and gel buffer consisted of TRIS-Base (1.38 M), EDTA (0.3%), SDS (0.4%), with a pH 8.8. For polymerization of resolving gel, TEMED (Tetramethylethylenediamine) (0.8 µl/ml) and 10% Amonium persulphate (APS) (17 µl /ml) were used. Staking gel was prepared with 4% acrylamide\bisacrylamide and gel buffer consisted of TRIS Base 0.45 M, EDTA (0.3%), SDS (0.4%), pH-6.8. For polymerization TEMED (3.5 µl /ml) 10% APS (10 µl/ml) were added.

2.3.2. Preparation of sample

SP sample was diluted with sample buffer for a final concentration of 50 µg of protein in 25µl . Sample buffer was prepared with TRIS-HCl (0.027 M, pH-6.8), SDS (2%), glycerol (25%), β-mercaptanol (5%), EDTA (0.018%), 1% bromophenol blue (0.1%). Diluted sample was boiled in water bath at 100°C for 5min for protein denaturation.

2.3.2.a. Electrophoresis

25µl of denaturated samples were loaded into gel well. 6 µl of Precision plus protein standard (Bio-Rad) was used to identify different protein bands. Protein standards was composed of protein bands of different molecular weight, such as, 250 kDa, 150 kDa, 100 kDa, 75 kDa, 50 kDa, 37 kDa, 25 kDa, 20 kDa, 15 kDa e10 kDa. Electrophoresis was conducted at 75 V for 3 hrs with PowerPac Basic Power supply (Bio-Rad).

2.3.2.b. Staining of gel

Gels were stained with Tincture solution, prepared with Comassie Brillian Blue (1%), tricholoroacetic acid (5. 4% w\v), acetic acid (5. 87% v/v) and methane (16. 8%v/v). Gels were incubated with staining solutions for a period of 6hrs and then washed with 7% acetic acid solution until no background was detectable.

2.3.2.c. Analysis of gel

Gel images were processed with Sante Dicom Viewer Image J program. Gel bands density was measured and analysed to get total protein % (TP%) and relative protein concentration (TP).

2.3.3 Statistical analysis

Two ways repeated ANOVA was performed where, treatment (1st and 2nd ejaculate, T and S) and breeds (Alpagota, Foza, Lamon, Padovana) were considered as independent variables, and semen quality parameters, biochemical and quantitative analysis were considered as dependent variables. Data were presented as mean±SEM. $P<0.05$ value was considered significant in this study. Moreover, Pearson correlation indices were calculated between all the parameters considered. All data were analyzed with Statistical software SIGMASTAT 2.03.

2.4. CHARACTERISTICS OF POST-THAWED EPIDIDYMAL SPERMATOZOA COLLECTED FROM ENDANGERED SHEEP BREEDS IN VENETO REGION

This experiment was performed to study post-thawed epididymal sperm quality collected from endangered Veneto sheep breeds.

2.4.1. Testes Collection

Testes of ram (n=10) of endangered Veneto breeds were collected from an abattoir and were transported to the laboratory with normal saline at a temperature that ranged between 20°C to 25°C within 4 to 6 hours postmortem. Immediately upon arrival to the laboratory, each pair of testes was dissected away from its scrotum and tunica vaginalis. Ram with anatomically abnormal testes were not used in this study.

2.4.2. Extender Preparation

TRIS- based (T) and milk based extender (S) were used for this study. Extender T was made of TRIS (2.42% w/v), citric acid (1.36%w/v), fructose (1%w/v), 20% (v/v) egg yolk, penicillin (100,000 IU) and streptomycin (100mg), and divided into Part-A (without glycerol) and Part-B (with 7% glycerol).

Extender S was prepared from non-fatty milk powder (11%, w/v) and distilled water, heated to 95°C for 10 min, and after cooling to room temperature (5%, v/v), penicillin (64.20mg) and streptomycin (100mg) were added. Extender S was divided into Part-E1 (without glycerol) and Part-E2 (with 7%glycerol). Extenders Part-B and part-E2 were kept at 4°C, whereas, Part-A and Part E1 were warmed up upto 37°C in water bath 30 min prior to epididymal sperm retrieval.

2. 4. 3. Epididymal Sperm Retrieval

Each cauda was dissected from the testis by cutting the vas deferens and corpus epididymis (Figure-2. 5). Upon dissection, each cauda was rinsed with normal saline to remove any remaining materials before epididymal sperm extraction. Specimens from each cauda were then allocated into two treatment groups (T and S). Epididymal sperm were harvested by making 5 to 6 incisions using a surgical blade in the cauda epididymides. The incisions were rinsed properly with dilution media in Petri dishes to collect sperm at maximum concentration. The sperm solution was transferred into a 15 ml plastic tube and placed in a water bath a 37°C for 15 minutes for sperm quality analyses.

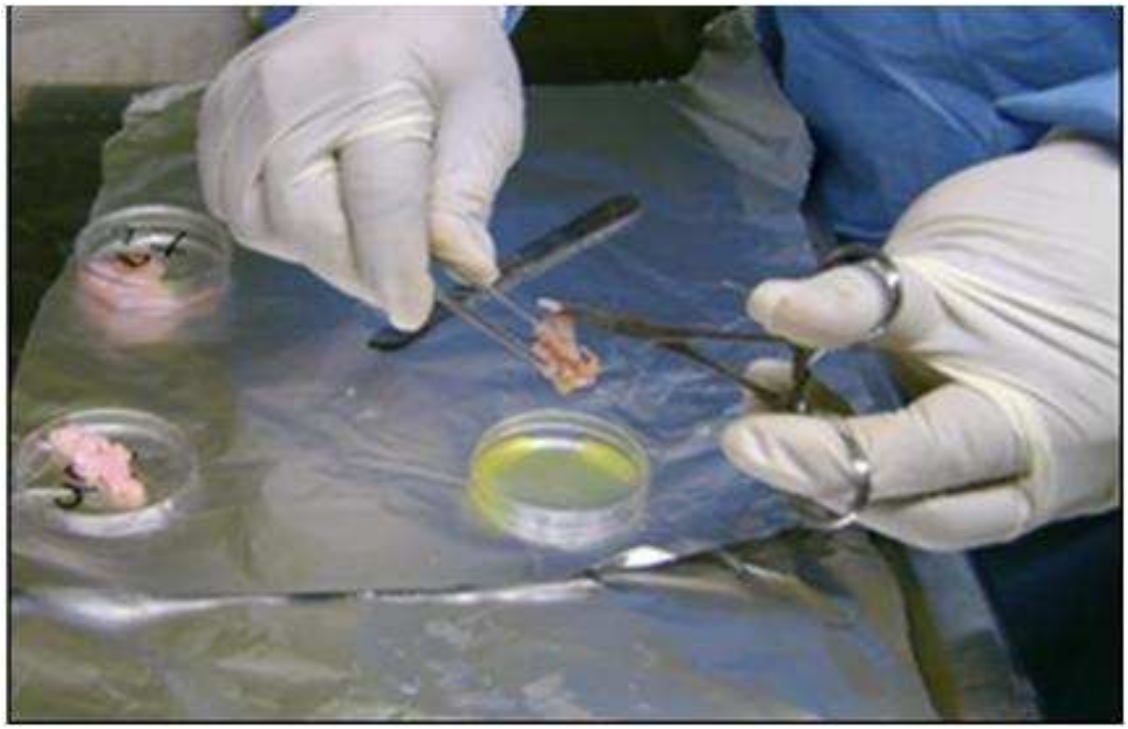


Figure 2.5. Collection of epididymal sperm.

2.4.5. Sperm evaluation

Evaluation parameters included concentration, motility and plasma membrane integrity. Sperm concentration was calculated with Bürker cell counting chamber with a dilution 1:200 in distilled water. To evaluate sperm motility, diluted sample (5µl) was placed on a pre-warmed (37 ◦C) slide, spread a cover slip and subjectively assessed under phase-contrast microscope at 40 X magnification.

2.4.5.a. Hypo-osmotic swelling test (HOST)

20µl of diluted sperm was mixed with 200µl hypo-osmotic solution, composed of 9g fructose and 4.9g sodium citrate in 1L distilled water, (100mOsm) and incubated at 37 °C, for 60 min. After incubation, 5µl of the mixture was spread with a cover slip on a warm slide. A total of 200 sperm were counted in different microscopic fields at 40X magnification. The percentage of sperm with curled tails was recorded as HOST+ve% cells (Revell and Mrode, 1994).

2.4.6. Epididymal sperm processing

Concentration was further adjusted to 400 x 10^6 sperm/ml by the addition of more extender. The plastic tube containing the epididymal sperm sample was placed in a glass beaker containing water and the beaker was placed in a refrigerator for cooling to 4°C for 2 hours. Cooled epididymal sperm diluted with part-A (T) and part-E1 (S) were mixed with part-B (T) and part-E2 (S) respectively, step by step over 15 min. All samples were loaded into 0.25ml straw with a concentration 100X10^6 sperm/straw. Straws were placed at 4°C for further

2 hrs to equilibrate. After equilibration straws were placed over (2-3 cm) liquid nitrogen vapors for 5 minutes and then plunged in liquid nitrogen and stored.

2.4.7. Post-thawed sperm assessment

2.4.7.a. Motility analysis with computer assisted semen analysis (CASA) system

5µl of freeze-thawed sperm sample were placed on a pre-warmed microscope slide covered with a pre-warmed cover slip and loaded into the analyzer.

The instrument settings were as follows: number of frames acquired-25; frames rate- 30 Hz; minimum cell size- 9 pixels, VAP 25 µ/s, STR- 80%VAP-cutoff -10µ/s. ; VSL cutoff- 15µ/s, minimum contrast-9; cell intensity- 15.

VAP (Average path velocity-µm/s), VCL (Curvilinear velocity- µm/s), VSL (Straight linear velocity-µm/s), ALH (Amplitude of lateral head displacement - µm), LIN% [Linearity-(VSL/VCL) X100)] and STR%[Straightness-(VSL/VAP)X100] were analyzed.

2.4.7.b. Cell viability

5µl of thawed sample were diluted with 195µl of saline extender (composed of 200 mM sucrose, 50 mM NaCl, 18.6 mM sodium lactate, 21 mM HEPES, 10 mM KCl, 4 mM $NaHCO_3$, 2.8 mM glucose, 0.4 mM $MgSO_4$, 0.3 mM sodium pyruvate, 0.3 mM $K2HPO_4$, 1.5 IU/mL penicillin, and 1.5 mg/mL streptomycin, pH 6.5). 2µl of 6-CFDA (1 mM dissolved in water), 2 µl of PI (0.75 mM dissolved in DMSO) and 2 µl of formaldehyde (12%w/v) were added into sample (modified from Martí et al.,2008). Stained samples were incubated at 37°C for 15 min in dark. Evaluation were performed with ultraviolet Fluorescence microscope.

Viable sperm% (CF+PI-, green), damaged sperm%(CF+PI+, green acrosome and red postacrosome) and dead%(CF-PI+, red) (Figure 2.1) were recorded.

2.4.7.c. Hypo-osmotic swelling test (HOST)

5 µl of thawed semen was added into 50 µl of fructose-sodium citrate solution (100 mOsm) and incubated for one hour at 37°C. 200 cells were counted where spermatozoa with curled tailed was considered as HOST+ve cell (Figure 2.2).

2.4.7.d. Apoptosis marker

50 µl thawed semen sample was mixed with 150 µl 1X buffer(Apoptosis detection kit, Abcam, UK and then stained with 2 µl 6-CFDA (1mM dissolved in water), 1µl Annexin V-Cy3. Sample was incubated for 15min in dark at room temperature and then 2 µl formaldehyde (12%w/v) was added to fix stain (modified from Martí et al.,2008). Samples were controlled with fluorescence microscope within short time after preparation. Viable cell % (CF+AN-,without translocated PS, green), apoptotic positive cells% (CF+AN+, green acrosome and dark postacrosome) and dead cells% (CF-AN+, dark) (Figure 2.3) were recorded.

2.3.3 Statistical analysis

One ways repeated ANOVA was performed where treatment (T and S) was considered as independent variable, and semen quality parameters, biochemical and quantitative analysis were considered as dependent variables. Data were presented as mean±SEM. $P<0.05$ value was considered significant in this study. Moreover, Pearson correlation indices were calculated between all the parameters considered. All data were analyzed with Statistical software SIGMASTAT 2.03.

2.5. STUDY OF BIO-CHEMICAL COMPOSITION OF SP AND THEIR INFLUENCES ON SEMEN QUALITY AND FREEZABILITY OF ALPACA SEMEN (DIET EFFECT ON SEMEN QUALITY, BIOCHEMICAL COMPOSITION AND PP OF SP IN ALPACA).

2.5.1. Animals

This research was conducted from April, 2009 to August, 2009 and five alpaca males (5-8 years old) were used. They belonged to a private farm (Agricole Poggio Piero) owned by Mr. Renè Steiger and were maintained in animal stall in the Department of Veterinary Clinical Sciences, University of Padova, Italy. Among five males, four were Hucaya and one male was Suri in phenotype. Before started the experiment, males were trained to copulate with artificial vagina in presence of two teaser females. All males were housed separately from female alpacas.

2.5.2. Experimental design

Total experimental period was divided into four periods, when animals were allowed to four different diets.

Table 2.1. Time schedule and diets for experimental periods.

Periods	Time	Diet
1°	02/03/09–12/04/09	Hay
2°	13/04/09– 24/05/09	Hay+pasture grazing
3°	25/05/09–05/07/09	Pasture grazing+ sheep concentrate
4°	06/07/09–16/08/09	Pasture grazing + horse concentrate

Sheep concentrate was composed of wheat flour, corn flour, corn seed, wheat bran, soyabin, calcium carbonate, sugar molasses . Feed integrity- crude protein 16%, crude fibre-10%, crude lipids-2.7%, ashes-7%.

Horse concentrate was composed of – wheat bran, maize bran, wheat's straw, wheat flour, rice polish, calcium carbonate, maize, sugar molasses, sodium chloride, soyabean crush. Feed integrity-crude protein 12%, crude lipids 3%, crud fiber 11%, ashes 7.5%.

In every period, semen was collected once in a week for four weeks. To mitigate effects of previous diet, semen collection was avoided for two weeks after starting with new diets. Before semen collection, testicular volume and BCS (Body condition score) were measured. Length, width and thickness of both testes were measured with a caliber (Gouletsou et al., 2008). Testicular volume was calculated as follows:

Testicular volume= length X widthXthicknessX0.5236

BCS was measured by palpating lumbar region as like other small ruminants.

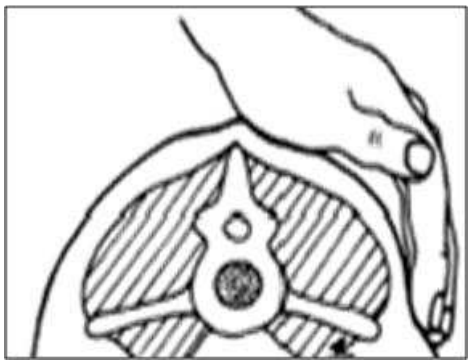


Figure 2.6. Schematic presentation of BCS measurement (Thompson and Meyer,1994).

2.5.3. Semen collection and Evaluation

Semen was collected with a modified ovine artificial vagina (AV) in presence of teaser female (Figure 2.8.). AV was prepared by placing a foam in the anterior inner latex to make a cervix like structure and a glass tube was attached with this part to collect semen (Figure 2.7.). During collection, AV was wrapped with an electric heating pad to warmth AV to maintain internal temperature of AV.

Figure 2.7. Modified AV used for this study.

Figure 2.8. Semen collection from alpaca semen.

Copulation time was recorded, (started when male mounted the female and considered ended, when male stood up and did not show interest to female). Semen was retrieved from the AV by repeated, sharp downwards thrusts to dislodge the viscous semen and then collected in a graduated test tube. Immediately after collection, semen tube was placed in a water bath at 37°C. Classical semen parameters such as volume, consistency (viscosity), concentration, motility percentage were evaluated. Ejaculate volume was recorded directly from the collecting tube. Consistency or viscosity of the semen was graded as:

1-viscous (when semen did not drop from a Pasteur pipette) (Figure 2.9.);

2- semiviscous (when some semen dropped from the Pasteur pipette to a glass slide);

3- liquid (when semen was fluid and dropped readily from the Pasteur pipette) (Bravo et al., (1997).

Figure 2.9. Viscous alpaca semen.

Sperm concentration was determined by the Cell VU® Sperm counting chamber and expressed as number of spermatozoa per ml. Total number of sperm was determined by multiplying sperm concentration by ejaculate volume. Use of cell VU® chamber was advantageous over Bürker chamber as dilution of sample were not required and sperm motility was also observed. For motility evaluation 10µl of semen was placed on pre-warmed glass slide and spread with cover slip and controlled with phase contrast microscope at 40X magnification. Sperm motility, detected as an oscillatory motion of the flagellum was determined as a percentage of motile sperm. After evaluation, semen samples

were centrifuged at 3500 rpm for 40 minutes and SP was separated and stored at -20°C for biochemical analysis.

2.5.4. Biochemical analysis and gel electrophoresis

Biochemical analysis was performed with an automatic analyzer using specific kit (Hitachi Boehering Mannheim, 912 Automatic Analyzer). SP enzymes such as alanine amino transfarase (ALT), γ-glutamyltransferase, alkaline phosphatase, acid phosphatase, creatine kinase, glucose, urea, creatinine, total protein, albumin, triglycerides, cholesterol, calcium were measured.

One dimensional Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to identify and quantify Protein Profiles (PP) in alpaca SP.

PAGE was performed with 16% polyacrylamide gels in the gel of separation according to Laemmli (1970). Detailed procedure was described in Experiment 1. 2b. Briefly, sample was diluted 1:20 with sample buffer. Total 15 µg protein was used for each sample. Protein standards (Broad Molecular Weight standards, Bio-Rad) composed of protein bands of different molecular weight; 200 kDa, 116.25 kDa, 97.4 kDa, 66.2 kDa, 45 kDa, 31 kDa, 21.5 kDa, 14.4 kDa e 6.5 kDa was used. Electrophoresis was conducted at 48 mA for 2 hrs with PowerPac Basic Power supply (Bio-Rad). Gels were stained with Comassie Brillian Blue.

Gel images were processed with Sante Dicom Viewer Image J program. Gel bands density was measured and analysed to get total protein % (TP%) and relative protein concentration (TP).

2. 5.5. Statistical analysis

Two ways repeated ANOVA was performed where, period and animals were considered as independent variables, and semen quality parameters, biochemical and semi-quantitative analysis were considered as dependent variables. Data were presented as mean±SEM. $P<0.05$ value was considered significant in this study. All data were analyzed with Statistical software SIGMASTAT 2.03.

2.6. STUDY OF BIO-CHEMICAL COMPOSITION OF SP AND ITS INFLUENCES ON SEMEN QUALITY AND FREEZABILITY OF ALPACA SEMEN (EFFECT OF DILUENTS ON FREEZABILITY OF SEMEN AND THEIR RELATIONSHIP WITH BIOCHEMICAL COMPOSITION AND PP OF SP).

2.6.1. Animals

Three alpaca males (6-8 years old) were used for this study and were housed in the animal stall of the Department of Veterinary Clinical Sciences, Faculty of Veterinary Medicine, University of Padova, Italy. During the study period, all three males were allowed to pasture grazing, and supplemented with hay and commercially formulated sheep concentrate. They had free access to water and were free of diseases.

2.6.2. Semen collection and evaluation

Semen was collected with a modified ovine artificial vagina in presence of teaser female, once a week, from April, 2010 to October, 2010.Immediately after collection, semen was transported to the Laboratory and kept at 37°C. Ejaculate volume was determined directly from the collecting tube. Viscosity of the semen was graded as 1, 2 and 3 according to Bravo et al., (1997). For motility evaluation, 10µl of semen was placed on pre-warmed glass slide and spread with cover slip and controlled with phase contrast microscope at 40X magnification. Sperm motility, detected as an oscillatory motion of the flagellum was determined as a percentage of motile sperm.

Sperm concentration was calculated by the Cell VU® sperm counting chamber.

2.6.2.a Sperm viability assessment

Staining method was adapted (modified from Martí et al., 2008) for alpaca semen before starting this experiment. 50µl of fresh semen were diluted with 250 µl of saline extender (composed of 200 mM sucrose, 50 mM NaCl, 18.6 mM sodium lactate, 21 mM HEPES, 10 mM KCl, 4 mM $NaHCO_3$, 2.8 mM glucose, 0.4 mM $MgSO_4$, 0.3 mM sodium pyruvate, 0.3 mM $K2HPO_4$, 1.5 IU/mL penicillin, and 1.5 mg/mL streptomycin, pH 6.5). Diluted samples were mixed with 3µl of 6-CFDA (1 mM dissolved in water), 3µl of PI (0.75 mM dissolved in DMSO) and 3 µl of formaldehyde (12%w/v) and were incubated at 37°C for 15 min in dark. Evaluation were performed with ultraviolet Fluorescence microscope and 100 cells were counted from different microscopic fields. Viable sperm% (CF+PI-,green), damaged sperm% (CF+PI+, green acrosome and red postacrosome) and dead% (CF-PI+, red) were recorded.

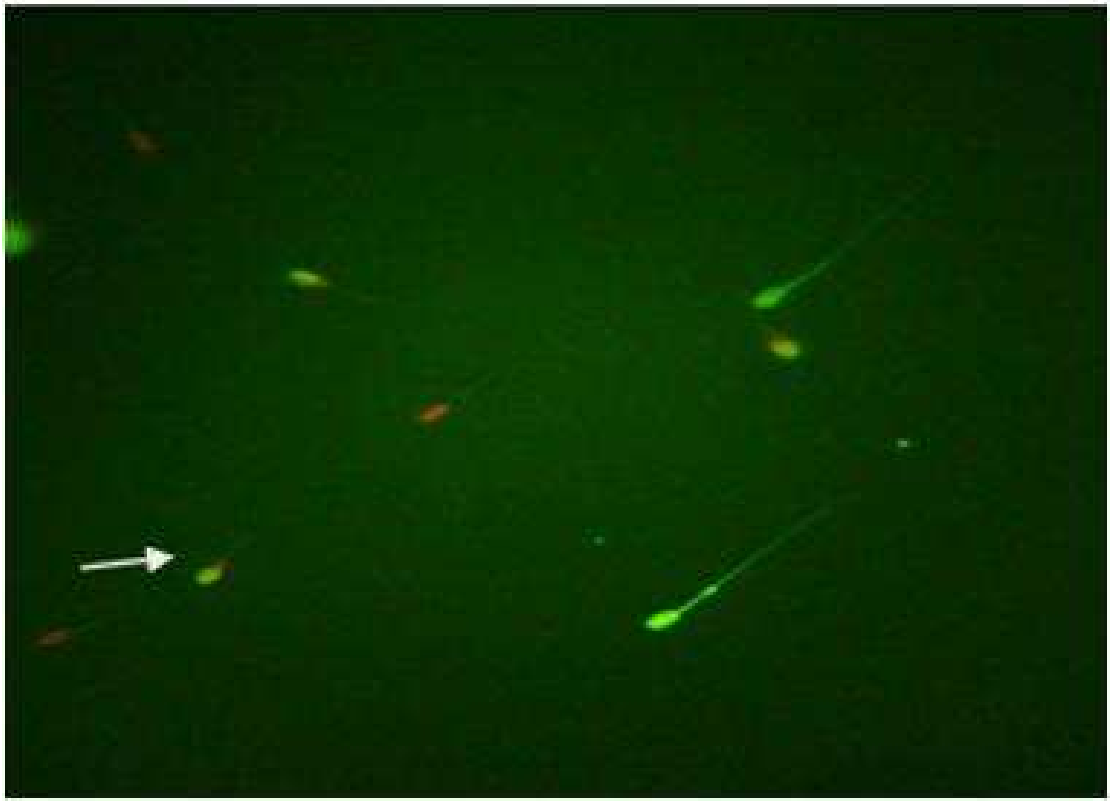


Figure-2.10: Alpaca spermatozoa with CFDA/PI stain. Viable sperm(CF+PI-, green), dead sperm (CF-PI+, red) and damaged sperm (CF+PI-, green acrosome and red postacrosome, marked with white arrow) (X50 magnification).

2.6.2.b. Hypo-osmotic swelling test (HOST)

50 µl of fresh semen was mixed into 200 µl of a hypo-osmotic solution of 2.45 mg fructose and 4.5 mg sodium citrate in 1ml distilled water (100 mOsm) and incubated for 20 min at 37°C. 100 cells were counted. Spermatozoa with curled tailed were considered as HOST+ve cell.

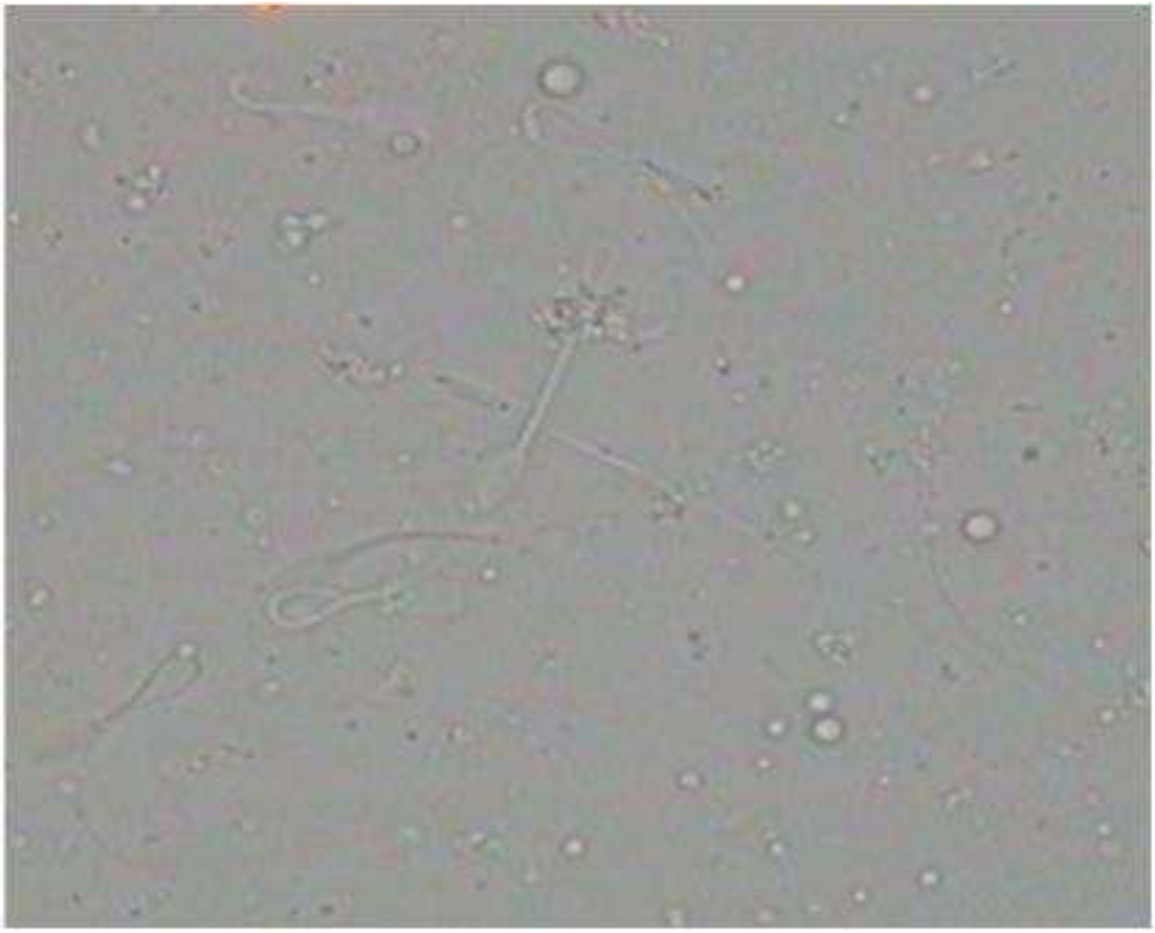


Figure 2.11: Alpaca spermatozoa with coiled tail (HOST+ve). Spermatozoa with straight tail considered as nonviable.

2.6.2.c. Apoptosis marker

This staining procedure was adapted for alpaca semen before starting the experiment. 50 µl fresh diluted semen sample was taken into 0.5ml eppendrof covered with aluminum paper (better to use colored eppendorf) into 250 µl 1X buffer(Apoptosis detection kit, Abcam, UK) and mixed properly by pippetting. First, 2.5 µl 6-CFDA (1mM dissolved in water), added into diluted sample and after 10 min 1µl Annexin V-Cy3 (Apoptosis detection kit, Abcam, UK) was added

into sample, and then incubated for 15 min in dark at room temperature. 2.5 µl formaldehyde (12%w/v) was used to fix stain. 100 sperm were counted with Ultraviolet fluorescence microscope from different microscopic fields within short time to avoid fluorescence loss. Viable cell % (CF+AN-, without PS translocation, green), apoptotic positive cells% (CF+AN+, green acrosome and dark postacrosome) and dead sperm% (CF-AN+, dark) were recorded.

2.6.3. Semen processing

2.6.3.a. Freezing with TRIS-citrate- egg yolk with glucose (G) and fructose (F)

Tris-citrate-egg yolk extender with glucose (G) and TRIS-citrate-egg yolk with fructose (F) were used for this study. Extender G based solution was prepared with 2.44% TRIS, 1.36 % citric acid, 0.82% glucose and 20 % (v/v) egg yolk, 62.3 mg penicillin and 1mg streptomycin. Extender F based solution was prepared with same materials except 0.82% fructose was used instead of glucose. Both extenders were divide into two parts, part-A kept at 37°C and part-B with 7% glycerol kept at 4°C.

Semen samples (n=24) were divided into three fractions, one fraction was centrifuged at 3500 rpm for 40 minutes to separate SP. Collected SP was preserved at -20°C for biochemical and gel electrophoresis. Other two fractions were diluted 1:1 with first part-A of F and G extenders and mix properly with pippetting. Samples were grouped as **G** and **F.** Test tubes containing diluted samples were placed in a glass beaker and placed in refrigerator at 4°C for 2 hrs to be cooled. Equal volumes of part-B of extender F and G were added step wise into cooled sample. Diluted sample was loaded into 0.25ml straws and kept at 4°C for 30 min. Before freezing cooled diluted sample were evaluated for motility and HOST analysis. Straws were exposed over liquid nitrogen vapor for 5 min

and then were plunged into liquid nitrogen for storage. For post-thawing evaluation, straws were thawed in a water bath at 37 °C for 30 seconds.

2.6.3.b. Freezing with TRIS-citrate- fructose- egg yolk (T) and skim milk (S) extender

Extender T consisted of TRIS (2.42% w/v), citric acid (1.36%w/v), fructose (1%w/v), 20% (v/v) egg yolk, penicillin (100.000 IU) and streptomycin (100mg), and divided into Part-A (without glycerol at 37°C) and Part-B (with 7% glycerol at 4°C).

Extender S was prepared with skim milk powder (11%, w/v), egg yolk (5%, v/v), penicillin (64.20mg) and streptomycin (100mg). Extender S was divided into Part-E1 (without glycerol maintained at 37°C) and Part-E2 (with 7%glycerol maintained at 4°C).

Semen samples (n=18) were divided into three fractions, one fraction was centrifuged at 3500 rpm for 40 minutes to separate SP. Collected SP was preserved at -20°C for biochemical analysis and gel electrophoresis. Other two fractions were diluted 1:1 with part-A (T) and Part-E1 (S) extenders and mix properly with pippetting. Samples were grouped as S and T. Test tubes containing T samples were placed in a glass beaker, whereas tube containing S samples were wrapped with paper, and then all samples were placed in refrigerator at 4°C for 2 hrs (cooling rate-1°C/3min). Equal volumes of part-B and part-E2 were added step wise into cooled sample T and B, respectively. Diluted samples were loaded into 0.25ml straws and kept at 4°C for 30 min. Before freezing cooled diluted sample were evaluated for motility and HOST analysis. Straws were exposed over liquid nitrogen vapor for 5 min and then were plunged into liquid nitrogen for storage. For post-thawing evaluation, straws were thawed in a water bath at 37 °C for 5 min to assess post-thawed quality.

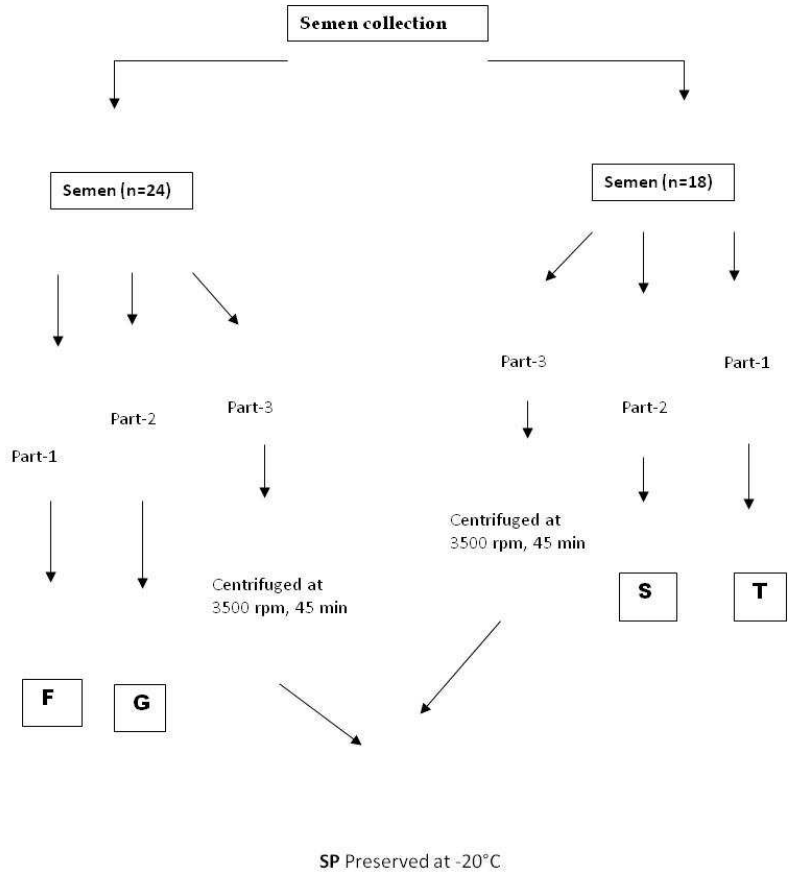


Figure 2.13. Experimental design used for this study.

2.6.4. Post-thawed sperm assessment

Motility% was evaluated subjectively with phase contrast microscope.

2.6.4.a. Sperm viability assessment

100µl of thawed sample were diluted with 100µl of saline extender (described for fresh semen) and 2µl of 6-CFDA, 2 µl of PI and 2 µl of formaldehyde were added into sample (adapted for alpaca frozen semen). Stained samples were incubated at 37°C for 15 min in dark. At least 100 sperm were counted with ultraviolet fluorescence microscope. Viable sperm% (CF+PI-

,green), damaged sperm%(CF+PI+, green acrosome and red postacrosome) and dead% (CF-PI+, red) (Figure 2.10) were recorded.

2. 6. 4. b. Hypo-osmotic swelling test (HOST)

5 µl of thawed semen was added into 30 µl of fructose-sodium citrate solution (100 mOsm) and incubated for one hour at 37°C. 100 cells were counted with phase contrast microscope at 40X. Sermatozoa with curled tailed was considered as HOST+ve cell (Figure 2.11).

2. 6. 4. c. Apoptosis marker

150 µl thawed semen sample was taken in 0.5ml eppendorf covered with aluminum paper and mixed with 50 µl 1X buffer and then stained with 1 µl 6-CFDA (1mM dissolved in water). After 5 min 1µl Annexine VCy3 was added into sample, mix properly with gentle moving and was incubated for 15 min in dark at room temperature. 1µl formaldehyde (12%, w/v) (modified from Martí et al., 2008). This staining procedure was adapted for alpaca semen, specially volume of 6-CFDA was reduced to avoid background color. Samples were controlled with ultraviolet fluorescence microscope (Diaplan, Leitz) Viable cell% (CF+AN-, without PS translocation, green), apoptotic positive cells% (CF+AN+, green acrosome and dark/red postacrosome) and dead cells% (CF-AN+, dark) (Figure-2.12) were recorded.

2.6.5. Biochemical analysis and gel electrophoresis

Biochemical analysis was performed with an automatic analyzer (Hitachi Boehering Mannheim, 912 Automatic Analyzer) using specific kits. SP enzymes such as alanine amino transfarase (ALT), alkaline phosphatase, glucose (Glu),

total protein (TP), triglycerides (TG), cholesterol (Chol), calcium (Ca), sodium (Na), potassium (K), magnesium (Mg), phosphorus (P) and chlorine (Cl) were measured.

To identify and quantify Protein Profiles (PP) in alpaca SP, one dimensional Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Detail procedure was described in 2.3. Briefly PAGE was performed with 16% polyacrylamide gels in the gel of separation according to Laemmli (1970). Sample was diluted 1:20 with sample buffer. Total 15 μg protein was used for each sample. Precision plus protein kaleidoscope standard (Bio-Rad) was used to identify different protein bands. Protein standards was composed of protein bands of different molecular weight, such as, 250 kDa, 150 kDa, 100 kDa, 75 kDa, 50 kDa, 37 kDa, 25 kDa, 20 kDa, 15 kDa e 10 kDa. Electrophoresis was conducted at 75 V for 3 hrs with Power Pac Basic Power supply (Bio-Rad). Gells were stained with Comassie Brilliant blue.

Gel images were processed with Sante Dicom Viewer Image J program. Gel bands density was measured and analysed to get total protein % (TP%) and relative protein concentration (TP).

2.6.6. Statistical analysis

One way ANOVA was performed where, treatment (S and T; F and G) were considered as independent variables, and semen quality parameters, biochemical and quantitative analysis were considered as dependent variables. Data were presented as mean±SEM. $P<0.05$ value was considered significant in this study. Besides Pearson's correlation indices are calculated for all the paramentrs considered. All data were analyzed with Statistical software SIGMASTAT 2.03.

CHAPTER 3. RESULTS

3.1. STUDY OF BIO-CHEMICAL COMPOSITION OF SP AND ITS INFLUENCES ON FREEZABILITY OF RAM SEMEN.

3.1.1. Comparison of effects of different ejaculate types on semen freezability in Sarda rams.

The results for the sperm parameters evaluated for fresh, cooled and thawed semen are shown in Figure 3.1 and 3.2. Sperm motility showed substantial variation, decreased significantly ($P<0.05$) post-thawing in all groups in respect to fresh semen. Post-thaw motility did not differ significantly (NS) between first (groups- F1) and second ejaculates (groups- S1) neither in terms of motility score nor percentage of motile sperm. Similar observation was present between pool of first and second ejaculates (group-P) and pool of ejaculates of different males (groups-P1).

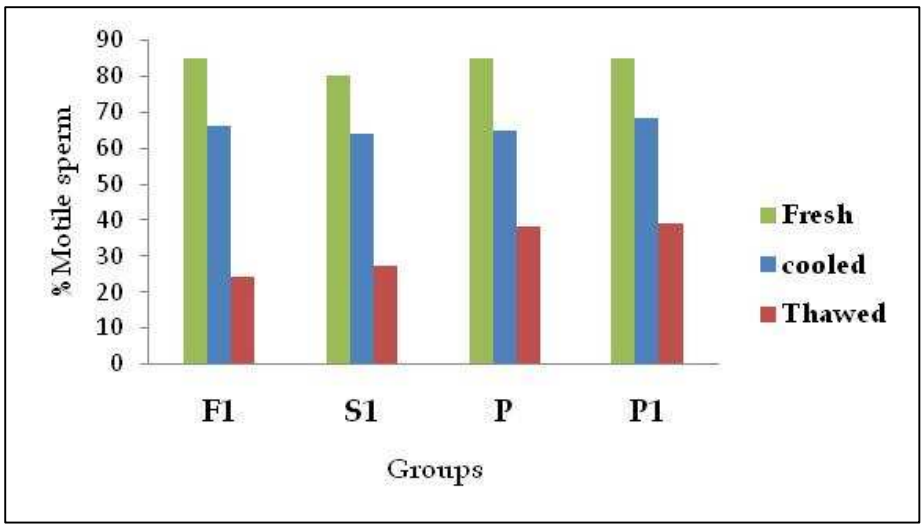


Figure 3.1. Changes in motility% in different groups after cryopreservation. F1- first ejaculate, S1- second ejaculate, P- pool of first and second ejaculate from a single ram, P1- pool of first ejaculates from different rams.

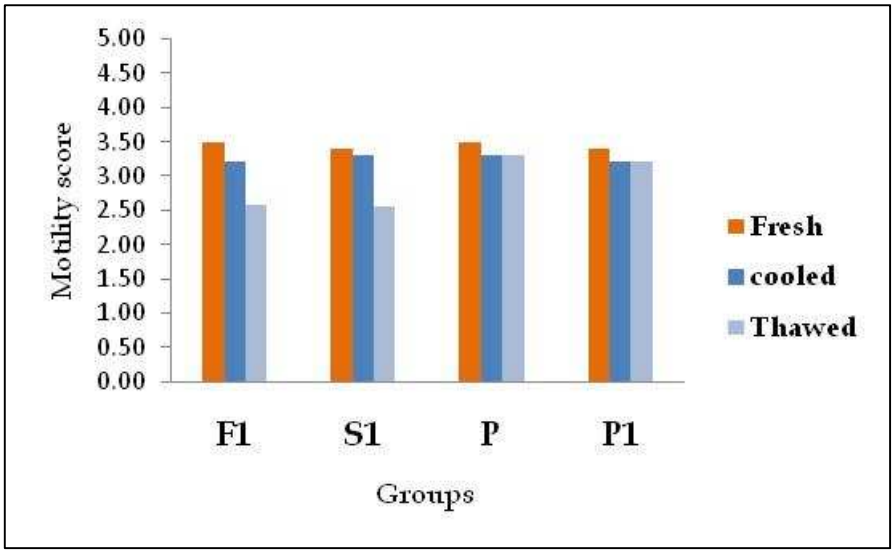


Figure 3.2. Changes in motility score for spermatozoa of different groups after cryopreservation. F1- first ejaculate, S1- second ejaculate, P- pool of first and second ejaculate from a single ram, P1- pool of first ejaculates from different rams.

In comparison to pool samples (P and P1) both motility score and the percentage of motile spermatozoa differed significantly ($P<0.05$) in both single ejaculate groups-F1 and S1. P1 displayed the highest value (40.5±3.2%) of motile spermatozoa with motility score 3.5 and spermatozoa frozen in F1 depicting the lowest value (24.2±1.6%). The result of this study reflected the fact that pool ejaculates from individual ram could maintain good motility after thawing.

3.1.2. Effects of ejaculates on the freezability of spermatozoa and the relationship with biochemical composition and Protein Profiles (PP) of seminal plasma (SP) with semen quality and freezability.

3.1.2a. Effects of ejaculates on semen freezability

Table -3.1 shows values (mean±S.E.M) of seminal parameters of ejaculates collected from different rams. The analysis of variance results reveal that ram had a significant effect on semen parameters in comparison to ejaculates within ram. Among four breeds, semen quality parameters were higher in Foza. In order to

derive conformity among different assays we observed Pearson's correlation coefficient among different quality parameters (Table 3.2.).

Semen parameters varied whit ejaculate slightly in volume and concentration whereas motility, viability and apoptosis free cells were comparatively higher in second ejaculates thought this variations was insignificant.

Table 3.1. Values (mean±S.E.M) of seminal parameters of ejaculates collected from different rams.

	Ejaculate	Alpagota	Foza	Padovana	Lamone
Volume (ml)	1°	1.11±0.1	1.57±0.1	0.90±0.1	0.80±0.1
	2°	0.92±0.1^{a}	1.44±0.1^{b}	0.97±0.1^{a}	0.71±0.1^{b}
Concentration (X10^{9}/ml)	1°	4.18±0.2°	4.14±0.2	4.56±0.2	3.81±0.2
	2°	3.25±0.2*^{a}	3.81±0.2^{ab}	4.02±0.2^{b}	3.71±0.2^{ab}
Motility%	1°	73.462±3.3^{a}	83.57±4.5^{b}	80.71±4.5^{ab}	77.14±4.5^{ab}
	2°	73.08±3.3^{a}	86.43±4.5^{b}	85.83±4.9^{ab}	81.43±4.5^{ab}
HOST+ve%	1°	64.63±3.5	73.34±4.8	69.66±4.8	64.43±4.8
	2°	64.53±3.5	73.50±4.8	76.12±5.2	71.51±4.8
CF+PI-%	1°	54.33±3.6	65.65±4.9	65.90±4.9	61.48±4.9
	2°	60.49±3.6	65.36±4.9	66.68±5.2	64.96±4.9
CF+PI+%	1°	19.41±2.5^{a}	21.11±3.5^{b}	14.49±3.5^{ab}	21.85±3.5^{ab}
	2°	17.12±2.5	20.11±3.5	12.28±3.7	17.23±3.5
CF-PI+%	1°	26.33±2.3	12.58±3.1	19.88±3.1	16.68±3.1
	2°	22.37±2.3	13.96±3.1	21.04±3.4	17.81±3.1
CF+AN-%	1°	50.27±4.7	60.38±6.3	50.67±6.3	54.40±6.3
	2°	55.49±4.7	69.50±6.3	61.83±6.8	54.36±6.3
CF+AN+%	1°	23.97±2.9^{a}	24.164±3.9^{a}	32.34±3.9^{b}	23.18±3.9^{a}
	2°	19.66±2.9	16.51±3.9	22.53±4.3	23.27±3.9
CF-AN+	1°	25.76±3.1	16.88±4.2	17.00±4.2	22.410±4.2
	2°	24.96±3.1	14.70±4.2	15.64±4.5	22.37±4.2

Different superscript letters indicate significant differences between animals, symbols indicate difference between ejaculates within males.

HOST+ve%- percent of Hypo-osmotic swelling test positive sperm, CF+PI-%- percent of viable sperm, CF+PI+%- percent of damaged sperm, CF-PI+%- percent of dead sperm, CF+AN+%- percent of viable cells without translocated PS, CF+AN+%- percent of apoptotic positive sperm, CF-AN+% percent of dead cells either by necrosis or apoptosis.

Table 3.2. Pearson's correlation of different quality parameters of fresh semen.

	HOST +ve	CF+PI-	CF+PI+	CF-PI+	CF+AN-	CF+AN+
MOT	0.492**	0.457**	0.146	-0.572**	0.508**	-0.479**
HOST +ve		0.659**	0.161	-0.803**	0.609**	-0.668**
CF+PI-			0.211	-0.875**	0.604**	-0.702**
CF+PI+				-0.723**	0.277	-0.555**
CF-PI+					-0.829**	0.545**
CF+AN-						-0.733**

According to the table 3.2, values of HOST+ve sperm and percent motile sperm moderately correlated significantly ($P<0.01$) with viable sperm%. A strong negative correlation was observed between damaged (CF-PI+%) and apoptotic (CF+AN+%) cells with both apoptotic free viable sperm (CF+AN-%) and HOST+ve sperm.

Regarding cryopreservation with two types of extender, (TRIS-based (T) and skim milk based (S)), the ram effect was very marked in different parameters. Differences in viability, apoptosis marker, motility% and HOST+ve% sperm among different rams are presented in Figures 3.3, 3.4 and 3.5 respectively. Whereas, Table 3.3 presents post-thaw semen quality parameter obtained from different assays, and Table 3.4 presents post-thaw CASA kinematics parameters in different treatment groups. Seminal parameters were higher in T-groups irrespective of ejaculate types in comparison to S- groups, but these differences was not significant among groups. Flow cytometric dot plot of fresh, viability (CFDA/PI) and Annexin V assays (CFDA/AN) of frozen-thawed semen are presented in Figure- 3.6, 3.7 and 3.8 respectively.

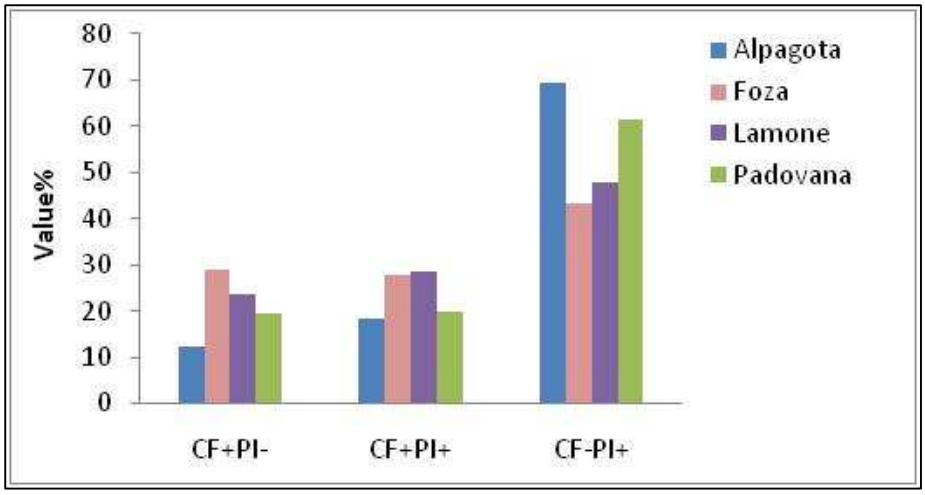


Figure 3.3 Values of viability assay (CFDA/PI) of post-thaw spermatozoa in different rams. CF+PI-%- percent of viable sperm, CF+PI+%- percent of damaged sperm, CF-PI+%-percent of dead sperm

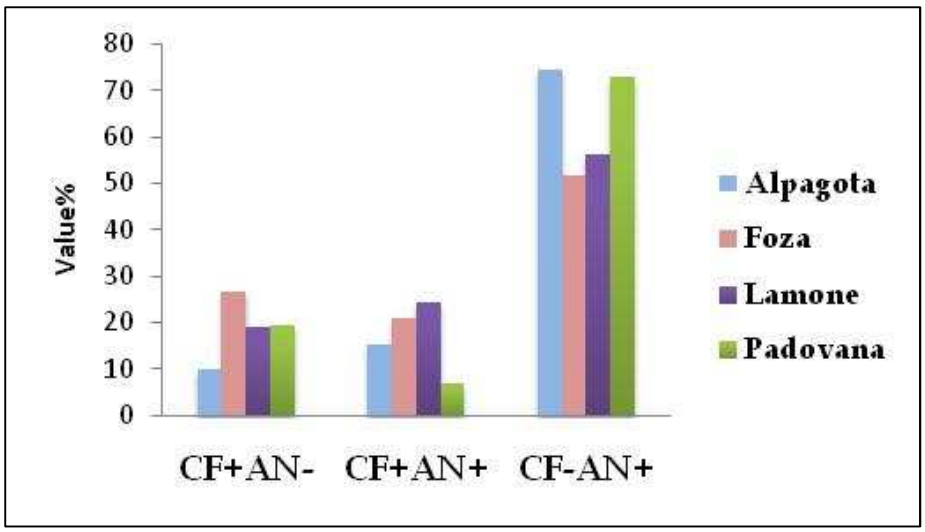


Figure 3.4. Values of Annexin V assay (CFDA/AN) of post-thaw spermatozoa in different rams CF+AN+%- percent of viable cells without translocated PS, CF+AN+%- percent of apoptotic positive sperm, CF-AN+% percent of dead cells either by necrosis or apoptosis.

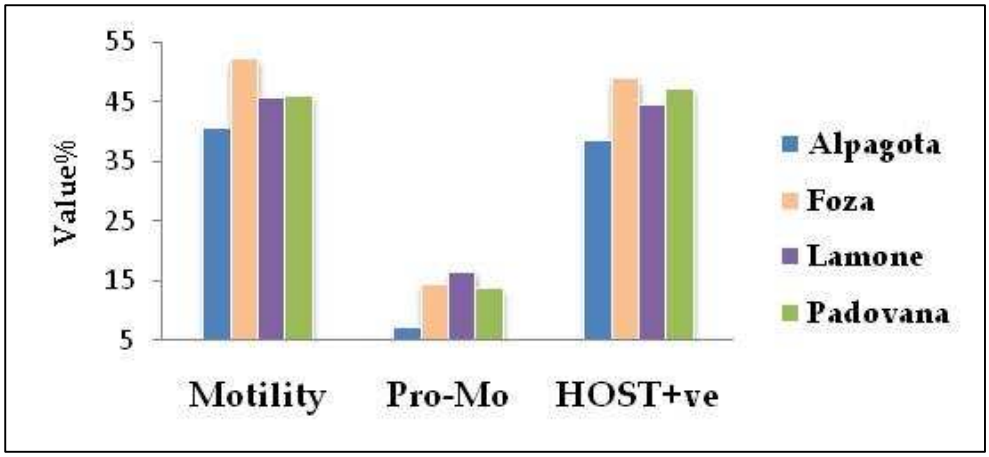


Figure 3.5. Percentage of post-thaw motile, progressively motile and HOST+ve sperm in different rams.

To understand the accuracy in evaluation of quality parameters with different assays, we examined Pearson's correlations among semen parameters presented in Table 3.3, and between CASA variables and semen quality parameters presented in Table 3.4. Relation among between evaluation parameters varied in magnitude from moderate to high, and from positive to negative. Among quality parameters the relation between plasma membrane intact sperms and percent of motile sperm were greater for post-thaw sperm than that observed in fresh semen (Table 3.3). Significant ($P<0.001$) correlation between viable sperm% (CF+PI-, CF+AN-, HOST+ve) and motility% reveals post-thaw sperm functionality depended on plasma membrane integrity. Considering CASA kinetic values, percent of motile (Motility%) sperm and progressively motile (Pro-Mo) were positively correlated with viable sperm% populations. LIN% showed positive correlation ($P<0.05$) with damaged sub populations (CF-PI+), in contrast VCL showed a positive correlation with apoptosis free viable sperm (CF+AN-%). Other kinetic values showed no significant relation with parameters of different assays.

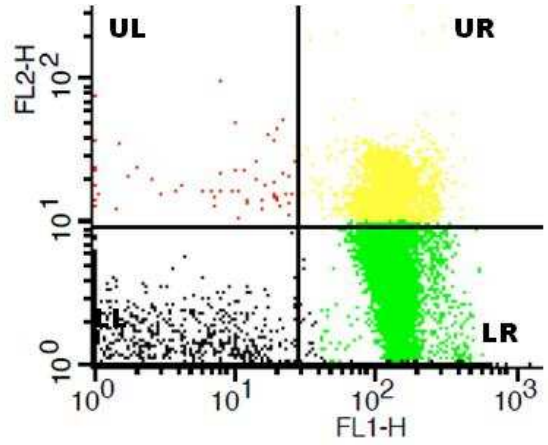


Figure- 3.6. Example of flow cytometric dot plot of fresh ram semen

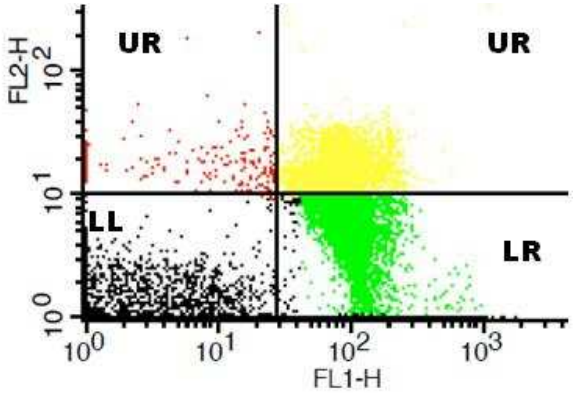


Figure 3.7. Example of flow cytometric dot plot of viability (CFDA/PI) of frozen-thawed ram semen

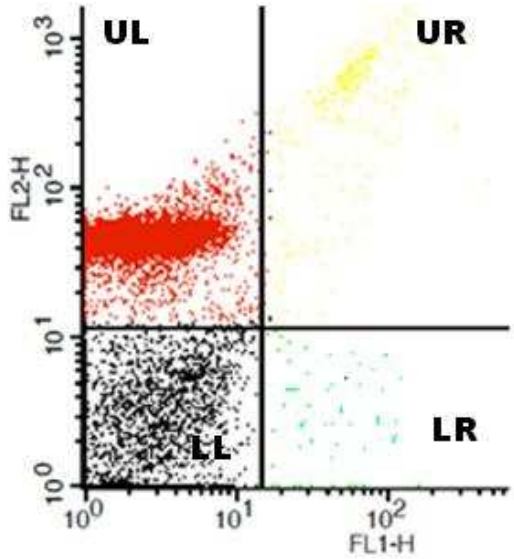


Figure 3.8. Example of flow cytometric dot plot of Annexin V assays (CFDA/AN) of frozen-thawed ram semen

Table 3.3. Post-thaw values (mean±S.E.M.) of semen parameters with different assays in different groups.

Group	HOST	CF+PI-	VLR	CF+PI+	VUR	CF-PI+	VUL	CF+AN-	ALR	CF+AN+	AUR	CF-AN+	AUL
1S	18.1±6.1	12.5±5.8	13.7±141	24.3±3.1	22.6±10.2	63.1±7.3	63.2±13.2	12.3±4.2	24.4±9.3	20.1±5.9	16.4±10.3	67.6±8.8	51.78±11.5
1T	30.6±6.1	32.6±5.8	24.3±14.1	23.4±3.1	11.4±10.2	43.9±7.3	63.0±13.2	25.6±4.2	27.3±9.1	13.5±5.9	11.9±5.9	60.9±8.8	63.6±11.5
2S	13.1±6.1	14.5±5.8	32.9±14.1	26.4±3.1	10.8±10.2	59.1±7.3	55.9±13.2	16.5±4.2	24.7±8.2	15.3±5.9	10.6±7.8	68.2±8.8	61.5±11.5
2T	26.8±6.1	25.9±5.8	29.8±14.1	18.3±3.1	19.3±10.2	55.8±7.3	50.6±13.2	20.4±4.2	34.7±8.8	21.4±5.9	19.7±8.8	58.2±8.8	51.3±11.5
3S	35±6.1	23.9±5.8	30.4±14.1	27.0±3.1	17.7±10.2	49.1±7.3	51.6±13.2	20.9±4.2	47.2±6.3	19.1±5.9	25.1±8.2	59.9±8.8	30.9±11.5
3T	36.9±6.1	25.5±5.8	13.1±14.1	28.8±3.1	13.1±10.2	45.7±7.3	73.5±13.2	24.8±4.2	48.6±17.1	12.0±5.9	2.8±1.8	63.2±8.8	50.6±11.5
4S	16.2±6.1	13.1±5.8	19.7±14.1	19.6±3.1	27.5±10.2	67.3±7.3	38.1±13.2	13.4±4.2	29.9±13.2	18.0±5.9	9.8±3.1	69.0±8.8	59.6±11.5
4T	30.6±6.1	20.3±5.8	24.4±14.1	21.7±3.1	19.3±10.2	59.9±7.3	63.2±13.2	16.6±4.2	25.7±8.4	16.7±5.9	15.5±6.2	65.±8.8	65.7±11.5

S- skim milk extender, T- TRIS-based extender, 1- first ejaculate, 2-second ejaculate, 3-pool of first and second ejaculates with seminal plasma, 4-pool of first and second ejaculates without seminal plasma; HOST+ve%- percent of Hypo-osmotic swelling test positive sperm, CF+PI-/VLR%- percent of viable sperm, CF+PI+/VUR%- percent of damaged sperm, CF-PI+/VUL%-percent of dead sperm, CF+AN-/ALR%- percent of viable cells without translocated PS, CF+AN+-AUR%- percent of apoptotic positive sperm, CF-AN+-AUL% percent of dead cells either by necrosis or apoptosis. CF indicates assessed by fluorescence microscope, A/V indicates assessed by flow cytometry.

Table 3.4. Post-thaw subjective motility and CASA kinetic values (mean±S. E. M.) of spermatozoa in different groups.

Groups	PTMo	Motility	Pro-Mo	VAP	VSL	VCL	ALH	BCF	STR	LIN
1S	18.1±6.1[a]	33.62±2.7	8.75±2.3[a]	87.78±4.6	66.15±4.2	137.41±7.6	16.40±1.1	15.39±0.9	73.50±1.6	49.25±2.1
1T	30.6±6.1[b]	40.18±2.7	17.25±2.3[b]	91.28±4.6	63.68±4.2	152.24±7.6	16.80±1.1	16.25±0.9	69.50±1.6	44.25±2.1
2S	13.1±6.1[a]	38.98±2.7	7.25±2.3[a]	91.60±4.6	68.56±4.2	141.34±7.6	17.25±1.1	14.73±0.9	73.50±1.6	49.75±2.1
2T	26.9±6.1[b]	43.64±2.8	18.63±2.3[b]	89.63±4.6	65.10±4.2	141.05±7.6	12.46±1.1	16.81±0.9	72.88±1.6	50.00±2.1
3S	35.0±6.1[b]	37.71±2.7	14.63±2.3[b]	89.13±4.6	63.49±4.2	147.10±7.6	17.08±1.1	15.85±0.9	69.63±1.6	44.75±2.1
3T	36.9±6.1[b]	44.30±2.7	15.13±2.3[b]	79.80±4.6	57.75±4.2	133.29±7.6	14.18±1.1	16.98±0.9	72.50±1.6	47.25±2.1
4S	16.3±6.1[a]	26.36±2.8	12.75±2.3	91.50±4.6	65.94±4.2	144.10±7.6	17.05±1.1	15.94±0.9	71.13±1.6	47.50±2.1
4T	30.6±6.1[b]	38.56±2.8	10.13±2.3[a]	75.91±4.6	58.30±4.2	119.09±7.6	9.91±1.1	14.41±0.9	77.88±1.6	52.00±2.1

Different superscripts indicate significant ($P<0.05$) difference among groups.

S- skim milk extender, T- TRIS-based extender, 1- first ejaculate, 2-second ejaculate, 3-pool of first and second ejaculates with seminal plasma, 4-pool of first and second ejaculates without seminal plasma; PTMo%-post-thaw motility assessed by microscope, Pro-Mo-Progressive motility %, VAP(Average path velocity) (μm/s), VCL(Curvilinear velocity) (μm/s), VSL(Straight linear velocity) (μm/s), ALH(Amplitude of lateral head displacement(μm), LIN (linearity) (%), STR (Straightness) (%).

Table 3.5. Pearson's correlation coefficients of agreement between proportions of post-thaw semen quality parameters assessed by different methods.

	VUR	**VLR**	**AUL**	**AUR**	**ALR**	**MOT**	**HOST +ve**	**CF+PI-**	**CF+PI+**	**CF-PI+**	**CF+AN-**	**CF+AN+**	**CF-AN+**
VUL	0.588	-0.128	0.483**	0.359*	-0.288	-0.533**	-0.519**	-0.507**	-0.0146	0.696**	-0.560**	-0.069	0.722**
VUR		-0.0926	0.692	0.653	-0.189	0.152	0.145	0.156	-0.205	-0.140	0.156	0.166	-0.308
VLR			0.220	0.580	-0.0188	0.081	0.553	0.325	-0.100	-0.0178	0.544	-0.0981	-0.0278
AUL				0.347**	-0.279	-0.440*	-0.420**	-0.453**	-0.024	0.622**	0.449**	-0.007	0.593**
AUR					-0.102	0.358*	0.299*	0.314*	0.309	-0.452*	0.333*	0.073	-0.486*
ALR						-0.334*	0.387*	0.352*	-0.123	-0.203	0.394*	-0.112	-0.207
MOT							0.642**	0.636**	-0.071	-0.858**	0.628**	0.493	-0.869**
HOST +ve								0.673**	-0.057	-0.917**	0.658**	0.668	-0.918**
CF+PI-									-0.062	-0.944**	0.647**	0.099	-0.921**
CF+PI+										-0.267	-0.019	0.178	-0.0839
CF-PI+											-0.893**	-0.222	0.636**
CF+AN-												-0.017	-0.926**
CF+AN+													-0.359*

** (P<0.001) *(P<0.05)HOST+ve%- percent of Hypo-osmotic swelling test positive sperm, CF+PI-/VLR%- percent of viable sperm, CF+PI+/VUR%- percent of damaged sperm, CF-PI+/VUL%-percent of dead sperm, CF+AN-/ALR%- percent of viable cells without translocated PS, CF+AN+-AUR%- percent of apoptotic positive sperm, CF-AN+-AUL% percent of dead cells either by necrosis or apoptosis. CF indicates assessed by fluorescence microscope, A/V indicates assessed by flow cytometry.

Table 3.6. Pearson's correlation coefficient between post-thaw seminal parameters and CASA kinetic parameters.

	VUL%	**VUR%**	**VLR**	**AUL**	**AUR**	**ALR**	**MOT%**	**HOST +VE**	**CF+PI-**	**CF+PI+**	**CF-PI+**	**CF+AN-**	**CF+AN+**	**CF-AN+**
Motility	-0.027	-0.127	-0.025	0.138	-0.089	-0.210	0.302*	0.422*	0.374*	0.149	-0.509*	0.424**	0.335	*-0.419
Pro-Mo	-0.044	-0.170	-0.103	0.122	-0.008	-0.171	0.335*	0.458*	0.391*	0.188	-0.555**	0.440**	0.073	*-0.476
VAP	-0.258*	0.678	0.101	0.564	-0.151	-0.379	0.440	0.184	0.190	-0.047	-0.199	0.252	0.174	-0.378
VSL	-0.294	0.149	0.114	0.162	-0.135	-0.361	-0.107	0.528*	0.676*	-0.222	0.135	0.108	0.206	-0.275
VCL	-0.183	-0.005	0.082	0.691	-0.154	-0.253	0.101	0.218	0.224	0.663*	-0.306	0.295*	0.118	-0.369
ALH	-0.222	0.140	0.653	0.091	-0.249	-0.340	-0.185	-0.163	-0.251	-0.057	0.126	-0.147	-0.002	0.084
BCF	0.587	-0.232	-0.335	0.611	-0.007	0.517	0.117	0.003	-0.080	-0.104	0.086	-0.166	-0.177	0.151
STR(%)	0.151	0.093	-0.137	-0.138	0.526	0.151	-0.215	-0.354	-0.292	-0.212	0.242	-0.399	-0.022	0.174
LIN(%)	0.194	0.113	-0.125	-0.154	0.660	0.074	-0.288	-0.363	-0.328	-0.363	0.305*	-0.406	0.110	0.170

** ($P<0.001$) *($P<0.05$). HOST+ve%- percent of Hypo-osmotic swelling test positive sperm, CF+PI-/VLR%- percent of viable sperm, CF+PI+/VUR%- percent of damaged sperm, CF-PI+/VUL%-percent of dead sperm, CF+AN-/ALR%- percent of viable cells without translocated PS, CF+AN+-AUR%- percent of apoptotic positive sperm, CF-AN+-AUL% percent of dead cells either by necrosis or apoptosis. CF indicates assessed by fluorescence microscope, A/V indicates assessed by flow cytometry. PTMo%-post-thaw motility assessed by microscope, Pro-Mo-Progressive motility %, VAP(Average path velocity) (µm/s), VCL(Curvilinear velocity) (µm/s), VSL(Straight linear velocity) (µm/s), ALH(Amplitude of lateral head displacement(µm), LIN (linearity) (%), STR (Straightness) (%).

3.1.2.b. Relation of semen freezability and biochemical and Protein Profiles (PP) of seminal plasma (SP)

SP contains different components such as macromolecules, proteins, enzymes, energy profiles. Table 3.7. shows concentration of different biochemical component of SP and Protein Profiles of SP in different males are summarized in Table 3.8.

Table 3.7. Concentration of different biochemical component of SP

	Ejaculate	**Alpagota**	**Foza**	**Lamone**	**Padovana**
Ca (mg/dl)	1°	13.44±1.5^{a}	13.38±1.7^{ab}	15.75±2.0^{ab}	23.13±2.2$^{b°}$
	2°	15.91±1.3	12.16±1.7	15.42±2.5	15.21±1.8*
Cl (mEq)	1°	4.69±2.7^{a}	10.50±2.9^{b}	2.50±3.5^{a}	9.38±3.9^{b}
	2°	7.96±2.3	6.21±2.9	8.33±4.5	6.25±3.2
Mg	1°	6.72±1.10	7.41±1.18	6.18±1.4	8.06±1.7
(mg/dl)	2°	5.98±0.9	6.33±1.2	6.21±1.8	6.04±1.3
P (mg/dl)	1°	22.97±3.6	21.96±3.8	19.25±4.5	20.00±5.0
	2°	21.48±3.0	16.13±3.8	17.08±5.8	18.33±4.1
K (mEq	1°	35.47±6.1	47.33±6.5	41.08±7.7	38.38±8.6
	2°	44.53±5.2	40.21±6.5	42.67±10.0	42.60±7.0
Na (mEq)	1°	164.38±10.5^{a*}	171.36±11.2^{a}	165.00±13.3^{a*}	200.00±14.8^{b}
	2°	185.23±8.9$^{a°}$	159.19±11.2^{b}	191.67±17.1$^{a°}$	183.33±12.1^{a}
CHOL	1°	60.50±6.4	63.71±6.8	50.00±13.0	76.00±12.7
(mg/dl)	2°	59.14±6.8^{a}	40.86±6.8^{a}	76.00±13.0^{b}	80.40±8.0^{b}
GLU	1°	3.0±0.4	2.57±0.4	3.00±0.8	3.00±0.8
(mg/dl)	2°	2.57±0.4	2.29±0.4	2.00±0.8	2.80±0.5
TP	1°	3.50±0.5	4.04±0.6	2.50±0.7	4.06±0.8
(g/dl)	2°	4.21±0.5	3.85±0.6	2.91±0.9	2.92±0.6
TG	1°	113.75±22.0^{a}	133.43±23.6^{a}	136.00±44.1^{a}	168.00±44.1^{b}
(mg)	2°	80.57±23.6^{a}	102±23.6^{ab}	65.00±44.1^{a}	166.00±27.7^{b}
GOT	1°	309.50±163.3	-	1057.00±326.5	7.00±326.5
(UI7l)	2°	3.43±±174.5	-	4.00±326.5	2.80±206.5
ALP	1°	9945.00±2527.4	13084.29±2701.9	21026.00±5054.7	8889.00±5054.7
(UI/l)	2°	7056.29±2701.9	6068.57±2701.9	10765.00±5054.7	10424.80±3196.9
LDH	1°	1240.75±563.4^{a*}	1797.71±602.3^{a}	2410.00±1126.8^{b}	993.00±1126.8$^{a°}$
(UI/l)	2°	2351.71±602.3°	2902.29±602.3	2974.00±1126.8	467.60±712.7*

Different superscript letters indicate significant differences between animals, symbols indicate difference between ejaculates within males (P<0.05).

Table 3.8. Protein Profiles of SP (relative protein concentration-mg/dl).

TP	Ejaculate	Alpagota	Foza	Padovana	Lamone
TP1	1°	2.04±2.3*	3.26±2.6	3.20±4.0	2.96±3.1*
(100 kDa)	2°	1.38±2.1[a]*	3.25±2.6[a]	4.00±3.5[a]	20.58±4.0[b]*
TPT2	1°	4.74±0.3	5.27±0.3	4.99±0.4	4.30±0.3
(75 kda)	2°	4.71±0.2	5.39±0.3	4.66±0.4	5.11±0.4
TP3	1°	4.09±0.4	5.07±0.5	5.57±0.7	4.42±0.6
(72 kDa)	2°	4.55±0.4	5.49±0.5	3.6±0.6	4.91±0.7
TP4	1°	4.04±0.5	4.93±0.6	5.94±0.9	4.63±0.7
(68 kDa)	2°	5.39±0.5	5.29±0.6	5.27±0.8	3.09±0.9
TP5	1°	7.12±0.5[a]	4.76±0.6[b]	6.98±0.9[ab]	4.16±0.7[b]
(50 kDa)	2°	7.46±0.5[a]	3.61±0.6[b]	6.30±0.8[ab]	6.35±0.9[ab]
TP6	1°	8.88±2.1[a]*	9.25±2.4[a]*	1.65±3.6[b]*	3.38±2.8[b]
(45 kDa)	2°	0.44±1.9°	3.35±2.4°	6.66±3.1°	5.55±3.6
TP7	1°	3.16±0.6	2.67±0.7	2.50±1.1*	2.79±1.0
(37 kDa)	2°	2.11±0.6	3.82±0.7	4.54±0.1°	2.68±1.1
TP8	1°	2.79±0.6[ab]	4.20±0.6[a]	7.77±1.0[b*]	3.60±0.8[ab]
(30 kDa)	2°	2.84±0.5	2.40±0.6	3.21±0.8°	2.62±1.0
TP9	1°	4.33±0.3	4.48±0.4	6.11±0.6	4.98±0.5
(25 kDa)	2°	4.70±0.3	4.90±0.4	4.70±0.5	5.98±0.6
TP10	1°	8.18±0.5[a]	5.80±0.5[b]	8.04±0-8[ab]	6.62±0.6[ab]
(20 kDa)	2°	7.74±0.4	6.58±0.5	6.65±0.7	6.88±0.8
TP11	1°	9.49±0.6	7.42±0.7	8.57±1.1	7.83±0.8
(15 kDa)	2°	8.70±0.6[a]	5.81±0.7[b]	7.09±01.1[ab]	8.47±1.1[ab]

We observed marked male difference in the concentrations of SP components rather than ejaculate differences. Among macro elements, Ca and Na showed significant ($P<0.05$) difference within ejaculates and between males. Significant variation also observed in Cholesterol (CHOL) and triglycerides (TG) concentrations. Regarding protein or enzymes, only LDH was variable in different males. On the other hand, same male different trend was observed in relative protein concentrations (PP). We found 11 proteins bands on gel. Almost all bands were present in SP of all males (Figure 3.9 a, b, c, d), except TP6 (45 kDa) was absent in both first and second ejaculate collected from Alpagota. Among different PP, TP1 of 100kDa, TP5 (50 kDa), TP8 (37kDa), TP9 (25kDA) and TP11(15kDA) were varied significantly ($P<0.05$) in different males.

Moreover, among different protein bands TP5 (50 kDa), TP6 (45kDa), TP10 (20kDa) and TP11(15kDa) concentration were higher.

It is note worthy to point out that our research objective was to study relationship between semen quality and biochemical components of SP. Therefore, we examined correlation coefficients among SP components and semen quality in fresh semen and results are shown in Table 3.9. and Table 3.10.

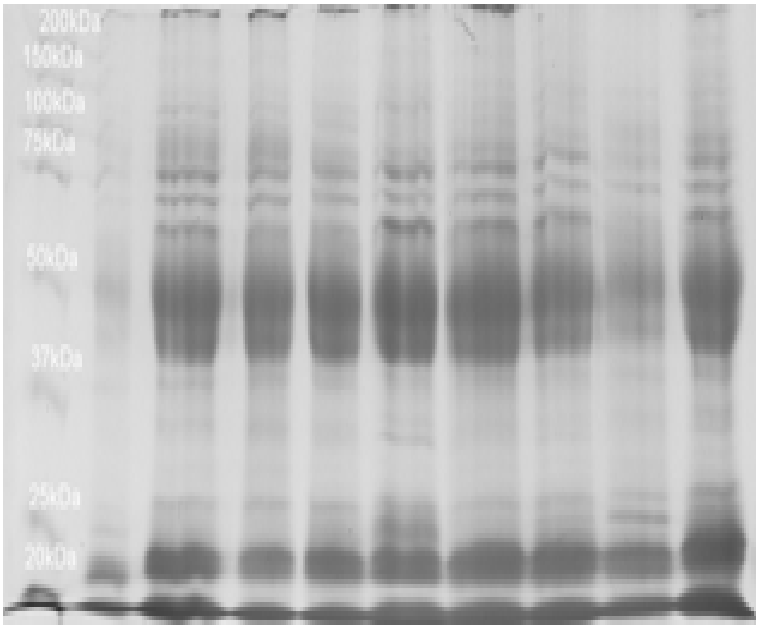


Figure 3.9a Quantitative protein composition of SP of Alpagota ram.

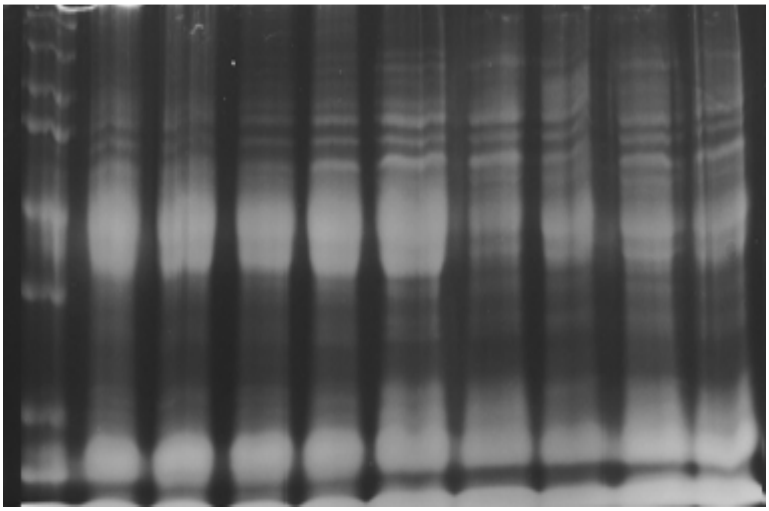


Figure 3.9b Quantitative protein composition of SP of Foza ram.

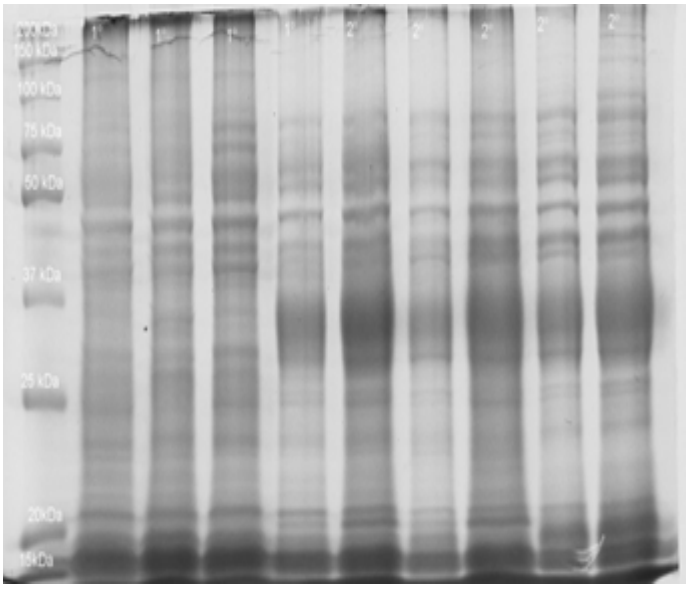


Figure 3.9c Quantitative protein composition of SP of Padovana ram.

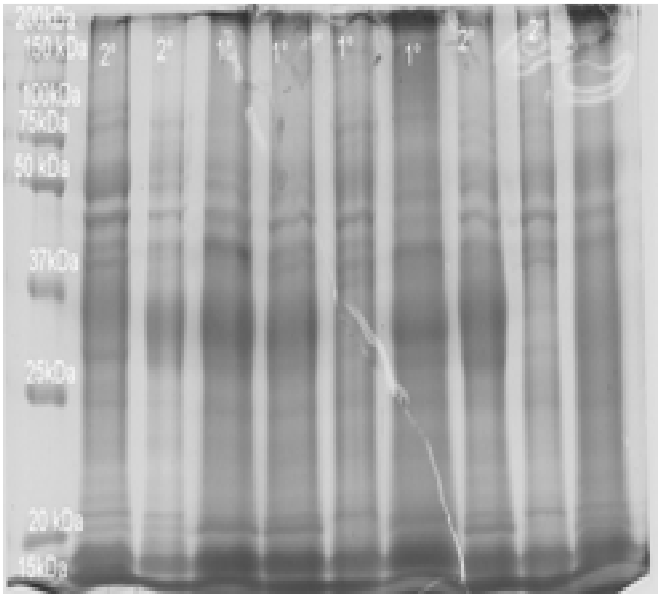


Figure 3.9d Quantitative protein composition of SP of Lamon ram.

Table 3.9. Pearson's correlation coefficient of SP elements and fresh semen parameters.

	GLU	**CHOL**	**TG**	**ALP**	**LDH**	**GOT**	**Cl**	**K**	**P**	**Mg**	**Ca**	**Na**	**TP**
VUL	-0,358	-0,363	-0,136	0,536*	0,264	-0,423	-0,0486	-0,117	-0,624*	-0,559*	-0,14	0,192	0,27
VUR	0,136	0,215	0,093	0,074	-0,279	0,543	0,090	-0,019	0,559*	0,487*	0,186	-0,176	-0,124
VLR	0,296	0,099	0,297	-0,190	0,172	-0,095	0,381	-0,212	-0,547*	-0,512*	0,266	0,143	0,384*
AUL	-0,333	-0,466	-0,073	-0,375	0,139	0,135	0,0207	-0,478*	-0,517*	-0,486*	-0,132	-0,101	0,013
AUR	0,004	0,234	0,119	-0,158	0,236	0,244	0,0513	-0,363	-0,252	-0,289	-0,195	-0,297	-0,4
ALR	0,157	-0,320	-0,050	0,265	-0,149	-0,383	-0,004	0,513*	0,442*	0,448*	0,158	0,241	0,352*
MOT	-0,223	-0,171	0,107	-0,267	0,007	-0,242	-0,169	-0,123	-0,189	-0,178	-0,145	-0,11	-0,292*
HOST +ve	-0,191	-0,285	-0,074	-0,079	0,042	-0,140	-0,083	-0,163	-0,245	-0,266*	-0,121	-0,073	-0,281*
CF+PI-	-0,073	-0,162	0,045	-0,032	0,107	-0,015	-0,247	-0,122	-0,273*	-0,207	-0,102	0,007	0,289*
CF+PI+	0,068	-0,009	-0,101	0,021	0,062	0,020	0,242	0,134	0,129	0,158	0,058	0,045	0,24
CF-PI+	0,054	0,239	0,033	0,014	-0,206	0,007	0,114	0,042	0,251	0,132	0,11	-0,013	0,173
CF+AN-	-0,220	-0,304*	-0,199	0,054	0,115	0,109	-0,066	0,140	0,237	0,275	-0,174	-0,139	0,285*
CF+AN+	0,377*	0,390*	0,478**	-0,132	-0,208	-0,181	0,097	0,0033	0,052	0,134	0,131	0,020	0,143
CF-AN+	-0,045	0,073	-0,185	0,008	0,045	0,002	0,0325	0,21	-0,315*	-0,293*	0,113	0,158	-0,266*

GLU-Glucose, CHOL-Cholesterol, TG-Triglyceride, ALP-Alkaline phosphatase, LDH- Lactose dehydrogenase, GOT- Glutamic oxalotransaminase, Cl- Chlorine, K- Potassium, P-Phosphorus, Mg-Magnesium, Ca-Calcium, Na-Sodium, TP-Total protein.

Table 3.10. Pearson's correlation coefficient of PP and semen parameters

	AUL	AUR	ALR	MOT%	HOST +VE	CF+PI-	CF+PI+	CF-PI+	CF+AN-	CF+AN+	CF-AN+
TP1(100 kDa)	0,197	-0,164	0,030	-0,111	0,219	0,049	-0,044	-0,020	0,169	-0,161	-0,126
TP2(75 kDa)	-0,261	-0,287	0,314	0,086	0,140	-0,006	0,011	-0,044	0,296*	-0,276*	-0,137
TP3(72kDa)	-0,176	-0,306	0,412	0,145	0,116	0,067	-0,026	-0,092	0,174	-0,126	-0,107
TP4 (68kDa)	-0,168	-0,240	0,355	0,076	-0,125	-0,158	0,035	0,152	-0,094	0,027	0,179
TP5(50 kDa)	0,376	0,193	-0,385	-0,056	0,087	-0,112	-0,028	0,210	-0,053	0,123	-0,083
TP6(45 kDa)	-0,403	-0,296	0,437	-0,036	-0,019	0,070	0,079	-0,472*	0,002	-0,094	0,082
TP737 kDa)	0,390	0,216	-0,276	0,153	0,070	0,093	0,034	-0,154	-0,072	0,137	-0,003
TP8(30 kDa)	-0,093	-0,100	0,031	-0,074	-0,112	0,107	0,044	-0,177	-0,160	0,151	0,113
TP9(25 kDa)	-0,482*	-0,167	0,347	-0,059	-0,083	-0,112	0,041	0,085	0,059	-0,188	0,135
TP10(20 kDa)	-0,197	-0,104	0,089	-0,006	0,044	-0,347**	-0,022	0,245	0,189	-0,271	-0,008
TP11 (15kDa)	0,206	0,186	-0,335	-0,068	0,016	-0,070	0,008	0,132	-0,303	-0,030	0,348

Low to moderate but significant negative correlation was observed between total protein, P, Mg, K with apoptotic free sperm subpopulation. On the other hand, most of the elements Na, K, Ca, P, Mg also total protein were insignificantly correlated with sperm motility. Among enzymes activities, GOT and ALP showed a significant positive relation with damaged sperms. Considering relation the energy profiles with semen quality, glucose, triglycerides and cholesterol showed a positive and significant correlation with apoptotic spermatozoa. Besides macromolecules, some protein bands such as TP2 (75kDa), TP6 (45 kDa) and TP9 (25 kDa) were negatively correlated with damaged and dead spermatozoa indicating their action in maintaining sperm viability. More important, TP2 was also correlated with apoptosis free viable sperm values.

Table 3.11. Values of post-thaw epididymal sperm quality parameters in two treatment groups.

Parameters	T	S
MOT	11.00±2.8	11.70±2.9
HOST+ve	26.95±4.4	22.23±3.8
CF+PI-	23.22±3.8	20.91±3.1
CF+PI+	13.44±3.0	20.54±3.2
CF-PI+	63.34±4.2	58.55±3.4
CF+AN-	19.85±3.3	19.30±3.2
CF+AN+	15.59±1.6	13.11±2.6
CF-AN+	64.55±3.7	67.59±4.3
Pro-Mo	10.60±1.6	9.70±2.0
VAP	80.04±1.8	87.95±3.7
VCL	126.98±2.4	135.20±6.0
VSL	59.11±1.5	66.29±2.9
ALH	16.03±0.6	17.12±0.5
LIN	48.30±1.2	50.50±1.6
STR	73.60±0.9	74.10±1.4

We found 65.47±4.2 % viable sperm and 52.50±3.9 motility percentage for the fresh epididymal sperm. There was not variations in different post-thawed epididymal parameters. In comparison to post-thawed semen motility was lower than viable cells. Regarding CASA kinematics parameters all CASA values were slightly higher in S groups, although these variations were not significant.

Table 3.12. shows the correlation between quality parameters and Table 3.13. shows relation between CASA kinematic and other paramenters considered. Correlation coefficients among different parameters revealed a strong index between different values, which was statistically highly significant (P<0.01).

Table 3.12. Pearson's correlation coefficient among different sperm quality parameters.

	HOST+ve	CF+PI-	CF+PI+	CF-PI+	CF+AN-	CF+AN+	CF-AN+
MOT	0.067	0.141	0.018	-0.141	0.198	0.036	-0.179
HOST+ve		0.890**	-0.052	-0.750**	0.929**	0.209	-0.862**
CF+PI-			-0.343	-0.601**	0.892**	0.109	-0.778**
CF+PI+				-0.545**	-0.079	0.275	-0.086
CF-PI+					-0.730**	-0.331	0.768**
CF+AN-						0.065	-0.842**
CF+AN+							-0.593**

Table 3.13. Pearson's correlation coefficient between CASA kinetics parameter and other epididymal sperm quality parameters.

	MOT	**Pro-Mo**	**VAP**	**VSL**	**VCL**	**ALH**	**BCF**	**STR**	**LIN**
MOT	0.411	0.472*	0.347	0.232	0.446*	-0.306	0.179	-0.395	-0.354
HOST+ve	0.463*	0.484*	0.219	0.058	0.324	-0.262	0.033	-0.460*	-0.389
PTMo	0.131	0.217	0.286	0.225	0.327	-0.142	0.087	-0.224	-0.191
PTHo	0.105	0.107	0.234	0.131	0.380	-0.268	-0.007	-0.343	-0.334
CF+PI-	-0.081	-0.045	0.221	0.202	0.265	-0.162	-0.055	-0.174	-0.160
CF+PI+	0.223	0.202	0.139	0.082	0.162	-0.171	-0.191	-0.059	0.002
CF-PI+	-0.117	-0.132	-0.316	-0.250	-0.374	0.290	0.212	0.206	0.142
CF+AN-	0.066	0.046	0.328	0.246	0.440*	-0.340	-0.150	-0.364	-0.302
CF+AN+	0.395	0.442*	0.261	0.115	0.353	-0.059	0.146	-0.316	-0.340
CF-AN+	-0.267	-0.276	-0.406	-0.261	-0.546	0.306	0.042	0.465*	0.427
MOT		0.977**	0.019	-0.329	0.391	-0.043	0.654	-0.887**	-0.874**
Pro-Mo			-0.002	-0.336	0.357	-0.028	0.720**	-0.836**	-0.851**
VAP				0.926**	0.895**	0.256	-0.426	-0.195	0.075
VSL					0.679**	0.201	-0.648**	0.163	0.409
VCL						0.193	-0.074	-0.578*	-0.357
ALH							0.137	0.036	0.166
BCF								-0.552**	-0.717**
STR									0.939**

3.2. Study of biochemical composition of seminal plasma and their influences on quality and freezability of alpaca semen.

3.2.1. Diet effect on semen quality, biochemical composition and Protein Profiles (PP) of seminal plasma in alpaca

There was a high variability in different parameters between alpaca males subjected to the same diets throughout the study period. Changes in semen quality parameters are shown in Figure 3.10., 3.11., 3.12, 3.13 and collection time, BCS and testicular volume recorded in different periods are shown in Figure 3.14., 3.15, 3.16a,b. Within four periods, no significant changes in semen collection time and viscosity parameter were observed in all males. Semen volume slightly increased in last two periods, where significantly increased ($P<0.05$) in Alpaca-1 during 4° period. A significant decline ($P<0.05$) in both motility and concentration was noted in all alpacas when they were supplied with sheep concentrates during 3°period and then increased significantly in 4° period. Among five males, ejaculates collected from alpaca-5 were free of spermatozoa all time. On the other hand, very low motility% was observed in semen of alpaca-3 during four periods. Both motility% and concentration were higher in ejaculates of alpaca-2 collected throughout the study period.

Neither right nor left testicular volume was affected with diets. In addition, BCS increased significantly ($P<0.05$) from 1° to 4° period (Figure 3.15.). Among five alpacas, alpaca-4 had larger testicles and alpaca-1 had a good (BCS 3.25).

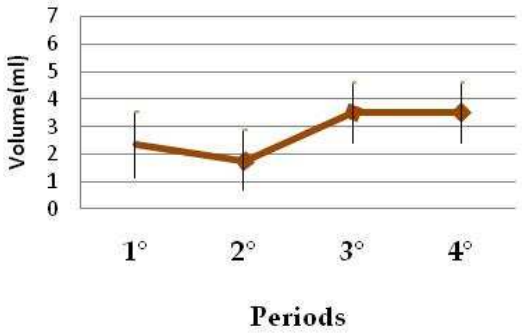


Figure 3.10. Changes in semen volume of alpacas during four periods.

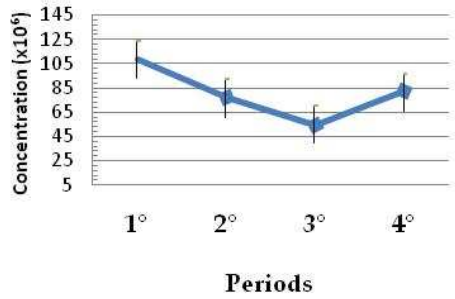


Figure 3.11. Changes in sperm concentrations during four periods

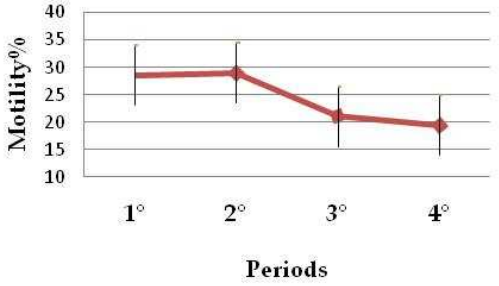


Figure 3.12. Changes (significant P<0.05) in motility parameters in different periods.

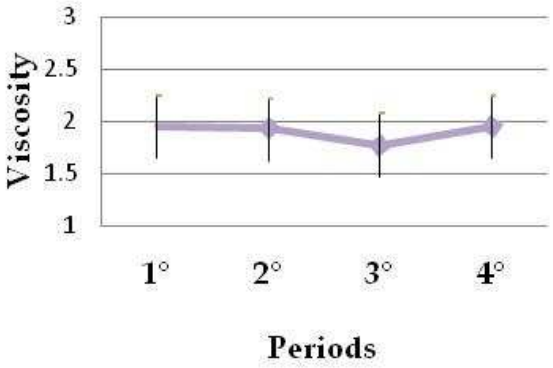


Figure 3.13. Changes in semen viscosity (NS) during four periods

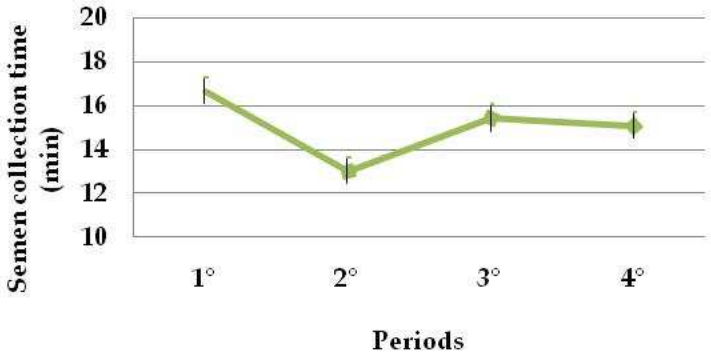


Figure 3.14. Changes (non significant) in semen collection times during four periods.

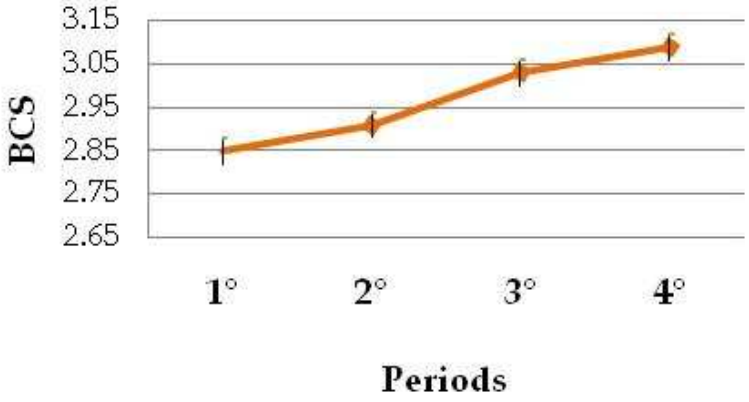


Figure 3.15. Changes in BCS (Body Condition Score) during four periods

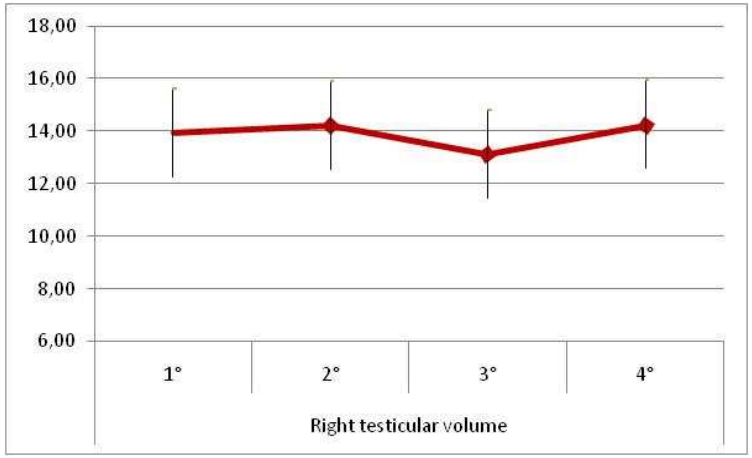


Figure 3.16a. Changes in Right Testicular volume (cm^3) during four periods.

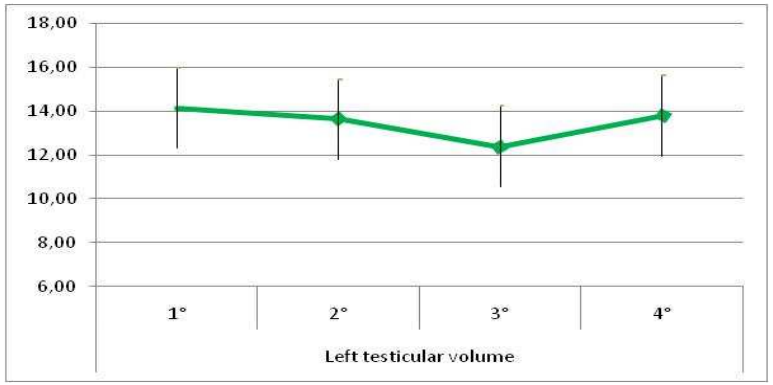


Figure 3.16b. Changes in Left Testicular volume (cm^3) during four periods.

Changes in alpaca seminal energy parameters, total protein (TP),and glucose are shown in Table 3.14. Regarding the energy profiles, triglycerides (TG) and cholesterol (CHOL) concentrations reduced significantly from 1° to 4°periods in four animals, in contrast increased significantly ($P<0.05$) in SP of alpaca-5. On the other hand, glucose (GLU) and total protein (TP) concentrations were not influenced by diets significantly. Among five males, energy profiles were higher in SP of alpaca-2 through the study period.

There was a significant effect ($P<0.05$) of diets on seminal enzymes parameters. Enzymes concentrations in SP of Alpaca males are presented in Table 3.15. GGT concentration was higher in SP of all alpacas collected during 2° period and then declined significantly ($P<0.05$). Also, ALT and ALP concentration decreased from 1° period to 4°period, except in alpaca-3 where these enzymes levels increased in SP collected during 3° and 4° period.

Moreover, there was a wide variation in values in terms of enzymes between alpacas irrespective of periods. ALP activities was higher in alpaca-1 and lower in alpaca- 4.

Table 3.14. Energy profiles and total protein (TP) concentrations (mean±S.E.M.) in Alpaca SP in four periods.

	Period	Alpaca				
		1	**2**	**3**	**4**	**5**
GLU	1°	1.67 ± 3.86	3.50 ± 4.73	1.33 ± 3.86	3.00 ± 4.73	2.00 ± 3.86
	2°	1.67 ± 3.86	3.50 ± 4.73	1.75 ± 3.35	2.00 ± 3.35	2.50 ± 4.73
	3°	1.25 ± 3.35	3.50 ± 4.73	1.75 ± 3.35	1.33 ± 3.86	2.50 ± 4.73
	4°	1.00 ± 3.86	1.50 ± 4.73	1.25 ± 3.35	1.00 ± 3.86	2.50 ± 4.73
TG	1°	68.67 ± 22.76*°	156.00 ± 27.87°	16.67 ± 22.76*	46.50 ± 27.88*°	20.00 ± 22.76*
	2°	37.67 ± 22.76*	149.50 ± 27.87°	48.50 ± 19.71*	97.00 ± 19.71*°	61.00 ± 27.87*°
	3°	5.00 ± 19.71*	122.00 ± 27.87°	10.25 ± 19.71*	45.33 ± 22.76*°	3.00 ± 22.76*
	4°	6.33 ± 22.76	76.50 ± 27.87	13.25 ± 19.71	22.67 ± 22.76	57.50 ± 19.71
CHOL	1°	1.33 ± 7.27	6.00 ± 8.91	0.67 ± 7.27	4.00 ± 8.91	1.67 ± 7.27
	2°	1.50 ± 8.91	4.50 ± 8.91	2.33 ± 7.27	3.00 ± 6.30	1.50 ± 8.91
	3°	-	3.00 ± 8.91	2.50 ± 8.91	1.50 ± 8.91	1.33 ± 7.27
	4°	0.50 ± 8.91	1.50 ± 8.91	0.50 ± 8.91	-	36.25 ± 6.30
TP	1°	5.67 ± 3.34*	25.00 ± 4.09°	4.33 ± 3.34*	3.00 ± 4.09 *	2.67 ± 3.34*
	2°	3.00 ± 3.34*	23.50 ± 4.09°	8.50 ± 2.89*	7.25 ± 2.89*	2.00 ± 4.09*
	3°	2.25 ± 2.89	16.00 ± 4.09	5.50 ± 2.89	3.33 ± 3.34	2.33 ± 3.34
	4°	2.33 ± 3.34	17.00 ± 4.09	5.50 ± 2.89	2.33 ± 3.34	14.50 ± 2.89

Table 3.15. Enzymes values (mean±S.E.M.) in Alpaca SP collected during study periods.

Enzymes	Periods	Alpaca				
		1	**2**	**3**	**4**	**5**
GGT	1°	476.33 ± 136.7	614.50 ± 167.54	93.00 ± 136.79	168.50 ± 167.54	169.00 ± 136.79
	2°	308.33 ± 136.79	705.50 ± 167.54	370.33 ± 136.79	573.00 ± 118.47	187.00 ± 167.54
	3°	94.25 ± 118.47	594.50 ± 167.54	234.25 ± 118.47	398.00 ± 136.79	167.67 ± 136.79
	4°	119.00 ± 136.79	468.50 ± 167.54	271.50 ± 118.47	199.00 ± 136.79	152.50 ± 118.47
ALP	1°	1842.33 ± 284.28* a	1487.00 ± 348.18*^	199.33 ± 284.28°	234.50 ± 348.18°^	441.67 ± 284.28°^
	2°	1145.67 ± 284.28 ab	1546.00 ± 348.18	392.00 ± 284.28	998.50 ± 246.20	301.50 ± 348.18
	3°	286.25 ± 246.20 b	810.50 ± 348.18	456.00 ± 246.20	212.00 ± 284.28	311.33 ± 284.28
	4°	401.00 ± 284.28 b	690.50 ± 348.18	463.25 ± 246.20	96.00 ± 284.28	226.50 ± 246.20
ALT	1°	13.33 ± 9.34*	75.50 ± 11.44° a	6.00 ± 9.34*	5.50 ± 11.44*	8.33 ± 9.34*
	2°	7.33 ± 9.34*	68.50 ± 11.44 °a	14.50 ± 8.09*	23.00 ± 9.34*	15.00 ± 11.44*
	3°	2.25 ± 8.09	9.50 ± 11.44 b	15.50 ± 8.09	18.50 ± 11.44	12.00 ± 9.34
	4°	3.33 ± 9.34	22.50 ± 11.44 b	13.75 ± 8.09	2.67 ± 9.34	5.75 ± 8.09

Different symbols indicate significant variation between males and letters indicate variation between periods (P<0.05).

We did one dimensional polyacrylamide gel electrophoresis in 16% gel of separation to study alpaca seminal Protein Profile. Assessment of the electrophoretic profile of SP proteins showed a total of 8 bands between molecular weights from 200-14.00 kDa. Surprisingly, all bands were not present in all alpacas (Figure 3.17a,b,c,d.). Protein Profiles of SP in alpaca-2 differed from other four males and showed protein fractions of 10-14kDa, whereas, protein fractions of around TP7(21 kDa) was absent in this animal during the study periods. Moreover, there were insignificant changes in the relative quantity of all Protein Profiles during four periods, though marked variation was observed in individual animals. Protein fractions of (TP3 ~60 kDa) were abundant. Changes in values of PP are shown in Table 3.16**.**

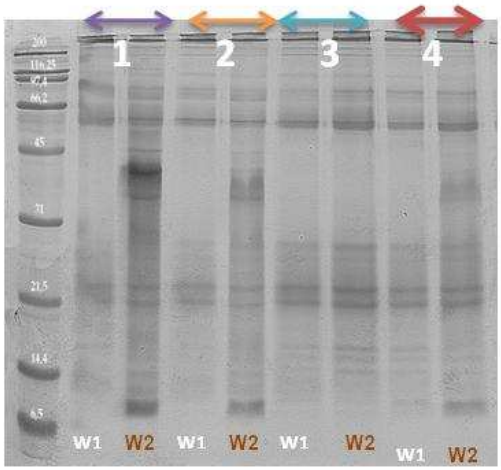


Figure 3.17a. Effect of diet on the quantitative protein composition of SP of Alpaca-1 obtained by 1-D SDS-PAGE gel electrophoresis.

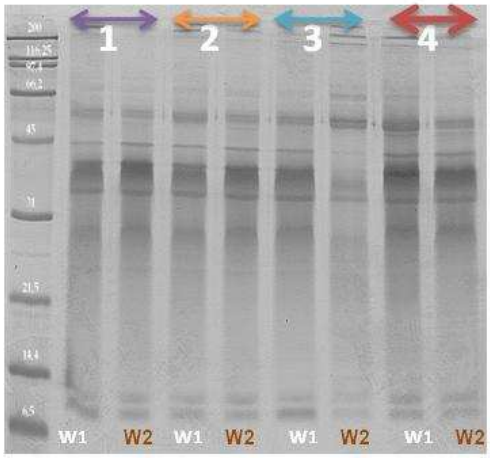


Figure 3.17b Effect of diet on the quantitative protein composition of SP of Alpaca-2 obtained by 1-D SDS-PAGE gel electrophoresis.

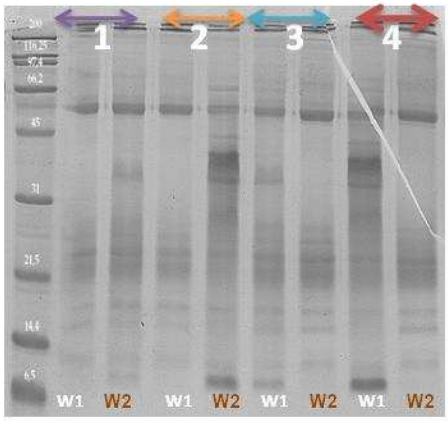


Figure 3.17c. Effect of diet on the quantitative protein composition of SP of Alpaca-3

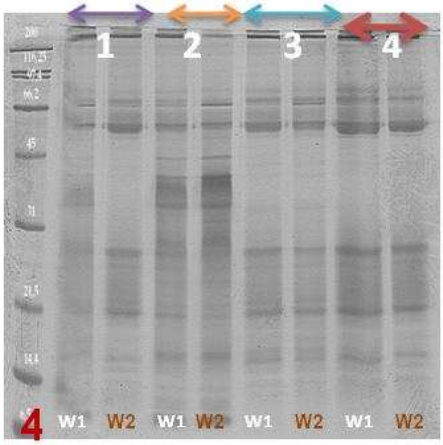


Figure 3.17d. Effect of diet on the quantitative protein composition of SP of Alpaca-4 obtained by 1-D SDS-PAGE gel electrophoresis.

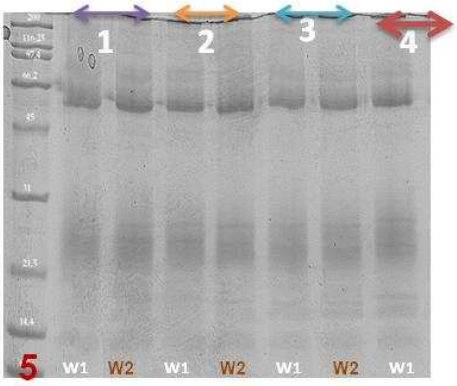


Figure 3.17e. Effect of diet on the quantitative protein composition of SP of Alpaca-5 obtained by 1-D SDS-PAGE gel electrophoresis.

Table 3.16. Effect of diet on SP Protein Profiles observed in alpaca.

TP	Period-1	Period-2	Period-3	Period-4
TP1 (200 kDa)	0.96±0.1	1.01±0.1	1.07±0.1	0.97±0.1
TP2 (97 kDa)	1.02±0.1	0.95±0.1	0.97±0.1	0.90±0.1
TP3 (60 kDa)	1.46±0.1	1.50±0.1	1.53±0.1	1.47±0.1
TP4 (45 kDa)	0.29±0.03	0.33±0.03	0.30±0.03	0.32±0.03
TP5 (45 kDa)	0.57±0.1	0.48±0.1	0.23±0.1	0.37±0.1
TP6 (31 kDa)	-	-	-	-
TP7 (21kDa)	-	-	-	-
TP8 (14 kDa9	0.20±0.1	0.55±0.1	0.71±0.1	0.65±0.1

Parameters for fresh semen collected from three alpacas used in this experiment is presented in Table 3.17. and correlation coefficients among these parameters are presented in Table 3.18. No significant correlation was observed between semen collection time and semen quality parameters. Whereas, semen volume showed a significant ($P<0.05$) negative correlation with semen motility and viscosity ($P<0.001$). More importantly a positive very significant ($P<0.001$) correlation observed between motility, cell viability, HOST+ve% and present of apoptosis free subpopulation. Higher negative correlation was observed between viscosity and sperm motility.

We studied the relationship between basic sperm parameters and SP components. The summary of the parameters compared are presented in Table 3.23. Macromolecules Ca, K,P, Mg, Cl had positive correlation with sperm quality, among which Ca, P, and Mg concentrations showed highly significant ($P<0.001$) correlation with sperm motility%, viability and apoptosis marker free sperm population, In contrary, Na concentration correlated negatively with spermatozoa quality parameters, though correlation was statistically insignificant. All most all ion concentrations were correlated positively with

semen viscosity, but correlation was highly significant (P<0.001) for Mg and Ca. Besides, significant (P<0.001) correlation was observed between semen volume and Na concentration in SP. Furthermore, negative correlation between Ca, P, Mg, K and non-viable or apoptotic cells observed this in this study indicated their possible role in maintaining sperm plasma membrane integrity .

Table 3.17. Values of alpaca fresh semen parameters.

	MEAN±SEM
CL TIME M	15.88±0.4
VOL	3.31±0.4
MOT%	33.34±3.9
CONCx10^6	58.75±8.5
VIS	1.73±0.1
HOST+VE	40.42±3.0
CF+PI^-	46.79±5.0
CF+PI^+	16.09±2.3
CF-PI^+	33.02±4.4
CF+AN^-	37.81±5.7
CF+AN^+	30.45±4.8
CF-AN^+	25.91±3.84

Table 3.18. Pearson correlation coefficients for alpaca fresh semen quality parameters and semen collection time.

	VOL.	**MOT%**	**CONC.X10^6**	**VIS**	**HOST+ve**	**CF+PI-**	**CF+PI+**	**CF-PI+**	**CF+AN-**	**CF+AN+**	**CF-AN+**
C.TIME	0,134	0,181	-0.0966	0.346	0,220	0,126	-0.233	-0.505	0,615	-0.433	-0.0119
VOL.		-0.450*	-0.346	-0,356**	-0.172	-0.375	-0.0601	0,106	-0.311	-0.158	0,107
MOT%			0,338**	-0.783***	0,508***	0,500**	-0.0945	-0.469	0,484**	-0.270	-0.296
CONC.X10^6				-0.389*	0,258	0,406	-0.177	-0.338	0,283	-0.113	-0.124
VIS					0.662**	0.804**	-0,463	-0,263	0.647*	-0,608	-0,079
HOST+						0,638***	-0.0982	-0.317	0,497**	-0.162	-0.0540
CF+PI-							-0.0760	-0.352	0,508**	-0.199	0,501
CF+PI+								0,216	0,097	0,294	-0.0714
CF-PI+									-0.305	0,372	0,355
CF+AN-										-0.460	-0.273
CF+AN+											0,303

Table 3.19. Quantitative (absolute and percentage) values of PP obtained by 1 DPAGE

TP	MEAN±SEM
TP1	0.20±0.11
TP1%	3.24±1.8
TP2	3.13±0.2
TP2%	33.47±1.6
TP3	0.97±0.3
TP3%	12.96±3.2
TP4	4.29±0.3
TP4%	45.81±2.3
TP5	0.76±0.2
TP5%	8.95±2.3
TP6	1.70±0.2
TP6%	22.35±3.0
TP7	1.80±0.3
TP7%	22.07±3.3
TP8	2.16±0.3
TP8%	31.36±4.7

Table 3.20. Biochemical parameters of fresh alpaca SP.

	MEAN±SEM
Cl(mEq)	81.77±12.0
K(mEq)	25.98±1.4
P(mg)	2.26±0.3
Mg(mg)	6.06±0.7
Ca(mg)	25.01±2.4
Na(mEq)	247.34±12.5
TP(g)	1.43±0.3
GLU(mg)	1.23±0.3
CHOL(mg)	1.61±0.24
TG(mg)	56.71±11.1
ALP(u)	191.16±30.8
LDH(u)	56.13±43.8
GOT(u)	2.13±1.2

Results regarding function of SP total protein and enzymes showed that Total protein, ALP and GOT had positive correlation with semen viscosity and sperm quality, where total protein concentration showed a positive relation with semen viscosity and sperm quality in terms of volume, apoptotic free population and cell viability, and this relation was statistically highly significant ($P<0.001$). In this study, observation was similar in terms of relation of LDH and glucose

with semen quality parameters. Both correlated negatively with semen volume, and viable cell populations, though this relation was not significant. Data on semen volume,SP proteins or energetic profiles revealed an insignificant negative correlation between them. Although, CHOL and TG were correlated insignificantly with sperm viability, absence or presence of apoptosis marker, a significant negative correlation ($P<0.05$) were observed between semen volume and CHOL.

Table 3.21. Correlation coefficients between quality parameters with macroelements of SP.

	Cl	K	P	Mg	Ca	Na
VOL.	0,515	-0.0511	-0.314	-0.340	-0.0647	0,387**
MOT%	0,078	0,324	0,298*	0,450**	0,326*	-0.167
CONC.X10^6	-0.0493	0,567	0,167	0,155	-0.0341	-0.170
VIS	0.0646	0.137	0.297	0.567**	0.445*	-0,075
HOST+VE	0,210	0,335	0,191	0,317*	0,240	0,086
CF+PI-	0,253	0,199	0,263	0,421*	0,184	-0.119
CF+PI+	-0.307	-0.287	-0.215	-0.172	0,120	-0.215
CF-PI+	-0.482	-0.0650	-0.285	-0.396	-0.418	0,608
CF+AN-	0,161	0,256	0,242	0,428*	0,327	0,270
CF+AN+	-0.228	-0.273	-0.246	-0.357	-0.129	-0.309
CF-AN+	-0.222	-0.0281	-0.0986	-0.178	-0.501*	0,200

Table 3.22. Relation of total protein(TP), energy profiles and enzymes of SP with semen quality parameters.

	TP	ALP	LDH	GOT	GLU	CHOL	TG
VOL.	-0.282	-0.214	-0.0852	-0.0889	-0.0532	-0.493*	-0.298
MOT%	0,444**	0,290	-0.0319	0,126	-0.102	0,143	0,103
CONC.X10^6	0,098	0,251	-0.0411	0,131	-0.0926	0,156	0,163
VIS	0.445*	0.484*	0.001	0.222	0,076	0.178	0.211
HOST+VE	0,251	0,225	-0.0995	-0.0148	-0.154	0,296	0,583
CF+PI-	0,367	0,342	0,206	0,424	-0.174	0,326	0,164
CF+PI+	0,365	-0.325	-0.258	-0.202	0,128	-0.303	-0.352
CF-PI+	-0.398	-0.348	-0.116	0,688	0,315	-0.132	0,551
CF+AN-	0,426*	0,201	0,174	0,155	0,676	0,275	0,070
CF+AN+	-0.370	-0.295	-0.273	-0.0192	0.00968	-0.260	-0.107
CF-AN+	-0.168	0,399	0,081	-0.218	0,079	0,676	0,181

Table 3.23. Values of post-thawed alpaca semen quality parameters.

Group	CF+PI-	CF+PI+	CF-PI+	CF+AN-	CF+AN+	CF-AN+
G	15.14±2.4	15.26±2.2	64.84±4.7	12.01±2.1	10.78±2.0	68.41±5.5
F	19.45±1.6	15.78±1.9	60.61±2.1	21.03±21.0	16.56±3.4	62.70±4.8
T	14.17±1.9	10.59±2.0	75.25±2.1	12.21±1.8	15.56±2.8	72.23±4.1
S	18.07±3.3	10.91±2.6	66.95±6.1	15.14±3.1	9.7±2.3	75.17±4.7

Figure 3.19. HOST+ve % in pre-freezing and post-thawing alpaca semen

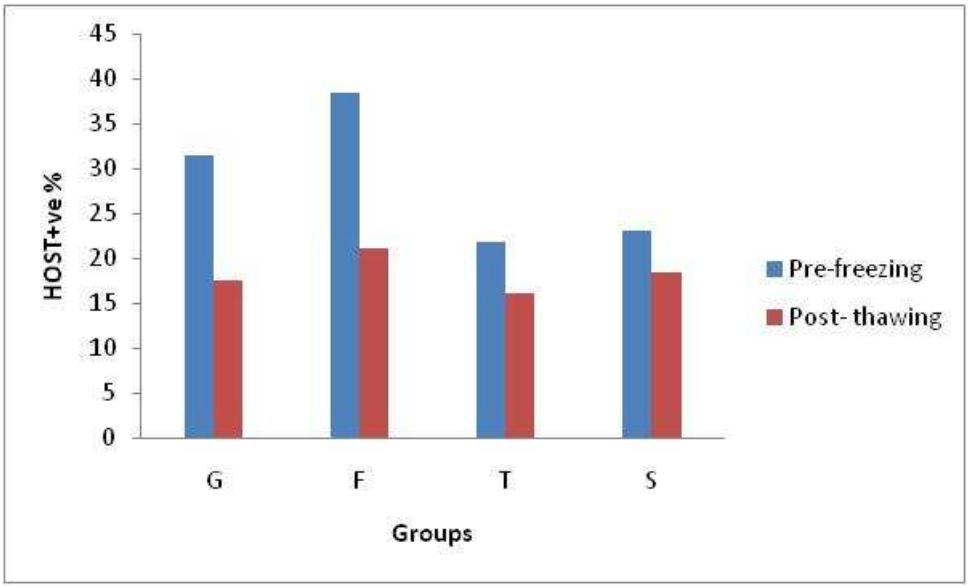
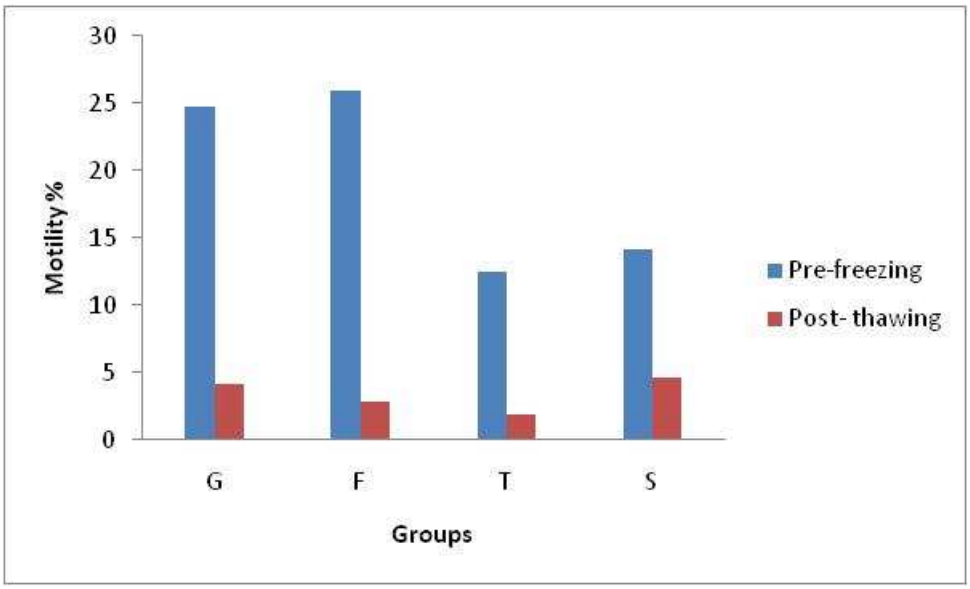


Figure 3.20. Motility (%) in pre-freezing and post-thawing alpaca semen



We used four diluents for cryopreservation. During cooling sperm motility and viability with HOST value were evaluated and motility decreased in all groups.

Pre-freezing and post-thawing viability and motility are presented in Figure 3.19 and 3.20. Whereas cell viability was not as affected as motility during cooling stage. Decreased motility% and HOST+ve% sperm were observed in group T. Consequently, after freezing thawing motility and viability decreased

significantly (P<0.05) in all groups (Table 3.19). Surprisingly, percentage of viable cells (HOST+ve, CF+PI) and apoptosis free viable (CF+AN-) sperm were higher than motility in all groups. Values of viability and Annexin V assay are presented in Table 3.21. Percentage of viable sperm were relatively higher in F group and lower values of seminal parameters are observed in groups T and G. These results revealed that extender with fructose can maintain post-thaw sperm viability better.

We examined correlations between cooled semen quality with SP components (Table 3.24. and 3.25).

In F group, P, Mg, Ca, Na, TP showed a positive correlation with percentage of motility% and HOST+ve% sperm and this relation was statistically insignificant. Significant(P<0.04) negative correlation was observed between semen volume, viscosity and sperm motility.

In group G, only significant(P<0.05) correlation was observed between P and motility. Whereas in group T and S, moderate but significant(P<0.001) correlation existed between P, Mg, Ca, TP and motility. There was no significant correlation between SP elements and HOST+ve sperm% in cooling stage. Considering the relation between PP ant semen quality, insignificant relation was observed.

TP2(Kda) and TP3 (KDa) showed a significant correlation with sperm motility and apoptosis cells.

In F group positive correlation observed between Na and motility % and a negative correlation observed between glucose and HOST%. A positive correlation observed between TP6 (75 kDa) with apoptosis cells.

For better screening of the relationship between post-thaw semen parameters and SP components we observed correlation coefficient in different groups and results are shown in Table 3.26., 3.27., 3.28., 3.29., 3.30., 3.31., 3.32., 3.33. No significant relation was observed between post-thaw semen characters

and SP components in groups F, T, G. In group S, we found significant correlation ($P<0.05$) between sperm motility and TP, Mg, K, P, Chol, TG. These elements also showed a positive correlation with apoptotic cells (CF+AN-). On the other hand, TP showed a positive correlation with apoptosis free viable cells as we observed in case of fresh alpaca semen.

Table 3.24. Pearson's correlation coefficient between cooled semen quality parameters and SP components in four groups

		Cl	K	P	Mg	Ca	Na	TP	GLU	CHOL	TG	ALP	LDH	GOT
F	MOT%	-0.052	-0.161	0.161	0.328	0.280	0.108	0.288	-0.158	-0.304	-0.031	0.141	-0.175	0.476
	HOST+VE	-0.185	0.173	0.228	0.292	0.132	0.193	0.149	-0.364	0.611	0.122	0.074	0.469	-0.0723
G	MOT%	-0.038	-0.248	0.554*	0.351	0.349	0.184	0.364	0.324	-0.118	-0.276	0.278	-0.153	0.288
	HOST+VE	-0.187	0.226	-0.248	-0.151	-0.177	-0.120	-0.188	-0.328	0.301	-0.249	-0.361	-0.088	-0.119
T	MOT%	0.269	0.378	0.435*	0.580**	0.503**	-0.271	0.588**	-0.156	0.381	0.151	0.328	0.258	0.177
	HOST+ve		-0.015	0.436	0.191	0.134	0.078	0.192	-0.176	0.122	-0.064	0.167	-0.053	0.108
S	MOT%	0.126	0.403	0.488**	0.573**	0.506**	-0.375	0.636**	-0.233	0.447**	0.229	0.299	0.314	0.181
	HOST+ve	0.259	0.094	0.209	0.328	0.292	-0.355	0.395	-0.0646	0.131	0.001	0.281	0.084	0.091

Table 3.25. Pearson's correlation coefficient between cooled semen quality parameters and SP Protein Profiles four groups.

		TP1	TP2	TP3	TP4	TP5	TP6	TP7	TP8
F	MOT%	-0.396	0.641	-0.241	0.330	-0.036	-0.345	0.076	0.092
	HOST+ve	-0.433	0.145	0.079	0.235	0.326	-0.270	0.088	-0.304
G	MOT%	-0.394	0.107	-0.263	0.360	0.260	-0.238	-0.031	-0.051
	HOST+ve	-0.116	-0.220	0.674*	-0.075	0.111	0.095	-0.220	0.102
T	MOT%	--	-0.196	0.407	-0.175	-0.367	0.106	-0.262	0.192
	HOST+ve	--	0.169	0.180	0.226	0.648	-0.147	-0.425	-0.088
S	MOT%	--	-0.195	0.490**	-0.213	-0.146	0.664	-0.334	0.133
	HOST+ve	--	0.303	0.190	0.316	-0.196	-0.183	-0.319	-0.319

Table 3.26. Pearson's correlation coefficient between post-thaw semen quality parameters and SP components in F-group.

	Cl	**K**	**P**	**Mg**	**Ca**	**Na**	**TP**	**GLU**	**CHOL**	**TG**	**ALP**	**LDH**	**GOT**
MOT%	-0.0250	-0.0410	-0.152	0.146	0.101	0.364	0.113	-0.182	0.203	-0.138	0.090	-0.285	-0.151
HOST+ve	-0.240	-0.231	-0.166	0.050	0.121	-0.058	0.424	-0.240	0.249	0.174	0.106	-0.059	-0.065
CF+PI-	-0.160	-0.103	0.240	0.082	0.501	0.609	0.097	-0.249	0.209	0.650	0.098	-0.201	0.263
CF+PI+	-0.213	-0.190	0.071	0.733	-0.006	-0.222	0.638	-0.105	0.120	0.094	0.378	0.176	-0.040
CF-PI+	0.317	0.628	0.192	0.566	0.087	-0.145	0.476	0.098	-0.270	0.009	-0.071	0.417	-0.019
CF+AN-	0.085	0.113	-0.111	0.458	0.149	0.185	-0.016	-0.300	0.530	-0.332	-0.146	-0.061	-0.099
CF+AN+	-0.188	0.073	-0.084	-0.057	0.085	-0.069	-0.089	-0.314	0.438	0.09	-0.299	-0.035	-0.210
CF-AN+	-0.385	-0.143	0.307	0.154	0.113	-0.212	0.159	0.133	-0.258	0.165	0.249	0.460	0.107

Table 3.27. Pearson's correlation coefficient between post-thaw semen quality parameters and SP Protein Profiles in F-group.

	TP1	**TP2**	**TP3**	**TP4**	**TP5**	**TP6**	**TP7**	**TP8**
MOT%	-0.330	0.125	-0.0587	0.303	-0.121	-0.0940	-0.240	-0.052
HOST+ve	-0.396	0.375	0.157	0.235	0.181	-0.450	-0.361	-0.372
CF+PI-	-0.272	0.213	0.235	0.093	0.177	-0.229	-0.225	-0.427
CF+PI+	-0.060	-0.147	-0.184	0.173	0.366	0.270	-0.321	-0.054
CF-PI+	0.203	0.213	0.527	-0.126	-0.212	-0.201	0.286	0.094
CF+AN-	-0.144	0.453	-0.0896	0.111	-0.183	0.126	0.222	-0.305
CF+AN+	-0.114	0.145	-0.123	0.153	0.159	0.665*	0.247	-0.301
CF-AN+	-0.196	0.442	0.092	0.264	0.184	-0.357	-0.144	0.201

Table 3.28. Pearson's correlation co-efficient between post-thaw semen quality parameters and SP components in G-group.

	Cl	**K**	**P**	**Mg**	**Ca**	**Na**	**TP**	**GLU**	**CHOL**	**TG**	**ALP**	**LDH**	**GOT**
MOT%	-0.0350	-0.0410	-0.142	0.126	0.111	0.264	0.113	-0.182	0.303	-0.118	0.090	-0.185	-0.121
HOST+ve	-0.289	-0.231	-0.166	0.070	0.121	-0.0585	0.424	-0.240	0.249	0.174	0.106	-0.059	-0.065
CF+PI-	-0.160	-0.103	0.240	0.082	0.501	0.609	0.097	-0.249	0.209	0.650	0.098	-0.201	0.263
CF+PI+	-0.423	-0.190	0.071	0.433	-0.006	-0.222	0.638	-0.105	0.120	0.094	0.378	0.176	-0.040
CF-PI+	0.417	0.628	0.192	0.566	0.087	-0.145	0.476	0.098	-0.270	0.009	-0.071	0.417	-0.019
CF+AN-	0.085	0.113	-0.111	0.658	0.149	0.185	-0.016	-0.300	0.530	-0.332	-0.146	-0.061	-0.099
CF+AN+	-0.188	0.073	-0.084	-0.057	0.085	-0.069	-0.089	-0.314	0.438	0.009	-0.299	-0.035	-0.210
CF-AN+	-0.385	-0.143	0.307	0.154	0.113	-0.212	0.159	0.133	-0.258	0.265	0.249	0.660	0.107

Table 3.29. Pearson's correlation co-efficient between post-thaw semen quality parameters and SP Protein Profiles in G-group

	TP1	**TP2**	**TP3**	**TP4**	**TP5**	**TP6**	**TP7**	**TP8**
MOT%	-0.330	0.125	-0.0587	0.303	-0.121	-0.0940	-0.240	-0.0529
HOST+ve	-0.396	0.375	0.157	0.235	0.181	-0.450	-0.361	-0.372
CF+PI-	-0.272	0.213	0.235	0.093	0.177	-0.229	-0.225	-0.427
CF+PI+	-0.0602	-0.147	-0.184	0.173	0.366	0.270	-0.321	-0.0542
CF-PI+	0.203	0.213	0.527	-0.126	-0.212	-0.201	0.286	0.094
CF+AN-	-0.144	0.453	-0.089	0.111	-0.183	0.126	0.222	-0.305
CF+AN+	-0.114	0.145	-0.123	0.153	0.159	0.665*	0.247	-0.301
CF-AN+	-0.196	0.442	0.092	0.264	0.184	-0.357	-0.144	0.201

Table 3.30. Pearson's correlation co-efficient between post-thaw semen quality parameters and SP components in T-group.

	Cl	**K**	**P**	**M**	**Ca**	**N**	**T**	**G**	**CH**	**T**	**A**	**L**	**G**
M	0.4	0.1	-	0.5	0.2	0.0	0.0	0.2	-	0.3	-	-	-
HO	-	0.0	0.0	0.1	-	-	0.1	-	0.26	0.1	0.1	-	-
CF	-	0.0	0.1	0.1	-	-	0.1	0.2	0.24	0.2	0.6	0.3	0.2
CF	-	-	-	-	-	0.1	0.2	-	0.00	0.1	-	0.6	-
CF-	0.0	-	-	-	0.1	0.2	-	0.1	-	-	0.6	-	0.1
CF	-	0.2	0.1	0.1	0.5	-	0.1	-	0.30	0.1	0.6	0.4	-
CF	0.0	0.0	-	-	-	0.1	-	-	0.15	-	-	-	-
CF-	0.4	-	-	-	0.4	-	-	0.3	-	-	0.1	0.1	0.1

Table 3.31. Pearson's correlation co-efficient between post-thaw semen quality parameters and SP Protein Profiles in T-group.

	TP1	**TP2**	**TP3**	**TP4**	**TP5**	**TP6**	**TP7**	**TP8**
MO	--	-0.042	0.453	-0.177	-0.275	0.314	0.553*	-0.013
HO	--	-0.110	0.288	-0.029	0.305	-0.218	-0.423	0.002
CF+	--	-0.371	0.426	-0.217	-0.058	0.250	-0.237	0.158
CF+	--	-0.092	-0.096	-0.125	0.185	0.131	-0.058	-0.041
CF-	--	0.197	-0.297	0.150	-0.152	-0.152	0.126	-0.107
CF+	--	-0.038	0.246	-0.001	0.319	-0.226	-0.454	-0.009
CF+	--	-0.218	-0.059	-0.097	0.242	-0.078	-0.113	0.093
CF-	--	0.116	-0.119	0.467	-0.447	0.108	0.196	-0.089

Table 3.32. Pearson's correlation co-efficient between post-thaw semen quality parameters and SP components in S-group.

	Cl	**K**	**P**	**Mg**	**C**	**Na**	**TP**	**G**	**CH**	**TG**	**A**	**L**	**G**
MOT%	0.1	0.4	0.56	0.47	0.	-	0.47	-	0.53	0.52	0.	0.	-
HOST	0.2	0.3	0.43	0.44	0.	-	0.48	0.	0.39	0.36	0.	0.	0.
CF+PI-	0.2	0.3	0.48	0.47	0.	-	0.47	0.	0.42	0.45	0.	0.	-
CF+PI	0.2	-	-	-	-	-	0.40	0.	-	-	-	-	-
CF-PI+	-	-	-	-	-	0.3	-	-	-	-	-	-	0.
CF+A	0.1	0.2	0.40	0.44	0.	-	0.50	0.	0.34	0.30	0.	0.	0.
CF+A	0.2	0.4	0.49	0.45	-	-	0.36	0.	0.56	0.50	0.	0.	0.
CF-	-	-	-	-	-	0.3	-	-	-	-	-	-	-

Table 3.33. Pearson's correlation co-efficient between post-thaw semen quality parameters and SP Protein Profiles in S-group.

	T	TP2	TP3	TP4	TP5	TP6	TP7	TP8
	--	-0.348	0.421	-0.284	-0.241	-0.0719	-0.153	0.299
HOST+VE	--	0.154	0.285	0.185	0.115	-0.203	-0.433	-0.152
CF+PI-	--	0.072	0.340	0.114	-0.215	-0.154	-0.287	-0.024
CF+PI+	--	0.216	-0.189	0.328	-0.158	-0.385	-0.082	-0.078
CF-PI+	--	-0.381	0.199	-0.276	0.201	-0.023	-0.056	0.210
CF+AN-	--	0.074	0.366	0.087	-0.137	-0.037	-0.410	-0.044
CF+AN+	--	-0.034	0.240	0.097	-0.175	-0.387	-0.127	0.106
CF-AN+	--	-0.053	-0.514	-0.150	0.122	0.149	0.229	-0.045

CHAPTER 4. DISCUSSION

4.1. STUDY OF BIO-CHEMICAL COMPOSITION OF SP AND ITS INFLUENCES ON FREEZABILITY OF RAM SEMEN.

4.1.1. Comparison of the effects of different ejaculate types on semen freezability in Sarda ram.

The main objective of semen analysis was to study the effect of the post-thaw quality in terms of freezability of different ejaculates using two parameters of sperm function, namely motility% and motility score. In routine laboratory test, post-thaw motility is considered as a valid test to valuated sperm quality from the point of view of cells functionality (Leboeuf, 1989). As expected, freezing-thawing process resulted in a drastic decrease in sperm motility. The mid piece and tail involved in the generation and propagation of tail movements is more vulnerable in ram spermatozoa during cryopreservation. Moreover, loss of motility is mainly related to a decrease in energy supply by the mitochondria due to ATP depletion and lipid peroxidation of the plasma membrane (De Lamirande and Gagnon, 1993). Some studies have demonstrated that post-thawing sperm survival is independent of pre-freezing motility (Fernandes et al., 1990) and sperm viability and motility do not necessarily have a good correlation (Valcircel et al., 1994).

In the present study, no significant variations in sperm quality parameters was observed between first and second ejaculates, though it has been reported that the second ejaculates have the best quality parameters (Ollero et al., 1996). Generally, for cryopreservation, pool of first and second ejaculates from different sires (Sanchez-Partida et al., 1999) or three first ejaculates (Abdelhakeam et al.,

1991) have been used, We observed that post-thaw semen quality was better in both pooled groups. This reflected the fact that pool of different successive ejaculates within ram could maintain the heterogeneity of spermatozoa. The wide variability of the biochemical components and properties of SP, even in successive sampling (Zedda et al., 1996) may be attributable to this response and comparatively higher quality values in pooled semen may be resulted from combined action of SP of ejaculates on spermatozoa. Results from this basic study let us to study biochemical composition in SP and their relation with semen preservation.

Cryopreservation process causes detrimental changes in sperm structure and function due to thermal, mechanical, chemical and osmotic stresses (Watson, 1995). It produces homogenous sperm samples which lead to a loss of functional versatility and capacity to respond to different situations reducing fertilizing capacity of spermatozoa (Ollero et al., 1998). Studying the causes of ejaculates variation in freezability would be helpful for cryoprotective strategies to improve post-thaw semen quality in order to establish the semen sample more suitable to freezing for the possible constitution of a sperm bank.

4.1.2. Effects of ejaculates on the freezability of spermatozoa and the relationship with biochemical composition and Protein Profiles (PP) of seminal plasma (SP) with semen quality and freezability.

The availability of semen quality semen results from the coordinated action of normal spermatogenesis, proper maturation during epididymal transit and composition of SP (Goeritz et al., 2003). The consideration of these interrelationship could allow the understanding of physiological changes of frozen-thawed spermatozoa. Therefore, our objective was concentrated on to study the relationship between sperm freezability and SP biochemical composition. We used two modern assays to evaluate sperm plasma membrane integrity (CFDA/PI) and plasma membrane architecture (CFDA/AN – apoptosis

marker) along with classical semen quality parameters to observe post-thaw semen quality inters of freezability.

In the present study, motility and viability, were significantly decreased after freezing and thawing procedure and an approximate reduction of 50% was observed in sperm viability and of 40% in sperm motility. We found 35-45% motile sperm in post-thaw sample. Motility value was lower than that of, reported by D'Alessandro et al. (2003) who observed about 60% post-thaw motile sperm using TRIS-based extender.

CASA findings on progressive motility revealed that progressive motility was markedly affected than individual motility (Figure 3.5). Anel et al (2003) also have similar observation and proposed that the physiological basis for progressive motility is more sensitive to cryobiological damage. Generally, flagellum of an activated sperm generates a symmetrical, lower amplitude waveform that drives the sperm in a relatively straight line. In contrast, the flagellar beat of hyperactivated sperm becomes asymmetrical with higher amplitude, results in circular or figure-eight trajectories (Yanagimachi, 1994). This kind of motility is mainly observed in freez-thawed spermatozoa indicating cryopreservation induced capacitation like process. It is well known that cryopreservation impaires cell structures (Watson, 1995). Changes in active transport and permeability of cell membrane in tail region, damage to axonemal elements, loss of mitochondrial structure and alteration in ATP production (Watson, 1995) might be responsible for altered flagelllar waves. Capacitated spermatozoa additionally have more rapid forward motility and greater lateral head displacement. It is, therefore, possible that using maximal sperm motility for formulating media for freezing semen may favor capacitated spermatozoa.

Computer Automated Sperm Analysis (CASA) has been introduced in the laboratory routine to improve the accuracy of data collection, to avoid errors due to subjective evaluation of different technicians and to reduce lime in the exam

performance (Amman and Hammerstedt, 1993). Results of CASA motility analysis are dependent on the type of equipment and the setting of the CASA instrument (Holt et al, 1994, 1996). . Specific motility characteristics related the quality of fresh or frozen ram semen is somewhat undefined. No international standardization in equipment settings has yet been implemented. Likewise, margins or threshold levels used to define sperm subpopulations vary between laboratories. In human spermatozoa,VCL±150µm/s, LIN ≤ 50% and ALH max±7. 0µm, are considered as hyperactivated motility (Mortimer et al., 1998).

To evaluate plasma membrane integrity we used fluorescence staining and hypo-osmotic swelling test (HOST). HOST appears to be a better indicator of cell membrane integrity than supravital stains because HOST reflects the biochemical activity required by the membrane to establish osmotic equilibrium (Neild et al., 1999). We found that spermatozoa having good motility did not show intact plasma membrane at the same level of motility in this study. This finding is in agreement with Valcárcel et al. (1994) who stated that spermatozoa having good motility showed membrane damage. Salamon and Maxwell, (2000) have also stated that motility is preserved better than the morphological integrity of spermatozoa. Very recently, Mocè et al., (2010) have proposed a technical cause of the discrepancy between the percentage of plasma membrane intact sperm and total motile sperm observed in most of these experiments (approximately 15% fewer plasma membrane intact sperm than motile sperm) and stated that the dilution process (4. 5 fold dilution) for the sperm viability evaluation induces osmotic stress to cell membranes, leading to membrane damage in some extent. Therefore, the functional integrity of plasma membrane should be evaluated and it has been found to be a better predictor of the fertilizing capacity of spermatozoa than motility in ram and bull semen (Vazquez et al., 1988 ;Correa and Zavos, 1994)

Considering the Annexin V assay for translocated PS, our results showed 20-35% apoptotic cells in fresh semen. This number is lower than that reported by Marti et al. (2008) who found 40% apoptotic cells in fresh sperm. Presence of

apoptotic cells in fresh ejaculate may be the remainders of an earlier abortive apoptotic population (Martin et al., 2004) and incidence of cells with translocated PS indicates viable sperm subpopulation with altered membrane function that will eventually undergo necrosis. The percentage of apoptotic cells was reduced to 13-20% in different treatment groups after freezing and thawing. Moreover, we found an increased tendency towards a higher percentage of dead spermatozoa either from necrosis or apoptosis. There is lacking of constant data regarding fate of apoptotic cells after cryopreservation ion of ram spermatozoa. In one study with ram semen, Ivanova-Kicheva et al. (2005) have observed both increased and decreased values of two types of diluents. However, our result is in contrast with some earlier findings in bulls (Anzar et al., 2002), human (Paasch et al., 2004) and boar (Pena et al., 2003), which revealed presence of increased number apoptotic sperm after cryopreservation. Cryopreservation induces a major increase in both types of necrotic cells. This difference among human, bull and ram semen can be explained by the higher sensitivity of ram spermatozoa to cryopreservation, compared to human and bull spermatozoa. Increasing number of dead cells and decreased apoptotic free viable cells might indicate the sensitivity of apoptotic cells to cryopreservation and simultaneously, cryopreservation induced apoptotic marker in new group of post-thaw live sperm. Increased Annexin-V binding in thawed spermatozoa probably results from plasma membrane damage incurred during freezing and thawing (Guthrie and Welch, 2005) and could be associated with increased exposure to ROS as the spermatozoa become increasingly isolated from the antioxidant and ROS-scavenging activity of SP (Aitken and Krausa, 2002; Muratori et al., 2003). It has been noted in humans spermatozoa that ROS level has a positive correlation with the extent of apoptotic sperms.. Translocation of PS also occurred during sperm capacitation, due to bicarbonate dependent changes in order for the spermatozoa to undergo the acrosome reaction to fertilize an oocyte (Harrison and Gadella, 2005). Further study is essential to find out the relationship of freeze-thawed induced apoptosis-like changes with sperm capacitation in ram semen.

In contrary, in a study with ram spermatozoa, Muller et al (1999) did not observe any apoptotic cells in live population of both fresh and freez-thawed spermatozoa and reported the presence of Annexin- V positive cells only in dead sperm population. PS is almost completely oriented to the cytoplasmic leaflet of intact spermatozoa, and is maintained by ATP-dependent aminophospholipid translocase . Muller et al., (1999) proposed that despite a reduction in the activity of the aminophospholipid translocase during cryopreservation process in intact cells it is capable of maintaining the asymmetric distribution of the aminophospholipids to a some extent.

Crystal ice formation during cryopreservation has been claimed for freezing induced membrane damage (Watson, 2000) In addition, Martin et al (2006) have proposed that alteration in membrane integrity might be dual effects of ice information and freezing induced apoptosis like changes. They have observed a sequential appearance of apoptotic characteristics in bull sperm during the cryopreservation process,; a decrease of the mitochondrial membrane potential immediately after dilution in the cryopreservation medium, caspase activation after equilibration and changes in membrane permeability after the complete freezing/thawing process. Therefore, the evaluation of sperm membranes with apoptosis marker is an appropriate indicator of the success of cryopreservation since sperm membranes are extremely susceptible to cryoinjury (Park and Graham, 1992; Holt and Modrano, 1997).

In our observations, results from the correlation coefficient values among different quality parameters is very promising. There was a very significant ($P<0.001$) relation between CF+PI- and CF+AN-. Several studies revealed a moderate correlation between the proportions of HOST positive and vital stain positive sperm (Correa and Zavos, 1994; Zou and Yang, 2000). In contrast, some researchers also reported a low or insignificant correlation between HOST and other vital stain results (Esteves et al., 1996; Neild et al., 1999). Moreover, the percentage of HOSt+ve spermatozoa was significantly correlated with motility% and progressive motility, but not with kinetic parameters VAP, VSL, or LIN in

our study. This results are in agreement with that of Anel et al. (2003). In contrary, Mandan et al (2003) observed a positive correlation between HOST+ve spermatozoa and kinetic parameters in buffalo spermatozoa.

The percent of HOST+ve sperm was higher than viable sperm without apoptotic marker, although both shared a very significant ($P< 0.001$) correlation between them. This findings can be supported by the observations of Martin et al., (1995) who proposed that during the early phases of disturbed membrane function, asymmetry of the membrane phospholipids occurs, before the integrity of the plasma membrane is progressively damaged (Martin et al., 1995). Moreover, our study revealed a significant ($P<0.001$) negative correlations between motility % and dead sperm, although a positive relation was observed between motility % and damaged sperm. In contrast, Januskauskas et al., (2003) found no relation between cell viability parameters and Annexine-+ve positive cells in post-thaw bull spermatozoa. Studies on this aspect in small ruminant is very scanty. It can be hypothesized, that Annexin-V binding assey, represent subpopulation of spermatozoa that have a reduced life span in vivo, could provide information for predicting the outcome of cryopreservation, as observed in case of human spermatozoa.

Regarding the extender effect, with the Skim milk extender the reduction in quality parameters in all S groups was even greater than with TRIS extenders (T groups). But, there was no significant difference in post-thawed sperm quality parameters in both extenders. Molinia et al. (1994) showed that TRIS-monosaccharide solutions gave the highest in vitro protection during the freeze-thawing process, as long as iso-osmolarity was maintained. The reduction of post-thaw sperm quality with milk extender might be related with the interaction of milk caseins and SP proteins. In a recent study, Bergeron and Manjunath, (2006) have reported that milk proteins such as casein micelles interact with BSP proteins. However, this difference between extenders might suggest that the TRIS based extender provides greater protection to spermatozoa and this variation might also result from the action of egg yolk as a cryoprotectant. Egg

yolk volume differs greatly in these of extender (TRIS contains 20% and milk extender 5%). Egg yolk low-density lipoprotein fraction (LDF) or phospholipids could merge with the sperm membrane, replacing some of the sperm phospholipids and, thereby, decreasing their phase-transition temperature (De Leeuw et al., 1993) and protect sperm membrane from cold shock damage (Graham and Foote, 1987). Phospholipid-mediated mechanism of egg yolk protection to sperm plasma membrane would be related to an increase of cholesterol and phospholipids ratios in the sperm led to decrease cold shock (Parks et al., 1981).

Among different treatment groups, all semen parameters were comparatively lower in goups 4S and 4T prepared with pool of centrifuged spermatozoa and frozen without SP. This group was included to augement the accuracy of our objective to study SP effect. Apart from elimination of SP centrifugation could be deleterious by itself, either by causing mechanical damage or by inducing generation of ROS and peroxidation of the sperm plasma membrane, as suggested in human (Parinaud et al., 1997). In comparison among groups pool of first and second ejaculates with SP showed comparatively higher values of quality parameters irrespective of diluents. Similar finding was observed in our first experiment where we found that pool of two ejaculates could maintain better post-thaw semen quality in Sarda rams. Moreover, the results of our study detected variation in post-quality semen parameters between, males, which supports previous findings of Watson, (1995) and Holt (2000) who have stated inter-male variability as a source of variation in sperm freezability.

4.1.2.a. Biochemical composition and Protein Profiles of SP

Cryopreservation induces cold shock, oxidative stress, cryoprotectant toxicity, intracellular ice crystal formation, and fluctuations in osmotic pressure (Watson, 1995; Isachenko, 2003) resulting in sperm plasma membrane alterations, thereby, leading to a reduction in sperm heterogeneity. An ejaculate consists of

heterogeneous sperm subpopulations, produced from different spermatogenic waves, matured and stored in epididymes as different cell cohorts, therefore, they yield different outcomes in a single treatment (Rodrı´guez-Martı´nez(, 2006). Response of spermatozoa to these induced insults depends on sperm inherent intrinsic and extrinsic factors. Sperm samples from a unique male and with similar sperm parameters have different post-thaw sperm survival patterns (Meseguer et al., 2004). The susceptibility of the sperm to cryodamage during the freezing process related with sperm intrinsic factors such as saturated vs. unsaturated fatty acids, together with low cholesterol content (White, 1993). A part from the extrinsic factors regarding technical procedure of cryopreservation, sperm contact with SP is also important, since SP can alter sperm properties. SP is a complex biological fluid, consisted of ions, Proteins, lipid, enzymes, energy substrate, hormones, cytokines, secreted from rete testis, epididymis and accessory sex glands (AGs) of the male reproductive tract (Mann and Lutwak-Mann, 1981). Recent years biochemical estimates of SP are also used for semen evaluation, since semen characteristics alone are not completely satisfactory for semen appraisal in the current practice of commercial AI (Ashwort et al., 1994; Barrios et al., 2000).

In this experiment, biochemical components of SP varied ejaculates (NS), and with individual, specially Ca, Na, cholesterol, triglycerides, and LDH varied significantly (P<. 05) with male (Table 3.7). Zedda et al. (1996) studied biochemical composition of SP in Sarda ram and some values of glucose, Ca, Mg, P, were higher in our case, in comparison to their observed value. Previous studies showed that the source of lipid components in SP might be epididymes and spermatozoa itself and their concentration varied with ejaculates (first ejaculate contain higher amount that the second ejaculates (Iqbal et al., 1984). In our case, such difference was less marked. Studies regarding SP components other than protein fractions are very limited in small ruminant. Moreover, discrepancy between different units it also makes difficult to compare our parameters with others. The composition of SP is determined by the size, storage capacity and secretary output of different organs of the male reproductive tract

and properties can be modified by seminal enzymes, sperm metabolism and the interaction of seminal constituents with sperm surface (Mann and Lutwak-Mann, 1981).

Correlation coefficient between semen quality parameters revealed some interesting features. Negative but significant correlation observed between all post-thaw semen motility and HOST+ve% parameters and total protein, K, Mg, P, Cholesterol, triglycerides were observed. More importantly, total protein, K, P, Mg showed negative significant ($P<0.01$) relation with apoptosis free viable sperm. TP, Na, K, Ca, and Mg in SP exert osmotic balance and are components of many important enzymes (Cevik et al., 2007) and maintained spermatozoa function (Mosaferi et al., 2005). Kaya et al (2002) have observed a negative correlation between sperm motility and Na and K concentration. Studies with human spermatozoa have shown that high sodium or potassium concentration in SP may induce spontaneous lipid peroxidation of the sperm membrane (Love at el., 2005). K is a natural metabolic inhibitor and higher K concentration in SP decreases sperm metabolism thereby, decreasing sperm motility (Massányi et al., 2003). Several studies reported relation total of total protein with fresh semen quality (Ashworth et al., 1994, Barrios et al., 2000). In addition, Barrios et al. (2000) also reported that the freezability of semen is positively correlated with total protein content in SP. However, variation in the relation of these components with different sperm action may indicate the complex interaction of these components on osmolarity imbalance.

Seminal enzyme such as GOT, ALP were positively correlated with damages or dead cells. This finding corresponds well with the reports of Sirat et al., (1995). Transaminases are located primarily in the midpiece of spermatozoa (Mann and Lutwak-Mann, 1981). ALP is a dephosphorylating enzyme has been reported in SP of all animals and is involved in sperm glycolytic reactions and fructose formation (Mann, 1964) and the measurement of these enzymes from SP is considered to be a sensitive indicator of the plasma membrane damage and altered membrane functions, which may occur due to inadequate epididymal

maturation associated with increased semen collection frequency (Sirat et al., 1995). Although, Ca, Na, LDH considered important for sperm function, their correlation with semen quality were not significant. Regarding the energy profiles of SP, glucose, cholesterol and triglycerides were correlated positively with apoptotic population. Meseguer et al., (2004) have demonstrated that an increase in both the intracellular and extracellular cholesterol concentrations in human sperm and SP is related with reduced survival rate during the freezing process. Presence of lipids in SP is important because of the ability of sperm cells to take up lipid components or fatty acids from the surrounding environment under determined circumstances (Cerolini et al., 2001). Besides, the transfer of proteins through sperm surface is modulated by the distribution of lipids along the membrane (Parks and Graham 1992). In this given circumstances, SP lipid favors peroxidation and lead to ROS production, which has a detrimental effect on sperm damage as well as induction of apoptosis like changes (Guthrie and Welch, 2005). Indeed, this hypothesis needs further confirmation.

Although the composition of SP can influence membrane stability, but studies on the effect of SP compounds on sperm function are contradictory (Bass et al., 1983). The role and ions channels in regulating sperm function has remained somewhat elusive. Interaction between spermatozoa and the surrounding medium is a crucial factor affecting sperm surface heterogeneity (Ollero et al., 1997), thereby, sperm function largely depend on the period of time that sperm are exposed to different fluids during epididymal transit, at ejaculation, diluents and fluids of the female reproductive tract. The effect of SP on ejaculated spermatozoa during the cryopreservation process has been studied by using different plasma removal techniques such as semen dialysis and centrifugation . Results have also been variable in these cases. Among different biochemical components, researches are mainly concentrated on SP protein parts. Several studies have described SP protein's function in the maintenance of plasma membrane stability (Desnoyers and Manjunath,1992), motility (Henricks et al., 1998; Sánchez-Luengo et al., 2004), capacitation (Therien et al., 1998), sperm-egg interaction and fertilization (Yanagimachi, 1994).

In our study with 1-D PAGE, we found total 11 protein bands (Figure 3.9 a, b, c, d) and almost all corresponded with Protein Profiles described by (Bergeron et al., 2005; Cardozo et al., 2006; Marco-Jime´nez et al. (2008). Among these proteins, TP5 (50 kDa), TP6 (45kDa), TP10 (20kDa) and TP11(15kDa) concentration were higher. In a recent study with ram semen, Marco-Jime´nez et al. (2008) have observed that around 80% of the total relative SP protein is represented by four protein fractions of molecular weight around 15 kDa, 21 kDa, 24 kDa and 50 kDa, and our finding correspond well with this observation. In addition, some proteins fraction such as,TP1 of 100kDa, TP5 (50 kDa), TP8 (37kDa), TP9 (25kDA) and TP11(15kDA) were varied significantly ($P<0.05$) with male (Table 3.8). In ram, previous studies have showed mostly two groups of proteins, one is aSFP (acidic seminal fluid protein) spermadhesins (12-15 kDa) (represent ~45% of total protein) (Teixeira et al., 2002) and another groups include heparine binding RSPs(ram seminal proteins) such as RSP- 15 kDa, RSP-16 kDa, RSP-22 kDa and RSP-24 kDa (represent ~ 20% of the total protein) (Bergeron et al., 2005). Spermadhesins secreted from seminal vesicles (Teixeira et al., 2006) exert an energy-preserving effect by restricting motility and protect sperm against lipid peroxidation maintaining the viability of spermatozoa (Schoneck et al., 1996).

Correlation coefficient between SP proteins and post-thaw semen quality revealed some interesting features. We found a moderate but significant ($P<0.01$) negative correlation between viable cells (CF+PI-) and TP10 of 20 kDa. RSP of 20 kDa is known to have protective effects against cold-shocked and can recover membrane integrity of spermatozoa (Perez-Pe et al., 2001b). In addition, RSPs share heparin and phosphorylcholine-binding properties and are thought to be involved in, first, sperm membrane stabilization **(**Barrios et al. (2005) as a "decapacitating factor", and later in membrane destabilization inducing phospholipid and cholesterol efflux from the plasma membrane for preparation of capacitation, similar to homologous BSPs (Bovine seminal Proteins) (Thérien et al., 1998; Manjunath and Thérien, 2002; (Villemure et al., 2003). Negative

correlation of TP10 (20 kDa) with viable cells may indicate their participation in capacitation process, but this hypothesis needs further confirmation.

On the other hand, some protein fractions such as TP2 (75 kDa), TP6 (45 kDa) and TP9 (25 kDa) were negatively correlated ($P<0.05$) with damaged and dead spermatozoa. More importantly, TP2 (75 kDa) showed a positive relation ($P<0.05$) with apoptosis free viable cells and negative correlation ($P<0.05$) with apoptotic cells (Table 3.10.). We are not aware of literatures presenting similar results or functions of these proteins. Many studies (Barrios B et al., 2000, Mortimer and Maxwell, 2004; Domı´nguez et al., 2008; Leahy et al., 2010) have reported a beneficial effect of addition of SP post-thaw sperm viability and motility and proposed that this action might be resulted from the stabilizing and protective action of some proteins of low molecular weight (15 to 25kDa). Jobim et al. (2004) reported a differences in SP Protein Profile from bulls with low and high semen freezability. On the basis of their study, the acidic protein of (13-16 kDa) could be used as markers of high semen freezability and protein of 25–26 kDa could be a marker of low semen freezability in bull.

However, observed correlation in our experiment strongly suggests future studies on proteins of high molecular weight. In addition, these findings would be of very important in improvement of media using directly protein fractions to maintain higher apoptosis marker free viable sperm subpopulation in cryopreserved semen. Previous research showed a seasonal effects on SP proteins composition and their action (Cardozo et al., 2006). The ability of ram SP proteins to repair and protect spermatozoa against cold-shock is somehow dependent on the season of the year (Pèrez-Pe et al. 2001a). Barrios et al. (2000) and (Dominguez et al (2008) have reported that addition of SP collected during winter or autumn significantly improves total and progressive motility of frozen-thawed ram spermatozoa. In addition, our findings revealed a significant ram variation in SP Protein Profiles which implies an importance of individual males during selection of good or bad freezer.

Sperm response to SP components varies with some factors duration of exposure time, handling and method of sperm preparation (washing; Perez-Pe et al., 2001) and presence and absence of natural and artificial media. The improvement of semen cryopreservation techniques requires in depth knowledge of the gamete physiology and the biochemical processes occurring during semen collection, processing, and freeze-thawing. Our findings hypothesize that SP composition should be considered as a determinate of good freezer and the characterization of "freezability" markers can help in the prediction of post-thawing results, which would be a useful tool in the selection and recruitment of semen donors introduction.

The composition of SP, therefore, is one factor that determines the suitability of individual rams for semen cryopreservation. Further studies will be necessary to clarify a possible physiological role of ions or organic molecules present in SP in the determination and regulation of semen quality, possibly secretary products of accessory sex glands. Moreover, the role of SP composition in the regulation of sperm functions should also be taken into account in the preparation of medium during assisted reproductive techniques in order to preserve and possibly enhance sperm fertilizing ability.

4.1.3. Characteristics of post-thawed epididymal spermatozoa collected from endangered sheep breeds in Veneto region.

The aim of this experiment was to study the post-thaw quality of epididymal sperm collected from Veneto sheep breeds. This study was conducted adjunct with second experiment to compare the sperm characteristics devoid of fluid secreted from accessory sex glands, with ejaculates contained whole SP.

We found 50-50% motile and 62-65% HOST+ve caudal epididymal sperm which were much lower than that of ejaculates. After cryopreservation with TRIS

and skim milk based extenders, all quality parameters were decreased and there was no variation among results from two different extenders. In a study with ram epididymal sperm, Ehling et al. (2006) have observed around 79.7± 0.1% motile sperm fresh sample and post-thawing motility 60.5%, which was much higher from our observation. In their study, sperm treatment was different and they used Salamon's one step freezing medium, which might be a cause of this variation. When, correlation coefficient is observed, motility%, HOST+ve %, viable sperm% showed very significant positive correlation ($P<0.001$) between them, compared with results obtained from ejaculated spermatozoa.

Reports on epididymal sperm are very conflicting. Morris et al. (2002) have stated that *in vivo* fertility of spermatozoa from cauda epididymis tends to be lower than that of ejaculated spermatozoa in cattle and sheep and these differences might be resulted from cell surface characteristics and low motility of epididymal spermatozoa. In stallion, Heise et al. (2010) have observed that exposure of epididymal spermatozoa to seminal plasma during harvesting from the tail of the epididymis increased the fertilising ability of epididymal spermatozoa. Some studies have also revealed no improvement in freezability or fertility of epididymal spermatozoa (Morris et al., 2002; Moore et al., 2005).

Our study reveals that epididymal spermatozoa does not possess similar characteristics of post-thaw ejaculated spermatozoa when undergo same treatment. Due to the significant changes that take place during the sperm maturation process, epididymal and ejaculated sperm would have different responses to various cryobiological stressors (Varisli et al., 2009). It is well documented that ejaculated spermatozoa differ from epididymal spermatozoa in pH (Hammerstedt et al, 1979), respiration, adenosine triphosphate synthesis and consumption (Cascieri et al, 1976), heparin-binding sites (Nass et al, 1990), and the profile of proteins bound to the plasma membrane (Dostalova et al, 1994; Calvete et al, 1996) and epididymal spermatozoa seem to be more resistant to cold shock (Johnson et al., 1980). Upon ejaculation, spermatozoa expose to fluid secreted form accessory sex glands and several beneficial proteins adsorbed onto

sperm surface. It is known that these proteins play an important role in the pprevention of premature activation during physiological transport of spermatozoa and stabilization of the plasma membrane with capacitation inhibitors (Desnoyers and Manjunath, 1992; Villemure et al., 2003). In bull, it was demonstrated that epididymal sperm retrieval using SP is beneficial to enhance sperm overall and progressive motility characteristics and to protect it from morphological abnormalities derived from the freezing process (Guerrero, 2006).

However, we did not examine the effect of SP on post-thaw epididymal sperm quality, which deserves an extensive research area. Optimal cryopreservation of epididymal spermatozoa would have great importance in the preservation of endangered species biodiversity. Therefore, further study is also needed to address how different components, secreted from accessory sex glands, alter the function of epididymal spermatozoa and thereby, it will help to understand the fate of seminal components in artificial media.

The precise nature of active constituents of SP that can help to improve post-thawed semen quality and fertility remains as a promising area of investigation. Proper understanding of beneficial effects of SP treatment for post-thawed spermatozoa could explore new approaches for cryopreservation, such as, widespread utilization of SP from vasectomized males, which can reduce extensive dilution of SP with commercial diluent media or can increase utilization of specific SP proteins to improve the function of semen extenders

4.2. Study of bio-chemical composition of seminal plasma and their influences on quality and freezability of alpaca semen.

4.2.1. Diet effect on semen quality, biochemical composition and Protein Profiles (PP) of seminal plasma(SP) in alpaca.

Reproductive performance is the culmination of many complex and integrated anatomic, developmental, physiologic, and behavioral processes and there is apparently a strong underpinning between nutritive status and reproductive performance (Van Saun, 2008). Various interactions between nutrition and reproduction have been the subject of numerous research in large ruminant. This would be necessary to understand the effect of different food intake and as consequence for optimizing the supply of nutrient in alpacas. There is few and fragmented information on the effects of diets on semen quality of alpaca. Therefore, this study was designed to determine the effect of diet on SP composition and semen quality. To our knowledge, there are no studies in male alpaca, based on diets, comparing the effect on reproductive function.

Data from our study revealed considerable animal to animal and week to week variations with regard to seminal attributes. Considerable variation in semen quality parameters in individual male was observed in the experiment, agreeing with previous reports on male variation (Buendia et al. 2002; Flores et al. 2002; Vaughan et al. 2003; Giuliano et al.,2008).

In the present study, the average time of semen collection by artificial vagina was 9-20 min. This confirms previous reports of the duration of semen collection in alpaca (10-20 min) reported by Brwon (2000). No significant changes was observed in collection time and mating length was some how constant for individual. Similar observation was reported by Tibary et al. (1999). The length of copulation is determined by the male and is affected by breed, age, season and frequency of use (Tibary et al. 1999). The effect of extended mating time on semen quality cannot be ignored in alpaca. The extended collection time results in semen being exposed to latex, high temperatures and atmospheric conditions leading to change in the ratio of the constituents and altering semen osmolarity and pH (Morton et al., 2010). Type of rubber in artificial vagina and length of

time semen stays in contact with the liner is said to have great effect on individual motility of spermatozoa (Deen and Sahani, 2000). During the study period, ejaculate volume ranged from 1-7 ml. This confirms previous report on alpaca semen volume ranged from 0.4 to 12.5 ml by Garnica et al. (1993).

Regarding the consistency of semen, the viscid nature of semen was specific for individual alpaca and did not change with diet. The degree of viscosity varies between males (Tibary et al. 1999) and decreases with increasing number of ejaculates on any day (Bravo et al.,1997b). The viscous nature of SP is a result of the presence of mucopolysaccharides from secretions of the bulbourethral glands or the prostate (Garnica et al., 1993), referred to as glycosaminoglycans; GAGs) which are made up of 95 % long chain polysaccharides and 5 % protein (Morton et al., 2009). Recent studies with human semen have demonstrated that the existence of a highly organized network of disulphide bonds, oligosaccharide and peptide chains in SP is responsible for the rheological characteristics of ejaculates with high viscosity and these molecules, that would be responsible for the hyperviscous rheological behavior, could be a key factor in sperm physiology and in sperm motility (Mendeluk et al., 2000). Viscous SP plays as reservoir for spermatozoa and the entrapped spermatozoa within viscous SP may be an adaptation in alpaca to ovulation, which occurs 36 h after copulation (Garnica et al., 1993).

A foam layer was found in most viscid ejaculate. Foam formation during ejaculation is well reported characters for camelids specially, when semen collected with artificial vagina (Ferre et al. 1996; Vaughan et al. 2003; Giuliano et al. 2008) and may result from males retrieving and reinserting their penises into the collection container (Ratto 2005).

Sperm concentration varied 20 to 240 million/ml which is slightly higher than that reported previously in this species (30,000 up to 150 million per ml) (Bravo 1995; Garnica et al. 1995; Gauly and Leidinger 1996). Such wide variations are attributed to differences in males, semen collection methods and ejaculate number (Tibary and Memon 1999). There was a marked decrease in sperm

concentration during 3° and 4° period when animals were allowed to pasture grazing and supplemented with sheep and horse concentrate respectively. In the mean time, changes in testicular size was less marked. This findings does not correspond with previous reports where authors observed a correlation between sperm production and testicular size in camelids (Tibary et al. 1999, Galloway 2000).

The motility of individual samples ranged between 5% and 80 % demonstrating high levels of variation between males, while the mean motility was low (< 35 %). The average sperm motility is within range (15. 3% to 63. 7%), reported for alpaca semen collected with artificial vagina (Bravo et al. 1997; Vaughan et al. 2003a). Only oscillatory motility were observed in all experiments. This is in agreement with previous studies which described only oscillatory motion (Garnica et al. 1993; Bravo et al., 2000; Giuliano et al., 2002; Vaughan et al.,2003). In contrast, Morton et al. (2010) have observed both oscillatory and progressive motility depending on viscid nature of the semen. Tibary and Memon (1999) also observed that sperm could gain progressive motility upon liquefaction of the semen. Motility% significantly decreased at last two periods (Figure 3.12.).

Results from biochemical analysis demonstrated that total protein and glucose of SP were not changes, while, concentrations of cholesterol, triglyceride, GOT, ALP and ALT were changed significantly ($P<0.05$) (Table 3.14 and 3.15).As stated in previous section, SP enzymes such as ALT, ALP, and GOT, are essential for metabolic processes, which provide energy for viability, motility and fertility of spermatozoa and the measurement of these enzymes from SP is considered to be a sensitive indicator of the plasma membrane damage (Sirat et al, 1998). The values obtained in our experiment were higher than that was reported previously (El-Manna et al. 1986; Zhao et al. 1992; Garnica et al. 1993; Bravo et al. 2000c; Agarwal et al. 2004). 1-D PAGE, showed eight protein bands,, and all bands were not present in all males (Figure 3.17 a, b, c, d, e). Among these protein profiles, TP3 of around 60 kDa was abundant. During, the study period, there

was insignificant change in protein profiles. In camelids, research for identification and quantification of protein has mainly been concentrated on a specific protein fraction of high molecular weight, which has GnRH like activities (Paolicchi et al, 1999; Pan et al, 2001), termed as ovulation inducing factor (OIF). Studies on proteins of smaller molecular weight are very limited. Moreover, seminal vesicle is absent in alpaca, which is known to be the main source of most of low molecular weight proteins, described for small ruminants and source of these proteins remain mystery. Unlike bull, ram, homologous proteins of RSPs or BSPs are still not confirmed. Our 1-D PAGE demonstrated the presence of TP8 of 14-15 kDa in alpaca SP which is known as to protect sperm cell from cold shock. Our previous study on alpaca SP protein have demonstrated that this protein fraction is related with alpaca semen freezability (Marion et al., 2010).

Our study revealed that sheep concentrate or horse concentrates exerted a negative effect on semen quality. The only positive effect found was increased volume of the semen. Some studies with other ruminants reported that young bulls kept on grazing plus concentrate feed showed better growth rate than those on grazing alone, with an earlier age at puberty and a greater scrota1 circumference (Perry et al. 1991; Tegegne et al. 1992). This response variation might be resulted from inherent metabolic systems of alpaca and may force to consider specific diets for alpaca. However, there is a true paucity of literature regarding nutritional mediation of reproductive performance of alpacas. The mechanism by which diet affects reproductive capacity is not clear. Diet induced changes might be resulted from variation in gonadotrophin levels, which would affect endocrine-gonadal function and secretions of accessory glands. The mechanisms underlying the changes in some reproductive traits in response to feeding regimen, would be related to changes in sexual hormones secretion, such as testosterone . Protein part of the diet could be responsible for the dietary effect and would act by a GnRH-independent pathway (Hötzel et al., 1995 & 1998). Several studies have shown the critical interaction between reproductive performances and protein status (Ferguson, 1989; Tamminga, 2006). Al-Haboby

et al. (1999) reported a negative effect of protein supplementation on semen quality, but this effect was presumably related to an excess of urea consumption, as the authors suggested. It has also been reported that under protein stress may result in smaller Leydig cells and seminiferous tubules, and decreased testosterone level conditions (Nolan et al., 1990). Research in other ruminants shows that both low protein diet and excess protein diets exert a diminished effect on pulsetile LH secretion through altered body metabolism (reviewed by Kaur and Arora, 1995). Presumably similar mechanisms are in place to describe the alteration in reproductive performances along with SP composition following diets treatment. But it demands further confirmation.

Energy intake has a greater effect on sexual activity than protein supply because this activity implies an important physical effort and therefore an important energy expenditure (Parker and Thwaites, 1972; Brown, 1994). High energy diet could exert a negative effect on sperm quality by increasing scrotal surface temperature leading to decreased sperm motility (Coulter et al., 1975). Beyond, protein, and their interaction, dietary status of many minerals and vitamins present in diets also influences reproductive performance (Smith and Akinbamiji, 2000; Wilde, 2006). Apart from the diet effect, time of the study period and environmental temperature cannot be ignored in alpaca. A seasonal variation in SP composition specially on proteins is well documented in small ruminants (Cardozo et al. 2006). These seasonal changes in Protein Profiles could be resulted from seasonal variation in gonadotrophin levels and their receptors in the testes (Xu et al., 1991) that affect endocrine gonadal function and secretions of epididymes and seminal vesicles. However, many previous studies referred to the variation in semen parameters within and between alpaca males, although none specifically investigated this variation. Our study could provide a base information regarding alpca diet formulation in improvement of reproductive performance. Future research sould be carried out to investigate the carry-over effects of different nutritional regimes on alpaca reproduction.

4.2.2. Effect of diluents on freezability of semen and their relationship with biochemical composition and Protein Profiles (PP) of SP

There is considerable interest in the use of AI in camelid breeding programs. this technique has not been widely applied to camelids because of difficulties in developing suitable protocols for dilution and conservation of alpaca semen to preserve the fertilising lifespan of semen in frozen form. Moreover, lacking of proper and effective diluents, and limitations in knowledge of biochemical structure of semen are also constrains for semen processing. This experiment was, therefore, conducted to study the relation to SP component with sperm freezability. We conducted two experiments using four types of extenders based on Tris and skim milk . Quality of both fresh and post-thaw semen was evaluates by assessing semen with fluorescence staining for plasma membrane integrity, Annexin V assays for apoptosis marker along with motility and hypo-osmotic swelling test.

In alpaca, assessment of sperm function has been limited to sperm motility. The viscosity makes semen samples hard to divide into aliquots, smear on slides (to assess integrity and viability and dilute with extenders (heterogeneous mixing results in poor post-thawing motility). SP also interferes with staining of the sperm making assessment of integrity and viability difficult (Morton et al., 2009). There are few reports on evaluation of viability, acrosome integrity using different stain (Aller et al., 2003; Morton et al., 2010) or other immuno-labeling technique (Valdivia et al., 2000;Alexi et al., 2005). Many of the sophisticated tests used to assess sperm from cattle, sheep and pigs had not been developed for alpacas. Very few tests have been established for alpacas, and those articles provided only limited information about the effects of certain procedures on sperm viability. The accurate measurement of basic semen parameters is vital if sperm function and integrity are to be determined. The examination of a number of parameters provides clues to the fertilising potential of the sperm for the development of artificial reproductive technologies. A wide range of functional

tests are now available for the assessment of livestock sperm (Gillan et al.,2005). The present study assessed the outcome of cryopreservation in alpaca semen as well as the utility of the Annexin-V binding assay in evaluating sperm quality.

There was significant ($p<0.05$) negetive correlation between sperm concentration and semen volume, semen viscosity (Table 3.23). Increased semen volume indicates functional status of accessory glands since major part of semen contributed by different accessory glands secretion.

Previous reports indicated that semen composition remains constant throughout ejaculation (Morton et al., 2008). While, others have observed that sperm concentration, percentage of active sperm, percentage of live sperm and percentage of normal sperm increased with time during a single copulation (Bravo et al. 2002).

After cryopreservation, motility drastically declined among all treatment groups with a percentage of motile sperm 2-5% (Figure 3.19.). Some studies differ from our results. In alpaca semen, motility after thawing was lower than 20 % (Valdivia et al., 2000). Whereas Bravo et al. (1996) reported motility 30- 40 % after freezing semen from alpacas. Most extenders used for the freezing of camelid semen have been prepared combining TRIS, citrate, glycerol and egg yolk (Valdivia et al., 2000,Aller et al., 2003). In relation to the extenders used, Alexi et al. (2005) have obtained 30% viability in post-thaw sperm diluted with skim milk based extender containing egg yolk, fructose and ethylene glycol. In the same experiment, they observed less than 10% motility when semen frozen with TRIS, citric acid, glucose and egg yolk with 7% glycerol. But in another study, extender-containing TRIS was reported to be superior than that containing 11% lactose (Vyas et al., 1998).

The structural viscosity make semen handling in the laboratory difficult (Tibary and Vaughan, 2006), hindering the separation of spermatozoa from SP and the separation of motile spermatozoa from immotile ones either using centrifugation or other methods (Maxwel et al., 2008).Therefore, different

enzymes (trypsin, collagenase, fibrolysin, hyaluronidase and papain) have been used to decrease SP viscosity camelids (Bravo et al.,2000; Giuliano et al., 2002; Poblete et al., 2003; Maxwel et al., 2008). Simultaneously, deleterious effects of these enzymes on spermatozoa depending on the time of incubation or the enzymes concentration (Tibary and Vaughan, 2006) is also reported. Owing to find out relationship of SP components with fresh and freez-thaw sperm quality we did not use any enzyme to liquefy semen in our experiment. Therefore, the lower motility might be resulted from the viscid nature of the fresh semen and negative correlation between these two parameters observed in our study confirms this hypothesis

The methodology applied to develop sperm cryopreservation protocols remains mostly empirical because of a lack of conformity between data derived from theoretical work and the observed results, despite the progress of fundamental cryobiology.

Although routine laboratory test can be used to evaluate sperm sample rapidly, they don't incorporate information on sub-cellular physical damage that occur during cryopreservation (Christensen et al., 2000). Combination of propidium iodide (PI) with carboxyfluorescein diacetate (CFDA) to evaluate sperm functions enables discrimination between live and dead or moribund spermatozoa but cannot detect early phases of disturbed membrane functions (Garner et al., 1986). The Annexin V- binding assay seems to provide additional information about plasma membrane deterioration besides conventional motility analysis and supravital staining.

We observed around 30% apoptotic cells in live population, that was hgher than we observed in ram and after cryopreservation, number decreased (Table 3.20). The present study is the first, to the best of our knowledge, which indicate presence of apoptotic sperm in alpaca semen and its correlation to sperm motility and plasma membrane integrity. urprisingly, we observed percentage value of viable and HOST+ve sperm was higher in comparison to sperm motility (Table 3.20).

Peroxidative damage to the sperm plasma membrane is thought to be an important cause reducing sperm motility, and sperm with unsaturated fatty acid enriched plasma membranes are particularly susceptible to oxidative stress (Jones et al. 1979). Unsaturated fatty acids give the plasma membrane the fluidity required to participate in fertilization and the fatty acid double bonds are attacked by ROS altering membrane fluidity which leads to a loss in sperm viability (Aitken and Krausz 2001). But, higher values of HOST+ve and viable sperm from two fluorescence assays revealed that this lower motility was due to their inherent characteristics to maintain energy to be alive. The higher percentage of viable cells in alpaca semen confirms the finding of Glander et al (1999) in human spermatozoa and suggests that not all immotile spermatozoa represent dead population. Genital fluids exert an influence on sperm motility. Some sperm that are immotile in vitro might regain motility in vivo, and vice versa (*Blach et al*. 1989).

Motility alone as a parameter, should not be used for alpaca semen appraisal as immotile sperm could maintain intact plasma membrane.

Bio-chemical composition

Most of the SP proteins are secretary product of seminal vesicle which is absent in alpaca. So, the presence and function of some low molecular SP proteins (identified in other ruminants) is questionable in alpaca. Moreover, there is lacking of data describing the relation of different macromolecules and enzymes with semen quality. Therefore, in the present study, we studied the relation of Protein Profiles (PP) and bio-chemical components with post-thawed sperm quality in alpaca. Biochemical characteristics of seminal fluid are different in the various portions of the male genital tract especially when considering ionic concentrations and pH (Tuck et al., 1970; Hinton et al., 1981). Such differences could play an important role in the regulation of important sperm functions as well as motility, capacitation and fertilizing ability acquisition (Eliasson, 1970; Eliasson and Lindholmer, 1972).

In this experiment, we also studied biochemical composition of SP and their relation with fresh and post-thaw sperm quality. The values of difrent components obtained in this study (Table 3.24) correspond to that of previously reported (El-Manna et al. 1986; Zha et al. 1992; Garnica et al. 1993; Bravo et al. 2000c; Agarwal et al. 2004). Correlation coefficient among different quality parameters and biochemical components revealed some interesting features. We found a positive significant correlation with total protein, Mg and apoptosis free viable cells, which was in contrast that we observed in ram semen. For screening the interrelationship among SP content and semen quality at different stages of cryopreservation, we examined relation between cooled semen quality and SP content (Table 3.25 and 3.26). There was insignificant relation between SP macroelements and sperm motility and plasma membrane integrity. Correlation was significant between sperm motility and P, Mg, Ca,TP in S and T groups. Consequently, an insignificant relation was existed between semen quality parameters and SP in F, G, and T groups. It is note worthy to point out that we observed a significant ($P<0.01$) correlation between post-thaw sperm motility and Mg, P, TP, Cholesterol, that we observed for fresh alpaca semen. More importantly, we found positive significant ($P<0.01$) correlation between TP and Mg with post-thaw apoptosis free viable population. This findings might indicate that skim milk based extender could maintain post-thaw sperm quality and less effect on SP induced environment beneficial for sperm survival.

In our study, we used three types of Tris based diluents containing 0.8% glucose (G), 0.8% fructose (F) and 1% fructose (T), whereas with skim milk based extender (S) we did not use fructose or glucose. Considering the extender, the post-thaw seminal parameters were higher in F group with 0.8% fructose, there was no wide difference between F and S groups. It is important to mention that SP, glucose is the principle sugar in Camelids, which is converted into fructose either by phosphorylated pathway or by monophosphorylated pathway through sorbitol dehydrogenase and aldose reductase (Agarwal et al, 2004). Information on total fructose concentration in SP of alpaca could help in the determination of appropriate concentration of fructose to be added into semen diluents.

Moreover, observation of correlation coefficient with post-thaw semen quality in S group is very promising and could help in the better formulation of extender specific for alpaca semen.

We conclude that analysis of both biophysical and biochemical characteristics of semen provide very useful information regarding semen freezability and male fertility.

The development of freezing technology in alpacas is mainly based on information derived from successes achieved in other species, from which, satisfactory result could not be obtained in alpaca. For successful cryopreservation we need to define a good ejaculate before processing. Determination of the biological make-up of the semen is very important for a better understanding of bio-physical and bio-chemical characters of seminal plasma (SP). This study could provide the basis for variation in semen quality between animal, which can help us in formulation of good diluents, to understand bio-chemical reactions that occur between SP and media and also to study the fate of spermatozoa in the media. It could help for utilization of specific SP proteins or elements to improve the function of semen extenders, which could explore new approaches for preservation of alpaca semen and also to overcome failures in reproductive function in South American Camelids.

CHAPTER 5. CONCLUSION

Spermatozoa undergo a dramatic loss of heterogeneity, viability, motility and positive response to HOST during cryopreservation process. We observed an interrelation between all of these seminal parameters, which indicate the necessity of fully potential and functional spermatozoa for successful fertilization with cryopreserved semen. Our results confirm that biochemical composition and SP function varies with diet, ejaculate within male and between males and species. Pool of first and second ejaculates promotes post-thaw sperm survivability when frozen with Tris-citrate-egg yolk based extender in ram semen. Our study revealed that Some SP components can be used freezability marker, and apoptotic free viable sperm population positively correlated with protein fraction of molecular weight 75 KDa and negatively correlated with total protein, magnesium in ram semen.

Alpaca spermatozoa are highly susceptible to cryopreservation. Our study suggest that skim milk based extender could maintain survivability of cryopreserved spermatozoa and maintained good relation between seminal plasma components and post-thaw semen quality. Annexin fluorescence assay can be used in this species to detect apoptotic sperm in viable sperm subpopulation. More importantly, we observed that Magnesium and total protein of SP showed a positive correlation with apoptosis free viable cells, which could be predictor for successful cryopreservation. The composition of SP, therefore, is one factor that determines the suitability of individual male for semen cryopreservation and should also be taken into account in the preparation of medium during assisted reproductive techniques in order to preserve and possibly enhance post-thaw sperm fertilizing ability.

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APPENDIX I – LIST OF FIGURES

APPENDIX II – LIST OF TABLES

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