COMPARATIVE STUDY ON CERTAIN FUNCTIONAL AND BIOCHEMICAL PARAMETERS IN FRESH AND FROZEN SEMEN OF BUFFALO BULLS WITH VARYING REPRODUCTIVE PERFORMANCE

THESIS

By

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CERTIFICATE I

This is to certify that the thesis entitled **"Comparative study on certain functional and biochemical parameters in fresh and frozen semen of buffalo bulls with varying reproductive performance "** submitted in partial fulfillment of the requirements for the award of the degree of **Master of Veterinary Science** in the discipline of **Animal Reproduction, Gynaecology and Obstetrics** of CSK Himachal Pradesh Krishi Vishvavidyalya, Palampur is a *bonafide* research work carried out by **Dr. Yogita Dhaka** (Admission No. V-2010-30-08) daughter of Smt. Bidami Devi and Sh. Hanuman Singh Dhaka under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been fully acknowledged.

(Madhumeet Singh)
Major Advisor

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CERTIFICATE II

This is to certify that the thesis entitled **"Comparative study on certain functional and biochemical parameters in fresh and frozen semen of buffalo bulls with varying reproductive performance"** submitted by **Dr. Yogita Dhaka** (Admission No. V-2010-30-08) daughter of Sh. Hanuman Singh Dhaka to the CSK Himachal Pradesh Krishi Vishvavidyalya, Palampur in partial fulfillment of the requirements for the award of the degree of **Master of Veterinary Science** in the subject of **Animal Reproduction, Gynaecology and Obstetrics** has been approved by the advisory committee after an oral examination of the student in collaboration with an External Examiner.

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CHAPTER 1

Introduction

Buffalo, through their potential for producing milk, meat and draft power, contribute significantly to the agricultural economy of many developing countries including India. Though, India ranks first in world buffalo population with 97.922 million buffaloes, (58.21 per cent of world buffalo population; 17th Livestock Census 2003), one of the major constraints in maximizing the production of buffalo is their inherent low reproductive efficiency.

Artificial breeding of buffaloes on large scale using semen from bulls with superior germplasm can solve the problem of low productivity as well as reproductive efficiency. Artificial insemination (AI) has been extensively exploited as a breeding tool for rapid improvement in the genetic potential in farm animals including buffaloes.

The success of AI technique is associated with effective prolongation of fertile life of spermatozoa obtained from genetically superior bulls under *in vitro* storage conditions. The quality of semen and seminal plasma are the main factors determining freezability and post-thaw fertility. The routine evaluation of semen in the laboratory of an AI stud include assessment of volume, pH, sperm concentration, mass activity and initial motility of the fresh semen and motility post-thaw and are valuable in order to access its quality and freezability. A high percentage of abnormal and dead spermatozoa in semen can have an adverse effect on their fertilizing potential (Heuer and Tahir 1982). The dead and abnormal spermatozoa are also known to exert toxic and lytic effect on motile sperm cells in the ejaculate (Shannon and Curson 1972), which leads to lower fertility. In addition, it has been established that sperm membrane functional status measurements as assessed by HOS test, seemed to be of clinical and practical importance in evaluating high and low fertility bulls and assessment of sperm membrane functional status should be considered as an additional parameter for the evaluation of viability and fertilizing capacity in frozen-thawed bovine spermatozoa used for all forms of Advanced Reproductive Technique (Correa *et al.* 1997).

During last three decades, extensive research work has been carried out both in India and abroad on various aspects for improving the freezing technology of buffalo bull semen. In spite of this improvement, the post-thaw semen quality of buffalo bulls is not as good as that of cattle (Dhami *et al.* 1995). The viability and fertility of frozen–thawed buffalo bull spermatozoa is considerably lower as these are more susceptible to hazards during freezing and thawing than cattle spermatozoa which create hurdles in extensive exploitation of production potential of buffalo. Moreover buffers, cryoprotectants, antibiotics, other agents and various cooling, freezing and thawing rates initially developed for cattle bull spermatozoa have been used for freezing of buffalo semen, at times, with contrasting results. Therefore, a better understanding of the fundamental principle of cryopreservation of buffalo spermatozoa is necessary as per its specific requirements.

Biochemical parameters of semen are of vital importance from its fertility and preservation aspects. There are specific biochemical factors that affect the ability of spermatozoa to prevent damages caused by the cryogenic procedures. The available data on the biochemical composition indicate that biochemical properties of buffalo bull semen differ from those of cattle semen in several aspects (Ganguli 1980). But there is scarcity of parallel information about the semen of the buffalo bulls. One of the many possible causes of lower freezability of buffalo bull semen compared to cattle bull can be due to the differences in the lipid ratio of the spermatozoa (Andrabi 2008). Freezing-thawing of buffalo spermatozoa is known to cause considerable damage to DNA, motility apparatus, plasma membrane and acrosomal cap (Rasul *et al.* 2001), leakage of intracellular enzymes (Dhami and Kodagali 1990) and thus, reduced fertility.

More than 40 per cent of AI sires may have one or the other problem affecting semen quality, freezability and fertility (Dhami and Shelke 2005). Although some of the bulls may apparently donate good quality semen but the freezability may be poor adversely affecting fertility, whereas not so poor quality semen may have acceptable freezability and fertility. Hence periodic detailed andrological investigation is prerequisite to successful breeding programme.

Evaluation of plasma membrane integrity, motility, vigour and morphology of fresh and frozen thawed semen along with the seminal characteristic as well as

biochemical and enzymatic profile of seminal plasma and their interrelation might establish a basis for the predicting the fertility potential of semen.

Hence the objectives of this investigation were;

a. To compare seminal attributes between the buffalo bulls of good and poor semen.

b. To determine the effect of freezing on biochemical characteristic of semen plasma.

c. To establish correlation between microscopic quality evaluation and biochemical analysis of buffalo bull semen.

CHAPTER 2

Review of literature

Cryopreservation has revolutionized the concept of storage of semen. Artificial insemination (AI) with frozen semen has proved as the most potent method for rapid genetic improvement, economization of breeding program and control of venereal diseases of domestic animals. This has been possible by selecting few highly fertile males to produce enough spermatozoa to inseminate thousands of females per year (Hafez 1993). So, a bull is aptly said to be half of the herd. To select breeding bulls, evaluation of semen is of prime importance.

The numerous effects that cryopreservation can induce in spermatozoa, range from lethal injuries to those that merely impair their subsequent function. In the last few years, a considerable increase in the understanding of both, the cell physiology of spermatozoa and the stress of cryopreservation, have contributed to a renewed interest in improving the performance of cryopreserved semen. Despite the significant progress, the post-thaw viability and fertility of the cryopreserved sperm is still reduced and is attributed to accumulated cellular injuries arising throughout the cryopreservation process. Many laboratory tests on semen samples validate to verify the detrimental effects and their origin during cryopreservation.

The inspection and handling of semen is considered a key and essential step for assessing fertility and the successful use of semen respectively. Knowledge of fertility in bulls is an objective of great importance for the production of semen, which is substantiated by good analysis of the semen. The ideal semen analysis should be simple and effective, allowing the breeding capacity of a particular ejaculate to be predicted. A fertile semen ejaculate must meet certain quality standards, pertaining to progressive motility, morphology, energy metabolism, structural integrity and functionality of the membrane, penetration capacity and optimum transfer of genetic material. It is assumed that these measurements provide information about the status of spermatogenesis in the male and fertilization potential by the sperm (Saacke 1983; Jeyendran *et al.* 1984; Amann 1989). The current work on semen analysis seeks to identify some kinetic, morphological or biochemical parameters indicating the status of

the sperm cell at any given time, concomitantly correlating with fertility and ejaculate quality. In routine production, the tests should be accurate, simple, fast and economical.

2.1 Initial parameters

Determining the initial quality of ejaculate is the first step in semen processing and should ensure that a high quality artificial insemination dose of semen will be produced after processing. Ideally, ejaculates that are thoroughly evaluated prior to processing help identify poor quality semen.

Semen evaluation must be done rapidly and in a short time after collection, to allow processing. A careful corroboration of amongst different test results offers selecting of high fertilization capacity ejaculate. Ejaculates are generally analyzed for colour, odour, viscosity, pH, concentration and motility, anomalies. Of these, concentration and motility are perhaps most routinely used for sorting ejaculates prior to processing since they require the least amount of time and decide semen doses per ejaculate. Measuring sperm concentration or total numbers of spermatozoa is not a sole component of semen quality evaluation, but more so, as a tool to monitor the health and productive output of the bull thereby optimizing the genetic potential of a single individual. Hence, the semen quality estimates, as discussed in the succeeding text, must be integrated into processing as a means to ensure that sufficient number of viable sperm are used for insemination

2.1.1 Volume

The volume of ejaculate varies from bull to bull and within each bull. In general, the volume of the semen increases with age, and also depends on the general and reproductive health of the animal and the frequency of its ejaculation.

The sexually matured and vigorous buffalo bulls produce considerably less semen as compared to cow bulls of similar status (Bhattacharya 1962). There are not significant breed differences in semen volumes of ejaculates in cow bulls. However, in buffalo bulls, variation in semen volume is attributed to reproductive health condition, age, frequency of collection, pooled volume, nutrition, season and management of bulls (Salisbury and Van Denmark 1962; Nazir 1988; Vale 1994). Contrarily, no differences have been found among buffaloes breeds in different countries (Alexiev *et al.* 1994; Mishra *et al.* 1994).

The ejaculated seminal volume varies depending on age of buffalo bull which shows increasing trend in swamp and riverine buffalo (Pant *et al.* 2003).

Similarly, Nordin *et al.* (1990) and Younis (1996) also reported higher ejaculatory volume in adult and old bulls than in young buffalo bulls. This indicates that buffalo bulls produce more volume of semen in older age as compared to younger age due to unexplained phenomenon. Contrarily, Javed *et al.* (2000) observed no significant difference in the volume of semen between postpubertal buffalo bulls of various age groups.

Seasonal variations have been observed in average semen volume (Chaudhary and Gangwar 1977). The mean value of semen volume per ejaculate was lowest during winters (mid November - mid February) and maximum during rainy season (mid July - mid November).

Several authors have estimated the average semen volumes in different buffalo breeds ranging from 1.5-5.0 ml in Murrah bulls (Sidhu and Guraya 1979; Pandey and Gupta 2004), 3.59 to 4.67 ml in Nili-Ravi bulls (Javed *et al.* 2000; Sajjad *et al.* 2007), 1.0 to 4.0 ml in Surti bulls (Kodagali *et al*. 1973), 5.02 to 5.32 ml in Jafarabadi bulls (Kerur 1971), 2.70 to 4.88 ml in Egyptian bulls (Abdou *et al*. 1977), 4.10 ml in Iraqi bulls (El-Wishy 1978) and 3 to 4 ml in Thai swamp buffalo bulls (Sukhato *et al.* 1988; Koonjaenak *et al.* 2007a).

2.1.2 Sperm concentration

Marked variations in the concentration of buffalo bull spermatozoa have been reported by various workers. The number of spermatozoa per unit volume may be as higher as 2000 X 10^6 sperms per ml in occasionally good samples with an average of 1200 X 10^6 spermatozoa per ml.

Sperm concentration in buffalo bulls ranges from 700 to 1600 million spermatozoa per ml (spz/ml) (Gopalakrishna and Rao 1978; Kunavongkrit and Bodhipaksha 1978; Heuer and Tahir 1982; Jainudeen *et al.* 1982; Mathias and Yusuf 1985; Sukhato *et al.* 1988; Pant *et al.* 2003). The older bulls having a higher sperm concentration (Nordin *et al.* 1990).

Sperm concentration in fresh semen of buffalo bulls in various experimental conditions have been tabulated in table R1.

Table R1: Sperm concentration in buffalo bull semen.

Species	Value (10^6 cells/mL)	References
Swamp buffalo (Thailand)	900-1000	Kunavongkrit and Bodhipaksha (1978)
Nili Ravi Buffalo	1000±500	Heuer and Tahir (1982)
Nili Ravi Buffalo	800-1000	Jainudeen *et al.* (1982)
Murrah buffalo	524.1±20.7	Rahman *et al*. (1991)
Surti Buffalo	940	Rahman *et al*. (1991)
Water buffalo (Italy)	690.6±187.9 to 1290.7±100	Galli *et al.* (1993)
Murrah buffalo	1031.4±28.7	Kumar *et al.* (1993)
Buffalo bull (Brazil)	1166.3±17.5	Aguiar *et al.* (1994)
Nili Ravi Buffalo	1060±500	Javed *et al.* (2000)
Nili Ravi Buffalo	1136±164	Kanwal *et al.* (2000)
Nili Ravi Buffalo	1005.45±69.06	Hussain *et al*. (2002)
Swamp buffalo (Thailand)	800 to 1200	Koonjaenak *et al.* (2007a)
Buffalo Bulls	1377.14±61.22	Shoushtari *et al.* (2009)
Kundhi Buffalo	11.35±1.25	Kunbhar *et al.* (2011)

In Murrah buffalo bulls, Kumar *et al.* (1993) estimated sperm concentrations range from 524.1 $\pm 20.7 \times 10^6$ to 1031.4 $\pm 28.7 \times 10^6$ cells/ml, whereas Pandey and Gupta (2004) recorded slightly higher concentration 1335.42 $\pm 58.36 \times 10^6$ cells/ml. Various workers (El-Wishy 1978; Nazir 1988; Raizada *et al.* 1988; Terezinha *et al.* 1991) reported higher sperm concentration (1650, 2900, 1150 and 1330 $\times 10^6$/ mL) in buffalo bulls.

Such variations can always be expected from workers working at different places with a variation in the number of animals selected, age groups etc. However, it can be

inferred from different reports that the buffalo bull produces semen with a sperm concentration ranging from of between 940 to 1650 X 10^6/ mL. (Javed *et al.* 2000)

A number of factors such as the sexual development and maturity of the bull, nutrition, reproductive health, size of testes, breed, age of the bull and climate affect the concentration of spermatozoa in ejaculates. Javed *et al.* (2000) observed that sperm concentration was lower in abnormal than in healthy bulls. Settergren (1994), Veeramachaneni *et al.* (1986) and Ahmad *et al.* (1988) also reported lower sperm concentration from bulls showing inflammatory, degenerative and hypoplastic conditions of testes. Rossi *et al.* (1975) related the decrease in sperm concentration with severity of testicular lesions. It can therefore, be inferred from these reports that the buffalo bulls upto the age of 15 years produce semen with a sperm concentration between 0.94 and 2.90 x10^6/µl. Javed *et al.* (2000) attributed lower sperm concentration in older than in younger bulls due to senility.

Routine measurement of sperm concentration is done either by spectrophotometer or haemocytometer. (Pant *et al.* 2003). Woelders (1991) suggested that the fluorimetric measurement of the amount of DNA might give a reliable estimate of concentration of spermatozoa.

2.1.3 Mass activity

Mass activity is a function of sperm concentration and percentage of motile spermatozoa. The mass activity recorded by different researchers in different breeds of buffalo bulls is presented in table R2

Table R2: Mass activity of spermatozoa in buffalo bull semen.

Species	Value	References
Buffalo bulls	2.93 (0-4 scale)	Heuer and Tahir (1982)
Buffalo bulls	2.53 (0-4 scale)	Vyawanare *et al.* (1989)
Nili-Ravi buffalo bulls	1.88±0.07(0-3 scale)	Younis (1996)
Nili-Ravi buffalo bulls	2.65±1.14(0-3 scale)	Javed *et al.*(2000)
Nili-Ravi buffalo	2.5 (0-4 scale)	Hussain *et al.* (2002)
Murrah buffalo	3.24±0.14 (0-5 scale)	Pandey and Gupta (2004)
Iran Buffalo Bulls	3.59±0.16(0-5 scale)	Eghbali *et al.* (2010)
Kundhi Buffalo bull	2.85±0.11(0-3 scale)	Kunbhar *et al.* (2011)

Mass activity is reported to be higher in adult than the older bulls (Saeed 1988; Younis 1996; Javed *et al.* 2000) probably due to higher sperm concentration and low sperm abnormalities in the former.

Mass activity from the adult healthy swamp buffalo exhibits quick to very quick waves of mass activity with the average percentage of initial progressive motile spermatozoa ranging from 65% to 80% depending on the age of the sires (Sukhato *et al.* 1988; Koonjaenak *et al.* 2007a).

2.1.4 pH of semen

pH of semen depends upon sperm concentration in the semen sample and samples with increased density have lower pH value (Ahmed *et al.* 2003). The normal pH of buffalo bull semen is 6.8 to 7.2. Excellent quality semen gives pH of 6.8 while poor quality shows pH ranging from 7.0 to 7.2. The poor quality semen contains larger amount of fluid from urethral and accessory glands (Salisbury and Van Denmark 1962). The pH of semen also decreases with time lapse between collection and measurement of pH since the fructose in the semen is metabolised to lactic acid by spermatozoa under anaerobic conditions.

According to Younis (1996), the pH of the semen was higher in low breeding season than the peak breeding season, probably due to higher sperm concentration noted in peak breeding season than low breeding season. The pH of semen is also closely related to mass activity and frequency of collection (Barnabe *et al.* 1992). Bovine semen quality was better in winter than in other seasons (Bhosreker *et al.* 1988).

Buffalo semen has a pH within the range of 6.25 to 7.2 (Alexander *et al.* 1971; Rattan 1990; Terezinha *et al.* 1991; Kumar *et al.* 1993; Aguiar *et al.* 1994; Younis 1996; Vale 1997; Sajjad *et al.* 2007). In Murrah buffalo bulls the recorded pH was 6.51±0.46 (Pandey and Gupta 2004) whereas it was 6.55±0.50 in Nili-Ravi buffalo bulls (Javed *et al.* 2000). The variation in overall semen pH may possibly be due to a difference in the duration of studies, number of observations made and quality of bulls.

2.2 Functional parameters

The traditional evaluation of the quality of ejaculate has been mainly based on routine semen analyses (motility, morphology and acrosomal integrity), but, these have a limited capacity for the prediction of the potential fertility of an ejaculate (Jeyendran *et*

al. 1984). The process of fertilisation involves complex biochemical and physiological events that cannot be measured by using the routine semen evaluation. Therefore, different tests have been developed to evaluate the functional and structural integrity of the sperm membrane including supra-vital staining, hypo osmotic swelling (HOS) test, water test, zona-free hamster ova test, and cervical mucus penetration test (Hafez 1993). Since the plasma membrane functional activity is crucial for the viability and fertilising ability of spermatozoa, its evaluation becomes an important criteria of semen evaluation (Hafez 1993).

2.2.1 Percent live spermatozoa

Plasma membrane integrity can be used to assess sperm viability (Revell and Mrode 1994; Garner *et al.* 1997; Januskauskas *et al.* 2000). The percentage of live spermatozoa determines the quality of the ejaculate. Semen with more than 30% initial dead spermatozoa may not be suitable for storage and freezing. Differential staining techniques have been used for determination of live and dead spermatozoa (Rochwerger and Cuaniscu 1992). The measure of the live-dead sperm ratio may be useful in conjunction with the motility examination for a more complete analysis. A certain percentage of dead sperm may not be apparent in the initial microscopic motility examinations, since these inactive sperm might be moved about merely by action of the live motile sperm. In addition, some live non-motile sperms may become motile after dilution or storage (Tomar *et al.* 1968, 1969). Differential live-dead staining may help reveal these differences, thus supplementing initial motility estimations and providing more conclusive results.

Fertile bulls have more viable spermatozoa than infertile or sub-fertile bulls (Rodriguez-Martinez and Barth 2007). A high percentage of abnormal and dead spermatozoa in buffalo bull semen can have an adverse effect on their fertilizing potential (Heuer and Tahir 1982). In addition to this, dead and abnormal spermatozoa have been known to exert toxic and lytic effect on motile sperm cells in the ejaculate (Shannon and Curson 1972), which leads to lower fertility. Therefore, the removal of non motile, dead and abnormal spermatozoa from the semen prior to its freezing might be another approach to increase post thaw motility and survivability of buffalo spermatozoa.

After dilution and cooling, certain changes take place within spermatozoa. Further deterioration in the quality is expected which may be due to loss of certain vital intracellular components in the dilution medium. The integrity of the plasma membrane reflects sperm viability and cryopreservation damages it (Watson and Stewart 1979; Lomeo and Giambersio 1991; Correa and Zavos 1994; Cormier *et al.* 1996; Correa *et al.* 1996; Hammadeh *et al.* 1999). Freezing and thawing procedures cause destabilization of plasma membrane and intracellular ice crystal formation (Steponkus *et al*. 1983) that decreases the number of viable spermatozoa (Coulter 1992; Shamsuddin and Rodriguez-Martinez 1994). Chaveiro *et al*. (2006) believed that cryopreservation process reduces sperm viability by 50 to 60 per cent. Further, incubation of semen causes progressive fall in sperm viability significantly correlated with incubation time (Kumar 2004).

The live sperm (%) as recorded in different studies in the semen of buffalo bulls during various stages of semen processing is presented in Table R3.

Table R3: Per cent live spermatozoa in the semen of buffalo bulls

Experimental conditions	Live spermatozoa (%)	References
Fresh		
	91.58	Kanwal *et al.* (2000)
	88.16	Singh and Raina (2000)
	84.30	Lodhi *et al. (*2008)
	85.26	Shoushtari *et al.* (2009)
	90.50	El-Sisy *et al.* (2010)
	89.68	Eghbali *et al.* (2010)
Post-equilibration		
	71.10	El-Sheshtawy *et al.* (2008)
	78.20	El-Sisy *et al.* (2010)
Post-thaw		
Effect of age of the bull		
<5 years	48.91	Abou-El-Roos *et al.* (2003)
5-10 years	49.75	
>10 years	43.73	
Effect of storage period		
1 year	46.74	Abou-El-Roos *et al.* (2003)
2 years	42.23	
>3 years	48.29	
Effect of post thaw incubation		

0hr incubation	58.90	Singh and Pant (2000)
1hr incubation	46.30	
2 hr incubation	36.50	
3 hr incubation	28.30	
4 hr incubation	23.50	
Effect of caffeine, cAMP and cattle seminal plasma		
Caffeine	57.80	Singh and Raina (2000)
cAMP	57.30	
Cattle seminal plasma	49.16	
Effect of different concentrations of vitamin E		
E 0.1 mM	66.86	Beheshti *et al.* (2011)
E 0.5 mM	71.03	
E 1 mM	73.03	
E 1.5 mM	74.70	

2.2.2 Progressively motile spermatozoa

Sperm motility is considered as one of the major criteria in semen analysis that predicts male fertility (Ying-Chu *et al.* 2003). Sperm motility of buffalo bull semen can be examined by using wet smears immediately after semen collection. Visual assessment of the proportion of motile spermatozoa is the most commonly used viability test to predict fertility. In Sweden, sperm motility is currently one of the quality criteria applied to detect viability of spermatozoa in frozen/thawed semen (Soderquist 1991).The decline in post-thaw motility and viability of spermatozoa and subsequent reduction in fertility have been recorded in frozen thawed human and rams semen (Alvarez and Storey 1993; Salamon and Maxwell 2003). Several reports have also shown that the freeze thawing procedures reduce the metabolic state of spermatozoa and damage their plasma membrane, resulting in a reduction in numbers of functional spermatozoa available for assisted reproduction (Hammerstedt *et al.* 1990).

Progressive motility in buffalo bulls ranges from 65% to 80% (Kunavongkrit and Bodhipaksha 1978**;** Jainudeen *et al.* 1982; Sukhato *et al.* 1988; Nordin *et al.* 1990), depending on the age of the sires. Kumar *et al.* (1993) recorded 60.8 ±1.5 to 69.0 ±4.0 percent progressive motility in semen of Murrah buffalo bulls bred in India, whereas Vyawanare *et al.* (1989), Suryaprakasam and Rao (1993) and Pandey and Gupta (2004) recorded 73.95±5.21, 69.00±0.86 and 72.92±2.70 per cent motility in Murrah buffaloes, respectively.

The percentage of progressive sperm motility of frozen-thawed Thai swamp buffalo semen under light microscope with phase contrast was about 40-45 % (Sukhato *et al*. 1988; Koonjaenak *et al*. 2007a). In a study, Koonjaenak (2007b) also observed 40-45 % average linear motility using computer-assisted sperm analysis (CASA). Moreover, Thai swamp buffalo spermatozoa still survived at 38˚C for 60 minutes with decrease in linear motility by about 10-15 %. Aguiar *et al.* (1994) observed 78.6 ±5.6 % motile spermatozoa in semen of Brazil buffalo bulls. Water buffaloes in Italy showed a variation in motility from 40±2 % to 82±5 % (Galli *et al*. 1993). Younis (1996) and Javed *et al.* (2000) observed the progressive motility of 60.45±0.48 and 56.89±0.65% respectively in Nili-Ravi buffalo bulls. Javed *et al.* (2000) observed lower mass activity and motility in abnormal than in healthy bulls. Settergren (1994) also reported very low mass activity and less than 50% motility from various types of abnormal bulls (hypoplastic, *Pasteurella* infection of testes etc.). Similar studies have been done by Veeramachaneni *et al.* (1986) indicating decreased motility with an increase in testicular lesions.

The motility scores recorded during various experimental conditions in the semen of buffalo bulls is presented in Table R4.

Table R4: Progressively motile spermatozoa in the semen of buffalo bulls.

Experimental conditions	Progressive Motility (%)	References
Fresh		
	87.20±1.06	Shoushtari *et al.*(2009)
	68.33±3.43	Kanwal *et al.* (2000)
	70.0±0.0	Yulnawati *et al.* (2010)
	71.75±2.62	Kunbhar *et al.* (2011)
Effect of showering and vitamin supplementation		
Single showering	70.83±2.41	Singh *et al.* (2001)
Multiple showering	74.11±1.56	
Effect of caffeine, cAMP and cattle seminal plasma		
Caffeine	75.55±1.67	Singh and Raina (2000)
cAMP	75.55±1.67	
Cattle seminal plasma	75.55±1.67	
Post-equiliberation		
Effect of different composition of extenders		
Andromed	60.00±0.00	Yulnawati *et al.*

	Andromed + dextrose 0.2%	63.33±2.36	(2010)
	Andromed + dextrose 0.4%	63.33±2.36	
Effect of showering and vitamin supplementation			
	Single showering	59.58±2.53	Singh *et al.* (2001)
	multiple showering	63.82±1.97	
Post-thaw			
Effect of different composition of extenders			
	Andromed	47.33±0.94	Yulnawati *et al.* (2010)
	Andromed + dextrose 0.2%	53.00±2.16	
	Andromed + dextrose 0.4%	54.67±0.94	
Effect of age of the bull			
	<5 years	37.27±0.46	Abou-El-Roos *et al.* (2003)
	5-10 years	40.42±2.64	
	>10 years	34.14±0.71	
Effect of storage period			
	1 year	35.86±1.14	Abou-El-Roos *et al.* (2003)
	2 years	32.69±1.93	
	>3 years	38.21±2.60	
Effect of post thaw incubation			
	0 hr incubation	41.1±2.3	Singh and Pant (2000)
	1 hr incubation	29.4±2.8	
	2 hr incubation	20.5±2.6	
	3 hr incubation	12.7±2.0	
	4 hr incubation	6.2±1.7	
	1 hr incubation	43.25±2.95	Kunbhar *et al.* (2011)
	2 hr incubation	31.58±2.05	
	3 hr incubation	19.68±2.65	
	4 hr incubation	7.80±0.86	
Effect of showering and vitamin supplementation			
	Single showering	32.91±1.09	Singh *et al.* (2001)
	multiple showering	34.41±2.59	
Effect of age and season			
<5 years	Dry summer	55.50±2.44	
	Humid summer	57.89±2.51	
	Autumn	57.63±2.51	
	Winter	52.95±2.33	
	Spring	52.00±2.44	
6-10 years	Dry summer	61.50±2.44	

	Humid summer	60.29±2.65	Javed *et al.* (2000)
	Autumn	62.00±2.44	
	Winter	52.70±2.23	
	Spring	59.70±2.44	
>11 years	Dry summer	54.50±2.44	
	Humid summer	57.50±2.44	
	Autumn	60.75±2.44	
	Winter	51.45±2.23	
	Spring	60.00±2.44	
Effect of caffeine, cAMP and cattle seminal plasma			
	Caffeine	48.00±4.51	Singh and Raina (2000)
	cAMP	50.50±4.04	
	Cattle seminal plasma	40.30±3.70	
Effect of different concentrations of vitamin E on motility			
	E 0.1 mM	56.98±0.05	Beheshti *et al.* (2011)
	E 0.5 mM	61.08±0.07	
	E 1.0 mM	63.16±0.17	
	E 1.5 mM	65.03±0.09	
Effect of different concentrations of selected amino acids			
Glutamine	25 mM	41.00±2.56	El-Sheshtawy *et al.* (2008)
	50 mM	36.00±2.45	
	100 mM	26.50±1.07	
Glycine	25 mM	42.50±2.59	
	50 mM	41.00±3.31	
	100 mM	33.00±1.86	
Alanine	25 mM	29.50±1.38	
	50 mM	31.00±2.08	
	100 mM	29.50±2.03	
Cystine	5 mM	42.50±3.96	

Sajjad *et al.* (2007) recorded the post thaw progressive sperm motility in the buffalo bulls to be 51.53±2.23 where as Khan and Ijaz (2007) observed the percentage motility of undiluted, diluted (cooled to 5˚C) and frozen-thawed sperm to be 81 ±1.57, 69.6 ±2.24 and 60.1 ±1.34%, respectively.

The difference in sperm motility in various reports could be due to variations in the judgement of motility, number of bulls studied, or difference of season of studies and

age of the bulls. Decreasing sperm motility in older buffalo bulls compared to young buffalo bulls has been observed by Younis (1996).

Abou-El-Roos *et al.* (2003) found that the percentage of post thaw motility of spermatozoa seems to be more fluctuating between periods of storage of the frozen semen of buffalo bulls, which might be attributed to the different trials adopted to establish the extender being used (Belorkar *et al*. 1993; El-Azab *et al*. 1998), the system of freezing (Shannon 1978**),** the technique of thawing (Hube *et al*. 1983; Yousef 1997) and handling of the frozen semen. Kumar *et al.* (2010) reported that cryopreservation of semen reduced the motility of spermatozoa. El-Sisy *et al.* 2010 recorded the mean sperm motility in freshly diluted (82.25%), post equilibrated (72.0%) and post thaw semen (36.6%).

2.2.3 HOS Reactive Spermatozoa Percentage

The role of plasma membrane in communication between the sperm cell and the external medium is essential (Calvete *et al*. 1996) and involves ion transport across membrane (Rodriguez-Gil and Rigau 1996; Caiza *et al*. 1997; Ishibashi *et al*. 1997; Kulkarni *et al*. 1997), the binding of different factors to specific receptors and the maintenance of membrane potential (Zeng *et al*. 1995). A physically intact plasmalemma does not ensure that it is functionally intact (Correa and Zavos 1994). An assay using hypo-osmotic swelling test (HOST) for membrane integrity was used by Jeyendran *et al.* (1984). They developed hypo-osmotic solution having 150 milliosmol (mOsmol) osmolarity, to assess the swelling of spermatozoa terminating into coiled tails of different shapes. This method is based on the ability of the membranes to allow passage of water in order to establish equilibrium between the fluid compartment within the spermatozoon and the external surroundings (Drevius 1972). The ability of sperm tail to swell up and coil in the presence of hypo-osmotic solution is a sign that the transport of water across the membrane occurs normally and that the tail membrane has normal functional activity. Since its development, HOST has been used in original or modified forms (Perez-Llano *et al*. 2001; Misro and Chaki 2008; Mokashi *et al.* 2008) for evaluation of sperm membrane integrity of both fresh and frozen semen in bovine (Rota *et al*. 2000; Lodhi *et al*. 2008), equine (Neild *et al*. 2000), canine (Rodriguez-Gil *et*

al. 1994), ovine (Soderquist *et al.* 1997), porcine (Perez-Llano *et al*. 2001) and caprine (Oliveira 2005).

In recent years, more attention has been given to evaluating sperm membrane integrity as it is of fundamental importance in the fertilization process. However, the results of HOST are not highly correlated to either *in-vitro* or *in-vivo* fertility (Van Der Van *et al.* 1986; England and Plummer 1993; Correa and Zavos 1994; Correa *et al*. 1996). Rota *et al*. (2000) failed to find any correlation between *in-vitro* fertility and HOS test results of bovine spermatozoa. On the other hand, it has been shown to be well correlated with non return rates after AI in studies conducted by Correa *et al*. (1997) and Januskauskas *et al*. (2000). Perez-Llano *et al*. (2001) observed that HOST results had a significant correlation with fertility and increasing proportions of HOST-responsive sperm in the ejaculate resulted in increased farrowing rates after AI in swine. In humans, a low proportion of HOST positive sperm in the ejaculate was associated with lower pregnancy and greater miscarriage rates (Buckett *et al*. 1997; Tartagni *et al*. 2002). Brito *et al*. (2003) suggested HOST to be the only plasmalemma evaluation method that significantly contributed to sperm quality tests in predicting *in-vitro* fertilization rates. Balakrishnan *et al*. (1992) suggested limited predictive value of HOST. However, they regarded less than 50 per cent threshold to be definite indicator of a male factor in infertility.

The percentage of HOST positive cells is usually lower than that of alive and motile cells. This could be an indication that this test is also able to discriminate a subpopulation of spermatozoa with a non-functional membrane in the population of viable spermatozoa (Perez-Llano *et al*. 2001). Thus, HOST is more conclusive than supravital staining as an indicator of biochemical activity that determines fertility in physically intact spermatozoa (Tartaglione and Ritta 2004). Mokashi *et al.* (2008) reported that distilled water gave highly significant correlation with post-thaw motility and the percentage reactive sperms was also higher as compared to 150 mOsmol HOST solution.

Although HOS test is used to determine the spermatozoa membrane integrity in various animal species and humans, the suggested osmotic pressure level for test application varies depending on species. The appropriate osmotic pressure to form

maximum tail curls are declared as 150 mOsm for humans (Jeyendran *et al.* 1984), bull (Kathiravan *et al.* 2008) and buffalo (Shukla and Misra 2007) sperm, 100 mOsm for ram (Aisen *et al.* 2005) sperm, 25-100 mOsm for stallion (Neild *et al.* 1999) sperm, and 60 mOsm for dog (Kumi-Diaka 1993) sperm.

Lodhi *et al.* (2008) conducted a study to determine the correlation of hypo-osmotic swelling test with conventional semen evaluation parameters of fresh semen collected from two Nili-Ravi buffalo and two Sahiwal cow bulls and documented that HOS test could be a valuable method for routine evaluation of semen for AI. Pant *et al.* (2002) employed HOST to assess frozen thawed buffalo semen and recorded mean values for ten bulls ranging between 22.6 ±3.9 and 58.8 ±6.3 with overall mean of 45.0 ±1.4. They further found that bull having the lowest mean value of swollen spermatozoa following HOST had the lowest fertility. Various swelling patterns of tail subjected to hypo osmotic solution have also been classified and reported to be significantly correlated with fertility, with type A (maximum swelling) swelling pattern showing highest correlation (Correa *et al.* 1997).

2.2.4 Spermatozoa with intact acrosome

Acrosome, carrying various enzymes, plays an important role in the events of fertilization. Detachment of acrosome or loss of acrosomal membrane integrity may results into decrease ATP and loss of intracellular enzymes and proteins. Spermatozoa, owing to loss of acrosomal intactness, could be highly motile but infertile. The assessment of acrosomal integrity is therefore, always a part of assessment of spermatozoa (Saacke *et al.* 1968). A higher percentage of normal acrosomes are desired in the semen, as it play important role in the process of fertilization. Acrosomal changes are highly correlated with fertility (Saacke and White 1972), and these are simpler and easier methods to evaluate the effect of freezing on acrosome.

The per cent intact acrosomes recorded in different breeds of buffalo bulls are presented in Table R5.

Table R5: Spermatozoa with intact acrosomes in the semen of buffalo bulls.

Experimental conditions		Intact Acrosome (%)	References
Fresh			
Effect of showering and vitamin supplementation			
	Single showering	76.63	Singh *et al.* (2001)
	multiple showering	79.16	
Post-equiliberation			
		65.30	El-Sheshtawy *et al.* (2008)
		79.6	El-Sisy *et al.* (2010)
Post-thaw			
Effect of age of the bull			
	<5 years	80.46	Abou-El-Roos *et al.* (2003)
	5-10 years	78.92	
	>10 years	78.56	
Effect of storage period			
	1 year	78.57	Abou-El-Roos *et al.* (2003)
	2 years	77.00	
	>3 years	80.14	
Effect of different concentrations of selected amino acids			
Glutamine	25 mM	59.00	El-Sheshtawy *et al.* (2008)
	50 mM	52.40	
	100 mM	39.00	
Glycine	25 mM	61.30	
	50 mM	57.60	
	100 mM	37.00	
Alanine	25 mM	51.005	
	50 mM	45.00	
	100 mM	38.30	
Cystine	5 mM	58.30	
Effect of showering and vitamin supplementation			
	Single showering	70.63	Singh *et al.* (2001)
	multiple showering	72.54	
Effect of different concentrations of vitamin E			
	E 0.1 mM	68.38	Beheshti *et al.* (2011)
	E 0.5 mM	68.03	
	E 1 mM	67.91	
	E 1.5 mM	68.26	

Various workers examined the acrosomal abnormalities by using the Giemsa stain technique (Raizada *et al*. 1990; Rao *et al*. 1990; Bhosrekar *et al*. 1994; Ramakrishnan and Ariff 1994) or fluoresceinated lectins (Cross and Meizel 1989; De Leeuw *et al*. 1991; Bawa *et al*. 1993; Chachur *et al*. 1997). More than 90% of spermatozoa were observed with intact acrosome in semen of buffalo bulls bred in Bahia, Brazil (Aguiar *et al*. 1994) and Murrah buffalo bulls after Giemsa staining (Kumar *et al*. 1993).

Talevi *et al*. (1994) reported similar results for water buffalo using a fluoresceinated lectin. Fabbrocini *et al*. (1996) used fluoresceinated lectin, FITC-labeled Maclura Pomifera Agglutinin (MPA) that binds to lectin-similar receptors on the cell surface to detect changes in the surface glycoconjugates. Three different sub-populations were found: (1) Cells with a coloured acrosome and tail. (2) Cells with the external border of the acrosome and the tail coloured. (3) Uncoloured cells. In semen of good quality, cells presenting pattern (1) were most common, while patterns (2) and (3) occurred in less than 20% of spermatozoa.

The percentage of cells that have an intact acrosome and are able to perform the acrosome reaction upon triggering is regarded as important semen characteristic (De leeuw *et al*. 1991; Wielders 1991). Thereby, a high correlation with fertility has been indicated for the percent intact acrosome of spermatozoa (Saake and White 1972). Stresses of freezing and thawing lead to acrosomal damage (Gilbert and Almquist 1978; Chaves 1979). A marked decline in the percentage of normal intact acrosome after freeze-thawing of bull (Werkmeister 1978) and buffalo spermatozoa (Patil *et al*. 1981; Kumar 1996; Singh 2002) has been reported.

2.3 Seminal plasma enzymes

In mammals, seminal plasma is a complex mixture of secretions from the epididymis and various accessory sex glands. The anatomy of accessory glands, as well as their chemical composition and the functions of their secretions vary among species (La Falci *et al*. 2002). A large variety of enzymes is present in seminal plasma, but in many instances the gland responsible for their production has not been identified. The enzyme levels of seminal plasma are very important for sperm metabolism as well as sperm function (Brooks 1990).

Enzymes such as, glutamic oxaloacetic transaminase (GOT)/ aspartate amino transferase (AST), glutamic pyruvic transaminase (GPT)/ alanine aminotransferase (ALT), lactate dehydrogenase, cholinesterases and alkaline or acid phosphatases, etc (Roberts 1971), proteolytic enzymes, phospholipases, transaminases like GOT, ATPase, glycosidase, dehydrogenases, nucleotidases, DNases, hyaluronidase have been recognized to be intimately related to the sperm cell and 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).

Buffalo bull spermatozoa are very fragile and have low survival and poor fertilizing ability during preservation as compared to that of bovine bull spermatozoa. Following dilution, freezing, thawing of bovine semen, and cold shock reactions, the activity of such enzymes have been reported to rise above their initial level in seminal plasma owing to increased cellular permeability which cause leakage of intracellular enzymes into the media concomitant to increased sperm abnormality and decreased sperm motility and viability (Mann and Lutwak-mann 1981**;** Dhami and Kodagali 1990**)**. Similar situation has been known in fresh semen containing a high proportion of abnormal sperms.

Among structural changes resulting from cold shock, the damages to the sperm plasmalemma and acrosome are particularly conspicuous and provide a sensitive indicator of injury to sperm cell (Mann 1964; Mann and Lutwak-Mann 1981). Permeability of spermatozoa that had sustained physical or chemical damage to the membrane in the plasmalemma, acrosomal or mitochondrial region, is markedly increased. Therefore irrespective of the underlying cause (senescence, storage, cold shock, freezing and thawing, exposure to toxic agents), such spermatozoa release their intracellular constituents and certain enzymes at a greatly enhanced rate.

Sidhu and Guraya (1979) studied the effect of cold shock on release of various enzymes from buffalo bull spermatozoa**.** The leakage of sperm cell enzymes into the seminal plasma following freezing and thawing could be used as marker for the sperm cell damage (Kakar and Anand 1984). Glycerolization and

freezing resulted in significant increases in semen hyaluronidase, aspartate amino transferase, lactic dehydrogenase and alkaline and acid phosphatase values, resulting in adverse effects on semen quality (Bhosrekar *et al.* 1994).

2.3.1 Alkaline Phosphatase (AKP)

Semen phosphatases play an important role in dephosphorylation during sperm metabolism. Alkaline and acid phosphatases in semen reflect the functional status of accessory sex glands and metabolic activity of spermatozoa. Epididymus along with ampulla is a main source of alkaline and acid phosphatase in semen.

Glogowski and Strzezek (1979) reported that AKP activity determined in fresh seminal plasma, plasma after dilution, diluted seminal plasma after 4 hour equilibration and after storage of semen in LN_2 for 24 hour revealed the activity to be 423.8, 137.9, 139.1 and 144.3 BU respectively. Chaudhary and Gangwar (1977) found the average values of AKP to be 1238 ±47.4 KAU/100 ml of seminal plasma while studying the seasonal variations in physico-biochemical determinants, they found activity of AKP varied significantly (P□ 0.01) between season being greater during both the cold and hot season.

Bhosrekar *et al.* (1994) reported drop in alkaline phosphatase on freezing of the semen, while Dhami and Kodagali (1988) documented that the activities of AKP and ACP were significantly higher in the seminal plasma after freezing than before freezing. They also found significant effect of extender on the leakage of these enzymes. Tris-fructose-yolk-glycerol extender provided better protection against leakage of phosphatases from the spermatozoa during freezing.

Season significantly influenced leakage of both phosphatases; the lowest leakage of both enzymes was in the hot season. The levels of AKP before and after freezing were negatively correlated with fertility (-0.572 and -0.628 resp.). The sperm concentration was negatively correlated with AKP levels of seminal plasma before and after freezing. The overall mean level of AKP in the pre-freeze and post-thaw plasma was 68.02 and 94.32 KAU/200 ml respectively (Dhami and Kodagali 1988).

Pangawakar *et al.* (1988) recorded the mean AKP activity as 717.21±20.31, 802.33±32.16 and 1002.65±31.22 KAU/100 ml in 3 different groups of semen with corresponding freezability of □ 85%, 65-85% and □ 65% of ejaculates, respectively

(p□ 0.01). This indication of high phosphatase activity in the least freezable groups reflects initial damage of the sperm membrane with subsequent increase in its permeability, resulting in leakage of enzymes into the seminal plasma.

Gomes De Castro *et al.* (1991) observed that semen quality was better in autumn than at other times in Murrah buffaloes. Chaudhary and Gangwar (1977) found the average AKP values of 1238±47.4 KAU/100 ml of seminal plasma while studying the seasonal variations. in physico-biochemical determinants, they found Activity of alkaline phosphates varied significantly (P□ 0.01) between periods being greater during both the cold and hot periods.

Various researchers studied the sperm motility and enzyme concentrations during pre and post freezing periods and recorded the overall mean sperm motility of 77.14±0.71% at pre-freeze stage, which declined significantly (p□ 0.01) to 44.89±1.11% at 7 days post-freezing. However, the levels of the five enzymes (GOT, GPT, AKP, ACP, LDH) increased significantly in post freeze seminal plasma. The overall average values of GOT, GPT and AKP in pre-freeze and post-thaw seminal plasma were 20.88±0.81 and 33.08±1.14 µmole/L, 7.05±0.32 and 11.44±0.47 µmole/L and 68.02±3.72 and 94.32±4.50 KAU/100 ml, respectively (Dhami and Kodagali 1990).

Dhami and Sahni (1993) reported that slow cooling of semen straws over a 2 hr period from 30˚C to 5˚C compared with that of faster cooling rates, plus at least 2 hr of equilibration at 5˚C, was required for optimal freezability, low enzyme leakage and higher fertilizing rates in buffalo bulls.

Season had a significant effect on various seminal attributes like ejaculate volume (greater in autumn than in summer), semen protein (highest in summer and autumn), alkaline phosphatase (greatest in spring and lowest in winter), semen fructose (greater in winter than in summer) and GOT (highest in summer) (Castro *et al.* 1994). Sperm concentration was significantly correlated with progressive and mass sperm motility (0.39 and 0.33 respectively) and alkaline phosphatase with GOT (0.17).

Oliver *et al*. (1997) reported that AKP in the semen after freezing and thawing were similar or slightly reduced.

El-Gawad and Allah (2007) reported that the pre and post-freezing motility, acrosomal integrity, AKP, AST leakages and fertility rate were significantly influenced by

different cooling rates and equilibration periods, diluents and their interactions. The mean pre-freeze and post-thawing motility following 2 hr of cooling was significantly higher than that following 1 hr of cooling, whereas the mean pre-freeze and post-thawing acrosomal defects following 2 hr of cooling was significantly less than that following 1 hr. The leakage of AST was significantly lower, leakage of AKP was significantly higher and the conception rates were higher for semen frozen after 2 hr (64.8%) than from 1 hr (38.4%).

Enzyme activity increased at pre-freezing and post-thawing stages when glycerol was added at room temperature. Cryopreservation of semen increased the concentrations of ALT and AKP, but no change in AST activity was recorded. It was concluded that backward motility of spermatozoa was associated with increased activities of ALT and AKP in the seminal plasma especially during the summer season (Sikka and Singh 2007).

Increased conception rates were noted in cows following inseminations with semen containing high level of AKP (Zvereva and Chuhrly 1972).

2.3.2 Transaminases (AST and ALT)

Transaminases in spermatozoa are intrinsically associated with their metabolic activity and functions as reservoir of energy for them (Flipse and Dietz 1966; Kumar *et al.* 1990).

Sharma *et al*. (2003) studied the Surti buffalo semen and reported that seminal enzymes like GOT, GPT and LDH in the seminal plasma significantly increased at post freezing and thawing and at different times of post thaw incubation indicating there was leakage of the spermatozoa. It is concluded that freezing and thawing of semen cause cryo-injury to the sperms, decrease in the percentage of intact acrosome percentage and leakage of semen enzymes.

Roychaudhary *et al*. (1974) and Chinnaiya *et al*. (1979**)** reported that level of acrosomal damage and extracellular release of AST and ALT enzymes were positively correlated. Like others they also reported that Tris-diluent is superior to Egg Yolk Citrate (EYC). AST has been reported to be directly correlated with the sperm concentration (Saxena *et al.* 1978) and acrosomal damage (Sharma *et al*. 2001).

Graham *et al.* (1974) and Kakar and Anand (1984) have stressed the importance of levels of AST-ALT enzymes in the seminal plasma, as an indicator of the quality of frozen semen. According to them, the amount of AST released is a measure of sperm membrane permeability and the extent of damage sustained by the spermatozoa during the process of deep-freezing. The AST-ALT release from spermatozoa has been reported as being influenced by a number of factors, such as cold shock (Tuli *et al*. 1988), speed of centrifugation (Gupta and Srivastava 1985), glycerol concentration and glycerolization temperature (Dutta *et al*. 1990) cooling rates, equilibration time and temperature (Pandit and Garg 1983), freezing rates (Jagmohan and Sharma 1988) and thawing temperatures (Kumar *et al.* 1990).

Kumar *et al.* (1990) observed that in buffalo frozen spermatozoa, the activity of AST and ALT increased on thawing, but the increase was lower in semen extended in Tris than in egg yolk-citrate. The leakage of both enzymes was lowest at 40 degrees and highest at 18 degrees. They also observed that ALT leakage was greater than AST leakage at all temperature.

Reddy *et al.* (1999) observed in Murrah buffalo semen that the overall mean activity of AST and ALT before freezing was 18.18±1.41 and 1.76±0.11 kinetic units (KU)/$60x10^6$ spermatozoa respectively. The post-thaw value for AST and ALT was 42.12±2.91 and 3.15±0.33, 38.33±4.03 and 3.13±0.38, 39.91±4.12 and 3.27±0.30 KU/$60x10^6$ spermatozoa following conventional freezing, moderate and slow rate of programmable freezing, respectively. It is concluded that an injury occurs subsequent to freezing and thawing of sperm cells resulting in an increase in the concentration of aminotransferase extracellularly. Gupta and Prasad (2008) reported the mean AST (63.15±2.69 IU/L) and ALT (15.08±0.54 IU/L), respectively.

The mean AST concentration in seminal plasma after dilution has been reported by Varshney *et al.* 1978 (83.50±3.00), Kumar 1986 (95.99±15.32) and Shukla *et al.* 2009 (87.45±13.52 IU/L) in buffalo bulls.

Dhami and Sahni (1994) studied the effect of cooling rates, equilibration periods, diluents and their interactions with freezability, enzyme AST leakage and fertility of frozen semen of three Murrah bulls. The pooled mean pre-freeze and post-thaw motility, AST leakage and fertility rates obtained were 70.85±0.31 and 37.84±0.56% (p□ 0.01);

18.95±0.32 and 29.03±0.45 µmole/L (p□ 0.01) and 64.52% respectively. The effect of bulls, cooling rates and equilibration periods influenced all these traits.

Buruiana *et al*. (1978) found an increase in AST activity from 52.5 to 80.2 units in fresh semen and 36.0 to 48.4 and 10.9 to 11.6 units for 1 and 13 month stored semen in liquid nitrogen, respectively. Bower *et al*. (1973) studied the effect of addition of glycerol on release of AST in extra cellular fluid and they found that addition of glycerol always increased the AST activity in extracellular fluid indicating damage to spermatozoa.

2.3.3 Hyaluronidase

Estimation of hyaluronidase has assumed a great importance in view of its place in spermatozoan system. This enzyme is present in acrosomal system of spermatozoa and nowhere else. Since integrity of acrosome is directly involved in fertilizing capacity of spermatozoa. This enzyme has assumed a great importance in estimating fertilizing capacity of semen. Sperm hyaluronidase is responsible for the penetration of the spermatozoa through the cumulus oophorus (Bedford 1968**;** Morton 1975). If there is damage to the acrosome it is presumed that this enzyme will leak out in extracellular fluid.

Hyaluronidase in buffalo bull spermatozoa is a readily released enzyme (Sidhu 1978; Sidhu and Guraya 1979). This enzyme is distributed on the outer acrosomal membrane of buffalo bull spermatozoa (Sidhu 1978).

The presence of hyaluronidase in the extracellular media after cold shock in the buffalo bull can be attributed to the detached acrosomes (Chinnaiya and Ganguli 1980). The hyaluronidase activity increased in extracellular fluid on freezing and thawing as compared to before freezing.

Strzezek *et al*. (1979) collected semen samples from 26 adult and 31 young bulls, which were frozen in liquid nitrogen and stored for 24 hours. For the 2 groups of bulls respectively, Hyaluronidase activity (in units/ 10^9 spermatozoa) averaged 5.35 and 6.35 in fresh semen, 10.5 and 7.0 after dilution, 13.2 and 10.9 after equilibration and 16.2 and 13.8 after thawing. Likewise other workers (Foulkes and Watson 1975) also estimated the hyaluronidase activity in bulls and it was 4.4, 5.7 and 18 % at post-dilution, post equilibration and after thawing of semen respectively. Tanyildizi and

Bozkurt (2002) estimated mean hyaluronidase activity (44.16 ± 3.83 µmol /L) in ram semen.

Ganguli and Kakar (1980) reported 3.34 fold higher leakage of hyaluronidase in seminal plasma when semen was treated by sonication than by freezing and thawing. They did not find any effect of diluents in release of hyaluronidase in seminal plasma.

Dhanda *et al* (1981) studied sorbitol dehydrogenase and hyaluronidase activity in buffalo semen. They reported marked variation amongst individuals. According to them motility of sperm is inversely related to hyaluronidase activity.

Danasouri (1988) observed loss of acrosomal enzymes namely acrosin and hyaluronidase, from spermatozoa after thawing at different temperatures and time. The highest acrosome activity remaining in the spermatozoa and the lowest hyaluronidase activity in the media were observed after thawing at 70° C.

Antonynk and Bezlyerdnikov (1983) have used hyaluronidase enzyme in different proportion in diluents for boar semen. They could not find any significant difference on conception rate in sows inseminated with diluted semen supplemented with hyaluronidase.

2.4 Seminal Plasma Mineral Profile

Seminal plasma of mammals is a complex fluid, which serves as a carrier for the spermatozoa on their journey from the male testes to their target i.e. oocyte. Seminal plasma contains a variety of biochemical components, some of which are relatively specific for the regulation of sperm function. Biochemical parameters of semen are of vital importance from its fertility and preservation aspects. Information on the extent of variation of seminal characteristics between bulls of different breeds is an essential pre-requisite for effective preservation of their germplasm to be used in AI program. A large number of reports on the biochemical composition of cattle semen have been published (Singh *et al.* 1969; Igboeli and Rakha 1971; Rattan 1990). The available data in this respect indicate that biochemical properties of buffalo bull semen differ from those of cattle semen in several aspects (Ganguli 1980). This may be one of the reasons of poor preservability of the buffalo bull semen, as most of the extenders designed for cow bull semen are being used as such for preservation of buffalo bull semen.

2.4.1 Calcium

Physiologically, calcium (Ca) is classified as either intracellular or extracellular. The skeleton is a major reservoir for providing Ca for both the extra and intracellular pools. Intracellular Ca has a key role in many important physiological functions including hormone secretion, glycogen metabolism and cell division. Extracellular Ca provides Ca ion for the maintenance of intracellular Ca, bone mineralization, blood coagulation and plasma membrane potential. Calcium stabilizes the plasma membrane and influences its permeability and excitability (Endres and Rude 2006). Calcium is a part of the second messenger system in many cell functions. In diacylglycerol inositol phosphate system induced by gonadotropins for instance, inositol phosphates are primarily involved in controlling calcium channels in the cell membrane and redirecting intracellular calcium, and allowing calcium to enter the cell (Ward *et al.* 1991).

The mean calcium concentration recorded in some species of animals is presented in Table R6.

Table R6: Calcium concentration in the semen of some species of animals

Species	Value (mg/dl)	Refrences
Buffalo bulls	43.45±1.49 mg/dL	Singh *et al.* (1970)
Buffalo bulls	41.11 mg/dL	Reddy and Raja (1979)
Buffalo bulls	45.6 mg/100ml.	Rattan (1990)
Buffalo bulls	29.97±5.15 mg/dL	Kanwal *et al.* (1998)
Buffalo bulls	32.42±3.10 mg/dL	Sansone *et al*. (2000)
Murrah Buffalo	44.95±0.96 mg %	Shukla *et al.* (2009)
Water buffalo bulls	22.36±0.52 mg/dL	Eghbali *et al*. (2010)
Cattle	21.26±0.56 mg/dL	Raval and Dhami (2006)
Ram	19.2±1.3 mg/dL	Abdel-Rahman *et al.* (2000)
Stallion	2.9 mmol/L (~11.6 mg/dL)	Pesch *et al*. (2006)
Stallion	5.75±4.2 mg/dL	Barrier-Battut *et al.* (2009)

Higher calcium content in the semen is reported to have depressing effect on sperm metabolism. The ions present in optimal concentrations in the semen of bulls helps in stimulating the motility and glycolysis and counteract the depressing effect of calcium present in the semen (Mann and Lutwak-Mann 1981).

A higher proportion of the non-skeletal Ca is present within cells than in extracellular fluids, but most of the intracellular Ca is bound to proteins in cell

membrane, mitochondria and nucleus. The concentration of Ca ion in intracellular fluid is reduced considerably by this binding (Kaplan *et al*. 1995).

2.4.2 Phosphorus

The phospholipid is required for the preservation of the sperm membrane integrity (Singh *et al.* 1969).

Singh *et al.* (1970), Dhami and Sahni (1993), Gupta and Singh (2007) and Shukla *et al.* (2009) reported the mean phosphorus concentration in buffalo semen to be 7.56±0.42, 12.28±0.37, 7.55±0.16 and 6.82±1.63 mg/dl respectively, where as Roy *et al.* (1960) recorded the average phosphorus to be 6.4±0.6 mg/dl bovine semen.

2.4.3 Magnesium

Magnesium (Mg) is the second most prevalent intracellular cation and is involved in the metabolic activity of the cell. Within the cell, most of the Mg is bound to proteins and negatively charged molecules. Nearly 80% of cytosolic Mg is bound to ATP and Mg-ATP is the substrate for numerous enzymes. The nucleus, mitochondria and endoplasmic reticulum contain significant amounts of Mg. Approximately 0.5% to 5.0% of the total cellular Mg is in free form. Transport of Mg across the cell membrane is regulated by a specific Mg transport system (Endres and Rude 2006).

Intracellular magnesium is involved in the activity of hormone receptor complex in the cell membrane. After the hormone binds to receptor, the affinity of G complex for Mg increases, catalyzing the exchange of GTP for GDP on the Gα protein. The Gα-GTP complex is now the 'active' form, and the active Gα-GTP may actually dissociate from the Gβγ complex and enter the cytoplasm (Ward *et al.* 1991). Low levels of Mg are associated with comparatively lower motility of spermatozoa (Singh *et al.* 1969).

The mean magnesium concentration recorded in different animals has been depicted in Table R7.

Table R7: Magnesium concentrations in semen of certain species of animals

Species	Value (mg/dl)	References
Water buffalo bulls	11.94±0.36 mg/dL	Eghbali *et al*. (2010)
Murrah Buffalo	6.61±0.49 mg/dL	Shukla *et al.*(2009)
Buffalo	5.24±0.49 mg/dL	Singh *et al.* (1970)
Buffalo	5.91 mg/dL	Reddy and Raja (1979)
Bovine	9.8±1.9 mg/dL	Cargle *et al.* (1958)

Ram	8.6±0.6 mg/dL	Abdel-Rahman *et al.* (2000)
Stallion	7.53 mg/dL	Pesch *et al.* (2006)
Stallion	3.63±1.9 mg/dL	Barrier-Battut *et al.* (2009)

2.4.4 Electrolytes

The sodium is an extracellular element while potassium is intracellular in nature and there is an opinion that normal ionic equilibrium and osmotic pressure are maintained by these ions (Hawk *et al.* 1964). A positive correlation of Na and K content in the semen is reported to be responsible for the maintenance of osmolarity and metabolic activity of the spermatozoa (Nath 1988).

Shukla *et al.* (2009) observed that the K was negatively correlated with sperm abnormalities, suggesting that the high K content in the seminal milieu might be responsible for maintenance of spermatozoa configuration (Nath 1988). Other researchers recorded no significant difference in successive ejaculates in semen concentration of Na and K in Murrah buffalo bulls (Chacur *et al.* 1994).

i. Sodium (Na)

Average Na concentrations in seminal plasma of buffalo bull reported by various workers are 186.89±10.90 mg% (Singh *et al.* 1969), 139.00±3.00 mg%, (Gupta and Tripathi 1983), 217.4 mg% (Rattan 1990), 319.2±51.5 mg% (Kanwal *et al.* 2000), 258.58±13.65 mg% (Sansone *et al.* 2000), 272.85±9.84 mg% (Gupta and Singh 2007). Similarly Kanakaraj and Easwaran 1991; Oba *et al.* 1994; Shukla *et al.* 2009 reported average sodium concentration of 243.0±4.09, 146.3±2.94 and 106.46±11.92 mg% respectively, in Murrah buffalo semen.

ii. Potassium (K)

Average K concentrations in seminal plasma of Murrah buffalo have been reported by various workers are 98.04±2.77 mg% (Kanakaraj and Easwaran 1991), 30.13 ±0.73 mg% (Oba *et al.* 1994), and 98.18±11.67 mg% (Shukla *et al.* 2009). Similarly Gupta and Singh (2007) observed the average K value of 75.78±3.23 mg %, in Tarai buffalo bulls, whereas Singh *et al.* 1969; Rattan 1990 and Dhami and Sahni 1993 observed average K concentration of 101.60±4.45, 150.3±2.30, 103.56±3.15 mg% in buffalo bull semen.

iii. Chloride (Cl)

Average Cl concentrations in seminal plasma of buffalo bulls reported by various workers are 347.50 mg% (Singh *et al.* 1969), 269.13±7.05 mg% (Dhami and Sahni 1993). Similarly average Cl value of 42.01 ±0.01 mg% (Gupta and Singh 2007) in Tarai buffalo, 366.73 + 53.69 (Shukla *et al.* 2009) in Murrah buffalo and 373.55 + 55.0 (Roy *et al.* 1960) in cattle semen

2.5 Correlation between functional and biochemical parameters

An adequate evaluation of semen for breeding purposes has always been of great significance. Semen analysis is a valuable diagnostic tool to assess the fertility status of the male. However, the prediction of potential fertility of a male on the basis of a single assay is not reliable. Conventional parameters used for evaluation of semen have limited application because they only help to assess the structural integrity of the cell (Neild *et al.* 1999). Each sperm cell consists of multiple sub cellular compartments with different functions, all of which must be intact for successful fertilization (Amann and Graham 1993). In recent years, more attention has been given to evaluating sperm membrane integrity as it is of fundamental importance in the fertilization process. Jeyendran *et al*. (1984) developed a hypo-osmotic swelling test (HOS) to evaluate sperm membrane function of human spermatozoa. Since its development, HOS test has been used for evaluation of sperm membrane integrity in bovines (Rota *et al*. 2000), equine (Neild *et al*. 2000), canine (Rodriguez-Gil *et al*. 1994), porcine (Perez-Llano *et al*. 2001)

Significant correlations have been reported to exist between various parameters that are related to sperm plasma membrane integrity. Barnabe *et al*. (1981) associated a higher initial progressive motility to a lower incidence of abnormal acrosomes, and reported a significant correlation between motility standards and sperm structure preservation. Kumar (2004) showed significantly positive correlation between sperm motility, live percentage and HOS reactive spermatozoa. The percentage of live sperms and live acrosome intact sperms has also been shown to be highly correlated with percentage of motile sperms (Kirk *et al*. 2005). Higher correlation is shown between motility and acrosome status or sperm viability (Kumar 2004) than HOS, probably because they are directly determined in fresh semen while HOS requires incubation. However, Correa *et al*. (1997) found similar correlations of all these tests with non return

rate after AI. Coetzee *et al*. (1989) observed in humans that the correlation between HOS and sperm viability was strong (r=0.76), moderate (r=0.50) between HOS and sperm morphology and weaker (r=0.42) with sperm penetration assay and IVF (r=0.24).

In an experiment conducted by Correa *et al*. (1997) to evaluate the relationships among frozen-thawed sperm characteristics, highly significant correlations were found among various sperm qualitative characteristics and overall fertility. The percentage of normal morphology and swollen spermatozoa showed the highest correlation coefficients. Based on the fertility levels, sperm characteristics from high fertility bulls were significantly correlated with fertility ratings. The percentage of motility was the single sperm characteristic with the highest correlation coefficient in high fertility bulls. The percentage of normal morphology and intact acrosomes were not significantly correlated with fertility rating in low fertility bulls ($P<0.05$).

Semen from triple crossbred (25% HF X 25% J X 50% Kankarej) bulls was evaluated by Raval and Dhami (2006) for physico-biochemical attributes and their interrelationship. The correlation matrix analysis revealed that the mass activity score showed significant positive correlation with initially motile sperms (r=0.81) and negative correlation with abnormal sperms(r=-0.46). The live sperms had highly significant ($P<0.01$) positive correlation with initial motility (r=0.54) and negative correlation with abnormal sperms (r=-0.60). Initial motility and abnormal sperm per cent were also negatively interrelated (r=-0.59).

In a retrospective study, Gillian *et al*. (2008) assessed various *in-vitro* sperm characteristics of Holstein Friesian bulls in relation to fertility. They found that adjusted *in-vivo* fertility correlated with subjectively assessed post-thaw motility (r=0.672, p=0.033), post-thaw sperm viability (r=0.635, p=0.048) and post-thaw sperm morphology (r=-0.762, p=0.010), while no correlation with acrosomal integrity was appreciated.

Comparing of various methods for evaluating sperm plasmalemma of bovine semen, Brito *et al*. (2003) found that the proportion of HOST responsive sperm was only moderately correlated with the proportion of plasmalemma-intact sperm identified by vital stains in contrast to high correlation reported by Correa and Javos (1994). Lodhi *et al*. (2008) reported significant correlation between HOST and progressive

motility(r=0.612; r=0.649), sperm viability (r=0.902; r=0.880) and morphologically normal sperms (r=0.661; 0.661) for Sahiwal cow bull and buffalo bull semen, respectively. Similar findings were reported in human (Jeyendran *et al*. 1984), equine (Mantovani *et al*. 2002) and fresh goat semen (Fonseca *et al*. 2005).

Mean Ca and Mg concentration was reported to be highly positively associated with sperm gross motility, progressive motility and viability. Seminal plasma calcium content was highly positively associated with seminal plasma magnesium content and was highly negatively associated with semen volume. Alternatively, seminal plasma Mg content was negatively associated with abnormal semen morphology and volume (Eghbali *et al.* 2010).

CHAPTER 3

Materials and Methods

3.1 EXPERIMENTAL ANIMALS AND THEIR MANAGEMENT

The study was conducted, from July to December, 2011, on 12 apparently healthy Murrah buffalo breeding bulls, aged between 5-7 years, maintained at Frozen Semen Bank, RCDF Ltd., Bassi, Jaipur. The selected bulls were divided into 2 groups, each comprising of 6 bulls according to their known ejaculate quality (donating good or poor quality semen, respectively) to compare their functional and biochemical attributes. Group 1 comprised those bulls, which were donating semen of excellent quality with good freezability and fertility parameters whereas group 2 included those bulls which were frequently donating either initial poor quality semen or higher degree of damage during processing (during equilibration or cryopreservation) but were otherwise healthy.

All the bulls were maintained under identical conditions, sexually mature and were regularly screened for sexually transmitted diseases to eliminate the possibility of any infection. The bulls were routinely vaccinated against contagious infections and deworming was done every six months using broad spectrum anthelmintics.

The bulls were reared in spacious individual pans with adequate loafing area, manger and water trough with access to *ad lib* drinking water and adequate drainage

for disposal of waste. Individual sheds in a single row were separated by concrete walls. Weekly spraying of Sodium Bicarbonate (40%) was routine practice for disinfection of the bull pens. The bulls were exercised daily for one hour and were groomed once a day.

Feeding of breeding bulls was purely on scientific basis according to the body weight. An admixture of the green and dry roughage along with concentrates and minerals was made available throughout the year and was fed as per the feeding schedule of semen laboratory depending upon body weight so as to contain Dry Matter 7.5-15 kg/day, Digestible Crude Protein 0.55-0.83 kg/day and Total Digestible Nutrients 4.0-6.1 kg/day. To meet the above mentioned requirement, each bull was provided with ~30 kg green fodder, ~6 kg dry fodder, ~3 kg concentrate feed and 50 gm mineral mixture per day.

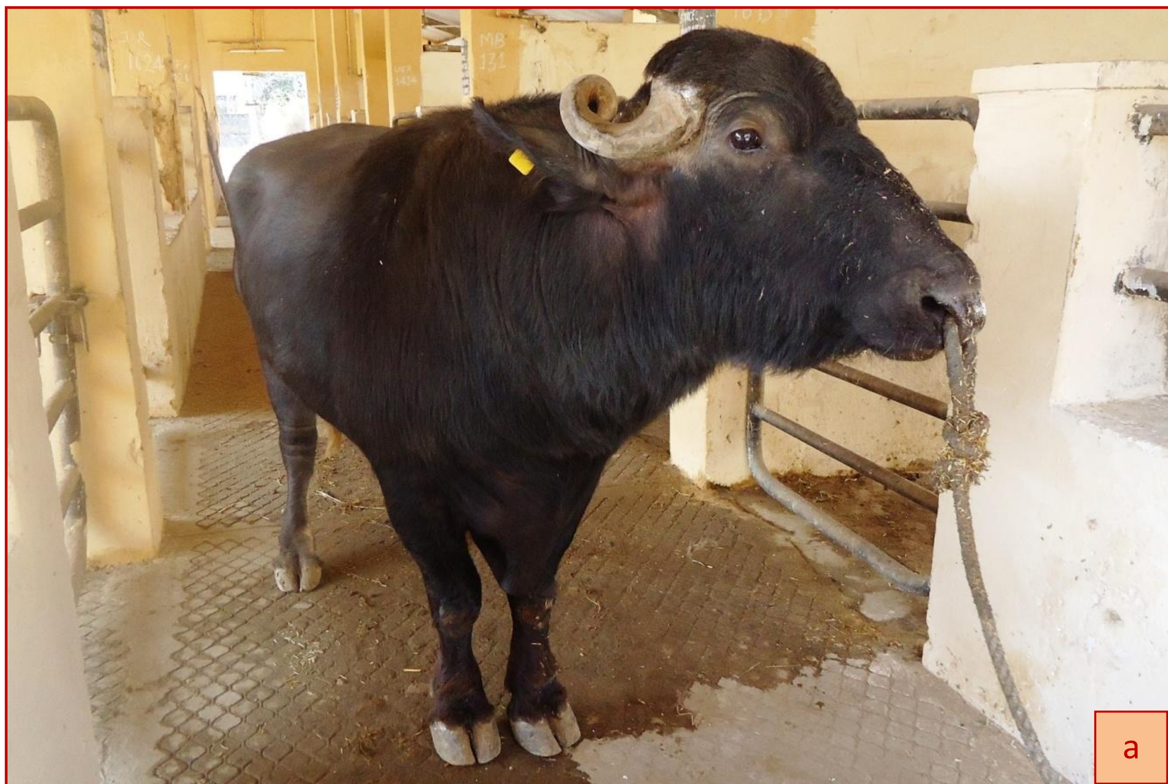
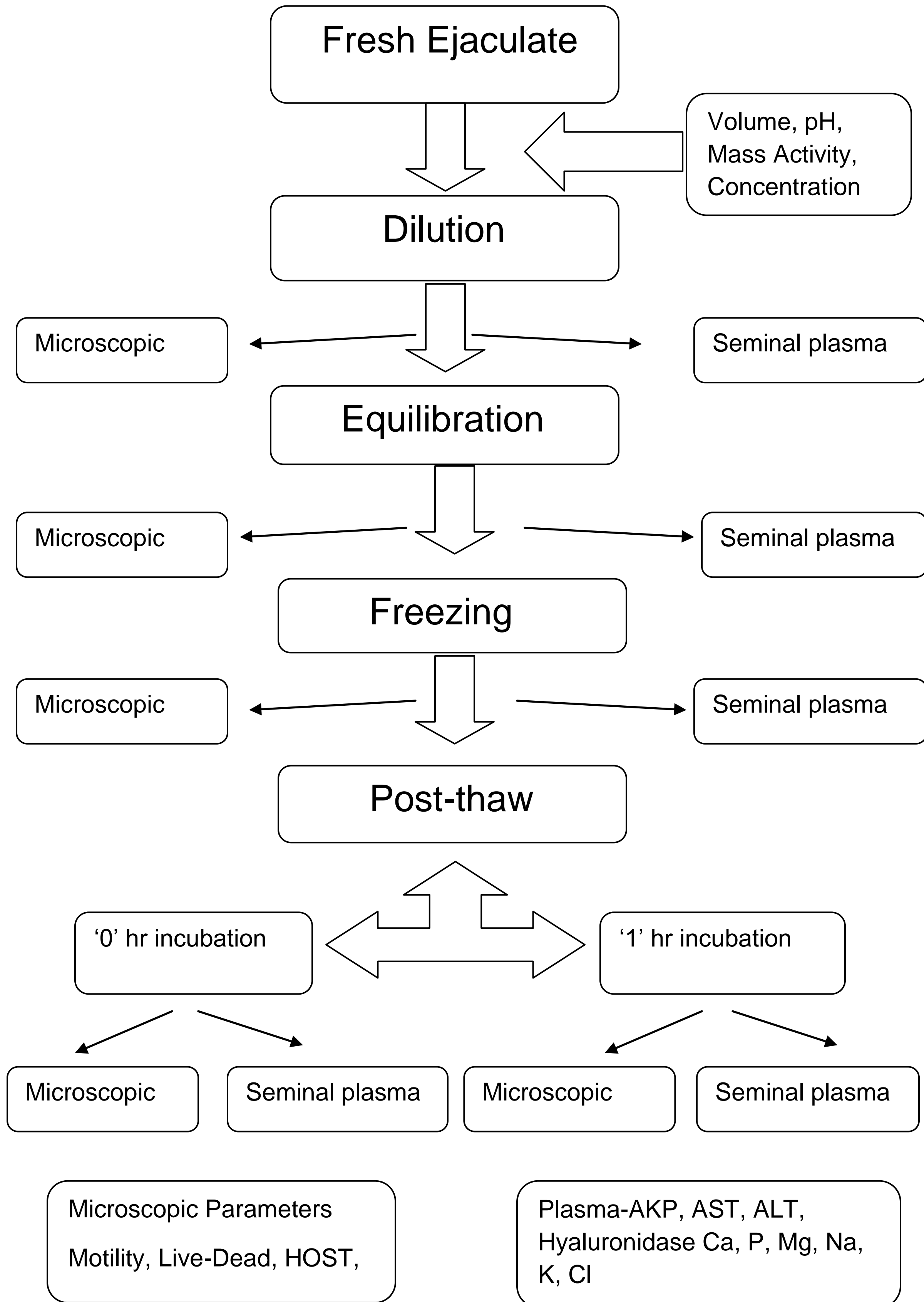


Plate 1a-b: Murrah buffalo bulls at Frozen Semen Bank, RCDF Ltd., Bassi, Jaipur

Diagrammatic illustration of the study plan



3.2 SEMEN COLLECTION

The bulls were washed half an hour before being taken to the site of collection adjacent to the semen processing laboratory in the morning hours beginning at 7 a.m. Semen was collected (Walton, 1945) using an artificial vagina (AV) of 9 inches size (Danish Model), on biweekly schedule. The temperature of AV was maintained between 42-45° C with adequate pressure. The artificial vagina had smooth lining and assembly had a dried and sterilized calibrated collection tube maintained at 37^0C packed in cotton stuffed plastic goblet and covered with an insulating rubber bag to prevent temperature fluctuations from collection of semen till its processing in the laboratory. The bulls were already trained for donating semen in artificial vagina. On each collection, two ejaculates were taken in succession and each ejaculate was preceded by a period of sexual preparation consisting of at least two to three false mounts over male dummy separated by about one minute restraint. The second ejaculate was collected about 15 minutes after the first collection. Strong pelvic thrust from the donor bull was considered as the most evident sign for successful ejaculation. Preferably first ejaculate was taken for the study. Immediately after collection, each ejaculate was placed in a water bath at 37°C and examined using various standard laboratory tests (mass activity, individual motility, live dead count, sperm abnormalities, acrosomal integrity etc.).

3.3 Initial Evaluation of Ejaculates: Before processing the collected semen sample were subjected to examination of following parameters;

i) Volume: The semen was collected in 15 ml graduated glass tube (0.1 ml accuracy). The total volume of an ejaculate was recorded directly from the collection tube in millilitre (mL). To eliminate any error, all the collection tubes were prior screened for their exact measure. Samples above 1 ml ejaculate volume were accepted.

ii) pH: pH of the fresh semen was determined within 1-2 minutes of collection with digital pH meter (EI 111).

iii) Sperm concentration: Sperm concentration was measured in already calibrated Bovine Photometer (IMV Hamilton Microlab 500 series) working on the principle of optical density as an indicator of sperm concentration of given sample. A minimum concentration of 500 million spermatozoa per ml of semen was acceptable for further processing.

iv) Mass Activity: Mass motility was assessed just after the semen collection. It was recorded by placing a small drop (~20 µl) of freshly collected undiluted semen on clean, grease free, pre-warmed glass slide, examining without cover slip, under a low power magnification (10X) of a phase contrast microscope (Nikon Eclipse E 400, Japan) fitted with a biotherm stage (37˚C). The mass activity of spermatozoa was graded from 0 to + 5 scale based on the appearance of waves and swirls (Salisbury *et al.* 1978).

0 = No mass activity

+ = Slow wave motion

++ = Rapid wave motion

+++ = Formation of eddies at the end of Wave

++++ = Eddies formation which end in between

++++ + = Churning of eddies.

Samples having +3 or more mass movement were considered for further processing in case of good quality ejaculates and below +3 were also considered in case of poor quality ejaculate evaluation.

3.5 PROCESSING AND ULTRA-FREEZING OF SEMEN

3.5.1 Semen dilution

After evaluating the semen, the selected samples were diluted with a calculated quantity of pre-warmed (37˚C) Tris citric acid egg yolk extender in sterilized conical flask so as to pack 20 million sperms/straw. Immediately after collection the semen samples were subjected to initial examination qualifying which they were diluted with pre-warmed (37°C) Tris citric acid egg yolk extender to make a final concentration of 80 million spermatozoa per ml of semen. Tris-egg-yolk-glycerol semen extender was used for the extension of all the ejaculates

Tris (Hydroxy methyl Amino Methane) (Sigma)	:	24.22 grams
Citric acid monohydrate (Rankem)	:	13.60 grams
D-Fructose (Loba)	:	10.0 grams
Glycerol (Rankem)	:	70 ml
Ultra pure water	:	730 ml
Streptomycin	:	1gm
Benzyl Penicillin	:	10 lac IU

Egg yolk : 200 mL

3.5.2 Filling, sealing and printing of straws

Diluted semen was filled in 0.25 ml French mini plastic straws, pre-sterilized by ultra-violet radiations, having one end plugged with a double layer of cotton enclosing a layer of polyvinyl chloride (PVC) powder and the other end was free. Filling of straws was done through suction and sealed at free end with the help of filling and sealing machine (IS4-Integrated System- IMV) and printing of straws was done by integrated inkjet printer.

3.5.3 Equilibration

Immediately after filling, sealing and printing, the straws were transferred in the cold handling cabinet (IMV) and were arranged on the freezing racks with the help of ramp which also facilitates easy counting. These semen straws were kept in the cold handling cabinet, maintained at 4˚C for 3 hours in order to bring down the temperature from 37°C to 4°C by gradual cooling.

3.5.4 Freezing of semen

After equilibration, racks were shifted to the programmable bio-freezer (IMV Digit cool 500) where nitrogen vapours were used to bring down the temperature to -140°C within 10 min. Thereafter, semen straws from the racks were shifted into goblets and plunged into liquid nitrogen where they could be stored for indefinite period of time for future use.

3.5.4 Thawing of semen

Thawing of frozen semen straws was done at 37°C for 30 seconds for post-thaw evaluation purpose.

3.6 SEMEN EVALUATION

The semen samples selected for processing after initial examination were evaluated for certain functional and biochemical parameters at four stages viz;

1. Post-dilution at 37°C
2. Post-equilibration at 4°C
3. 0 hour post-thaw
4. 1 hour post-thaw

At all these stages the semen samples were evaluated for motility, live and dead count, reaction to hypo-osmotic solution and acrosomal integrity. Additionally, diluted semen was centrifuged at all the four stages to separate seminal plasma for biochemical estimation.

3.6.1 Live and Dead Count of Spermatozoa

To ascertain the percentage of live spermatozoa, one drop of semen was mixed with five drops of Eosin-Nigrosin stain (Eosin 1.67gm, Nigrosin 10 gm, Distilled water 100 ml). Smears were drawn on a clean grease free glass slide, dried at room temperature and examined under high power objective. All stained and partially stained spermatozoa were considered dead. The percentage of live spermatozoa was determined by counting at least 200 spermatozoa (Sharma 2011).

3.6.2 Motility

A drop of semen was taken on a pre-warmed clean, grease free slide. A cover slip was placed over it to allow uniform spreading of the drop under it. Three fields were examined randomly under the high power objective of a warm stage (37°C) phase contrast microscope (Nikon Eclipse 50 Japan). All non-motile sperms and sperms with circular, backward and oscillating movements were counted. The slide was then passed over flame to kill all the sperms and total number was counted randomly (Sharma 2011). The percentage of progressively motile sperms was calculated as;

$$\frac{\text{Total sperms - sperms counted before passing over flame}}{\text{Total sperms}} \times 100$$

3.6.3 Hypo Osmotic Swelling Test (HOST)

Hypo-osmotic swelling test was performed as described by Pant et al. (2002) with slight modifications. Hypo-osmotic solution of 100 mOsm/L and control solution of 300 mOsm/L were prepared as under:

<u>Hypo-osmotic swelling Test Solution</u>

Trisodium citrate (analytical reagent) : 0 .450g
($Na_3C_6H_50_7.2H_20$; MW 294.10, Qualigens)

D-fructose (analytical reagent) : 0.990g

($C_6H_{12}0_6$; MW 180.16, Merck)

Double distilled water	:	up to 100 ml
Osmolarity	:	100 mOsm/L

Control Solution

Trisodium citrate (analytical reagent) ($Na_3C_6H_50_7.2H_20$; MW 294.10, Qualigens)	:	2.94g
D-fructose (analytical reagent) ($C_6H_{12}0_6$; MW 180.16, Merck)	:	5.4g
Double glass distilled water	:	up to 100 ml
Osmolarity	:	300 mOsm/L

To perform this test, 0.1 ml of extended semen was mixed with 1 ml of pre incubated hypo-osmotic solution (100 mOsm/L) in sugar tube. A control was set by mixing 1 ml of control solution (300 mOsm/L) with 0.1 ml of same semen in another sugar tube. Both tubes were then incubated in water bath at 37°C for 30 minutes. A drop from each solution of incubated semen was examined under phase contrast microscope at 400x magnification for swelling (ballooning or curling) of sperm tails. A minimum of 100 spermatozoa were counted. The proportion of swollen spermatozoa in control sample was subtracted from the proportion of swollen spermatozoa in hypo-osmotic solution. The resultant figure was considered as per cent HOST reactive spermatozoa.

3.6.4 Percent Intact Acrosome

For evaluation of acrosomal integrity, smears of extended semen were drawn on a clean grease free glass slide and were air dried. Dried smears were then fixed with formal saline (1 part formalin and 4 parts normal saline) for 20-30 minutes at room temperature. Slides were then washed under running tap water for 10 to 15 minutes, dried and dipped in the jar containing Giemsa staining solution overnight. Thereafter the slides were rinsed in distilled water and dried (Singh 1986).

Giemsa staining solution was prepared by mixing 3 ml of Sorenson's buffer working solution, 4.5 ml Giemsa stock solution and 42.5 ml distilled water. Sorenson's buffer working solution was prepared as under:

<u>Sorenson's Buffer solution preparation</u>

Solution A

Potassium dihydrogen orthophosphate (LR) (KH_2PO_4, MW 136.09, s.d.fine-chem)	:	1.316 grams
Double distilled water	:	100 ml

Solution B

di- Sodium hydrogen orthophosphate (AR) (Na_2HPO_4 MW 141.96, s.d.fine chem.)	:	1.414 grams
Double distilled water	:	100 ml

Working solution was prepared by mixing 1.7 ml of solution A and 3.3 ml of solution B.

Stained spermatozoa were examined under 10x100 magnifications under oil immersion. Spermatozoa having ruffled acrosomes, devoid of acrosomes, swollen acrosomes and having abnormal contour were counted by examining at least 100 spermatozoa.

3.7 Estimation of Biochemical Parameters of Seminal Plasma

Certain enzymes and minerals were estimated in the seminal plasma separated at all the four stages.

3.7.1 Separation of seminal plasma

For biochemical (mineral and enzyme) estimation, 2 ml diluted semen was taken at initial post dilution stage and 10 straws were cut at each of post-equilibration and post-thaw stages. Semen was collected in glass vials and centrifuged@ 268 g for 25 minutes (2000 rpm), seminal plasma was separated and stored at -20°C till further analysis.

3.7.2 Enzymes estimation

The enzymes estimated in this study were Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and Alkaline Phosphatase (AKP). These were estimated in seminal plasma using ELISA (Vet Test 8008). AST and ALT was

estimated using the diagnostic kit (Cogent, Span diagnostics) as per the method of Reitman and Frankel (1957), whereas, AKP was estimated by using diagnostic kit (Cogent, Span diagnostics) as per the method of Kind and King, (1954). The wavelength used for estimation of AST and ALT was 505 nm, whereas, for AKP it was 510nm.

3.7.3 Minerals estimation

The minerals estimated were Calcium (Ca), Phosphorus (P), Magnesium (Mg), Sodium (Na), Potassium (K) and Chloride (Cl). All the mineral estimations, except Magnesium, were done using the diagnostic kit (Span diagnostics) with the help of spectrophotometer (PI based double beam UV spectrophotometer). The wavelengths used for estimation of Ca, P, Na, K and Cl were 578 nm, 340 nm, 550 nm, 630 nm and 505 nm, respectively. Magnesium was estimated using atomic absorption spectrophotometer (Perkin Elmer AAnalyst 400) at wave length of 285.2 nm.

3.8 STASTICAL ANALYSIS

The data obtained were analysed using SAS statistical package version 9.2. Generalized Linear Model of one way ANOVA based on Fisher's Least Significant Difference method was used to determine the individual bull variation with regard to the various seminal attributes at different stages of processing. Multivariate analysis was used to determine the correlations and to frame regression equations and their significance were tested by ANOVA.

CHAPTER 4

Results and Discussion

4.1 Initial and functional parameters of good and poor quality semen of Murrah buffalo bulls

4.1.1 Comparison of initial parameters of good versus poor quality semen of Murrah buffalo bulls

Comparative values of various initial parameters of good versus poor quality semen of Murrah buffalo bulls have been shown in Table 1 and Figures 1.1, 1.2, 1.3 and 1.4, respectively. Bull wise initial parameters in good quality semen of individual bulls have been depicted in Annexure 1.

Table 1: Comparison of initial parameters (mean±SE) of good versus poor quality semen of Murrah buffalo bulls.

Semen Quality	Volume (ml)	Sperm concentration ($X10^6$)	Mass activity (0-5 Scale)	pH
Good	3.92 ± 0.08^a (3.0-5.0)	1593.88 ± 24.22^a (1257-1947)	4.30 ± 0.08^a (3.0-5.0)	6.90 ± 0.003^a (6.84-6.93)
Poor	3.38 ± 0.13^b (1.5-5.5)	1503.10 ± 24.60^b (1242-1887)	1.66 ± 0.083^b (0.0-3.0)	7.35 ± 0.027^b (6.95-7.82)

Figures within parenthesis indicate range
Figures with different superscripts (a, b) within a column differ significantly ($p<0.01$)

i. Ejaculate Volume

Ejaculate volume varies, depending on breed and age of the bull (Vale 1994). In general, the volume of the semen increases with age, and also depends upon the general and reproductive health of the animal and the frequency of its ejaculation. It is inferred that an increase or decrease in volume of semen is not usually correlated with fertility or sterility in a male unless ejaculation fails to occur (Roberts 1971).

In present study, the mean ejaculate volume was 3.92±0.08 and 3.38±0.13 ml for good and poor quality ejaculates, respectively. The observed values in present study corresponded to those reported by Sukhato *et al.* (1988), Koonjaenak *et al.* (2007a) and Sajjad *et al.* (2007). Relatively higher ejaculate volume have also been reported by El-Wishy (1978) in Iraqi (4.10 ml) and by Javed *et al.* (2000) in Nili-Ravi (4.67±1.62 ml) buffalo bulls. Contrarily, significantly lower semen volume has been reported by

Chaudhary and Gangwar (1977) and Pandey and Gupta (2004) in Murrah buffalo bulls (1.64 ml and 1.82±0.16 ml, respectively).

ii. Sperm Concentration

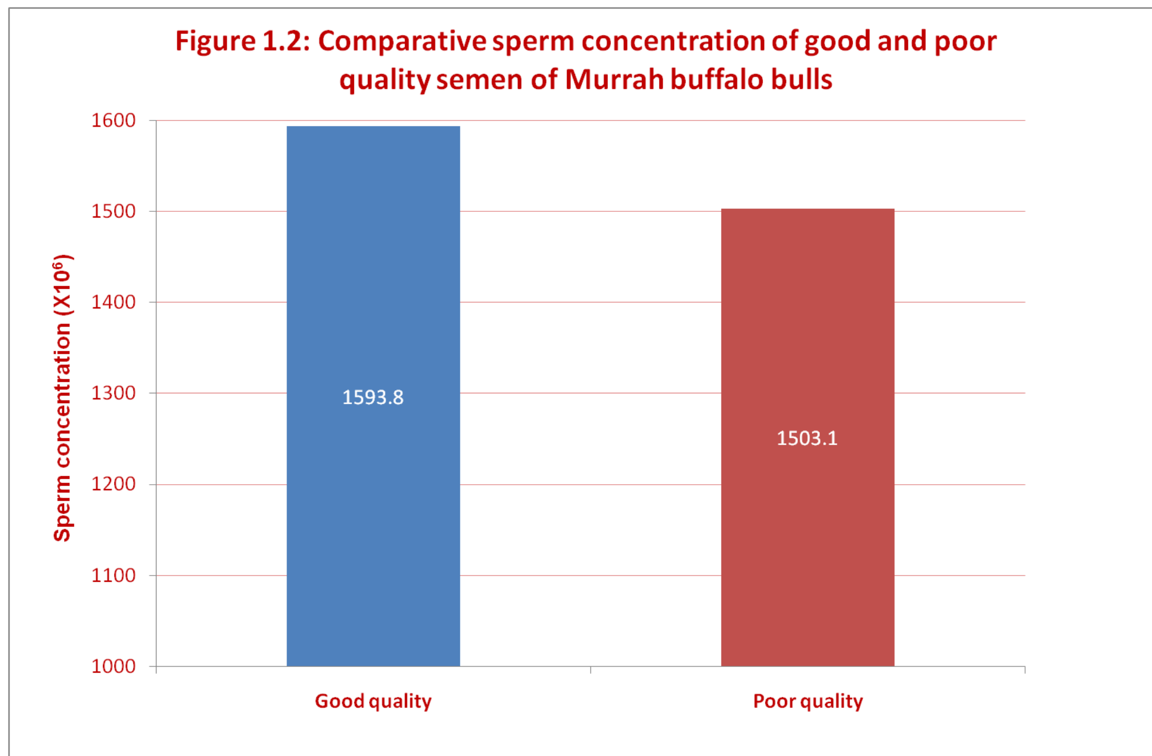
Sperm concentration is a breed character. In a given breed, a number of factors such as sexual development and maturity of the bull, nutrition, reproductive health, size of testes, breed, age of the bull and climate affect the concentration of spermatozoa in ejaculates. The lower sperm concentration in old bulls could be due to senility (Javed *et al.* 2000).

In present study, the overall mean concentration in good quality ejaculates was 1593.88±24.22x10^6 whereas it was 1503.10±24.60x10^6 per ml in poor quality ejaculates.

Relatively lower sperm concentrations of 690.6±187.9 to 1290.7±100 x10^6/ml (Galli *et al.* 1993), 524.1±20.7-1031.4±28.7x10^6/ml (Kumar *et al.* 1993), 1166.3±17.5x10^6/ml (Aguiar *et al.* 1994), 1335.42±58.36x10^6/ml (Pandey and Gupta 2004), 1000±0.50x10^6/ml (Javed *et al.* 2000), 1150x10^6/ml (Nazir 1988) and 1330x10^6/ml (Terezinha *et al.* 1991) have been recorded by others. However, El-Wishy

(1978) and Raizada *et al.* (1988) reported higher sperm concentration of $1650x10^6$/ml and $2900x10^6$/ml, respectively in the semen of buffalo bulls.

The breed variations in sperm concentration have also been documented. The sperm concentrations were $970x10^6$/ml in Swamp (Jainudeen *et al*. 1982), 1060×10^6/ml in Nili-Ravi (Heuer and Tahir 1982), $940x10^6$/ml in Surti and $1050x10^6$/ml in Murrah (Rahman *et al*. 1991) buffalo bulls.



iii. Mass Activity

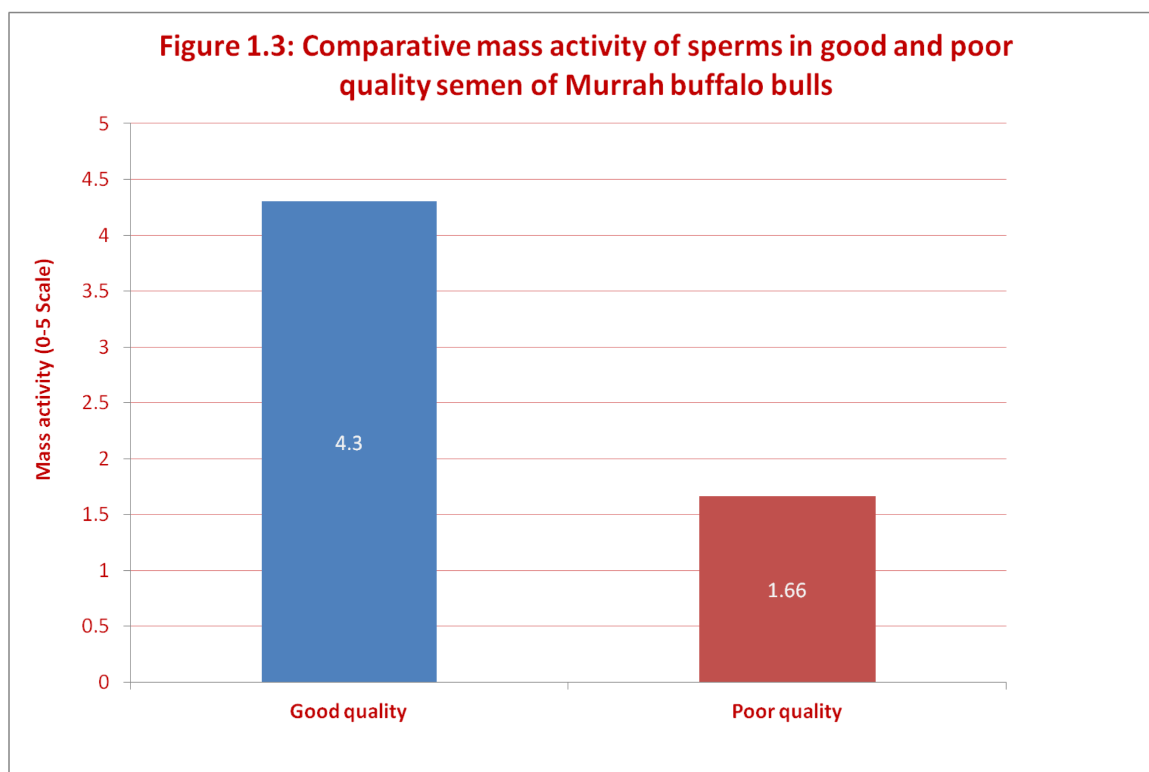
Mass activity represents a qualitative assessment of freshly ejaculated semen. The quality of sperm movement is usually graded according to the type of movement made by the largest proportion of the total spermatozoa population.

In present study the mean mass activity of good quality ejaculates (in a scale of 0-5) was 4.30±0.08 whereas, in case of poor quality ejaculates it was 1.66±0.08. The mean mass activity was significantly higher ($p<0.01$) in good quality ejaculates.

Mean mass activity recorded in good quality ejaculates of present study was higher than that reported by others (2.93, Heuer and Tahir 1982; 2.53, Vyawanare *et al*. 1989; 3.24, Pandey and Gupta 2004; 3.59, Eghbali *et al*. 2010). However, some other studies have reported relatively lower mass activity in buffalo bulls. Younis (1996) and

Javed *et al.* (2000) recorded mass activity of 1.88±0.07 and 2.65±1.14, respectively, in Nili-Ravi buffalo bulls. Kunbhar *et al.* (2011) recorded mass activity of 2.85±0.11 in Kundhi buffalo bulls.

The variation in mass activity could be due to higher sperm concentration, low sperm abnormalities and age of the bulls (Saeed 1988; Younis 1996; Javed *et al.* 2000) and also due to individual, season, management and collection procedure (Sagdeo *et al.* 1990; Sharma *et al.* 1991).



iv. pH

The pH of the ejaculate is regulated predominantly by the basic seminal vesicles and acidic prostatic secretions. Acidic ejaculate (lower pH value) may indicate one or both of the seminal vesicles are blocked. A basic ejaculate (higher pH value) may indicate an infection. A pH value outside the normal range is harmful to sperm.

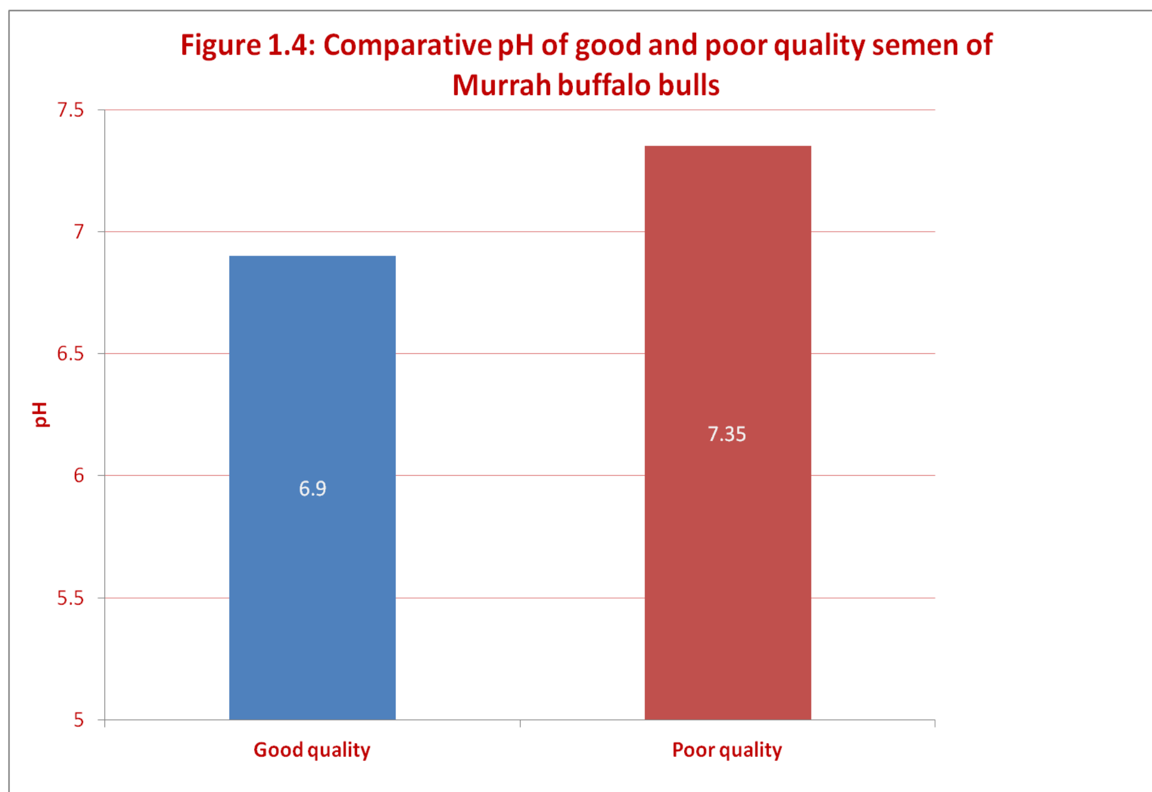
In the present study, the mean pH of good quality ejaculates was 6.90±0.003 where as it was 7.35±0.027 in poor quality ejaculates, the difference being highly significant ($p<0.01$).

In the present experiment, the pH recorded in good quality ejaculate was in accordance with the mean pH reported by other researchers (6.4–7.0, Rattan 1990;

Kumar *et al.* 1993; Aguiar *et al.* 1994; Vale 1997). Similarly, Javed *et al.* (2000) recorded pH of 6.55±0.50 in good quality ejaculate in Nili Ravi, whereas Pandey and Gupta (2004) reported it to be 6.51±0.46 in Murrah buffalo bulls.

However, lower pH values of 5.82±0.09 (Kunbhar *et al.* 2011), 6.51 (Alexander *et al.* 1971), 6.26±0.05 (Terezinha *et al.* 1991) and 6.38±0.19 (Younis 1996) and higher mean pH of 7.01 ± 0.08 (Sajjad *et al.* 2007) were observed by others in the semen of buffalo bulls. The differences in pH could be due to variations of breed, sperm concentration, per cent viability, season, frequency of collection and number of observations.

In buffaloes, good quality semen has pH of 6.8, while poor quality shows pH of 7.0 to 7.2, attributed to larger amount of fluid from urethral and accessory glands in poor quality semen (Salisbury and Van Denmark 1962). The pH of semen also decreases with time lapse between collection and measurement of pH since the fructose in the semen is metabolised by spermatozoa to lactic acid under anaerobic condition. The anaerobic conditions are usually expected to exist in a narrow collection tube.



4.1.2 Comparison of functional parameters of good versus poor quality semen of Murrah buffalo bulls

Comparative functional semen evaluation parameters of good versus poor quality semen of Murrah buffalo bulls have been shown in Table 2 and Figures 2.1, 2.2, 2.3 and 2.4, respectively. Bull wise functional parameters in good quality semen of individual bulls have been depicted in Annexure 2 to 5.

Table 2: Comparative functional parameters (mean±SE) of good versus poor quality semen during various stages of processing in Murrah buffalo bulls.

Parameter (n=48)	Quality	Stage of semen processing				LSD
		Fresh Diluted	Equilibration	0 hr Post-thaw	1 hr Post-thaw	
Live spermatozoa (%)	Good	90.88±0.26 $^{a\,A}$ (86-94)	86.44±0.25 $^{a\,B}$ (82-90)	78.29±0.31 $^{a\,C}$ (73-83)	74.00±0.36 $^{a\,D}$ (69-80)	0.827
	Poor	64.42±0.91 $^{b\,A}$ (49-79)	48.77±1.11 $^{b\,B}$ (32-63)	29.42±1.10 $^{b\,C}$ (15-44)	10.75±0.78 $^{b\,D}$ (02-25)	2.7497
Progressive motile spermatozoa (%)	Good	86.15±0.34 $^{a\,A}$ (79-90)	73.17±0.38 $^{a\,B}$ (68-80)	63.5±0.29 $^{a\,C}$ (60-68)	40.46±0.50 $^{a\,D}$ (32-49)	1.0825
	Poor	44.48±0.75 $^{b\,A}$ (30-54)	31.25±0.69 $^{b\,B}$ (20-43)	18.15±0.69 $^{b\,C}$ (08-29)	5.42±0.58 $^{b\,D}$ (00-13)	1.9025
HOS Reactive Spermatozoa (%)	Good	89.19±0.26 $^{a\,A}$ (84-92)	85.27±0.23 $^{a\,B}$ (81-89)	77.05±0.31 $^{a\,C}$ (72-82)	72.90±0.39 $^{a\,D}$ (68-79)	0.8497
	Poor	63.17±0.93 $^{b\,A}$ (47-77)	47.58±1.09 $^{b\,B}$ (31-61)	28.44±1.09 $^{b\,C}$ (14-43)	9.94±0.79 $^{b\,D}$ (00-24)	2.7466
Intact Acrosome (%)	Good	91.21±0.30 $^{a\,A}$ (87-95)	85.48±0.37 $^{a\,B}$ (80-90)	78.77±0.35 $^{a\,C}$ (74-83)	70.79±0.32 $^{a\,D}$ (67-76)	0.9316
	Poor	65.33±0.88 $^{b\,A}$ (46-80)	49.65±1.04 $^{b\,B}$ (34-61)	32.06±0.81 $^{b\,C}$ (19-43)	15.88±0.64 $^{b\,D}$ (05-24)	2.3898

Figures within parenthesis indicate range
Figures with different superscripts (a, b) within a column differ significantly ($p<0.01$)
Figures with different superscripts (A, B) within a row differ significantly ($p<0.01$)

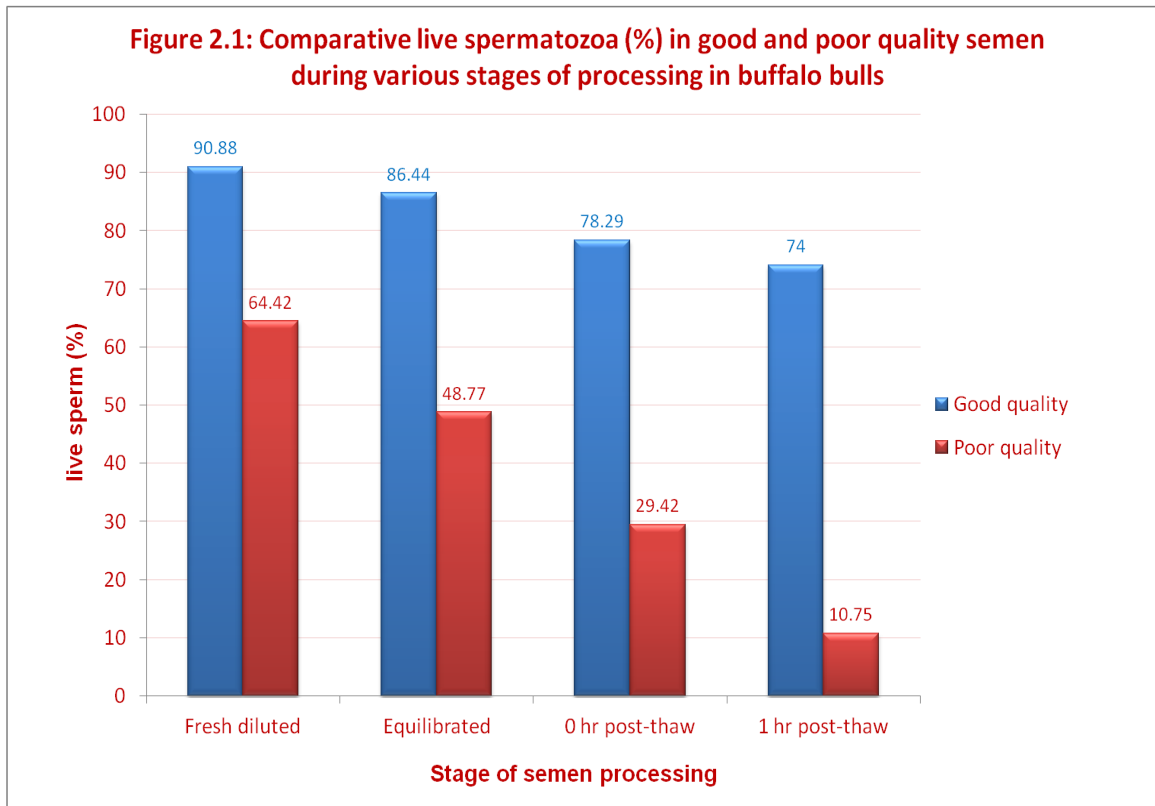
i. Live Spermatozoa

Intactness of the spermatozoa plasma membrane is a prerequisite for ideal sperm metabolism and function (Harrison 1997). In order to fertilize an oocyte, a sperm must have an intact and competent plasma membrane. Once damaged, spermatozoa are not able to reseal the compromised plasma-lemma (Woelders 1991), and therefore, fail to maintain the ions and co-factor concentrations essential to sperm survival (Januskauskas and Zilinskas 2002). It is generally accepted that semen with more than 30 per cent initially dead spermatozoa is not to be recommended for the preservation and such semen is liable to reduce the fertility (Lasley 1951).

As evident from the Table 2, the mean livability was significantly higher ($p<0.01$) in good quality ejaculates at all the four stages of processing. At post-dilution stage it was 90.88±0.26 per cent in good quality ejaculates, whereas it was 64.42±0.91 per cent in poor quality ejaculates. Subsequent processing of semen lead to a significant ($p<0.01$) progressive decline in mean livability.

The per cent live spermatozoa in freshly diluted semen in present study is in accordance with those reported by other researchers (90.50%, El-Sisy *et al.* 2010; 89.68±0.94%, Eghbali *et al*. 2010), whereas it was higher than other reports (84**.**3%, Lodhi *et al.* 2008; 85.26%, Shoushtari *et al.* 2009). This variation could be attributed to breed (Hazarika *et al*. 1988; Singh and Pangaonkar 1990), age (Sudheer 2000) and seasons (Dhami *et al*. 1998). At equilibration stage, lower percentages of live spermatozoa (71.10% and 78.2%) were reported by El-Sheshtawy *et al.* (2008) and El-Sisy *et al.* (2010), respectively. These differences may be due to variation in the cooling and equilibration time (Mathur *et al*. 1991) and glycerolization (Veeraiah *et al*. 1997).

Per cent livability, in current study, at post-thaw stage was significantly higher than that reported by other workers (41.10±1.66%, El-Sheshtawy *et al.* 2008; 59.0%, El-Sisy *et al.* 2010; 61.7±1.7%, Singh 2010; 55.51±0.16, Beheshti *et al*. 2011). This variation can be due to variable glycerol concentration (Robbins *et al*. 1976), thawing procedure (Yott and Wells 1978; Bhosrekar *et al*. 1984), freezing rate and time of equilibration (Mathur *et al*. 1991) and also age of egg used in diluent (Veeraiah *et al*. 1997).



ii. Progressive motility of spermatozoa

Motility is essential for the transport of sperm through the female reproductive tract and for penetration of oocyte during fertilization. Motility is commonly believed to be one of the most important characteristics associated with the fertilizing ability of sperm and is an expression of viability and structural integrity of spermatozoa. Superiority of semen depends on the number of progressively motile spermatozoa present in it. Weakly motile spermatozoa may generally be senile or crippled, and hence, may not be very successful in the fertilization.

Perusal of Table 2 shows that the mean progressive motility was significantly higher ($p<0.01$) at all the four stages of semen evaluation in good quality ejaculates. A significant decline ($p<0.01$) was observed in overall mean progressive motility in semen at different processing stages in both good and poor quality ejaculates. The overall mean progressive motility recorded at post-dilution stage in good quality ejaculates was 86.15±0.34 per cent, where as it was 44.48±0.75 per cent in poor quality ejaculates, which further declined to 73.17±0.38 in good quality and 31.25±0.69 per cent in poor quality ejaculates at post-equilibration stage. Immediately after post-thaw, it was

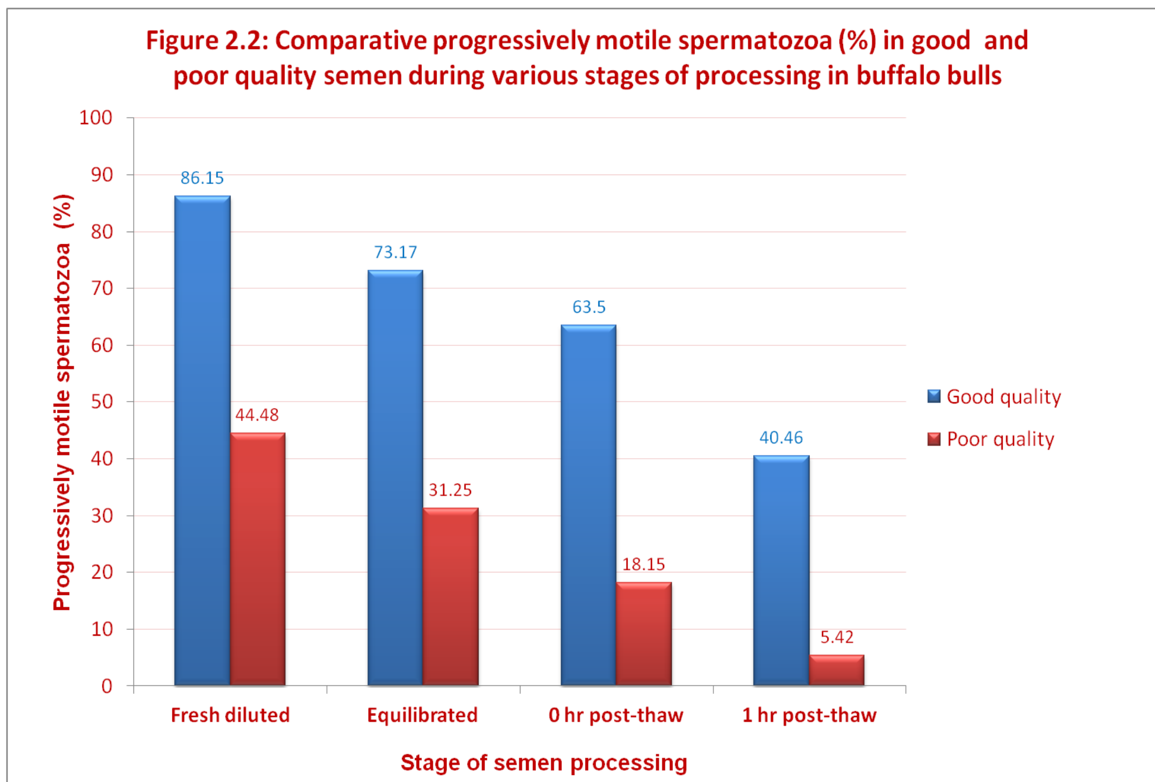
63.50±0.29 per cent and 18.15±0.69 per cent in good and poor quality ejaculates, respectively. After 1 hr incubation post-thaw, progressive motility further declined and it was 40.46±0.50 and 5.42±0.58 per cent in good and poor quality ejaculates, respectively.

Mean progressive motility in freshly diluted semen recorded in present study (86.15±0.34%) was similar to 87.02±1.06 per cent reported by Eghbali *et al.* (2010), but was higher than that reported by others (80.75%, Lodhi *et al.* 2008; 81.66±0.62%, Shoushtari *et al.* 2009; 82.33±0.88%, Raj Kumar *et al.* 2010; 82.25%, El-Sisy *et al.* 2010, 71.75±2.62%, Kunbhar *et al.* 2011; 64.53±0.76%, Rehman *et al.* 2012). This variability of progressive motility, amongst the other factors, could be due to innate difference in the process of spermatogenesis and adaptability of bulls to environmental fluctuation (Rodriguez-Martinez 2007).

At post-equilibration stage, the mean progressive motility was recorded to be 73.17±0.38 per cent, which was similar to 72.0 and 69.64 per cent reported by El-Sisy *et al. (*2010) and Khan and Ijaz (2007), respectively, but was higher than 60.50±1.74 per cent reported by El-Sheshtawy *et al.* (2008). Immediately after thawing of semen, the mean progressive motility recorded was 63.5±0.29 per cent, which was in accordance to 60.1±1.34 per cent, reported by Khan and Ijaz (2007), but was significantly higher than that reported by other researchers (31.66±1.66%, Raj Kumar *et al.* 2010; 36.6%, El-Sisy *et al.* 2010; 48.1±1.9%, Singh 2010; 45.86±0.18%, Beheshti *et al.* 2011). This variation may be due to kind of dilutor (Rao *et al.* 2002), pre-freezing holding time (Dhami and Kodagali 1991), glycerol concentration (Sagdeo *et al.* 1991), initial sperm motility (Mohanty 1999), age of egg used in extender (Sudheer 2001) and thawing procedures (Singh *et al.* 1993).

After 1 hr incubation, mean per cent progressive motility was recorded to be 40.46±0.50 per cent, which was comparable to 43.25±2.95 per cent and 40.6 per cent, reported by Kunbhar *et al.* (2011) and Rehman *et al.* (2012). However, it was higher than 29.4±1.2 per cent as reported by Singh (2010). This variation to withstand the incubation effect might be due to the difference in thawing procedures (Singh 1986), incubation medium (Singh 1996), and equilibration time (Dhami *et al.* 1995). The significant decline in progressive motility over different stages of processing has been documented by Sugulle *et al.* (2006).

Spermatozoal motility is known to be dependent on mitochondrial function. The ATP generated by oxidative phosphorylation in the inner mitochondrial membrane is transferred to the microtubules to drive motility. Hence reduced sperm motility induced by cryopreservation is believed to be mainly associated with mitochondrial damage (Ruiz-Pesini *et al*. 2001; Januskauskas and Zillinskas 2002). This could be due to death of spermatozoa during freezing, reduction of cAMP (Chaudhary and Anand 1977; Kakar and Anand 1984), and decrease in ATP level due to inability of the mitochondrial enzymes to produce ATP (Heath and Gupta 1980).



iii. HOS reactive spermatozoa percentage

The HOS test was originally designed for evaluation of biochemical activity of the physically intact human sperm membrane (Jeyendran *et al*. 1984). An intact and functionally active membrane is a prerequisite for metabolism, capacitation, acrosome reaction, attachment and penetration of oocyte (Jayendran *et al*. 1984). Deterioration of spermatozoa function due to change in structural components occurs during the process of semen processing, freezing, cryostorage and thawing (Graham 1978; Zavos 1983, 1990a; Centola *et al*. 1992). In hypo-osmotic solution, water enters into the sperm through

the membrane to establish equilibrium between extra- and intracellular fluid. As a result the head of the sperm becomes swollen and the membrane covering the tail expands, causing the flagellum to coil inside it. Spermatozoa with damaged or inactive membrane are unable to support osmotic swelling and hence do not respond to HOS test.

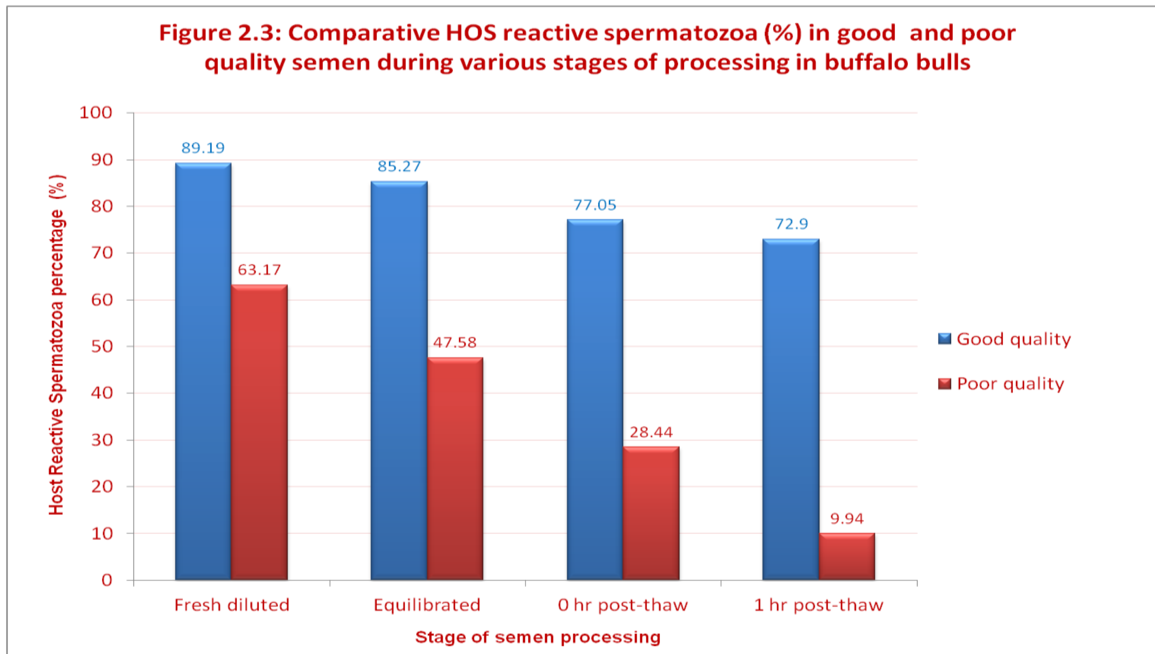
Significantly higher ($p<0.01$) HOS reactivity was observed at all the stages of semen processing in good quality ejaculates. Evaluation of HOS reactivity at various stages of processing showed that the HOS reactive spermatozoa percentage declined significantly ($p<0.01$) from post-dilution (89.19±0.26 and 63.17±0.93%) to post-equilibration (85.27±0.23 and 47.58±1.09%) and then at post-thaw stages (77.05±0.31 and 28.44±1.09% immediately after thawing and 72.90±0.39 and 9.94±0.79% after 1 hr post-thaw incubation) in good and poor quality ejaculates, respectively.

The mean percentage of HOS reactive spermatozoa at post-dilution stage recorded was 89.19±0.26 per cent in current experiment which was in agreement to 88.35 per cent (El-Sisy *et al.* 2010), where as it was higher than 85.25 and 78.33 per cent, reported by Lodhi *et al.* (2008) and Raj Kumar *et al.* (2010), respectively.

At post-equilibration stage, the mean HOS reactive sperm percentage was 85.27±0.23, which was higher than 79.6 per cent reported by El-Sisy *et al. (*2010).

El-Sisy *et al. (*2010) and Raj Kumar *et al*. (2010) recorded 56.9 per cent and 38.1 per cent mean HOS reactivity, respectively, immediately after thawing, which was lower than the present study.

Deterioration of spermatozoa function due to change in structural components occurs during the process of semen processing, freezing, cryo-storage and thawing (Graham 1978; Zavos 1983; Centola *et al*. 1992). This accounted for the gradual fall in the percentage of sperms responsive to HOS test post-thaw.



iv. Percent intact acrosome spermatozoa

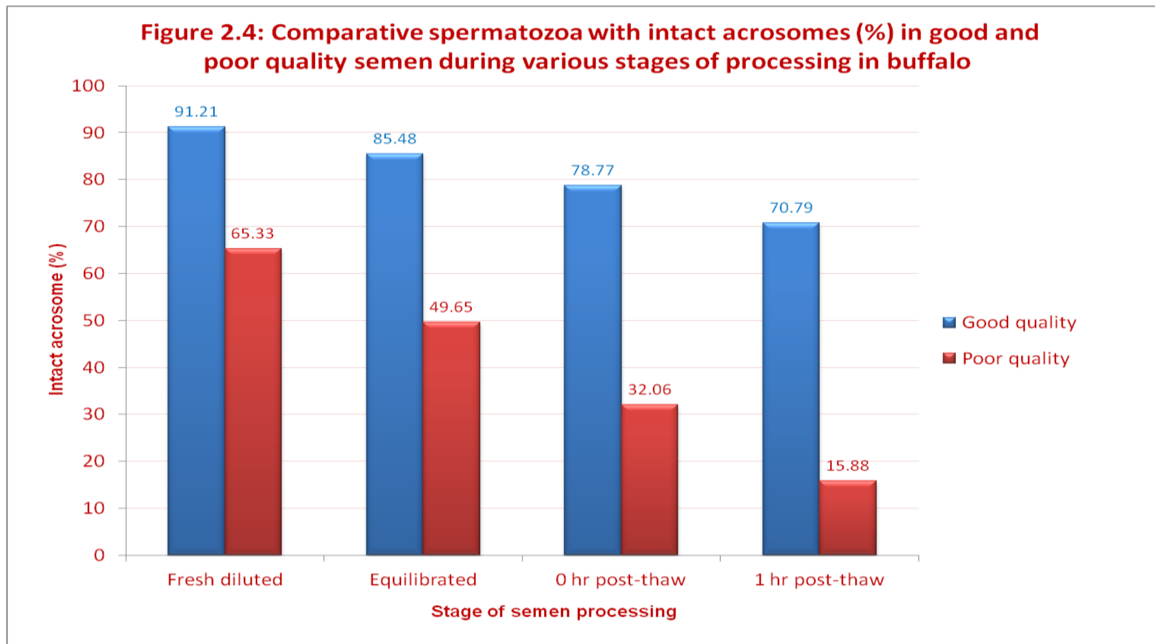
Acrosome, carrying various enzymes, plays an important role in the process/events of fertilization. Detachment of acrosome or loss of its membrane integrity may result into decrease ATP and loss of intracellular enzymes and proteins. Spermatozoa with a loss of acrosomal intactness could be highly motile but infertile. The assessment of acrosomal integrity is, therefore, an important assessment of spermatozoa (Saacke *et al.* 1968). A higher percentage of normal acrosomes are desirable in the semen. Acrosomal changes are highly correlated with fertility (Saacke and White 1972) and are simpler and easier methods to evaluate the effect of freezing on acrosome intactness.

Significantly higher (p<0.01) intact acrosome percentage was noticed in good quality ejaculates as compared to poor quality ejaculates at all the four stages of semen evaluation. The overall mean intact acrosomes recorded at post-dilution stage in good quality ejaculates was 91.21±0.30 per cent, where as this was 65.33±0.88 per cent in poor quality ejaculates which declined to 85.48±0.37 per cent in good quality and 49.65±1.04 per cent in poor quality ejaculates at post-equilibration stage, respectively. Immediately after post-thaw, it was 78.77±0.35 and 32.06±0.81 per cent in good and poor quality ejaculates, respectively. After 1 hr incubation post-thaw, mean per cent intact acrosome further declined to 70.79±0.32 and 15.88±0.64 per cent in good and poor

quality ejaculates, respectively. The decline in per cent intact acrosome spermatozoa at different stages was significant ($p<0.01$). (Table 2)

El-Sisy *et al.* (2010) reported 88.32 per cent intact acrosome in freshly diluted semen which was similar to the present study but was higher than 55.63 per cent, reported by Rehman *et al.* (2012). At post-equilibration stage the mean intact acrosome per cent recorded was 85.48±0.37 per cent, which was higher than 65.30 and 79.6 per cent reported by El-Sheshtawy *et al.* (2008) and El-Sisy *et al.* (2010) respectively. Immediately after post-thaw, the mean intact acrosome per cent in present study recorded was 78.77±0.35, which were significantly higher than that reported earlier (50.30%, El-Sheshtawy *et al.* 2008; 57.9%, El-Sisy *et al.* 2010; 68.38 %, Beheshti *et al.* 2011), whereas after 1 hr incubation the mean intact acrosome per cent was recorded as 70.79±0.32 which was higher than 35.3 per cent reported by Rehman *et al.* (2012). The variation in the observations could be due to the variability of response of the cell membrane and some primary defects in spermatogenesis between different breeds and individual bulls (Mann and Lutwak-Mann 1981).

Stresses of freezing and thawing lead to acrosomal damage (Gilbert and Almquist 1978; Chaves 1979). A marked decline in normal intact acrosome after freeze-thawing of bull (Kumar 2004) and buffalo spermatozoa (Patil *et al*. 1981; Kumar 1996; Singh 2002) has been reported. Yulnawati *et al*. (2010) observed that there was a change in osmotic pressure of cytoplasmic membrane during freezing process. Difference in osmotic pressure between intra and extra cellular medium damages the cytoplasmic membrane, which may be the cause of lowered percentage of cytoplasmic membrane integrity after freezing and thawing process.



4.2 Enzymatic profile of good and poor quality semen of Murrah buffalo bulls

Enzymes, such as Aspartate amino transferase (AST), Alanine aminotransferase (ALT), lactate dehydrogenase, cholinesterases and phosphatases (alkaline and acid, Roberts 1971), proteolytic enzymes, phospholipases, transaminases, ATPase, glycosidase, dehydrogenases, nucleotidases, DNAses, hyaluronidase (Bhosrekar *et al.* 1994) have been recognized to be intimately related to the sperm cell and 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). These have been known to leak into seminal plasma when there is damage to the sperm cells.

Following dilution, freezing, thawing and cold shock reactions, the activity of such enzymes have been reported to rise above their initial level in seminal plasma owing to increased cellular permeability, which cause leakage of intracellular enzymes into the media concomitant to increased sperm abnormality and decreased sperm motility and viability (Mann and Lutwak-mann 1981; Dhami and Kodagali 1990). Similar situation have been recorded in fresh semen containing a high proportion of abnormal sperm (Mann and Lutwak-Mann 1981). Such damage to the sperm cells and

leakage of vital enzymes has been known to lower post-thaw recovery and fertility (Dhami and Kodagali 1990). For the said reasons, enzyme determination of semen plasma is essential in measuring the degrees of damage to the sperm cells before ejaculation and during the freezing process (Roberts 1971).

Comparative enzymatic profile of good versus poor quality semen of Murrah buffalo bulls have been shown in Table 3 and Figures 3.1, 3.2, 3.3 and 3.4, respectively. Bull wise enzymatic profiles in good quality semen of individual bulls have been depicted in Annexure 6 to 9.

Table 3: Comparative concentrations of various enzymes (mean±SE) in seminal plasma of good and poor quality semen during various stages of processing in Murrah buffalo bulls

Parameter (n=48)	Quality	Stage of semen processing				LSD
		Fresh Diluted	Equilibration	0 hr Post-thaw	1 hr Post-thaw	
Alkaline Phosphatase (KAU/dl)	Good	147.75±1.63 $^{a\,C}$ (134.89-164.33)	152.13±1.65 $^{a\,BC}$ (138.56-167.22)	155.94±1.69 $^{a\,B}$ (144.00-172.89)	163.22±2.59 $^{a\,A}$ (146.33-176.33)	5.39
	Poor	113.40±0.74 $^{b\,D}$ (104.00-121.33)	117.62±0.73 $^{b\,C}$ (109.77-127.55)	121.13±0.79 $^{b\,B}$ (114.55-130.99)	126.09±0.91 $^{b\,A}$ (118.11-138.00)	2.22
AST (U/L)	Good	87.22± 0.19^{aD} (83.45-89.54)	93.34± 0.25^{aC} (91.03-96.45)	103.08±0.21^{aB} (100.32-106.00)	107.97± 0.18^{aA} (105.00-109.96)	0.59
	Poor	122.55±0.74^{bD} (113.90-131.37)	127.51±0.80^{bC} (117.30-137.90)	134.39±0.69^{bB} (125.23-142.90)	146.26±1.34^{bA} (130.49-160.34)	2.59
ALT (U/L)	Good	14.75± 0.12^{aD} (12.94-16.43)	15.63± 0.11^{aC} (13.98-17.34)	16.92± 0.14^{aB} (15.22-18.93)	17.73± 0.11^{aA} (16.16-19.29)	0.34
	Poor	25.43±0.47^{bD} (19.85-32.12)	33.56±0.36^{bC} (25.68-38.58)	39.47±0.43^{bB} (31.20-44.39)	44.87±0.43^{bA} (34.58-49.59)	1.18
Hyaluronidase (U/ml)	Good	216.54±1.36 $^{a\,D}$ (197-233)	222.81±1.26 $^{a\,C}$ (204-237)	237.56±1.22 $^{a\,B}$ (221-251)	245.65±1.16 $^{a\,A}$ (230-259)	3.49
	Poor	451.54±2.12 $^{b\,D}$ (428-461)	463.08±2.31$^{b\,C}$ (432-491)	492.23±2.38 $^{b\,B}$ (460-517)	501.13±2.63 $^{b\,A}$ (466-525)	6.60

Figures within parenthesis indicate range
Figures with different superscripts (a, b) within a column differ significantly ($p<0.01$)
Figures with different superscripts (A, B) within a row differ significantly ($p<0.01$)

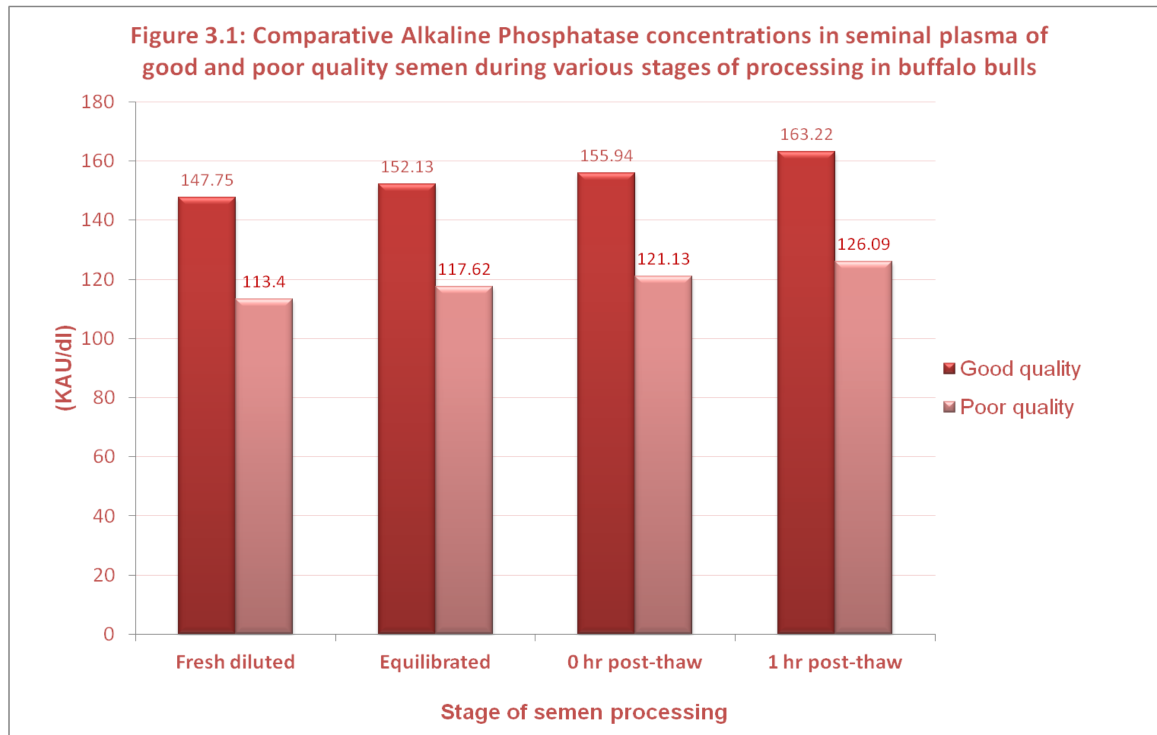
4.2.1 Alkaline Phosphatase (AKP)

Significantly higher ($p<0.01$) level of alkaline phosphatase (AKP) was recorded in good quality ejaculates in comparison to poor quality ejaculates at all the four stages of evaluation. The concentrations of AKP increased significantly ($p<0.01$) as the processing stages advanced in both good and poor quality semen. At post-dilution stage it was 147.75±1.63 and 113.40±0.74 KAU/dL and this concentration increased at equilibration to 152.13±1.65 and 117.62±0.73 KAU/dL in good and poor quality ejaculates, respectively. Immediately after post-thaw, AKP concentration was 155.94±1.69 and after 1 hr incubation it was 163.22±2.59 KAU/dL in good quality ejaculates whereas in poor quality ejaculates this concentration was 121.13±0.79 and 126±0.91KAU/dL at respective post-thaw stages.

Bhosrekar *et al.* (1994) reported drop in AKP on freezing of the semen, while Dhami and Kodagali (1988) reported increase of the enzyme activity on freezing semen in the extracellular fluid. They also found significant effect of extender on the leakage of these enzymes. Chaudhary and Gangwar (1977) found the average AKP values 1238±47.4

KAU/dl of seminal plasma. Dhami and Kodagali (1988) reported the overall mean level of AKP in the pre-freeze and post-thaw plasma 68.02 and 94.32 KAU/200 ml, respectively, which were significantly lower than the present results. Glogowski and Strzezek (1979) determined the AKP activity in fresh, after dilution, diluted seminal plasma after 4 hour equilibration and after storage of semen in LN_2 for 24 hour and recorded the corresponding activity to be 423.8, 137.9, 139.1 and 144.3 BU, respectively, where as Juyena and Stelletta (2012) found AKP values 315 BU/dL in seminal plasma of fresh ejaculate of buffalo bulls. Pangawakar *et al.* (1988) recorded the mean alkaline phosphatase activity as 717.21±20.31, 802.33±32.16, and 1002.65±31.22 KAU/100 ml in 3 different groups of ejaculates with freezablity of □ 85, 65-85 and □ 65 per cent, respectively.

Semen phosphatases play an important role in dephosphorylation during sperm metabolism. Alkaline and Acid phosphatases in semen reflect the functional state of accessory sex glands and metabolic activity of spermatozoa. Zvereva and Chuhrly (1972) reported increase in the conception rates in cows following inseminations with semen containing increasing order of alkaline phosphatase. According to Ibrahim *et al.* (1985) though epididymis represents a main source of Alkaline and Acid phosphatase in semen, ampullae share a great part in contributing these elements. At least some might be the results of leakage from sperm cells (Mahmoud *et al.* 1986). Hence higher AKP in good quality semen of present study might have originated from epididymal and/ or ampullae.



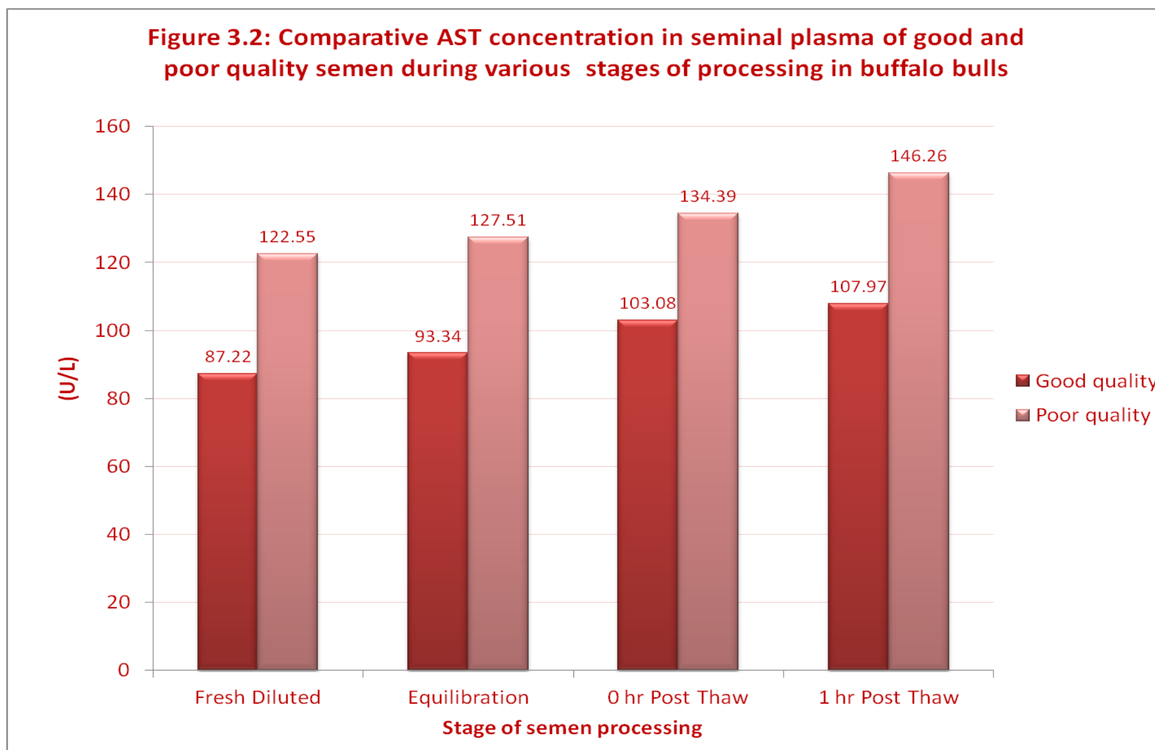
4.2.2 Aspartate aminotransferase (AST)

Significantly higher AST ($p<0.01$) concentrations were recorded in poor quality ejaculates at all the four stages of semen processing. The AST concentration increased significantly ($p<0.01$) from post-dilution to 1 hr incubation post-thaw in both good and poor quality ejaculates. As evident from Table 2, the AST concentration was 87.22±0.19, 93.34±0.25, 103.08±0.21 and 107.97±0.18 U/L at post-dilution, post-equilibration, post-thaw and 1 hr incubation stages in good quality ejaculates, respectively, whereas in poor quality ejaculates it was 122.55±0.74, 127.51±0.80U/L at post-dilution and post-equilibration stages and 134.39±0.69 and 146.26±1.34 U/L at 0 and 1 hr post-thaw stages, respectively.

Mean AST concentration recorded in fresh seminal plasma was 87.22±0.19 U/L, which corresponds to the earlier studies (52.5-80.2 IU/L, Buruiana *et al.* 1978; 83.50±3.00 IU/L, Varshney *et al.* 1978; 95.88±15.32 IU/L, Kumar 1986 and 87.45±13.52 U/L, Shukla *et al.* 2009). Contrarily, higher mean AST values of 166 U/L have also been reported by Juyena and Stelletta (2012) in buffalo seminal plasma.

The mean AST levels increased significantly ($p<0.01$) during different stages of semen processing. Similarly, increases in mean AST concentration from pre-freeze to post-freeze have been documented earlier (Dhami and Kodagali 1990; Dhami and Sahni 1994; Reddy *et al.* 1999).

The AST is a purely cellular enzyme and is not found in seminal plasma (Pace and Graham 1970). The presence of this enzyme in plasma indicates damage to spermatozoa, the assay of this enzyme has been used for evaluation of the quality of semen. (Flipse 1960; Crabo *et al.* 1971; Jani *et al.* 1983; Bhosrekar *et al.* 1988 and Bhosrekar *et al.* 1994). Bower *et al.* (1973) observed that addition of glycerol always increased the AST activity in extracellular fluid thereby indicating damage to spermatozoa.



4.2.3 Alanine aminotransferase (ALT)

Significant lower ($p<0.01$) concentrations of ALT were recorded in good quality ejaculates. The ALT concentrations in seminal plasma increased significantly ($p<0.01$) with advancement in the semen processing stages both in good and poor quality ejaculates. At post-dilution stage, the mean ALT concentration was 14.75±0.12 and

25.43±0.47 U/L which increased to 15.63±0.11 and 33.56±0.36 U/L at post equilibration stage in good and poor quality ejaculates, respectively. Immediately after post-thaw the concentration was 16.92±0.14 whereas, it was 17.73±0.11 U/L in good quality ejaculates after 1 hr incubation the corresponding values being 39.47±0.43 and 44.87±0.43 U/L at different post-thaw stages, in poor quality ejaculates.

The mean ALT concentration (14.75±0.12) in freshly diluted semen was in accordance with value of 15.08±54 IU/litre obtained earlier by Gupta and Prasad (2008), but was significantly lower than 34 U/L, recorded by Juyena and Stelletta (2012).

There was a significant increase ($p<0.01$) in mean ALT concentration with subsequent stages of semen processing. Similar increment in ALT concentration has been reported by earlier researchers (Dhami and Kodagali 1990; Juyena and Stelletta 2012; Reddy *et al.* 1999) in buffalo bulls

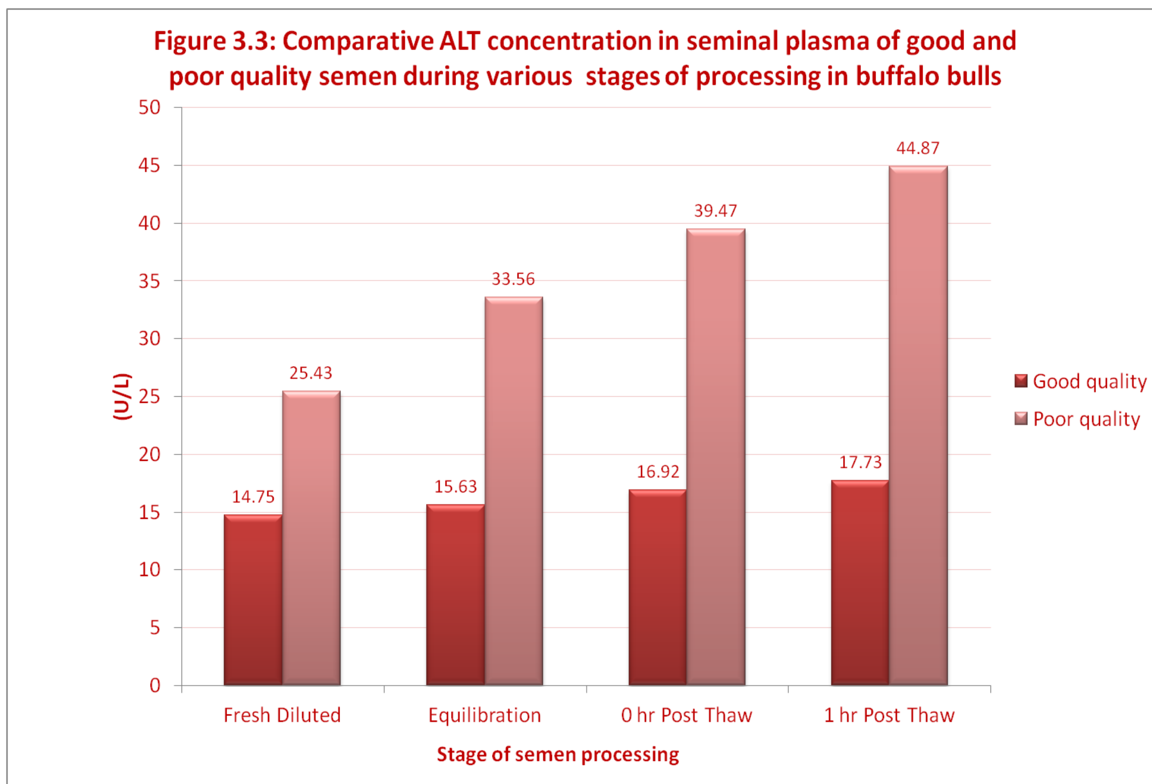


Figure 3.3: Comparative ALT concentration in seminal plasma of good and poor quality semen during various stages of processing in buffalo bulls

4.2.4 Hyaluronidase

Significantly higher ($p<0.01$) hyaluronidase concentrations were recorded in poor quality ejaculates and the concentration increased significantly ($p<0.01$) with semen processing in good as well as poor quality ejaculates. In good quality ejaculates, the

value was 216.54±1.36 U/ml in freshly diluted semen which increased to 222.81±1.26 U/ml at post-equilibration stage. However, at corresponding stages these values were 451.54±2.12 U/ml and 463±2.31 U/ml in poor quality ejaculates. Immediately post-thaw, the concentrations were 237.56±1.22 and 492.23±2.38 U/ml, which increased to 245.65±1.16 and 501.13±2.63 U/ml after 1 hr incubation in good and poor quality ejaculates, respectively.

There was a significant increase ($p<0.01$) in mean hyaluronidase concentration with subsequent stages of semen processing, which corroborated with earlier findings (Foulkes and Watson 1975; Strzezek *et al*. 1979).

Estimation of hyaluronidase has assumed a great importance in view of its place in spermatozoan system. This enzyme is present in acrosomal system of spermatozoa and nowhere else. Since integrity of acrosome is directly involved in fertilizing capacity of spermatozoa, this enzyme has assumed a great importance in estimating fertilizing capacity of semen. If there is damage to the acrosome, it is presumed that this enzyme will leak out in extracellular fluid.

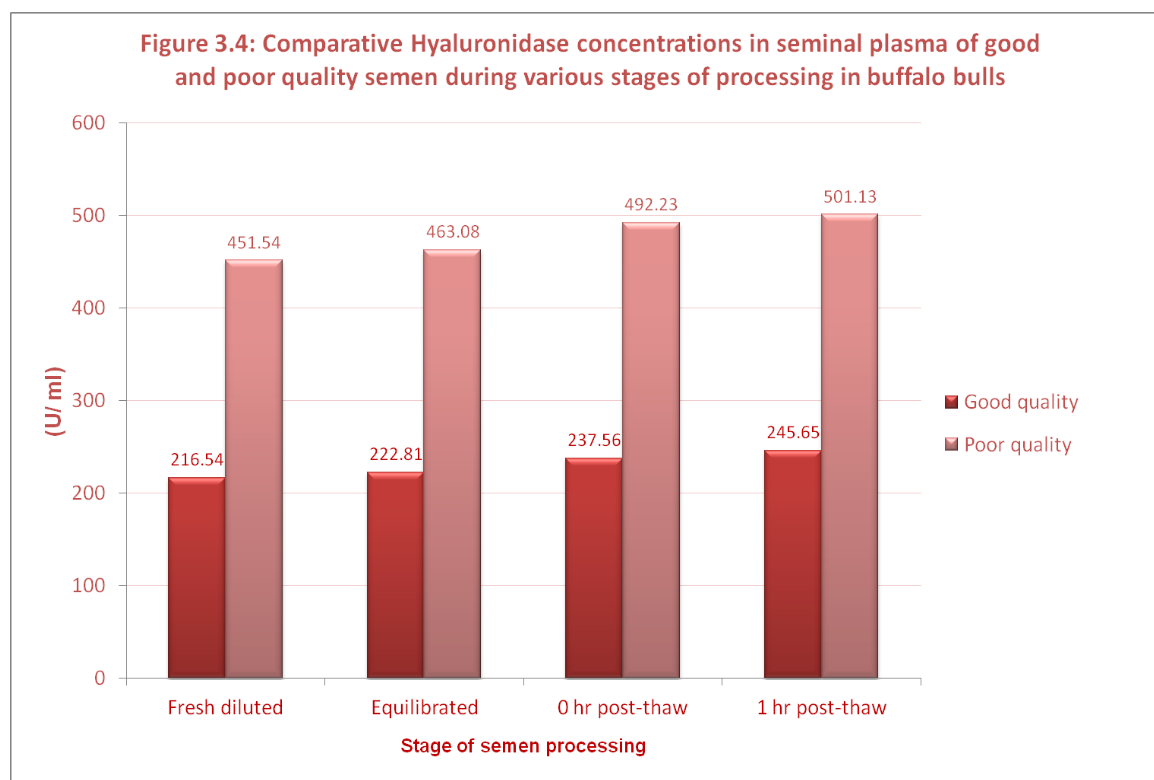


Figure 3.4: Comparative Hyaluronidase concentrations in seminal plasma of good and poor quality semen during various stages of processing in buffalo bulls

The effects of cryopreservation on sperm function and fertility have been widely studied, particularly in bovine. Various sperm organelles have been known to be

affected due to the detrimental effects of cryopreservation. Induction of premature acrosomal reaction, altered mitochondrial function, and reduction of motility and failure of chromatin decondensation, all of which are known to influence the viability and fertility of the sperm cells (Chaveiro *et al.* 2006; Cooter *et al.* 2005; Watson. 2000; Wongtawan *et al.* 2006).

Cooling is a major stressor, as a result of which membrane bound phospholipids reorient themselves into a different configuration that disrupt membrane function and permeability (Amman and Graham 1993; Lessard *et al.* 2000). The stress response shown by spermatozoa as a reaction to a drop in temperature is referred as cold shock. Generally, cold shock damage manifests itself as a decline in cell metabolism, altered membrane permeability, loss of intracellular components, irreversible loss of spermatozoan motility and an increase in the number of dead spermatozoa. The damage to the cellular membranes is of most significance because it has a carry-over effect on other cellular structures and functions.

There is no method to predict the fertilizing capacity of semen. Motility under microscope is the most widely used technique for assessing the quality of semen, but with the advent of deep frozen semen, the recovery rates though present after thawing has resulted in either very low fertility or no fertility at all. This may be due to damage of spermatozoa during the process of freezing. To record the extent of damage occurred to spermatozoa, there is no satisfactory method except to depend upon biochemical test to estimate the presence of enzymes (limited only to cells), in seminal plasma after freezing.

The probable reason for increased concentrations of intracellulaer enzymes in the seminal plasma with advancement of semen processing stages may be an irreversible loss of sperm plasma membrane integrity, which depends on the individual bull, breed, diluents used, and freezing rates, thawing temperature and thawing time. The loss of acrosomal integrity is significantly higher in poor freezer than in good freezer semen ejaculates of crossbred bulls (Perumal 2012).

4.3 Mineral profile of good and poor quality semen of Murrah buffalo bulls

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 seminal plasma. Cations such as sodium, potassium, calcium, and magnesium in seminal plasma influence osmotic balance and are components of many important enzymes as well (Cevik *et al.* 2007). Sodium is the principle cation in seminal plasma, with an exception in bull semen where calcium concentration is very high (Setchell and Brooks 1988). Calcium triggers the acrosome reaction in mammalian spermatozoa and is also involved with sperm motility. Sodium ion is an important element for spermatozoa function (Mosaferi *et al.* 2005). Potassium is a natural metabolic inhibitor and higher potassium concentration in seminal plasma decreases sperm metabolism thereby, decreasing sperm motility (Massanyi *et al.* 2003).

Comparative mineral profile of good versus poor quality semen of Murrah buffalo bulls have been shown in Table 4 and Figures 4.1, 4.2, 4.3, 4.4, 4.5 and 4.6, respectively. Bull wise mineral profiles in good quality semen of individual bulls have been depicted in Annexure 10 to 15.

Table 4: Comparative concentrations of various mineral (mean±SE) in seminal plasma of good and poor quality semen during various stages of processing in Murrah buffalo bulls

Parameter (n=48)	Quality	Stage of semen processing				LSD
		Fresh Diluted	Equilibration	0 hr Post-thaw	1 hr Post-thaw	
Calcium (mg/dl)	Good	45.18±0.29 $^{a A}$ (41.37-47.49)	44.96±0.30 $^{a AB}$ (41.55-47.29)	44.23±0.36 $^{a B}$ (40.32-46.98)	44.04±0.37 $^{a B}$ (40.06-46.81)	0.92
	Poor	41.95±0.18 $^{b A}$ (39.40-43.40)	41.77±0.18 $^{b A}$ (39.35-43.21)	40.90±0.18 $^{b B}$ (38.40-42.37)	40.72±0.17 $^{b B}$ (38.28-42.09)	0.49
Phosphorus (mg/dl)	Good	9.98±0.11 $^{a A}$ (8.40-11.43)	9.67±0.11 $^{a B}$ (8.23-11.02)	9.21±0.11 $^{a C}$ (7.28-10.85)	8.78±0.11 $^{a D}$ (7.00-10.54)	0.31
	Poor	6.70±0.10 $^{b A}$ (5.23-8.09)	6.36±0.087 $^{b B}$ (5.02-7.51)	6.08±0.08 $^{b C}$ (4.97-7.11)	5.75±0.08 $^{b D}$ (4.56-6.87)	0.24
Magnesium (mg/dl)	Good	7.21±0.11 $^{a A}$ (6.09-8.21)	7.13±0.11 $^{a A}$ (6.00-8.18)	7.09±0.11 $^{a A}$ (5.93-8.15)	7.05±0.12 $^{a A}$ (5.89-8.12)	0.31
	Poor	6.25±0.05 $^{b A}$ (5.80-6.69)	6.16±0.05 $^{b AB}$ (5.71-6.59)	6.11±0.04 $^{b B}$ (5.72-6.46)	6.06±0.04 $^{b B}$ (5.67-6.40)	0.12
Sodium (mg/dl)	Good	357.77±2.39 $^{a A}$ (329.20-385.49)	330.54±2.26 $^{a B}$ (301.29-348.93)	310.81±4.06 $^{a C}$ (246.84-340.39)	295.31±4.89 $^{a D}$ (220.92-338.39)	9.98
	Poor	353.14±2.05 $^{a A}$ (335.40-388.74)	316.10-1.94 $^{b B}$ (298.20-342.28)	281.62±3.29 $^{b C}$ (245.30-325.28)	258.17±3.44 $^{b D}$ (217.28-304.35)	7.78
Potassium (mg/dl)	Good	99.84±0.25 $^{a A}$ (96.75-103.93)	98.34±0.22 $^{a B}$ (95.02-101.28)	96.55±0.20 $^{a C}$ (93.98-98.99)	95.15±0.20 $^{a D}$ (92.01-97.59)	0.61
	Poor	69.37±0.37 $^{b A}$ (63.45-73.39)	67.56±0.34 $^{b B}$ (62.34-70.57)	65.47±0.33 $^{b C}$ (61.23-68.86)	63.62±0.32 $^{b D}$ (59.02-67.02)	0.96
Chloride (mg/dl)	Good	369.40±0.81 $^{a A}$ (355.75-383.6)	361.99±0.95 $^{a B}$ (353.45-381.09)	355.52±1.02 $^{a C}$ (339.49-364.43)	346.85±0.98 $^{a D}$ (332.76-352.84)	2.63
	Poor	274.42±0.98 $^{b A}$ (256.34-287.45)	267.59±0.91 $^{b B}$ (251.54-279.76)	260.99±0.90 $^{b C}$ (249.39-272.45)	254.43±0.94 $^{b D}$ (241.45-263.03)	2.61

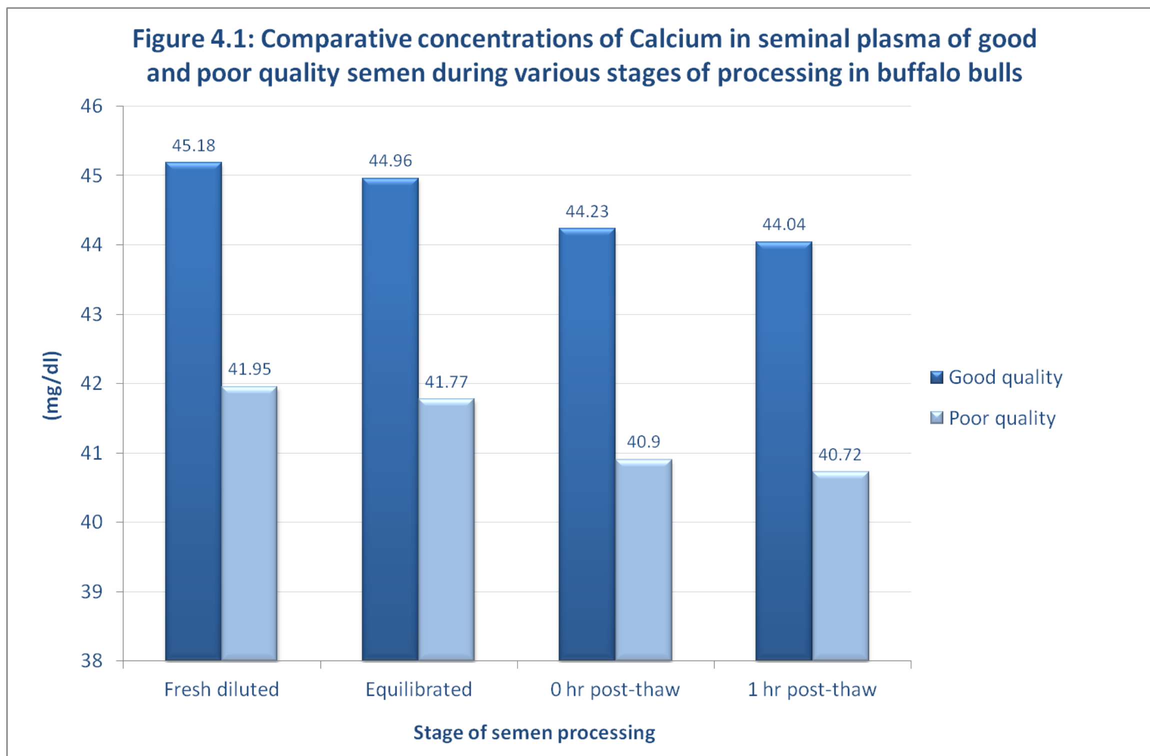
Figures within parenthesis indicate range
Figures with different superscripts (a, b) within a column significantly ($p<0.01$)
Figures with different superscripts (A, B) within a row differ significantly ($p<0.01$)

Kaya *et al*. (2002) and Meseguer *et al.* (2004) found that calcium concentrations in seminal plasma are good predictors of post-thaw semen quality. Magnesium, 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). Assumpcao *et al.* (2005) observed a negative correlation of abnormal sperm morphology with Phosphorus, Calcium and Sodium concentrations and a positive correlation with Potassium concentration.

4.3.1 Calcium

The calcium concentrations were significantly higher (p<0.01) in good quality seminal plasma at all the stages of evaluation. There was a significant decline (p<0.01) from post-dilution (45.18±0.29 and 41.95±0.18 mg/dl) to post-thaw (44.23±0.36 and 40.90±0.18 mg/dl) stage in good and poor quality ejaculates, respectively. A non-significant difference was noticed immediately after post-thaw and 1 hr incubation post-thaw in good as well as poor quality ejaculates.

The overall mean calcium concentration in the present study was comparable to the previous reports (40.0 mg/dl, Roy *et al.* 1960; 43.45 mg/dl, Singh *et al.* 1970; 41.11 mg/dl, Reddy and Raja 1979; 45.6 mg/dl, Rattan 1988; 42.01 mg/dl, Gupta and Singh 2007 and 44.95 mg/dl, Shukla *et al.* 2009) but was higher than recorded by other researchers (30.0±5.2 mg/dl, Kanwal *et al.* 2000; 32.42±3.10 mg/dl, Sansone *et al.* 2000 and 22.36±0.52 mg/dl, Eghbali *et al.* 2010). Low serum calcium levels have been correlated to poor seminal characteristics and fertility in buffalo bulls (Bagha 2003).

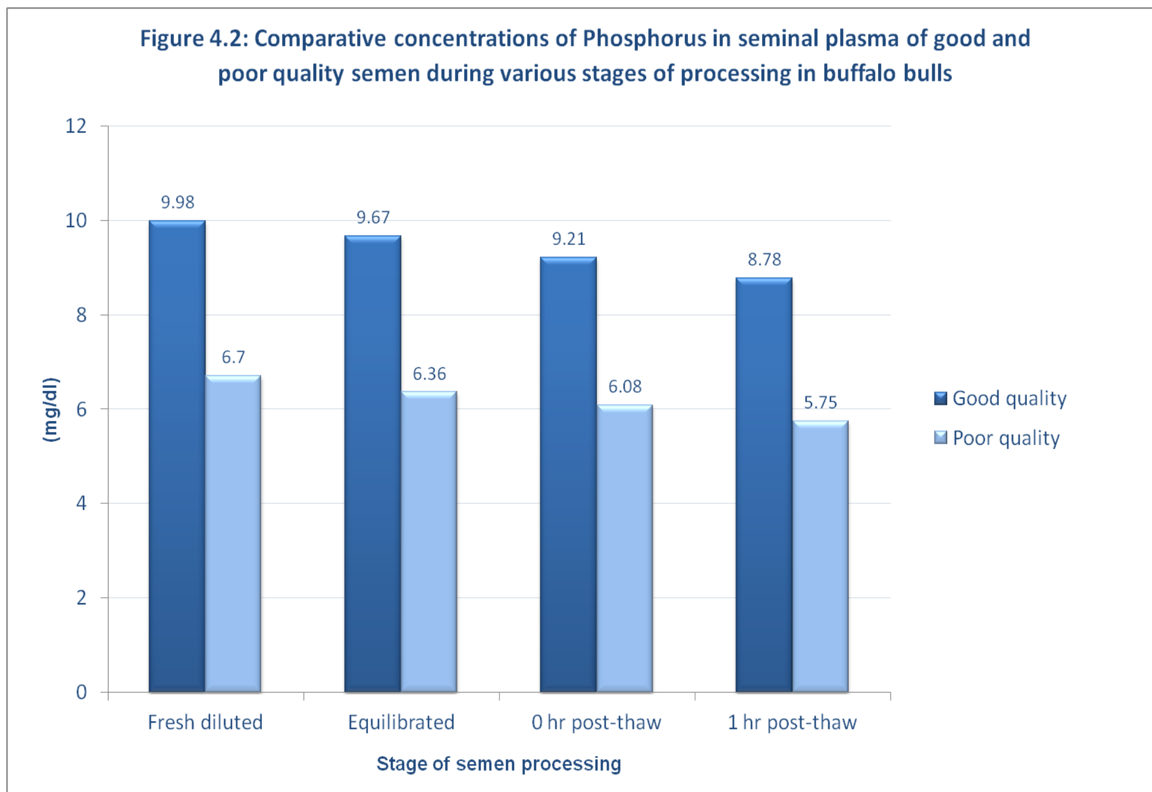


4.3.2 Phosphorus

Significantly lower (p<0.01) phosphorus concentrations were recorded in poor quality ejaculates. A significant decline (p<0.01) was observed in phosphorus concentration as the semen processing stages advanced in both good and poor quality

ejaculates. At post-dilution stage the mean value was 9.98±0.11 which decreased to 9.67±0.11 mg/dl at post-equilibration stage in good quality ejaculates and from 6.70±0.10 to 6.36±0.09 mg/dl at corresponding semen processing stages in poor quality ejaculates. Immediately after post-thaw the mean phosphorus concentrations were 9.21±0.11 and 6.08±0.08 mg/dl and after 1 hr incubation post-thaw these were 8.78±0.11 and 5.75±0.08 mg/dl in good and poor quality ejaculates, respectively.

The mean phosphorus concentration recorded (9.67±0.11 mg/dl) in the seminal plasma of the buffalo bulls in present study was higher than the previous reports (6.4±0.6 mg/dl, Roy *et al.* 1960; 7.56±0.42 mg/dl, Singh *et al.* 1970; 7.55±0.16 mg/dl, Gupta and Singh 2007 and 6.82±1.63 mg/dl, Shukla *et al.* 2009) whereas higher concentration of phosphorus was reported (12.28±0.37 mg/dl) by Dhami and Sahni (1993). Previously, poor seminal characteristics and low fertility has been correlated to low serum phosphorus in buffalo bulls (Bagha 2003). The phospholipid is required for the maintenance of the sperm membrane integrity (Singh *et al.* 1969).

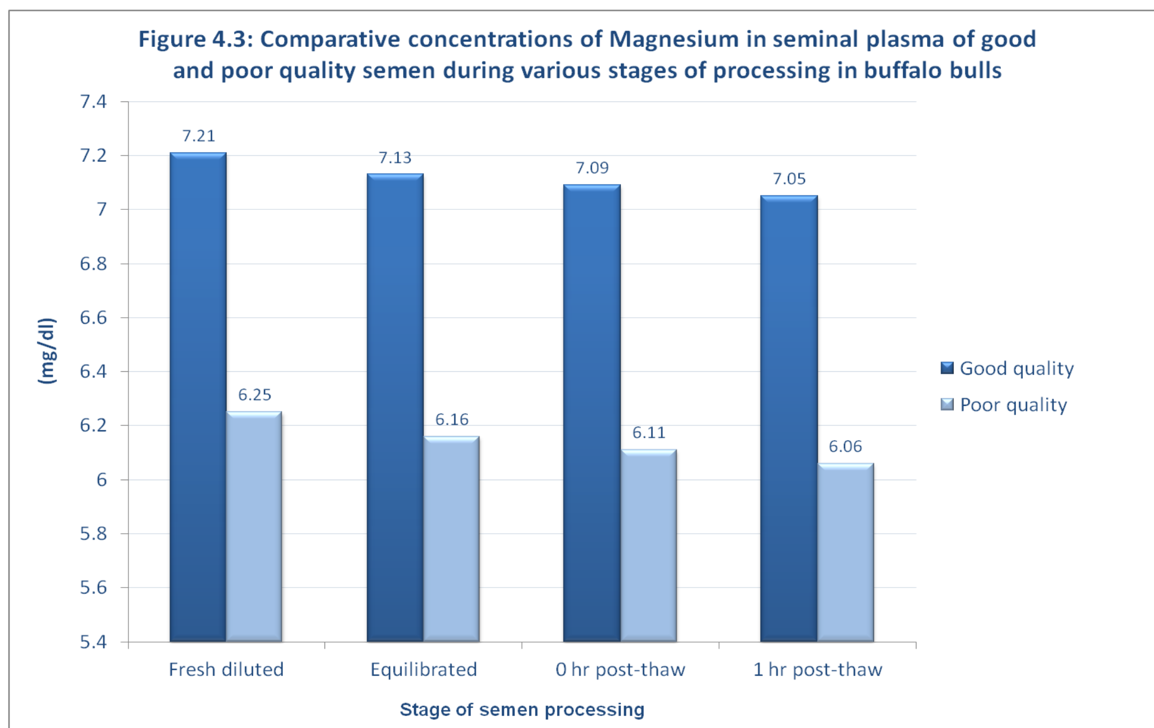


4.3.3 Magnesium

Perusal of table 4 shows that the mean magnesium levels were significantly higher ($p<0.01$) in good quality ejaculates at all the four stages of semen evaluation. In case of

good quality ejaculates, there was a non-significant decline from post-dilution (7.21±0.11mg/dl) to 1 hr incubation post-thaw (7.05±0.12 mg/dl), where as in poor quality ejaculates, this decline was significant ($p<0.01$) from 6.25±0.05 post-dilution to 6.06±0.04 mg/dl after 1 hr incubation post-thaw.

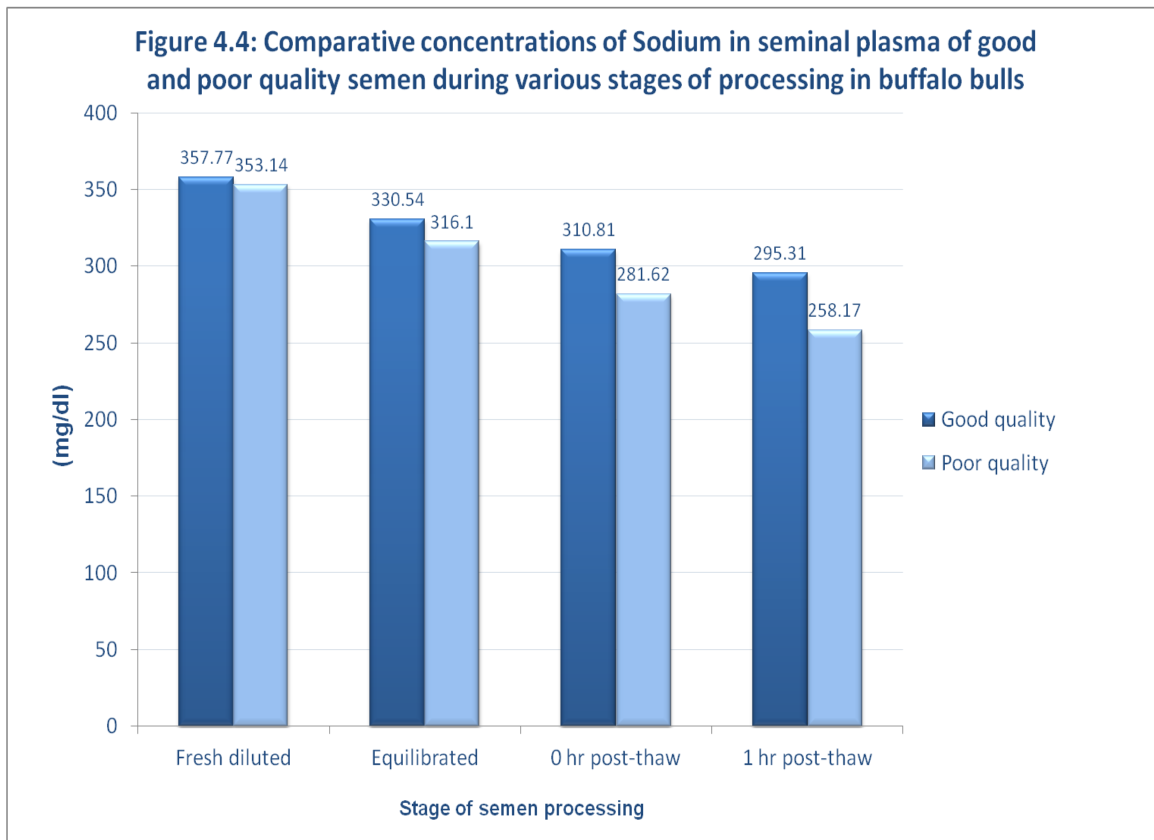
The magnesium content in the seminal plasma of the Murrah bulls in the present study was in accordance with earlier findings (6.46±0.39 mg/dl, Sansone *et al.* 2000; 6.61±0.49 mg/dl, Shukla *et al.* 2009). Lower (5.24±0.49 mg/dl, Singh *et al.* 1970; 5.91 mg/dl, Reddy and Raja 1979; 5.00±0.9 mg/dl, Kanwal *et al* 2000) and higher (11.94mg/dl, Eghbali *et al.* 2010) concentration has also been documented.



4.3.4 Sodium

A significant decline was observed in sodium concentration ($p<0.01$) with processing stages in good as well as poor quality ejaculates. There was a non-significant difference in corresponding post-dilution stage in good and poor quality ejaculates. A significant ($p<0.01$) decline was observed from 357.77±2.39 and 353.14±2.05 mg/dl (post-dilution) to 310.81±4.06 and 281.62±3.29 mg/dl immediately post-thaw in good and poor quality ejaculates, respectively

The mean sodium concentration in freshly diluted semen recorded was 357.77±2.39 mg/dl, which was higher than previous reports (186.89 mg/dl, Singh *et al.* 1970; 139.00 mg/dl, Gupta and Tripathi, 1983; 243.0 mg/dl, Kanakaraj and Krishnamurthy 1984; Kanakaraj and Easwaran 1991; 217.4 mg/dl, Rattan 1990; 319.2 mg/dl, Kanwal *et al*. 2000; 258.58 mg/dl, Sansone *et al*. 2000; 272.85 mg/dl, Gupta and Singh 2007; 106.46 mg/dl, Shukla *et al*. 2009).



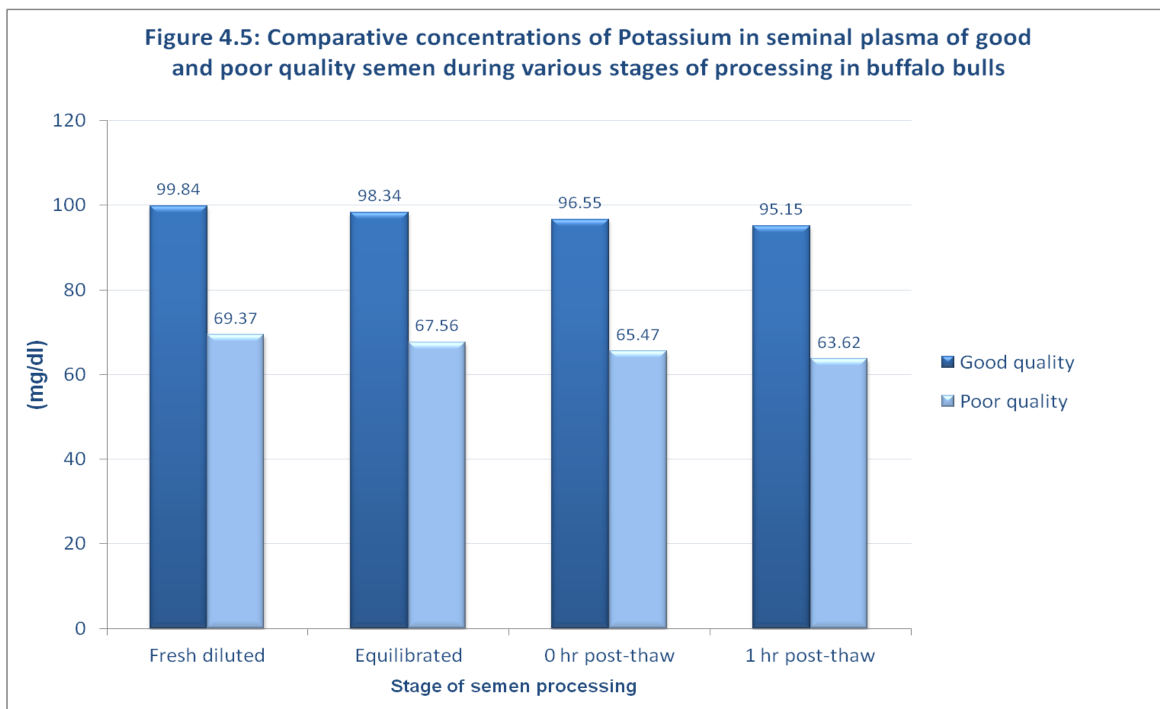
4.3.5 Potassium

Significantly higher ($p<0.01$) potassium concentration was observed in good quality ejaculates. At post-dilution stage, the mean potassium concentration was 99.84±0.25 in good quality ejaculates and 69.37±0.37 mg/dl in poor quality ejaculates. Immediately after post-thaw, it was 96.55±0.20 and 65.47±0.33 mg/dl in good and poor quality ejaculates, respectively.

The average potassium content (99.84±0.25 mg/dl) in the present experiments was comparable to the findings of the earlier researchers (101.60±4.45 mg/dl, Singh *et*

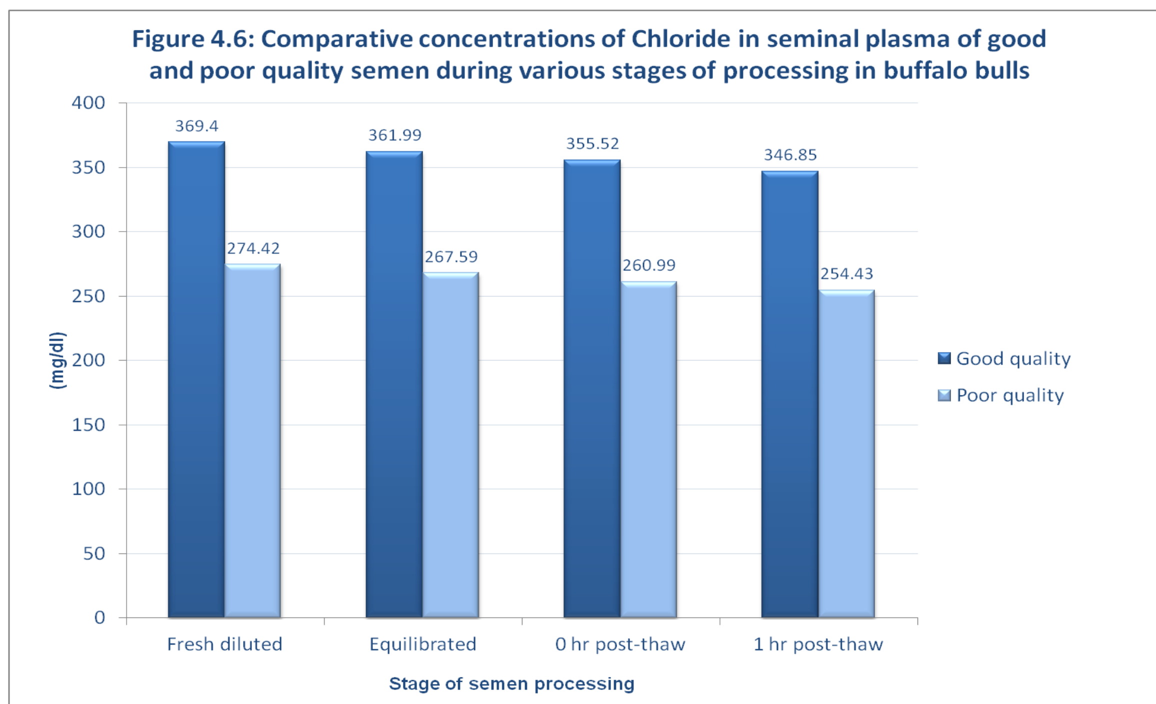
al. 1970; 98.04±2.77 mg/dl, Kanakaraj and Easwaran 1991; 103.56±3.15 mg/dl, Dhami and Sahni 1993 and 98.18±11.67 mg/dl, Shukla *et al.* 2009) but higher than other reports (75.78±3.23 mg/dl, Gupta and Singh 2007; 64.5±16.1 mg/dl, Kanwal *et al.* 2000) in buffalo bulls.

Significantly higher mean potassium concentrations of 150.3 and 154.83 mg/dl had been reported by Rattan (1990) and Sansone *et al.* (2000), respectively in buffalo bull seminal plasma.



4.3.6 Chloride

The mean chloride concentration was significantly higher ($p<0.01$) in good quality ejaculates and there was a significant decline in the mean concentration values with advance in semen processing stages. At post-dilution stage the mean chloride concentration in good quality ejaculate was 369.40±0.81 and in poor quality ejaculate it was 274.42±0.98 mg/dl, which declined significantly ($p<0.01$) to 355.52±1.02 and 260.99±0.90 mg/dl at immediately post-thaw stage in good and poor quality ejaculates, respectively.



The mean chloride content (369.40±0.81 mg/dl), in the present study, was in agreement with the previous reports (373.55±55.0 mg/dl, Roy *et al.* 1960; 347.50 mg/dl, Singh *et al.* 1970 and 366.73±53.69 mg/dl, Shukla *et al.* 2009), but was considerably higher than 269.13±7.0 and 224.06±2.60 mg/dl reported by Dhami and Sahni (1993) and Sansone *et al.* (2000), respectively. Variation in seminal plasma mineral concentration may be due to breed and individual variation, seasonal impact and the assay technique used.

Macro-minerals (sodium, potassium and chloride) are essential in the biological fluids to maintain osmotic pressure within and outside the cells, and potassium especially, is important for flagellar motion of spermatozoa. Significantly lower values of these macro-minerals in the seminal plasma might be the probable reason of poor quality semen ejaculates (Dhami and Shelke, 2005)

The magnesium, a co-factor and found in nearly all enzymatic systems, plays an important role in sperm motility. Lower magnesium concentration may be a probable reason of initial poor quality of semen.

The phosphorus is one of the important components in many enzymes involved in sperm glycolysis (Flerchinger and Erb 1955). Comparatively lower concentration of

phosphorus in ejaculates may lead to significantly lowered viable spermatozoa percentage.

There is a constant decline in mean concentration of minerals with advancement of semen processing stage. The likely reason behind this decline might be that there is an increase in dead percentage of spermatozoa and with passive diffusion of minerals through the plasma membrane of these dead spermatozoa. A lower mineral concentration in semen may be the probable reason of poor freezability of ejaculates.

4.4 Correlation between functional and biochemical parameters

4.4.1 Relationship between sperm livability and biochemical parameters

The overall inter-relationship of the sperm livability with various functional and biochemical parameters along with their regression equations for good versus poor quality semen of Murrah buffalo bulls have been depicted in Table 5 and Figures 5.1 to 5.13, respectively. This relationship within freshly diluted and post-thaw good versus poor quality semen has been depicted in Annexure 17 and Annexure 18, respectively.

Table 5: Relationship of live sperms with certain functional and biochemical parameters in seminal plasma of good versus poor quality Murrah buffalo bull semen

Sr. No.	Relationship between Parameters (n=192)		Quality	Correlation coefficient	Regression Estimate	Regression Equation
1	Livability	Motility	Good	0.92738**	0.38 ±0.01	y=57.39+0.38x
			Poor	0.99934**	1.31±0.00	y= 5.76+1.31x
2		HOST	Good	0.98934**	1.01±0.01	y= 0.43+1.01x
			Poor	0.99934**	1.00±0.00	y= 0.79+1.00x
3		Acrosome	Good	0.92725**	0.81±0.02	y=16.52+0.81x
			Poor	0.98273**	1.07±0.01	y= -5.43+1.07x
4		AKP	Good	-0.47586 **	-0.23±0.03	y= 117.78-0.23x
			Poor	-0.59034**	-1.75±0.17	y= 247.75-1.75x
5		AST	Good	-0.9416**	-0.79±0.02	y=160.01- 0.79x
			Poor	-0.8358**	-1.63±0.08	y=253.99-1.63x
6		ALT	Good	-0.7907**	-3.86±0.22	y=145.18-3.86x
			Poor	-0.9165**	-2.51±0.08	y=128.21-2.51x
7		Hyaluronidase	Good	-0.78965**	-0.38±0.02	y= 170.17-0.38x
			Poor	-0.74037**	-0.61±0.04	y= 327.24-0.61x
8		Calcium	Good	0.21353**	0.64±0.21	y= 53.84+0.64x
			Poor	0.35028**	5.62±1.09	y= -194.17+5.62x

9		Phosphorus	Good	0.43048^{**}	3.39±0.52	y= 50.50+3.39x
			Poor	0.43474^{**}	13.32±2.00	y= -44.54+13.32x
10		Magnesium	Good	0.18175^{*}	1.63±0.63	y= 70.79+1.63x
			Poor	0.14500^{*}	9.91±4.90	y= -22.56+9.91x
11		Sodium	Good	0.69600^{**}	0.14±0.01	y= 36.31+0.14x
			Poor	0.84324^{**}	0.44±0.20	y= -95.08+0.44x
12		Potassium	Good	0.76092^{**}	2.27±0.14	y= -139.14+2.27x
			Poor	0.72068^{**}	4.81±0.34	y= -281.32+4.81x
13		Chloride	Good	0.77688^{**}	0.51±0.03	y= -101.15+0.51x
			Poor	0.70555^{**}	1.53±0.11	y= -365.94+1.53x

* ($P<0.05$) ** ($P<0.01$) NS (not significant)

A suitable evaluation of semen for breeding and in vitro fertilization purposes has always been of great importance. Evaluation of sperm quality usually is linked with the desire for predicting fertility in a clinical setting or to enable maximum number of offspring from a valuable sire (Amann and Graham 1993; Neild *et al.* 1999). The traditional evaluation of the quality of ejaculate has been mainly based on routine semen analyses (motility, morphology and acrosomal integrity) which have a limited capacity for the prediction of the potential fertility of an ejaculate (Jeyendran *et al.* 1984). Significant correlations between various kinematic parameters of bull spermatozoa and *in-vivo* fertility have been described by a number of workers (Farrell *et al*. 1998; Zhang *et al*. 1998; Januskauskas *et al*. 2001, 2003; Cseh *et al*. 2004), but no single *in-vitro* diagnostic test has completely accounted for the variation associated with fertility. However, when a number of these tests were combined into a mixed model, the correlation with fertility was extremely high. Additional tests of sperm function added to the routine spermiogram substantially increased the predictive value (Gillian *et al*. 2008). It has also been suggested that assays, that determine if a cell is alive (motility and viability), appear to be most informative, as they consistently appear in multivariate correlations with fertility (Moce and Graham 2008).

The clinical predictive value of this 'functional' test of viability, however, is still being debated (Avery *et al*. 1990; DY Liu and Baker 1992).The relationship between the fertility and sperm motility (Popwell and Flowers 2004; Januskauskas *et al.* 2000) and morphology (Popwell and Flowers 2004) is not consistent. Although these laboratory tests can be used to rapidly evaluate a semen sample, they do not incorporate

information on sub-cellular physical damage that can occur during cryopreservation. With an aim to simplify sperm evaluation, the correlation between these characteristics could be evaluated and instead of evaluation all of them, one of them could be evaluated and evaluation process could be simplified. In terms of prediction, if two variables were correlated perfectly, then knowing the value of one score permits a perfect prediction of the score on the second variable. Generally, whenever two variables are significantly correlated, the researcher may use the score on one variable to predict the score on the second (R Ho 2006). Thus, with calculation of correlation between the routine sperm analysis parameters, would not be necessary to assay of other related parameters and instead of them, other tests could be used to evaluate the functional characteristics of sperm like Zona-free hamster ova test and cervical mucus penetration test (Hafez 1993).

Perusal of the Table 5 indicates that live sperm percentage was significantly ($P<0.01$) correlated with progressively motile spermatozoa ($r=0.92738$ and 0.99934, $n=192$) in good and poor quality ejaculates. The overall regression coefficient of progressive motility on live sperm percentage was 0.38 ± 0.01 and 1.31 ± 0.00 in good and poor quality ejaculates, respectively (Figure 5.1).

Similarly, sperm livability percentage was significantly ($P<0.01$) correlated with HOS responsive spermatozoa ($r=0.98934$ and 0.99934, $n=192$) and the overall regression coefficients of HOST on live sperm (%) were 1.01 ± 0.01 and 1.00 ± 0.00 (Figure 5.2) in good and poor quality semen, respectively. The correlation between live sperm percentage and acrosomal integrity ($r=0.92725$ and 0.98273) was also significant ($P<0.01$) with the regression coefficients of acrosomal integrity on livability calculated to be 0.81 ± 0.02 and 1.07 ± 0.01 (Figure 5.3) in good and poor quality ejaculates, respectively.

Figure 5.1: Relationship between live and progressively motile spermatozoa of good and poor quality Murrah buffalo bull semen

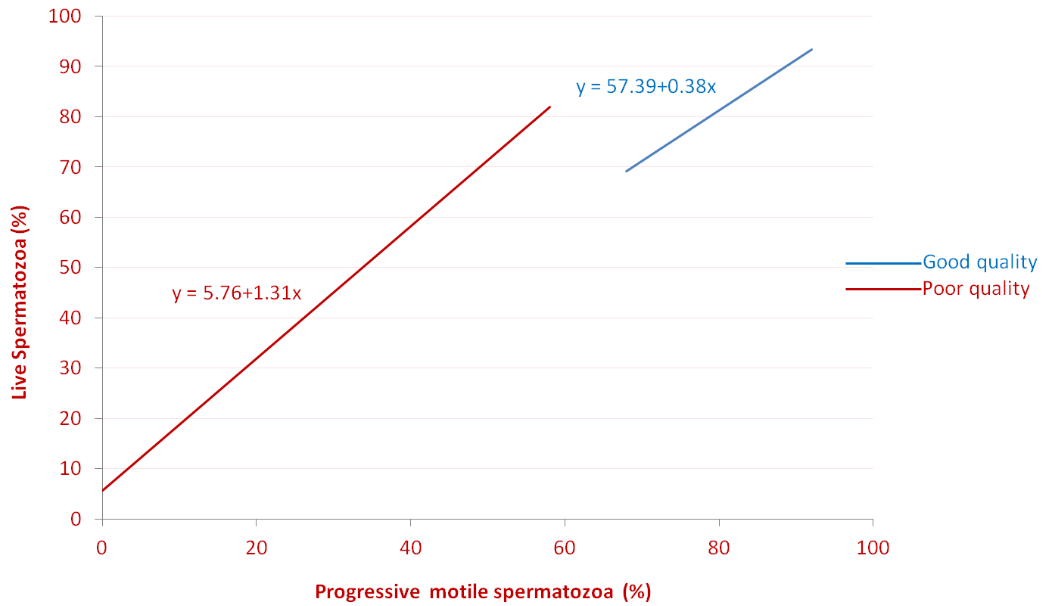
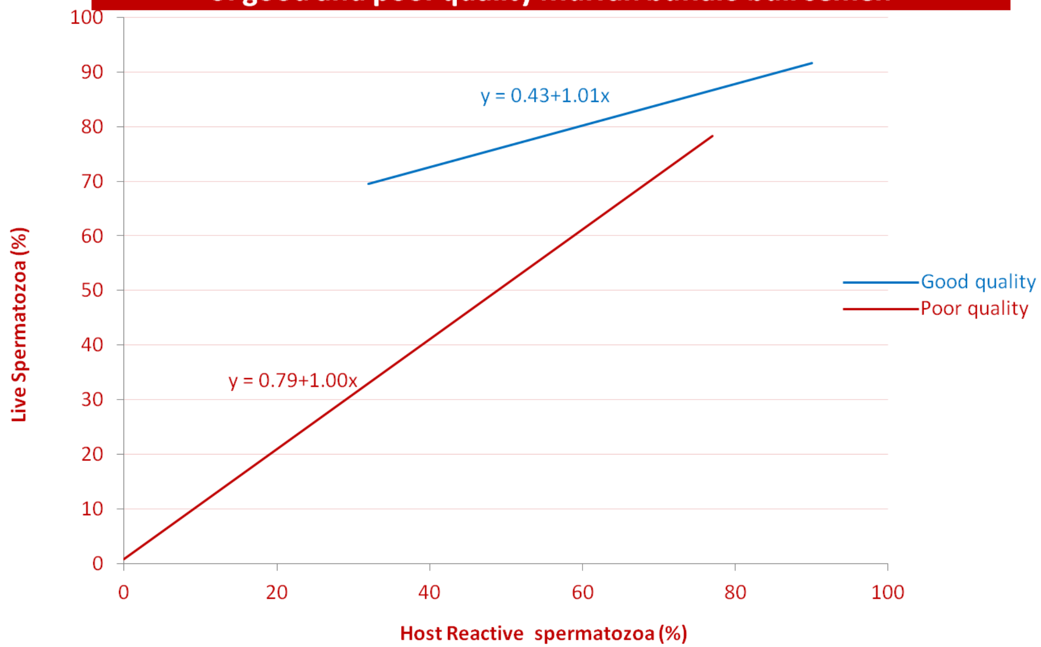
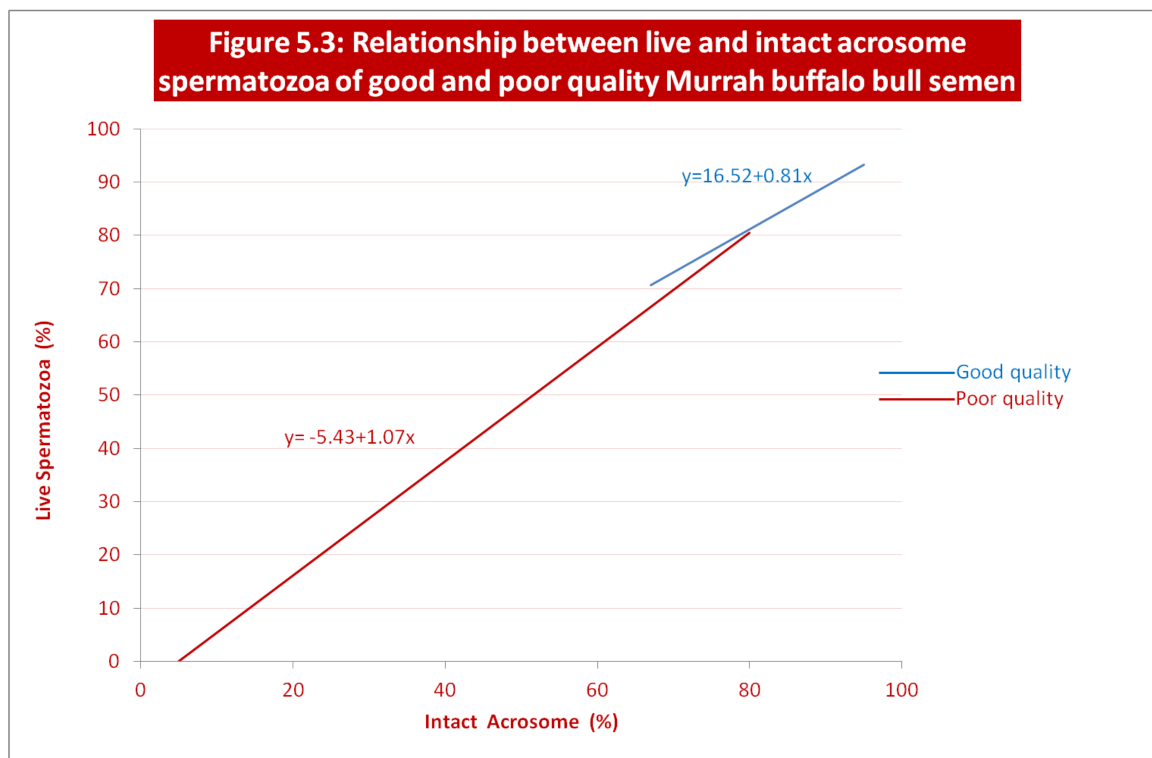


Figure 5.2: Relationship between live and HOST reactive spermatozoa of good and poor quality Murrah buffalo bull semen





A significant (P<0.01) negative relationship was observed between livability and AKP (r=-0.47586 and -0.59034) and AST (r=-0.9416 and -0.8358) leakage in good and poor quality semen, respectively. The regression coefficients of AKP and AST on livability calculated were -0.23±0.03 and -0.79±0.02 in good quality ejaculates and -1.75±0.17 and -1.63±0.08 in poor quality ejaculates (Figures 5.4 and 5.5), respectively.

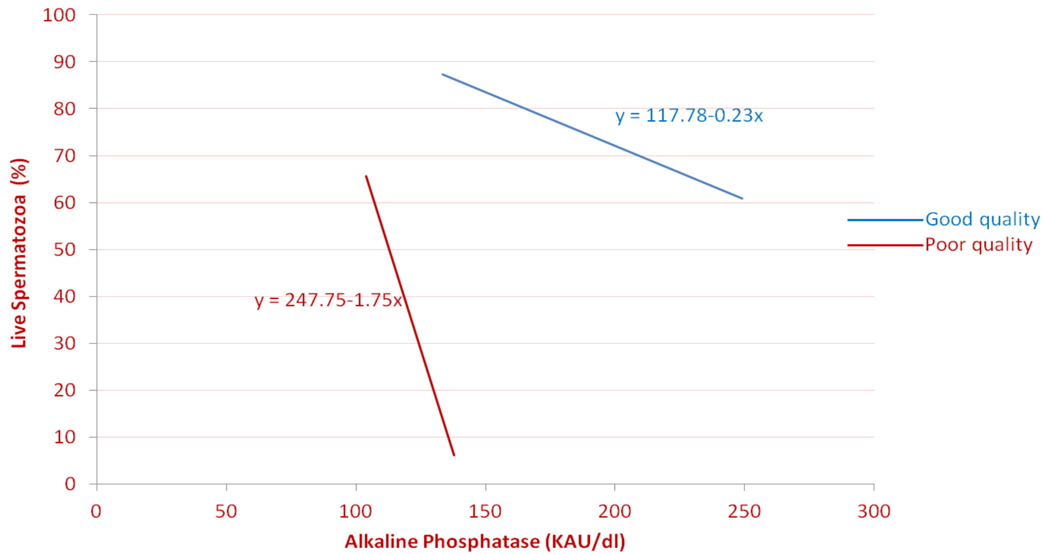


Figure 5.4: Relationship between live spermatozoa and concentration of alkaline phosphatase in the seminal plasma of good and poor quality Murrah buffalo bull semen

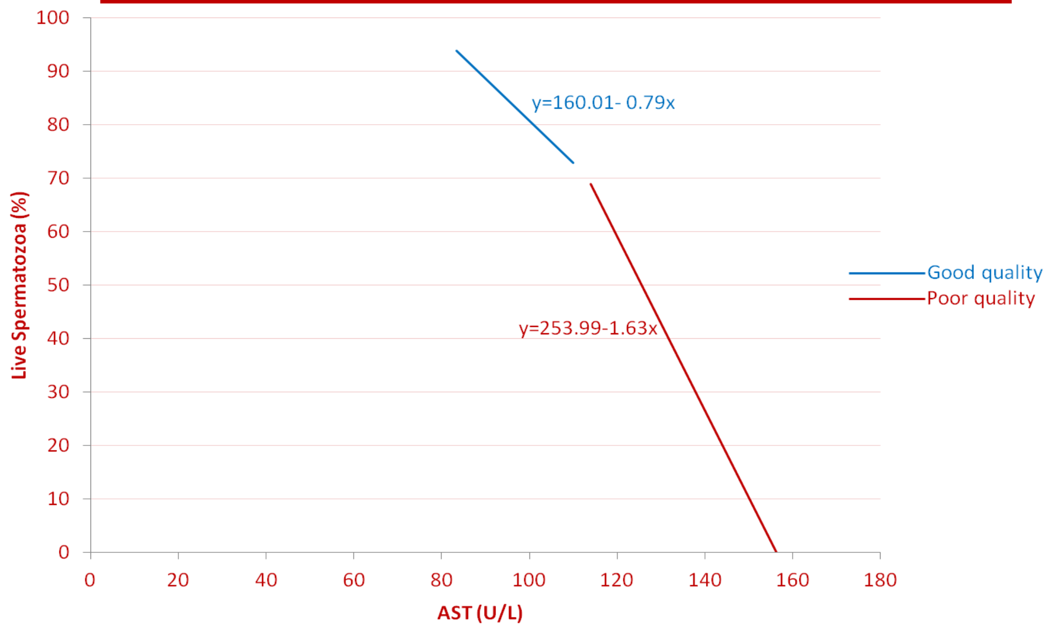


Figure 5.5: Relationship between live spermatozoa and concentration of AST in the seminal plasma of good and poor quality Murrah buffalo bull semen

Highly negative significant ($P<0.01$) correlation was observed between per cent livability and leakage of ALT (r= -0.7907 and -0.9165) and hyaluronidase (r= -0.78965 and -0.74037) in good and poor quality ejaculates, respectively, with regression coefficients of ALT and hyaluronidase on livability being 3.86±0.22 and -0.38±0.02 in

good quality ejaculates and -2.51±0.08 and -0.61±0.04 in poor quality ejaculates (Figures 5.6 and 5.7), respectively.

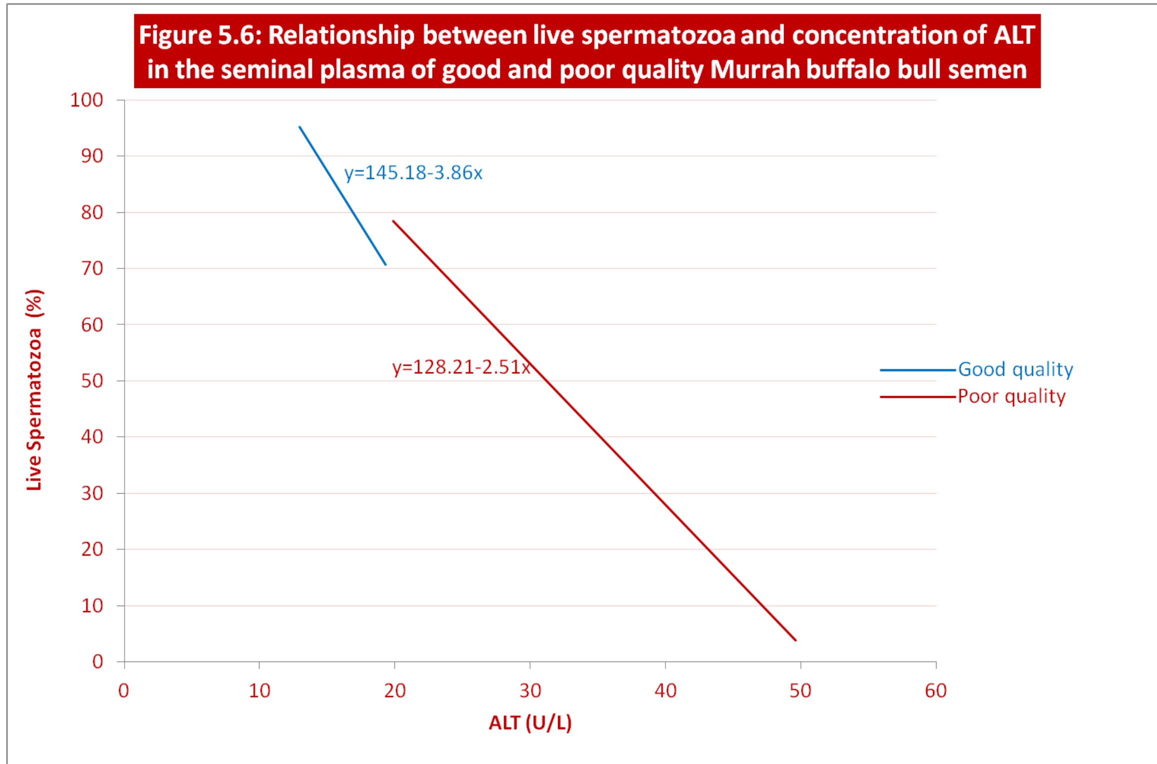


Figure 5.6: Relationship between live spermatozoa and concentration of ALT in the seminal plasma of good and poor quality Murrah buffalo bull semen

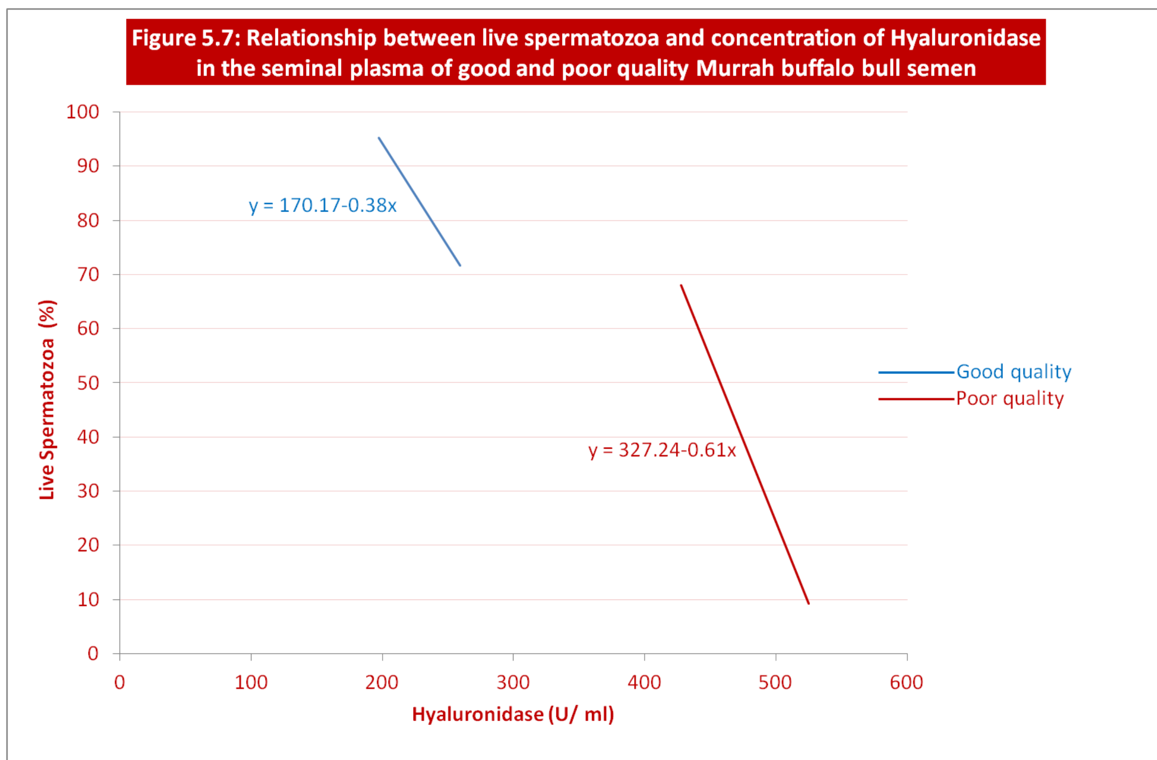


Figure 5.7: Relationship between live spermatozoa and concentration of Hyaluronidase in the seminal plasma of good and poor quality Murrah buffalo bull semen

Live sperm percentage was significantly (P<0.01) correlated with calcium (r=0.21353 and 0.35028) and phosphorus (r=0.43048 and 0.43474) with regression coefficients of calcium being 0.64±0.21 and 5.62±1.09 and of phosphorus 3.39±0.52 and 13.32±2.00 on livability in good and poor quality ejaculates (Figures 5.8 and 5.9), respectively.

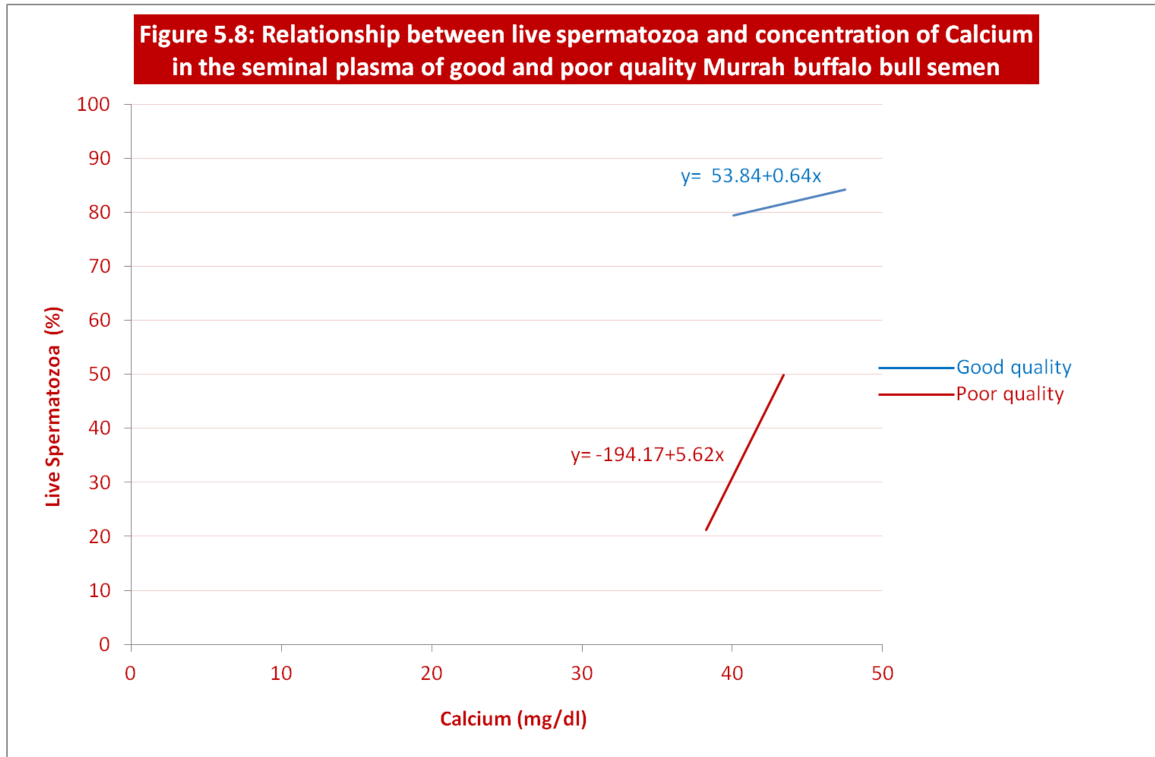
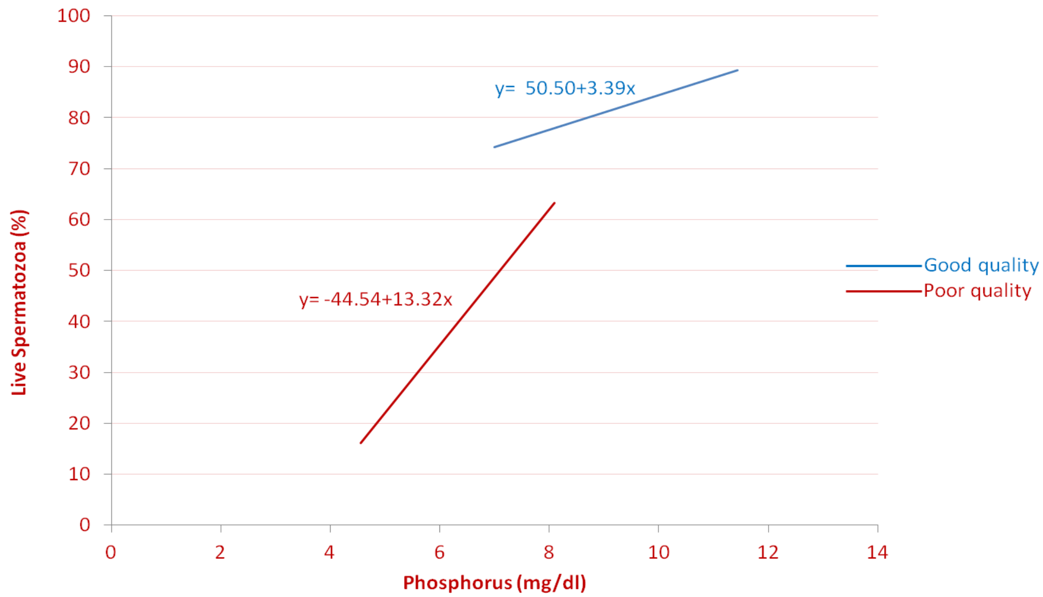
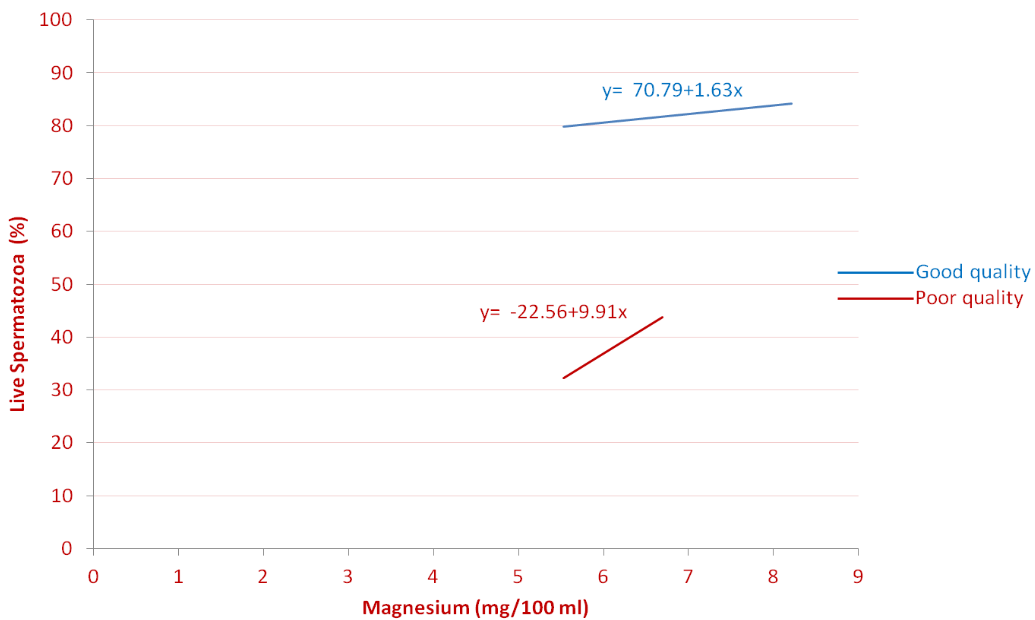


Figure 5.8: Relationship between live spermatozoa and concentration of Calcium in the seminal plasma of good and poor quality Murrah buffalo bull semen



Similarly, a significant (P<0.05) relationship was observed between sperm livability and magnesium concentration (r=0.18175 and 0.14500). The regression coefficient of magnesium on livability was 1.63±0.63 and 9.91±4.90 in good and poor quality ejaculates, respectively (Figure 5.10).



Live sperm percentage was significantly (P<0.01) correlated with sodium (r=0.69600 and 0.84324), potassium (r=0.76092 and 0.72068) and chloride (r=0.77688 and 0.70555) in good and poor quality ejaculates, respectively. The regression coefficient of sodium, potassium and chloride on live per cent spermatozoa in good and poor ejaculates were 0.14±0.01 and 0.44±0.20 (Figure 5.11), 2.27±0.14 and 4.81±0.34 (Figure 5.12) and 0.51±0.03 and 1.53±0.11 (Figure 5.13), respectively.

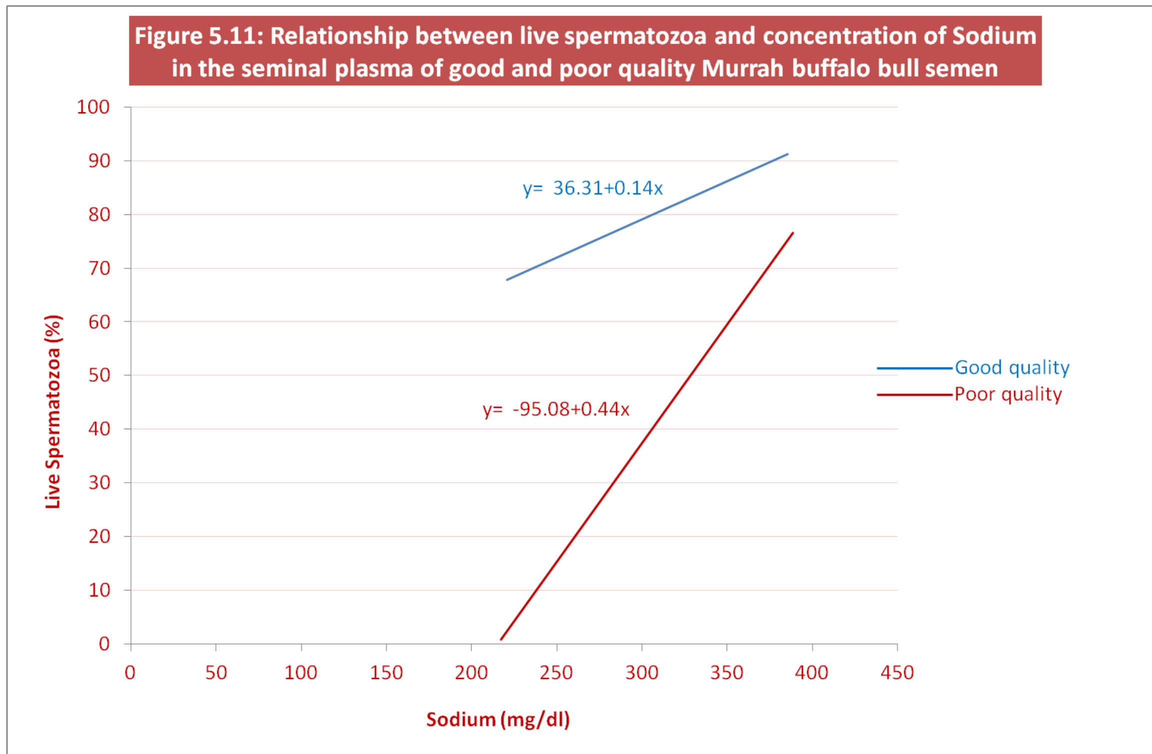


Figure 5.11: Relationship between live spermatozoa and concentration of Sodium in the seminal plasma of good and poor quality Murrah buffalo bull semen

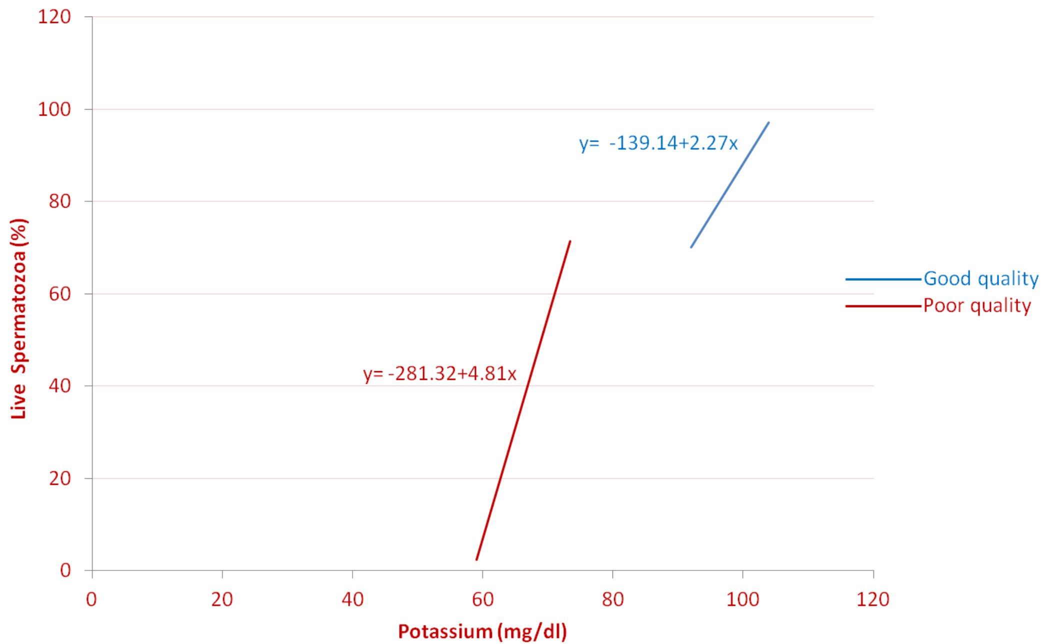


Figure 5.12: Relationship between live spermatozoa and concentration of Potassium in the seminal plasma of good and poor quality Murrah buffalo bull semen

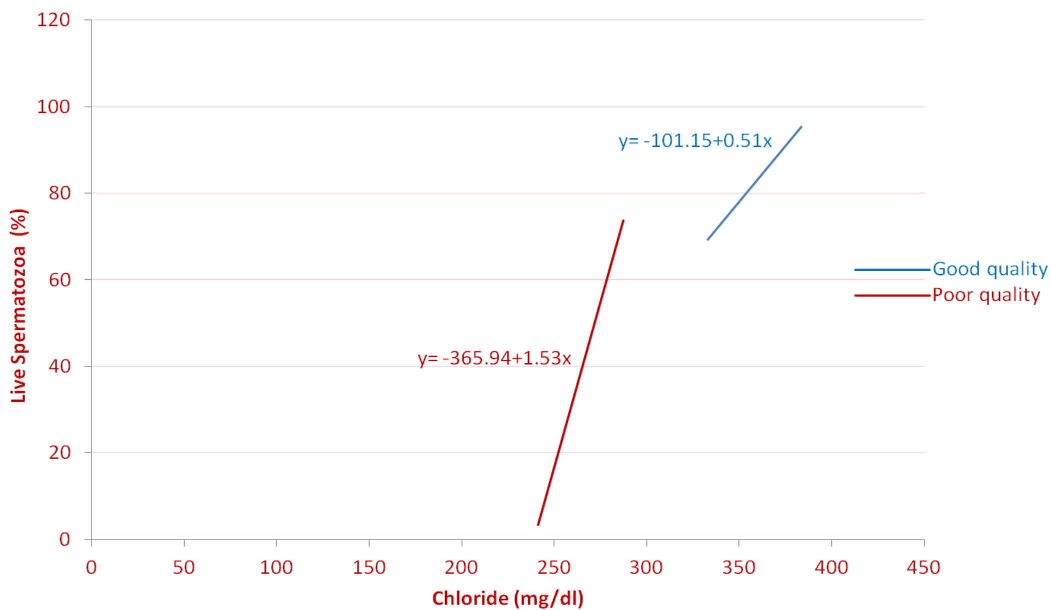


Figure 5.13: Relationship between live spermatozoa and concentration of Chloride in the seminal plasma of good and poor quality Murrah buffalo bull semen

El-Sisy *et al.* (2010) showed a significant correlation between live spermatozoa per cent with HOS reactive (r=0.681) and acrosomal abnormality (r=0.220) in buffalo

bull spermatozoa. The percentage of live sperms and acrosome intact sperms has also been shown to be highly correlated with percentage of motile sperms (Kumar 2004; Kirk *et al*. 2005).

Raval and Dhami (2006) reported a significant negative relationship ($p<0.05$) of live sperm percentage with sperm abnormality ($r=-0.60$) and a non significant correlation between live sperm percentage and AST ($r=-0.09$), ALT ($r=0.21$) AKP ($r= 0.05$), calcium ($r=0.21$), phosphorus ($r=0.42$) and magnesium ($r=-0.01$) in triple crossbred (25% HF X 25% J X 50% Kankarej) bulls.

4.4.2 Relationship between progressive motility and biochemical parameters

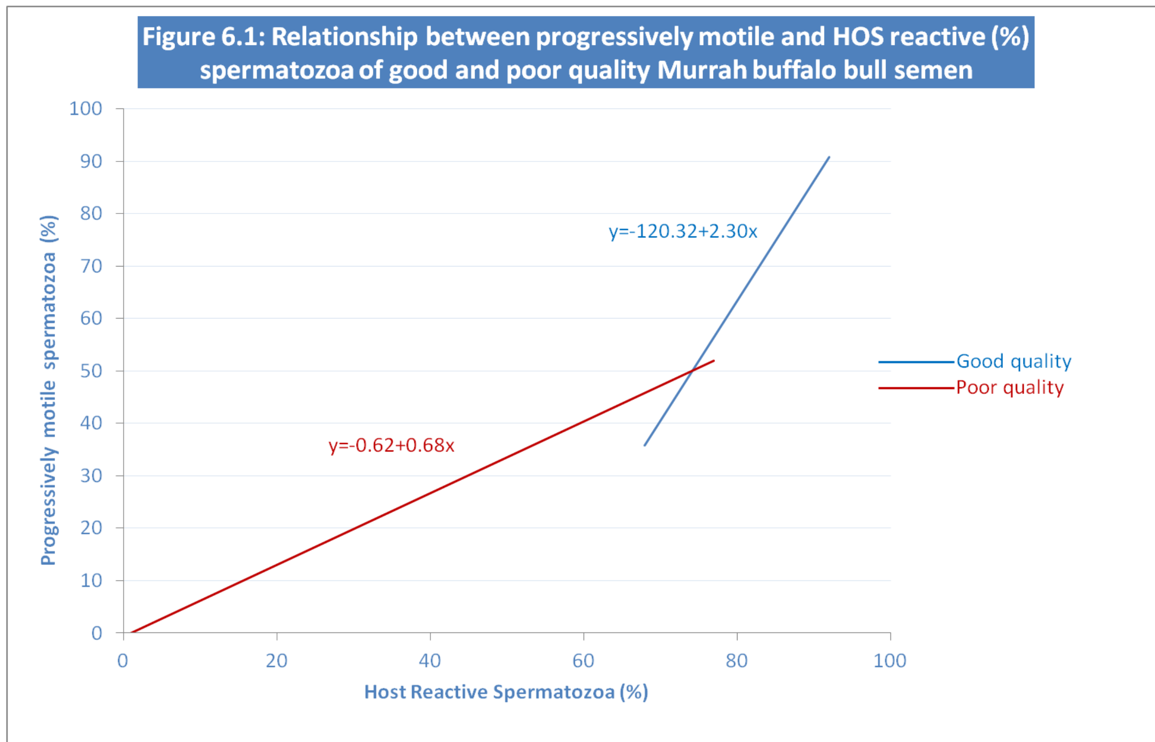
The overall inter-relationship of the progressive motility of sperms with various functional and biochemical parameters along with their regression equations for good versus poor quality semen of Murrah buffalo bulls have been depicted in Table 6 and Figures 6.1 to 6.12. This relationship within freshly diluted and post-thaw good and poor quality semen has been depicted in Annexure 17 and Annexure 18, respectively.

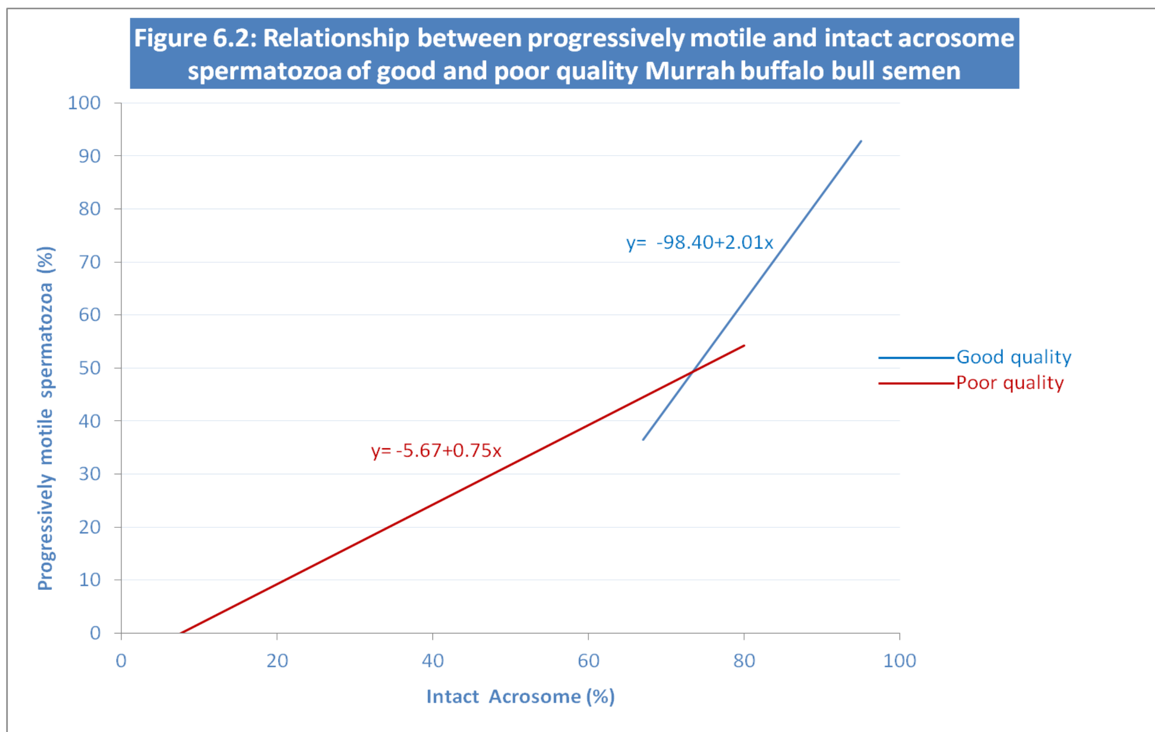
Table 6: Relationship of progressive motility of spermatozoa with certain functional evaluation parameters and various biochemicals in seminal plasma of good and poor quality Murrah buffalo bull semen

Sr. No.	Relationship between Parameters (n=192)		Quality	Correlation Coefficient	Regression Estimate	Regression Equation
1	Progressive Motility	HOST	Good	0.92052**	2.30±0.07	y=-120.32+2.30x
			Poor	0.94212**	0.68±0.02	y=-0.62+0.68x
2		Acrosome	Good	0.94709**	2.01±0.05	y= -98.40+2.01x
			Poor	0.95266**	0.75±0.02	y= -5.67+0.75x
3		AKP	Good	-0.45140**	-0.53±0.08	y= 147.71-0.53x
			Poor	-0.66370**	-1.42±0.12	y= 194.04-1.42x
4		AST	Good	-0.9292**	-1.91±0.06	y=252.71-1.91x
			Poor	-0.7337**	-1.03±0.07	y=160.87-1.03x
5		ALT	Good	-0.7787**	-9.28±0.54	y=216.70-9.28x
			Poor	-0.8954**	-1.76±0.06	y=87.94-1.76x
6		Hyaluronidase	Good	-0.76605**	-0.90±0.05	y= 273.62-0.90x
			Poor	-0.68714**	-0.40±0.03	y= 217.55-0.40x
7		Calcium	Good	0.21883**	1.60±0.52	y= -5.61+1.60x
			Poor	0.33624**	3.88±0.79	y= -135.60+3.88x
8		Phosphorus	Good	0.46707**	8.98±1.23	y= -18.64+8.98x
			Poor	0.51561**	11.35±1.37	y= -45.83+11.35x
9		Magnesium	Good	0.12246NS	2.68±1.58	y= 46.73+2.68x
			Poor	0.20495**	10.07±3.49	y= -37.05+10.07x
10		Sodium	Good	0.67512**	0.34±0.03	y= -43.29+0.34x
			Poor	0.89136**	0.34±0.01	y= -76.54+0.34x
11		Potassium	Good	0.73808**	5.38±0.36	y= -458.61+5.38x
			Poor	0.61697**	2.96±0.27	y= -171.87+2.96x
12		Chloride	Good	0.78694**	1.30±0.07	y= -387.93+1.27x
			Poor	0.71250**	1.11±0.08	y= -268.63+1.11x

* (P<0.05) ** (P<0.01) NS (not significant)

Perusal of the Table 6 shows that per cent progressive motility was significantly (P<0.01) correlated with HOS reactive (r=0.92052 and 0.94212) and per cent intact acrosome (r=0.94709 and 0.95266) in good and poor quality ejaculates, respectively. The regression coefficients of HOS reactive spermatozoa and spermatozoa with intact acrosome on progressive motility were 2.30±0.07 and 0.68±0.02 (Figure 6.1) and 2.01±0.05 and 0.75±0.02 (Figure 6.2) in good and poor quality ejaculates, respectively.





Similarly, a significant (P<0.01) negative relationship was observed between progressive motility and AKP (r=-0.45140 and -0.66370) and AST (r=-0.9292 and -0.7337) leakage in good and poor quality ejaculates, respectively. The regression coefficients of AKP and AST on progressive motility were -0.53±0.08 and -1.91±0.06 in

good quality ejaculates and -1.42±0.12 and -1.03±0.07 in poor quality ejaculates, respectively (Figure 6.3 and 6.4).

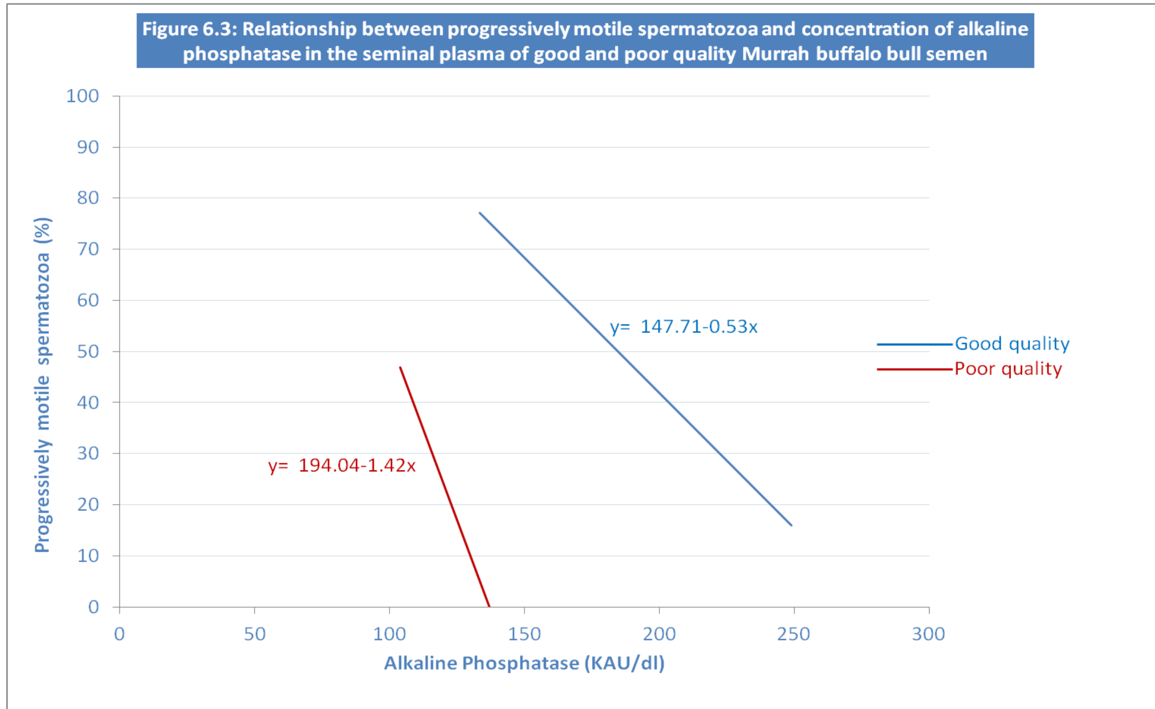


Figure 6.3: Relationship between progressively motile spermatozoa and concentration of alkaline phosphatase in the seminal plasma of good and poor quality Murrah buffalo bull semen

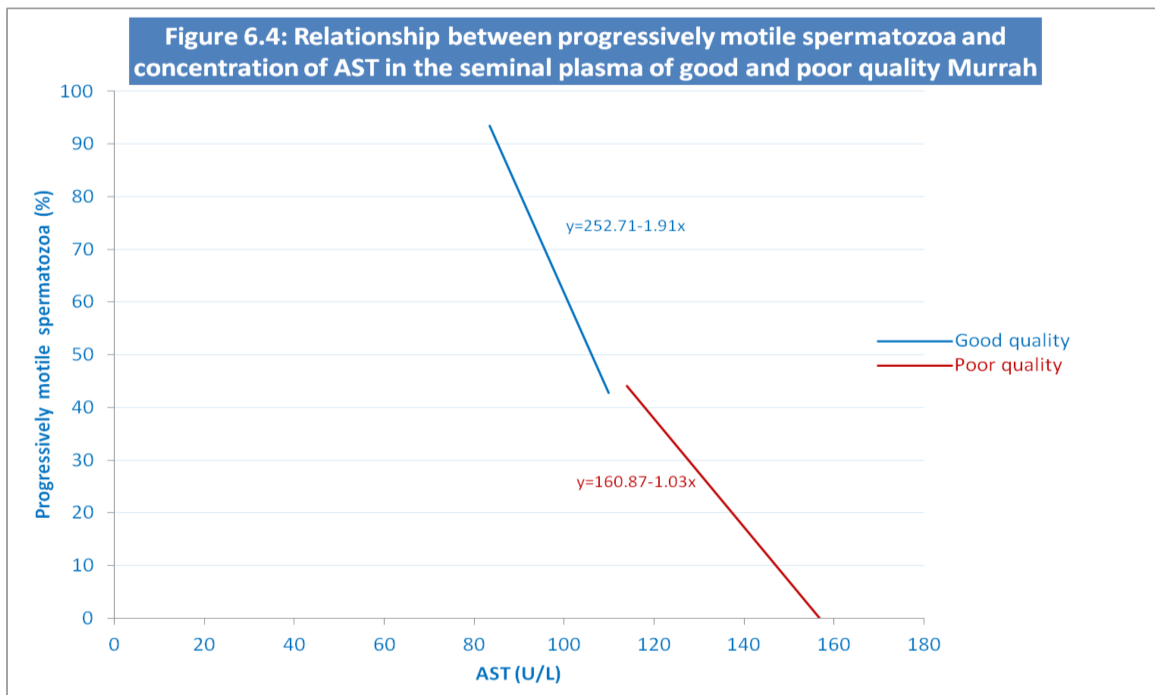
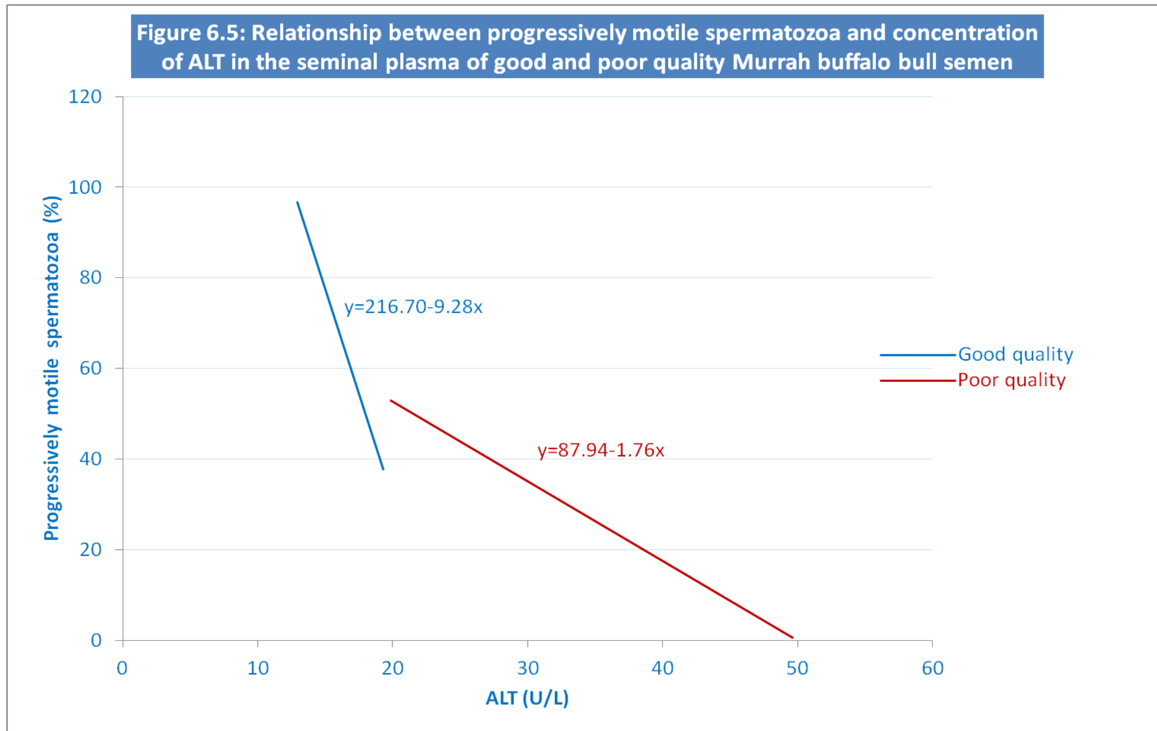
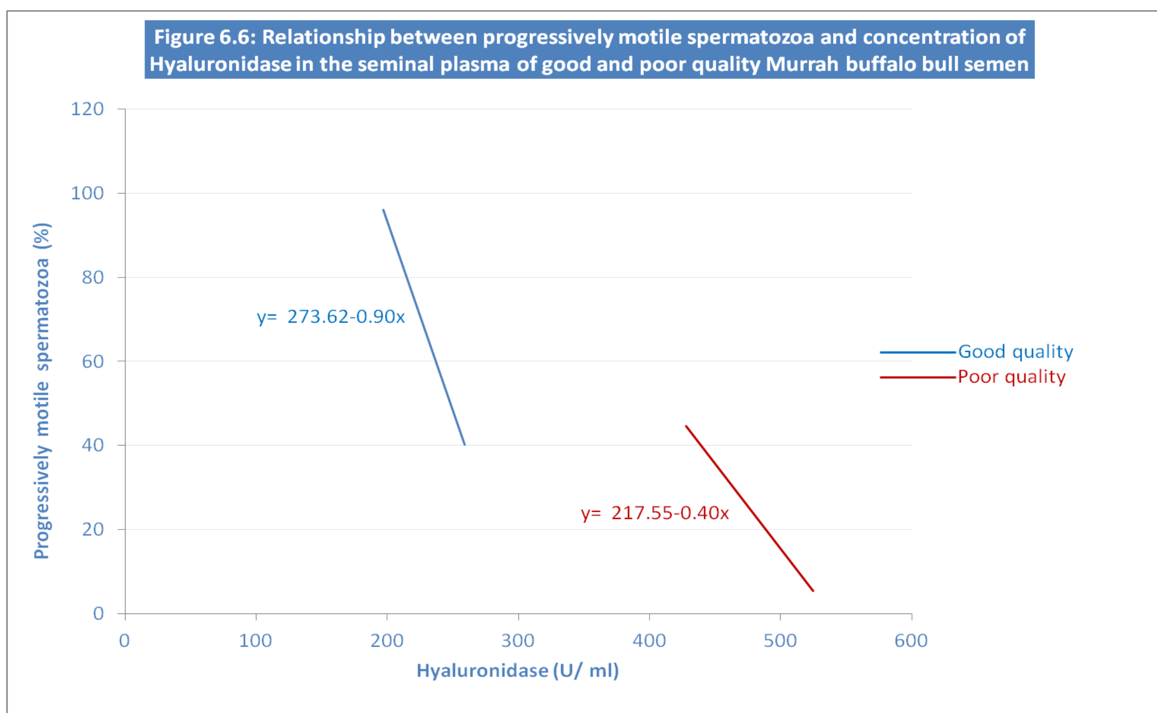


Figure 6.4: Relationship between progressively motile spermatozoa and concentration of AST in the seminal plasma of good and poor quality Murrah

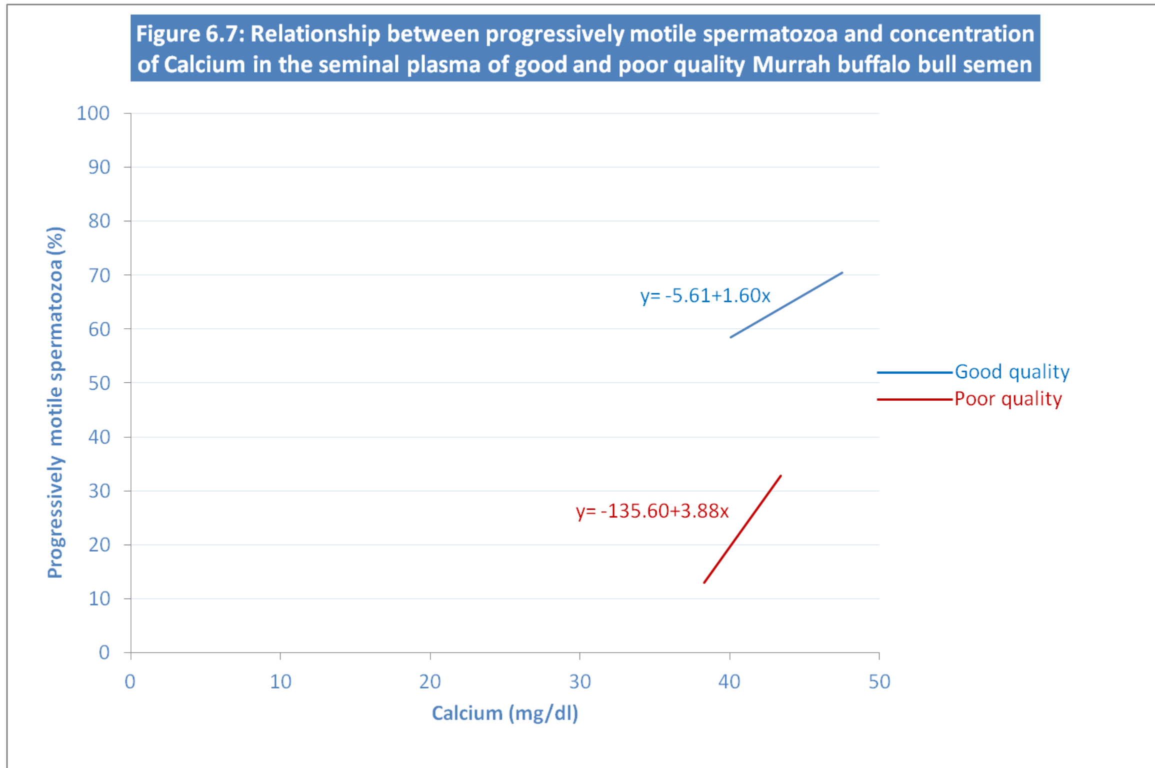
Highly negative significant (P<0.01) correlation was observed between per cent progressive motility and leakage of ALT (r= -0.7787 and -0.8954) and hyaluronidase (r= -0.76605 and -0.68714) in good and poor quality ejaculates, respectively, with

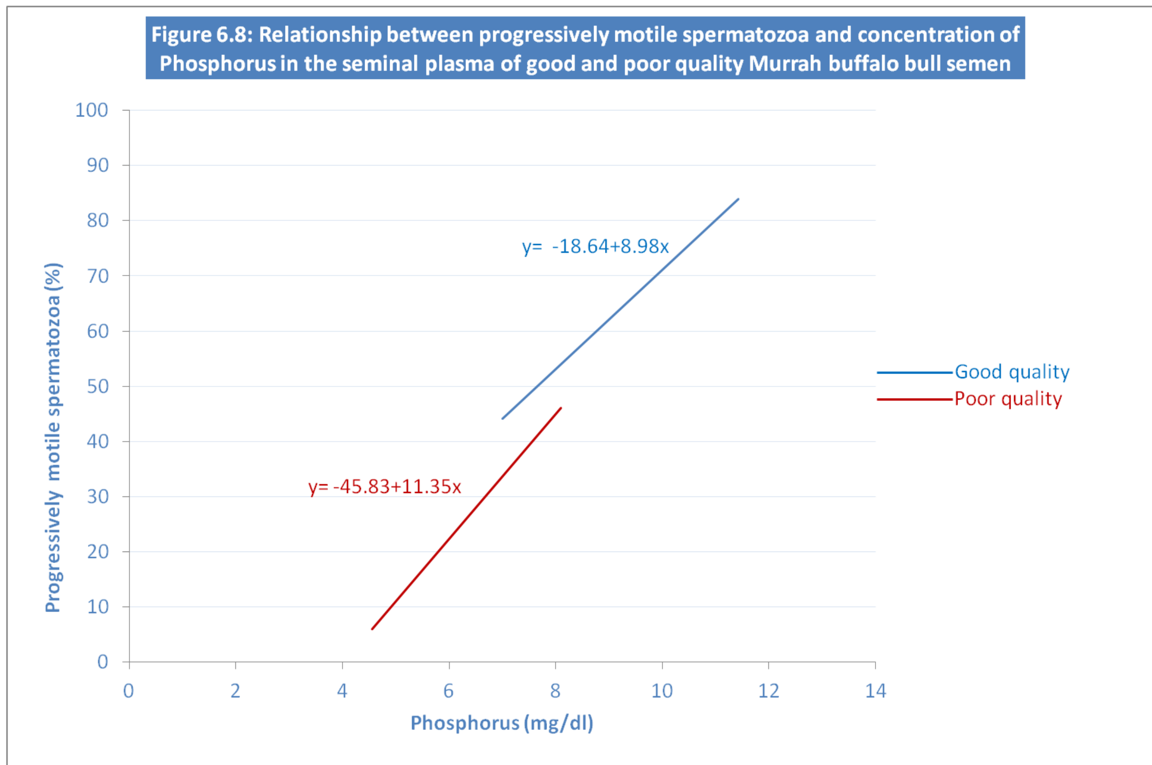
regression coefficients of ALT and hyaluronidase on progressive motility being -9.28±0.54 and -0.90±0.05 in good quality ejaculates and -1.76±0.06 and -0.40±0.03 in poor quality ejaculates, respectively (Figures 6.5 and 6.6, respectively).



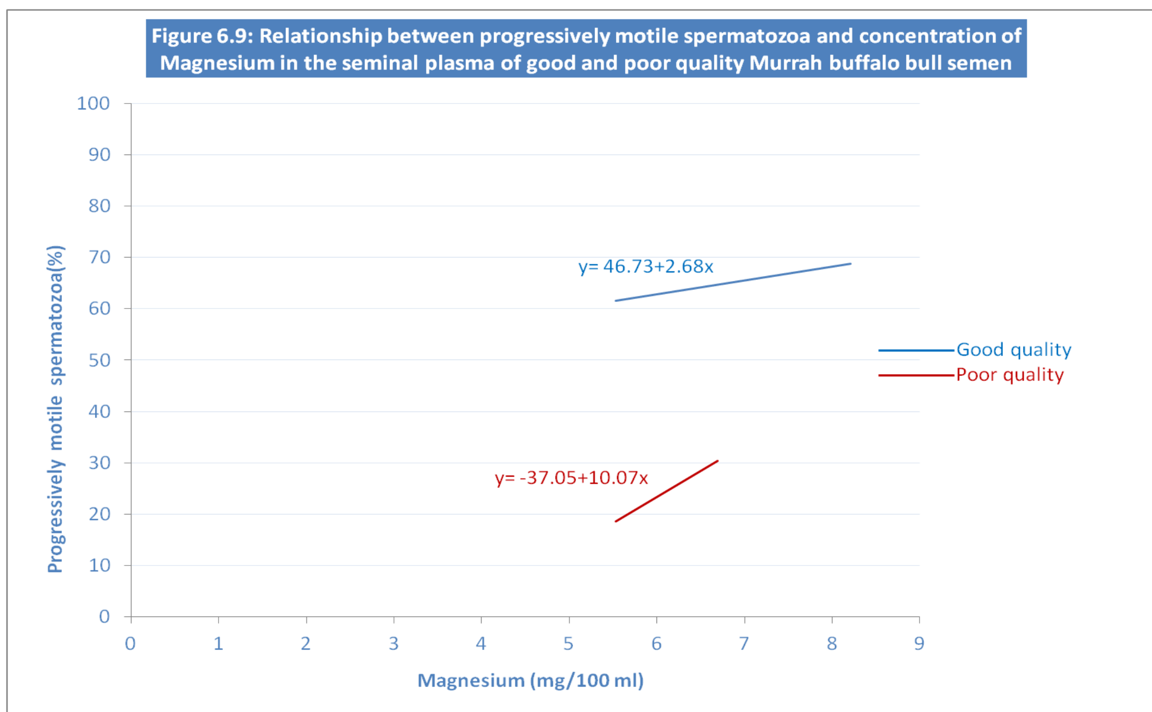


Progressive motility was significantly ($P<0.01$) correlated with calcium (r=0.21883 and 0.33624) and phosphorus (r=0.46707 and 0.51561) with regression coefficients of calcium being 1.60±0.52 and 3.88±0.79 (Figure 6.7) and phosphorus 8.98±1.23 and 11.35±1.37 (Figure 6.8) on progressive motility in good and poor quality ejaculates, respectively.





A non-significant relationship was observed between progressive motility and magnesium (r=0.12246) with regression coefficient of 2.68±1.58 in good quality ejaculate, where as in poor quality ejaculate, these exhibited a significant ($P<0.01$) relationship (r=0.20495) with a regression coefficient of 10.07±3.49 (Figure 6.9).



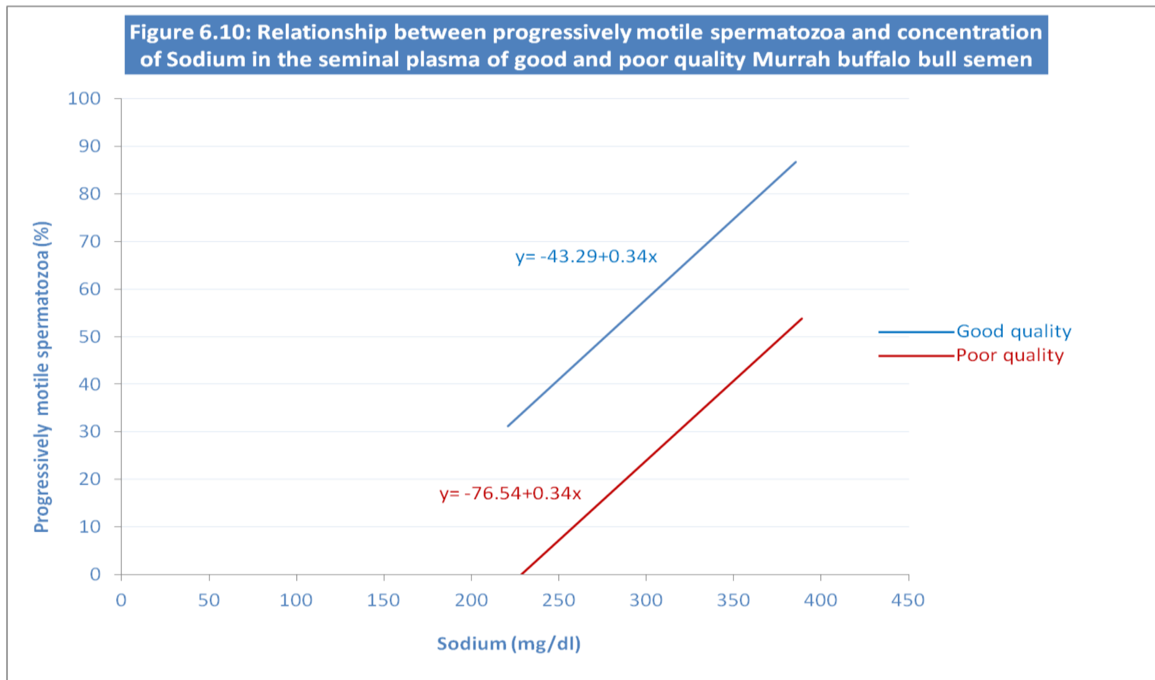
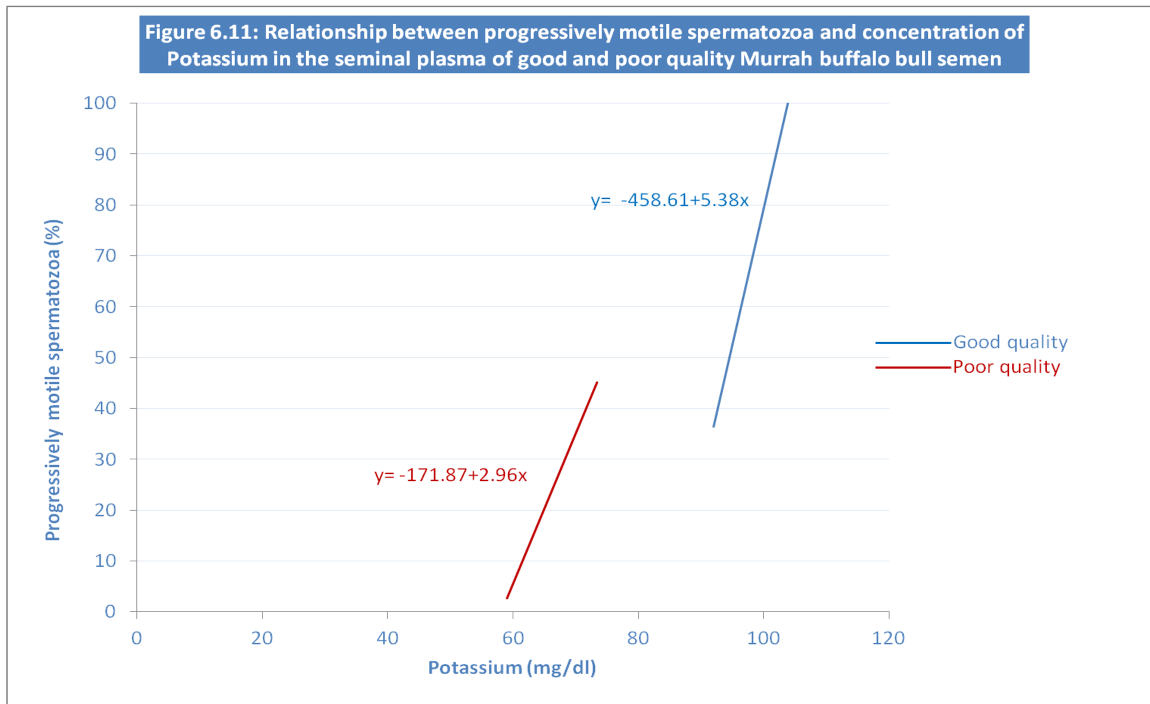
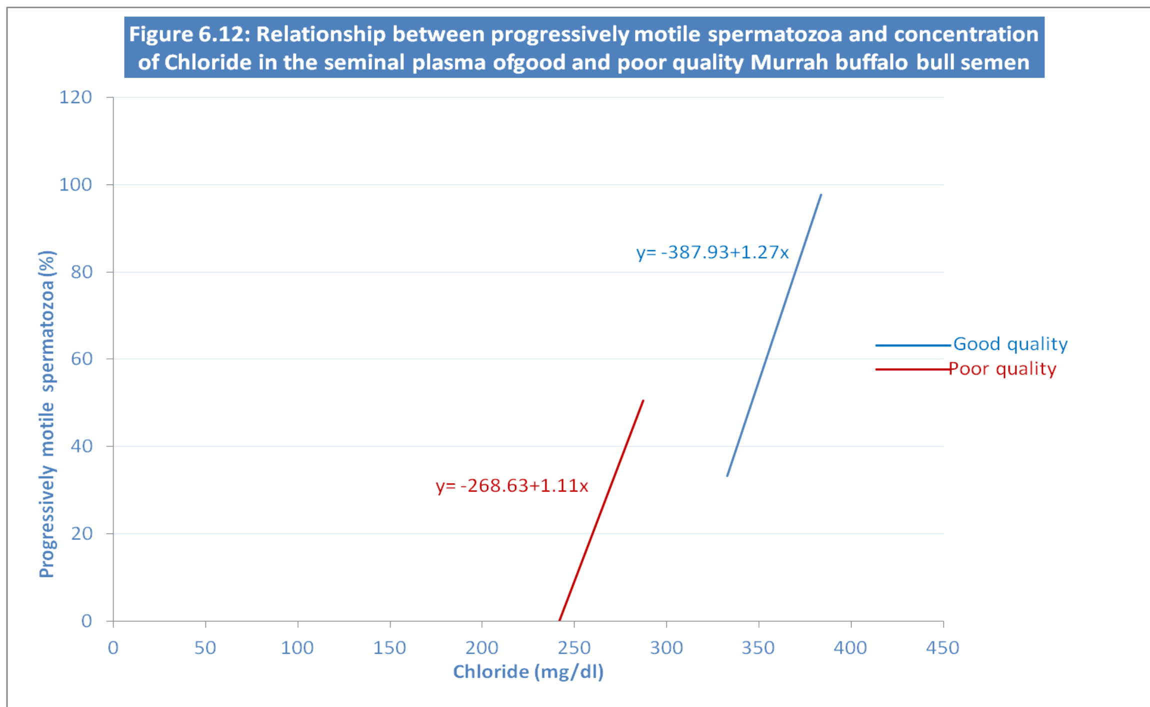


Figure 6.10: Relationship between progressively motile spermatozoa and concentration of Sodium in the seminal plasma of good and poor quality Murrah buffalo bull semen

The correlations of progressive motility with sodium (r=0.67512 and 0.89136), potassium (r= 0.73808 and 0.61697) and chloride (r=0.78694 and 0.71250) were significant ($P<0.01$) in good and poor quality ejaculates. The regression coefficient of sodium (0.34±0.03 and 0.34±0.01), potassium (5.38±0.36 and 2.96±0.27) and chloride (1.30±0.07 and 1.11±0.08) on progressively motile spermatozoa in good and poor quality ejaculates, respectively, were also significant (Figures 6.10, 6.11 and 6.12, respectively).





Sperm motility is a fairly reliable indication of the viability of fresh and frozen semen (Saacke and White 1972; Graham *et al.* 1980). Barnabe *et al.* (1981) associated a higher initial progressive motility to a lower incidence of abnormal acrosomes. El-Sisy *et al.* (2010) reported a significant correlation of motility with live (r= 0.728), HOS reactive (r=0.918) and abnormal acrosome (r=0.277) in buffalo bull spermatozoa. Kumar (2004)

reported a significantly positive correlation between sperm motility, live percentage and HOST reactive spermatozoa. Raval and Dhami (2006) reported a significant ($p<0.05$) correlation between initial motility with live sperm (r=0.54), sperm abnormality (r=-0.59) but a non significant relationship with AST (r=-0.10), ALT (r=0.34), AKP (r=-0.15) calcium (r= 0.01), phosphorus (r=0.21) and magnesium (r=0.21) in triple crossbred bulls. Dogan *et al.* (2009) reported a significant correlation between progressive motility and HOST (r=0.56), but a non significant correlation with AST (r=-0.34) and AKP (r=0.15) in stallion semen.

4.4.3 Relationship between HOS reactive spermatozoa and biochemical parameters

The overall inter-relationship of the HOS reactive sperms with various functional and biochemical parameters along with their regression equations for good versus poor quality semen of Murrah buffalo bulls have been depicted in Table 7 and Figures 7.1 to 7.11. This relationship within freshly diluted and post-thaw good and poor quality semen has been depicted in Annexure 17 and Annexure 18, respectively.

Table 7: Relationship of HOS reactive spermatozoa with certain functional evaluation parameters and various biochemicals in seminal plasma of good and poor quality Murrah buffalo bull semen

Sr. No.	Relationship between Parameters (n=192)		Quality	Correlation Coefficient	Regression Estimate	Regression Equation
1	HOST	Acrosome	Good	0.91623**	0.78±0.24	y= 17.38+0.78x
			Poor	0.98272**	1.07±0.01	y= -6.15+1.07x
2		AKP	Good	-0.47729**	-0.22±0.03	y= 115.83-0.22x
			Poor	-0.58627**	-1.73±0.17	y= 243.63-1.73x
3		AST	Good	-0.9360**	-0.77±0.02	y=156.60-0.77x
			Poor	-0.8376**	-1.62±0.08	y=251.71-1.61x
4		ALT	Good	-0.7965**	-3.81±0.21	y=143.00-3.81x
			Poor	-0.9177**	-2.49±0.08	y=126.58-2.49x
5		Hyaluronidase	Good	-0.78737**	-0.37±0.02	y= 166.76-0.37x
			Poor	-0.74026**	-0.60±0.04	y= 323.90-0.60x
6		Calcium	Good	0.19565**	0.57±0.21	y= 55.49+0.57x
			Poor	0.35496**	5.66±1.08	y= -196.51+5.66x
7		Phosphorus	Good	0.42809**	3.30±0.51	y= 50.05+3.30x
			Poor	0.43346**	13.18±1.98	y= -44.71+13.18x
8		Magnesium	Good	0.19163**	1.68±0.63	y= 69.12+1.68x
			Poor	0.14716*	9.98±4.87	y= -24.05+9.98x
9		Sodium	Good	0.70064**	0.14±0.01	y= 35.68+0.14x

			Poor	0.84402**	0.44±0.02	y= -95.22+0.44x
10		Potassium	Good	0.75416**	2.21±0.14	y= -133.82+2.21x
			Poor	0.72238**	4.78±0.33	y= -280.65+4.78x
11		Chloride	Good	0.77414**	0.50±0.03	y= -97.92+0.50x
			Poor	0.70352**	1.51±0.11	y= -362.71+1.51x

Perusal of the Table 7 indicates that HOS reactive spermatozoa percentage was significantly (P<0.01) correlated with per cent intact acrosome spermatozoa (r=0.91623 and 0.98272, n=192) in good and poor quality ejaculates, respectively. The overall regression coefficient of per cent intact acrosome spermatozoa on HOS reactive spermatozoa percentage was 0.78±0.24 and 1.07±0.01 in good and poor quality ejaculates, respectively (Figure 7.1).

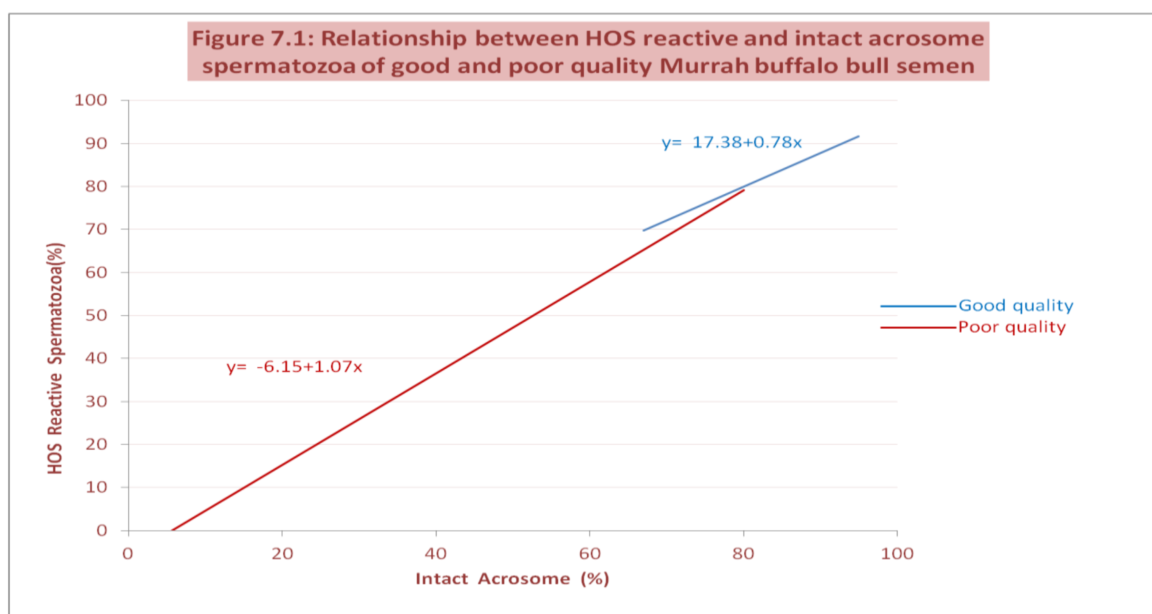


Figure 7.1: Relationship between HOS reactive and intact acrosome spermatozoa of good and poor quality Murrah buffalo bull semen

The correlation of HOS reactive spermatozoa with AKP (r=-0.47729 and-0.58627) and AST (r=-0.9360 and -0.8376), as well as the regression coefficient of AKP (-0.22±0.03 and -1.73±0.17), and AST (-0.77±0.02 and -1.62±0.08) on HOS reactive spermatozoa were significant (P<0.01) in good and poor quality ejaculates, respectively (Figures 7.2 and 7.3, respectively).

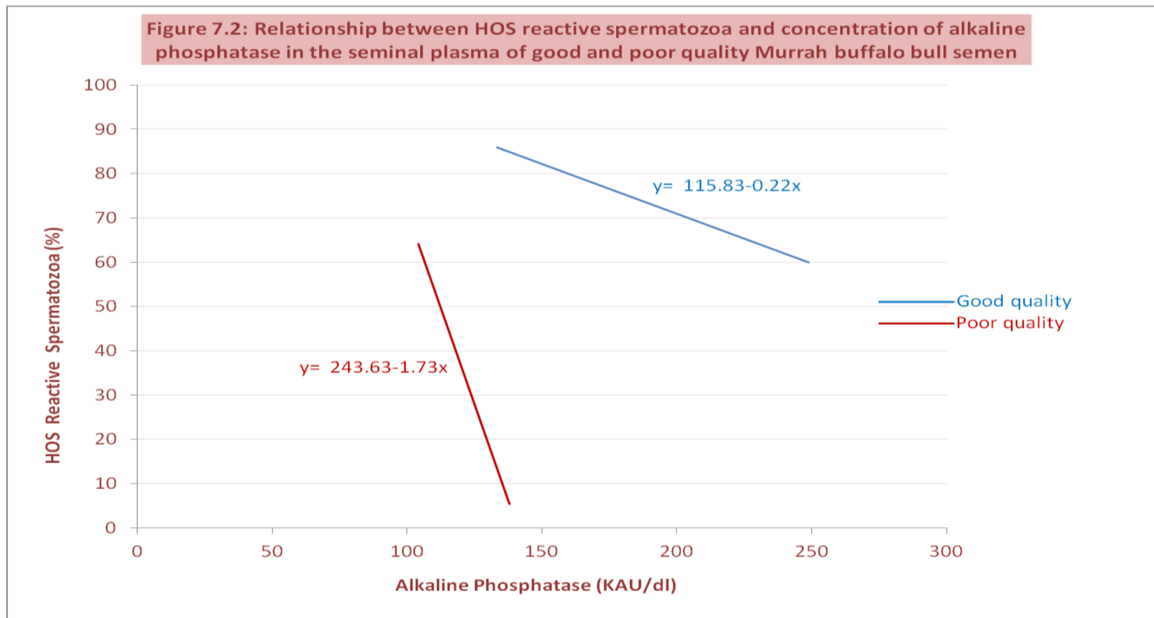


Figure 7.2: Relationship between HOS reactive spermatozoa and concentration of alkaline phosphatase in the seminal plasma of good and poor quality Murrah buffalo bull semen

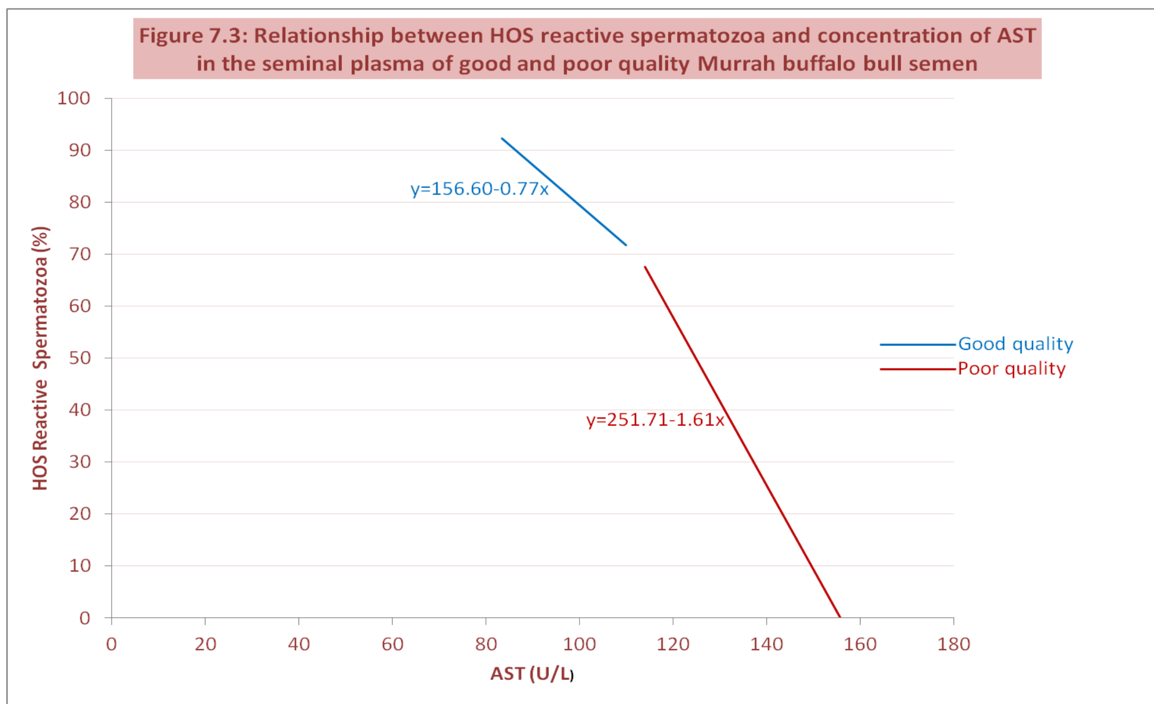
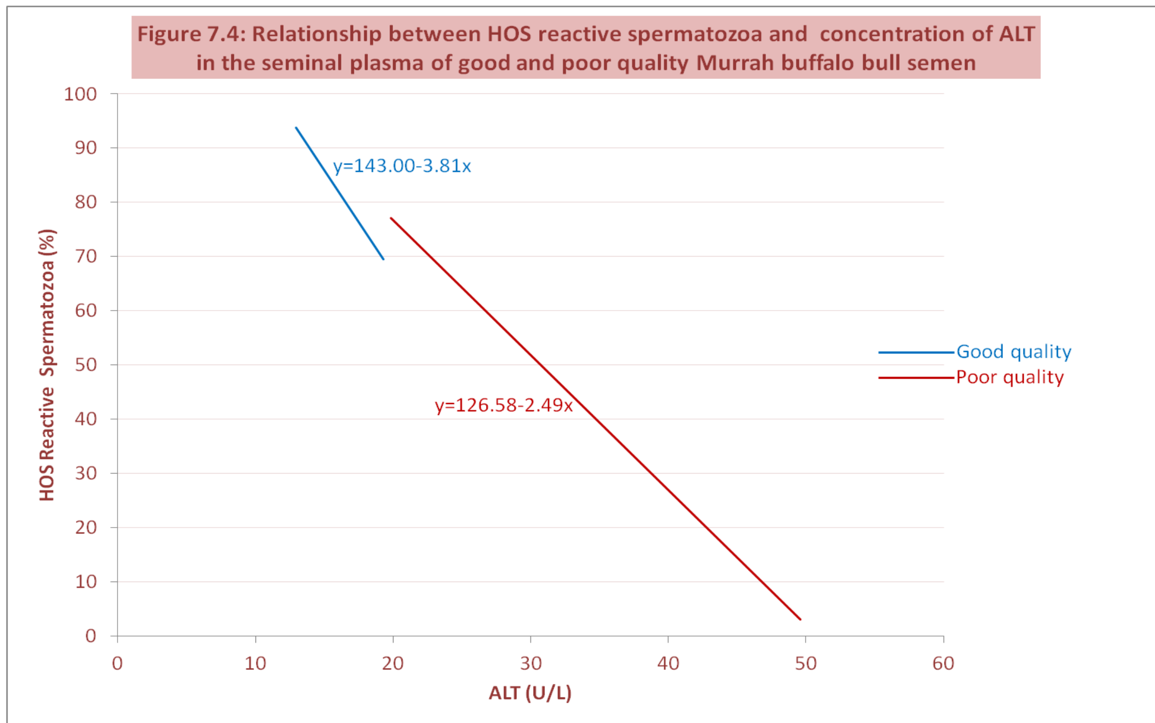
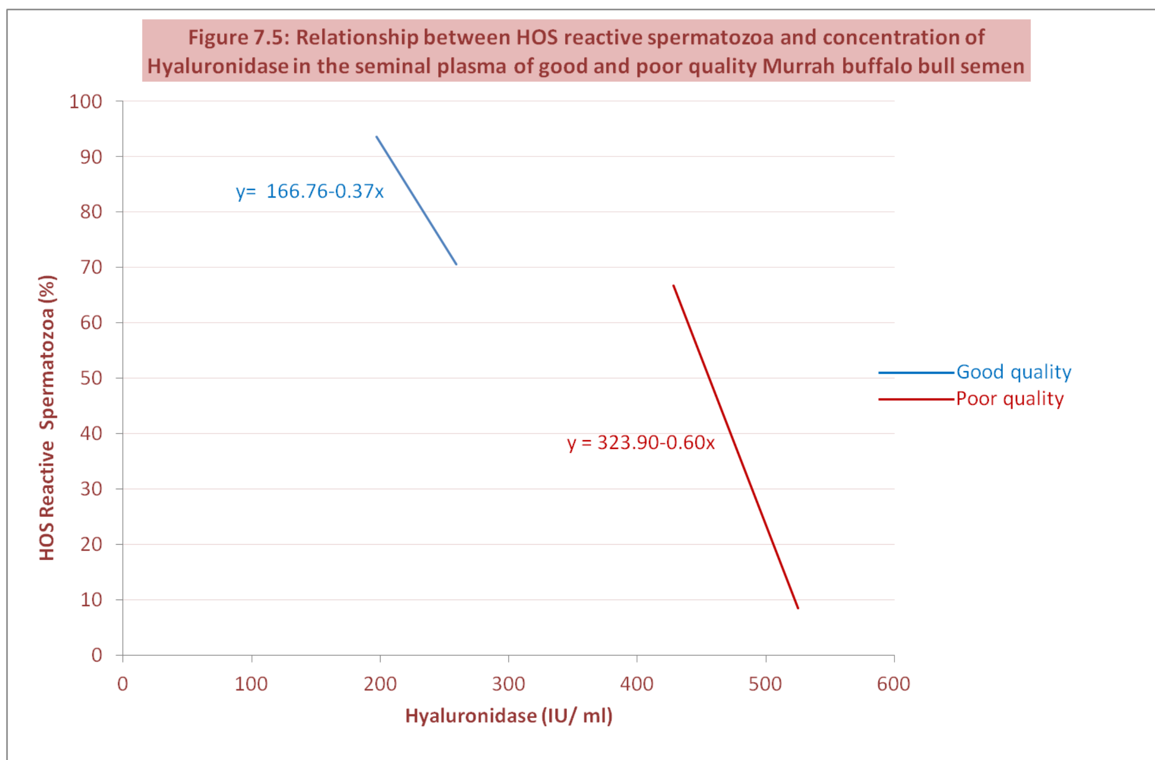


Figure 7.3: Relationship between HOS reactive spermatozoa and concentration of AST in the seminal plasma of good and poor quality Murrah buffalo bull semen

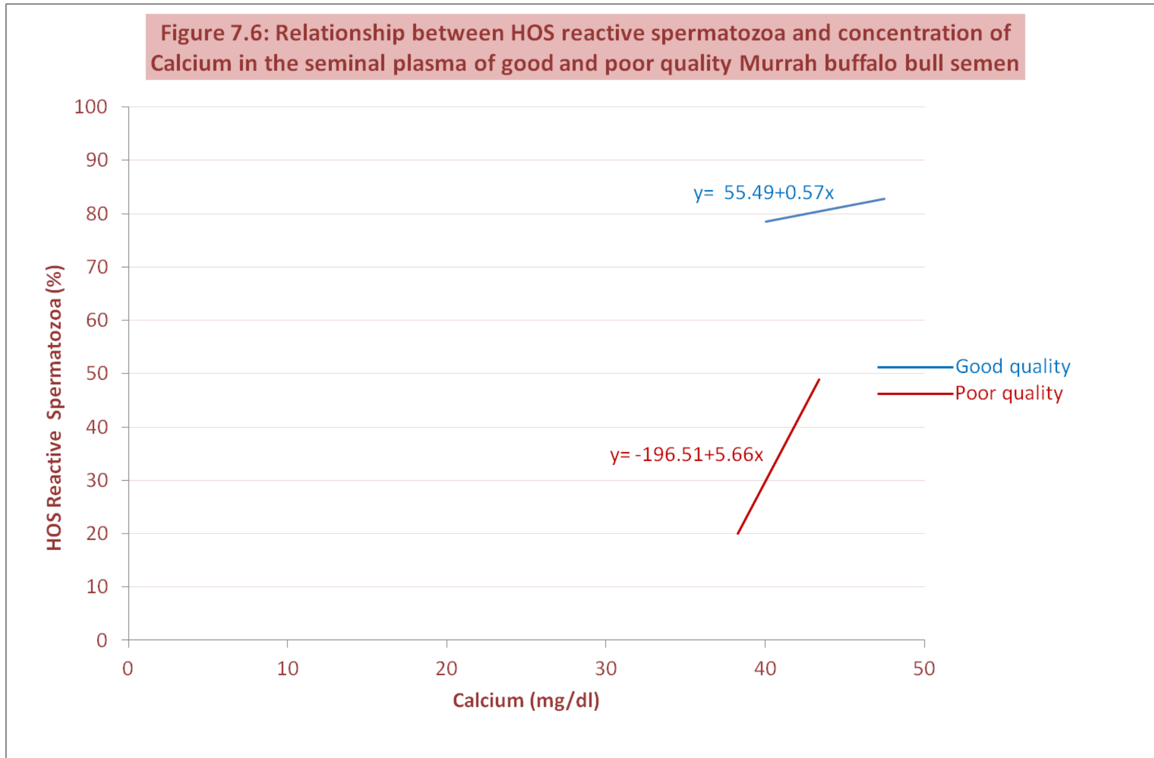
Highly negative and significant ($P<0.01$) correlation was observed between HOS reactive spermatozoa and leakage of ALT ($r= -0.7965$ and -0.9177) and hyaluronidase ($r= -0.78737$ and -0.74026) in good and poor quality ejaculates, respectively, with regression coefficients of ALT and hyaluronidase on HOS reactive spermatozoa being -3.81±0.21 and -0.37±0.02 in good quality ejaculates and -2.49±0.08 and -0.60±0.04 in poor ejaculates, respectively (Figure 7.4 and 7.5, respectively).

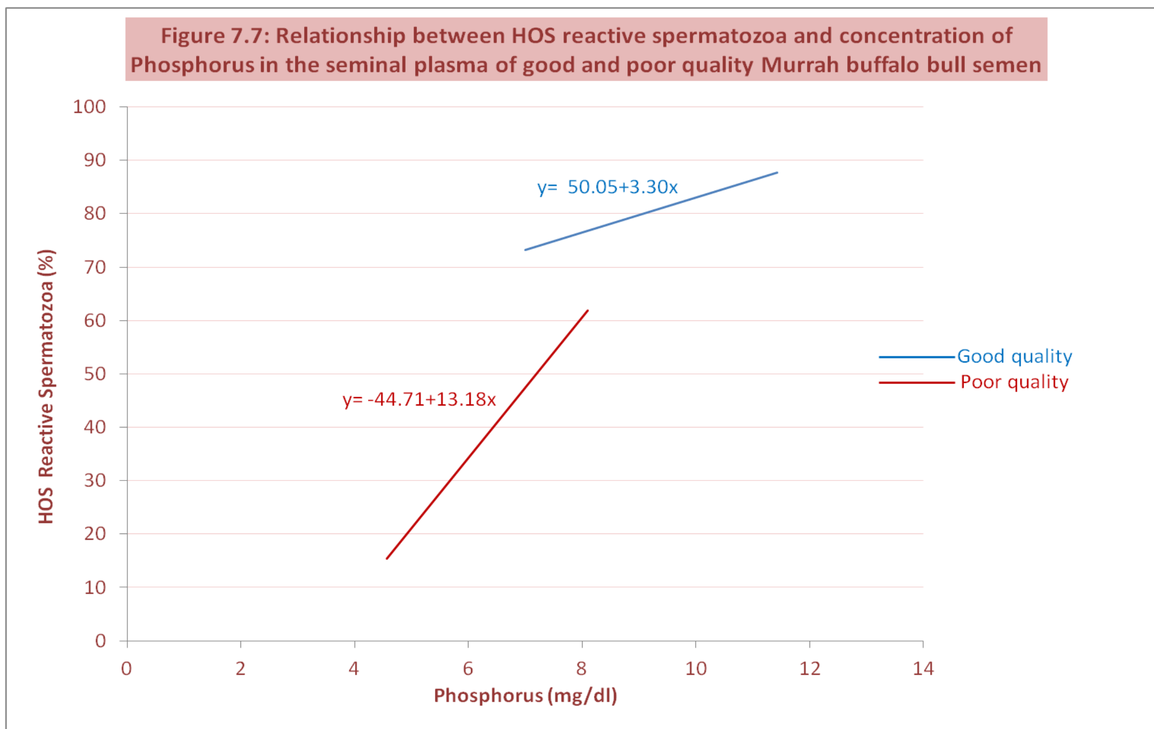




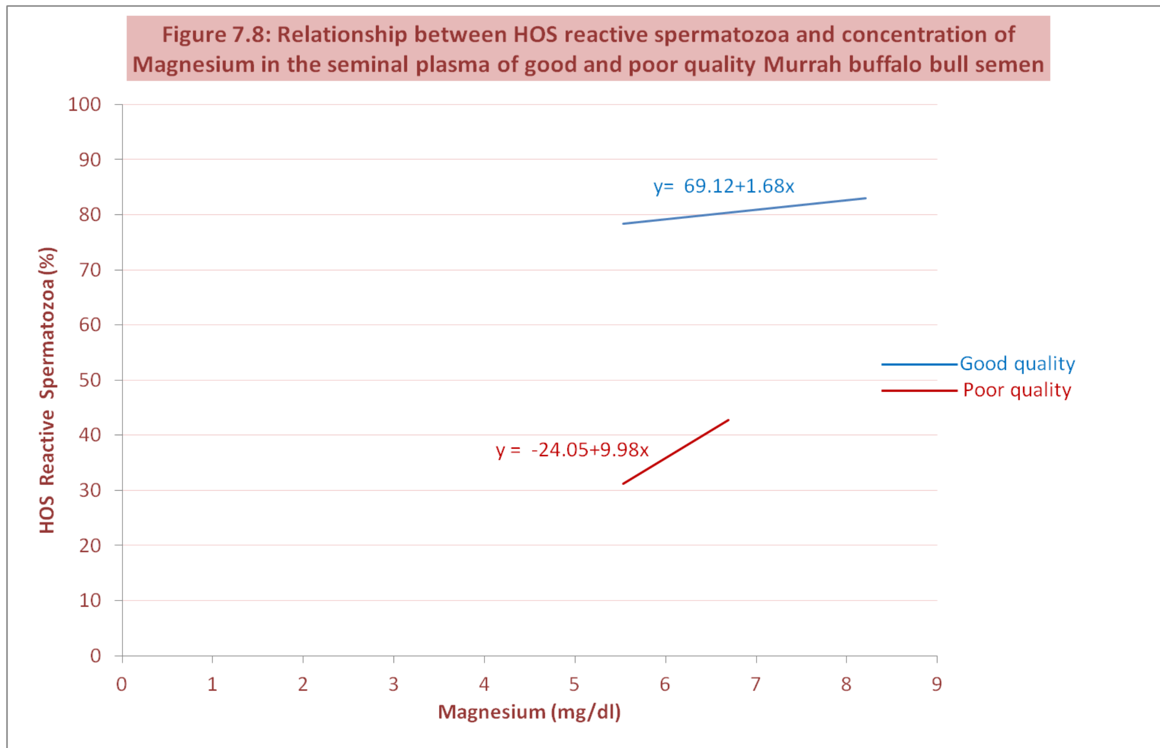
Mean HOS reactive spermatozoa was significantly ($P<0.01$) correlated with calcium (r=0.19565 and 0.35496) and phosphorus (r=0.42809 and 0.43346) with regression coefficients of calcium being 0.57±0.21 and 5.66±1.08 (Figure 7.6) and

phosphorus 3.30±0.51 and 13.18±1.98 (Figure 7.7) in good and poor quality ejaculates, respectively.





A significant (P<0.01) relationship was recorded between HOS reactive spermatozoa and magnesium (r=0.19163) in good quality ejaculate with regression coefficient of magnesium on HOS reactive spermatozoa being 1.68±0.63. Whereas, in poor quality ejaculate, there was a significant (P<0.05) relationship (r=0.14716) of magnesium on HOS reactive spermatozoa percentage with a regression coefficient of 9.98±4.87 (Figure 7.8).



Mean HOS reactive spermatozoa was significantly (P<0.01) correlated with sodium (r=0.70064 and 0.84402), potassium (r=0.75416 and 0.72238) and chloride (r=0.77414 and 0.70352) in good and poor quality ejaculates, respectively. The regression coefficient of sodium, potassium and chloride on HOS reactive spermatozoa in good and poor quality ejaculates were 0.14±0.01 and 0.44±0.02 (Figure 7.9), 2.21±0.14 and 4.78±0.33 (Figure 7.10) and 0.50±0.03 and 1.51±0.11 (Figure 7.11), respectively.

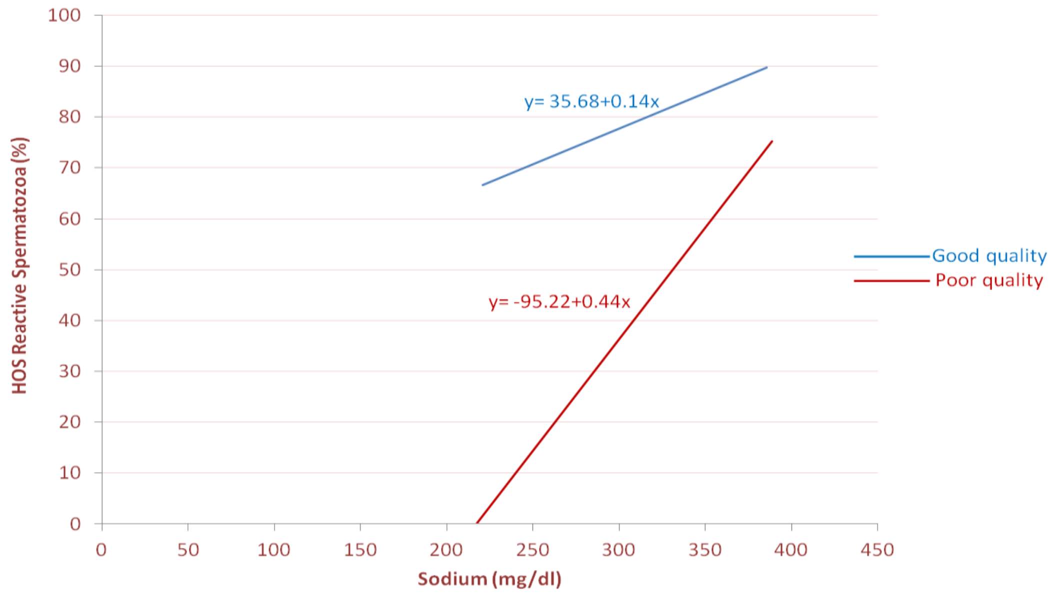


Figure 7.9: Relationship between HOS reactive spermatozoa and concentration of Sodium in the seminal plasmaof good and poor quality Murrah buffalo bull semen
HOS Reactive Spermatozoa (%)
100
90
80
70
60
50
40
30
20
10
0
0
50
100
150
200
250
300
350
400
450
Sodium (mg/dl)
y= 35.68+0.14x
y= -95.22+0.44x
Good quality
Poor quality

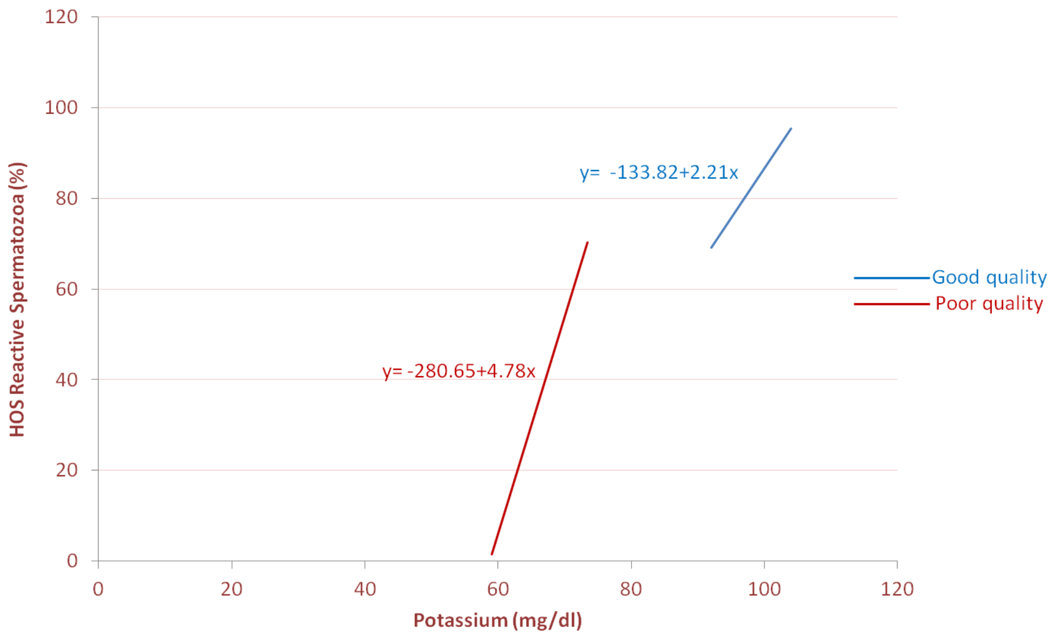
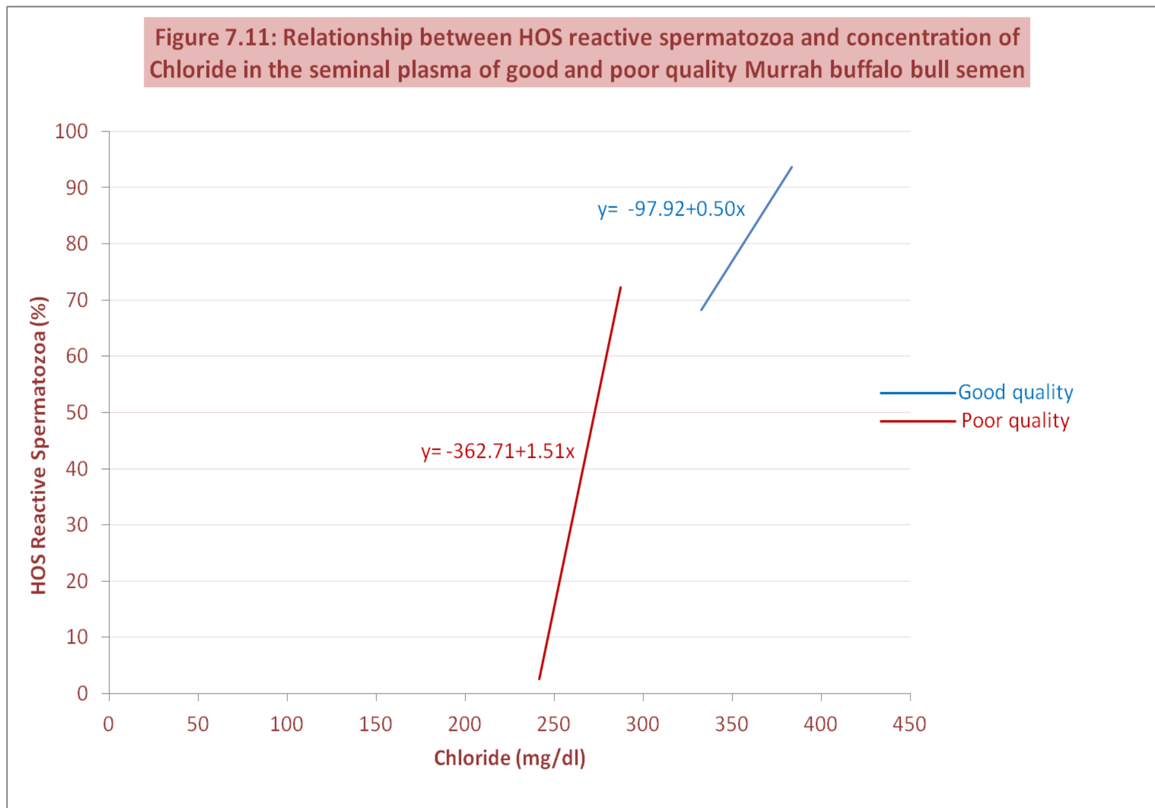


Figure 7.10: Relationship between HOS reactive spermatozoa and concentration of Potassium in the seminal plasma of good and poor quality Murrah buffalo bull semen
HOS Reactive Spermatozoa (%)
120
100
80
60
40
20
0
0
20
40
60
80
100
120
Potassium (mg/dl)
y= -133.82+2.21x
y= -280.65+4.78x
Good quality
Poor quality



Lodhi *et al.* (2008) reported a positive and significant (P<0.05) correlation between HOST and progressive motility (r=0.649), sperm viability (r=0.880) and morphologically normal spermatozoa (r=0.611) in buffalo bull spermatozoa. Similar findings were reported in human (Jeyendran *et al.* 1984), equine (Mantovani *et al.* 2002) and fresh goat semen (Fonseca *et al.* 2005). The percentage of positive sperms to HOS test varies with bull (Prasad *et al.* 1999), season (Kale *et al.* 2000), mass activity, progressive motility, sperm count and total sperm with intact acrosome (Prasad *et al.* 1999).

Comparing of various methods for evaluating sperm plasmalemma of bovine semen, Brito *et al.* (2003) found that the proportion of HOS responsive sperm was only moderately correlated with the proportion of plasmalemma-intact sperm identified by vital stains in contrast to high correlation reported by Correa and Javos (1994). Dogan *et al.* (2009) reported a non significant correlation between HOST and AST (r=-0.31) and AKP (r=-0.17) in Arabian horses.

4.4.4 Relationship between intact acrosomes and biochemical parameters

The overall inter-relationship of the sperms with intact acrosomes with various biochemical parameters along with their regression equations for good versus poor quality semen of Murrah buffalo bulls have been depicted in Table 8 and Figures 8.1 to 8.10. This relationship within freshly diluted and post-thaw good and poor quality semen has been depicted in Annexure 17 and Annexure 18, respectively.

Table 8: Relationship of spermatozoa with intact acrosomes and certain functional evaluation parameters and various biochemicals in seminal plasma of good and poor quality Murrah buffalo bull semen

Sr. No.	Relationship between Parameters (n=192)		Quality	Correlation coefficient	Regression Estimate	Regression Equation
1	Intact Acrosome	AKP	Good	-0.51428**	-0.28±0.03	y= 125.45-0.28x
			Poor	-0.60056**	-1.63±0.16	y= 235.55-1.63x
2		AST	Good	-0.9268**	-0.90±0.03	y=169.25-0.90x
			Poor	-0.8076**	-1.44±0.08	y=231.27-1.44x
3		ALT	Good	-0.7724**	-4.33±0.26	y=151.97-4.33x
			Poor	-0.9081**	-2.27±0.08	y=122.17-2.27x
4		Hyaluronidase	Good	-0.85093**	-0.47±0.02	y= 190.14-0.47x
			Poor	-0.76107**	-0.57±0.04	y= 312.32-0.57x
5		Calcium	Good	0.18750**	0.65±0.25	y= 52.77+0.65x
			Poor	0.38229**	5.61±0.98	y= -191.33+5.61x
6		Phosphorus	Good	0.47493**	4.29±0.58	y= 41.16+4.29x
			Poor	0.44462**	12.46±1.82	y= -36.78+12.45x
7		Magnesium	Good	0.15049*	1.55±0.73	y= 70.53+1.55x
			Poor	0.16756*	10.47±4.47	y= -23.63+10.47x
8		Sodium	Good	0.62641**	0.15±0.13	y= 33.94+0.15x
			Poor	0.84825**	0.40±0.02	y= -82.00+0.40x
9		Potassium	Good	0.75116**	2.58±0.16	y= -169.50+2.58x
			Poor	0.69577**	4.24±0.32	y= -241.50+4.24x
10		Chloride	Good	0.74740**	0.57±0.04	y= -121.16+0.57x
			Poor	0.72969**	1.45±0.09	y= -341.64+1.45x

* (P<0.05)   ** (P<0.01)   NS (not significant)

A significant (P<0.01) negative relationship was recorded between per cent intact acrosome spermatozoa with AKP (r= -0.51428 and -0.60056) and AST (r= -0.9268

and -0.8076) leakage in good and poor quality ejaculates, respectively. The regression coefficients of AKP and AST on per cent intact acrosome spermatozoa were -0.28±0.03 and -0.90±0.03 in good quality ejaculates and -1.63±0.16 and -1.44±0.08 in poor ejaculates, respectively (Figures 8.1 and 8.2, respectively).

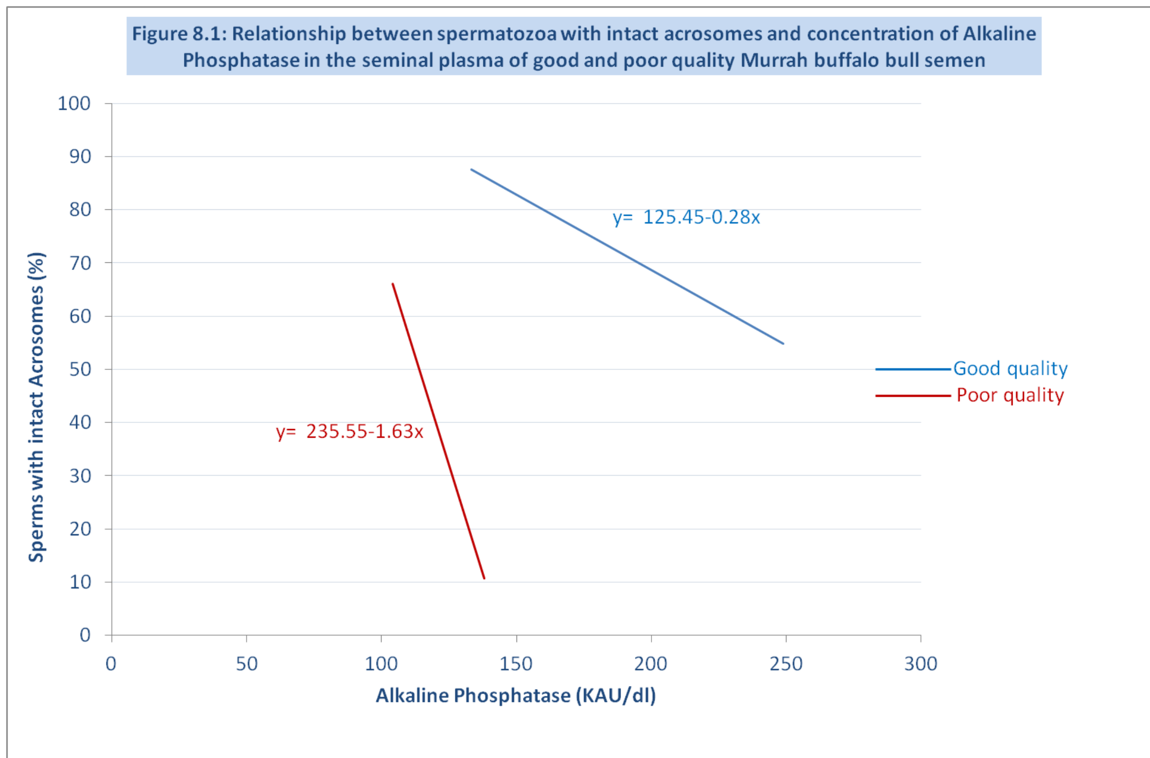
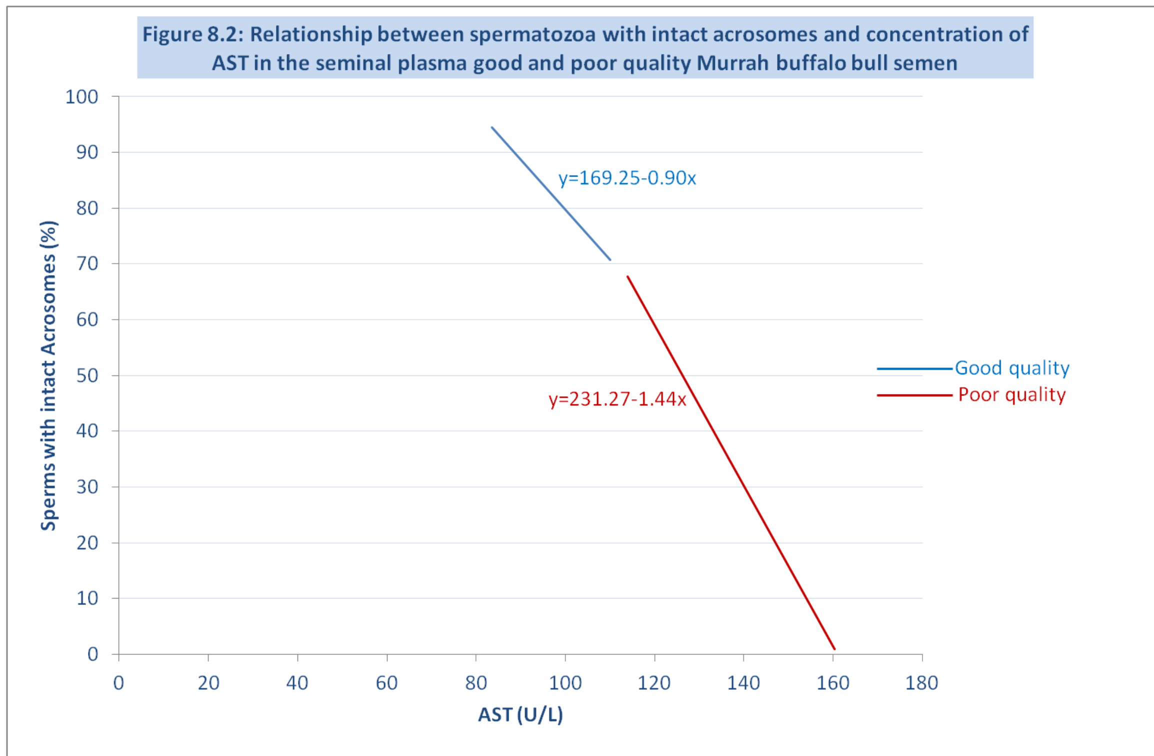
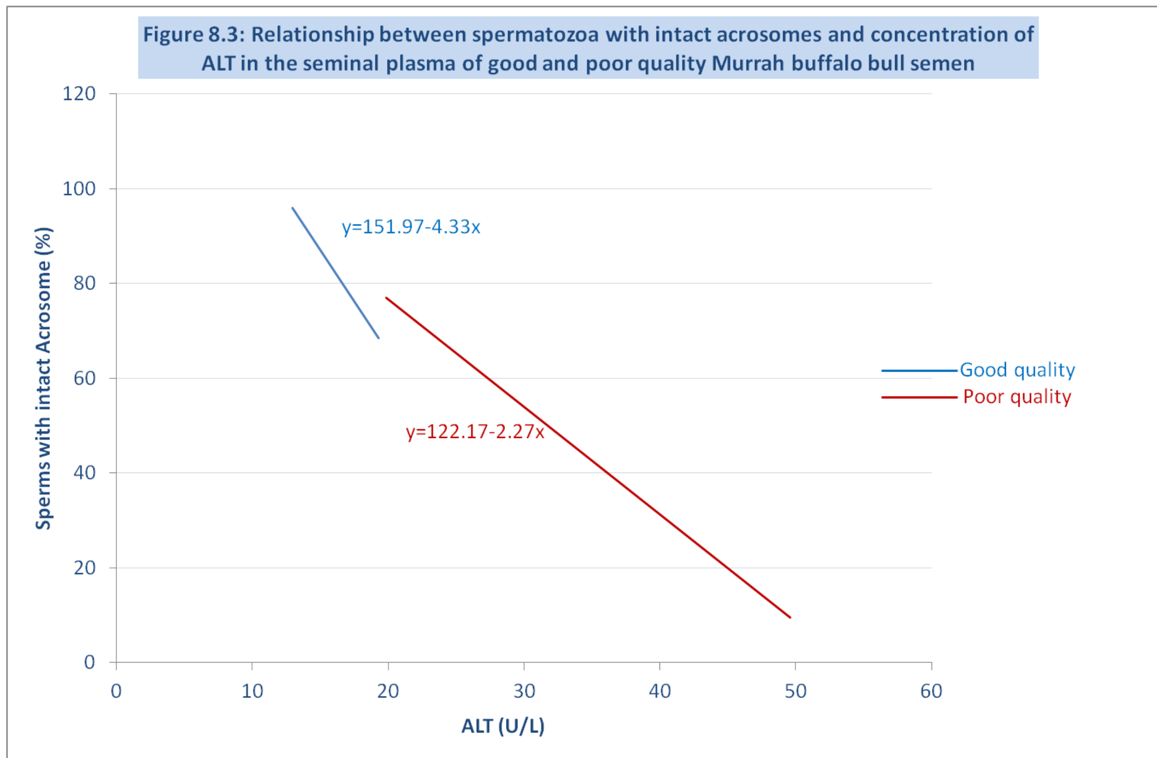
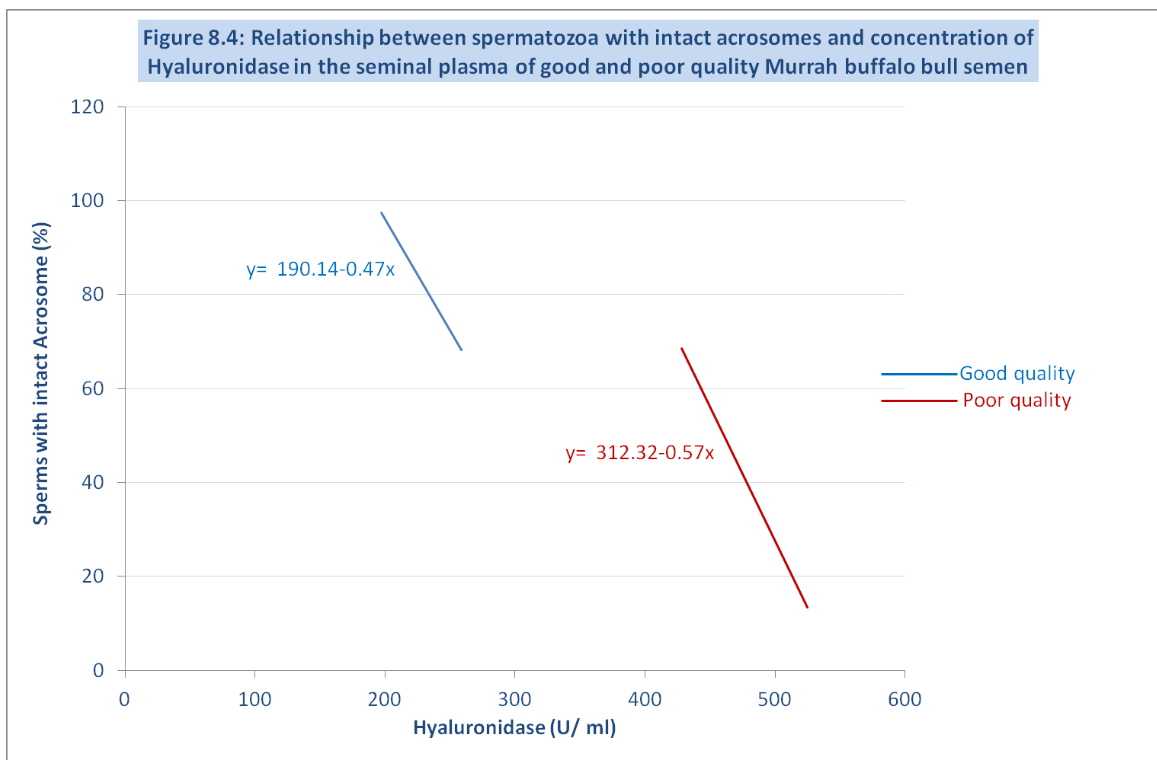


Figure 8.1: Relationship between spermatozoa with intact acrosomes and concentration of Alkaline Phosphatase in the seminal plasma of good and poor quality Murrah buffalo bull semen



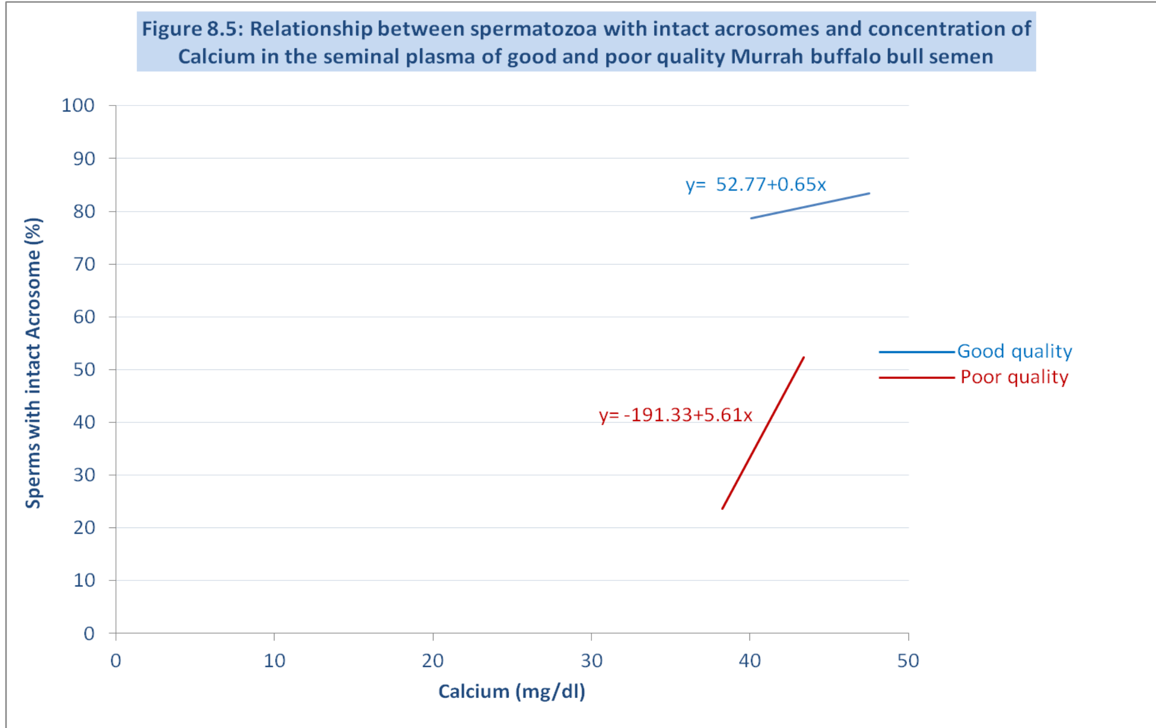
Highly significant (P<0.01) and negative correlation was observed between intact acrosome spermatozoa with leakage of ALT (r= -0.7724 and -0.9081) and hyaluronidase (r= -0.85093 and -0.76107) in good and poor quality ejaculates, respectively, with regression coefficients of ALT and hyaluronidase on per cent intact acrosome spermatozoa being -4.33±0.26 and -0.47±0.02 (Figure 8.3) in good quality ejaculates and -2.27±0.08 and -0.57±0.04 (figure 8.4) in poor quality ejaculates, respectively.

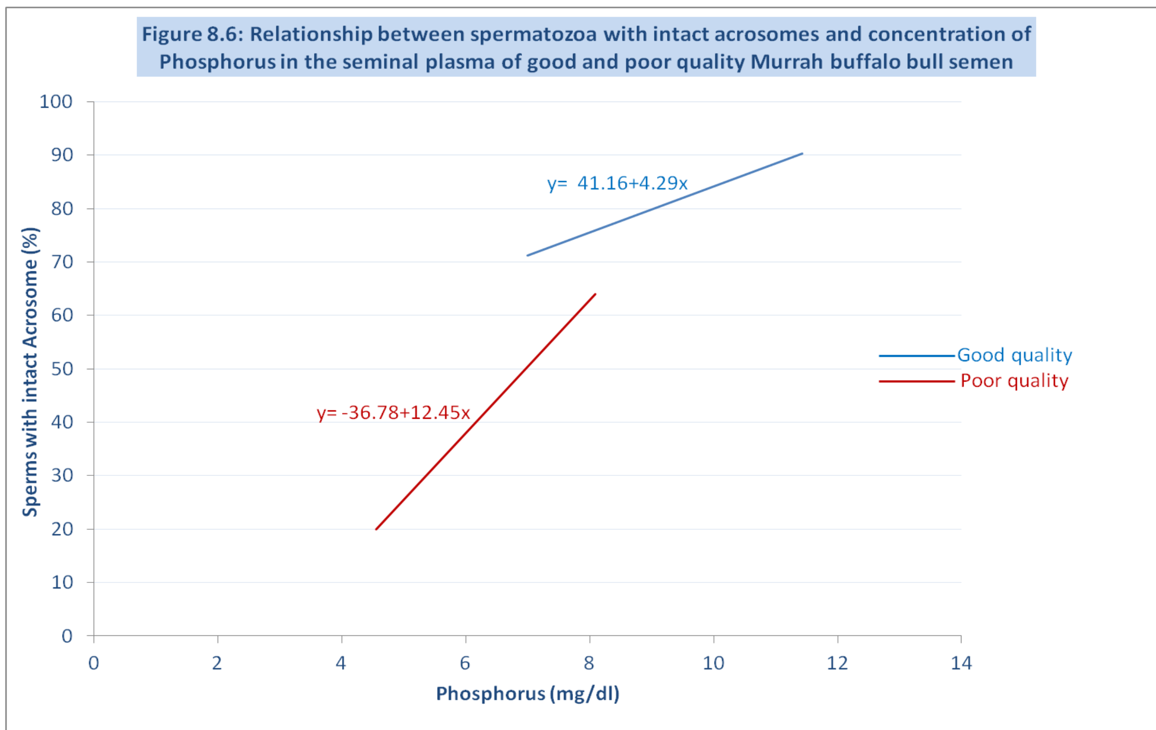




Intact acrosome spermatozoa were significantly ($P<0.01$) correlated with calcium ($r=0.18750$ and 0.38229) and phosphorus ($r=0.47493$ and 0.44462). Regression coefficients of calcium and phosphorus on per cent intact acrosome spermatozoa were

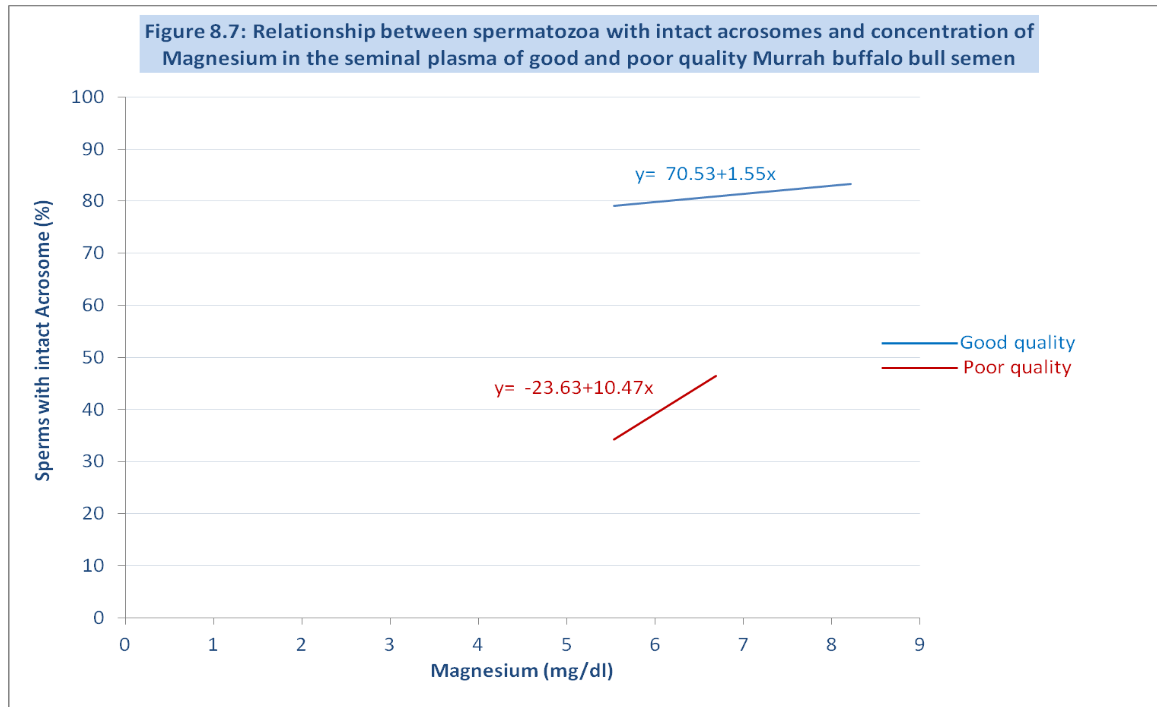
0.65±0.25 and 5.61±0.98 (Figure 8.5) and 4.29±0.58 and 12.46±1.82 (Figure 8.6) in good and poor quality ejaculates, respectively.





Similarly, a significant (P<0.05) relationship was observed between intact acrosome spermatozoa and magnesium (r=0.15049 and 0.16756) with regression

coefficient of magnesium on intact acrosome spermatozoa to be 1.55±0.73 and 10.47±4.47 in good and poor quality ejaculates, respectively (Figure 8.7).



The correlations of per cent intact acrosome spermatozoa with sodium (r=0.62641 and 0.84825), potassium (r= 0.75116 and 0.69577) and chloride (r=0.74740 and 0.72969) as well as regression coefficient of sodium (0.15±0.13 and 0.40±0.02; Ffigure 8.8), potassium (2.58±0.16 and 24.24±0.32; Ffigure 8.9) and chloride (0.57±0.04 and 1.45±0.09; figure 8.10) on per cent intact acrosome spermatozoa in good and poor ejaculates were significant ($P<0.01$) in good and poor quality ejaculates, respectively.

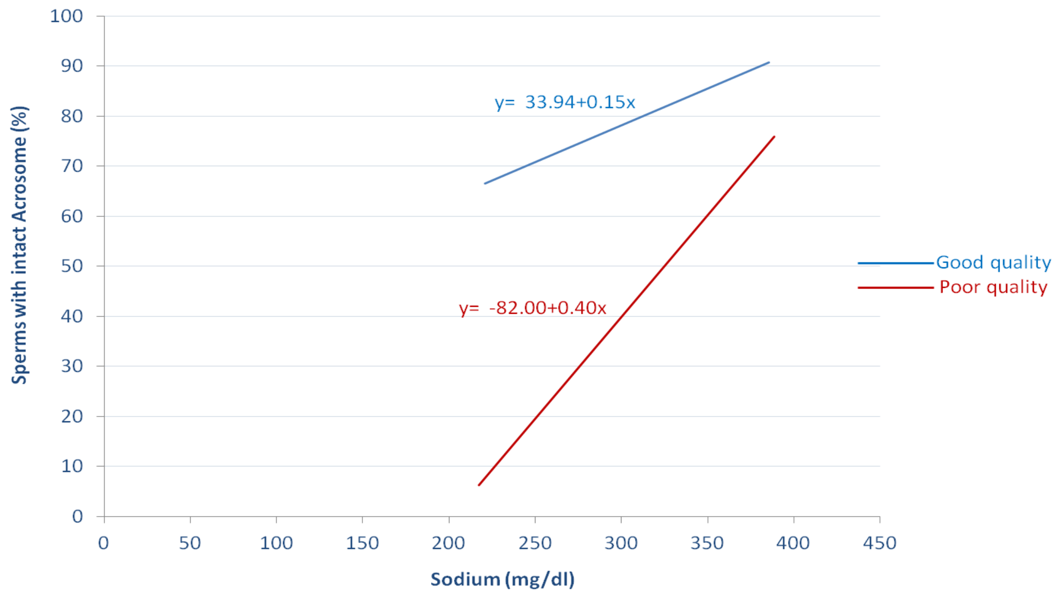


Figure 8.8: Relationship between spermatozoa with intact acrosomes and concentration of Sodium in the seminal plasma of good and poor quality Murrah buffalo bull semen
Sperms with intact Acrosome (%)
100
90
80
70
60
50
40
30
20
10
0
y= 33.94+0.15x
y= -82.00+0.40x
Good quality
Poor quality
0
50
100
150
200
250
300
350
400
450
Sodium (mg/dl)

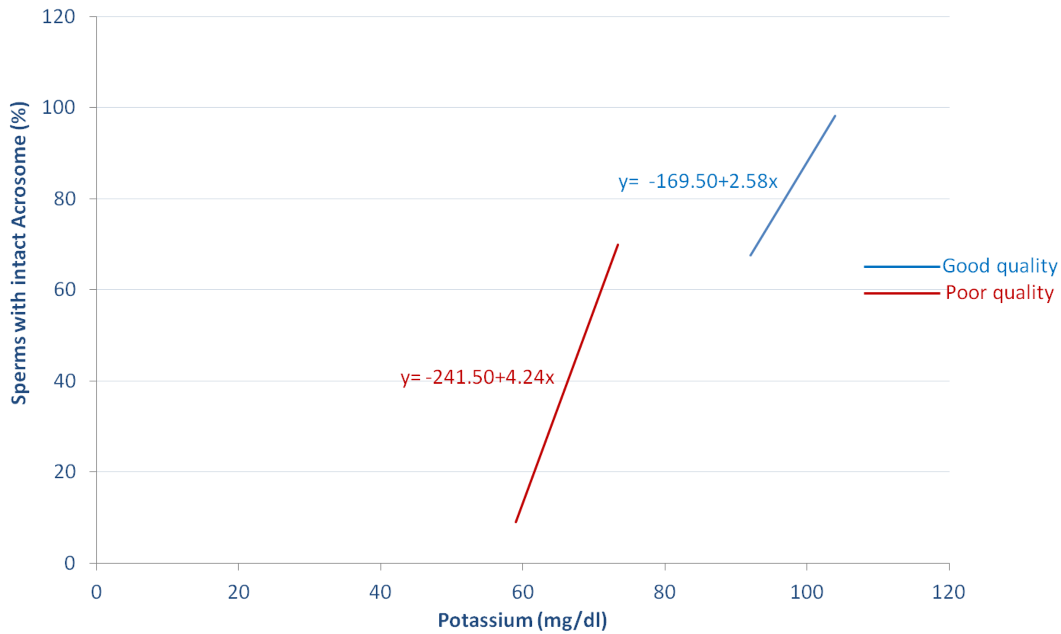


Figure 8.9: Relationship between spermatozoa with intact acrosomes and concentration of Potassium in the seminal plasma of good and poor quality Murrah buffalo bull semen
Sperms with intact Acrosome (%)
120
100
80
60
40
20
0
y= -169.50+2.58x
y= -241.50+4.24x
Good quality
Poor quality
0
20
40
60
80
100
120
Potassium (mg/dl)

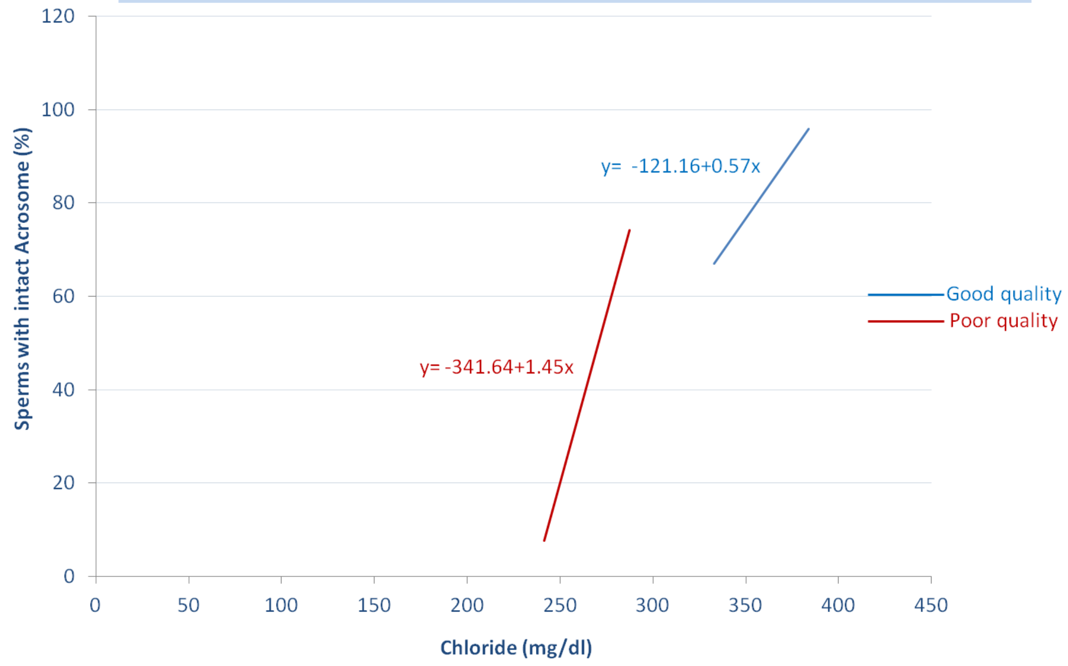


Figure 8.10: Relationship between spermatozoa with intact acrosomes and concentration of Chloride in the seminal plasma of good and poor quality Murrah buffalo bull semen
Sperms with intact Acrosome (%)
120
100
80
60
40
20
0
0
50
100
150
200
250
300
350
400
450
Chloride (mg/dl)
y= -121.16+0.57x
y= -341.64+1.45x
Good quality
Poor quality

CHAPTER 5

Summary and Conclusions

The study was conducted on 12 apparently healthy Murrah buffalo breeding bulls, maintained at Frozen Semen Bank, RCDF Ltd., Bassi, Jaipur. The selected bulls were divided into 2 groups, each comprising of 6 bulls, according to their known ejaculate quality (donating good or poor quality semen, respectively) to compare their functional and biochemical attributes. Group 1 comprised those bulls, which were donating semen of excellent quality with good freezability and fertility parameters whereas group 2 included those bulls which were frequently donating either initial poor quality semen or higher degree of damage occured during processing (during equilibration or cryopreservation) but were otherwise healthy.

After collection, initial parameters of ejaculate were recorded. Then the semen was diluted and further processing and freezing was done as per laboratory schedule. Thawing of semen straws was done at 37°C for 30 seconds. Semen evaluation was done at 4 different stages *i.e.* at fresh diluted, post-equilibration, 0 hr and 1 hr post-thaw. At all these stages the semen samples were evaluated for motility, live and dead count, reaction to hypo-osmotic solution and acrosomal integrity. Additionally, 2 ml diluted semen at all the four stages was centrifuged @ 268 g (2000 rpm) for 25 minutes to separate seminal plasma for biochemical estimation (enzymes and minerals) and this was stored at -20˚C pending analysis.

Enzymes (AKP, AST, ALT and hyaluronidase) and minerals (Calcium, phosphorus, magnesium, sodium, potassium and chloride) were estimated in the seminal plasma separated at all the four stages.

5.1 Initial parameters of good and poor quality semen of Murrah buffalo bulls

In present study, the mean ejaculate volume was 3.92±0.08 and 3.38±0.13 ml for good and poor quality ejaculates, respectively. The overall mean sperm concentration in good quality ejaculates was 1593.88±24.22 whereas it was 1503.10±24.60 million per ml in poor quality ejaculates. The mean mass activity of good quality ejaculates was 4.30±0.08 where as in case of poor quality ejaculates it was 1.66±0.08. The mean pH of

good quality ejaculates was 6.90±0.003 where as it was 7.35±0.027 in poor quality ejaculates. The differences in mass activity and pH were highly significant ($p<0.01$).

5.2 Functional parameters of good and poor quality semen of Murrah buffalo bulls

The mean live per cent was significantly higher ($p<0.01$) in good than poor quality ejaculates at all the four stages. At fresh diluted stage it was 90.88±0.26 per cent in good quality ejaculates whereas in poor quality ejaculates, it was 64.42±0.91 per cent. Subsequent processing of semen lead to a significant ($p<0.01$) progressive decline in mean livability. At post equilibration, the mean live percentage was 86.44±0.25 and 48.77±1.11 which further declined to 78.29±0.31 and 29.42±1.10 in good and poor quality ejaculates, respectively. The mean progressive motility was significantly higher ($p<0.01$) in good quality ejaculates at all the four stages of semen evaluation. A significant decline ($p<0.01$) was observed in overall mean progressive motility with semen processing stages. The overall mean progressive motility recorded at fresh diluted stage in good quality ejaculates was 86.15±0.34 per cent, where as it was 44.48±0.75 per cent in poor quality ejaculates which declined to 73.17±0.38 in good quality and 31.25±0.69 per cent in poor quality ejaculates at post-equilibration stage. Immediately after post-thaw, it was 63.50±0.29 per cent and 18.15±0.69 per cent in good and poor quality ejaculates, respectively. After 1 hr incubation post-thaw, progressive motility further declined and it was 40.46±0.50 and 5.42±0.58 per cent in good and poor quality ejaculates, respectively. Significantly higher ($p<0.01$) HOS reactivity was observed in good quality ejaculates at all the stages of semen processing. Evaluation of HOS reactivity at various stages of processing showed that the HOS reactive spermatozoa percentage declined significantly ($p<0.01$) from fresh diluted (89.19±0.26 and 63.17±0.93%) to post-equilibration (85.27±0.23 and 47.58±1.09%) and then at post-thaw stages (77.05±0.31 and 28.44±1.09% immediately after thawing and 72.90±0.39 and 9.94±0.79% after 1 hr incubation) in good and poor quality ejaculates, respectively. The intact acrosome percentage was higher ($p<0.01$) in good quality ejaculates at all the four stages of semen evaluation. The overall mean intact acrosomes recorded at fresh diluted stage in good quality ejaculates was 91.21±0.30 per cent, where as this was 65.33±0.88 per cent in poor quality ejaculates which declined to 85.48±0.37 per cent in good quality and 49.65±1.04 per cent in poor quality

ejaculates at post-equilibration stage, respectively. Immediately after post-thaw, it was 78.77±0.35 and 32.06±0.81 per cent in good and poor quality ejaculates, respectively. After 1 hr incubation post-thaw, mean per cent intact acrosome further declined to 70.79±0.32 and 15.88±0.64 per cent in good and poor quality ejaculates, respectively. The decline in percent intact acrosomes through various stages of evaluation within good and poor quality ejaculates was significant ($p<0.01$).

5.3 Enzymatic profile of good and poor quality semen of Murrah buffalo bulls

Concentration of alkaline phosphatase (AKP) recorded in good quality ejaculates was significantly higher ($p<0.01$) in comparison to poor quality ejaculates at all the four stages of evaluation. The concentrations of AKP increased significantly ($p<0.01$) as the processing stages advanced in both good and poor quality semen. At fresh diluted stage it was 147.75±1.63 and 113.40±0.74 KAU/dL and this concentration increased at equilibration to 152.13±1.65 and 117.62±0.73 KAU/dL in good and poor quality ejaculates, respectively. Immediately after post-thaw this concentration was 155.94±1.69 and after 1 hr incubation it was 163.22±2.59 KAU/dL in good quality ejaculates whereas in poor quality ejaculates this concentration was 121.13±0.79 and 126±0.91KAU/dL at respective post-thaw stages. Similarly, significantly higher AST ($p<0.01$) concentrations were recorded in poor quality ejaculates at all the four stages of semen processing. The AST concentration increased significantly ($p<0.01$) from fresh diluted to 1 hr post-thaw incubation in both good and poor quality ejaculates. The AST concentration was 87.22±0.19, 93.34±0.25, 103.08±0.21 and 107.97±0.18 U/L at fresh diluted, post-equilibration, post-thaw and 1 hr incubation stages in good quality ejaculates, respectively, whereas in poor quality ejaculates it was 122.55±0.74, 127.51±0.80 U/L at fresh diluted and post-equilibration stages and 134.39±0.69 and 146.26±1.34 U/L at 0 and 1 hr post-thaw stages. Significant lower ($p<0.01$) concentrations of ALT were recorded in good quality ejaculates. The ALT concentrations in seminal plasma increased significantly ($p<0.01$) with advancement in the semen processing stages both in good and poor quality ejaculates. At fresh diluted stage, the mean ALT concentration was 14.75±0.12 and 25.43±0.47 U/L which increased to 15.63±0.11 and 33.56±0.36 U/L at post equilibration stage in good and poor quality ejaculates, respectively. Immediately after post-thaw the concentration was

16.92±0.14 and after 1 hr incubation, it was 17.73±0.11 U/L in good quality ejaculates whereas these were 39.47±0.43 and 44.87±0.43 U/L at respective post-thaw stages in poor quality ejaculates. Higher ($p<0.01$) concentrations of hyaluronidase were recorded in poor quality ejaculates and the concentration increased significantly ($p<0.01$) with semen processing in good as well as poor quality ejaculates. In good quality ejaculates, it was 216.54±1.36 U/ml in freshly diluted semen which increased to 222.81±1.26 U/ml at post-equilibration stage whereas these values were 451.54±2.12 U/ml and 463±2.31 U/ml at respective semen processing stages in poor quality ejaculates. Immediately post-thaw the concentrations were 237.56±1.22 and 492.23±2.38 U/ml in good and poor quality ejaculates, respectively, and these increased to 245.65±1.16 and 501.13±2.63 U/ml after 1 hr incubation in good and poor quality ejaculates, respectively.

5.4 Mineral profile of good and poor quality semen of Murrah buffalo bulls

The calcium concentrations were significantly higher ($p<0.01$) in good quality seminal plasma at all the stages of evaluation. There was a significant decline ($p<0.01$) from fresh diluted (45.18±0.29 and 41.95±0.18 mg/dl) to post-thaw (44.23±0.36 and 40.90±0.18 mg/dl) stage in good and poor quality ejaculates, respectively. A non-significant difference was noticed immediately post-thaw and 1 hr incubation post-thaw in good as well as poor quality ejaculates. Similarly, significantly higher ($p<0.01$) phosphorus concentrations were recorded in good quality ejaculates. A significant decline ($p<0.01$) was observed in phosphorus concentration as the semen processing stages advanced in both good and poor quality ejaculates. At fresh diluted stage the mean value was 9.98±0.11 which decreased to 9.67±0.11 mg/dl at post-equilibration stage in good quality ejaculates and from 6.70±0.10 to 6.36±0.09 mg/dl at respective semen processing stages in poor quality ejaculates. Immediately after post-thaw the mean phosphorus concentrations were 9.21±0.11 and 6.08±0.08 mg/dl and after 1 hr incubation post-thaw these were 8.78±0.11 and 5.75±0.08 mg/dl in good and poor quality ejaculates, respectively. The mean magnesium levels were significantly higher ($p<0.01$) in good quality ejaculates at all the four stages of semen evaluation. In case of good quality ejaculates, there was a non-significant decline from fresh diluted (7.21±0.11mg/dl) to 1 hr incubation post-thaw (7.05±0.12 mg/dl), where as in poor quality ejaculates, this decline was significant ($p<0.01$) from 6.25±0.05 fresh diluted to

6.06±0.04 mg/dl 1 hr incubation post-thaw. A significant decline was observed in sodium concentration ($p<0.01$) with processing stages in good as well as poor quality ejaculates. There was a non-significant difference in fresh diluted stage in good and poor quality ejaculates. A significant ($p<0.01$) decline was observed from 357.77±2.39 and 353.14±2.05 mg/dl (fresh diluted) to 310.81±4.06 and 281.62±3.29 mg/dl immediately post-thaw in good and poor quality ejaculates, respectively. Significantly higher ($p<0.01$) potassium concentration was observed in good quality ejaculates. At fresh diluted stage, the mean potassium concentration was 99.84±0.25 in good quality ejaculates and 69.37±0.37 mg/dl in poor quality ejaculates. Immediately post-thaw, it was 96.55±0.20 and 65.47±0.33 mg/dl in good and poor quality ejaculates, respectively. The mean chloride concentration was significantly higher ($p<0.01$) in good quality ejaculates and there was a significant decline in the mean concentration values with advancing semen processing stages. At fresh diluted stage the mean chloride concentration in good quality ejaculate was 369.40±0.81 and in poor quality ejaculate it was 274.42±0.98 mg/dl which declined significantly ($p<0.01$) to 355.52±1.02 and 260.99±0.90 mg/dl at immediately post-thaw stage in good and poor quality ejaculates, respectively.

5.5 Correlation between functional and biochemical parameters

The live sperm percentage was significantly ($P<0.01$) correlated with progressively motile spermatozoa ($r=0.92738$ and 0.99934, $n=192$) in good and poor quality ejaculates. The overall regression coefficient of progressive motility on live sperm percentage was 0.38 ±0.01 and 1.31±0.00 in good and poor quality ejaculates, respectively. Similarly, sperm livability percentage was significantly ($P<0.01$) correlated with HOS responsive spermatozoa ($r=0.98934$ and 0.99934, $n=192$) and the overall regression coefficients of HOST on live sperm (%) were 1.01±0.01 and 1.00±0.00 in good and poor quality semen, respectively. The correlation between live sperm percentage and acrosomal integrity ($r=0.92725$ and 0.98273) was also significant ($P<0.01$) with the regression coefficients of acrosomal integrity on livability calculated to be 0.81±0.02 and 1.07±0.01 in good and poor quality ejaculates, respectively.

A significant ($P<0.01$) negative relationship was observed between livability and AKP ($r=-0.47586$ and -0.59034) and AST ($r=-0.9416$ and -0.8358) leakage in good and

poor quality semen, respectively. The regression coefficients of AKP and AST on livability calculated were -0.23±0.03 and -0.79±0.02 in good quality ejaculates and -1.75±0.17 and -1.63±0.08 in poor quality ejaculates, respectively. Highly negative significant ($P<0.01$) correlation was observed between per cent livability and leakage of ALT (r= -0.7907 and -0.9165) and hyaluronidase (r= -0.78965 and -0.74037) in good and poor quality ejaculates, respectively, with regression coefficients of ALT and hyaluronidase on livability being 3.86±0.22 and -0.38±0.02 in good quality ejaculates and -2.51±0.08 and -0.61±0.04 in poor quality ejaculates, respectively.

Live sperm percentage was significantly ($P<0.01$) correlated with calcium (r=0.21353 and 0.35028) and phosphorus (r=0.43048 and 0.43474) with regression coefficients of calcium being 0.64±0.21 and 5.62±1.09 and of phosphorus 3.39±0.52 and 13.32±2.00 on livability in good and poor quality ejaculates, respectively. Similarly a significant ($P<0.05$) relationship was observed between sperm livability and magnesium concentration (r=0.18175 and 0.14500). The regression coefficient of magnesium on livability was 1.63±0.63 and 9.91±4.90 in good and poor quality ejaculates, respectively. Live sperm percentage was significantly ($P<0.01$) correlated with sodium (r=0.69600 and 0.84324), potassium (r=0.76092 and 0.72068) and chloride (r=0.77688 and 0.70555) in good and poor quality ejaculates, respectively. The regression coefficient of sodium, potassium and chloride on live per cent spermatozoa in good and poor ejaculates were 0.14±0.01 and 0.44±0.20, 2.27±0.14 and 4.81±0.34 and 0.51±0.03 and 1.53±0.11, respectively.

The per cent progressive motility was significantly ($P<0.01$) correlated with HOS reactive (r=0.92052 and 0.94212) and per cent intact acrosome (r=0.94709 and 0.95266) in good and poor quality ejaculates, respectively. The regression coefficients of HOS reactive spermatozoa and spermatozoa with intact acrosome on progressive motility were 2.30±0.07 and 0.68±0.02 and 2.01±0.05 and 0.75±0.02 in good and poor quality ejaculates, respectively.

Similarly, significant ($P<0.01$) negative relationship was observed between progressive motility and AKP (r=-0.45140 and -0.66370) and AST (r=-0.9292 and -0.7337) leakage in good and poor quality ejaculates, respectively. The regression coefficients of AKP and AST on progressive motility were -0.53±0.08 and -

1.91±0.06 in good quality ejaculates and -1.42±0.12 and -1.03±0.07 in poor quality ejaculates, respectively. Highly negative significant (P<0.01) correlation was observed between per cent progressive motility and leakage of ALT (r= -0.7787 and -0.8954) and hyaluronidase (r= -0.76605 and -0.68714) in good and poor quality ejaculates, respectively, with regression coefficients of ALT and hyaluronidase on progressive motility being -9.28±0.54 and -0.90±0.05 in good quality ejaculates and -1.76±0.06 and -0.40±0.03 in poor quality ejaculates, respectively).

Progressive motility was significantly (P<0.01) correlated with calcium (r=0.21883 and 0.33624) and phosphorus (r=0.46707 and 0.51561) with regression coefficients of calcium being 1.60±0.52 and 3.88±0.79 and phosphorus 8.98±1.23 and 11.35±1.37 on progressive motility in good and poor quality ejaculates, respectively. A non-significant relationship was observed between progressive motility and magnesium (r=0.12246) with regression coefficient of 2.68±1.58 in good quality ejaculate, where as in poor quality ejaculate, there was a significant (P<0.01) relationship (r=0.20495) with a regression coefficient of 10.07±3.49. The correlations of progressive motility with sodium (r=0.67512 and 0.89136), potassium (r= 0.73808 and 0.61697) and chloride (r=0.78694 and 0.71250) were significant (P<0.01) in good and poor quality ejaculates. The regression coefficient of sodium (0.34±0.03 and 0.34±0.01), potassium (5.38±0.36 and 2.96±0.27) and chloride (1.30±0.07 and 1.11±0.08) on progressively motile spermatozoa in good and poor quality ejaculates, respectively, were also significant.

The HOS reactive spermatozoa percentage was significantly (P<0.01) correlated with per cent intact acrosome spermatozoa (r=0.91623 and 0.98272, n=192) in good and poor quality ejaculates, respectively. The overall regression coefficient of per cent intact acrosome on percentage HOS reactive spermatozoa percentage was 0.78±0.24 and 1.07±0.01 in good and poor quality ejaculates, respectively.

The correlation of HOS reactive spermatozoa with AKP (r=-0.47729 and-0.58627) and AST (r=-0.9360 and -0.8376), as well as the regression coefficient of AKP (-0.22±0.03 and -1.73±0.17), and AST (-0.77±0.02 and -1.62±0.08) on HOS reactive spermatozoa were significant (P<0.01) in good and poor quality ejaculates, respectively. Highly negative significant (P<0.01) correlation was observed between HOS reactive spermatozoa and leakage of ALT (r= -0.7965 and -0.9177) and hyaluronidase (r= -

0.78737 and -0.74026) in good and poor quality ejaculates, respectively, with regression coefficients of ALT and hyaluronidase on HOS reactive spermatozoa to be -3.81±0.21 and -0.37±0.02 in good quality ejaculates and -2.49±0.08 and -0.60±0.04 in poor ejaculates, respectively.

Mean HOS reactive spermatozoa was significantly (P<0.01) correlated with calcium (r=0.19565 and 0.35496) and phosphorus (r=0.42809 and 0.43346) with regression coefficients of calcium being 0.57±0.21 and 5.66±1.08 and phosphorus 3.30±0.51 and 13.18±1.98 in good and poor quality ejaculates, respectively. A significant (P<0.01) relationship was recorded between HOS reactive spermatozoa and magnesium (r=0.19163) in good quality ejaculate with regression coefficient of magnesium on HOS reactive spermatozoa being 1.68±0.63. Whereas, in poor quality ejaculate, there was a significant (P<0.05) relationship (r=0.14716) of magnesium on HOS reactive spermatozoa percentage with a regression coefficient of 9.98±4.87. Mean HOS reactive spermatozoa was significantly (P<0.01) correlated with sodium (r=0.70064 and 0.84402), potassium (r=0.75416 and 0.72238) and chloride (r=0.77414 and 0.70352) in good and poor quality ejaculates, respectively. The regression coefficient of sodium, potassium and chloride on HOS reactive spermatozoa in good and poor quality ejaculates were 0.14±0.01 and 0.44±0.02, 2.21±0.14 and 4.78±0.33 and 0.50±0.03 and 1.51±0.11, respectively.

A significant (P<0.01) negative relationship was recorded between per cent intact acrosome spermatozoa with AKP (r= -0.51428 and -0.60056) and AST (r= -0.9268 and -0.8076) leakage in good and poor quality ejaculates, respectively. The regression coefficients of AKP and AST on per cent intact acrosome spermatozoa were -0.28±0.03 and -0.90±0.03 in good quality ejaculates and -1.63±0.16 and -1.44±0.08 in poor ejaculates, respectively. Highly significant (P<0.01) negative correlation was observed between intact acrosome spermatozoa with leakage of ALT (r= -0.7724 and -0.9081) and hyaluronidase (r= -0.85093 and -0.76107) in good and poor quality ejaculates, respectively, with regression coefficients of ALT and hyaluronidase on per cent intact acrosome spermatozoa being -4.33±0.26 and -0.47±0.02 in good quality ejaculates and -2.27±0.08 and -0.57±0.04 in poor quality ejaculates, respectively.

Intact acrosome percentage was significantly (P<0.01) correlated with calcium (r=0.18750 and 0.38229) and phosphorus (r=0.47493 and 0.44462). Regression coefficients of calcium and phosphorus on per cent intact acrosome spermatozoa were 0.65±0.25 and 5.61±0.98 and 4.29±0.58 and 12.46±1.82 in good and poor quality ejaculates, respectively. Similarly, a significant (P<0.05) relationship was observed between intact acrosome spermatozoa and magnesium (r=0.15049 and 0.16756) with regression coefficient of magnesium on intact acrosome spermatozoa to be 1.55±0.73 and 10.47±4.47 in good and poor quality ejaculates, respectively. The correlations of per cent intact acrosome spermatozoa with sodium (r=0.62641 and 0.84825), potassium (r= 0.75116 and 0.69577) and chloride (r=0.74740 and 0.72969) as well as regression coefficient of sodium (0.15±0.13 and 0.40±0.02;), potassium (2.58±0.16 and 24.24±0.32) and chloride (0.57±0.04 and 1.45±0.09) on per cent intact acrosome spermatozoa in good and poor ejaculates were significant (P<0.01) in good and poor quality ejaculates, respectively.

Conclusions

1. Significantly higher mass activity and lower pH values were recorded in good quality semen.
2. There was a significantly higher percentage of live, progressively motile, HOS reactive and intact acrosome spermatozoa in good quality semen.
3. Greater loss of plasma membrane integrity and higher leaching of intracellular enzymes is detected in poor quality semen.
4. Lower AST, ALT and Hyaluronidase and higher AKP concentrations were recorded in good quality semen.
5. Relatively lower calcium, phosphorus, magnesium, sodium, potassium and chloride levels were recorded in poor quality semen.
6. There was a strong correlation between functional and biochemical parameters of good as well as poor quality semen of Murrah buffalo bulls.

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Annexure 1: Bull wise initial parameters in good quality semen of Murrah buffalo bulls.

Bull no.	Parameters			
	Volume (ml.)	Sperm concentration($X10^6$)	Mass activity (0-5 Scale)	pH
B-122 (n=8)	3.69 ± 0.16^{a} (3.0-4.5)	1478.25 ± 33.85^{b} (1360-1615)	4.50 ± 0.13^{ab} (4.0- 5.0)	6.89 ± 0.00^{d} (6.88-6.90)
B-123 (n=8)	4.12 ± 0.18^{a} (3.5- 5.0)	1522.38 ± 74.22^{b} (1257-1870)	4.62 ± 0.13^{a} (4.0-5.0)	6.89 ± 0.00^{cd} (6.88-6.91)
B-125 (n=8)	4.00 ± 0.19^{a} (3.5-5.0)	1618.63 ± 34.49^{ab} (1489-1770)	4.09 ± 0.13^{b} (3.5-4.5)	6.90 ± 0.00^{bc} (6.89-6.91)
B-126 (n=8)	3.75 ± 0.14^{a} (3.5-4.5)	1691 ± 56.56^{a} (1467-1947)	4.25 ± 0.21^{ab} (3.5-5.0)	6.86 ± 0.00^{e} (6.84-6.87)
B-128 (n=8)	3.81 ± 0.25^{a} (3.0-5.0)	1751.63 ± 50.23^{a} (1587-1940)	4.19 ± 0.19^{ab} (3.5-5.0)	6.91 ± 0.00^{ab} (6.89-6.93)
B-131 (n=8)	4.12 ± 0.23^{a} (3.0-5.0)	1501.38 ± 34.87^{b} (1387-1660)	4.12 ± 0.26^{ab} (3.0-5.0)	6.91 ± 0.00^{a} (6.90-6.93)
L.S.D.	0.5546	141.67	0.5213	0.0102

Figures within parenthesis indicate range
Figures with different superscripts (a, b) within a column differ significantly ($p<0.01$)

Annexure 2: Bull wise live sperms (%) in good quality semen of Murrah buffalo bulls during different stages of processing.

Bull no.	Stage of semen processing			
	Fresh Diluted	Equilibration	0 hr Post-thaw	1 hr Post-thaw
B-122 (n=8)	90.12 ± 0.44^{ab} (88-92)	84.87 ± 0.61^{b} (82-87)	76.75 ± 0.67^{b} (74-80)	72.75 ± 0.67^{b} (70-76)
B-123 (n=8)	91.75 ± 0.59^{a} (89-94)	86.87 ± 0.44^{a} (85-89)	79.87 ± 0.64^{a} (78-83)	75.50 ± 0.87^{a} (73-79)
B-125 (n=8)	91.12 ± 0.52^{ab} (89-93)	87.12 ± 0.40^{a} (86-89)	79.12 ± 0.40^{a} (77-81)	75.37 ± 0.73^{a} (71-77)
B-126 (n=8)	91.62 ± 0.50^{a} (90-94)	86.75 ± 0.53^{a} (85-90)	79.37 ± 0.65^{a} (77-83)	75.25 ± 0.80^{a} (73-80)
B-128 (n=8)	91.12 ± 0.52^{ab} (89-93)	87.12 ± 0.40^{a} (86-89)	78.50 ± 0.42^{a} (77-80)	73.50 ± 0.63^{ab} (71-76)
B-131 (n=8)	89.5 ± 0.89^{b} (86-93)	85.87 ± 0.91^{ab} (82-89)	76.12 ± 0.74^{b} (73-80)	71.62 ± 0.71^{b} (69-75)
L.S.D	1.6769	1.6468	1.7197	2.1048

Figures within parenthesis indicate range
Figures with different superscripts (a, b) within a column differ significantly ($p<0.01$)

Annexure 3: Bull wise progressive motile spermatozoa (%) in good quality semen of Murrah buffalo bulls during different stages of processing.

Bull no.	Stage of semen processing			
	Fresh Diluted	Equilibration	0 hr Post-thaw	1 hr Post-thaw
B-122 (n=8)	83.25±0.84^{b} (79-86)	71.87±0.72^{bc} (69-75)	63.88±0.74^{b} (60-67)	36.88±0.95^{d} (32-40)
B-123 (n=8)	87.50±0.65^{a} (86-90)	75.00±1.18^{a} (71-80)	66.00±0.57^{a} (64-68)	44.38±1.60^{a} (34-49)
B-125 (n=8)	86.62±0.65^{a} (84-90)	74.87±0.77^{a} (70-77)	63.75±0.45^{b} (62-66)	39.13±0.79^{cd} (37-43)
B-126 (n=8)	87.37±0.32^{a} (86-89)	73.62±0.68^{ab} (72-78)	63.88±0.30^{b} (63-65)	42.25±0.41^{ab} (40-44)
B-128 (n=8)	86.50±0.78^{a} (84-90)	72.75±0.84^{abc} (68-75)	62.25±0.41^{c} (61-64)	41.50±0.42^{bc} (40-43)
B-131 (n=8)	85.62±0.84^{a} (82-89)	70.87±0.58^{c} (69-73)	61.25±0.25^{c} (60-62)	38.63±0.68^{d} (35-41)
L.S.D	2.0128	2.3303	1.3773	2.582

Figures within parenthesis indicate range

Figures with different superscripts (a, b) within a column differ significantly ($p<0.01$)

Annexure 4: Bull wise HOS reactive spermatozoa (%) in good quality semen of Murrah buffalo bulls during different stages of processing.

Bull no.	Stage of semen processing			
	Fresh Diluted	Equilibration	0 hr Post-thaw	1 hr Post-thaw
B-122 (n=8)	87.87±0.74^{b} (84-90)	83.62±0.61^{b} (81-86)	75.25±0.59^{b} (73-78)	71.12±0.85^{b} (69-74)
B-123 (n=8)	89.87±0.55^{a} (88-92)	85.25±0.31^{a} (84-86)	78.62±0.63^{a} (76-81)	74.37±0.89^{a} (71-78)
B-125 (n=8)	89.87±0.61^{a} (87-92)	86±0.38^{a} (85-88)	77.75±0.62^{a} (76-80)	74.62±0.78^{a} (71-77)
B-126 (n=8)	89.62±0.50^{ab} (88-92)	85.87±0.55^{a} (84-89)	78.25±0.65^{a} (76-82)	74.12±0.91^{a} (70-79)
B-128 (n=8)	89.37±0.53^{ab} (87-92)	85.87±0.30^{a} (85-87)	77.12±0.40^{a} (75-78)	72.62±0.68^{ab} (70-76)
B-131 (n=8)	88.50±0.76^{ab} (85-91)	85.0±0.78^{ab} (82-88)	75.25±0.73^{b} (72-79)	70.50±0.73^{b} (68-74)
L.S.D.	1.7778	1.4699	1.739	2.3166

Figures within parenthesis indicate range

Figures with different superscripts (a, b) within a column differ significantly ($p<0.01$)

Annexure 5: Bull wise spermatozoa with intact acrosome (%) in good quality semen of Murrah buffalo bulls during different stages of processing.

Bull no.	Stage of semen processing			
	Fresh Diluted	Equilibration	0 hr Post-thaw	1 hr Post-thaw
B-122 (n=8)	91.75±0.45^{ab} (89-93)	86.12±0.40^{b} (85-88)	78.62±0.46^{b} (76-80)	69.75±0.53^{cd} (67-71)
B-123 (n=8)	91.00±0.65^{bc} (88-93)	86.37±0.56^{b} (84-89)	79.00±0.68^{ab} (77-82)	71.62±0.94^{b} (68-76)
B-125 (n=8)	93.12±0.40^{a} (92-95)	88.00±0.46^{a} (86-90)	80.50±0.76^{a} (76-83)	73.62±0.56^{a} (71-76)
B-126 (n=8)	92.25±0.92^{ab} (88-95)	86.75±0.88^{ab} (83-90)	80.50±0.60^{a} (78-83)	70.62±0.32^{bc} (69-72)
B-128 (n=8)	89.87±0.52^{cd} (88-92)	84.00±0.27^{c} (83-85)	79.12±0.40^{ab} (78-81)	70.62±0.32^{bc} (69-72)
B-131 (n=8)	89.25±0.45^{d} (87-91)	81.62±0.38^{d} (80-83)	74.87±0.30^{c} (74-76)	68.50±0.38^{d} (67-70)
L.S.D	1.6931	1.5106	1.585	1.5802

Figures within parenthesis indicate range
Figures with different superscripts (a, b) within a column differ significantly ($p<0.01$)

Annexure 6: Bull wise Alkaline Phosphatase concentration (KAU/dl) in good quality semen of Murrah buffalo bulls during different stages of processing.

Bull no.	Stage of semen processing			
	Fresh Diluted	Equilibration	0 hr Post-thaw	1 hr Post-thaw
B-122 (n=8)	155.53±0.21^{c} (154.34-156.11)	162.14±0.20^{b} (161.22-162.89)	164.76±0.19^{b} (163.98-165.56)	175.03±0.16^{a} (174.33-175.67)
B-123 (n=8)	138.45±0.22^{d} (137.45-139.00)	142.33±0.13^{c} (141.89-142.89)	145.40±0.24^{c} (144.33-146.56)	147.29±0.24^{c} (146.33-148.55)
B-125 (n=8)	136.41±0.27^{e} (135.34-137.56)	140.79±0.30^{d} (139.89-142.33)	145.18±0.31^{cd} (144.00-146.45)	149.06±0.45^{bc} (147.00-150.78)
B-126 (n=8)	135.68±0.16^{e} (134.89-136.33)	139.60±0.34^{e} (138.56-141.11)	143.47±1.47^{d} (144.00-146.11)	161.99±12.42^{ab} (148.56-151.22)
B-128 (n=8)	157.28±0.45^{b}	162.33±0.23^{b}	165.84±0.29^{b}	170.82±0.32^{a}

	(155.56-159.00)	(161.22-163.11)	(164.78-167.56)	(169.56-172.00)
B-131 (n=8)	163.11 ± 0.34^{a} (161.89-164.33)	165.59 ± 0.33^{a} (164.23-167.22)	170.99 ± 0.48^{a} (169.00-172.89)	175.62 ± 0.21^{a} (174.78-176.33)
L.S.D.	0.8329	0.7611	1.8986	14.489

Figures within parenthesis indicate range
Figures with different superscripts (a, b) within a column differ significantly ($p<0.01$)

Annexure 7: Bull wise AST concentration (U/L) in good quality semen of Murrah buffalo bulls during different stages of processing.

Bull no.	Stage of semen processing			
	Fresh Diluted	Equilibration	0 hr Post-thaw	1 hr Post-thaw
B-122 (n=8)	86.59±0.12[a] (86.06-87.04)	93.86±0.69[ab] (91.34-96.45)	102.02±0.46[c] (101.22-104.45)	108.21±0.37[a] (106.84-109.32)
B-123 (n=8)	87.31±0.21[a] (86.45-88.00)	93.03±0.50[ab] (91.03-94.58)	102.66±0.44[bc] (101.72-104.32)	107.95±0.34[a] (106.47-109.54)
B-125 (n=8)	87.72±0.70[a] (83.45-89.54)	94.55±0.44[a] (92.39-96.34)	102.50±0.34[bc] (101.40-103.34)	107.78±0.43[a] (105.45-109.36)
B-126 (n=8)	87.57±0.17[a] (87.02-88.19)	92.94±0.66[ab] (89.46-95.45)	102.78±0.31[bc] (101.89-104.45)	107.68±0.62[a] (105.00-109.37)
B-128 (n=8)	86.67±0.69[a] (84.38-89.39)	92.19±0.78[b] (91.34-94.87)	105.02±0.37[a] (103.82-106.83)	107.86±0.46[a] (105.52-109.60)
B-131 (n=8)	87.52±0.44[a] (85.48-89.76)	93.49±0.78[ab] (91.37-95.35)	103.50±0.40[b] (102.23-105.28)	108.31±0.53[a] (106.28-109.96)
L.S.D.	1.3023	1.6997	1.1166	1.3339

Figures within parenthesis indicate range

Figures with different superscripts (a, b) within a column differ significantly ($p<0.01$)

Annexure 8: Bull wise ALT concentration (U/L) in good quality semen of Murrah buffalo bulls during different stages of processing.

Bull no.	Stage of semen processing			
	Fresh Diluted	Equilibration	0 hr Post-thaw	1 hr Post-thaw
B-122 (n=8)	15.43±0.21[a] (15.37-16.43)	16.27±0.22 [a] (15.34-17.34)	17.33±0.32[a] (16.28-18.94)	18.09±0.28[ab] (17.23-19.29)
B-123 (n=8)	15.35±0.19[a] (14.78-15.98)	15.93±0.18[ab] (15.34-16.82)	17.50±0.30[a] (16.28-18.93)	18.28±0.23[a] (17.20-19
B-125 (n=8)	14.44±0.17[b] (13.84-15.12)	15.07±0.16[c] (14.20-15.72)	16.39±0.29[b] (15.22-17.45)	17.41±0.21[bc] (16.45-18.49)
B-126 (n=8)	14.56±0.17[b] (13.87-15.29)	15.57±0.16[bc] (14.34-16.05)	17.27±0.29[a] (16.22-18.85)	17.89±0.30[abc] (16.98-18.93)
B-128 (n=8)	13.43±0.11[c] (12.92-13.76)	15.13±0.30[c] (13.98-16.43)	16.16±0.31[b] (15.28-17.94)	17.28±0.29[c] (16.20-18.27)
B-131 (n=8)	15.30±0.18[a] (14.73-15.91)	15.27±0.27[ab] (14.98-16.90)	16.88±0.33[ab] (15.24-17.92)	17.45±0.24[bc] (16.16-18.35)
L.S.D.	0.4985	0.6356	0.8779	0.7435

Figures within parenthesis indicate range

Figures with different superscripts (a, b) within a column differ significantly ($p<0.01$)

Annexure 9: Bull wise Hyaluronidase concentration (U/ml) in good quality semen of Murrah buffalo bulls during different stages of processing.

Bull no.	Stage of semen processing			
	Fresh Diluted	Equilibration	0 hr Post-thaw	1 hr Post-thaw
B-122 (n=8)	213.00±0.71[d] (210-216)	218.87±0.91[c] (216-224)	229.62±0.68[c] (226-232)	238.50±0.71[c] (235-241)
B-123 (n=8)	219.37±0.73[c] (217-223)	227.12±1.08[b] (223-231)	242.75±1.03[a] (239-248)	250.87±1.26[a] (247-259)
B-125 (n=8)	200.50±0.76[e] (197-203)	208.37±1.13[d] (204-212)	226.50±1.49[c] (221-232)	235.37±1.45[c] (230-241)
B-126 (n=8)	213.87±0.72[d] (211-218)	220.12±1.06[c] (215-225)	234.75±1.31[b] 230-241)	242.25±1.13[b] (238-248)
B-128 (n=8)	228.12±1.7[a] (223-233)	232.37±1.07[a] (228-237)	246.00±1.28[a] (240-250)	253.62±1.4[a] (249-259)
B-131 (n=8)	224.37±1.48[b] (219-230)	230.00±1.34[ab] (225-235)	245.75±1.33[a] (240-251)	253.25±1.18[a] (248-258)
L.S.D	2.7743	3.1541	3.4689	3.4593

Figures within parenthesis indicate range

Figures with different superscripts (a, b) within a column differ significantly ($p<0.01$)

Annexure 10: Bull wise Calcium concentrations (mg/dl) in good quality semen of Murrah buffalo bulls during different stages of processing.

Bull no.	Stage of semen processing			
	Fresh Diluted	Equilibration	0 hr Post-thaw	1 hr Post-thaw
B-122 (n=8)	46.35±0.03[b] (46.24-46.45)	46.11±0.02[b] (46.00-46.21)	45.80±0.02[b] (45.72-45.86)	45.59±0.01[b] (45.53-45.64)
B-123 (n=8)	47.40±0.02[a] (47.36-47.50)	47.23±0.01[a] (47.17-47.27)	46.87±0.02[a] (46.81-46.98)	46.74±0.01[a] (46.70-46.81)
B-125 (n=8)	42.29±0.01[e] (41.37-42.48)	41.95±0.06[e] (41.55-42.10)	40.38±0.02[e] (40.32-40.43)	40.12±0.01[e] (40.06-40.16)
B-126 (n=8)	47.40±0.03[a] (47.31-47.49	47.23±0.01[a] (47.16-47.29)	46.87±0.02[a] (46.79-46.96)	46.74±0.01[a] (46.71-46.80)
B-128 (n=8)	43.32±0.03[d] (43.23-43.41)	43.11±0.02[d] (43.01-43.21)	42.22±0.03[d] (42.08-42.35)	41.99±0.02[d] (41.87-42.05)
B-131	44.32±0.03[c]	44.11±0.02[c]	43.24±0.03[c]	43.06±0.03[c]

(n=8)	(44.26-44.43)	(44.03-44.19)	(43.13-43.33)	(42.89-43.13)
L.S.D.	0.1661	0.0841	0.065	0.0518

Figures within parenthesis indicate range
Figures with different superscripts (a, b) within a column differ significantly (p<0.01)

Annexure 11: Bull wise Phosphorus concentration (mg/dl) in good quality semen of Murrah buffalo bulls during different stages of processing.

Bull no.	Stage of semen processing			
	Fresh Diluted	**Equilibration**	**0 hr Post-thaw**	**1 hr Post-thaw**
B-122 (n=8)	10.23 ± 0.18^{b} (9.43-11.02)	10.07 ± 0.19^{ab} (9.12-10.98)	9.40 ± 0.18^{b} (9.12-9.98)	8.92 ± 0.13^{b} (8.20-9.34)
B-123 (n=8)	9.05 ± 0.13^{d} (8.40-9.56)	8.79 ± 0.12^{e} (8.10-9.11)	8.24 ± 0.18^{d} (7.28-8.89)	7.76 ± 0.14^{d} (7.00-8.17)
B-125 (n=8)	9.46 ± 0.20^{cd} (8.56-10.11)	9.15 ± 0.22^{de} (8.23-9.78)	8.71 ± 0.21^{c} (8.01-9.34)	8.28 ± 0.22^{c} (7.45-9.02)
B-126 (n=8)	11.07 ± 0.09^{a} (10.67-11.43)	10.51 ± 0.15^{a} (10.01-11.02)	10.20 ± 0.15^{a} (9.76-10.85)	9.91 ± 0.15^{a} (9.34-10.54)
B-128 (n=8)	9.89 ± 0.15^{bc} (9.34-10.54)	9.53 ± 0.13^{cd} (9.19-10.15)	9.23 ± 0.11^{b} (9.00-9.89)	8.81 ± 0.07^{b} (8.45-9.06)
B-131 (n=8)	10.17 ± 0.18^{b} (9.43-10.45)	9.98 ± 0.19^{bc} (9.21-10.89)	9.45 ± 0.14^{b} (8.93-9.89)	9.01 ± 0.11^{b} (8.45-9.45)
L.S.D.	0.4499	0.4844	0.4651	0.4139

Figures within parenthesis indicate range
Figures with different superscripts (a, b) within a column differ significantly (p<0.01)

Annexure 12: Bull wise Magnesium concentrations (mg/dl) in good quality semen of Murrah buffalo bulls during different stages of processing.

Bull no.	Stage of semen processing			
	Fresh Diluted	**Equilibration**	**0 hr Post-thaw**	**1 hr Post-thaw**
B-122 (n=8)	6.14 ± 0.01^{f} (6.09-6.19)	6.06 ± 0.01^{f} (6.00-6.10)	5.98 ± 0.01^{f} (5.93-6.01)	5.91 ± 0.01^{f} (5.89-5.93)
B-123 (n=8)	6.91 ± 0.02^{d} (6.80-6.99)	6.87 ± 0.02^{d} (6.78-6.93)	6.84 ± 0.02^{d} (6.75-6.89)	6.79 ± 0.01^{d} (6.69-6.81)

B-125 (n=8)	7.36±0.01^{c} (7.31-7.40)	7.31±0.01^{c} (7.27-7.33)	7.27±0.01^{c} (7.24-7.28)	7.23±0.01^{c} (7.21-7.26)
B-126 (n=8)	8.19±0.01^{a} (8.16-8.21)	8.16±0.01^{a} (8.13-8.18)	8.13±0.01^{a} (8.11-8.15)	8.10±0.00^{a} (8.08-8.12)
B-128 (n=8)	8.04±0.02^{b} (7.98-8.11)	7.97±0.01^{b} (7.93-8.04)	7.94±0.01^{b} (7.90-8.00)	7.91±0.01^{b} (7.88-7.93)
B-131 (n=8)	6.64±0.01^{e} (6.59-6.69)	6.41±0.13^{e} (6.50-6.59)	6.40±0.01^{e} (6.36-6.43)	6.34±0.01^{e} (6.31-6.39)
L.S.D.	0.0389	0.1502	0.0305	0.0242

Figures within parenthesis indicate range

Figures with different superscripts (a, b) within a column differ significantly ($p<0.01$)

Annexure 13: Bull wise Sodium concentration (mg/dl) in good quality semen of Murrah buffalo bulls during different stages of processing.

Bull no.	Stage of semen processing			
	Fresh Diluted	**Equilibration**	**0 hr Post-thaw**	**1 hr Post-thaw**
B-122 (n=8)	345.62 ± 1.80^{d} (341.92-355.04)	305.83 ± 1.23^{e} (301.29-312.92)	254.27 ± 1.76^{d} (246.84-260.39)	227.05 ± 1.91^{f} (220.92-228.92)
B-123 (n=8)	377.55 ± 1.83^{a} (369.39-385.49)	335.14 ± 1.44^{c} (328.29-340.39)	316.70 ± 1.34^{b} (309.39-320.10)	297.20 ± 1.54^{d} (290.92-302.29)
B-125 (n=8)	334.88 ± 1.44^{e} (329.20-341.20)	313.70 ± 1.04^{d} (310.20-318.29)	303.80 ± 1.05^{c} (300.20-308.39)	293.64 ± 0.89^{e} (290.20-297.90)
B-126 (n=8)	358.86 ± 1.41^{b} (352.29-363.83)	340.26 ± 1.12^{b} (338.26-345.90)	335.00 ± 1.40^{a} (328.29-340.39)	333.40 ± 0.92^{a} (329.09-338.39)
B-128 (n=8)	378.19 ± 1.25^{a} (371.29-381.92)	344.47 ± 1.01^{a} (340.39-348.93)	336.58 ± 0.82^{a} (332.29-339.39)	317.82 ± 0.85^{b} (312.92-320.29)
B-131 (n=8)	351.49 ± 0.82^{c} (348.40-355.09)	343.85 ± 0.97^{a} (340.38-348.38)	318.54 ± 0.90^{b} (314.62-321.29)	302.73 ± 0.65^{c} (299.87-305.36)
L.S.D.	4.1804	3.2714	3.5839	3.4618

Figures within parenthesis indicate range

Figures with different superscripts (a, b) within a column differ significantly ($p<0.01$)

Annexure 14: Bull wise Potassium concentration (mg/dl) in good quality semen of Murrah buffalo bulls during different stages of processing.

Bull no.	Stage of semen processing			
	Fresh Diluted	**Equilibration**	**0 hr Post-thaw**	**1 hr Post-thaw**
B-122 (n=8)	98.74 ± 0.11^{d} (98.39-99.11)	97.83 ± 0.25^{bc} (97.19-99.01)	95.54 ± 0.14^{d} (95.03-96.02)	94.50 ± 0.13^{b} (94.20-94.89)
B-123 (n=8)	99.56 ± 0.28^{c} (98.29-100.83)	98.52 ± 0.23^{b} (97.39-99.38)	96.52 ± 0.23^{c} (95.39-97.49)	94.70 ± 0.33^{b} (93.20-95.84)
B-125 (n=8)	102.34 ± 0.27^{a} (101.54-103.93)	100.30 ± 0.22^{a} (99.38-101.28)	98.41 ± 0.17^{a} (97.56-98.82)	96.90 ± 0.20^{a} (95.89-97.59)
B-126 (n=8)	97.79 ± 0.29^{e} (96.75-99.34)	96.44 ± 0.31^{d} (95.02-97.62)	94.72 ± 0.20^{e} (93.98-95.39)	93.40 ± 0.26^{c} (92.01-94.20)
B-128 (n=8)	101.59 ± 0.23^{b} (100.64-102.83)	99.60 ± 0.22^{a} (98.74-100.01)	97.79 ± 0.23^{b} (97.20-98.99)	96.50 ± 0.18^{a} (95.90-97.29)
B-131 (n=8)	98.99 ± 0.27^{cd} (97.94-100.20)	97.31 ± 0.29^{c} (96.20-98.67)	96.34 ± 0.23^{c} (94.98-96.87)	94.88 ± 0.33^{b} (93.29-95.93)
L.S.D.	0.7095	0.7303	0.5722	0.7203

Figures within parenthesis indicate range

Figures with different superscripts (a, b) within a column differ significantly ($p<0.01$)

Annexure 15: Bull wise Chloride concentrations (mg/dl) in good quality semen of Murrah buffalo bulls during different stages of processing.

Bull no.	Stage of semen processing			
	Fresh Diluted	**Equilibration**	**0 hr Post-thaw**	**1 hr Post-thaw**
B-122 (n=8)	361.23 ± 1.07^{c} (355.75-364.44)	353.36 ± 1.05^{d} (349.78-357.57)	344.58 ± 1.62^{d} (339.49-350.94)	338.38 ± 1.14^{d} (332.76-342.76)
B-123 (n=8)	371.09 ± 1.88^{ab} (364.45-381.90)	361.11 ± 1.01^{bc} (356.56-364.76)	355.54 ± 0.96^{bc} (352.44-358.45)	343.90 ± 1.49^{c} (340.33-350.95)
B-125 (n=8)	369.42 ± 1.14^{b} (364.90-373.99)	359.70 ± 1.22^{c} (353.67-364.44)	354.17 ± 1.06^{c} (347.78-356.93)	343.21 ± 1.59^{c} (341.43-348.94)
B-126 (n=8)	374.09 ± 1.75^{a} (368.23-383.6)	370.81 ± 1.88^{a} (363.54-381.09)	363.86 ± 2.07^{a} (356.65-374.26)	354.92 ± 0.94^{a} (351.44-358.45)
B-128 (n=8)	369.78 ± 1.15^{b} (365.95-374.94)	362.91 ± 1.67^{bc} (353.45-369.40)	356.01 ± 1.25^{bc} (347.76-359.47)	347.80 ± 1.70^{b} (340.43-352.84)
B-131 (n=8)	370.74 ± 1.58^{ab} (363.40-377.25)	364.07 ± 1.67^{b} (355.89-371.40)	358.97 ± 1.47^{b} (351.47-364.43)	352.87 ± 0.95^{a} (348.98-357.76)
L.S.D.	4.1739	4.1605	4.1524	3.8175

Figures within parenthesis indicate range

Figures with different superscripts (a, b) within a column differ significantly ($p<0.01$)

Annexure 16: Inter-relationship between some biochemicals in seminal plasma of good and poor quality Murrah buffalo bull semen (n=192).

Sr. No.	Relationship between Parameters (n=192)		Quality	Correlation coefficient	Regression Estimate	Regression Equation
1.	AKP	AST	Good	0.3892**	0.68±0.12	y=87.99+0.68x
			Poor	0.4599**	0.30±0.04	y=79.57+0.30x
2.		ALT	Good	0.2710**	2.76±0.71	y=109.97+2.76x
			Poor	0.6574**	0.61±0.05	y=97.83+0.61x
3.		Hyaluronidase	Good	0.53833**	0.54±0.06	y= 30.19+0.54x
			Poor	0.28671**	0.08±0.02	y= 81.85+0.08x
4.		Calcium	Good	-0.21771**	-1.36±0.44	y= 215.41-1.36x
			Poor	0.21656**	1.17±0.38	y= 71.11+1.17x
5.		Phosphorus	Good	-0.04153NS	-0.68±1.19	y= 161.19-0.68x
			Poor	-0.54572**	-5.64±0.63	y= 154.62-5.64x
6.		Magnesium	Good	-0.34124**	-6.37±1.27	y= 200.15-6.37x
			Poor	0.18184*	4.19±1.64	y= 93.81+4.19x
7.		Sodium	Good	-0.33702**	-0.14±0.03	y= 201.25-0.14x
			Poor	-0.74178**	-0.13±0.01	y= 159.11-0.13x
8.		Potassium	Good	-0.32946**	-2.05±0.43	y= 354.51-2.05x
			Poor	-0.31704**	-0.71±0.15	y= 166.95-0.71x
9.		Chloride	Good	-0.39387**	-0.54±0.09	y= 348.55-0.54x
			Poor	-0.43714**	-0.32±0.05	y= 203.98-0.32x
10.	AST	ALT	Good	0.7820**	1.10±0.06	y=93.19+1.10x
			Poor	0.7829**	4.53±0.26	y=24.14+4.53x
11.		Hyaluronidase	Good	0.7860**	0.45±0.03	y=5.89+0.45x
			Poor	0.7931**	0.34±0.02	y=26.46+0.34x
12.		Calcium	Good	-0.2206**	-0.79±0.25	y=132.95-0.79
			Poor	-0.3188**	-2.63±0.57	y=241.48-2.63x
13.		Phosphorus	Good	-0.5047**	-4.72±0.59	y=142.33-4.72x
			Poor	-0.1874**	-2.95±1.12	y=151.05-2.95
14.		Magnesium	Good	-0.0765*	-0.81±0.77	y=103.71-0.81x
			Poor	0.1112 NS	3.90±2.53	y=108.66+3.90x
15.		Sodium	Good	-0.6676**	-0.16±0.01	y=150.42-0.16x
			Poor	-0.6171**	-0.17±0.02	y=182.89-0.17x
16.		Potassium	Good	-0.7445**	-2.64±0.17	y=355.42-2.64x
			Poor	-0.8483**	-2.90±0.13	y=326.17-2.90x
17.		Chloride	Good	-0.6676**	-0.59±0.04	y=310.22-0.59x
			Poor	-0.6448**	-0.72±0.06	y=322.67-0.72x
18.	ALT	Hyaluronidase	Good	0.6334**	0.06±0.01	y=1.84+0.06x
			Poor	0.7153**	0.21±0.02	y=66.16+0.21x
19.		Calcium	Good	0.0881**	0.06±0.04	y=13.84+0.06x
			Poor	-0.3672**	-2.15±0.40	y=124.89-2.15x

20.		Phosphorus	Good	-0.4285^{**}	-0.69±0.11	y=22.76-0.69x
			Poor	-0.4457^{**}	-4.99±0.73	y=66.88-4.99x
21.		Magnesium	Good	-0.2752^{**}	-0.51±0.13	y=19.85-0.51x
			Poor	-0.0434^{*}	-1.08±1.81	y=42.48-1.08x

cont.

Annexure 16 cont.

22.	ALT	Sodium	Good	-0.6282^{**}	-0.03±0.00	y=24.77-0.03x
			Poor	-0.9005^{**}	-0.17±0.01	y=87.89-0.17x
23.		Potassium	Good	-0.7531^{**}	-0.46±0.03	y=61.15-0.46x
			Poor	-0.7347^{**}	-1.79±0.12	y=154.90-1.79x
24.		Chloride	Good	-0.6901^{**}	-0.09±0.01	y=49.64-0.09x
			Poor	-0.6052^{**}	-0.48±0.05	y=162.54-0.48x
25.	Hyaluronidase	Calcium	Good	-0.09635^{NS}	-0.60±0.45	y= 257.38-0.60x
			Poor	-0.58519^{**}	-11.49±1.15	y= 951.81-11.49x
26.		Phosphorus	Good	-0.42329^{**}	-6.92±1.07	y= 295.72-6.92x
			Poor	-0.13333^{NS}	-4.99±2.69	y= 508.06-4.99x
27.		Magnesium	Good	-0.01903^{NS}	-0.35±1.35	y= 233.16-0.35x
			Poor	0.04773^{NS}	3.99±6.05	y= 452.49+3.99x
28.		Sodium	Good	-0.31741^{**}	-0.13±0.03	y= 274.26-0.13x
			Poor	-0.52713^{**}	-0.34±0.04	y= 578.94-0.34x
29.		Potassium	Good	-0.65762^{**}	-4.08±0.34	y= 627.93-4.08x
			Poor	-0.83672^{**}	-7.82±0.32	y= 930.65-7.82x
30.		Chloride	Good	-0.52843^{**}	-0.72±0.08	y= 489.71-0.72x
			Poor	-0.60619^{**}	-1.61±0.15	y= 901.58-1.61x
31.	Calcium	Phosphorus	Good	0.26895^{**}	0.71±0.18	y= 37.96+0.71x
			Poor	-0.13139^{NS}	-0.25±0.14	y= 42.89-0.25x
32.		Magnesium	Good	-0.10061^{NS}	-0.30±0.22	y= 46.74-0.30x
			Poor	0.40534^{**}	1.72±0.28	y= 30.73+1.72x
33.		Sodium	Good	0.13527^{NS}	0.01±0.00	y= 41.61+0.01x
			Poor	0.29898^{**}	0.01±0.00	y= 38.39+0.01x
34.		Potassium	Good	-0.31222^{**}	-0.31±0.07	y= 74.91-0.31x
			Poor	0.44801^{**}	0.19±0.03	y= 28.96+0.19x
35.		Chloride	Good	0.20194^{**}	0.04±0.02	y= 28.69+0.04x
			Poor	0.28382^{**}	0.04±0.01	y= 31.21+0.04x
36.	Chloride	Phosphorus	Good	0.56207^{**}	6.72±0.72	y= 295.26+6.72x
			Poor	0.20567^{**}	2.91±1.00	y= 246.27+2.91x
37.		Magnesium	Good	0.38610^{**}	5.25±0.91	y= 321.03+5.25x
			Poor	0.33928^{**}	10.70±2.15	y=198.61+10.70x
38.		Sodium	Good	0.80883^{**}	0.25±0.01	y= 277.28+0.25x

Sr. No.	Relationship between Parameters		Quality	Correlation coefficient	Regression Estimate	Regression Equation
			Poor	0.50820**	0.12±0.02	y= 227.26+0.12x
39.		Potassium	Good	0.54668**	2.48±0.28	y= 116.97+2.48x
			Poor	0.47091**	1.45±0.20	y= 167.99+1.45x
40.	Magnesium	Phosphorus	Good	0.21315**	0.19±0.06	y= 5.36+0.19x
			Poor	0.16607*	0.07±0.03	y= 5.68+0.07x
41.		Sodium	Good	0.52155**	0.01±0.00	y= 3.27+0.01x
			Poor	0.14825*	0.001±0.00	y= 5.80+0.001x
42.		Potassium	Good	0.14404*	0.05±0.02	y= 2.44+0.05x
			Poor	-0.26058**	-0.03±0.01	y= 7.83-0.03x
43.	Phosphorus	Sodium	Good	0.41278**	0.01±0.00	y= 5.94+0.01x
			Poor	0.55401**	0.01±0.00	y= 3.36+0.01x
44.		Potassium	Good	0.11969NS	0.05±0.03	y= 4.98+0.05x
			Poor	0.05545NS	0.01±0.02	y= 5.42+0.01x
45.	Potassium	Sodium	Good	0.51659**	0.04±0.00	y= 86.02+0.04x
			Poor	0.53594**	0.04±0.00	y= 53.79+0.04x

* (P<0.05) ** (P<0.01) NS (not significant)

Annexure 17: Inter-relationship between some evaluation parameters and biochemicals in seminal plasma of freshly diluted good and poor quality semen of Murrah buffalo bulls.

Sr. No.	Relationship between Parameters (n=48)		Quality	Correlation coefficient	Regression Estimate	Regression Equation
1	Livability	Motility	Good	0.90315**	0.58±0.04	y=40.76+0.58x
			Poor	0.63213**	0.76±0.14	y= 30.42+0.76x
2		HOST	Good	0.93348**	0.94±0.05	y=6.66 +0.94 x
			Poor	0.99209**	0.97±0.018	y= 3.00+0.97x
3		Acrosome	Good	0.57791**	0.54±0.11	y=41.16 +0.54x
			Poor	0.90992**	0.94±0.06	y= 2.81+0.94x
4		AKP	Good	-0.40789**	-0.90±0.29	y= 103.98-0.90x
			Poor	0.52727**	0.65±0.15	y= -9.22+0.65x
5		AST	Good	0.1797**	0.22±0.20	y= 69.52+0.22x
			Poor	-0.3188**	-0.39±0.17	y=112.33-0.39x
6		ALT	Good	-0.2220**	-0.46±0.30	y=97.66-0.46x
			Poor	-0.3453 NS	-0.67±0.27	y=81.56-0.67x
7		Hyaluronidase	Good	-0.26718NS	-0.07±0.04	y= 105.99-0.07x
			Poor	-0.28640*	-0.12±0.06	y= 120.10-0.12x
8		Calcium	Good	0.06336 NS	0.81±0.19	y=86.97+0.81x
			Poor	0.28280NS	1.43±0.72	y= 4.41+1.43x
9		Phosphorus	Good	-0.18840NS	-0.63±0.48	y=96.89-0.63x
			Poor	-0.1501NS	-1.42±1.37	y=73.90-1.42x
10		Magnesium	Good	0.37293**	1.26±0.46	y=81.59+1.26x
			Poor	-0.0123NS	-0.23±2.79	y=65.87-0.23x

11		Sodium	Good	0.60514**	0.07±0.01	y=66.64+0.07x
			Poor	0.08275^{NS}	0.04±0.07	y= 51.40+0.04x
12		Potassium	Good	0.33170*	0.46±0.19	y=45.16+0.46x
			Poor	0.23001^{NS}	0.56±0.35	y=25.28+0.56x
13		Chloride	Good	0.55824**	0.21±0.05	y= 12.70+0.21x
			Poor	-0.1773^{NS}	-0.16±0.13	y=109.64-0.16x
14	Motility	HOST	Good	0.89201**	1.02±0.12	y=-4.39+1.02x
			Poor	0.61303**	0.50±0.09	y=13.10+0.50x
15		Acrosome	Good	0.50559**	0.74±0.18	y=18.53+0.74x
			Poor	0.59814**	0.51±0.10	y=10.99+0.51x
16		AKP	Good	-0.39367**	-0.14±0.05	y=105.73-0.14x
			Poor	0.21094^{NS}	0.21±0.15	y=20.12+0.21x
17		AST	Good	0.3127**	0.57±0.26	y=36.22+0.57x
			Poor	-0.3211**	0.32±0.14	y=84.39-0.33x
18		ALT	Good	-0.2783**	-0.78±0.39	y=97.59-0.77x
			Poor	-0.0028^{NS}	-0.00±0.24	y=44.59-0.00x
19		Hyaluronidase	Good	-0.18888^{NS}	-0.08±0.06	y=102.57-0.08x
			Poor	-0.0661^{NS}	-0.02±0.05	y=55.10-0.02x
20		Calcium	Good	-0.01430^{NS}	-0.02±0.29	y=86.97-0.02x
			Poor	0.01769^{NS}	0.07±0.62	y=41.38+0.07x
21		Phosphorus	Good	-0.11663^{NS}	-0.60±0.75	y=91.73-0.60x
			Poor	-0.2190^{NS}	-1.71±1.12	y=55.92-1.71x
22		Magnesium	Good	0.44129**	2.30±0.68	y=69.12+2.30x
			Poor	0.00555^{NS}	0.09±2.30	y=43.94+0.09x

cont.

Annexure 17 cont.

23	Motility	Sodium	Good	0.67475**	0.12±0.02	y=44.20+0.12x
			Poor	-0.1417^{NS}	-0.05±0.05	y=62.91-0.05x
24		Potassium	Good	0.35398*	0.76±0.29	y=10.38+0.76x
			Poor	0.04610^{NS}	0.09±0.30	y=37.99+0.09x
25		Chloride	Good	0.69131**	0.41±0.06	y=-64.12+0.41x
			Poor	0.33461*	0.26±0.10	y=-26.10+0.26x
26	HOST	Acrosome	Good	0.48501**	0.45±0.19	y= 47.90+0.45x
			Poor	0.92161**	0.97±0.06	y=-0.50+0.97x
27		AKP	Good	-0.37201**	-0.08±0.03	y=100.99-0.08x
			Poor	0.56671**	0.71±0.15	y=-17.59+0.71x
28		AST	Good	0.2136**	0.30±0.20	y=62.96+0.30x
			Poor	-0.3313**	-0.41±0.17	y=113.98-0.41x
29		ALT	Good	-0.2393**	-0.51±0.31	y=96.75-0.51x
			Poor	-0.3585^{NS}	-0.71±0.27	y=81.33-0.71x
30		Hyaluronidase	Good	-0.27339^{NS}	-0.07±0.04	y=104.50-0.07x

			Poor	-0.31394*	-0.14±0.06	y=125.45-0.14x
31		Calcium	Good	-0.05475^{NS}	-0.07±0.19	y=92.05-0.07x
			Poor	0.31818*	1.64±0.72	y=-5.71+1.64x
32		Phosphorus	Good	-0.23771^{NS}	-0.79±0.47	y=96.77-0.79x
			Poor	-0.16456^{NS}	-1.58±1.40	y=73.78-1.58x
33		Magnesium	Good	0.36307*	1.21±0.45	y=80.25+1.21x
			Poor	0.01568^{NS}	0.30±2.84	y=61.28+0.30x
34		Sodium	Good	0.54696**	0.06±0.01	y=67.51+0.06x
			Poor	0.06005^{NS}	0.03±0.07	y=53.53+0.03x
35		Potassium	Good	0.34179*	0.47±0.19	y=42.62+0.47x
			Poor	0.24028^{NS}	0.60±0.36	y=21.45+0.60x
36		Chloride	Good	0.60419**	0.22±0.04	y=5.54+0.22x
			Poor	-0.16062^{NS}	-0.15±0.14	y=104.97-0.15x
37	Acrosome	AKP	Good	-0.52683**	-0.12±0.03	y=109.31-0.12x
			Poor	0.59711**	0.71±0.14	y=-15.14+0.71x
38		AST	Good	0.1842**	0.30±0.23	y=65.46+0.30x
			Poor	-0.1883**	-0.22±0.17	y=92.65-0.22x
39		ALT	Good	-0.0463**	-0.11±0.36	y=92.88-0.11x
			Poor	-0.3071^{NS}	-0.58±0.27	y=80.05-0.58x
40		Hyaluronidase	Good	-0.58237**	-0.16±0.03	y=126.59-0.16x
			Poor	-0.36782*	-0.15±0.06	y=134.34-0.15x
41		Calcium	Good	0.01027^{NS}	0.01±0.20	y=90.33+0.01x
			Poor	0.40067**	1.96±0.66	y=-16.70+1.96x
42		Phosphorus	Good	0.01061^{NS}	0.04±0.51	y=90.58+0.04x
			Poor	-0.12902^{NS}	-1.17±1.33	y=73.20-1.17x
43		Magnesium	Good	0.21034^{NS}	0.75±0.51	y=85.55+0.75x
			Poor	0.05148^{NS}	0.94±2.68	y=59.47+0.94x
44		Sodium	Good	0.25686^{NS}	0.03±0.02	y=80.14+0.03x
			Poor	-0.05126^{NS}	-0.02±0.06	y=73.11-0.02x
45		Potassium	Good	0.26121^{NS}	0.38±0.21	y=52.91+0.38x
			Poor	0.22422^{NS}	0.53±0.34	y=28.52+0.53x
46		Chloride	Good	0.39549**	0.16±0.05	y=32.28+0.16x
			Poor	-0.12963^{NS}	-0.11±0.13	y=97.24-0.11x

cont.

Annexure 17 cont.

47	AKP	AST	Good	-0.1844**	-1.60±1.26	y=287.73-1.60x
			Poor	-0.0625**	-0.06±0.15	y=121.01-0.06x
48		ALT	Good	0.0361**	0.48±1.95	y=140.68+0.48x
			Poor	-0.0620^{NS}	-0.10±0.23	y=115.88-0.10x
49		Hyaluronidase	Good	0.64306**	0.77±0.13	y=-19.14+0.77x

			Poor	-0.38702**	-0.14±0.05	y=174.47-0.14x
50		Calcium	Good	-0.25630^{NS}	-1.47±0.81	y=24.64-1.47x
			Poor	0.57301**	2.35±0.49	y=14.69+2.35x
51		Phosphorus	Good	0.08952^{NS}	1.35±2.19	y=134.63+1.35x
			Poor	-0.25245^{NS}	-1.93±1.09	y=126.33-1.93x
52		Magnesium	Good	-0.43237**	-6.58±2.00	y=195.39-6.58x
			Poor	0.51605**	7.92±1.94	y=63.91+7.92x
53		Sodium	Good	-0.11397^{NS}	-0.06±0.07	y=168.51-0.06x
			Poor	-0.43261**	-0.16±0.05	y=168.62-0.16x
54		Potassium	Good	-0.11851^{NS}	-0.74±0.90	y=221.50-0.74x
			Poor	0.05841^{NS}	0.12±0.29	y=105.32+0.12x
55		Chloride	Good	-0.36595**	-0.63±0.23	y=378.93-0.63x
			Poor	0.05460^{NS}	0.11±0.11	y=102.08+0.11x
56	AST	ALT	Good	0.0991**	0.15±0.22	y=85.00+0.15x
			Poor	0.4151**	0.66±0.21	y=105.75+0.66x
57		Hyaluronidase	Good	-0.1810^{NS}	-0.02±0.02	y=92.64-0.02x
			Poor	0.5826**	0.20±0.04	y= 30.20+0.20x
58		Calcium	Good	-0.0179^{NS}	-0.01±0.09	y=87.75-0.01x
			Poor	0.0099^{NS}	0.04±0.61	y=120.84+0.04x
59		Phosphorus	Good	0.0068^{NS}	0.01±0.25	y=87.11+0.01x
			Poor	0.5557^{NS}	4.27±0.94	y= 93.92+4.27x
60		Magnesium	Good	0.0685**	0.12±0.26	y=86.36+0.12x
			Poor	0.5271^{NS}	8.12±1.93	y= 71.80+8.12x
61		Sodium	Good	-0.1339**	-0.01±0.01	y=90.99-0.01x
			Poor	0.1087^{NS}	0.04±0.05	y= 108.61+0.04
62		Potassium	Good	0.0013*	0.00±0.11	y=87.13+0.00x
			Poor	-0.7126**	-1.43±0.21	y= 221.41-1.43x
63		Chloride	Good	0.1694**	0.04±0.03	y=72.67+0.04x
			Poor	-0.3495^{NS}	-0.26±0.10	y= 195.23-0.26x
64	ALT	Hyaluronidase	Good	-0.1151*	0.01±0.01	y= 17.01-0.01x
			Poor	0.5620*	0.12±0.03	y= -30.53+0.12x
65		Calcium	Good	0.4487^{NS}	0.19±0.06	y= 6.17+0.19x
			Poor	-0.5743^{NS}	-1.49±0.31	y= 87.83-1.49x
66		Phosphorus	Good	-0.0724^{NS}	-0.08±0.16	y= 15.55-0.08x
			Poor	0.3246^{NS}	1.57±0.67	y= 14.93+1.57x
67		Magnesium	Good	-0.6887**	-0.79±0.12	y= 20.47-0.79x
			Poor	0.4613^{NS}	4.46±1.27	y= -2.47+4.46x
68		Sodium	Good	-0.2531**	-0.01±0.01	y= 19.42-0.01x
			Poor	-0.3641*	-0.08±0.03	y= 54.75-0.08x
69		Potassium	Good	-0.4454**	-0.22±0.06	y= 36.36-0.22x
			Poor	-0.6875*	-0.86±0.13	y= 85.33-0.86x
70		Chloride	Good	-0.2599**	-0.04±0.02	y= 29.42-0.04x
			Poor	0.2881*	0.14±0.07	y=-12.20+0.14x

cont.

Annexure 17 cont.

71	Hyaluronidase	Calcium	Good	0.13647^{NS}	0.65±0.69	y=187.32+0.65x
			Poor	-0.44809**	-5.26±1.54	y=672.33-5.26x
72		Phosphorus	Good	0.04576^{NS}	0.58±1.83	y=211.12+0.58x
			Poor	0.49249**	10.78±2.81	y=379.27+10.78x
73		Magnesium	Good	0.01904^{NS}	0.24±1.85	y=215.12+0.24x
			Poor	0.45649**	20.03±5.75	y=326.35+20.03x
74		Sodium	Good	0.34093*	0.14±0.06	y=165.97+0.14x
			Poor	0.30645*	0.32±0.15	y=339.61+0.32x
75		Potassium	Good	-0.26519^{NS}	-1.37±0.73	y=353.79-1.37x
			Poor	-0.81516**	-4.64±0.49	y=773.65-4.64x
76		Chloride	Good	-0.04833^{NS}	-0.07±0.21	y=242.27-0.07x
			Poor	-0.06493^{NS}	-0.14±0.32	y=490.00-0.14x
77	Calcium	Phosphorus	Good	0.22844^{NS}	0.60±0.37	y=39.20+0.60x
			Poor	-0.34839*	-0.65±0.26	y=46.31-0.65x
78		Magnesium	Good	-0.14378^{NS}	-0.38±0.38	y=47.93-0.38x
			Poor	0.30346*	1.13±0.52	y=34.87+1.13x
79		Sodium	Good	0.21252^{NS}	0.02±0.01	y=38.57+0.02x
			Poor	-0.04386^{NS}	-0.00±0.01	y=43.32-0.00x
80		Potassium	Good	-0.73802**	-0.80±0.11	y=124.76-0.80x
			Poor	0.31150*	0.15±0.07	y=31.47+0.15x
81		Chloride	Good	0.00702^{NS}	0.00±0.04	y=44.42+0.00x
			Poor	-0.07824^{NS}	-0.01±0.03	y=45.90-0.01x
82	Chloride	Phosphorus	Good	0.02550^{NS}	0.23±1.29	y=366.60+0.23x
			Poor	-0.44147**	-4.48±1.34	y=304.43-4.48x
83		Magnesium	Good	0.53475**	4.76±1.09	y=334.65+4.76x
			Poor	0.08236^{NS}	1.67±2.99	y=263.95+1.67x
84		Sodium	Good	0.54582**	0.16±0.04	y=311.71+0.16x
			Poor	-0.50027**	-0.24±0.06	y=359.07-0.24x
85		Potassium	Good	0.18161^{NS}	0.66±0.52	y=303.08+0.66x
			Poor	-0.02077^{NS}	-0.05±0.39	y=278.22-0.05x
86	Magnesium	Phosphorus	Good	0.23644^{NS}	0.23±0.14	y=4.85+0.23x
			Poor	0.14332^{NS}	0.07±0.07	y=5.77+0.07x
87		Sodium	Good	0.40761**	0.01±0.04	y=2.39+0.01x
			Poor	-0.21149^{NS}	-0.00±0.00	y=8.00-0.00x
88		Potassium	Good	0.26233^{NS}	0.11±0.06	y=-3.49+0.11x
			Poor	-0.66289**	0.09±0.01	y=12.22-0.09x
89	Phosphorus	Sodium	Good	-0.09972^{NS}	0.00±0.00	y=11.16-0.00x
			Poor	0.27988^{NS}	0.01±0.01	y=2.03+0.01x

90		Potassium	Good	-0.44988**	-0.19±0.05	y=28.44-0.19x
			Poor	-0.44420**	-0.11±0.03	y=14.72-0.11x
91	Potassium	Sodium	Good	0.18696^{NS}	0.02±0.01	y=94.35+0.02x
			Poor	0.04926^{NS}	0.01±0.03	y=66.21+0.01x

* (P<0.05)   ** (P<0.01)   NS (not significant)

Annexure 18: Inter-relationship between some functional parameters and biochemicals in seminal plasma of post-thaw good and poor quality semen of Murrah buffalo bulls.

Sr. No.	Relationship between Parameters (n=48)		Quality	Correlation coefficient	Regression Estimate	Regression Equation
1	Livability	Motility	Good	0.53047**	0.57±0.14	y=41.97+0.57x
			Poor	0.21542 NS	0.34±0.23	y=23.16+0.34x
2		HOST	Good	0.85688**	0.86±0.77	y= 11.98+0.86x
			Poor	0.99507**	1.00±0.01	y=0.82+1.00x
3		Acrosome	Good	0.43047**	0.38±0.12	y=48.37+0.38x
			Poor	0.87913**	1.19±0.09	y=-8.75+1.19x
4		AKP	Good	-0.59153**	-0.11±0.02	y=95.23-0.11x
			Poor	0.04899NS	0.07±0.20	y=21.16+0.07x
5		AST	Good	0.0314 NS	0.05±0.22	y=73.49+0.05x
			Poor	-0.3965**	-0.63±0.22	y=114.53-0.63x
6		ALT	Good	-0.0779**	-0.17±0.33	y=81.21-0.17x
			Poor	-0.5268**	-1.34±0.32	y=82.16-1.34x
7		Hyaluronidase	Good	-0.08948NS	-0.02±0.04	y=83.69-0.02x
			Poor	0.06555NS	0.03±0.07	y=14.49+0.03x
8		Calcium	Good	0.10394NS	0.09±0.13	y=74.39+0.09x
			Poor	-0.16262NS	-0.01±0.90	y=70.89-1.01x
9		Phosphorus	Good	-0.18937NS	-0.54±0.42	y=83.25-0.54x
			Poor	-0.19164NS	-2.51±1.90	y=44.69-2.51x
10		Magnesium	Good	0.44306**	1.23±0.37	y=69.53+1.23x
			Poor	-0.27959NS	-7.57±3.83	y=75.66-7.57x
11		Sodium	Good	0.29931*	0.02±0.01	y=70.78+0.02x
			Poor	0.12327 NS	0.04±0.05	y=17.81+0.04x
12		Potassium	Good	0.20323NS	0.32±0.23	y=47.60+0.32x
			Poor	0.41314**	1.37±0.44	y=-60.15+1.37x
13		Chloride	Good	0.31679*	0.01±0.04	y=42.92+0.10x
			Poor	-0.00211NS	-0.00±0.18	y=30.09-0.00x
14	Motility	HOST	Good	0.47647**	0.44±0.12	y= 29.33+0.44x
			Poor	0.21470NS	0.14±0.09	y=14.29+0.14x
15		Acrosome	Good	0.30898*	0.25±0.12	y=43.57+0.25x
			Poor	0.33172*	0.28±0.12	y=9.14+0.28x
16		AKP	Good	-0.56934**	-0.10±0.02	y=78.60-0.10x
			Poor	-0.37516**	-0.32±0.12	y=57.67-0.32x
17		AST	Good	-0.2695	-0.37±0.20	y=101.77-0.37x
			Poor	0.3377 NS	0.34±0.14	y=27.16+0.34x
18		ALT	Good	0.2036**	0.42±0.30	y=56.43+0.42x
			Poor	-0.1921**	-0.30±0.23	y=30.17-0.30x
19		Hyaluronidase	Good	-0.25388NS	-0.06±0.03	y=77.66-0.06x

			Poor	0.36554*	0.11±0.04	y=-33.87+0.11x
20		Calcium	Good	0.41093**	0.33±0.11	y=48.98+0.33x
			Poor	-0.07898^{NS}	-0.31±0.57	y=30.74-0.31x
21		Phosphorus	Good	-0.33264*	-0.88±0.37	y=71.55-0.88x
			Poor	0.29965*	2.46±1.15	y=3.22+2.46x
22		Magnesium	Good	-0.00080^{NS}	-0.00±0.38	y=63.50-0.00x
			Poor	-0.02950^{NS}	-0.50±2.49	y=21.20-0.50x

cont.

Annexure 18 cont.

23	Motility	Sodium	Good	-0.14807^{NS}	-0.01±0.01	y=66.93-0.01x
			Poor	0.63448**	0.13±0.02	y=-19.21+0.13x
24		Potassium	Good	-0.16276^{NS}	-0.24±0.21	y=86.28-0.24x
			Poor	-0.22940^{NS}	-0.47±0.30	y=49.23-0.47x
25		Chloride	Good	-0.05739^{NS}	-0.02±0.04	y=69.43-0.02x
			Poor	-0.29587*	-0.23±0.11	y=76.87-0.23x
26	HOST	Acrosome	Good	0.31709*	0.28±0.12	y=55.10+0.28x
			Poor	0.88104**	1.18±0.09	y=-9.40+1.18x
27		AKP	Good	-0.54623**	-0.10±0.02	y=92.61-0.10x
			Poor	0.02991^{NS}	0.04±0.20	y=23.45+0.04x
28		AST	Good	-0.0399 ^{NS}	-0.06±0.22	y=83.12-0.06x
			Poor	-0.3980**	-0.63±0.21	y=112.95-0.63x
29		ALT	Good	-0.0752**	-0.17±0.32	y=79.84-0.17x
			Poor	-0.5346**	-1.34±0.31	y=81.39-1.34x
30		Hyaluronidase	Good	-0.02066^{NS}	-0.00±0.03	y=78.28-0.00x
			Poor	0.08143^{NS}	0.04±0.07	y=10.09+0.04x
31		Calcium	Good	0.11583^{NS}	0.10±0.13	y=72.65+0.10x
			Poor	-0.17700^{NS}	-1.09±0.90	y=73.10-1.09x
32		Phosphorus	Good	-0.25187^{NS}	-0.71±0.41	y=83.59-0.71x
			Poor	-0.16657^{NS}	-2.16±1.89	y=41.57-2.16x
33		Magnesium	Good	0.45265**	1.25±0.37	y=68.12+1.25x
			Poor	-0.28368^{NS}	-7.60±3.79	y=74.86-7.60x
34		Sodium	Good	0.34980*	0.03±0.01	y=68.30+0.03x
			Poor	0.14179^{NS}	0.05±0.05	y=15.24+0.05x
35		Potassium	Good	0.13479^{NS}	0.20±0.23	y=56.77+0.20x
			Poor	0.41175**	1.35±0.44	y=-59.88+1.35x
36		Chloride	Good	0.30984*	0.10±0.04	y=42.60+0.10x
			Poor	-0.01211^{NS}	-0.01±0.18	y=32.24-0.01x
37	Acrosome	AKP	Good	-0.61149**	-0.13±0.02	y=98.59-0.13x
			Poor	0.05226^{NS}	0.05±0.15	y=25.56+0.05x
38		AST	Good	-0.1034**	-0.17±0.25	y=96.70-0.17x

			Poor	-0.3257**	-0.38±0.16	y=83.69-0.38x
39		ALT	Good	0.0582**	0.15±0.37	y=76.30+0.15
			Poor	-0.4126**	-0.77±0.25	y=62.57-0.77x
40		Hyaluronidase	Good	-0.48053**	-0.14±0.04	y=111.54-0.14x
			Poor	-0.04987^{NS}	-0.02±0.05	y=40.45-0.02x
41		Calcium	Good	0.04108^{NS}	0.40±0.15	y=76.99+0.40x
			Poor	-0.04529^{NS}	-0.21±0.68	y=40.59-0.21x
42		Phosphorus	Good	0.08669^{NS}	0.28±0.48	y=76.20+0.28x
			Poor	-0.15540^{NS}	-1.51±1.41	y=41.21-1.51x
43		Magnesium	Good	0.47925**	1.51±0.41	y=68.02+1.51x
			Poor	-0.27841^{NS}	-5.56±2.83	y=66.07-5.56x
44		Sodium	Good	0.04003^{NS}	0.00±0.01	y=77.63+0.00x
			Poor	0.12358^{NS}	0.03±0.04	y=23.47+0.03x
45		Potassium	Good	0.04142^{NS}	0.07±0.26	y=71.68+0.07x
			Poor	0.36144*	0.88±0.34	y=-25.80+0.88x
46		Chloride	Good	0.09373^{NS}	0.03±0.05	y=66.91+0.03x
			Poor	0.05123^{NS}	0.05±0.13	y=20.05+0.05x

cont.

Annexure 18 cont.

47		AST	Good	0.3166 ^{NS}	2.58±1.14	y=-109.66+2.58x
			Poor	-0.1186*	-0.14±0.17	y=139.41-0.14x
48		ALT	Good	-0.1654**	-2.00±1.76	y=189-2.00x
			Poor	0.4020*	0.73±0.25	y=92.20+0.73x
49		Hyaluronidase	Good	0.42502**	0.59±0.19	y=16.50+0.59x
			Poor	-0.57508**	-0.19±0.04	y=215.20-0.19x
50		Calcium	Good	-0.25054^{NS}	-1.18±0.68	y=207.67-1.18x
			Poor	0.66791**	2.99±0.49	y=-1.28+2.99x
51	AKP	Phosphorus	Good	0.14366^{NS}	2.22±2.82	y=135.31+2.22x
			Poor	-0.37766**	-3.56±1.29	y=142.75-3.56x
52		Magnesium	Good	-0.43272**	-6.55±2.03	y=202.33-6.55x
			Poor	0.38104**	7.41±2.65	y=75.84+7.41x
53		Sodium	Good	-0.18364^{NS}	-0.08±0.06	y=180.82-0.08x
			Poor	-0.59429**	-0.14±0.03	y=161.35-0.14x
54		Potassium	Good	0.04930^{NS}	0.42±1.27	y=115.22+0.42x
			Poor	0.31394*	0.75±0.33	y=72.22+0.75x
55		Chloride	Good	-0.23279^{NS}	-0.40±0.25	y=297.19-0.40x
			Poor	0.12442 ^{NS}	0.11±0.13	y=92.74+0.11x
56	AST	GPT	Good	-0.2762 ^{NS}	-0.41±0.21	y=110.04-0.41x
			Poor	0.3051**	0.48±0.22	y=115.26+0.48x
57		Hyaluronidase	Good	0.4387 ^{NS}	0.07±0.02	y=85.38+0.07x

			Poor	0.5174 NS	0.15±0.04	y=60.64+0.15x
58		Calcium	Good	-0.2691 NS	0.16±0.08	y=109.96-0.16x
			Poor	0.2852 NS	1.11±0.55	y=88.85+1.11x
59		Phosphorus	Good	0.0086 NS	0.02±0.27	y=102.93+0.02x
			Poor	0.1143 NS	0.93±1.20	y=128.68+0.94x
60		Magnesium	Good	0.3420 NS	0.63±0.25	y=98.63+0.63x
			Poor	0.6955 NS	11.79±1.80	y=62.36+11.79x
61		Sodium	Good	0.4706 NS	0.02±0.01	y=95.62+0.02x
			Poor	0.4222 NS	0.09±0.03	y=109.49+0.09x
62		Potassium	Good	0.3128 NS	0.33±0.15	y=71.50+0.33x
			Poor	-0.6794 NS	-1.41±0.22	y=226.60-1.41x
63		Chloride	Good	0.2270*	0.04±0.05	y=86.64+0.04x
			Poor	0.0294 NS	0.02±0.11	y=128.55+0.02x
64	ALT	Hyaluronidase	Good	-0.0543**	-0.01±0.02	y=18.39-0.01x
			Poor	-0.1870 NS	-0.03±0.03	y=56.25-0.03x
65		Calcium	Good	0.4762 NS	0.18±0.05	y=8.74+0.18x
			Poor	0.3011 NS	0.74±0.35	y=9.19+0.74x
66		Phosphorus	Good	-0.0038 NS	-0.00±0.19	y=16.96-0.00x
			Poor	-0.0516 NS	-0.27±0.76	y=41.08-0.27x
67		Magnesium	Good	-0.2193**	-0.27±0.18	y=18.83-0.27x
			Poor	0.5527 NS	5.89±1.31	y=3.42+5.89x
68		Sodium	Good	-0.1704 NS	-0.00±0.00	y=18.74-0.00x
			Poor	-0.4943**	-0.07±0.02	y=57.81-0.07x
69		Potassium	Good	-0.3920 NS	-0.28±0.10	y=43.50-0.28x
			Poor	-0.2272 NS	-0.30±0.19	y=58.88-0.30x
70		Chloride	Good	-0.0614 NS	-0.01±0.02	y=19.90-0.01x
			Poor	0.3487 NS	0.17±0.07	y=4.18+0.17

cont.

Annexure 18 cont.

71	Hyaluronidase	Calcium	Good	0.09847 NS	0.34±0.50	y=222.87+0.34x
			Poor	-0.47730**	-6.44±1.75	y=755.43-6.44x
72		Phosphorus	Good	-0.03142NS	-0.35±1.67	y=240.92-0.35x
			Poor	0.42440**	12.04±3.79	y=419.10+12.04x
73		Magnesium	Good	0.09070 NS	0.10±1.63	y=230.60+0.10x
			Poor	0.30341*	17.76±8.22	y=383.72+17.76x
74		Sodium	Good	0.54373**	0.17±0.04	y=183.80+0.17x
			Poor	0.62826**	0.45±0.08	y=364.29+0.45x
75		Potassium	Good	0.00631 NS	0.04±0.92	y=233.92+0.04x
			Poor	-0.64040**	-4.58±0.81	y=792.40-4.58x
76		Chloride	Good	0.34241*	0.42±0.17	y=86.73+0.42x

			Poor	-0.06655^{NS}	-0.17±0.39	y=537.92-0.17x
77	Calcium	Phosphorus	Good	0.16310^{NS}	0.54±0.49	y=39.25+0.54x
			Poor	-0.42577**	-0.90±0.28	y=46.34-0.90x
78		Magnesium	Good	-0.10475^{NS}	-0.34±0.48	y=46.60-0.34x
			Poor	0.40895**	1.78±0.58	y=30.05+1.78x
79		Sodium	Good	-0.07589^{NS}	-0.01±0.01	y=46.41-0.01x
			Poor	-0.10587^{NS}	-0.01±0.01	y=42.50-0.01x
80		Potassium	Good	-0.79058**	-1.43±0.17	y=182.74-1.43x
			Poor	0.24707^{NS}	0.13±0.08	y=32.31+0.13x
81		Chloride	Good	0.08075^{NS}	0.03±0.05	y=33.74+0.03x
			Poor	-0.03477^{NS}	-0.01±0.03	y=42.67-0.01x
82	Chloride	Phosphorus	Good	0.36809*	3.33±1.26	y=325.16+3.33x
			Poor	-0.25933^{NS}	-2.80±1.54	y=277.97-2.80x
83		Magnesium	Good	0.55328**	4.90±1.10	y=320.91+4.90x
			Poor	0.33158*	7.37±3.10	y=215.90+7.37x
84		Sodium	Good	0.71677**	0.18±0.03	y=298.47+0.18x
			Poor	-0.52494**	-0.14±0.03	y=301.62-0.14x
85		Potassium	Good	-0.14433^{NS}	0.72±0.74	y=425.25-0.72x
			Poor	-0.10557^{NS}	-0.29±0.40	y=279.80-0.29x
86	Magnesium	Phosphorus	Good	0.27667^{NS}	0.28±0.15	y=4.52+0.28x
			Poor	-0.01042^{NS}	-0.01±0.07	y=6.14-0.01x
87		Sodium	Good	0.77778**	0.02±0.00	y=0.10+0.02x
			Poor	-0.09009^{NS}	-0.00±0.00	y=6.42-0.00x
88		Potassium	Good	0.09991^{NS}	0.06±0.08	y=1.69+0.06x
			Poor	-0.50808**	-0.06±0.02	y=10.18-0.06x
89	Phosphorus	Sodium	Good	0.16923^{NS}	0.00±0.00	y=7.69+0.00x
			Poor	0.29305*	0.01±0.00	y=3.97+0.01x
90		Potassium	Good	-0.53891**	-0.30±0.07	y=37.82-0.30x
			Poor	-0.47333**	-0.12±0.03	y=13.90-0.12x
91	Potassium	Sodium	Good	0.10250^{NS}	0.01±0.01	y=95.93+0.01x
			Poor	-0.27773^{NS}	-0.03±0.01	y=73.37-0.03x

* ($P<0.05$) ** ($P<0.01$) NS (not significant)