STUDIES ON ROLE OF SOME NON-ENZYMATIC ANTI-OXIDANT ADDITIVES ON THE CRYOPRESERVATION OF BOVINE SEMEN

By

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Faculty of Veterinary Sciences & Animal Husbandry
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Division of Veterinary Gynaecology & Obstetrics

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Main Campus, Chatha, Jammu-180009

2021

CERTIFICATE-I

This is to certify that the thesis entitled "**Studies on role of some non-enzymatic anti-oxidant additives on the cryopreservation of bovine semen**" submitted in partial fulfillment of the requirements for the degree of **Master of Veterinary Science in Veterinary Gynaecology and Obstetrics** to the Faculty of Veterinary Sciences & Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, is original work and has similarities with published work not more than minor similarities as per UGC norms of 2018 adopted by the University. Further, the level of minor similarities has been declared after checking the manuscript with **Urkund** software provided by the University.

The work has been carried out by **Mr. Mohammad Shaheem Butt** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma. It is further certified that help and assistance received during the course of thesis investigation have been duly acknowledged.

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ABSTRACT

Title of the thesis	:	**Studies on role of some non-enzymatic anti-oxidant additives on the cryopreservation of bovine semen**
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Registration No.	:	**J-19-MV-584**
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Abstract

The present study was undertaken at Division of V.G.O., F.V.Sc & A.H, SKUAST-J, R.S. Pura, Jammu in collaboration with CABS, Hakkal, Jammu The main objective of this study was to ascertain the effects of different antioxidants (Ascorbic acid, Vitamin-E, Cysteine, Taurine, BHT and Trehalose) on cryopreservation of semen. Semen samples from cattle bull were used to evaluate the effect of additives on cryopreservation of semen. The complete experiment was divided into 3 groups. Group I: Control, TEYCAFG+ cysteine, TEYCAFG+ taurine. Group II: Control, TEYCAFG+ ascorbic acid, TEYCAFG+ vitamin E. Group III: Control, TEYCAFG+ trehalose, TEYCAFG+ BHT.

The results indicated that the progressive motility with taurine in group I, ascorbic acid and vitamin E in group II differed significantly ($P<0.05$) from control. The live sperm count with taurine in group I, ascorbic acid and vitamin E in group II differed significantly ($P<0.05$) from control. The acrosomal integrity at post-thaw stage with cysteine and taurine in group I, ascorbic acid in group II differed significantly ($P<0.05$) from control. The HOST with ascorbic acid in group II differed significantly ($P<0.05$) from control. Lipid peroxidation with cysteine and taurine in group I, ascorbic acid and vitamin E in group II and BHT in group III differ significantly ($P<0.05$) from control. The catalase with taurine in group I differ significantly ($P<0.05$) from control and cysteine, ascorbic acid in group II and both trehalose and BHT in group III differ significantly ($P<0.05$) from control. SOD with taurine in group I, ascorbic acid in group II & both trehalose and BHT in group III added semen differed significantly ($P<0.05$) from control. It was concluded that in group I the taurine, in group II ascorbic acid and in group III BHT added semen sample improved the quality of post-thaw semen though the increase was non-significant.

Keywords: Physio-morphological, Antioxidant, Oxidative stress, Cryopreservation.

Signature of Major Advisor

Shaheem

Signature of Student

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ABBREVIATIONS

%	Per cent
&	And
@	At the rate of
<	Less than
>	Greater than
=	Equals to
^{o}C	Degree centigrade
IU/ml	International unit per millilitre
ml	Millilitre
µl/ml	Microliter per millilitre
nmol	Nano moles
µmol/ml	Micro mole per millilitre
ng	Nano gram
pmol	Pico mole
dL	Decilitre
U/ml	Unit per millilitre
kU/g	Kilo unit per gram
U/g	Unit per gram
U/mg	Unit per milligram
µkat/g	Micro katal unit per gram
mmol	Milli mole
µM	Micro mole
mU/ml	Milli unit per millilitre
%inh	Per cent inhibition
µg	Micro gram
kU/L	Kilo unit per litre
U/L	Unit per litre
10^{6}	1 million
10^{9}	1 billion
g	Gram

mg	Milligram
Kg	Kilogram
µl	Microliter
et al.,	et alli (and others)
ad lib.	ad libitum
H	Hours
Min.	Minutes
Sec.	seconds
n	Number
pH	Potential of hydrogen
ROS	Reactive oxygen species
TEYCAFG	Tris-egg yolk-citric acid-fructose-glycerol
HOST	Hypo-osmotic Swelling Test
HOS	Hypo-osmotic Solution
PUFA	Polyunsaturated fatty acid
BHT	Butylated hydroxy toluene
LPO	Lipid peroxidation
CAT	Catalse
SOD	Superoxide dismutase
H_2O_2	Hydrogen peroxide
mM	Milli-mole
MT	Metric Ton
S.S.	Sum of square
ANOVA	Analysis of variance
i.e.	That is
vs	Versus
Na_2HPO_4	Disodium hydrogen phosphate
SE	Standard error
KH_2PO_4	Potassium dihydrogen phosphate
O_2	Oxygen
Viz	As follow
HF	Holstein Friesian

Chapter-I

Introduction

CHAPTER-I

INTRODUCTION

India is pioneer in milk production with production of about 187.7 Million Tonnes (MT) in year 2018-19 in which the contribution of Jammu and Kashmir (J&K) was only 2.54 MT (NDDB, 2020). As per 2019 census the cattle population in India was 192.5 million, whereas in J&K it was 1231 thousand (NDDB, 2020). To achieve the aim of sustainable milk production, major requirement is to improve the gene pool of an animal (Singh *et al.,* 2020). The best proven method to improve the gene pool is artificial insemination (AI) with the semen from the bulls of superior germplasm.

An Italian scientist Abbe Lazzaro Spallanzani in 1780 was the first to provide the documented proof of Artificial Insemination (AI) successfully in bitch (Hezavehei *et al.,* 2018). Artificial insemination is one of the most important proven reproductive biotechnology to improve the gene pool. It causes the extensive propagation of the semen from superior male by preventing the spread of sexually transmitted diseases and have prime focus on genetic improvement programmes in livestock (Andrabi *et al.,* 2008; Bucak *et al.,* 2009a; Hafez, 2013). AI requires fresh or well-preserved semen and 95% of AI is done by using preserved semen (Raheja *et al.,* 2018).

The semen is preserved in an ideal dilutor or extender to maintain the quality of semen. The extender is the important factor in preservation of semen which should have adequate pH, buffering capacity, appropriate osmolality, etc (Raheja *et al.,* 2018; Rammutla, 2018; Hernandez-Aviles *et al.,* 2020), it also preserve and maintain sperm metabolic processes by controlling the pH of medium before and after freezing, controlling bacterial contamination and transmission, and reduce cryo-injuries to semen and thus improves fertilization (Malik *et al.,* 2018; Raheja *et al.,* 2018). Egg yolk semen extender is most commonly used in the laboratory and for the field techniques as for its suitable results (Layek *et al.,* 2016). For experimental purpose different types of cryoprotective agents were used in semen extender like DMSO and propanediol but glycerol remains the choice of cryoprotectant for semen cryopreservation as glycerol crosses the cell membrane (Forouzanfar *et al.,* 2010).

Semen cryopreservation is the method of choice for its long term storage and preserving animal genetic resources for the development of genetically superior sires through AI (Lardy and Phillips, 1939) and before cryopreservation semen undergo sequence of stages that include semen dilution in extender, equilibration, freezing, cold storage and post-thawing evaluation (Haris *et al.*, 2020), but cryopreservation generates sub-lethal injury to sperm due to chemical, osmotic, thermal and mechanical stresses and semen may produce changes like loss of motility, viability, membrane integrity, damage to DNA and destruction of acrosomal membrane (Rasul *et al.*, 2001; Bucak *et al.*, 2007).

The oxidative stress (OS) is main causative reason for the infertility and it is the outcome of instability in the reactive oxygen species (ROS) and antioxidants which leads to sperm damage, deformity and finally male infertility (Bansal and Bilaspuri, 2011). The formation of ROS by sperm is regular physiological event, which is destructive to the sperm and it is associated with male infertility (Sharma and Agarwal, 1996). At physiological levels, ROS influence and mediate the gametes and vital reproductive process (Gagon *et al.,* 1991; Aitken, 1997; Attaran *et al.,* 2000). Cryopreservation causes functional and structural damage to spermatozoa that is related with reactive oxygen species (ROS) production, impairment of sperm motility and decreased fertilizing potential causing female infertility.

Sperm cells are well furnished with a dominant defence system of antioxidant against ROS (Sikka, 1996). Spermatozoa and seminal plasma maintain an antioxidant system which contain taurine, reduced glutathione (GSH), glutathione peroxidise (GSH-Px), catalase (CAT) and superoxide dismutase (SOD) to avoid oxidative damage (Aurich *et al.*, 1997; Storey, 1997; Lapointe and Bilodeau, 2003), but after freezing and post thawing antioxidant ability in spermatozoa may not be enough to prevent oxidative stress (Ernster *et al.*, 1992; Najafi *et al.*, 2014; Masoudi *et al.*, 2016).

Antioxidants are the key defence factor present in semen beside the oxidative stress that occurs due to presence of free radicals (Sikka, 1996). Antioxidant like ascorbic acid, catalase, alpha- tocopherol, taurine etc added in freezing diluent have a protective effect against OS like lipid peroxidation, thereby preserving the metabolic activity and cellular viability of bovine semen (Beconi *et al.,* 1993; Agarwal *et al.,* 2004).

Antioxidant like vitamin E is known for its capability to provide the protection to the sperm membrane by inhibiting the production of ROS and lipid peroxidation (Ugur *et al.,* 2019). It is also known as an "anti-sterility factor X", so the presence of this vitamin is essential for common purpose of male reproductive system (Moumeni *et al.,* 2009). Addition of vitamin E in the semen dilutor increases the sperm fight against lipid peroxidation (Cerolini *et al.,* 2000).

The role of an ascorbic acid is well known for tolerating the negative effects of reactive oxygen species and nitrogen radicals (Padayatty *et al.,* 2003). It acts as a free radical scavenger due to production of monodehydroascorbate (MDHA) radicals that are not able to react with other molecules or oxygen to produce more reactive radicals (Cullen and Buettner, 2012). It has an antioxidant property and is available in the epididymal secretions and semen of different species and have a protective actions in the epididymis (Chinoy, 1972). It has an important role in preventing sperm from ROS and maintains the hereditary respectability of sperm cells by reducing oxidative damage to sperm DNA (Fraga *et al.,* 1991).

Cysteine is a sulphur containing amino acid, and is exist normally in fundamental seminal plasma and sperm nucleic acid that keep up the integrity of the DNA and furthermore acts as an antioxidant directly and/or indirectly through intracellular antioxidant which protects semen from ROS-mediated harmful effects (Topraggaleh *et al.,* 2014). It is the precursors of intracellular glutathione biosynthesis (Meister and Anderson, 1983). It contains thiol groups, which act as non-enzymatic antioxidant and without difficulty penetrates into the sperm (Coyan *et al.,* 2011). It has been reported that the cysteine in cryopreservation media enhances sperm motility and viability in frozen-thawed semen of bull (Sariozkan *et al.,* 2009a), boar (Kaeoket *et al.,* 2010) and buck (Kulaksiz and Daskin, 2010). It has been reported that the better effects of cysteine for safety of sperm chromatin have been proved during chilled preservation of boar (Szcesniak-Fabianczyk *et al.,* 2003) and freezing of bull semen (Tuncer *et al.,* 2010).

Taurine is an amino acid which can be used as an antioxidant (Neamah and Houbi, 2020). It's a sulfonic amino acid that is non-enzymatic scavenger which plays a major role in prevention of spermatozoa against ROS, in case if spermatozoa make

contact to aerobic conditions and storage 4^{o}C under refrigeration (Alvarez and Storey, 1983; Bucak and Tekin, 2007; Baran *et al.,* 2009; Perumal *et al.,* 2013). In recent time, taurine is used as an antioxidant in semen dilutor and has been adding in the semen of boar (Funahashi and Sano, 2005), bull (Uysal *et al.,* 2007), human (Lopes *et al.,* 1998), ram (Bucak *et al.,* 2007) and goat (Atessahin *et al.,* 2008) to improve motility, viability, membrane integrity and fertility by inhibiting lipid peroxidation and protecting cells against ROS (Chen *et al.,* 1993; Foote *et al.,* 1993). It also regulates mitochondrial protein synthesis by protecting mitochondria against superoxide generation (Jong *et al.,* 2012).

Butylated hydroxytoluene (BHT) is an antioxidant which is a synthetic analogue of vitamin E used to protect auto-oxidation of lipid bi-layer and membrane of sperm cells (Hammerstedt *et al.,* 1990). It readily incorporates into sperm membrane and prevents the sperm from cold shock during cryopreservation (Anderson *et al.,* 1994). It preserves liquid and frozen semen (Ijaz *et al.,* 2009) that improves sperm survival and membrane integrity and reduce the loss of motility (Uysal and Bucak, 2007). BHT in semen extender have been found to have beneficial effects at optimum concentration for sperm motility and viability in certain breeds such as Holstein (Ansari *et al.,* 2011) and Sahiwal (Asadpour *et al.,* 2012) bulls. The better result of BHT in cryopreservation have been observed in goat spermatozoa using different extender which have shown the enhancement in motility parameters in BHT treated groups as compare to control groups (Khalif and El-Saidy, 2008; Memon *et al.,* 2012; Naijian *et al.,* 2013; Ogretmen and Inanan, 2014). It has been reported that different concentration of BHT in different extender enhances the post-thaw quality of canine (Ziaullah *et al.,* 2012), ram (Farshad *et al.,* 2010) goat and turkey (Donoghue and Donoghue, 1997) semen. BHT reduces the ROS from the surroundings of the sperm and converts these molecules into hydroperoxides (Merino *et al.,* 2015) thus reducing the harmful effects of ROS on sperm during freezing process (Khumran *et al.,* 2015).

Trehalose is a sugar that binds two D-glucose molecules and stabilizes membrane phospholipids by binding to their polar head groups (Ozturk *et al.,* 2020). However if the membrane is not stabilized, several cracks occurs in the outer and inner layers of the cell

membrane that leads to cellular dehydration and osmotic imbalance (Ozturk *et al.*, 2020). It stabilizes the cell membrane and prevents cell dehydration (De Leeuw *et al.*, 1993). Trehalose protects the extracellular environment and reduces the size of water molecules by binding with hydrogen atoms (Rudolph and Crowe, 1985; Ozturk *et al.*, 2019). Due to its antioxidant properties it affects the quality and in-vitro fertilizing potentials of spermatozoa and deteriorating the lipid peroxidation of the cryopreserved semen (Badr *et al.*, 2014). Trehalose supplementation shows greater cryoprotective capacity and property to improve the sperm motility, membrane integrity and acrosomal integrity parameters (Hu *et al.*, 2009). It also shows the relation with phospholipid polar head groups during freezing and reduces the Van der Waals interaction between the hydrocarbon chains (Iqbal *et al.*, 2018).

Keeping in the view, the present research was designed with the following objectives:

1. To study the effect of cryopreservation on different physio-morphological characteristics of the semen.

2. To ascertain the effects of different antioxidants (Ascorbic acid, Vitamin-E, Cysteine, Taurine, Butylated hydroxy toulene and Trehalose) on cryopreservation of semen.

3. To evaluate the oxidative stress to the spermatozoa.

Chapter-II

Review of Literature

CHAPTER-II

REVIEW OF LITERATURE

2.1 Semen Characteristics

2.1.1 Volume

Semen volume is essentially the amount of semen produced by various species like human, animal etc during ejaculation. 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). The ejaculated volume significantly increased with age and the same was increased linearly with age along with motile spermatozoa and sperm concentration, which was correlated with total sperm count (Abdel-Raouf, 1965; Almquist and Cunningham, 1967; Colchen-Bourlaoud and Thibier, 1973; Rao and Rao, 1979). There is positive correlation between total number of sperm and semen volume (Nishiyama *et al.*, 1968).

Mathevon *et al.* (1998) reported the mean ejaculated volume in young and mature HF bull semen as 5.48 ±1.83 and 6.73 ±1.99 ml, respectively.

Javed *et al.* (2000) reported the mean ejaculatory volume of bull semen as 4.67±1.62 ml.

Rana and Dhami (2004) reported the average ejaculated volume in Gir bull and Jaffarabady buffalo bull as 7.03 ± 0.44 ml and 6.36 ± 0.33 ml, respectively.

Mandal *et al.* (2005) reported the mean value of ejaculate volume in Sahiwal bull as 3.15 ± 0.13 ml.

Fuerst-Waltl *et al.* (2006) reported that the average ejaculate volume in Austrian Simmental bulls as 4.8 ± 1.9 to 6.9 ± 2.5 ml, respectively.

Munsi *et al.* (2007) reported the mean volume of semen in Holstein Friesian crossbred bull as 4.5 ± 0.4 ml.

Sarder (2007) reported the volume of ejaculate semen in bovines as 7.23 ml and 7.09 ml at >29^{o}C and 25-29^{o}C, respectively.

Uysal *et al.* (2007) reported that the mean ejaculate volume in HF bull as 5.03 ± 0.2 ml.

Bhoite *et al.* (2008) reported the mean value of ejaculate volume in Friesian x Gir cross bred and JY x Friesian cross bred bull semen as 5.278 ± 0.056 and 4.473 ± 0.083 ml, respectively.

Shaha *et al.* (2008) reported the ejaculate volume of semen in Holstein Friesian cross with Zebu cattle as 4.1-7.6 ml.

Koivisto *et al.* (2009) reported the average value of ejaculate volume of semen in *Bos indicus* and *Bos Taurus* as 5.1±0.03 and 6.5±0.01 ml, respectively.

Fiaz *et al.* (2010) reported the mean value of ejaculate volume in H.F. and Jersey bull semen as 4.150 ± 0.05 and 3.025 ± 0.03 ml, respectively.

Hossain *et al.* (2012) reported the mean volume in Local, Friesian and Sahiwal bull semen as 9.3±0.01, 11.5±0.06, and 9.8±0.10 ml, respectively.

Patel and Siddiquee (2013) reported the mean value of ejaculate volume in Kankrej bull semen as 4.84 ± 0.01 ml.

Bhakat *et al.* (2014) reported the average value of ejaculate volume of Karan Fries bull semen as 4.45 ± 0.10 ml.

Srivastava and Kumar (2014) reported the mean volume in fresh semen of HF and Crossbred bull as 4.9 ± 1.9 and 5.6 ± 1.3 ml, respectively.

Matharoo (2015) reported the mean volume in crossbred bull semen as 4.61±0.11 ml.

Sannat *et al*. (2015) reported the mean volume in Sahiwal, Holstein-Friesian cross, Jersey and Gir bull semen as 4.5 ± 0.29, 7.6 ± 0.33, 4.9 ±0.23 and 5.3 ± 0.25 ml, respectively.

Rehman *et al.* (2016) reported the mean volume in Friesian, Jersey, Achai, Crossbred and Sahiwal bull semen as 6.82± 0.56, 6.42± 0.69, 6.00± 0.43, 7.30± 0.36 and 5.50± 0.04 ml, respectively.

Chaudhary *et al.* (2017) reported the mean ejaculate volume in Gir bull semen as 6.20±0.42 ml.

Chauhan *et al.* (2017) reported the mean ejaculate volume in Frieswal bull semen as 5.60±0.16 ml.

D'Andre *et al.* (2017) reported the mean volume in Friesian, Jersey and Inyambo bull semen as 5.76±0.08, 4.29±0.09, 3.37±0.1 ml, respectively.

Baloch *et al.* (2019) reported the mean ejaculated volume in Holstein-Friesian bull semen as 7.103±0.14 ml.

Haris *et al.* (2020) reported the mean ejaculate volume in Simmental bull fresh semen as 6.90 ± 0.91ml.

Pal *et al.* (2020b) reported the average ejaculate volume in two Hariana bull fresh semen as 5.38± 0.20 and 5.04± 0.34 ml.

2.1.2 Mass activity

The collective movement of sperms or their wave motion is called as mass activity. The mass activity is estimated and graded based on the power of wave development and disintegration in undiluted semen and observed under microscope (low power) without cover slip (10x) and expressed on 0 to 5 scale (Tomar, 1997).

Rana and Dhami (2004) reported the mean mass activity in Gir bull and Jaffarabady buffalo bull semen as 3.33 ± 0.11 and 2.80 ±0.06.

Mandal *et al.* (2005) reported the overall mean mass activity value in Sahiwal bull semen as 2.66 ± 0.007.

Munsi *et al.* (2007) reported the mean mass activity of HF Crossbred bull semen as 3.8 ± 0.3.

Sarder (2007) reported the average mass activity in bovine semen as 2.96 ± 0.1.

Koivisto *et al.* (2009) reported the average value of mass activity in *Bos indicus* and *Bos taurus* as 3.3 ± 0.1 and 2.8 ± 0.1, respectively.

Fiaz *et al.* (2010) reported the mean value of mass activity in HF and Jersey bull semen as 1.92 ± 0.07 and 2.21 ± 0.04, respectively.

Ansari (2011) reported that the mass activity in 2 Holstein Friesian (HF) cross bred bull semen as 3.22 ± 0.12 and 3.18 ± 0.10, respectively.

Patel and Siddiquee (2013) reported the mean value of mass activity in Kankrej bull semen as 3.72 ±0.02.

Srivastava and Kumar (2014) reported the mean mass motility (0-5) in fresh semen of HF and Crossbred bull as 4.0 ± 0.4 and 3.0 ± 0.2, respectively.

Matharoo (2015) reported the average value of mass activity in crossbred bull semen as 2.39±0.03.

Chaudhary *et al.* (2017) reported the average mass activity in Gir cattle and Surti buffalo semen as 3.44±0.09 and 3.42±0.08, respectively.

D'Andre *et al.* (2017) reported the mean mass activity in Friesian, Jersey and Inyambo bull semen as 2.67±0.03, 3.05±0.04 and 2.90±0.06, respectively.

Baloch *et al.* (2019) reported the mean mass activity in Holstein Friesian bull semen as 3.6±0.052.

Pal *et al.* (2020b) reported the mean mass motility (0-5) in two Hariana bull fresh semen as 3.69 ±0.09 and 3.63± 0.08.

2.1.3 Sperm concentration

Sperm concentration refers to the number of sperm per unit volume (ml) of semen.

Ulfina and Raina (2003) observed the mean value of sperm concentration in Sahiwal bull semen as was 1641.35 ± 735.83 millions/ml.

Rana and Dhami (2004) reported the mean sperm concentration in Gir and Jaffarabady Buffalo bull semen as 2608 ± 53 and 1384 ±40 millions/ml, respectively.

Mandal *et al.* (2005) reported the average value of spermatozoa concentration in Sahiwal bull semen as 1335.25 ± 51.00 $x10^6$ /ml.

Munsi *et al.* (2007) reported the mean spermatozoa concentration in HF Crossbred bull fresh semen as 1410.0 ± 70.0 $x10^6$ /ml.

Sarder (2007) reported the mean sperm concentration Hostein Friesian bull semen as 1331.0 million/ml.

Uysal *et al.* (2007) reported the mean value concentration of spermatozoa in HF bull fresh semen as 1480.0 ± 70.0 $x10^6$ /ml.

Abdel-Khalek *et al.* (2008) reported the mean concentration of spermatozoa in Hostein bull neat semen as 1.77 ± 14.4 x 10^9 sperms/ ml.

Shaha *et al.* (2008) reported the mean sperm concentration in Friesian cross with Zebu fresh semen as 1000 million/ml.

Koivisto *et al.* (2009) reported the mean sperm concentration in *B. indicus* and *B. taurus* semen as 1423.8 $x10^6$ and 1613.8 $x10^6$ ⁄ml, respectively.

Hossain *et al.* (2012) reported the mean concentration of spermatozoa in bull fresh semen as 1286.6 to 1858.6 million/ml.

Patel and Siddiquee (2013) reported the average sperm concentration in Kankrej bull fresh semen as 1253.83 ±14.68 $x10^6$.

Srivastava and Kumar (2014) reported the mean sperm concentration in fresh semen of HF and Crossbred bull as 917.5 ± 127.9 and 968.1 ± 111.3 millions /ml, respectively.

Rehman *et al.* (2016) reported the mean value of spermatozoa concentration in Friesian, Jersey, Achai, Crossbred and Sahiwal fresh semen as 1351.7±24.50,

1560.0±59.00 $x10^6$, 1333.3±62.20 $x10^6$, 1315.8±30.27 $x10^6$ and 1300.0±131.94 $x10^6$, respectively.

Chaudhary *et al.* (2017) reported the average semen concentration in Gir cattle and Surti buffalo fresh semen as 1169.44±61.71 $x10^6$ and 846.30±54.82 $x10^6$, respectively.

Chauhan *et al.* (2017) reported the mean concentration in Frieswal bull semen as 1076.1±32.56 million/ml.

D'Andre *et al.* (2017) reported the average value of sperm concentration in Friesian, Jersey and Inyambo bull semen as 2.3.12±0.03 $x10^9$, 3.31±0.05 $x10^9$ and 3.29± 0.07 $x10^9$, respectively.

Baloch *et al.* (2019) reported the mean sperm concentration in HF bull semen as 1407.789±11.42 $x10^6$.

Haris *et al.* (2020) reported the mean sperm concentration in Simmental bull fresh semen as 1.864 ±170.56 $x10^6$sperm/ml.

2.1.4 Progressive sperm motility

Progressive motility refers to sperm that are swimming in a mostly straight line or large circle and it describe the ability of sperm to move properly through the female reproductive tract to reach the ovum.

The progressive forward motility reflects the physiological status of bull spermatozoa after semen collection or cryopreservation and is normally used as an indicator of sperm cell population (Selvaraju *et al.*, 2009).

Rasul *et al.* (2001) reported the mean initial motility in buffalo bull diluted and post thawed semen as 77.3±2.3 and 53.0± 4.6 per cent, respectively.

Rana and Dhami (2004) reported the average sperm motility in Gir bull and Jaffarabady buffalo bull fresh semen as 70.50 ± 0.89 and 66.75 ±1.0 per cent, respectively.

Mandal *et al.* (2005) reported the overall mean value of sperm initial motility in Sahiwal bull post thawed semen as 52.7 ± 1.31 per cent.

Munsi *et al.* (2007) reported the mean value of sperm motility in HF Crossbred bull fresh semen as 77.5 ± 1.5 per cent.

Sarder (2007) reported the mean sperm motility in bull fresh and post thawed semen as 63.95±4.61 and 55.09±5.63 per cent, respectively.

Uysal *et al.* (2007) reported the average value of sperm motility in HF bull fresh semen as 83.5 ± 2.4 per cent.

Shaha *et al.* (2008) reported the average sperm motility in Holstein-Friesian × Zebu and Jersey × Zebu bull fresh semen as 66.2 to 76.0 and 56.6 to 60 per cent, respectively.

Koivisto *et al.* (2009) reported the mean progressive sperm motility in *B. indicus* and *B. taurus* fresh semen as 56.5±0.1 and 58.5±0.3 per cent, respectively.

Fiaz *et al.* (2010) observed the average initial motility in HF and Jersey bull fresh semen as 70.95 ± 0.7 and 73.49 ± 0.8 per cent, respectively.

Reddy *et al.* (2010) reported the mean motility in buffalo fresh semen as 81.33 ± 0.88 percent, respectively.

Hossain *et al.* (2012) reported the mean sperm motility in bull before freezing and after freezing semen as 63.7 and 62.2-63.6 per cent, respectively.

Patel and Siddiquee (2013) observed the average progressive motility in Kankrej bull fresh, diluted, equilibrated and post thawed semen as 86.15 ± 0.30, 80.59 ± 0.43, 74.73 ± 0.58 and 56.83 ± 0.34 per cent, respectively.

Kadirvel *et al.* (2014) reported the mean individual motility in buffalo fresh and frozen thawed semen as 81.45±2.40 and 56.72±4.80 per cent, respectively.

Srivastava and Kumar (2014) reported the mean individual motility in fresh and post-thawed semen in HF semen as 82.2 ± 3.6 and 30.31 ± 1.25; in crossbred bull semen as 78.1 ± 6.3 and 28.1 ± 1.4 per cent, respectively.

Matharoo (2015) reported the mean initial motility in crossbred bull post thaw semen as 67.60±0.47 per cent.

Sannat *et al*. (2015) reported the average progressive sperm motility in Sahiwal, Gir, Jersey and HF bull fresh semen as 73 ± 2.1, 71 ± 2.1, 75 ± 2.3, 74 ± 1 per cent, respectively.

Li *et al*. (2016) reported the mean sperm motility in bull semen with high to low motility in fresh semen as 85.1 ± 0.3 to 55.2 ± 1.8 and in post-thawed semen as 36.5 ± 1.8 to 14.7 ± 1.4 per cent, respectively.

Pawshe *et al*. (2016) reported the mean value of sperm motility in HF bull post thawed semen as 61.7 ± 2.6 per cent.

Rehman *et al.* (2016) reported the mean sperm motility in Friesian, Jersey, Achai, Crossbred and Sahiwal fresh semen as 77.51± 0.65, 76.94± 0.81, 78.88± 1.67, 80.90± 1.58 and 76.50± 3.55 per cent, respectively.

Chaudhary *et al.* (2017) reported the average sperm motility in Gir bull and Surti buffalo bull fresh as 75.00±0.95, 80.42±0.73; in pre freezing semen as 69.38±0.92, 74.38±0.81 and in post thawed as 40.42±1.50, 39.58±1.85 per cent, respectively.

Chauhan *et al.* (2017) reported the mean value of individual motility in Frieswal bull cryopreserved semen as 60.39±0.04 per cent.

D'Andre *et al.* (2017) reported the average sperm motility in Friesian, Jersey, Inyambo bull in fresh semen as 62.04±0.6, 68.14±0.9, 66.01±1.04 and in post frozen semen as 40.43±0.5, 43.45±0.9, 42.20±1.1 per cent, respectively.

Ratnani *et al.* (2017) reported the mean sperm motility in Maduran bull post freezing semen as 54.00 ± 3.94 per cent.

Khalil *et al.* (2018) reported the mean value of sperm motility in HF bull fresh, diluted, equilibrated and post thawed semen as 77 ± 1.7, 71 ± 0.8, 63 ± 1.7 and 50.8 ± 2.7 per cent**,** respectively.

Khan *et al.* (2018) reported the average sperm motility in HF, Sahiwal, Crossbred bull fresh semen as 84.16 ± 0.54, 85.12 ± 0.51, 82.08 ± 0.91 and frozen semen as 71.08 ± 0.29, 76.54 ± 0.55, 40.00 ± 0.35 per cent, respectively.

Lone *et al.* (2018) reported the mean value of sperm motility in buffalo bull fresh, pre- freezing and post-freezing semen as 85.27 ± 5.25, 74.58 ± 5.63 and 51.35 ± 3.38 per cent, respectively.

Baloch *et al.* (2019) reported the mean value of sperm motility in Holstein Friesian bull fresh and post-thaw semen as 74.58± 1.75 and 51.87± 0.648 per cent, respectively.

Haris *et al.* (2020) observed the average individual motility in Simmental bull neat semen as 73.01 ± 1.78 per cent.

Khan *et al.* (2021b) reported the mean individual motility in equilibrated semen at 2hr, 4hr and 6hr in Jersey bull semen as 40.54± 0.51, 74.16± 0.23 and 54.41± 0.32; in HF bull semen as 40.16± 0.44, 71.08± 0.29 and 51.70± 0.35; in Nili-ravi bull semen as 38.62± 0.42, 68.91 ± 0.38 and 50.54± 0.38 per cent, respectively.

2.1.5 Live spermatozoa count

Live spermatozoa/ Sperm viability refer to the percentage of live sperm present in the semen sample. The different staining method are used for differentiating live and dead spermatozoa and was proposed by using eosin-nigrosin stain in which eosin stain the dead sperm cells and nigrosin for the background stain. This mixture gave an identical preparation with stained (dead) cells clearly from the live or unstained cells. The nigrosin provides a smooth background stain than fast green, opal blue, or aniline blue (Blom, 1950).

Rana and Dhami (2004) reported the mean live sperm in Gir and Jaffarabady buffalo bull fresh semen as 71.85 ± 1.49 and 77.90 ± 2.08 per cent, respectively.

Mandal *et al.* (2005) reported the overall mean value of live spermatozoa in Sahiwal bull fresh semen as 70.40 ± 3.91 per cent.

Uysal *et al.* (2007) reported the mean value of total viability in HF bull fresh semen as 82.30 ±2.8 per cent.

Abdel-Khalek *et al.* (2008) observed the average live sperm in H.F. bull diluted semen as 73.0 ± 1.3 per cent.

Bhoite *et al.* (2008) observed the mean live spermatozoa in crossbred bulls having 50 % Brown Swiss, Friesian and Jersey cross bull fresh semen as 78.58 ± 0.288, 76.59 ± 0.14 and 73.84 ± 0.29 per cent, respectively.

Shaha *et al.* (2008) reported the mean value of dead sperm in Friesian cross x Zebu bull semen as 18.4 to 19 per cent.

Reddy *et al.* (2010) reported the mean viability in buffalo fresh semen 83.00 ± 0.57 per cent, respectively.

Mishra *et al.* (2013) reported the value of mean live spermatozoa in as crossbred bull, Red Sindhi, Haryana and Jersey as 88.73±0.47, 88.90±0.47, 88.70±0.59 and 89.53±0.59 per cent, respectively.

Patel and Siddiquee (2013) reported the average of value live spermatozoa in Kankrej bull fresh, diluted, equilibrated and post thawed semen as 90.58 ± 0.20, 87.24 ± 0.44, 79.91 ± 0.24 and 58.22 ± 0.24 per cent, respectively.

Kadirvel *et al.* (2014) observed the mean viability in buffalo fresh and post thawed semen as 88.43±0.70 and 59.68±4.20 per cent, respectively.

Srivastava and Kumar (2014) reported the mean live sperm in fresh and post-thawed semen in HF bull semen as 87.4 ± 1.4 and 55.5 ± 1.0; in crossbred bull semen as 84.1 ± 2.3 and 45.9 ± 1.3 per cent, respectively.

Rehman *et al.* (2016) reported the mean value of dead spermatozoa in Friesian, Jersey, Achai, Cross and Sahiwal bull fresh semen as 18.32± 0.47, 17.68± 0.58, 17.55± 1.19, 25.80± 1.13 and 19.00± 2.54 per cent, respectively.

Chaudhary *et al.* (2017) reported the mean live spermatozoa in Gir bull; Surti buffalo bull fresh semen as 76.50±1.30; 81.75±0.70, pre-freezing stage as 69.92±1.74; 77.04±0.63 and in post thawed semen as 48.92±1.94; 52.38±1.49 per cent, respectively.

Chauhan *et al.* (2017) reported the mean live sperm in Frieswal bull fresh semen as 78.93±0.004 per cent.

D'Andre *et al.* (2017) reported the mean dead spermatozoa in Friesian, Jersey and Inyambo bull fresh as 35.96±0.5, 29.37±0.7 and 34.12±1.05 per cent, respectively.

Rao *et al.* (2017) reported the average live sperm in crossbred bull post thawed semen as 56.24 ± 0.01 per cent.

Khalil *et al.* (2018) reported the mean dead spermatozoa count in HF bull fresh, diluted, equilibrated and post thawed semen as 23 ± 0.7, 26 ± 0.4, 35 ± 1.3, 45 ± 2.2 per cent, respectively.

Khan *et al.* (2018) reported the mean dead spermatozoa in HF, Sahiwal, crossbred bull fresh semen as13.04 ±0.44, 12.62 ±0.44, 14.12 ±0.83 and in frozen semen as 22.20 ±0.32, 19.04 ±0.50, 51.87 ±0.50 per cent, respectively.

Lone *et al.* (2018) reported the mean live spermatozoa in buffalo bull fresh, pre-freezing and post-freezing as 89.45 ± 4.70, 78.33 ± 5.24 and 56.89 ± 3.03 per cent, respectively.

Pal *et al.* (2020b) observed the mean viability in two Hariana bull fresh semen as 87.13 ±1.49 and 86.13± 1.27 per cent.

Khan *et al.* (2021b) reported the mean dead sperm in equilibrated semen at 2hr, 4hr and 6hr in Jersey bull semen as 50.91 ± 0.55, 19.70± 0.32 and 37.54± 0.28; in HF bull semen as 52.37± 0.56, 22.20± 0.32 and 39.50± 0.44; in Nili-ravi bull semen as 53.29± 0.67, 26.08± 0.35 and 42.16± 0.31 per cent, respectively.

2.1.6 Acrosomal integrity

Acrosomal integrity is one of the most important aspects that determine the success of fertilization. The acrosome should be intact so that it undergo capacitation and acrosomal changes in the female reproductive tract to attain fertilizing ability. The optimum fertility depends on the acrosome being structurally and biochemically intact (Binsila *et al.*, 2018).

Rasul *et al.* (2001) observed the mean intact acrosome in bull diluted and post thawed semen as 73.2± 2.4 and 61.8± 2.4 per cent, respectively.

Rana and Dhami (2004) reported the overall mean intact acrosome in Gir and Jaffarabady bull fresh semen as 84.80 ± 0.89 and 83.50 ± 1.24 per cent, respectively.

Munsi *et al.* (2007) reported the mean value of normal intact acrosome in HF crossbred bull fresh semen as 93.5 ± 0.5 per cent.

Reddy *et al.* (2010) reported the mean abnormal acrosome in buffalo fresh semen 5.66 ± 0.88 per cent, respectively.

Mishra *et al.* (2013) reported the mean acrosomal integrity in crossbred, Red Sindhi, Haryana and Jersey bull fresh semen as 82.20±0.47, 80.16±0.47, 81.00±0.69 and 81.73±0.69 per cent, respectively.

Patel and Siddiquee (2013) reported the mean acrosomal integrity in Kankrej bull fresh, diluted, equilibrated and post thawed semen as 81.71 ± 0.11, 75.13 ± 0.42, 68.16 ± 0.80 and 53.65 ± 0.58 per cent, respectively.

Kadirvel *et al.* (2014) observed the average acrosomal integrity in buffalo fresh and post thawed semen as 90.70±1.20 and 71.84±4.4 per cent, respectively.

Mittal *et al.* (2014) observed the mean intact acrosome in Bhadawari bull diluted, equilibrated and post thawed semen as 80.16±0.50, 74.59±0.51 and 69.41±0.54 per cent, respectively.

Srivastava and Kumar (2014) reported the mean intact acrosome in fresh and post-thawed semen in HF bull semen as 89.9 ± 1.2 and 70.5 ± 0.8; in crossbred bull semen as 87.4 ± 2.3 and 67.7 ± 1.2 per cent, respectively.

Sandeep *et al.* (2015) observed the acrosomal integrity in Murrah bull post thaw semen as 36.25 ± 0.91 per cent.

Chaudhary *et al.* (2017) reported the mean acrosomal integrity in Gir bull; Surti buffalo bull in fresh semen as 94.50±0.40; 94.04±0.36, in pre-freezing stage as 90.50±0.45; 90.04±0.39 and in post thawed semen as 79.50±0.59; 79.04±0.44 per cent, respectively.

Dhami *et al.* (2017) reported the mean acrosomal integrity in Gir bull fresh semen as 95.59±0.35 per cent.

Rao *et al.* (2017) reported mean acrosomal integrity in crossbred bull diluted semen as 85.77 ± 0.01 and in post thawed semen as 56.24 ± 0.01 per cent, respectively.

Khan *et al.* (2018) reported the mean value of acrosomal integrity in HF, Sahiwal, Crossbred bull in fresh semen as 71.50 ± 0.53, 79.91 ± 0.51, 62.58 ± 0.67 and in frozen semen as 63.20 ± 0.31, 70.95 ± 0.47, 35.83 ± 0.56 per cent, respectively.

Lone *et al.* (2018) observed the mean acrosomal integrity in buffalo bull diluted semen as 80.17 ± 3.26 per cent.

Kumar *et al.* (2019) reported the mean acrosomal integrity in Hariana bull post thaw semen as 63.14 ± 4.03 per cent.

Khan *et al.* (2021b) reported the mean acrosomal integrity in equilibrated semen at 2hr, 4hr and 6hr in Jersey bull semen as 37.79 ±0.47, 68.41±0.32 and 50.83±0.37; in HF bull semen as 50.83±0.37, 63.20±0.31 and 47.66±0.44; 31.04±0.48; in Nili-ravi bull semen as 25.2±0.41, 59.29±0.26 and 42.12±0.35 per cent, respectively.

2.1.7 Sperm abnormalities

Abnormalities are a measure of the spermatozoa in an ejaculate that are unusual or have undesirable characteristics. Abnormalities generally have estimates that are low to moderately heritable. Abnormalities can be further divided into primary, secondary or tertiary and are further classified as percentage of spermatozoa with an abnormal head, tail and/or cytoplasmic droplet (Butler *et al.,* 2020).

Mandal *et al.* (2005) reported the overall mean value of total abnormal sperm in Sahiwal bull fresh semen as 18.40 ± 3.03 per cent.

Munsi *et al.* (2007) reported the mean value of sperm abnormalities in HF bull fresh semen as 6.5 ± 1.0 per cent.

Uysal *et al.* (2007) reported the mean value of total sperm abnormalities in HF bull fresh semen as 5.40 ± 0.6 per cent.

Koivisto *et al.* (2009) reported the sperm abnormalities in *B. indicus* and *B. Taurus* bull fresh semen as 9.0 to 28.4 and 6.9 to 12.3 per cent, respectively.

Patel and Siddiquee (2013) reported the mean sperm abnormalities in Kankarej bull fresh, diluted, equilibrated and post thawed semen as 4.24 ± 0.03, 6.13 ± 0.07, 7.56 ± 0.15 and 13.52 ± 0.35 per cent, respectively.

Mittal *et al.,* (2014) observed average sperm abnormalities in Bhadawari bull diluted and post thawed semen as 4.91 ± 0.14 and 7.75 ± 0.17 per cent, respectively.

Srivastava and Kumar (2014) observed the average sperm abnormalities in fresh semen of HF and Crossbred bull as 10.1 ± 1.5 and 12.6 ± 2.3 per cent, respectively.

Chaudhary *et al.* (2017) reported the mean value abnormal spermatozoa in Gir bull; Surti buffalo bull in fresh semen as 5.25±0.36; 4.67±0.37, in pre-freezing stage as 7.26±0.42; 6.00±0.39 and in post thawed semen as 11.06±0.34; 10.13±0.33 per cent, respectively.

Rao *et al.* (2017) observed the mean sperm abnormalities in Crossbred bull diluted and in post thawed semen as 7.28 ± 0.02 and 18.36 ± 0.04 per cent, respectively.

Khalil *et al.* (2018) observed the average abnormal sperm in HF bull fresh, diluted, equilibrated and post thawed semen as 19 ± 0.8, 20 ± 1.2, 24 ± 0.5 and 29 ± 1.5 per cent, respectively.

Pal *et al.* (2020b) reported the mean abnormal sperm in two Hariana bull fresh semen as 3.50±0.42 and 3.75±0.49 per cent.

2.1.8 Hypo-osmotic swelling test (HOST)

The hypo-osmotic swelling test (HOST) is used for determining sperm quality by evaluating the membrane integrity of spermatozoa of various farm animals including cattle,

horses and swine and also serve as a useful indicator of fertility potential of spermatozoa (Zubair *et al.*, 2015).

Andrabi *et al*. (2001) observed the average HOST value in Buffalo and Sahiwal bull fresh semen as 41.66 and 69.60 per cent, respectively.

Rasul *et al*. (2001) reported the HOST in bull diluted, equilibrated and post thawed semen as 80.2±3.9, 60.4±5.6, 32.6± 3.8 per cent respectively.

Pant *et al.* (2002) reported the mean percentage of HOST in buffalo bull post thaw semen following conventional, moderate rate and slow rate programmable freezing as 45.0 ± 0.44, 57.5 ± 0.48 and 45.5 ± 0.41 per cent, respectively.

Uysal *et al.* (2007) reported the mean HOST in bull fresh semen as 83.80 ± 3.3 per cent.

Reddy *et al.* (2010) reported the mean HOST positive sperm in buffalo fresh semen 84.00 ± 2.08 per cent, respectively.

Mishra *et al.* (2013) observed the mean HOST in Crossbred, Red Sindhi, Haryana and Jersey bull fresh semen as 83.90±0.51, 81.16±0.51, 83.53±0.66 and 84.36±0.66 per cent, respectively.

Kadirvel *et al.* (2014) observed the average HOST in buffalo fresh and post thawed semen as 74.40±0.80 and 47.75±0.97 per cent, respectively.

Srivastava and Kumar (2014) observed the average HOST in post thaw semen of HF and Crossbred bull as 21.8 ± 0.5 and 21.0 ± 0.6 per cent, respectively.

Rehman *et al.* (2016) reported the mean HOST in Friesian, Jersey, Achai, Cross and Sahiwal bull fresh semen as 71.69± 0.79, 77.42± 0.97, 72.44± 2.00, 68.20± 1.90 and 74.50± 4.25 per cent, respectively.

Chaudhary *et al.* (2017) reported the mean HOST in Gir bull; Surti buffalo bull in fresh semen as 76.92±1.27; 82.25±0.62, in pre-freezing stage as 71.79±1.44; 77.04±0.56 and in post thawed semen as 28.67±1.29; 28.04±1.3 per cent, respectively.

Chauhan *et al.* (2017) reported the overall mean Hypo-osmotic swelling positive spermatozoa in bull fresh semen as 73.76±0.47 per cent.

Dhami *et al.* (2017) reported the mean HOST positive sperm in Gir bull fresh semen 80.30±1.90 per cent.

Ratnani *et al.* (2017) reported the average value of HOST in Maduran bull post freezing semen as 58.50± 1.87 per cent.

Khan *et al.* (2018) reported the mean HOST in HF, Sahiwal, Crossbred bull in fresh semen as 81.66 ± 0.48, 82.25 ± 0.43, 80.62 ± 0.69 and in frozen semen as 69.20 ± 0.39, 78.58 ± 0.45, 38.29 ± 0.45 per cent, respectively.

Lone *et al.* (2018) reported the average HOST in buffalo bull fresh, pre-freezing and post thawed semen as 77.37 ± 5.38, 70.91 ± 5.92 and 51.30 ± 4.43 per cent, respectively.

Baloch *et al.* (2019) observed the average HOST positive sperm in HF bull fresh semen and post thawed semen as 58.37 and 44.79 per cent, respectively.

Khan *et al.* (2021b) reported the mean HOST positive sperm in equilibrated semen at 2hr, 4 hr and 6 hr in Jersey bull semen as 40.91± 0.49, 70.95± 0.33 and 51.79± 0.33; in HF bull semen as 40.75± 0.45, 69.20± 0.31 and 49.91± 0.41; in Nili-ravi bull semen as 37.12± 0.31, 66.95± 0.38 and 48.70± 0.32 per cent, respectively.

2.2 Cryopreservation of semen

2.2.1 Cryopreservation

Artificial insemination is a widely used technique in animals that mostly uses cryopreserved semen to handle the speed of genetic improvement by inseminating selected or multiple females with the semen collected from a male of good genetic quality. (Flores *et al.,* 2011; Comizzoli, 2015; Masoudi *et al.*, 2016).

The initiation of semen cryopreservation was done 200 years backs by Lazaro Spallanzani in 1776 and preserves spermatozoa by cooling it in snow (Royere *et al.*, 1996). The cryopreservation of spermatozoa was introduced in the 1960s to preserve fertility

(Hezavehei *et al.*, 2018). The scientific advancement was made noticeably later with Polge's discovery of glycerol's which have a cryoprotective properties and observed that the invention of glycerol made a turning point within the sphere of fertility preservation which considerable improves the techniques for cryopreservation of semen of various species (Polge *et al.*, 1949). The sperm cryobanks were deployed in the 1960s for cattle and in the 1970s for humans (Sanger *et al.*, 1992). In present days, animal and human assisted reproductive technology regularly uses cryopreserved semen (Kopeika *et al.*, 2015; Yeste, 2016).

The freezing of spermatozoa may be a right solution to take care of fertility and the frozen-thawed semen is used for intrauterine insemination (IUI), IVF or intracytoplasmic sperm injection (Dohle, 2010).

The cellular damage due to cooling or freezing affect both the structure and performance of the cells (Watson, 1990).

The adding of cryoprotectant agent (CPA) in semen provides some defense to spermatozoa and minimize the harmfull effects of cryopreservation (Katila, 1997).

2.2.2 Effects of Cryopresentation

Under ideal conditions, it is predictable that little damage will occur to spermatozoa during the freezing process (Andrabi, 2007). However, the spermatozoa undergo some stressful condition that includes cold shock, osmotic stress, and ice crystals formation when expose to cryopreservation (Celeghini *et al.*, 2008).

The plasma membrane of the spermatozoa is the main site of injury during cryopreservation (Tvrda *et al.*, 2011). The cryopreservation causes excessive production of reactive oxygen species (ROS) due to various stresses it must endure like atmospheric oxygen exposure, thermal shock, removal of seminal plasma (Bailey *et al.*, 2003).

Throughout the cryopreservation steps, the mammalian spermatozoa must tolerate various types of stresses caused by ice crystals formation, chemical toxicity, and oxidative stress, which mainly damage cytoplasm membrane, consequently resulting in a lower post-thawed quality and fertility (Salamon and Maxwell, 1995; Watson, 1995; Holt, 2000).

2.3 Oxidative stress (OS)

MacLeod (1943) reported toxic effects of O_2 on sperm and observed that experiments disbursed *in vitro* that increased O_2 concentrations reduces sperm motility and suggested that H_2O_2, generated by cells from O_2, was the actual toxic agent.

Tosic and Walton (1946) observed the damaging effects of H_2O_2 on bovine sperm motility and viability.

Sikka *et al.* (1995) stated that oxidative stress may be a condition related to an increased rate of cellular damage induced by oxygen and oxygen derived oxidants commonly referred to as ROS.

Bilodeau *et al.* (2000) and Gürler *et al.* (2015) stated that the cattle semen cryopreservation is well known to cause excessive production of ROS and to decrease antioxidative activity.

Agarwal and Saleh (2002) and Aitken and Baker (2006) stated that OS decreases sperm quality and male fertility in vivo.

Wathes *et al.* (2007) stated that the key reason for incidence of OS in semen is depletion of seminal antioxidants and excess generation of free radicals by sperm.

Agarwal *et al.* (2008) stated that each one cellular components including lipids, proteins, nucleic acids, and sugars are potential targets of oxidative stress.

Bucak *et al.* (2010) stated that OS is the most significant factors causing poor quality semen and has been reported to be oxidative stress.

Desai *et al.* (2010) stated that production of ROS that exceed the antioxidant ability of the seminal plasma which ends up in oxidative stress (OS) which is injurious to spermatozoa.

Agarwal and Majzoub (2017) stated the free radicals appear to have an important role in cell damage after freezing and thawing, as antioxidant supplementation improves the quality of cryopreserved sperm.

Bollwein and Bittner (2018) stated that oxidative stress (OS) has a main role in pathophysiology of nearly all biological systems.

2.3.1 Effect of Reactive Oxygen Species (ROS)

Aitken (1995) reported that peroxide (H_2O_2), gas (NO), and anion (O_2–) have positive eff ects on intracellular signaling, sperm capacitation, and acrosome reactions.

Lamirande and Gagnon (1995) and Agarwal and Prabakaran (2005) stated that ROS are formed as a necessary by-products throughout the conventional enzymatic reactions of inter- and intracellular signaling.

Lamirande and Gagnon (1995) and Agarwal and Prabakaran (2005) stated that mammalian spermatozoa represent a growing list of cell types that exhibit a capacity to get ROS when incubated under aerobic conditions, such as, oxide (H_2O_2), the superoxide ($\bullet O_2^-$), the chemical group (OH•), and hypochlorite radical.

Lamirande and Gagnon (1995) and Agarwal and Prabakaran (2005) stated that thanks to high reactive nature of spermatozoa, ROS can combine readily with other molecules, directly causing oxidation that may result in structural and functional changes and lead to cellular damage.

Medeiros *et al.* (2002) reported that the precise mechanism of ROS generation and performance haven't been fully characterized in sperm.

Said *et al.* (2010) reported that in cryopreservation, any changes in mitochondrial membrane fluidity may lead to the discharge of ROS and changes within the membrane potential.

Tvrda *et al.* (2011) observed that the ROS attacks the phospholipid and cholesterol of the cell wall causing lipid peroxidation and cholesterol efflux during cryopreservation resulting in disturbances within the cholesterol and phospholipid ratio.

Tvrda *et al.* (2011) observed that successive insults faced by sperm cells within the kind of lipid peroxidation, ice crystallization, changes in pH, and force per unit area will cause debilitated motility and membrane integrity, DNA damage, and necrobiosis and these

factors will eventually result in necrobiosis and compromise the fertilization capacity of sperm.

Ugur *et al.* (2019) stated that the suitable levels of molecules like peroxide (H_2O_2), gas (NO), and superoxide (O_2–) play a significant role in sperm physiology, namely capacitation and acrosome reaction, they're detrimental to sperm function at high concentrations because of toxicity.

Khan *et al.* (2021) reported that when reactive oxygen species (ROS) exceeds the defense mechanisms of sperm, consequent damage occurs within the semipermeable membrane structure and molecular modification additionally.

2.3.2 Lipid peroxidation (LPO)

The lipid peroxides, assayed in terms of malanaldehyde (MDA) concentration is reason for membrane damage and reduced motility (Muzafer *et al.,* 2012).

The way of ROS induced damage to spermatozoa include an oxidative attack on the sperm membrane lipids follows in start of lipid peroxidation (LPO) fall (Sharma and Agarwal, 1996).

The mammalian spermatozoa are well-known to be at risk of loss of motility within the exogenous oxidant, as a results of LPO (Rao *et al.,* 1989).

Bucak *et al.* (2009b) reported that MDA level in Angora goat post thawed semen as 1.54±0.18 nmol/2X10^8 spermatozoa.

Sarıözkan *et al.* (2009a) reported the mean MDA levels in bull post thawed semen of three groups, namely taurine (2 mM), cysteine (2 mM), and control as 1.5 ± 0.2, 0.8 ±0.2 and 0.7 ±0.2 nmol/ml respectively.

Sariözkan *et al.* (2009b) reported the mean MDA level in bull post thawed semen as 0.70 ± 0.13 nmol/ml.

Bucak *et al.* (2010) reported the mean LPO level in bull post thawed semen as 20.42 ± 1.92 µM 10^9cell/ml.

Domínguez-Rebolledo *et al.* (2010) reported the mean MDA level in deer post thawed semen as 10.8 ± 1.2 nmol MDA/10^8 spermatozoa.

Paudel *et al.* (2010) reported the mean MDA level in Swedish bull post thawed semen at 10 mM ascorbic acid as 712.1 ± 49.1 nmol⁄10^9sperms.

Sarıözkan *et al.* (2010) reported the mean LPO production in Angora goat post thawed semen as 2.82 ± 0.28 nmol/L.

Tuncer *et al.* (2010) reported the mean MDA level in HF bull post thawed semen at different concentration of cysteine *i.e.* 5, 10 mM and control as 2.30 ±0.11, 4.99 ±0.44 and 1.44 ±0.08 nmol/mL, respectively.

Sicherle *et al.* (2011) reported the mean LPO level in ram post thawed semen control, catalase and trolox as 1.2 ± 0.18, 1.2 ± 0.25 and 1.4 ± 0.20 nmol/10^8 sperm, respectively.

Asadpour *et al.* (2012) reported the MDA levels in HF bull post thawed semen at different concentration of BHT *i.e.* 0.5, 1.0, 2.0, 4.0 mM and control as 3.90 ± 0.38, 5.4 ± 0.17, 5.54 ± 2.31, 6.07 ± 0.44 and 6.84 ± 0.29 nmol/dL, respectively.

Chhillar *et al.* (2012) reported the mean MDA level in Karan Fries bull fresh and post thawed semen as 1.43 ± 0.12 and 2.61 ± 0.33 nmol/10^8 cells respectively.

Taşdemir *et al.* (2012) reported the mean LPO level in HF bull frozen thawed semen as 0.94 ± 0.20 µm/ml 10^9cell/ml.

Kadirvel *et al.* (2014) reported the mean LPO production in buffalo fresh and post thawed semen as 278.78±18.28 and 364.67±22.40 nmol MDA/ 10^9Spermatozoa, respectively.

Karaji *et al.* (2014) reported the mean MDA level in HF and Simmental bull post thawed semen as 12.21 ±1.07 and 15.31 ±0.74 pmol mg-1 respectively.

Sandeep *et al.* (2015) reported the mean MDA level in Murrah buffalo bull diluted semen as 784.50 ± 8.31 ng/120 million sperm.

Sarıözkan *et al.* (2015) reported the mean MDA level in Brown swiss bull post thawed semen as 2.31 ±0.76 nmol ml.

Bansal and Cheema (2016) reported the mean MDA level in buffalo 1, 2 and 3 post thaw semen as 406.87+45.03, 196.83+4.89 and 201.83+3.56 nmoles of MDA /µg protein/ml, respectively.

Büyükleblebici *et al.* (2016) reported the mean MDA level in Brown Swiss post thawed semen as 0.50±0.27 µm/ml-10^9 cell/ml.

Motemani *et al.* (2017) reported the average MDA level in HF bull post thawed semen at different concentrations of α-tocopherol *i.e.* in control, Vit E 1.2 mM, Vit.E 2.4 mM and Vit E 4.8 mM as 7.2±0.6, 8.1±0.6, 8±0.6 and 6.1±0.6 nmol/mL, respectively.

Chikhaliya *et al.* (2018) reported the mean value of MDA level in Gir bull post thawed semen at different concentration of Taurine *i.e.* 25 mM, 50 mM, 75 mM and Control as 24.53 ± 0.63, 15.95 ± 0.74, 28.48 ± 0.55 and 28.07 ± 1.26 µmol/ml, respectively.

Lone *et al.* (2018) observed the mean MDA level in buffalo bull diluted and post thawed semen as 297.87 ± 22.61 and 496.02 ± 39.28 nmol MDA/ 10^9 spermatozoa, respectively.

Singh *et al.* (2020) reported the mean MDA level in crossbred bull post thawed semen control group and ascorbic acid (5mM) group as 3.49 ± 0.19 and 1.70 ± 0.04 nmol/10^8 Spermatozoa, respectively.

2.3.3 Catalase (CAT)

The catalase (CAT) is the most resourceful natural enzyme when catalysing the split of H_2O_2 into H_2O and O_2; H_2O_2 is one of the reactive oxygen species (ROS) linked in oxidative stress.

Bucak *et al.* (2009b) reported that CAT activity in Angora goat post thawed semen as 6.063±0.65 kU/2X10^8 spermatozoa.

Sarıözkan *et al.* (2009a) reported the mean CAT level in bull post thawed semen in taurine (2 mM), cysteine (2 mM), and control as 35.1 ± 8.1, 11.0 ± 2.6 and 6.8 ± 1.8 kU/g, respectively.

Hu *et al*. (2010a) reported the average value of CAT in bull post thawed semen at different concentrations of ascorbic acid *i.e.* with 0, 2.5, 4.5, 6.5 and 8.5mg/ml as 2.02 ± 0.15, 2.08 ± 0.28, 3.87 ± 0.36, 2.03 ± 0.45 and 1.39 ± 0.51 U/ml, respectively.

Hu *et al.* (2010b) reported the mean CAT level in bull diluted semen with an extender containing trehalose at different concentration *i.e.* with 0, 25, 50, 100, and 200 mM as 2.01 ± 0.12, 2.25 ± 0.31, 2.19 ± 0.27, 3.17 ± 0.56 and 3.06 ± 0.49 U/mL, respectively.

Taşdemir *et al.* (2012) reported the mean CAT level in HF bull frozen thawed semen as 12.9 ± 1.49 µm/ml 10^9cell/ml.

Ashrafi *et al.* (2013) reported that the mean CAT level in HF bull post thawed semen as 0.0 ± 0.0 U/ml.

Kadirvel *et al.* (2014) reported the mean CAT level in buffalo fresh and post thawed semen as 0.80±0.01 and 0.00±0.00 U/10^9 spermatozoa, respectively.

Zhao *et al.* (2015) reported the mean CAT level in Qinchuan bull frozen thawed semen as 1.16 ± 0.82 U/ml.

Sharma *et al.* (2016) reported the catalase level in buffalo bull post thawed semen in rainy, winter and summer season as 23.36 ± 0.25, 24.25 ± 0.30 and 24.22 ± 0.56 µmol H_2O_2 decomosed/ min/ 10^8 spermatozoa, respectively.

Lone *et al.* (2018) reported the catalase activity in buffalo bull diluted semen as 0.001 ± 0.00 nM/mg protein.

Singh *et al.* (2020) reported the mean catalase level in crossbred bull post-thaw semen control group and ascorbic acid (5mM) group as 1.18 ± 0.36 and 1.31 ± 0.73 µmol/10^8 Spermatozoa, respectively.

2.3.4 Superoxide dismutase (SOD)

The superoxide dismutase (SOD) is a major part of antioxidative defense systems and plays a defensive role for spermatozoa from oxidative damage (Yan *et al.*, 2014). The superoxide is a free radical and that is converted to oxygen and hydrogen peroxide by the dismutation activity of antioxidant agent superoxide dismutase (Tariq *et al.*, 2015). The SOD protect spermatozoa against natural O_2 toxicity and LPO (Saraswat *et al*., 2016).

Magnes and Li (1980) reported the mean SOD level in bovine post thawed semen as 41 U/10^9 spermatozoa.

Beorlegui *et al.* (1997) reported the SOD level in bull post thawed semen ranging between 44 to 144U/10^{10} spermatozoa.

Bucak *et al.* (2009b) reported that SOD level in Angora goat post thawed semen as 1.64±0.16 U/2X10^8 spermatozoa.

Sarıözkan *et al.* (2009a) reported the mean SOD level in bull post thawed semen in taurine (2 mM), cysteine (2 mM), and control as 2.1 ± 0.4, 21.4 ± 2.9 and 7.2 ± 1.8 U/g protein, respectively.

Sariözkan *et al.* (2009b) reported the mean SOD level in bull post thawed semen as 0.11 ± 0.024 µkat/g protein.

Hu *et al*. (2010a) reported the mean SOD level in bull post thawed semen in ascorbic acid at different concentrations *i.e*. with 0, 2.5, 4.5, 6.5 and 8.5mg/ml as 1.57 ± 0.11, 1.36 ± 0.13, 1.42 ± 0.09, 1.50 ± 0.07 and 1.46 ± 0.02 U/ml, respectively.

Hu *et al.* (2010b) reported the mean SOD level in bull post thawed semen in trehalsoe at different concentration *i.e.* with 0, 25, 50, 100, and 200 mM as 1.58 ± 0.08, 1.46 ± 0.11, 1.58 ± 0.07, 1.38 ± 0.09 and 1.60 ± 0.10 U/mL, respectively.

Sarıözkan *et al.* (2010) reported the mean SOD activity in Angora goat post thawed semen as 0.12 ± 0.01 U/mg protein.

Ashrafi *et al.* (2013) reported the mean SOD level in HF bull frozen semen as 5.5 ± 1.7 % inhibition.

Kadirvel *et al.* (2014) reported the mean SOD activity in buffalo fresh and post thawed semen as 23.34±0.77 and 11.78±0.88 U/10^9 spermatozoa, respectively.

Sarıözkan *et al.* (2015) reported the mean SOD level in Brown swiss bull post thawed semen as 2.75 ±0.24 U/ ml.

Zhao *et al.* (2015) reported the mean SOD level in Qinchuan bull frozen thawed semen as 1.9 ± 0.11U/ml.

Bansal and Cheema (2016) reported the mean SOD activity in 3 buffalo bull semen post thawed semen as 96+0.94, 174.74+4.86 and 267.85+6.05 IU/ 10^9/ min, respectively.

Liu *et al.* (2017) reported the mean SOD activity in boar post thawed semen 318.5±31.4 U/10^9spermatozoa.

Lone *et al.* (2018) observed the level of superoxide dismutase in buffalo bull diluted and post thawed semen as 0.39 ± 0.06 and 0.16 ± 0.03 Units/mg protein respectively.

Singh *et al.* (2020) reported the mean SOD level in crossbred bull post-thaw semen in control group and ascorbic acid (5mM) group as 0.95 ± 0.06 and 1.31 ± 0.05 Units/10^8 spermatozoa, respectively.

2.4 Antioxidants

The antioxidants may be a protective *viz*. catalase, transferring and EDTA, which protects the formation of ROS, or scavenging antioxidants such as ascorbic acid and vitamin E, that remove the existing ROS (Lampiao, 2012).

Antioxidants are agents that can neutralize or reduce the formation of free radicals, breakdown the substrates of oxidation, and decrease the risk of injuries to spermatozoa during cryopreservation (Roca *et al.*, 2004).

These antioxidants prevent the sperm cells from deleterious lipid peroxidation and prevent its integrity and viability (Perumal *et al.*, 2013).

Some of the primary antioxidants naturally present in mammalian semen are glutathione, reduced glutathione, glutathione peroxidase, superoxide dismutase, catalase Vit E and C, melatonin, etc (Perumal *et al.*, 2013).

An antioxidants can also be categorized into enzymatic antioxidants viz. GSH, catalase, SOD and non-enzymatic antioxidants viz. carotenoids, vitamins C and E, taurine, albumin, cysteines (Bansal and Bilaspuri, 2011).

Bilodeau *et al.* (2000) reported that there is a significant reduction in the level of intracellular antioxidants following a freeze/thaw cycle thus, antioxidant supplementation is beneficial to defend the sperm cells from oxidative stress during the cryopreservation process.

2.4.1 Cysteine

The cysteine is an amino acid containing thiol and is an antecedent of intracellular glutathione (Uysal and Bucak, 2007) and it was seen that cysteine infiltrates the cell membrane layer effectively, improves the intracellular GSH biosynthesis both *in vivo* and *in vitro* and ensures the membrane lipids and proteins because of indirect radical scavenging properties (Hendin *et al.*, 1999).

Holt (1997) stated that cysteine is one of the additives that have been used in freezing extender of human, boar, goat, bull to improve post-thaw sperm parameters.

Atessahin *et al.* (2008) reported in frozen semen of Angora goat with cysteine (0, 5, 10 and 15 mM), the value of mean MDA level as 6.27 ± 0.18, 7.54 ± 0.48, 9.02 ± 0.51 and 8.66 ± 0.69 nmol/ml, respectively and value of mean CAT activity as 638.2 ± 10.2, 373.2 ± 12.1, 945.9 ± 77.0 and 872.8 ± 89.1 kU/L, respectively.

Bucak *et al.* (2008) reported in frozen thawed semen of ram in control and with cysteine (5mM) added group, the value of mean MDA level as 9.20 ± 0.51 and 9.96 ± 0.34 nmol/ml, respectively and value of mean CAT activity as 738.81 ± 39.69 and 842.40 ± 90.42 kU/L, respectively.

El-Sheshtawy *et al.* (2008) reported the mean per cent value in bull post thawed semen cysteine (5mM) as 65.00±2.47 for initial motility, 72.80±2.48 of HOST and 69.60±1.33 for acrosomal integrity.

Michael *et al.* (2009) reported that L-cysteine had very partial positive effects on chilled canine semen parameters.

Sarıözkan *et al.* (2009a) reported in post thawed semen of bull with cysteine (2 mM) and control, the value of average motility as 57.8 ± 1.2 and 49.6 ± 1.1 per cent, respectively, value of average HOST positive sperm as 48.4 ± 1.9 and 43.8 ± 2.1 per cent, respectively and value of average sperm abnormalities as 8.0 ± 0.5 and 15.0 ± 1.1 per cent, respectively.

Andreea and Stela (2010) reported the average lipid peroxidation level in post thaw semen of ram A in control and cysteine (10mM) added group as 5.55±0.55 and 4.06±0.35 and in post thaw semen of ram B as 5.61±0.51 and 3.72±0.31 MDA nmol/10^8 spermatozoa, respectively.

Tuncer *et al.* (2010) reported in post thawed semen of HF bull with cysteine at 5, 10 mM concentration and control, the value of mean motility as 48.61 ± 4.60, 48.06 ± 4.69 and 52.22 ± 2.46 per cent, respectively and value of sperm abnormalities as 10.00 ± 0.56, 9.11 ± 0.60 and 8.83 ± 0.57 per cent, respectively.

Beheshti *et al.* (2011b) stated that the addition of additives such as cysteine to the semen freezing extender, prevents cryodamage to spermatozoa metabolism an antioxidant capacities.

Memon *et al*. (2011) stated that cysteine has cryoprotective impact on the functional integrity of axosome and mitochondria further developing frozen thawed semen motility in various species.

Perumal *et al.* (2011) reported the addition of Cysteine (5mM) non-significantly lowers ($p = 0.15$) lipid peroxide (lipid peroxidation (nmol/10^8 sperm) levels when compared to control in crossbred jersey bull.

Topraggaleh *et al.* (2013) reported in post thawed semen of buffalo with cysteine (5mM, 7.5mM, 10mM) and control group, the value of mean individual motility as 63.55 ± 2.00, 67.15 ± 1.46, 53.43 ± 2.58 and 56.16 ± 1.67 per cent, respectively and the value of HOST as 65.75 ± 1.02, 70.97 ± 0.85, 64.32 ± 0.87 and 61.17 ± 0.98 per cent, respectively.

Büyükleblebici *et al.* (2014) reported in post thawed semen of HF bull with control and cysteine (5mM) added semen, the value of mean individual motility as 46.63±2.40 and 49.00±3.10 per cent, respectively, value of mean abnormal acrosome as 2.25±0.25 and 2.50±0.33 per cent, respectively, value of mean total abnormalities as 11.13± 0.77 and 12.25±1.21 per cent, respectively, value of mean HOST positive sperm as 41.50±2.32 and 37.75±2.38 per cent, respectively and value of mean LPO level as 2.01±0.26 and 3.21±0.27 nmol/ml, respectively.

Tasdemir *et al.* (2014) reported the mean individual motility, sperm abnormalities, abnormal acrosome, LPO level and CAT activity in HF bull post thawed semen in control 51.9±3.89, 17.5±0.57, 5.8±0.45 per cent, 0.5±0.27 µm/ml-10^9 cell/ml and 18.9±4.94 µm/ml-10^9 cell/ml, respectively and with cysteine (5mM) added group as 52.5±5.00, 9.8±0.80, 3.4±0.53 per cent, 0.44±0.28 µm/ml-10^9 cell/ml and 15.7±4.73 µm/ml-10^9 cell/ml, respectively.

Varghese *et al.* (2015) reported the mean motility, viability, sperm abnormalities, acrosomal integrity and HOST positive sperm in buffalo semen in control after dilution as 75.12 ±0.77, 82.75±0.76, 4.93±0.25, 92.85 ±0.34 and 82.72 ±0.91 per cent, respectively; at pre-freeze stage as 65.37±0.84, 72.75±0.99, 6.35±0.27, 89.70±0.39 and 70.82±0.92 per cent, respectively; after thawing as 38.37±0.95, 44.82±1.02, 9.10±0.22, 82.43±0.32 and 42.52±1.04 per cent, respectively. In cysteine HCl (0.5 mg/ml) after dilution as 77.75±0.69, 84.27±0.66, 4.68±0.26, 93.08±0.32 and 83.40±0.70 per cent, respectively; at pre-freeze stage as 68.37±0.81, 70.62±0.81, 5.73±0.28, 90.58±0.34 and 74.17± 0.93 per cent, respectively; after thawing as 39.50±0.80, 46.37±0.99, 8.15±0.26, 84.40±0.18 and 45.10±1.04 per cent, respectively. In cysteine HCl (1.0 mg/ml) after dilution as 80.62±0.65, 86.00±0.49, 4.53±0.25, 93.28±0.33 and 85.85±0.67 per cent, respectively; at pre-freeze stage as 72.62±0.69, 78.97±0.93, 5.45±0.25, 90.90±0.35 and 78.12±0.79 per cent,

respectively; after thawing as 46.50± 0.72, 52.97±0.79, 7.10±0.26, 85.73±0.18 and 51.62±0.82 per cent, respectively.

Wadood *et al.* (2015) reported mean motility, live sperm, HOST positive sperm, acrosomal integrity and LPO level in buffalo bull post thawed semen in control as 43.00±3.22, 61.69±3.32, 52.55±3.38, 54.56±3.48 per cent and 31.59±3.95 nm, respectively; with cysteine (2.0mM) as 47.00±2.33, 65.27±2.10, 56.24±3.49, 57.55±2.05 per cent and 35.17±5.23 nm, respectively; with cysteine (5.0mM) as 43.67±1.79, 65.70±2.16, 56.27±3.07, 53.20±3.78 per cent and 29.61±3.99 nm, respectively; with cysteine (8.0mM) as 40.33±2.69, 62.97±3.02, 53.88±2.37, 56.40±3.70 per cent and 27.05±2.22 nm, respectively.

Ansari *et al.* (2016) detailed the average initial motility and HOST positive sperm in buffalo bull semen were higher in extender containing 1.0mM cysteine than those with 0.5mM or 0.0mM at 0, 2 and 4 hours frozen-thaw at 37°C.

Ansari *et al.* (2016) observed the average live sperm in buffalo bull semen was higher in extender containing 0.5mM and 1.0mM cysteine than those with 0.0mM cysteine at 0, 2 and 4 hours frozen-thaw at 37°C.

Bhardwaz *et al.* (2016) reported the mean progressive motility, live sperm, acrosomal integrity and sperm abnormalities in Jersey bull post thawed semen control as 53.88±0.43, 54.38±0.49, 74.83±0.44 and 10.38±0.28 per cent, respectively; with cysteine (10mM) as 63.92±0.44, 64.67±0.49, 78.17±0.4 and 9.43±0.28 per cent, respectively; with cysteine (12mM) as 50.05±0.43, 50.83±0.49, 69.83±0.44 and 11.05±0.28 per cent, respectively; with cysteine (15mM) as 40.05±0.43, 40.83±0.49, 59.83±0.44 and 22.11±0.28 per cent, respectively.

Patel *et al.* (2016) reported the mean motility, livability and sperm abnormalities in post thawed semen of Mehsani buffalo bull in control as 52.83±0.52, 78.70±0.21 and 5.43±0.21 per cent, respectively and in cysteine HCl (1mg/ml) added semen as 57.83±0.52, 82.33±0.23 and 5.03±0.17 per cent, respectively.

Al-Dahan *et al.* (2020) reported the mean MDA level in HF bull post thawed semen control and cysteine (5mM) as 1.025 ± 0.38 and 1.06 ± 0.12 mmol/ ml, respectively.

Ali and Banana (2020) reported the mean motility, viability, HOST positive sperm and acrosomal integrity in HF bull equilibrated semen in control as 50.71±2.45, 92.97±0.77, 90.00±0.57 and 93.42±0.42 per cent, respectively; with cysteine (2mM) as 52.57±3.40, 93.50±0.77, 92.57±0.64 and 94.85±0.55 per cent, respectively; with cysteine (4mM) as 53.85±3.42, 93.65±0.80, 92.85±0.70 and 94.85±0.57 per cent, respectively.

Pal *et al.* (2020a) reported the mean motility, live sperm, acrosomal integrity and HOST positive sperm in Hariana bull semen in control after dilution as 70.88±0.61, 83.50±0.90, 82.25±0.94 and 80.25±0.98 per cent respectively; after equilibration as 65.00±1.10, 78.88±0.79, 77.13±0.91 and 74.38±0.70 per cent, respectively; after thawing as 58.25±1.54, 71.00±1.65, 69.00±1.84 and 68.38±1.67 per cent respectively. In cysteine (5mM) after dilution as 73.75±0.52, 86.37±0.86, 83.75±0.88 and 82.75±0.98 per cent, respectively; after equilibration as 68.38±1.16, 81.88±0.66, 78.50±0.90 and 76.75±0.59 per cent, respectively; after thawing as 62.75±1.37, 75.88±0.35, 73.13±0.69 and 72.88±0.39 per cent, respectively.

Khan *et al.* (2021a) reported the mean motility, HOST positive sperm, acrosomal integrity and live sperm in Sahiwal × Holstein-Friesian bull frozen thawed semen in control as 50.00±1.60, 42.29±1.63, 40.83±1.56 and 49.87±1.50 per cent, respectively; cysteine (5mM) as 52.80±1.44, 45.56±1.77, 43.41± 1.60 and 52.66± 1.44 per cent, respectively; with cysteine (7.5mM) as 60.60±1.89, 54.48± 1.74, 54.11±2.33 and 66.35± 1.65 per cent, respectively; with cysteine (10mM) as 51.12±2.20, 43.82± 1.62, 40.15±1.45 and 50.50± 1.73 per cent, respectively.

Wafa *et al.* (2021) reported the mean HOST positive sperm, individual motility, live sperm, abnormal sperm and intact acrosome in buffalo bull post thawed semen in control group as 56.83±0.90, 48.33±1.06, 54.58±1.16, 35.75±1.51 and 34.33±1.04 per cent, respectively and with cysteine (1mM) added semen as 66.33±0.98, 58.92±1.68, 64.33±1.02, 25.75±1.07 and 24.75±0.95 per cent, respectively.

2.4.2 Taurine

The taurine is a sulfonic amino acid which act as a non-enzymatic scavenger that plays a major role in the defence of spermatozoa against ROS in case of exposure to aerobic condition and the freezing-thawing process. It display an antioxidant properties by increasing catalase level in close union with SOD concentration (Reddy *et al.*, 2010).

Bucak and Tekin (2007) reported the average per cent value in ram semen at 5^{o}C added with Taurine (50 mM), the initial motility was 76.4 ± 2.4, abnormal sperm was 7.9 ± 1.0 and live spermatozoa was 78.3 ± 2.6 in ram semen.

Bucak *et al.* (2007) reported in frozen semen of ram with taurine (0, 25 and 50mM) as 9.2 ± 0.5, 11.4 ± 1.1, 11.2 ± 0.9 nmol/ml, respectively and value of CAT activity as 738.8 ± 39.7, 1139.6 ± 128.6, 1037.5 ± 68.2 kU/L, respectively.

Atessahin *et al.* (2008) reported in frozen thawed semen of Angora goat with taurine (0, 25, 50 and 75 mM), the value of mean MDA level as 6.27 ± 0.18, 5.54 ± 0.31, 9.80 ± 0.51, 4.46 ± 0.31 nmol/ml, respectively and value of CAT activity as 638.2 ± 10.2, 744.9 ± 78.6, 866.7 ± 129.5, 856.4 ± 101.4 kU/L, respectively.

Sarıözkan *et al.* (2009a) reported in post thawed semen of bull with taurine (2 mM) and control, the value of average motility as 43.3 ± 2.4 and 49.6 ± 1.1 per cent, respectively, value of average HOST positive sperm as 46.6 ± 1.9 and 43.8 ± 2.1 per cent, respectively and value of average sperm abnormalities as 14.0 ± 0.7 and 15.0 ± 1.1 per cent, respectively.

Reddy *et al.* (2010) observed the average motility in buffalo post thawed semen in control and with addition of taurine (50mM) as 31.67±1.67 and 48.33±1.67 per cent, respectively.

Chhillar *et al.* (2012) reported in post thawed semen of Karan fries bull in control and taurine (50mM) added semen, the value of mean MDA level as 2.61 ± 0.33 and 1.49 ± 0.14 nmol MDA/10^{8} cells, respectively, value of mean individual motility as 36 ±3.05 and 51 ±1.52 per cent, respectively, value of mean sperm viability as 38.66 ± 1.2 and

59.00±1.73 per cent, respectively and value of mean HOST positive sperm as 37.667 ± 3.38 and 54 ± 1.15 per cent, respectively.

Oh *et al.* (2012) reported in post thawed semen of Korean jeju black bull in control and with taurine (20mM) added semen, the value of average progressive motility as 64.00 ± 7.42 and 69.00 ± 5.12 per cent, respectively, value of mean viability as 58.25 ± 6.03 and 63.80 ± 7.50 per cent, respectively and value of HOST as 51.90 ± 9.99 and 60.50 ± 6.86 per cent, respectively.

Singh *et al.* (2012) reported the mean motility in buffalo bull fresh semen as 85.00 ± 2.88 whereas, in post thaw semen the value in control and taurine (50mM) are as 36.00 ± 3.05 and 61.66 ± 2.02 per cent, respectively.

Beheshti *et al.* (2013) reported the mean per cent value in bull post thawed semen of live spermatozoa, acrosomal integrity and initial motility with taurine as semen additive at 50 mM concentration as 73.98 ± 0.62, 67.57 ± 0.33 and 56.90 ± 0.19, respectively.

Kumar *et al.* (2013) reported the mean motility and HOST positive sperm in buffalo bull in fresh semen as 87.0 ± 3.78 and 76.00 ± 3.05 per cent, respectively and in post thawed control, taurine (50mM) added semen as 40.33 ± 3.17 and 37.66 ± 0.33, 59.33 ± 5.2 and 61.66 ± 2.03 whereas in Karan fries bull fresh semen as 83.66 ± 2.72 and 72.00 ± 2.88 per cent, respectively and in post thawed control and taurine (50mM) added semen as 37.66 ± 4.33 and 37.33 ± 3.28, 55.66 ± 4.25 and 56.00 ± 2.08 per cent, respectively.

Mughal *et al.* (2013) reported in post thawed semen of buffalo bull with taurine (0, 20, 40 and 60 mM), the value of mean initial motility as 49.7 ± 2.5, 49.0 ± 1.8, 45.3 ± 2.0 and 41.0 ± 2.0 per cent, respectively, value of mean live sperm as 57.9 ± 3.3, 58.4 ± 2.4, 57.7 ± 2.6 and 57.4 ± 2.3 per cent, respectively, value of mean acrosomal integrity as 69.8 ± 2.2, 72.4 ± 2.3, 71.5 ± 1.7 and 70.6 ± 2.0 per cent, respectively, value of mean HOST positive sperm as 59.6 ± 3.4, 57.2 ± 2.5, 62.9 ± 1.4 and 52.9 ± 3.7 per cent, respectively and mean LPO production as 65.9 ± 8.9, 57.7 ± 10.6, 63.8 ± 8.2 and 61.6 ±8.3 nm, respectively.

Perumal *et al.* (2013) reported the mean abnormal sperm in Mithun bull cooling semen taurine (0, 25, 50 and 100mM) as 8.38± 0.26, 7.84± 0.74, 5.75± 0.32 and 7.62± 1.23 per cent respectively.

Singh *et al.* (2014) reported the mean LPO level in buffalo bull post thawed semen in control and taurine (50mM) added semen as 2.4870.10 and 1.4770.16 nmol MDA/10^8cells, respectively.

Tirpák *et al.* (2015) reported the initial motility in bovine post thawed semen taurine (50mM) has higher significance level as compare to control.

Varghese *et al.* (2015) reported the mean motility, viability, sperm abnormalities, acrosomal integrity and HOST positive sperm in buffalo semen in control after dilution as 75.12 ±0.77, 82.75±0.76, 4.93±0.25, 92.85 ±0.34 and 82.72 ±0.91 per cent, respectively; at pre-freeze stage as 65.37±0.84, 72.75±0.99, 6.35±0.27, 89.70±0.39 and 70.82±0.92 per cent, respectively; after thawing as 38.37±0.95, 44.82±1.02, 9.10±0.22, 82.43±0.32 and 42.52±1.04 per cent, respectively. In taurine (4.0 mg/ml) after dilution as 81.75±0.79, 86.17±0.65, 4.60±0.24, 92.23±0.39 and 86.25±0.64 per cent, respectively; at pre-freeze stage as 73.25±0.81, 79.37±0.88, 5.80±0.28, 88.53±0.46 and 78.08±0.95 per cent, respectively; after thawing 50.00±0.62, 55.02±0.80, 7.90±0.29, 82.75±0.30 and 53.73±0.69 per cent, respectively. In taurine (6.0 mg/ml) after dilution as 81.00±0.82, 84.80±0.83, 4.93±0.20, 90.55±0.44 and 85.63±0.86 per cent, respectively; at pre-freeze stage as 71.12±1.02, 76.32±1.14, 6.38±0.29, 86.28± 0.45 and 75.03±1.08 per cent, respectively; in thawing as 34.25±0.71, 41.07±0.99, 8.90±0.31, 79.98±0.35 and 39.10±0.91 per cent, respectively.

Baber *et al.* (2016) reported in post thawed semen of Sahiwal bull with taurine (0.0mM, 20mM, 40mM and 60mM) added group, the value of mean motility as 45.50±0.74, 50.50±0.56, 45.25±0.76 and 42.50±0.74 per cent, respectively, value of mean viability as 58.00±1.21, 62.75±1.23, 56.75±1.23 and 57.25±0.93 per cent, respectively, value of mean HOST positive sperm as 58.00±0.63, 63.75±0.55, 55.50±0.59 and 51.50±0.42 per cent, respectively and value of mean acrosomal integrity as 61.50±0.94, 65.00±0.79, 61.00±0.79 and 58.25±0.73 per cent, respectively.

Banday *et al.* (2017) reported the mean MDA level in ram post thawed semen control and taurine (40mM) as 4.43 ±0.62 and 3.29 ±0.21 nmol/10^8 spermatozoa, respectively.

Chaturvedi *et al.* (2020) reported the average motile spermatozoa in control and taurine (4mg/ml) in Gir bull diluted semen as 85.50±0.92, 85.43±1.48 and in frozen thawed semen as 48.76±1.69, 48.53±2.75 per cent, respectively whereas, in Murrah bull diluted semen as 85.03±0.72, 84.56±1.27 and in frozen thawed semen as 52.61±1.46, 51.98±2.87 per cent, respectively.

Hegazy *et al.* (2020) reported the mean motility, live sperm and sperm abnormalities in buffalo bull semen in control after dilution as 70.12 ±0.51, 65.12±1.44 and 28.75±1.54 per cent, respectively; after equilibration as 63.00±0.84, 59.75±1.95 and 35.00±1.16 per cent, respectively; after thawing as 42.87±1.43, 45.00±1.41 and 39.00±1.22 per cent, respectively. In taurine (20ng/ml) after dilution as 72.75 ±1.06, 69.75±1.62 and 23.12±1.00 per cent, respectively; after equilibration as 66.87±1.31, 63.87±1.63 and 29.75±1.19 per cent, respectively; after thawing as 47.62±1.43, 46.37±1.49 and 35.50 ±1.34 per cent, respectively. In taurine (40ng/ml) after dilution as 78.00 ±0.70, 76.75±0.97 and 16.87±0.71 per cent, respectively; after equilibration as 72.75±1.08, 72.12±1.10 and 20.87±0.66 per cent, respectively; after thawing as 55.62 ±1.84, 52.62±1.16 and 21.37±0.82 per cent, respectively whereas, the value of HOST in post thaw semen in control, taurine (20 and 40 ng/ml) added semen as 39.12±1.00, 45.62±1.14 and 48.12±0.91 per cent, respectively.

El-Seadawy *et al.* (2021) reported the mean progressive motility, HOST positive sperm, live sperm, abnormal sperm and acrosomal integrity in buffalo bull semen in control after equilibration as 88.33± 1.67, 70.33± 3.18, 86.67± 2.03, 18.67± 0.67 and 91.33± 2.33 per cent, respectively; in post thawed semen as 42.50± 2.50, 49.00± 5.32, 71.25± 2.39, 8.00± 1.29 and 81.75±1.18 per cent respectively. In taurine (50mM) added semen after equilibration as 91.67± 0.88, 83.00± 2.08, 94.00±1.53, 5.00± 1.15 and 92.67± 1.45 per cent, respectively; in post thaw semen as 60.00±5.00, 57.50± 2.10, 74.00±0.82, 7.75± 0.85 and 80.75± 0.48 per cent, respectively.

Sakr *et al.* (2021) reported the mean motility, acrosomal integrity and HOST positive sperm in buffalo semen in control post- dilution as 71.0 ±2.0, 78.6±1.4 and 70.6±1.1 per cent, respectively; in post equilibration as 54.5±1.5, 70.0±1.9 and 58.0±1.7 per cent, respectively; in post-thawing as 37.5±1.5, 63.0±2.5 and 45.0±2.3 per cent, respectively. In taurine (25mM) post-dilution as 74.8±2.3, 78.2±0.9 and 78.2±0.7 per cent, respectively; in post-equilibration as 60.0±1.2, 74.2±1.2 and 69.2±1.5 per cent, respectively; in post-thawing as 47.4±1.8, 64.3±3.1 and 56.3±3.1 per cent, respectively. In taurine (50mM) post-dilution as 75.9±1.5, 79.4±1.7 and 79.4 ±1.3 per cent, respectively; in post-equilibration as 59.0±1.4, 75.6±1.8 and 68.6±1.5 per cent, respectively; in post thawing as 47.0±1.1, 67.3±2.8 and 57.3±2.1 per cent, respectively. In taurine (75mM) post-dilution as 73.5±0.8, 80.4±1.1 and 72.4±1.4 per cent, respectively; in post-equilibration as 48.1±1.8, 72.6±2.3 and 60.6±2.1 per cent, respectively; in post-thawing as 34.5±1.8, 63.7±3.4 and 50.7±2.9 per cent, respectively. In taurine (100mM) post-dilution as 74.0±1.4, 76.8±1.6 and 71.8±1.7 per cent, respectively; in post-equilibration as 48.3±2.0, 72.8±2.1 and 61.8±2.4 per cent, respectively; in post-thawing as 35.6±0.8, 62.2±3.8 and 49.2±2.8 per cent, respectively.

2.4.3 Ascorbic acid

The vitamin C acts as water- soluble antioxidant in blood plasma and seminal plasma and this positive impact of adding Vitamin C to diluted semen appears to identify with a decrease in DNA damage of spermatozoa and it not only improve viability but it also protected acrosome and membrane integrity (Maia *et al.,* 2010).

Beconi *et al.* (1993) demonstrated that good quality bull semen appears to benefit more from ascorbate, Vitamin E and SOD than poor quality semen.

It was reported that ascorbic acid supplementation reduced ROS generation (Beconi *et al.,* 1993; Geva *et al.,* 1996; Hsu *et al.,* 1998; Mirzoyan *et al.,* 2006).

Sharma and Agarwal (1996) stated that vitamin C plays an important role in combating oxidative stress in the seminal plasma.

Aitken and Krausz (2001) reported that cryopreservation reduces the functional integrity of bull spermatozoa, however, inclusion of vitamin C reduces the oxidative stress in equilibration and thawing of semen thus protecting the sperm and improves quality of semen.

Foote *et al.* (2002) reported that higher concentration of ascorbic acid was detrimental to sperm motility of frozen thawed bull semen.

Agarwal *et al*. (2004) stated that vitamin C prevents the sperm agglutination.

Hu *et al.* (2010a) reported in post thawed semen of cattle bull with different concentrations of ascorbic acid (0, 2.5, 4.5, 6.5 and 8.5mg/ml), the value of mean motility as 36.18 ± 1.53, 38.34 ± 1.35, 50.61 ± 1.62, 42.36 ± 1.83 and 35.30 ± 1.37 per cent, respectively, value of mean acrosomal integrity as 52.3± 1.58, 55.6± 2.30, 64±1.20, 58.4±0.89 and 52.6±1.41 per cent, respectively and value of HOST as 45.1± 1.23, 48± 0.89 52.6±1.51, 49.2± 1.33 and 44.3± 1.69 per cent, respectively.

Paudel *et al.* (2010) reported the mean value of HOST in Swedish bull post thawed semen at 10 mM ascorbic acid as 35.9 ± 1.3.

Asadpour *et al.* (2011) reported the MDA level in bull post thawed semen control and vitamin C (1, 2mM) as 5.90 ± 0.42 and 5.74 ± 0.33, 6.45 ± 0.96 nmol 10^9, respectively.

Barati *et al.* (2011) observed that the inclusion of vitamin C reduces the oxidative stress during cooling of semen thus protecting the bull semen during the final step of cryopreservation improving the longevity and quality of semen.

Batool *et al.* (2012) reported in post thawed semen of zebu cattle with ascorbic acid (0.0, 0.5, 1.5, 2.5 and 3.5mM), the value of mean progressive motility as 33.3 ± 1.7, 40.0 ± 2.9, 38.3 ± 1.7, 36.7 ± 3.3 and 38.3 ± 1.7 per cent, respectively and the value of mean viability as 59.3 ± 3.7, 62.7 ± 1.8, 66.3 ± 2.0, 56.7 ± 1.5 and 61.7 ± 5.5 per cent, respectively.

Lampiao *et al*. (2012) stated that it reacts with OH, O_2 and H_2O_2 in the extracellular fluid, thus protecting sperm viability and motility.

Azawi and Hussein (2013) observed that addition of Vit.C to semen extender will overcome the harmful effect of the oxidant, which could be due to inhibition or stopped lipid peroxidation damage by the effect of this antioxidant.

Rao *et al.* (2013) reported the mean motility in bull semen in control at pre-freeze stage as 76.88±0.02 per cent; in post thawed stage as 43.75 ±0.01 per cent and with vitamin C (5mM) at pre-freeze stage as 78.05±0.02 per cent; in post thawed stage as 46.83 ±0.01 per cent.

Ahmad *et al.* (2014) observed that the increase level of vitamin C as antioxidant increased the sperms individual motility.

Mittal *et al.* (2014) reported the mean progressive motility, live sperm, abnormal sperm and intact acrosomal sperm in Bhadawari bull semen in control after dilution as 63.44±0.82, 81.03±0.41, 4.91±0.14 and 80.16±0.50 per cent, respectively; after equilibration as 58.13±0.80, 75.81±0.42, 5.03±0.18 and 74.59±0.51 per cent, respectively; after thawing as 53.22±0.81, 70.13±0.37, 7.75±0.17 and 69.41±0.54 per cent, respectively. In ascorbic acid (5mM) after dilution as 70.28±0.97, 83.19±0.42, 4.06±0.09 and 84.50±0.41 per cent, respectively; after equilibration as 64.88±0.92, 78.28±0.48, 3.97±0.11 and 79.22±0.51 per cent, respectively; after thawing as 59.72±0.87, 72.97±0.41, 6.22±0.11 and 74.00±0.49 per cent, respectively.

Srivastava and Kumar (2014) reported the mean motility, livability, intact acrosome and HOST positive sperm in post thawed semen of HF bull in control as 29.2 ± 5.2, 55.2 ± 4.6, 69.2 ± 4.4 and 21.4 ± 2.0 per cent, respectively. In ascorbic acid (10mM) added semen as 47.8 ± 5.8, 72.6 ± 3.1, 80.3 ± 3.5 and 30.4 ± 3.0 per cent, respectively.

Sandeep *et al.* (2015) reported the mean progressive motility, live sperm, intact acrosome and MDA level in buffalo semen in control in pre-freezing as 67.70±1.12, 70.77±1.10, 73.00±1.10 per cent and 784.50±8.31 concentration ng/120 million sperms, respectively; in post freezing as 36.25±0.91, 39.36±0.90, 41.93±0.94 per cent and 691.25 ±8.23 concentration ng/120 million sperms, respectively. With ascorbic acid (2.5mM) in pre-freezing as 73.33±1.07, 76.21±1.01, 76.50±2.13 per cent and 610.42±12.07 concentration ng/120 million sperms, respectively; in post-freezing as 45.62 ±0.69,

48.21±0.75, 50.68±0.81 per cent and 521.16±8.23 concentration ng/120 million sperms, respectively.

Sohail *et al.* (2015) reported in post thaw semen of Sahiwal bull semen with ascorbic acid (0.0, 1.0, 2.0, 3.0 and 4.0mg/ml) added semen, the value of mean motility as 50.63±3.50, 53.67±4.12, 63.93±3.87, 64.97±3.27 and 62.73±2.80 per cent, respectively, value of mean viability as 61.27±6.15, 60.43±3.17, 65.27±2.97, 69.10±3.76 and 62.73±2.80 per cent, respectively and value of HOST positive sperm as 49.97±3.62, 51.37±3.98, 58.27±4.48, 60.10 ±3.35 and 54.20±3.68 per cent, respectively.

Patel *et al.* (2016) reported the mean motility, livability and sperm abnormalities in post thawed semen of Mehsani buffalo bull in control as 52.83±0.52, 78.70±0.21 and 5.43±0.21 per cent, respectively and in ascorbic acid (0.2mg/ml) added semen as 57.83±0.52, 81.73±0.22 and 5.23±0.18 per cent, respectively.

Singh and Sharma (2018) reported the mean live sperm, motility, HOST positive and acrosomal integrity in Jersey bull semen in neat semen as 81.39±0.88, 75.42±0.55, 66.42±1.12 and 87.28±0.94 per cent, respectively; in control after dilution as 80.53±0.76, 74.31±0.41, 67.47±0.05 and 86.17±0.94 per cent, respectively; after equilibration as 76.77±0.68, 71.06±0.65, 63.31±1.11 and 82.47±1.05 per cent, respectively; after thawing as 53.16±1.19, 47.58±1.08, 40.78±0.93 and 67.92±0.93 per cent, respectively. In ascorbic acid (0.02%) after dilution as 80.49±0.82, 74.14±0.39, 69.17±0.92 and 86.89±0.92 per cent, respectively; after equilibration as 78.86±0.76, 71.61±0.52, 64.58±0.98 and 84.47±0.86 per cent, respectively; after thawing as 57.39±1.02, 51.28±0.90, 45.56±0.99 and 69.97±0.56 per cent, respectively.

Page and Rosenkrans (2019) reported the mean motility in bull post thawed semen with ascorbic acid (0, 5, 10 and 20mM) added semen as 35.9, 32.9, 32.7 and 30.7 per cent, respectively.

Pinto *et al.* (2020) reported the mean motility, mean major; minor sperm defects and acrosomal integrity in frozen semen of Nellore bull in control as 36.66±10.30, 6.27±0.60; 3.22±0.75 and 38.66±13.45 per cent, respectively. In vitamin C (2.5mM) added semen as 43.33±7.90, 6.5±0.63; 2.72±0.41 and 41.44±16.82 per cent, respectively.

Singh *et al.* (2020) reported the mean acrosomal integrity, live spermatozoa, sperm abnormalities, HOST positive sperm and progressive motility in crossbred bull in control post-dilution as 79.7 ± 1.52, 74.4 ± 1.82, 7.2 ± 0.63, 71.0 ± 1.10 and 67.0± 1.52 per cent, respectively; in post thawing as 53.9 ± 0.94, 50.8 ± 1.17, 16.8 ± 0.61, 45.4 ± 0.93 and 48.0± 2.49 per cent, respectively. In ascorbic acid (5mM) post-dilution as 78.7 ± 1.78, 72.4 ±1.69, 6.8 ± 0.83, 71.5 ± 1.69 and 68.0± 1.33 per cent, respectively; in post thawing as 64.3 ± 2.42, 55.4 ± 0.88, 13.2 ± 0.79, 49.4± 0.87 and 50.0± 2.11 per cent, respectively.

Saurabh *et al.* (2021) reported the mean individual motility, viability, abnormal sperm, intact acrosome and HOST positive sperm in murrah buffalo bull semen in control after dilution as 70.33±0.23, 82.79±0.29, 8.08±0.18, 90.31±0.18 and 50.67±0.27 per cent, respectively; after thawing as 45.50±0.34, 64.27±0.41, 16.27±0.27, 71.23±0.33 and 32.98±0.45 per cent, respectively. In ascorbic acid (0.20mg/ml) after dilution as 72.71±0.25, 83.85±0.23, 8.04±0.12, 90.13±0.19 and 52.77±0.28 per cent, respectively; after thawing as 58.13±0.37, 72.06±0.41, 11.58±0.16, 80.33±0.44 and 41.27±0.33 per cent, respectively. In ascorbic acid (0.5 mg/ml) after dilution 72.31 ± 0.25, 83.79±0.21, 8.04±0.10, 90.31 ± 0.20 and 52.63±0.24 per cent, respectively; after thawing as 58.77±0.40, 71.27±0.49, 12.83±0.22, 81.42 ±0.44 and 40.46±0.39 per cent, respectively.

2.4.4 Vitamin E

The useful effects of vitamin E on sperm parameters have been reported, it acts as lipophillic compound results in scavenging of reactive oxygen species (ROS) and inhibit the propagation of lipid peroxidation which result in increase of intracellular ATP level, decreased abnormal acrosomal reaction and semen motility increased (Breininger *et al.*, 2005).

Beconi *et al.* (1991) demonstrated that vitamin E prevents lipid peroxidation of frozen bovine semen and this also improves sperm quality and fertility in human sperm.

Brzezinska–Slebodzinska *et al.* (1995) stated that vitamin E supplementation considerably increases the number of spermatozoa.

It was observed that addition of vitamin E had a beneficial effect on sperm motility in liquid ram semen, fresh human semen and to little effect in equine chilled semen respectively (Upreti *et al.,* 1997; Donnelly *et al.,* 1999; Ball *et al.,* 2001).

Dal *et al.* (1998) stated that in some studies it has been documented low levels of vitamin E would allow for production of physiological level of ROS that are necessary for capacitation, acrosome reaction and *in vitro* fertilization.

It was observed that addition of antioxidants such as vitamin E and vitamin C to the semen freezing diluent, may prevent or diminishes cryodamage to spermatozoa metabolism and antioxidant capacities (Andrabi *et al.,* 2008; Anghel *et al,* 2009; Beheshti *et al.* 2011a).

Asadpour *et al.* (2011) reported in post thawed semen of HF bull with control and vitamin E (0.1, 0.2 mM) added semen, the value of mean MDA level as 6.07 ± 0.54 and 5.45 ± 1.42, 7.69 ± 0.16 nmol 10^9, respectively, value of mean viability as 50 ± 5 and 55 ± 4, 46 ± 3 per cent, respectively and value of mean motility as 43 ± 6 and 50 ± 5, 40 ± 1 per cent, respectively.

Asadpour *et al.* (2011) observed that the elevated doses of vitamin E advance the bull sperm quality parameters i.e. motility and viability of sperms after freezing.

Beheshti *et al.* (2011a) observed that the inclusion of various degree of vitamin E to semen extender prior to freezing causes huge improvement in sperm quality parameters such as motility and viability of sperms than control group.

Hu *et al.* (2011) reported in post thawed semen of bovine with vitamin E (0, 0.5, 1, 1.5 and 2mg/ml) added semen, the value of mean motility as 42.67 ±2.27, 44.45 ±2.48, 53.21 ±2.61, 53.86 ±2.86 and 42.71 ±2.38 per cent, respectively, value of mean SOD level as 1.35±0.09, 1.69 ±0.12, 1.72±0.14, 1.89±0.19 and 1.31±0.07 U/ml, respectively and value of CAT activity as 3.89±0.65, 4.26±0.71, 5.97±0.86, 6.08±1.07 and 4.03±0.59 U/ml, respectively.

Batool *et al.* (2012) reported in post thaw semen of zebu cattle with vitamin E (0.0, 0.5, 1.5, 2.5 and 3.5mM) added semen, the value of mean motility as 40.0 ±0.0, 45.0 ±8.7,

43.3 ±2.9, 40.0 ±0.0 and 31.7 ± 2.9 per cent, respectively and value of mean viability as 59.3. ± 3.7, 72.3 ± 3.1, 59.0 ± 2.6, 59.3 ± 1.5 and 60.7 ± 1.5 per cent, respectively.

Muzafer *et al.* (2012) reported in frozen thawed semen of crossbred bull the mean progressive motility in bull frozen thawed semen in control and vitamin E (0.3mg/ml) added semen, the value of mean progressive motility as 44.44±1.96 and 56.39±1.54 per cent, respectively, value of mean live sperm as 51.91±1.56 and 61.08±1.01 per cent, respectively, value of mean sperm abnormalities as 18.00±0.49 and 15.14±0.46 per cent, respectively, value of mean acrosomal integrity as 79.69 ±0.75 and 84.72 ±0.48 per cent, respectively, value of HOST positive sperm as 37.86 ±1.39 and 48.12±0.97 per cent, respectively and value of MDA production as 0.60 ±0.06 and 0.12 ±0.01 µmol/ml, respectively.

Towhidi and Parks (2012) reported in post thaw semen of bull with vitamin E (0, 0.2 and 0.4 mM) added semen, the value of mean motility as 36.5±0.4, 41.4±0.4 and 42.1±0.4 per cent, respectively and value of mean live sperm as 41.9±0.4, 45.1±0.4 and 46.0±0.4 per cent, respectively.

Rao *et al.* (2013) observed the average individual motility in bull semen in control at pre-freeze stage as 76.88±0.02 per cent; in post thawed stage as 43.75 ±0.01 per cent and with vitamin E (1mg/ml) at pre-freeze stage as 81.09±0.01 per cent; in post thawed stage as 50.69 ±0.02 per cent.

Lukman *et al.* (2014) reported in cooling (5^{o}C) semen of Bali ball with vitamin E (0.0, 0.2, 0.4 and 0.6 g/100ml), the value of mean motility as 78.8±2.22, 79.5±1.5, 79.5±1.5 and 77.8±4.1 per cent, respectively and value of mean viability as (0.0, 0.2, 0.4 and 0.6 g/100ml) as 91.7±3.13, 90.6± 3.38, 94.4± 2.35 and 90.3± 3.01 per cent, respectively.

Mittal *et al.* (2014) reported the mean progressive motility, live sperm, abnormal sperm and intact acrosomal sperm in Bhadawari bull semen in control after dilution as 63.44±0.82, 81.03±0.41, 4.91±0.14 and 80.16±0.50 per cent, respectively; after equilibration as 58.13±0.80, 75.81±0.42, 5.03±0.18 and 74.59±0.51 per cent, respectively; after thawing as 53.22±0.81, 70.13±0.37, 7.75±0.17 and 69.41±0.54 per cent, respectively. In vitamin E (5mM) after dilution as 66.59±0.99, 82.06±0.38, 4.28±0.10 and 81.75±0.47

per cent, respectively; after equilibration as 61.31±0.94, 76.75±0.46, 4.44±0.16 and 76.91±0.51 per cent, respectively; after thawing as 56.75±0.75, 71.25±0.46, 6.84±0.12 and 71.59±0.48 per cent, respectively.

Motemani *et al.* (2017) reported in post thawed semen of HF bull with different concentrations of α-tocopherol (control, 1.2 mM, 2.4 mM and 4.8 mM), the value of mean motility as 61.3±1.67, 64.1±1.67, 4.2±1.67 and 75.9±1.67 per cent, respectively, value of mean HOST as 60.5±1.6, 61.7±1.6, 73.0±1.6 and 70.5±1.8 per cent, respectively and value of mean live sperm as 61.3±1.8, 64.1±1.8, 76.1±1.8 and 78.2±1.8 per cent, respectively.

Kumar *et al.* (2018a) reported in frozen thawed semen of buffalo bull with vitamin E (0.0, 0.5, 1.0, 1.5 and 2 mg/ml) added semen, the value of mean motility as 36.7 ± 2.5, 37.5 ± 1.4, 40.0 ± 2.5, 38.9 ± 2.1 and 37.3 ± 1.4 per cent, respectively, value of mean live sperm as 56.5 ± 2.1, 61.3 ± 2.2, 64.6 ± 3.0, 58.7 ± 2.0 and 56.6 ± 1.4 per cent, respectively, value of mean HOST positive sperm as 28.7 ± 2.1, 30.6 ± 2.1, 35.5 ± 2.7, 32.2 ± 2.7 and 32.0 ± 2.2 per cent, respectively and value of mean acrosomal integrity as 60.0 ± 2.6, 60.4 ± 3.2, 62.3 ± 2.0, 62.3 ± 1.7 and 61.7 ± 3.3 per cent, respectively whereas, the value of lipid peroxidation in control and with vitamin E (1mg/ml) as 245.5 ± 25.9 and 187.0 ± 19.8 MDA µmole/10^9 spermatozoa, respectively and mean value of SOD activity as 272.6 ± 31.1, 324.4 ± 29.1 IU/10^9 spermatozoa /minute, respectively.

Dheerib *et al.* (2020) reported the mean sperm abnormalities and mean live sperm in HF bull semen in control after cooling as 15.31±0.77 and 56.66±2.81; after cryopreservation for 48hrs as 20.86±0.39 and 49.57±2.36 per cent, respectively. In vitamin E (0.8mM) after cooling as 13.16±0.64 and 58.24±1.52; after 48 hr of cryopreservation as 15.57±0.72 and 51.42±1.50 per cent, respectively.

Haris *et al.* (2020) reported in post thawed semen of Simmental bull with vitamin E (0.0, 0.134, 0.268 and 0.402 gram/100 ml extender) added semen, the value of mean individual motility as 41.68, 45.06, 44.73 and 40.91 per cent, respectively, value of mean dead sperm as 11.80, 7.79, 8.27 and 12.92 per cent, respectively and value of mean sperm abnormalities as 8.55, 5.48, 5.12 and 9.59 per cent, respectively.

Pal *et al.* (2020a) reported the mean motility, live sperm, acrosomal integrity and HOST positive sperm in Hariana bull semen in control after dilution as 70.88±0.61, 83.50±0.90, 82.25±0.94 and 80.25±0.98 per cent respectively; after equilibration as 65.00±1.10, 78.88±0.79, 77.13±0.91 and 74.38±0.70 per cent, respectively; after thawing as 58.25±1.54, 71.00±1.65, 69.00±1.84 and 68.38±1.67 per cent respectively. In vitamin E (2.5mM) after dilution as 75.12±0.61, 87.38±1.00, 84.75±0.79 and 84.25±0.98 per cent, respectively; after equilibration as 70.00±0.98, 83.13±0.74, 74.75±0.56 and 78.50±0.79 per cent, respectively; after thawing as 64.75±1.23, 77.63±0.18, 79.75±1.03 and 74.25±0.49 per cent, respectively.

Al-Dean *et al.* (2021) observed the limited (minimized) dilution rate with high concentration level of the antioxidant (vitamin E) improves the individual motility of spermatozoa after freezing, this improvement suggests positive affect on the fertilization index especially for poor quality semen.

Hassan & Eidan (2021) reported in post thaw semen of HF bull in control and vitamin E (2mM), the value of mean progressive motility as 55.71±2.02 and 60.00±5.00 per cent, respectively, value of mean live sperm as 79.64±0.90 and 85.00±2.00 per cent, respectively and value of sperm abnormalities as 7.22±0.89 and 4.75±0.75 per cent, respectively.

Khan *et al.* (2021a) reported the mean motility, HOST positive sperm, acrosomal integrity and live sperm in Sahiwal × Holstein-Friesian bull frozen thawed semen in control as 50.00±1.60, 42.29±1.63, 40.83±1.56 and 49.87±1.50 per cent, respectively; with vitamin E (1.2mM) as 51.90±1.66, 44.38± 1.43, 41.00±1.40 and 50.98± 1.88 per cent, respectively; with vitamin E (2.4mM) as 53.65±1.98, 46.45± 1.89, 43.00±1.80 and 51.44±1.40 per cent, respectively; with vitamin E (4.8mM) as 59.40±1.44, 53.12 ± 1.77, 52.00±2.15 and 62.66± 1.60 per cent, respectively.

Tudu *et al.* (2021) reported the mean SOD level in black Bengal goat post thawed semen with vitamin E (0, 1 and 2 mg/ml) added semen as 0.339 ± 0.006, 0.125 ± 0.006 and 0.125 ± 0.002 U/mg of protein, respectively.

2.4.5 Trehalose

The trehalose is a non-reducing disaccharide consisting of two glucose moieties joined together by an alpha-1, 1 glucosidic bond (Patist and Zoerb, 2005). It uphold the osmotic pressure of the diluents and it has a stabilizing result on both cellular protein and plasma membrane (Aboagla and Terada, 2003) and also acts as cryoprotectant and decreasing the extent of cell injury by reducing the intracellular ice formation (Storey *et al.*, 1998).

Fukuhara and Nishikawa (1973) stated that trehalose have various functions in sperm extenders, plus provide energy substrate to the sperm cell during incubation.

Bucak and Tekin (2007) reported the mean per cent value in ram semen at 5^{o}C with trehalose (100 mM), the motility as 69.3 ± 3.4, abnormal sperm as 9.3 ± 1.1 and live sperm as 79.1 ± 2.5.

Bucak *et al.* (2007) reported in frozen semen of ram with trehalose (0, 50 and 100mM) as 9.2 ± 0.5, 8.6 ± 0.2 and 8.6 ± 0.2 nmol/ml, respectively and value of CAT activity as 738.8 ± 39.7, 815.0 ± 64.9 and 1064.8 ± 27.8 kU/L, respectively.

Hu *et al.* (2010b) reported in post thawed semen of cattle bull with trehalose (0, 25, 50, 100 and 200mM) added semen, the value of mean sperm motility as 36.88 ± 1.53, 38.34 ± 1.35, 44.36 ± 1.83, 46.61 ± 1.62 and 35.30 ± 1.37 per cent, respectively, value of mean SOD level as 1.58 ± 0.08, 1.46 ± 0.11, 1.58 ± 0.07, 1.38 ± 0.09 and 1.60 ± 0.10 U/mL, respectively and value of mean CAT activity as 2.01 ± 0.12, 2.25 ± 0.31, 2.19 ± 0.27, 3.17 ± 0.56 and 3.06 ± 0.49 U/mL, respectively.

Reddy *et al.* (2010) reported the mean motility in buffalo post thawed semen in control and trehalose (100mM) as 31.67±1.67 and 41.67±1.67 per cent, respectively.

Chhillar *et al.* (2012) reported in post thaw semen of Karan fries bull control and trehalose (100mM) added semen, the value of mean MDA level as 2.61 ± 0.33 and 1.51 ± 0.16 nmol MDA/10^{8} cells, respectively, value of mean individual motility as 36 ±3.05 and 47 ±1.15 per cent, respectively, value of mean HOST as 50 ± 1.52 and 37.667 ± 3.38 per

cent, respectively and value of mean sperm viability as 52.00±1.15 and 38.66 ± 1.2 per cent, respectively.

Oh *et al.* (2012) reported in post thawed semen of Korean jeju black bull in control and with trehalose (20mM) added semen, the value of average progressive motility as 64.00 ± 7.42 and 65.00 ± 4.47 per cent, respectively, value of mean viability as 58.25 ± 6.03 and 59.15 ± 6.09 per cent, respectively and value of HOST as 51.90 ± 9.99 and 57.95 ± 9.28 per cent, respectively.

Singh *et al.* (2012) reported the mean motility in buffalo bull fresh semen as 85.00 ± 2.88 whereas, in post thaw semen the value in control and trehalose (100mM) are as 36.00 ± 3.05 and 54.33 ± 2.60 per cent, respectively.

Kumar *et al.* (2013) reported the mean motility and HOST positive sperm in buffalo bull in fresh semen as 87.0 ± 3.78 and 76.00 ± 3.05 per cent, respectively and in post thawed control, trehalose (100mM) added semen as 40.33 ± 3.17 and 37.66 ± 0.33, 53.33 ± 4.4 and 50.33 ± 2.8 whereas in Karan fries bull fresh semen as 83.66 ± 2.72 and 72.00 ± 2.88 per cent, respectively and in post thawed control and trehalose (100mM) added semen as 37.66 ± 4.33 and 37.33 ± 3.28, 47.0 ± 1.15 and 52.33 ± 2.79 per cent, respectively.

Badr *et al.* (2014) observed the mean value in bull post thawed semen with trehalose (100 mM), the motility as 51.66±7.27 per cent, acrosomal integrity as 14.33±2.40 per cent, SOD as 48.33±6.02 U/L and MDA as 11.33±1.85 nmol/ml.

Büyükleblebici *et al.* (2014) reported in post thawed semen of HF bull with control and trehalose (25mM) added semen, the value of mean individual motility as 46.63±2.40 and 49.75± 4.67 per cent, respectively, value of mean abnormal acrosome as 2.25±0.25 and 2.88±0.30 per cent, respectively, value of mean total abnormalities as 11.13± 0.77 and 13.38±0.96 per cent, respectively, value of mean HOST positive sperm as 41.50±2.32 and 39.13±3.17 per cent, respectively and value of mean LPO level as 2.01±0.26 and 2.27±0.23 nmol/ml, respectively.

Singh *et al.* (2014) reported the mean LPO level in buffalo bull post thawed semen in control and trehalose (100mM) added semen as 2.4870.10 and 1.4870.02 nmol MDA/10^8cells, respectively.

El-Sheshtamy *et al.* (2015) reported the mean HOST positive sperm and acrosomal integrity in cattle bull in control after equilibration as 79.00± 1.00 and 72.00± 1.59 per cent, respectively; in post thaw semen as 50.80± 1.91 and 52.40± 1.03 per cent, respectively. In trehalose (100mM) after equilibration as, 83.40± 2.69 and 78.00± 1.10 per cent, respectively; in post thaw semen as 60.20± 1.83 and 49.00± 1.87 per cent, respectively.

Gamal *et al.* (2016) reported in post thawed semen of buffalo bull control and trehalose (4.9g/100ml) supplemented semen, the value of mean motility as 33.00 ± 2.26 and 37.00 ± 4.29 per cent, respectively, value of mean live sperm as 71.50 ± 1.60 and 68.40 ± 3.14 per cent, respectively, value of mean abnormal sperm as 11.00 ± 1.45 and 16.30 ± 0.90 per cent, respectively and value of mean HOST positive sperm as 64.00 ± 2.08 and 68.70 ± 1.94 per cent, respectively.

Iqbal *et al.* (2016) reported in post thawed semen of Nili Ravi buffalo bull with trehalose (0, 15, 30, 45 and 60 mM) added semen, the value of mean SOD level as 41.23 ± 0.81, 41.77 ± 2.08, 50.50 ± 0.80, 49.80 ± 0.71 and 49.96 ± 0.85 U/mL, respectively and value of CAT activity as 1.52 ± 0.06, 1.82 ± 0.12, 3.68 ± 0.49, 1.58 ± 0.04 and 1.92 ± 0.19 U/mL, respectively.

Shaikh *et al.* (2016) reported the mean motility, viability, abnormal sperm, acrosomal integrity and MDA level in Kankrej bull fresh semen as 89.02±0.19, 89.97±0.21, 3.08±0.15, 90.72±0.25 per cent and 49.96 ± 0.06 µmol/ml, respectively; after thawing in control as 53.91±0.57, 60.33±0.45, 5.08±0.22, 68.83±0.38 per cent and 31.98 ± 0.07 µmol/ml, respectively. In trehalose (50mM) after thawing as 58.00±0.42, 65.58±0.35, 4.75±0.18, 72.41±0.37 per cent and 32.15 ± 0.09 µmol/ml, respectively. In trehalose (100mM) after thawing as 64.16±0.52, 71.41±0.31, 5.00±0.21, 80.91±0.43 per cent and 20.06 ± 0.13 µmol/ml, respectively. In trehalose (150mM) after thawing as 57.58±0.35, 65.83±0.40, 4.50±0.15, 72.91±0.39 and per cent and 28.04 ± 0.08 µmol/ml, respectively.

Al-Badrany *et al.* (2017) reported the mean sperm abnormality, dead sperm and initial motility in HF bull semen with trehalose (100mM) after dilution as 7.97 ± 0.65, 26.44± 1.09 and 55.67 ± 1.61 per cent, respectively; at pre-freezing stage as 9.10 ± 0.78, 28.62± 1.33 and 51.33 ± 1.3 per cent, respectively; in post-freezing 10.43 ± 0.89, 30.69± 1.26 and 54.67 ± 1.03 per cent, respectively.

Öztürk *et al.* (2017) reported in post thawed semen of HF bull with control and trehalose (25mM) added semen, the value of mean motility as 43 ± 2.73 and 50 ± 3.53 per cent, respectively, value of mean acrosomal integrity as 43.5 ± 4.46 and 48.2 ± 8.70 per cent, respectively, value of HOST positive sperm as 31.7 ± 3.94 and 44.1 ± 2.18 per cent, respectively and value of mean LPO level as 19.47 ± 7.36 and 19.82 ± 8.96 μM × 10^9, respectively.

Varela *et al.* (2019) observed in frozen thawed semen of HF bull in control and trehalose (100mM) added semen, the value of mean SOD activity as 1144.8 ± 826.6, 3324 ± 3233.5 U/ml, respectively and value of mean CAT level as 84829.5 ± 72368.8, 18875 ± 41432.0 U/ml, respectively.

Al-Badrany *et al.* (2020) reported the mean motility, dead sperm and sperm abnormalities in HF bull in control after dilution as 63.33±0.57, 22.68±3.42 and 6.99±0.55 per cent, respectively; after cooling as 53.77±0.75, 27.73±0.27 and 9.55±0.47 per cent, respectively; after thawing as 52.55±0.62, 35.99±1.24 and 14.67±0.45 per cent, respectively. In trehalose (50mM) after dilution as 61.94±0.59, 19.18±5.49 and 6.25 ±0.24 per cent, respectively; after cooling as 57.33±0.81, 25.38±0.37 and 10.56±0.37 per cent, respectively; after thawing as 53.33±0.90, 30.06±0.17 and 13.50±0.47 per cent, respectively. In trehalose (100mM) after dilution as 70.83±0.83, 14.15±5.36 and 4.22±0.32 per cent, respectively; after cooling as 66.38±0.88, 21.80±0.33 and 5.77±0.27 per cent, respectively; after thawing as 65.55±0.89, 25.96±0.54 and 10.04±0.31 per cent, respectively. In trehalose (150mM) after dilution as 56.94±0.91, 24±1.46 and 8.04±0.32 per cent, respectively; after cooling as 45.00±0.80, 32.15±0.55 and 10.75±0.58 per cent, respectively; after thawing as 46.66±0.70, 38.92±0.47 and 15.63±0.56 per cent, respectively. In trehalose (200mM) after dilution as 53.33±0.57, 28±2.99 and 12.65±0.43 per cent, respectively; after cooling as 45.50±1.31, 37.43±0.41 and 15.66±0.57 per cent,

respectively; after thawing as 46.61±1.32, 41.60±0.66 and 21.12±0.31 per cent, respectively.

2.4.6 Butylated hydroxytoluene (BHT)

The BHT has an brilliant antioxidant capacity and it behave as an artificial analogue of vitamin E, mainly acting as terminating agent which suppresses autoxidation converting peroxy radicals to hydroperoxides (Fujisawa *et al*., 2004). It simply penetrates the sperm membrane increasing their fluidity (influencing the membrane phase transition) to decrease ice-crystal structure within the cell thereby protecting the sperm (Naijian *et al.,* 2013).

Shoae and Zamiri (2008) reported the mean progressive motility, live sperm and intact acrosome in HF bull semen in control after equilibration as 62.1 ± 2.5, 70.5 ± 1.1 and 70.0 ± 1.1 per cent, respectively; after thawing as 33.3 ± 0.9, 51.7 ± 0.9 and 50.5 ± 1.0 per cent, respectively. In BHT (0.5mM) after equilibration as 67.9 ± 3.2, 73.6 ± 1.2 and 73.2 ± 1.2 per cent, respectively; after thawing as 32.9 ± 0.7, 56.9 ± 0.8 and 56.4 ± 0.9 per cent, respectively. In BHT (1mM) after equilibration as 60.8 ± 2.4, 71.5 ± 1.0 and 70.8 ± 0.9 per cent, respectively; after thawing as 44.2 ± 1.3, 48.9 ± 0.9 and 48.7 ± 0.9 per cent, respectively. In BHT (2mM) after equilibration as 60.0 ± 1.9, 73.3 ± 1.5 and 72.3 ± 1.6 per cent, respectively; after thawing as 32.9 ± 1.4, 45.3 ± 1.8 and 44.4 ± 1.8 per cent, respectively. In BHT (4mM) after equilibration as 54.6 ± 1.6, 64.1 ± 1.6 and 63.1 ± 1.5 per cent, respectively; after thawing as 32.9 ± 1.4, 44.8 ± 1.7 and 43.9 ± 1.7 per cent, respectively.

Ijaz *et al.* (2009) reported in post thawed semen of buffalo bull with BHT (0, 0.5, 1, 2 and 3 mM) supplemented semen, the value of mean motility as 44.6 ± 3.1, 48.7 ± 3.5, 58.2 ± 2.6, 58.5 ± 3.5 and 54.1 ± 4.4 per cent, respectively, value of mean acrosomal integrity as 21.0 ± 3.1, 24.1 ± 3.3, 27.8 ± 2.9, 28.7 ± 2.1 and 26.1 ± 1.4 per cent, respectively, value of mean HOST positive sperm as 44.5 ± 3.0, 45.3 ± 4.0, 51.3 ± 3.9, 52.3 ± 3.4 and 46.5 ± 3.9 per cent, respectively and value of mean live sperm as 59.7 ± 5.3, 58.2 ± 3.1, 63.1 ± 2.9, 67.6 ± 2.9 and 60.7 ± 3.5 per cent, respectively.

Asadpour *et al.* (2012) reported in post thawed semen of HF bull in control, 0.5, 1.0, 2.0 and 4.0 mM BHT, the value of mean sperm motility as 35 ± 1, 40 ± 3, 41 ± 1, 32

± 1 and 30 ± 1 per cent, respectively and value of mean sperm viability as 42 ± 1, 40 ± 1, 37 ± 2, 40 ± 1 and 35 ± 2 per cent, respectively.

Muzafer *et al.* (2012) reported in frozen thawed semen of crossbred bull the mean progressive motility in bull frozen thawed semen in control and BHT (2mM) added semen, the value of mean progressive motility as 44.44±1.96 and 55.55±1.51 per cent, respectively, value of mean live sperm as 51.91±1.56 and 58.19±0.93 per cent, respectively, value of mean sperm abnormalities as 18.00±0.49 and 15.69±0.49 per cent, respectively, value of mean acrosomal integrity as 79.69 ±0.75 and 82.86 ±0.69 per cent, respectively, value of HOST positive sperm as 37.86 ±1.39 and 49.21 ±0.89 per cent, respectively and value of MDA production as 0.60 ±0.06 and 0.19±0.02 µmol/ml, respectively.

Naijian *et al*. (2013) stated that being lipid soluble, BHT functions as an antioxidant within and outside the sperm membrane; hence it is favored over other antioxidants.

Patel *et al.* (2015) reported the average initial motility, live spermatozoa and HOST positive sperm in Hariana bull semen in control after dilution as 69.46±0.63, 80.59±0.56 and 78.11±0.58 per cent, respectively; after equilibration as 63.54±0.60, 73.93±0.71 and 72.46±0.97 per cent, respectively; after thawing as 49.17±0.57, 65.39±0.63 and 57.34±0.74 per cent, respectively. In BHT (0.5mM) after dilution as 73.08±0.59, 83.89±0.54 and 81.05±0.59 per cent, respectively; after equilibration as 67.75±0.55, 78.42±0.64 and 75.90±0.67 per cent, respectively; after thawing as 54.50±0.50, 59.95±0.70 and 62.94±0.64 per cent, respectively. In BHT (1.0mM) after dilution as 77.50±0.70, 88.10±0.64 and 84.92±0.92 per cent, respectively; after equilibration as 71.92±0.65, 82.44±0.67 and 79.51±0.69 per cent, respectively; after thawing as 59.13±0.60, 70.72±0.78 and 67.92±0.73 per cent, respectively.

Wadood *et al.* (2016) observed in frozen thawed semen of buffalo bull with BHT (1.75, 2, 2.25 and 0 mM) added semen, the value of mean progressive motility as 36.67±2.79, 46.33±2.36, 38.00±2.33 and 43.33±3.22 per cent, respectively, value of mean live sperm as 63.06±2.73, 58.89±2.39, 54.26±2.60 and 61.69±3.32 per cent, respectively, value of mean HOST positive sperm as 50.62±2.16, 57.76±3.06, 47.94±3.16 and

52.55±3.38 per cent, respectively and value of mean LPO level as 10.61±2.34, 8.22±1.06, 12.61± 3.60 and 31.59±3.95 nM/ml, respectively.

Khumran *et al.* (2017) reported in diluted semen of crossbred bull with control, BHT (0.5, 1, 1.5, 2 and 3mM) added semen, the value of mean motility as 58.47±4.00, 61.93±3.81, 61.07±4.22, 55.87±4.16, 53.33±4.38 and 51.73±4.69 per cent, respectively, value of mean live sperm as 59.50±2.73, 66.83±2.59, 67.83±1.26, 65.00±2.58, 54.67±0.93 and 49.67±0.51 per cent, respectively, value of mean abnormal acrosome as 9.83±2.28, 12.67±2.44, 4.50±0.97, 9.17±2.13, 16.67±1.45 and 17.17±1.60 per cent, respectively and value of mean normal sperm as 67± 0.77, 90.82± 0.68, 94.50± 0.54, 91.33± 1.15, 87.83± 0.82 and 86.83± 0.59 per cent, respectively.

Singh *et al.* (2017) reported the mean progressive motility, live sperm and HOST positive sperm in crossbred bull semen in control after dilution as 73.92±2.15, 81.56±2.30 and 77.67±1.81 per cent, respectively; after equilibration as 64.28±2.96, 72.50±2.63 and 69.92±2.30 per cent, respectively; after thawing as 49.53±2.00, 59.14±2.18 and 60.56±2.93 per cent, respectively. In BHT (0.5mM) after dilution as 75.22±2.15, 82.69±4.47 and 80.28±1.79 per cent, respectively; after equilibration as 66.42±2.89, 77.06±2.59 and 72.53±2.26 per cent, respectively; after thawing as 52.78±1.63, 64.31±2.19 and 65.31±3.35 per cent, respectively. In BHT (1mM) after dilution as 77.33±2.24, 86.58±2.34 and 83.36±1.95 per cent, respectively; after equilibration 68.47±2.58, 81.33±2.77 and 76.61±2.20 per cent, respectively; after thawing as 56.58±1.38, 69.22±2.99 and 70.31±3.27 per cent, respectively.

Kumar *et al.* (2018b) reported in post thawed semen of Frieswal with BHT (0, 2.5, 5 and 10mM) added semen, the value of mean MDA production as 3.69±0.41, 2.64± 0.09, 1.26± 0.07 and 2.74± 0.21 µmol/mL, respectively, value of mean SOD level as 21.45± 0.49, 38.82± 0.94, 43.16± 1.24 and 39.61± 1.14 U/mL, respectively and value of mean CAT activity as 0.002± 0.0, 0.004±0.0, 0.02±0.0 and 0.005±0.0 U/mg, respectively.

Kumar *et al.* (2018b) reported the mean sperm abnormalities in Frieswal bull at 0, 2.5, 5 and 10mM BHT after dilution as 9.85± 2.12, 10.14± 1.02, 9.34± 2.11 and 9.24±

0.98; after equilibration as 12.12± 1.11, 12.45± 0.88, 10.04± 2.31 and 11.24± 1.02; after thawing as 15.12± 1.75, 14.45± 0.26, 11.04± 2.14 and 14.71± 1.11 per cent, respectively.

Mostafa *et al.* (2019) reported the mean per cent value in buffalo bull post thawed semen with BHT (1.0 mM/mL), the initial motility as 47.25 ± 4.66, live spermatozoa as 55.43 ± 5.50, normal sperm as 74.81 ± 7.50, HOST as 72.24 ± 7.25 and acrosomal integrity as 63.28 ± 6.28.

Tudu *et al.* (2021) reported the mean SOD level in black Bengal buck post thawed semen BHT (0, 1 and 2mM) as 0.333 ± 0.004, 0.169 ± 0.003 and 0.121 ± 0.003 U /mg of protein, respectively.

Chapter-III

Materials and Methods

CHAPTER-III

MATERIALS AND METHODS

3.1 Experimental Animal

Semen samples (n=30; 10 in each group) from mature cattle bull stationed at Central Artificial Breeding Station (CABS) Frozen semen processing laboratory, Department of Animal Husbandary, Hakkal, U.T. of Jammu & Kashmir were taken to evaluate the effect of additives on the cryopreservation of the semen.

3.2 Extension of Semen Samples

After collection and evaluation of the fresh semen, each ejaculate was extended with Tris Egg Yolk Citric acid Fructose Glycerol (TEYCAFG) extender divided into three aliquots: A (Control), B (Additive 1), C (Additive 2). All the aliquots of the single ejaculate were used for the evaluation of the same group.

Group I (Amino Acids)	A. TEYCAFG + Control	B. TEYCAFG+ Cysteine (5mM)	C. TEYCAFG+ Taurine (50mM)
Group II (Vitamins)	A. TEYCAFG+ Control	B. TEYCAFG+ Ascorbic acid (0.5 mg/ml)	C. TEYCAFG+ Vitamin E (4 mg/ml)
Group III (Miscellaneous)	A. TEYCAFG+ Control	B. TEYCAFG+ Trehalose (100mM)	C. TEYCAFG+ Butylated hydroxyltoluene (1mM)

3.3 Place of Experiment

Experiment was carried out at Central Artificial Breeding Station (CABS), Frozen semen processing laboratory, Department of Animal Husbandary, Hakkal, U.T. of Jammu & Kashmir, Division of V.G.O. and Division of V.P.T., F.V.Sc & A.H., SKUAST- J, R.S.PURA Jammu during the period between November 2020 to July 2021.

3.4 Housing of Animal

The bulls were housed in individual pens with proper spaced area.

3.5 Feeding and Management

The bulls were maintained under scientific and uniform conditions of feeding and management throughout the experiment period. The dairy bull ration consisted of 2-3 kg high protein feed, 30 g mineral mixture, 30-50 kg green fodder and 6 to 10 kg wheat bhossa. Water was provided *ad lib*.

3.6 Collection of Semen

Semen collection from donor bulls was made twice a week (Tuesday and Saturday) with the help of artificial vagina (AV) on dummy animals between 8.30 to 9.30 am in winters and 7:30 to 8:30 am in summers. The temperature of AV was maintained at 42°C by filling with warm water and prior to collection of semen the preputial sheath of the bull was cleaned to avoid contamination of semen with dung, mud or urine by using normal saline. Each bull was given one or two false mount before collection.

3.7 Evaluation of Semen

The semen analysis was done in neat, diluted, equilibrated and frozen-thawed samples as follows:

3.7.1 Volume

The volume of neat semen sample was directly read from the graduated collecting tube in millilitre (ml) with an accuracy of 0.10 ml.

3.7.2 Mass activity

Mass activity was evaluated as per process described by Tomar *et al.* (1966). A drop of neat semen was placed on warm (37°C) slide and examined under low power (10x) objective of the microscope. Mass activity score was noted on the basis of swirls activity of semen. Semen was graded on 0-5 scale as follows:

S.No.	Semen activity characteristics	Motility score
1	Very quick waves and swirl's and it is difficult to trace the point of their formation and disintegration, in which about 80-100 per cent of spermatozoa are progressively motile	+5
2	Rapid swirl's and eddies, in which about 60-80 per cent of spermatozoa are progressively motile	+4
3	The swirl's are slow & scattered in the field, in which about the 40-60 per cent of spermatozoa are progressively motile	+3
4	Swirl's or wave motion absent, individual movements of the spermatozoa are evident in which about 20-40 per cent spermatozoa are progressively motile	+2
5	No wave motion are observed in which about 10-20 per cent of spermatozoa are progressively motile	+1
6	Spermatozoa are immobile	0

3.7.3 Sperm concentration

The concentration of spermatozoa was observed by Accucell (calibrated by using haemocytometer).

3.7.4 Progressive motility

The progressive motility of sperm was determined using the procedure as described by Dhurvey *et al*. (2012)**.** The progressive motility of fresh, diluted, equilibrated and frozen thawed semen was assessed after covering the minute drop of semen on slide with a thin cover slip at 37˚C and observed under high power magnification (60x). The motility was recorded in percentage of motile spermatozoa.

$$\text{Progressive motility (\%)} = \frac{\text{Number of progressively motile spermatozoa}}{\text{Total number of spermatozoa observed}} \times 100$$

3.7.5 Live spermatozoa count

To determine the percentage of live and dead spermatozoa in semen samples, Eosin-nigrosin staining technique was used as per the method described by Hancock

(1951) in which dead spermatozoa were differentiated by their ability to obtain stained by eosin dye. The live spermatozoa are impermeable to eosin stain and colourless whereas Nigrosine provides a blue black background.

Composition of the stain used was:

Eosin (Water soluble)	1.67 g
Nigrosin (Water soluble)	10.00 g
Sodium citrate dihydrate	2.9 g
Double glass distilled water upto	100 ml

Ingredients of Eosin- nigrosin stain were accurately weighed on a chemical balance and triturated by using glass pestle and mortar, the final volume of solution is made up to 100 ml in a volumetric flask by adding double glass distilled water. The stain was kept under incubation at 37°C for 2 days. The supernatant was then carefully decanted and stored in a tight stopper glass bottle.

Procedure

At the time of evaluation a small drop of semen was added to 5-6 drops of Eosin-Nigrosin stain in a watch glass kept at 37^{o}C and mixed gently. Thin smear was prepared on clean glass slides within 30 seconds after mixing.

At least 200 spermatozoa were counted for estimating percentage of live spermatozoa under oil immersion objective (100X). The spermatozoa which were stained appears pink either completely or partially were classified as dead and unstained spermatozoa counted as live.

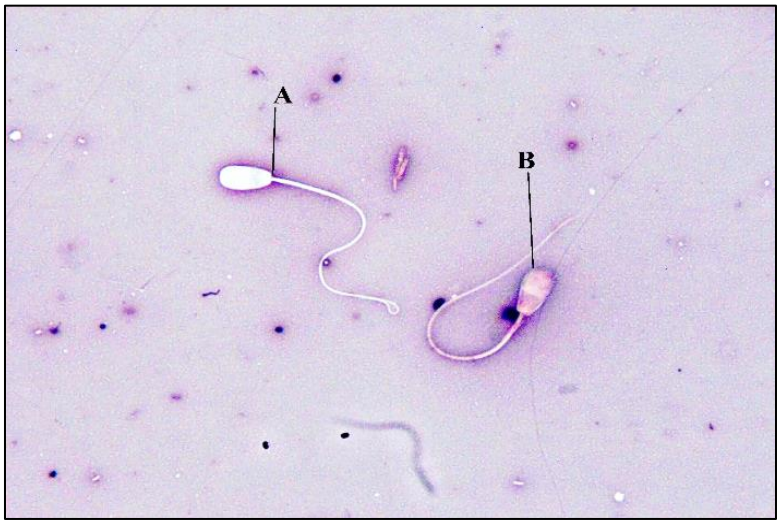
$$\text{Live spermatozoa (\%)} = \frac{\text{Number of unstained sperm cells}}{\text{Total number of sperm cell counted}} \times 100$$

3.7.6 Acrosomal integrity

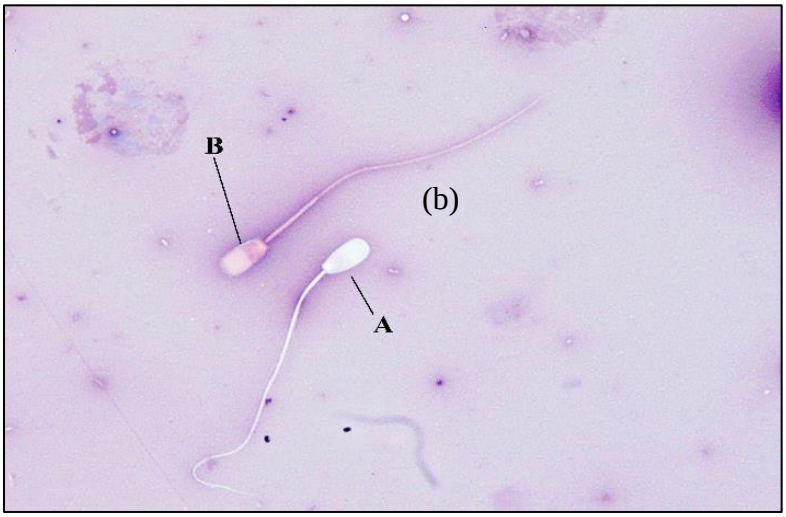
Sperm acrosomal integrity was determine by using the procedure as described by Hancock (1952).

Plate 1: Accucell (IMV) for evaluation of sperm concentration

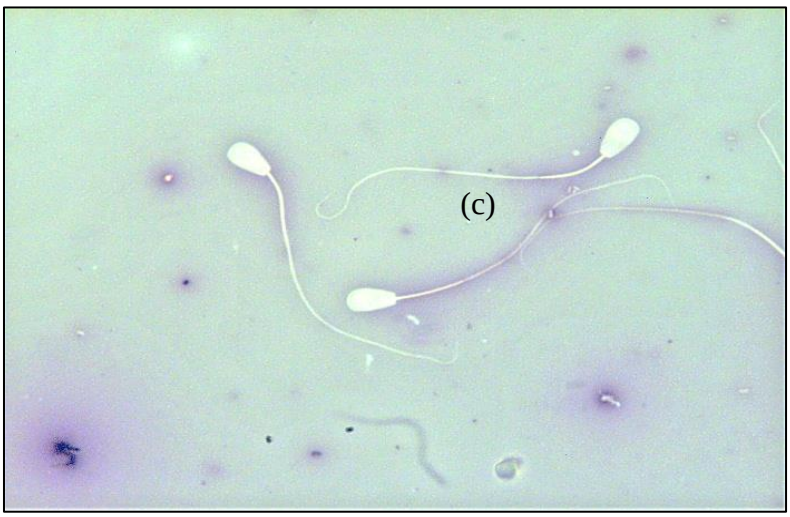
Plate 2: Photographs of live and dead spermatozoa



(a) Live spermatozoa [A], Dead Spermatozoa [B]

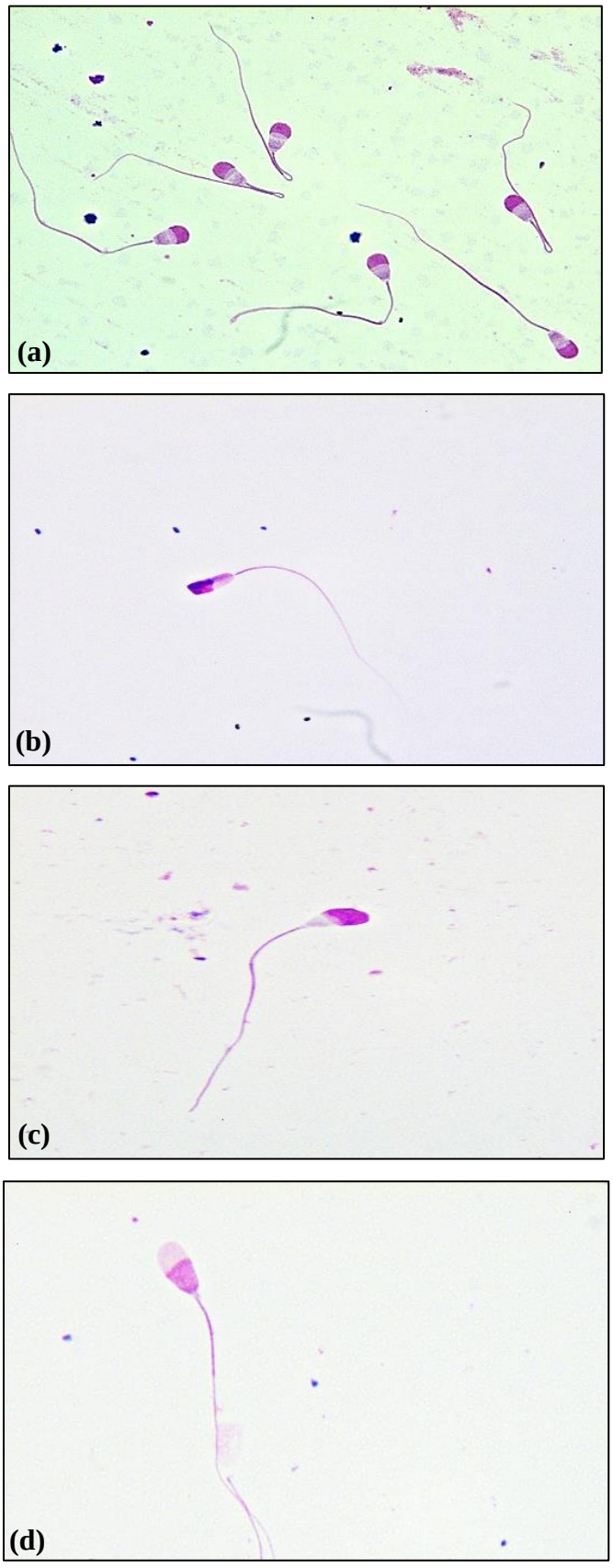


(b) Live spermatozoa [A] and Dead Spermatozoa [B]



(c) Live spermatozoa

Plate 3: Photographs of intact acrosome and damaged acrosome spermatozoa



(a) Intact acrosome, (b), (c) and (d) Abnormal sperm

Prepration of stock solution

Giemsa stock solution (Watson, 1995), Sorenson phosphate buffer (Sol A and Sol B)

- Sol A- Potassium Dihydrogen Phosphate (KH_2PO_4)
- Sol B- Sodium Dihydrogen Phosphate (NaH_2PO_4)

Fixative: 10% formalin solution was used for fixation of spermatozoa.

Working solution

Stock Giemsa stain	3ml
Sorensen 0.1m phosphate buffer (at 7 pH)	2ml
Distilled water	100ml

Staining procedure

1. A thin smear of diluted semen drop was made on a clean grease free slide.
2. The smear was air dried and fixed with 10% formalin solution for 30 mins at 37^{o}C in an incubator.
3. The slides were rinsed with distilled water and air dried.
4. 2 ml of Sorenson's buffer was taken in coupling jar and 3ml of Giemsa stain was added to it drop by drop with continuous stirring.
5. 45 ml of distilled water (freshly boiled for 15 minutes to remove carbon dioxide and than cooled) was than added slowly with continues stirring.
6. Fixed slides were placed in coupling jar containing solution and incubated at 37°C for 4 hours.
7. After incubation, slides were first rinsed with tab water and air dried.
8. 100 spermatozoa were examined under oil immersion objective (100X).
9. The damaged acrosome i.e. bubbled or swelled acrosome, separated from head or entirely lost were calculated.

$$\text{Acrosomal integrity (\%)} = \frac{\text{Number of acrosome stained spermatozoa}}{\text{Total number of spermatozoa counted}} \times 100$$

3.7.7 Sperm abnormalities

Sperm abnormalities were determined with slight modification than as described by Kumar *et al.* (1993). Eosin- nigrosin were used to determine the sperm abnormalities.

Staining technique

A small drop of semen was added to 5-6 drops of Eosin-Nigrosin stain in a watch glass kept at 37ºC and mixed gently. A thin smear was prepared on a clean glass slides within 30 seconds after mixing. At least 200 spermatozoa were examined to determine percentage of abnormal spermatozoa i.e. head, mid-piece and tail abnormalities under oil immersion objective (100X).

$$\text{Total sperm abnormalities (\%)} = \frac{\text{Total number of abnormal spermatozoa}}{\text{Total number of spermatozoa counted}} \times 100$$

3.7.8 Hypo-osmotic swelling test

Hypo osmotic swelling test (HOST) was determined using the procedure as described by Jeyendran *et al*. (1984). Hypo-osmotic solutions of 150 mOsm/kg were prepared as follow:

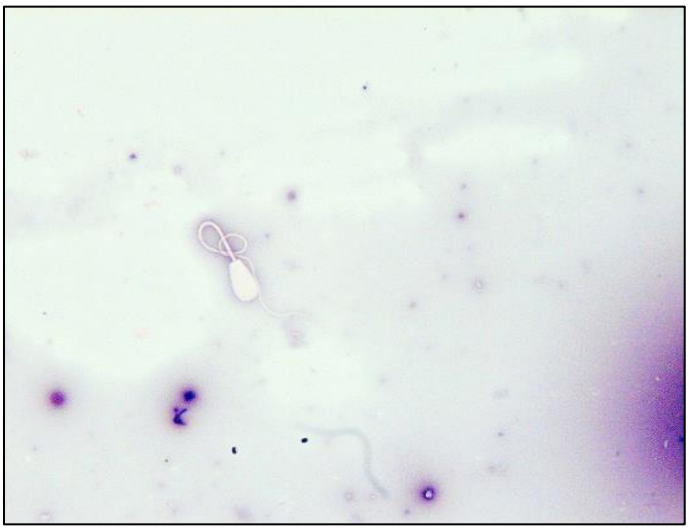
Sodium citrate	0.735g
Fructose	1.351g
Distilled water	100ml

Procedure:

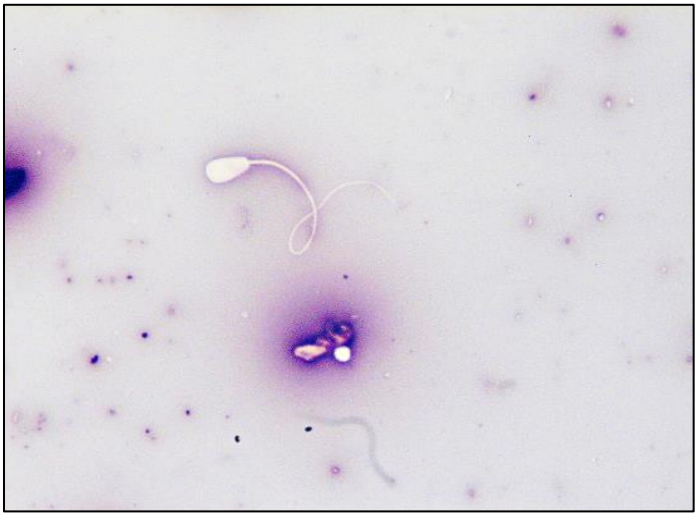
One ml of hypo-osmotic solution was mixed with 100µl of the semen sample and then incubated at 37˚C for 1 hour. Following incubation, sperm swelling were examined using a drop of sample on a glass slide covered with a cover slip. A total of at least 100 spermatozoa were counted using a warm stage microscope and different types of swollen and curled tail spermatozoa were determined and expressed in percentage.

$$\text{HOST positive spermatozoa (\%)} = \frac{\text{Number of HOST positive spermatozoa}}{\text{Total number of spermatozoa observed}} \times 100$$

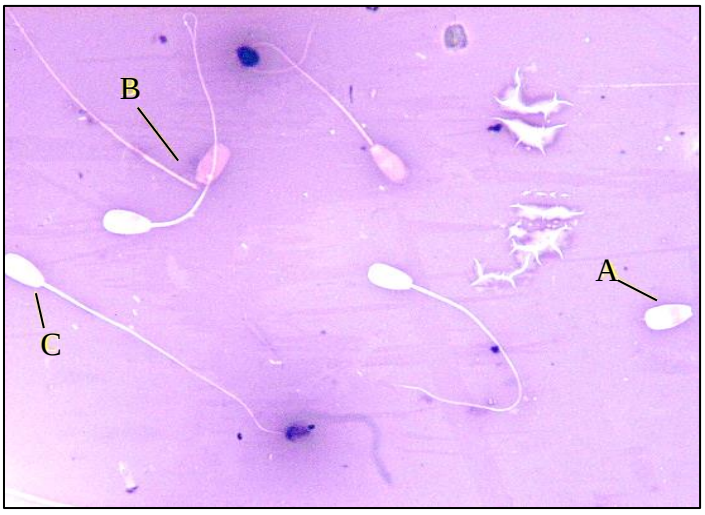
Plate 4: Photographs of sperm abnormalities (normal and abnormal spermatozoa)



(a) Abnormal sperm [Tail defect]



(b) Tail defect



(c) Free headed sperm [A]; Broken tail sperm [B]; Normal spermatozoa [C]

3.8 Cryopreservation of Semen

3.8.1 Extension of semen samples

The semen samples were initially diluted in Tris-egg yolk-citric acid-fructose-glycerol (TEYCAFG) extender kept at 37ºC containing ingredient in 1:1 dilution and the initial evaluation was done for the estimation of dilution rate (Singh *et al*., 2020; Deka, 1984).

Composition of TEYCAFG extender

Tris	2.42g/ 100 ml
Citric acid	1.38g/100 ml
D-fructose	1.00g/100 ml
Glycerol	7 ml
Egg yolk	20 ml

With a single step addition of glycerol into the extender, final dilution was made so that each ml of extended semen contained at least 40×10^6 spermatozoa (Anel *et al*., 2003; Singh *et al*., 2020) and labeled accordingly.

After dilution, the semen sample were divided into three aliquots for each group. In every group the first part was considered as control group while the other two remaining parts were added with different additives separately. Thus the neat semen was extended in three ways in three different groups:

Group I (Amino Acids)	A. TEYCAFG + Control	B. TEYCAFG+ Cysteine (5mM)	C. TEYCAFG+ Taurine (50mM)
Group II (Vitamins)	A. TEYCAFG+ Control	B. TEYCAFG+ Ascorbic acid (0.5 mg/ml)	C. TEYCAFG+ Vitamin E (4 mg/ml)
Group III (Miscellaneous)	A. TEYCAFG+ Control	B. TEYCAFG+ Trehalose (100mM)	C. TEYCAFG+ Butylated hydroxyltoluene (1mM)

The pH of all the prepared extenders were adjusted within the range of 7.2 to 7.4.

3.8.2 Filling of semen in straws

Polyvinyl straws of 0.5 ml capacity were filled with diluted semen manually with the help of straw filling assembly. The straws were then placed on the comb to drain out the excessive semen.

3.8.3 Sealing of the straws

The open ends of the filled straws were perfectly sealed with polyvinyl alcohol powder, and the adhered extra powder was wiped with the help of cotton.

3.8.4 Equilibration of the straws

Sealed straws were arranged on a rack and equilibrated for 4 h at 4°C in cold handling unit.

3.8.5 Freezing of straws

Freezing of straw was done through forced vapour freezing in a bio-freezer. The computer programme was controlled by Eurotherm controller console. Starting with temperature of 20°C, it was than cooled gradually with automatic controlled programme as follows:

1. The machine was put on at 20°C and brought down at 10°C per minute till 4°C.
2. The machine was then closed, once the temperature was stabilized at 4°C it was started again.
3. The rate of fall of temperature @ 5°C per minute from 4°C to -10°C.
4. From -10°C the rate of the fall have been programmed at @40°C per minute up to - 100°C.
5. Finally from -100°C to -140°C @ 20°C per minute.
6. After -140°C the freezer was opened and the straws were removed from the racks and are collected in pre-cooled goblets and then plunged in to liquid nitrogen at -196°C.
7. These pre cooled goblets were then transferred to pre cooled storage tank @ -196°C.

Plate 5: Staw filling assembly and French medium PVC straw (0.5ml)

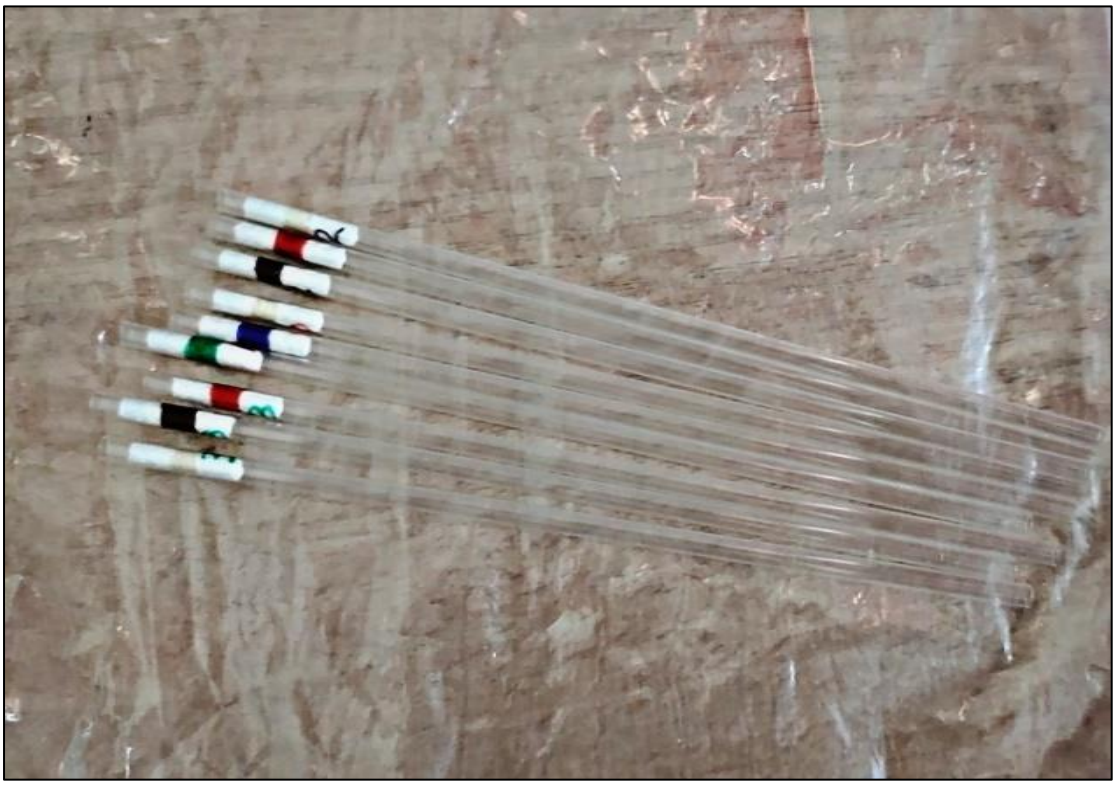


Plate 6: Cooling chamber for equilibration (4°C)

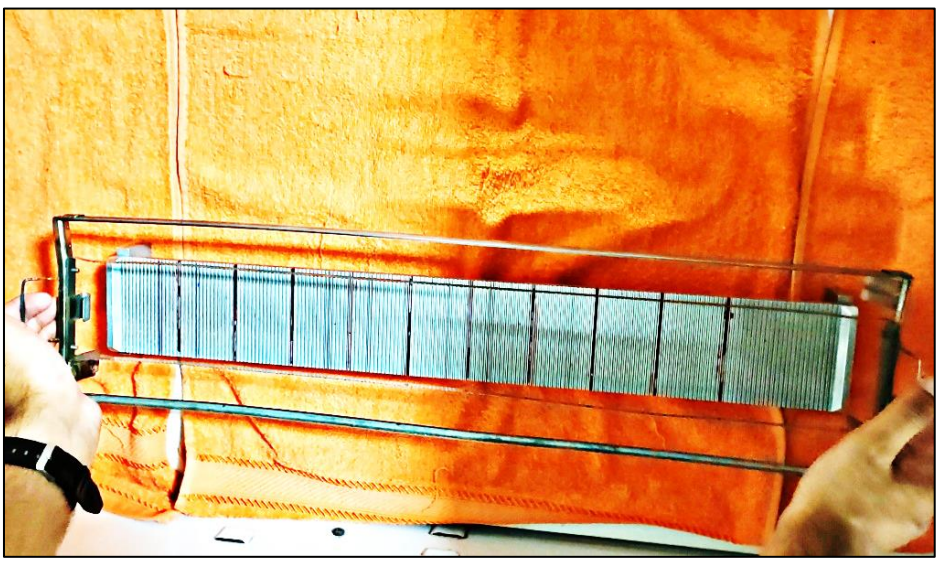


Plate 7: Graphical representation of rate of freezing

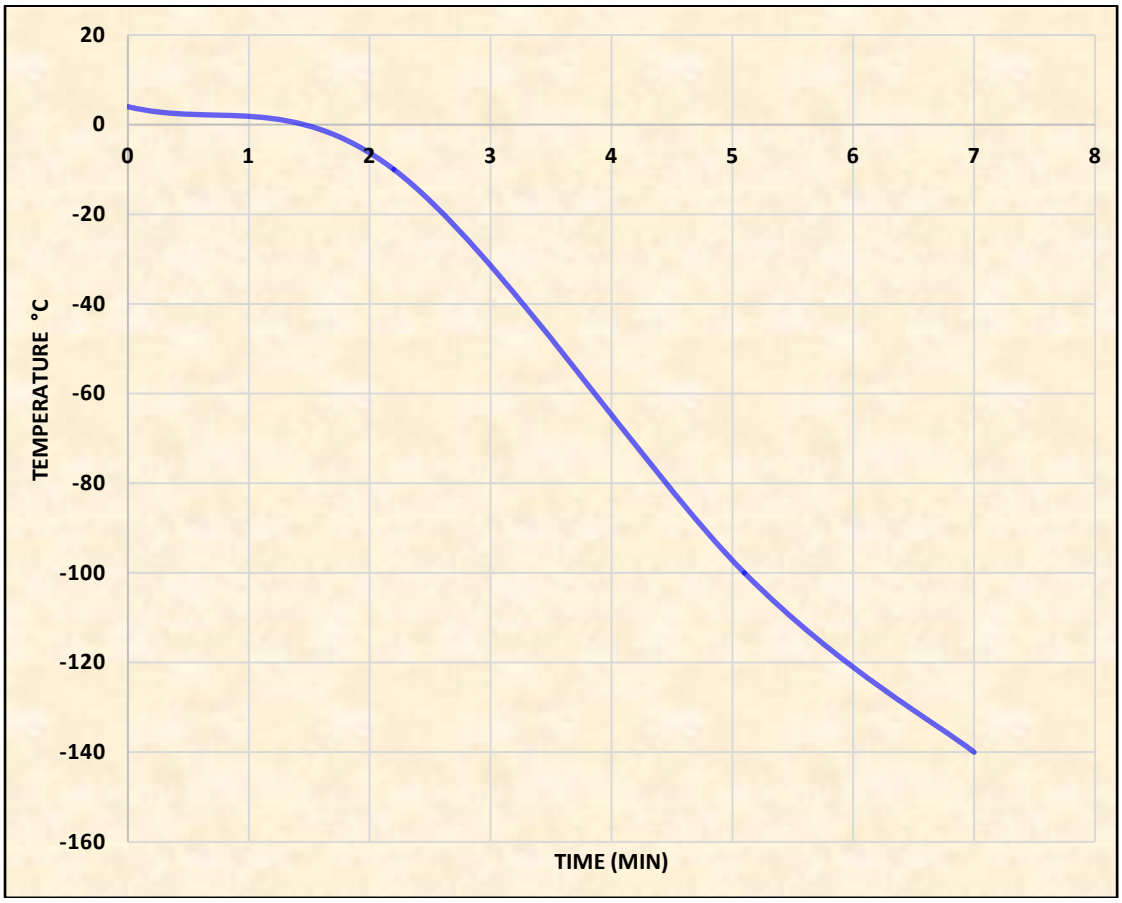


Plate 8: Bio-freezer (Digit cool-5300, IMV Technologies, France) for freezing of semen

3.8.6 Storage of semen straws

After freezing semen straws were stored in liquid nitrogen filled cryocan till post-thaw evaluation of the semen.

3.8.7 Thawing of frozen semen straws

Semen straws kept in the cryocan were thawed in a thawing unit maintained at 37°C for 30 secs for post-thaw evaluation of the semen.

3.8.8 Evaluation of diluted, equilibrated and frozen thawed semen

Semen was evaluated at these stages for progressive motility, live sperm percentage, acrosomal integrity, sperm abnormalities, HOST, while the oxidative stress parametres like Malondialdhyde (MDA), Superoxide dismutase (SOD) and Catalase (CAT) were evaluated only in the post thawed semen samples.

3.9 Evaluation of Oxidative Stress to the Semen

3.9.1 Malondialdhyde (MDA):

The activity of lipid peroxidation (LPO) in spermatozoa was calculated by the method as described by Shafiq-ur-Rehman (1984). It is determined in terms of Malondialdehyde (MDA) production which is the end product of Lipid peroxidation (LPO) that is measured through thiobarbituric acid (TBA) assay.

Reagents: 0.67 % Thiobarbituric acid (TBA) and 10% Trichloroacetic acid (TCA)

Procedure:

Test	**Blank**
Take 1 ml 1% lysate or 10% tissue homogenate	Take 1 ml distilled water
1 ml 10% TCA	Add 1 ml 10 % TCA
Vortex it, then centrifuge at 3000 rpm for 10min	Vortex it, then centrifuge at 3000 rpm for10 min
Supernatant was collected and sediment was rejected.	Keep it in boiling water, cool it and add 1 ml distilled water
Take 1 ml supernatant and add 1 ml 0.67% TBA to it and keep it in boiling water bath for 10 min	
Cool it and dilute with 1 ml distilled water and read the absorbance at 535 nm	Read the absorbance at 535 nm

3.9.2 Catalase (CAT)

The activity of catalase in the semen samples was determined according to the method described by Aebi (1983). It is the common enzyme generally found in all living organisms which are exposed to oxygen. It catalyzes the hydrogen peroxide to water and oxygen. It prevents the cell from oxidative damage by ROS.

Reagents:

1. 50 mM Phosphate buffer, pH 7.0
2. Hydrogen peroxide, 30 mM: 0.34 ml of 30 % H_2O_2 was diluted with buffer.
3. The optical density of diluted H_2O_2 at 240 nm should be around 1.5.
4. Buffered H_2O_2 solution was prepared fresh.

Procedure:

Test	**Blank**
2 ml Phosphate buffer	2 ml phosphate buffer
Add 20µl sample	
Add 1 ml H_2O_2	
Read absorbance at 240 nm every 10 seconds interval for 1 min. (There is decrease in absorbance)	Set zero base

3.9.3 Superoxide dismutase (SOD)

The extent of SOD in semen was determined by the method explained by Marklund and Marklund (1974). Superoxide dismutase (SOD) is an antioxidant which has properties to catalyzes dismutation of superoxide into oxygen and hydrogen peroxide. Superoxide is produced as a by-product of oxygen metabolism and, if it is not regulated, it causes various types of cell damage.

Reagents:

1. Pyrogallol, 0.6 mM: Dissolve 76 mg of pyrogallol in 100 ml of water, store in brown bottle. The solution was prepared fresh before use.
2. EDTA, 6mM: Dissolve 223 mg EDTA disodium salt in 100 ml distilled water.
3. Tris-HCl buffer 100 mM: Dissolve 1.21 g Tris in 80 ml of distilled water.
4. Adjust pH to 8.2 with 10 mM HCl and make volume to 100 ml.

Procedure:

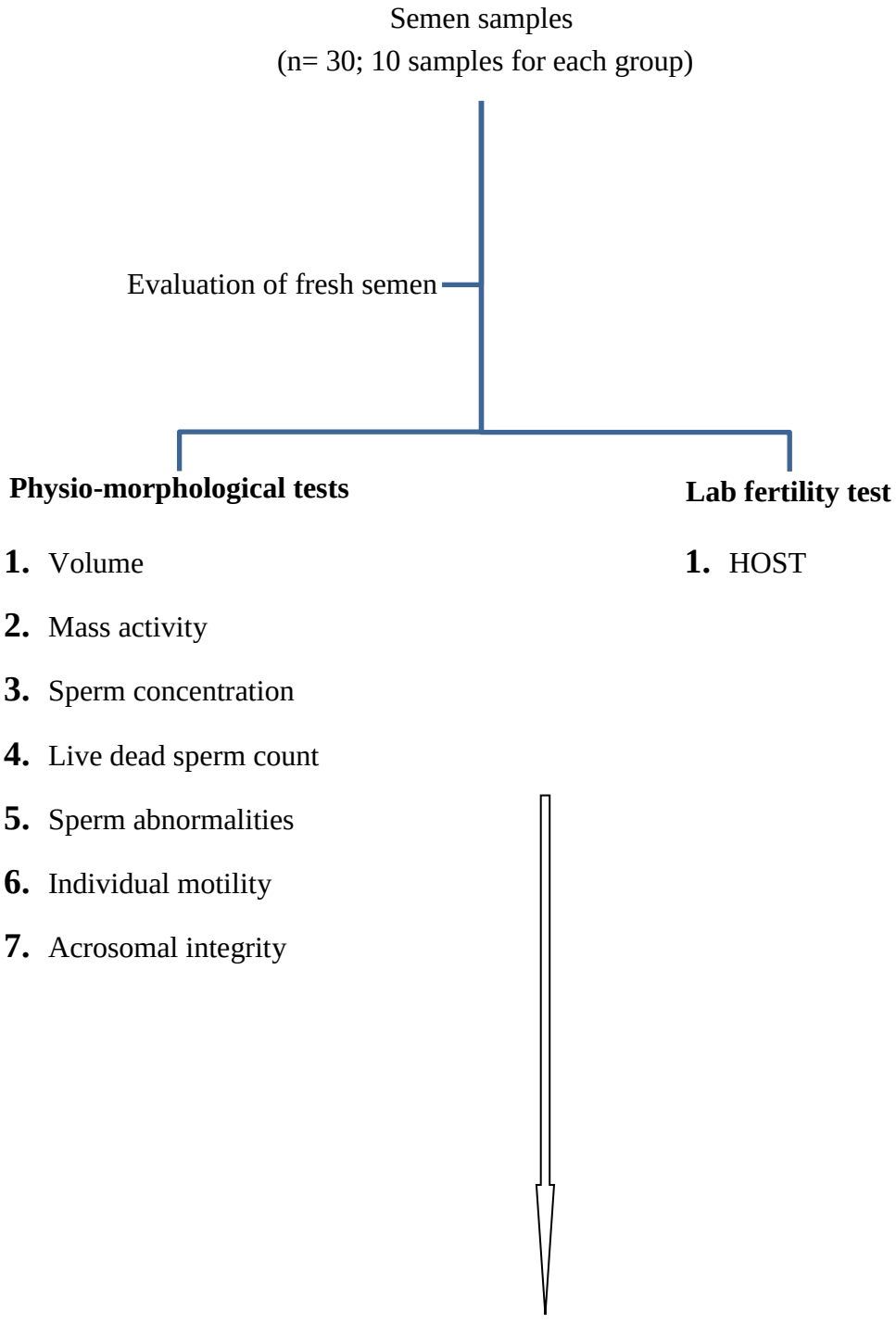
Reagents	**Control**	**Test**	**Blank**
Tris HCl buffer	1.5 ml	1.5 ml	1.5 ml
EDTA	0.5 ml	0.5 ml	0.5 ml
Pyrogallol	1 ml	1 ml	-
Enzyme preparation (1% lysate or tissue homogenate)	-	20µl	-

Read absorbance at 420 nm. Every 30 seconds up to 4 min. The unit of enzyme activity is defined as the amount of enzyme causing 50% inhibition of auto-oxidation of pyrogallol observed in control.

3.10 Statistical Analysis

All the data were analysed statistically using Analysis of Variance (ANOVA) as per Snedecor and Cochran, 1989.

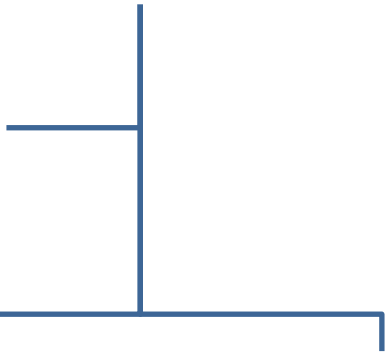
Plan of work



Contd.

Evaluation of diluted semen

Group 1: A. Control; B. Cysteine; C. Taurine
Group 2: A. Control; B. Ascorbic acid; C. Vitamin E
Group 3: A. Control; B. BHT; C. Trehalose



Physio-morphological tests

1. Live dead sperm count
2. Sperm abnormalities
3. Individual motility
4. Acrosomal integrity

Lab fertility test

1. HOST

↓

Equilibration

Evaluation: Live dead sperm count, Sperm abnormalities, Individual motility, Acrosomal integrity and HOST

↓

Freezing of straw

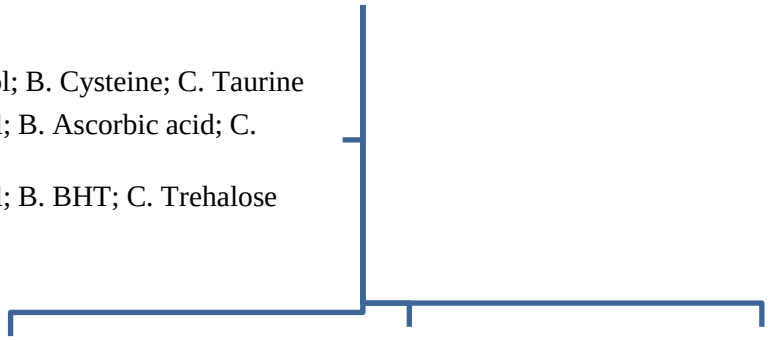
↓

Thawing (37˚c for 30 sec)

↓

Evaluation of Thawed semen

Group 1: A. Control; B. Cysteine; C. Taurine
Group 2: A. Control; B. Ascorbic acid; C. Vitamin E
Group 3: A. Control; B. BHT; C. Trehalose



Physio-morphological tests

1. Live dead sperm count
2. Sperm abnormalities
3. Individual motility
4. Acrosomal integrity

Lab fertility test

1. HOST

Oxidative stress test

1. MDA
2. SOD
3. Catalase

Chapter-IV

Results

CHAPTER-IV

RESULTS

4.1 Evaluation of Semen in Group I

4.1.1 Ejaculate volume

Perusal of Table (4.1), it was observed that mean volume of ejaculated semen in cattle bulls was 5.46 ± 1.62 ml with a range of 3.5 to 8.5 ml.

4.1.2 Mass activity (0-5)

Viewing the Table (4.1), it was observed that mean mass activity (0-5 scale) of ejaculated semen in cattle bulls was 3.65 ± 0.11 with a range of 3.5 to 3.8.

4.1.3 Sperm concentration (10^6/ml)

As evident from Table (4.1), it was observed that mean sperm concentration (millions/ml) of ejaculated semen in cattle bulls was 806.76 ± 65.92 millions/ml with a range of 584 to 1125 millions/ml.

Table 4.1: Seminal attributes in neat semen

Parameter	Mean ± S.E	Range
Volume (ml)	5.46 ± 1.62	3.5 to 8.5
Mass activity (0-5)	3.65 ± 0.11	3.5 to 3.8
Sperm concentration (million/ml)	806.76 ± 65.92	584 to 1125

- Values are given as mean ± S.E of 10 animals unless and otherwise stated.

4.1.4 Progressive motility

Table 4.2: Progressive motility (%) in neat, diluted, equilibrated and post-thaw semen

Stage	Progressive motility (%)			
	Neat semen	Control	Cysteine	Taurine
Diluted	$84.19^{Cc} \pm 0.37$	$69.01^{Ab} \pm 0.29$	$70.06^{Bb} \pm 0.25$	$70.66^{Bb} \pm 0.32$
Equilibrated		$62.31^{Ab} \pm 0.54$	$63.71^{ABb} \pm 0.53$	$64.16^{Bb} \pm 0.58$
Post thaw		$47.72^{Aa} \pm 0.79$	$50.1^{ABa} \pm 1.03$	$50.7^{Ba} \pm 1.09$

- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar **capital** and **small** letter superscripts within the **row** and **column,** respectively, differ significantly (P<0.05).

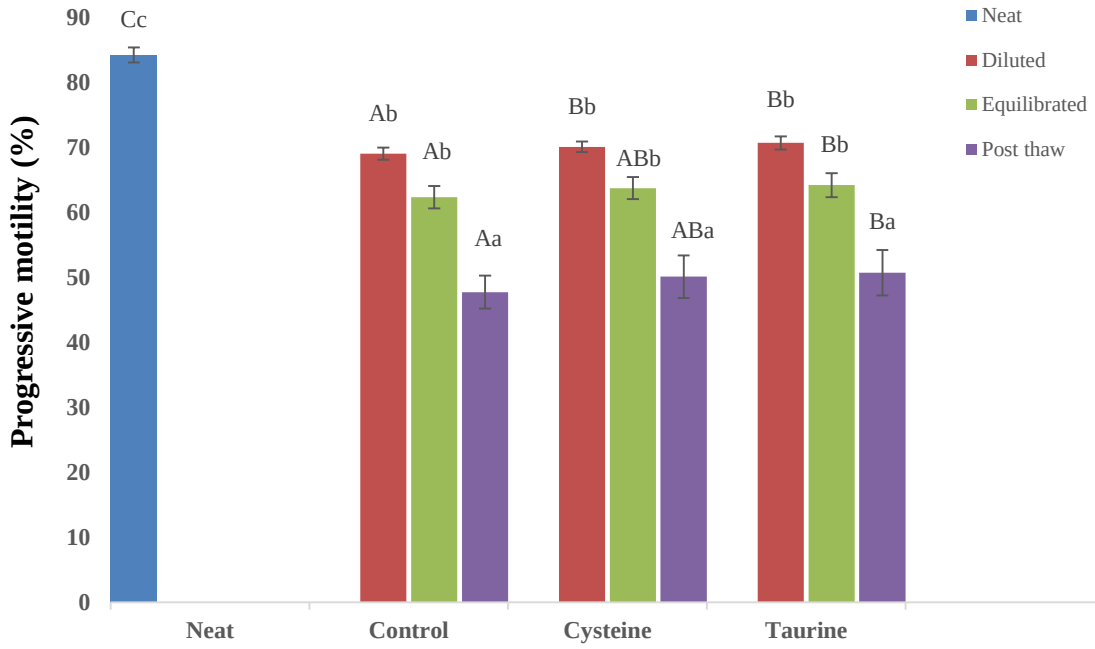
As evident from the Table (4.2), the per cent progressive motility of neat semen was 84.19 ± 0.37. Its values following dilution, equilibration and thawing were 69.01 ± 0.29, 62.31 ± 0.54 and 47.72 ± 0.79, respectively in control aliquot; 70.06 ± 0.25, 63.71 ± 0.53 and 50.1 ± 1.03, respectively in cysteine added aliquot; 70.66 ± 0.32, 64.16 ± 0.58 and 50.7 ± 1.09, respectively in taurine added aliquot.

Viewing the Table (4.2), it was observed that the highest value of progressive motility (%) was observed in the neat semen, which declined after dilution and further declined in equilibrated and post thaw semen samples, respectively. This trend of decline was observed in all the 3 aliquots of the group *i.e.* in control, cysteine and taurine added aliquots.

Comparing the Table (4.2), progressive motility (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, cysteine and taurine supplemented aliquots, a significant (P<0.05) difference was observed within and between all the aliquots at all the stages. It was also observed that the progressive motility (%) of post thaw semen sample differed significantly (P<0.05) within all aliquots *i.e.* control, cysteine and taurine added semen from diluted and equilibrated semen. Whereas,

within all the aliquots, the diluted semen differed non-significantly (P<0.05) from the equilibrated semen.

Perusal of Table (4.2), it was observed that the progressive motility (%) between different stage *i.e.* diluted, equilibrated and post thaw stages, the cysteine and taurine added aliquot differed significantly (P<0.05) from control aliquots at diluted stage, whereas between cysteine and taurine added aliquots there was no significant (P<0.05) difference at diluted stage. Among equilibrated stage the taurine supplemented aliquots differed significantly (P<0.05) from control, whereas there was no significant difference between cysteine and taurine supplemented aliquots and also between control and cysteine supplemented aliquots at equilibration stage. Between the post thaw stage of all the aliquots the taurine supplemented aliquots differed significantly (P<0.05) from control, whereas non-significantly from cysteine added semen sample. Moreover, between control and cysteine added semen sample there was no significant difference at post thaw stage.



- Means with dissimilar **capital** and **small** letter superscripts between different aliquots and within same stage, respectively, differ significantly (P<0.05).

Figure 1: Bar diagram showing progressive motility (%) in neat, diluted, equilibrated and post thaw semen

4.1.5 Live Sperm

Table 4.3: Live sperm (%) in neat, diluted, equilibrated and post-thaw semen

Stage	Live sperm (%)			
	Neat semen	**Control**	**Cysteine**	**Taurine**
Diluted	$90.10^{Cd} \pm 0.67$	$78.73^{Ac} \pm 1.46$	$79.52^{Ac} \pm 0.48$	$80.27^{Ac} \pm 0.95$
Equilibrated		$71.22^{Ab} \pm 1.4$	$74.28^{Ab} \pm 0.78$	$73.63^{Ab} \pm 1.03$
Post thaw		$59.59^{Aa} \pm 0.69$	$62.18^{ABa} \pm 0.95$	$62.61^{Ba} \pm 0.98$

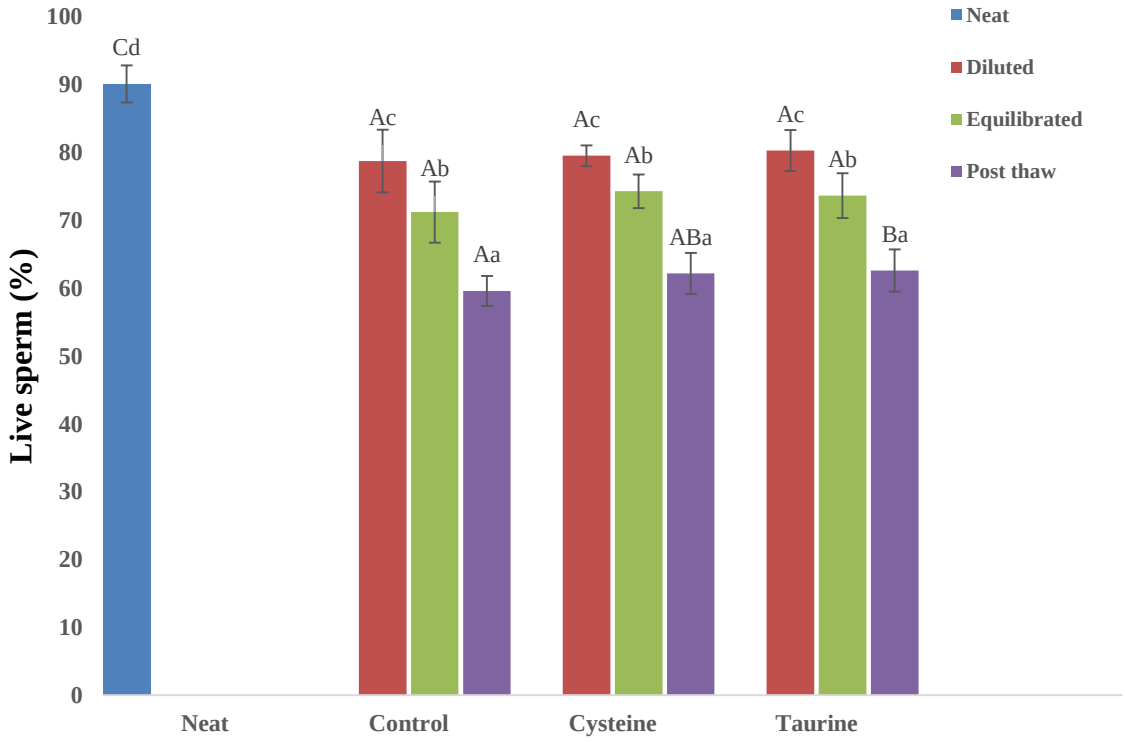
- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar **capital** and **small** letter superscripts within the **row** and **column,** respectively, differ significantly (P<0.05).

As evident from the Table (4.3), the per cent live sperm in the neat semen was 90.10 ± 0.67. Its values following dilution, equilibration and thawing were 78.73 ± 1.46, 71.22 ± 1.4 and 59.59 ± 0.69, respectively in control aliquot; 79.52 ± 0.48, 74.28 ± 0.78 and 62.18 ± 0.95, respectively in cysteine added aliquot; 80.27 ± 0.95, 73.63 ± 1.03 and 62.61 ± 0.98, respectively in taurine added aliquot.

Viewing the Table (4.3), it was observed that the highest value of live sperm (%) was observed in the neat semen which declined after dilution and further declined in equilibrated and post thaw semen samples, respectively. This trend of decline was observed in all the 3 aliquots of the group *i.e.* in control, cysteine and taurine supplemented aliquots.

Comparing the Table (4.3), live sperm (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, cysteine and taurine supplemented aliquots, a significant (P<0.05) difference was observed within and between all the aliquots at all the stages. It was also observed that the live sperm (%) of all the stages *i.e.* diluted, equilibrated and post thaw stages differed significantly (P<0.05) within all aliquots *i.e.* control, cysteine and taurine supplemented aliquots.

Perusal of Table (4.3), it was observed that the live sperm (%) between different stages *i.e.* diluted, equilibrated and post thaw stages, the control, cysteine and taurine supplemented aliquots were non-significant (P<0.05) at diluted stage and equilibrated stage. At the post thaw stage the taurine supplemented aliquots differed significantly (P<0.05) from control, whereas non-significantly from cysteine supplemented semen sample. Moreover, between control and cysteine added aliquots there was no significant difference at post thaw stage.



- Means with dissimilar **capital** and **small** letter superscripts between different aliquots and within same stage, respectively, differ significantly (P<0.05).

Figure 2: Bar diagram showing live sperm (%) in neat, diluted, equilibrated and post thaw semen

4.1.6 Acrosomal integrity

Table 4.4: Acrosomal integrity (%) in neat, diluted, equilibrated and post-thaw semen

Stage	Acrosomal integrity (%)			
	Neat semen	Control	Cysteine	Taurine
Diluted	$84.39^{Cd} \pm 1.25$	$74.44^{Ac} \pm 1.43$	$77.32^{Ac} \pm 1.11$	$77.18^{Ac} \pm 0.82$
Equilibrated		$67.33^{Ab} \pm 1.14$	$70.18^{ABb} \pm 1.00$	$71.26^{Bb} \pm 1.15$
Post thaw		$59.53^{Aa} \pm 0.49$	$62.63^{Ba} \pm 0.86$	$63.86^{Ba} \pm 0.84$

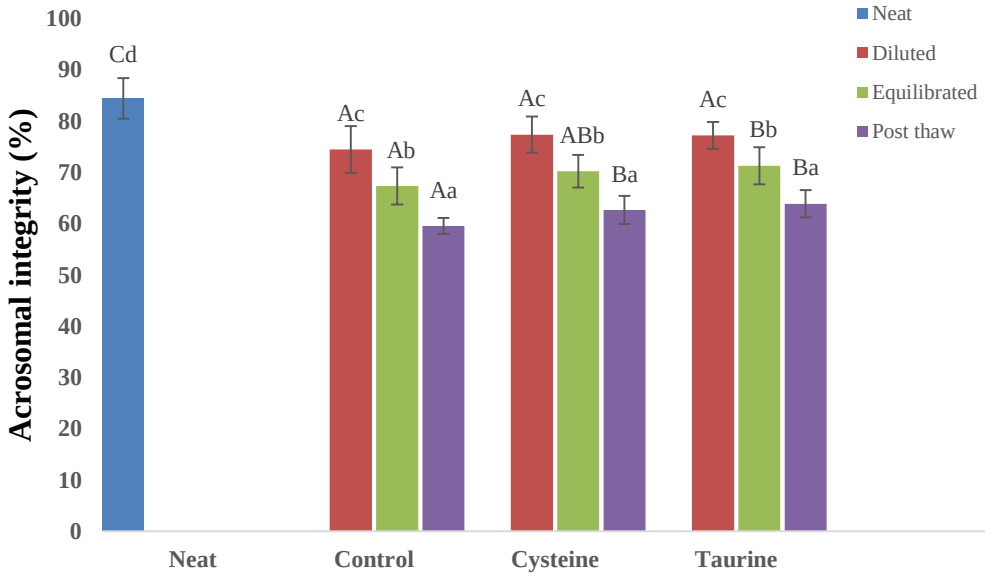
- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar **capital** and **small** letter superscripts within the **row** and **column,** respectively, differ significantly (P<0.05).

As evident from the Table (4.4), the per cent acrosomal integrity of neat semen was 80.39 ± 1.25. Its values following dilution, equilibration and thawing were 74.44 ± 1.43, 67.33 ± 1.14 and 59.53 ± 0.49, respectively in control aliquot, 77.32 ± 1.11, 70.18 ± 1.00 and 62.63 ± 0.86, respectively in cysteine added aliquot; 77.18 ± 0.82, 71.26 ± 1.15 and 63.86 ± 0.84, respectively in taurine added aliquot.

Viewing the Table (4.4), it was observed that the highest value of acrosomal integrity (%) was observed in the neat semen which declined after dilution and further declined in equilibrated and post thaw semen samples, respectively. This trend of decline was observed in all the 3 aliquots of the group *i.e.* in control, cysteine and taurine supplemented aliquots.

Comparing the Table (4.4), acrosomal integrity (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, cysteine and taurine supplemented aliquots, a significant (P<0.05) difference was observed within and between all the aliquots at all stages. It was also observed that the acrosomal integrity (%) of all the stages *i.e.* diluted, equilibrated and post thaw stages differed significantly (P<0.05) within all aliquots *i.e.* control, cysteine and taurine supplemented aliquots.

Perusal of Table (4.4), it was observed that the acrosomal integrity (%) between different stages i.e. diluted, equilibrated and post thaw stages, the control, cysteine and taurine supplemented aliquots were non-significant (P<0.05) at diluted stage. Among equilibrated stage the taurine added semen sample differed significantly (P<0.05) from control, whereas there was no significant difference between cysteine and taurine supplemented aliquots and also between control and cysteine supplemented semen sample at equilibration stage. Between the post thaw stage of all the aliquots the cysteine and taurine added aliquots differed significantly (P<0.05) from control. Moreover, between cysteine and taurine supplemented aliquots there was no significant difference at post thaw stage.



- Means with dissimilar **capital** and **small** letter superscripts between different aliquots and within same stage, respectively, differ significantly (P<0.05).

Figure 3: Bar diagram showing acrosomal integrity (%) in neat, diluted, equilibrated and post thaw semen

4.1.7 Sperm abnormalities

Table 4.5: Sperm abnormalities (%) in neat, diluted, equilibrated and post-thaw semen

Stage	Sperm abnormalities (%)			
	Neat semen	**Control**	**Cysteine**	**Taurine**
Diluted	$3.30^{Aa} \pm 0.30$	$6.22^{Bb} \pm 0.38$	$5.40^{Bb} \pm 0.44$	$5.67^{Bb} \pm 0.48$
Equilibrated		$8.42^{Bc} \pm 0.65$	$7.78^{Bb} \pm 0.62$	$7.62^{Bb} \pm 0.63$
Post thaw		$12.31^{Bd} \pm 0.59$	$11.62^{Bc} \pm 0.54$	$11.34^{Bc} \pm 0.38$

- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar **capital** and **small** letter superscripts within the **row** and **column,** respectively, differ significantly ($P<0.05$).

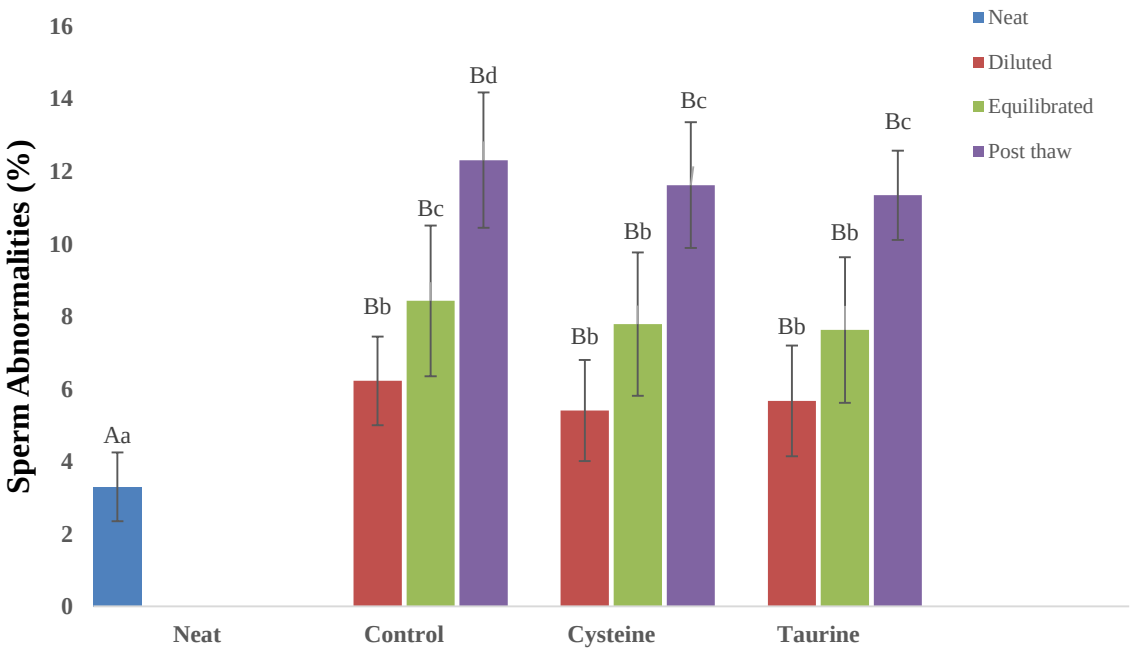
As evident from the Table (4.5), the per cent sperm abnormalities in neat semen was 3.30 ± 0.30. Its values following dilution, equilibration and thawing were 6.22 ± 0.38, 8.42 ± 0.65 and 12.31 ± 0.59, respectively in control aliquot; 5.40 ± 0.44, 7.78 ± 0.62 and 11.62 ± 0.54, respectively in cysteine added aliquot; 5.67 ± 0.48, 7.62 ± 0.63 and 11.34 ± 0.38, respectively in taurine added aliquot.

Viewing the Table (4.5), it was observed that the lowest value of sperm abnormalities (%) was observed in the neat semen which inclined after dilution and further inclined in equilibrated and post thaw semen samples, respectively. This trend of incline was observed in all the 3 aliquots of the group *i.e.* in control, cysteine and taurine supplemented aliquots.

Comparing the Table (4.5), sperm abnormalities (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, cysteine and taurine added aliquots, a significant ($P<0.05$) difference was observed within and between the all the aliquots at all stages and also it was observed that the sperm abnormalities (%) of post thaw semen sample differed significantly ($P<0.05$) within all the aliquots *i.e.* control cysteine and taurine added semen from diluted and equilibrated semen. Whereas, within control semen sample, the diluted semen differed significantly

(P<0.05) from the equilibrated semen. Moreover, within cysteine and taurine supplemented aliquots, the diluted semen was non-significant from the equilibrated semen.

Perusal of Table (4.5), it was observed that the sperm abnormalities (%) between all the different stages *i.e.* diluted, equilibrated, post thaw stages and between all the aliquots *i.e.* control, cysteine, taurine supplemented aliquots differed non-significantly.



- Means with dissimilar **capital** and **small** letter superscripts between different aliquots and within same stage, respectively, differ significantly (P<0.05).

Figure 4: Bar diagram showing sperm abnormalities in neat, diluted, equilibrated and post thaw semen

4.1.8 Hypo-osmotic swelling test (HOST)

Table 4.6: Hypo-osmotic swelling test (HOST) positive (%) in neat, diluted, equilibrated and post-thaw semen

Stage	HOST (%)			
	Neat semen	Control	Cysteine	Taurine
Diluted	$82.20^{Bc} \pm 1.00$	$74.06^{Ab} \pm 0.97$	$76.76^{Ab} \pm 0.97$	$75.28^{Ab} \pm 1.19$
Equilibrated		$68.83^{Ab} \pm 1.32$	$70.9^{Ab} \pm 1.51$	$70.25^{Ab} \pm 1.58$
Post thaw		$57.61^{Aa} \pm 0.93$	$59.94^{Aa} \pm 1.09$	$59.65^{Aa} \pm 1.53$

- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar **capital** and **small** letter superscripts within the **row** and **column,** respectively, differ significantly ($P<0.05$).

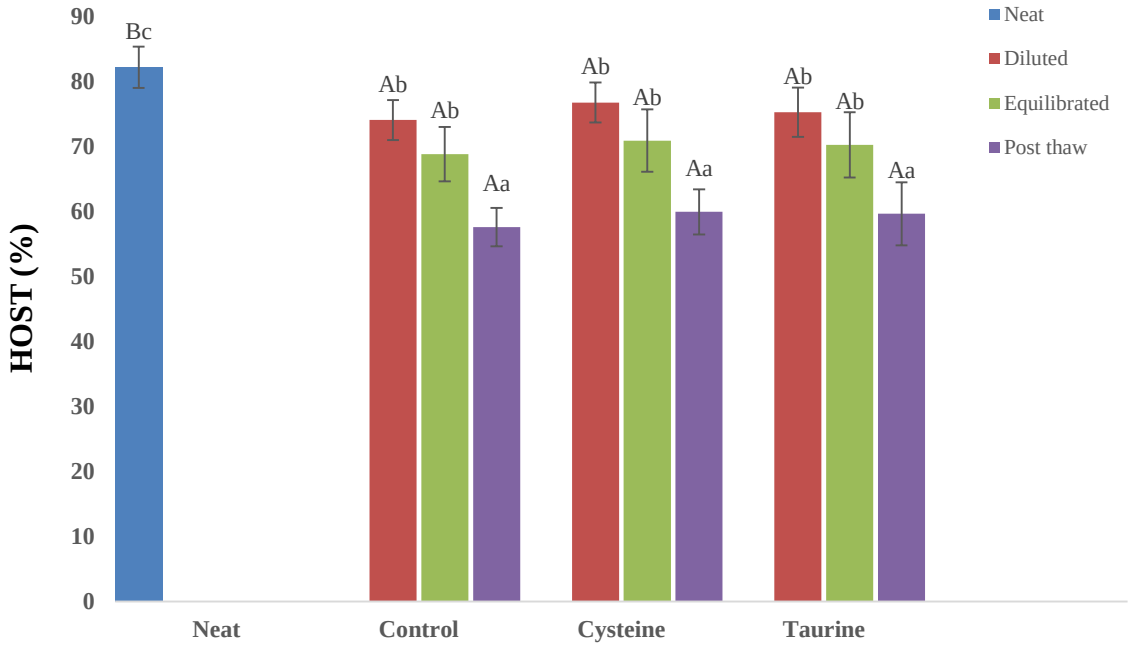
As evident from the Table (4.6), the per cent HOST positive (%) in neat semen was 82.20 ± 1.00. Its values following dilution, equilibration and thawing were 74.06 ± 0.97, 68.83 ± 1.32 and 57.61 ± 0.93, respectively in control aliquot; 76.76 ± 0.97, 70.9 ± 1.51 and 59.94 ± 1.09, respectively in cysteine added aliquot; 75.28 ± 1.19, 70.25 ± 1.58 and 59.65 ± 1.53, respectively in taurine added aliquot.

Viewing the Table (4.6), it was observed that the highest value of HOST positive (%) was observed in the neat semen which declined after dilution and further declined in equilibrated and post thaw semen samples, respectively. This trend of decline was observed in all the 3 aliquots of the group *i.e.* in control, cysteine and taurine supplemented aliquots.

Comparing the Table (4.6), HOST positive (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, cysteine and taurine added aliquots, a significant ($P<0.05$) difference was observed within and between all the aliquots at all the stages and it was also observed that the HOST positive (%) of post thaw semen sample differed significantly ($P<0.05$) within all aliquots *i.e.* control, cysteine and taurine supplemented aliquots from diluted and equilibrated semen.

Whereas, within all the aliquots, the diluted semen differ non-significantly (P<0.05) from the equilibrated semen.

Perusal of Table (4.6), it was observed that the HOST positive (%) between all the different stages *i.e.* diluted, equilibrated, post thaw stages and between all the aliquots *i.e.* control, cysteine, taurine supplemented aliquots differed non-significantly.



- Means with dissimilar **capital** and **small** letter superscripts between different aliquots and within same stage, respectively, differ significantly (P<0.05).

Figure 5: Bar diagram showing hypo-osmotic swelling test (HOST) positive (%) in neat diluted, equilibrated and post thaw semen

4.1.9 Lipid peroxidation (LPO)

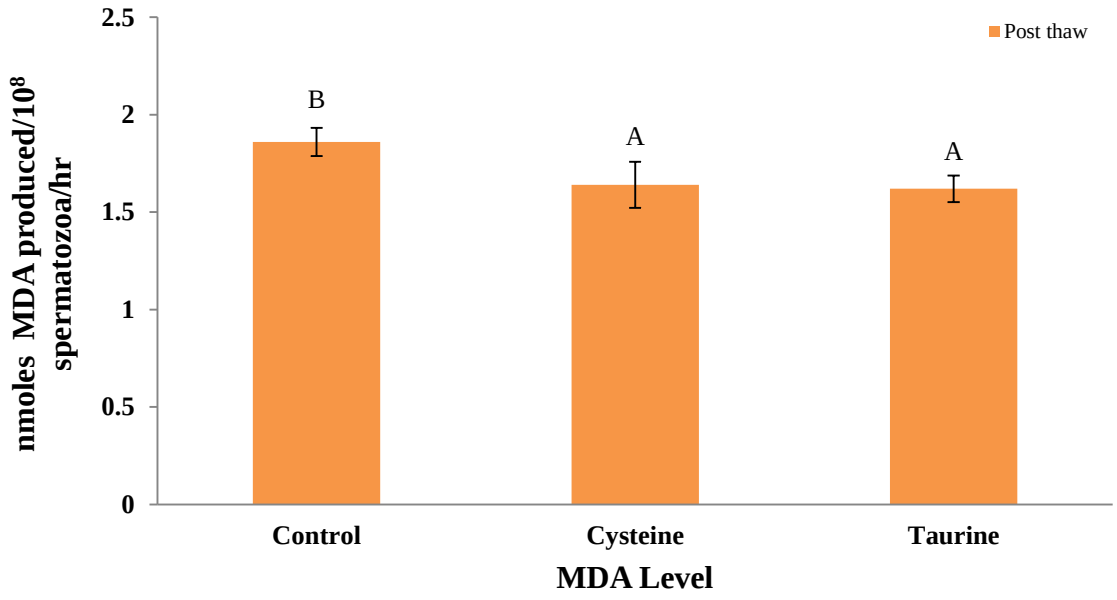
Table 4.7: Lipid peroxidation (LPO) measured as level of MDA in post thaw semen

Stage	MDA level (nmoles of MDA produced/ 10^8 spermatozoa/hr)		
	Control	Cysteine	Taurine
Post thaw	$1.86^{B} \pm 0.02$	$1.64^{A} \pm 0.03$	$1.62^{A} \pm 0.02$

- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar letter superscripts in the **row** differ significantly (P<0.05).

As evident from the Table (4.7), the mean value of MDA level (nmoles of MDA produced/ 10^8 spermatozoa/hr) in post thaw semen was 1.86 ± 0.02 in control aliquot, 1.64 ± 0.03 in cysteine added aliquot and 1.62 ± 0.02 in taurine added aliquot.

Comparing the Table (4.7), it was observed that the MDA level between the post thaw semen sample with or without additives *i.e.* both cysteine and taurine supplemented semen differed significantly (P<0.05) from control. Whereas, cysteine added differed non-significantly from taurine supplemented aliquots.



- Means with dissimilar letter superscripts in the bar differ significantly (P<0.05).

Figure 6: Bar diagram showing lipid peroxidation (LPO) measured as level of MDA in post thaw semen

4.1.10 Catalase (CAT)

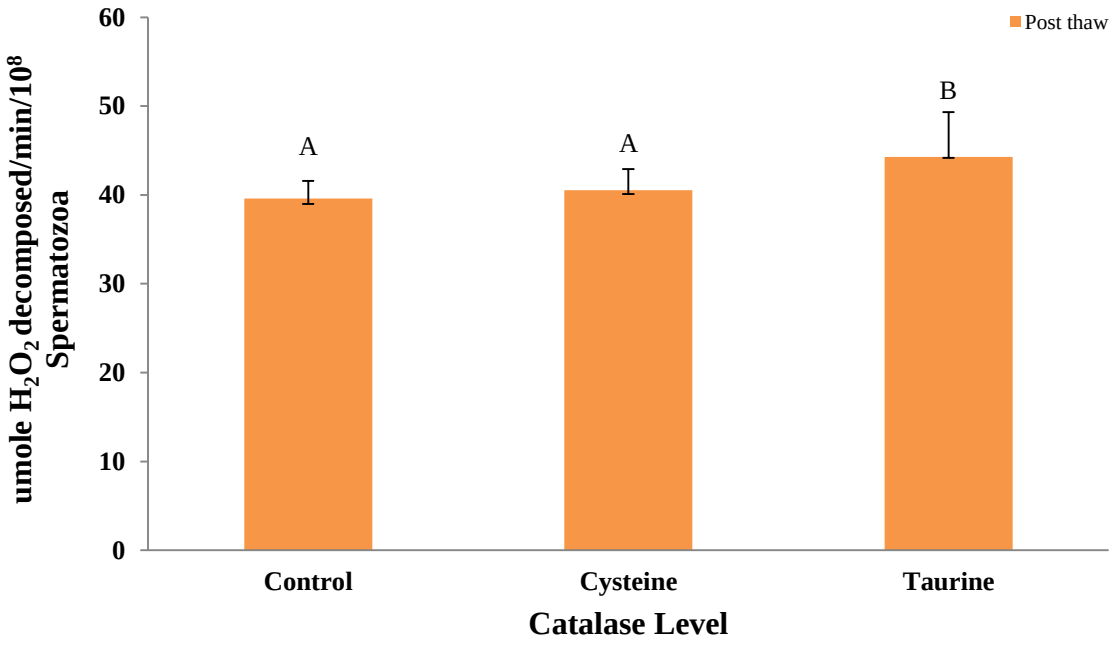
Table: 4.8: Catalase (CAT) level in post thaw semen

Stage	Catalase (µmol H_2O_2 decomposed/min/10^8 spermatozoa)		
	Control	Cysteine	Taurine
Post thaw	39.61^{A}± 0.62	40.52^{A} ± 0.75	44.29^{B} ± 1.59

- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar letter superscripts in the **row** differ significantly (P<0.05).

As evident from the Table (4.8), the mean value of catalase (µmol H_2O_2 decomposed/min/10^8 spermatozoa) in post thaw semen was 39.61 ± 0.62 in control aliquot, 40.52 ± 0.75 in cysteine added aliquot and 44.29 ± 1.59 in taurine added aliquot.

Comparing the Table (4.8), it was observed that the catalase level between the post thaw semen with or without additive *i.e.* taurine added aliquots differ significantly (P<0.05) from control and cysteine supplemented aliquots. Whereas cysteine added differed non-significantly from control supplemented semen.



- Means with dissimilar letter superscripts in the bar differ significantly (P<0.05).

Figure 7: Bar diagram showing catalase (CAT) levels in post thaw semen

4.1.11 Superoxide dismutase (SOD)

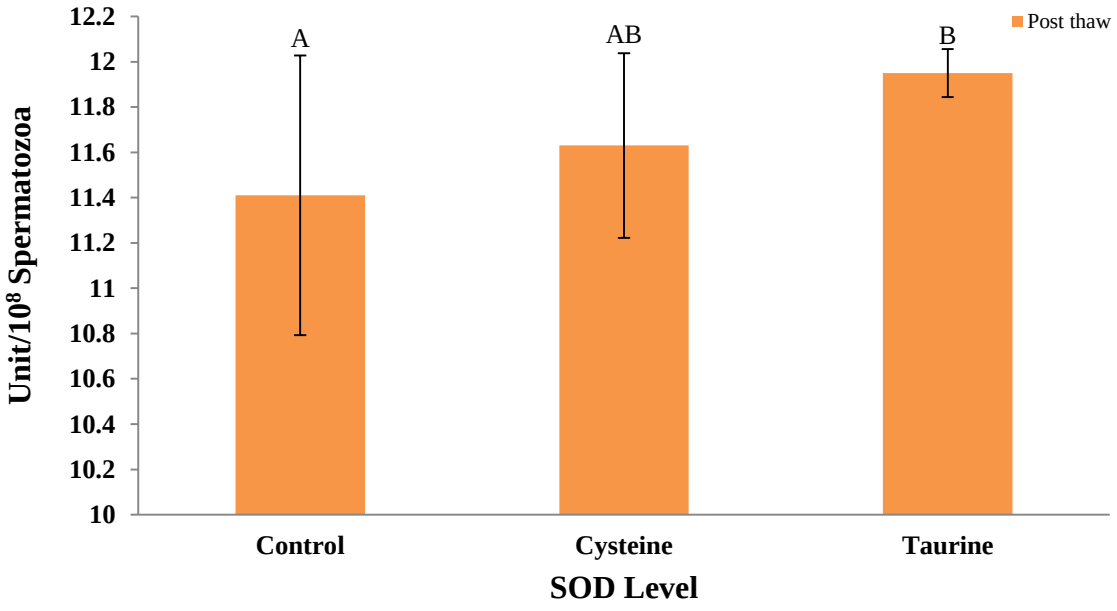
Table 4.9: Superoxide dismutase (SOD) level in post thaw semen

Stage	Superoxide dismutase (Unit/ 10^8 spermatozoa)		
	Control	Cysteine	Taurine
Post thaw	$11.41^{A} \pm 0.19$	$11.63^{AB} \pm 0.12$	$11.95^{B} \pm 0.03$

- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar letter superscripts in the **row** differ significantly (P<0.05).

As evident from the Table (4.9), the mean value of SOD level (Unit/ 10^8 spermatozoa) in post thaw semen was 11.41 ± 0.19 in control aliquot, 11.63 ± 0.12 in cysteine added aliquot and 11.95 ± 0.03 in taurine added aliquot.

Comparing the Table (4.9), it was observed that the SOD level between the post thaw semen sample with or without additive *i.e.* taurine supplemented aliquots differ significantly (P<0.05) from the control semen sample. Whereas, cysteine added differed non-significantly from both control and taurine supplemented semen.



- Means with dissimilar letter superscripts in the bar differ significantly (P<0.05).

Figure 8: Bar diagram showing superoxide dismutase (SOD) level in post thaw semen

4.2 Evaluation of Semen in Group II

4.2.1 Ejaculate volume

Perusal of Table (4.10), it was observed that mean volume of ejaculated semen in cattle bulls was 5.79 ± 0.51 ml with a range of 4 to 8.5 ml.

4.2.2 Mass activity (0-5)

Viewing the Table (4.10), it was observed that mean mass activity (0-5 scale) of ejaculated semen in cattle bulls was 3.64 ± 0.42 with a range of 3.4 to 3.8.

4.2.3 Sperm concentration (10^6/ml)

As evident from Table (4.10), it was observed that mean sperm concentration (millions/ml) of ejaculated semen in cattle bulls was 877.56 ± 67.53 millions/ml with a range of 642 to 1178 millions/ml.

Table 4.10: Seminal attributes in neat semen

Parameter	Mean ± S.E	Range
Volume (ml)	5.79 ± 0.51	4 to 8.5
Mass activity (0-5)	3.64 ± 0.42	3.4 to 3.8
Sperm concentration (million/ml)	877.56 ± 67.53	642 to 1178

- Values are given as mean ± S.E of 10 animals unless and otherwise stated.

4.2.4 Progressive motility

Table 4.11: Progressive motility (%) in neat, diluted, equilibrated and post-thaw semen

Stage	Progressive motility (%)			
	Neat semen	**Control**	**Ascorbic acid**	**Vitamin E**
Diluted	$84.33^{Cd} \pm 0.36$	$68.7^{Ac} \pm 0.28$	$70.39^{Bc} \pm 0.31$	$69.68^{Bc} \pm 0.23$
Equilibrated		$63.12^{Ab} \pm 0.49$	$64.51^{Ab} \pm 0.63$	$64.27^{Ab} \pm 0.50$
Post thaw		$48.97^{Aa} \pm 0.56$	$51.47^{Ba} \pm 0.77$	$51.13^{Ba} \pm 0.77$

- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar **capital** and **small** letter superscripts within the **row** and **column,** respectively, differ significantly ($P<0.05$).

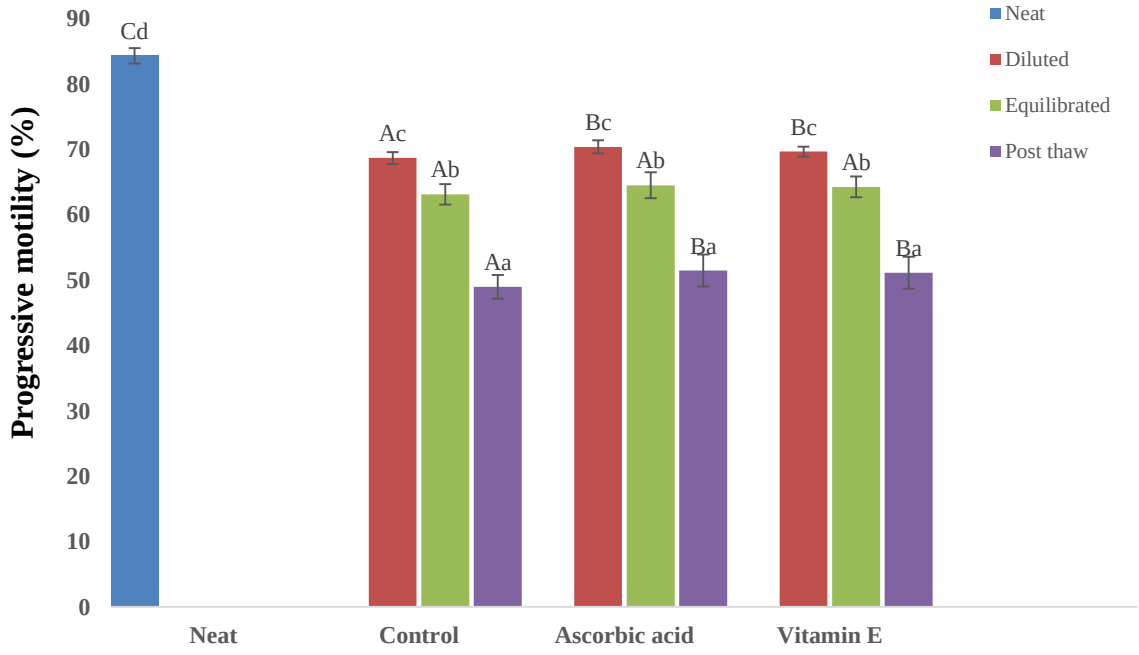
As evident from the Table (4.11), the per cent progressive motility of neat semen was 84.33 ± 0.36. Its values following dilution, equilibration and thawing were 68.7 ± 0.28, 63.12 ± 0.49 and 48.97 ± 0.56, respectively in control aliquot; 70.39 ± 0.31, 64.51 ± 0.63 and 51.47 ± 0.77, respectively in ascorbic acid (vitamin C) added aliquot; 69.68 ± 0.23, 64.27 ± 0.23 and 51.13 ± 0.77, respectively in vitamin E added aliquot.

Viewing the Table (4.11), it was observed that the highest value of progressive motility (%) was observed in the neat semen, which declined after dilution and further declined in equilibrated and post thaw semen samples, respectively. This trend of decline was observed in all the 3 aliquots of the group *i.e.* in control, ascorbic acid and vitamin E supplemented aliquots.

Comparing the Table (4.11), progressive motility (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, ascorbic acid and vitamin E added aliquots, a significant ($P<0.05$) difference was observed within and between all the aliquots at all the stages. It was also observed that the progressive motility

(%) of all the stages *i.e.* diluted, equilibrated and post thaw stages differed significantly (P<0.05) within all aliquots *i.e.* control, ascorbic acid and vitamin E added semen.

Perusal of Table (4.11), it was observed that the progressive motility (%) between different stage *i.e.* diluted, equilibrated and post thaw stages, the ascorbic acid and vitamin E supplemented aliquots differed significantly (P<0.05) from control semen aliquots at diluted stage, whereas between ascorbic acid and vitamin E added aliquots there was no significant (P<0.05) difference at diluted stage. Among equilibrated stage, all the aliquots *i.e.* control, ascorbic acid and vitamin E supplemented aliquots differed non-significantly. Between the post thaw stage of all the aliquots the ascorbic acid and vitamin E added semen differed significantly (P<0.05) from control. Moreover, between ascorbic acid and vitamin E supplemented aliquots there was no significant difference at post thaw stage.



- Means with dissimilar **capital** and **small** letter superscripts between different aliquots and within same stage, respectively, differ significantly (P<0.05).

Figure 9: Bar diagram showing progressive motility (%) in neat, diluted, equilibrated and post thaw semen

4.2.5 Live sperm

Table 4.12: Live sperm (%) in neat, diluted, equilibrated and post-thaw semen

Stage	Live sperm (%)			
	Neat semen	**Control**	**Ascorbic acid**	**Vitamin E**
Diluted	$89.03^{Cd} \pm 0.88$	$76.84^{Ac} \pm 1.23$	$78.46^{Ac} \pm 1.12$	$77.97^{Ab} \pm 1.24$
Equilibrated		$70.16^{Ab} \pm 0.82$	$72.35^{Ab} \pm 1.08$	$72.81^{Ab} \pm 0.94$
Post thaw		$57.88^{Aa} \pm 1.34$	$62.57^{Ba} \pm 0.95$	$62.12^{Ba} \pm 0.82$

- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar **capital** and **small** letter superscripts within the **row** and **column,** respectively, differ significantly ($P<0.05$).

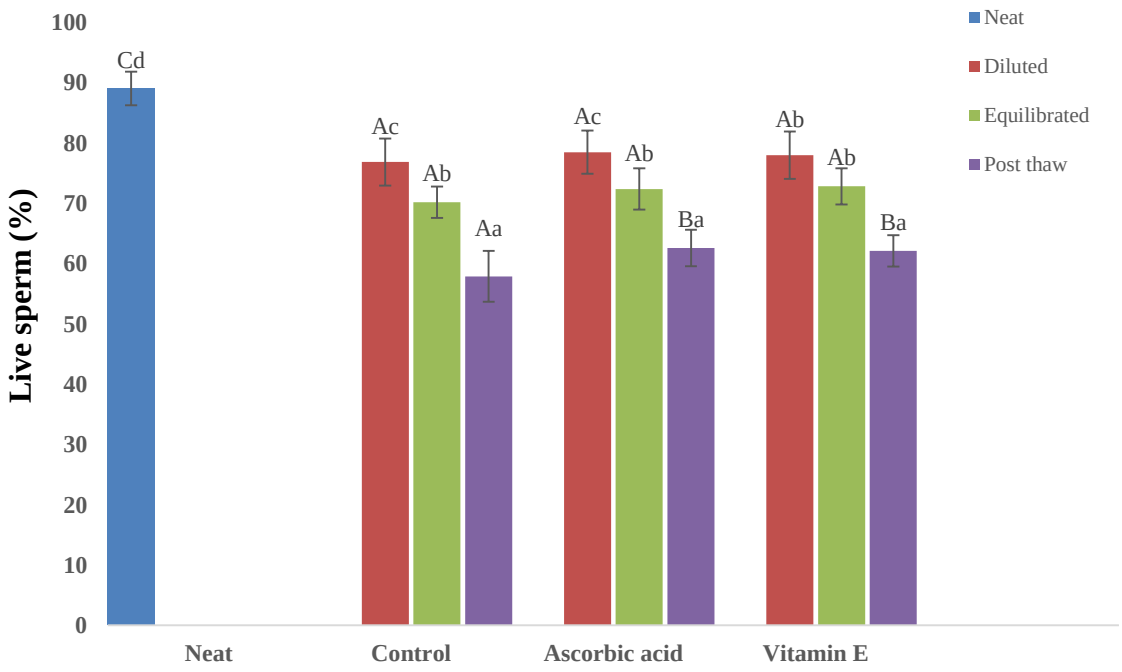
As evident from the Table (4.12), the per cent live sperm in neat semen was 89.03 ± 0.88. Its values following dilution, equilibration and thawing were 76.84 ± 1.23, 70.16 ± 0.82 and 57.88 ± 1.34, respectively in control aliquot; 78.46 ± 1.12, 72.35 ± 1.08 and 62.57 ± 0.95, respectively in ascorbic acid added aliquot; 77.97 ± 1.24, 72.81 ± 0.94 and 62.12 ± 0.82, respectively in vitamin E added aliquot.

Viewing the Table (4.12), it was observed that the highest value of live sperm (%) was observed in the neat semen, which declined after dilution and further declined in equilibrated and post thaw semen samples, respectively. This trend of decline was observed in all the 3 aliquots of the group *i.e.* in control, ascorbic acid and vitamin E supplemented aliquots.

Comparing the Table (4.12), live sperm (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, ascorbic acid and vitamin E supplemented aliquots, a significant ($P<0.05$) difference was observed within and between all the aliquots at all the stages. It was also observed that the live sperm (%) of post thaw semen sample differed significantly ($P<0.05$) within all aliquots *i.e.* control, ascorbic acid and vitamin E supplemented aliquots from diluted and equilibrated semen. Whereas, within control and ascorbic acid the diluted semen differed significantly

(P<0.05) from the equilibrated semen. Moreover, within vitamin E, the diluted semen differed non-significantly (P<0.05) from the equilibrated semen.

Perusal of Table (4.12), it was observed that the live sperm (%) between different stage *i.e.* diluted, equilibrated and post thaw stages, all the aliquots *i.e.* control, ascorbic acid and vitamin E supplemented aliquots differed non-significantly (P<0.05) at diluted and equilibrated stage. Between the post thaw stage of all the aliquots the ascorbic acid and vitamin E added semen differed significantly (P<0.05) from control. Moreover, ascorbic acid supplemented semen differed non-significantly from vitamin E supplemented aliquots at post thaw stage.



- Means with dissimilar **capital** and **small** letter superscripts between different aliquots and within same stage, respectively, differ significantly (P<0.05).

Figure 10: Bar diagram showing live sperm (%) in neat, diluted, equilibrated and post thaw semen

4.2.6 Acrosomal integrity

Table 4.13: Acrosomal integrity (%) in neat, diluted, equilibrated and post-thaw semen

Stage	Acrosomal integrity (%)			
	Neat semen	Control	Ascorbic acid	Vitamin E
Diluted	$83.82^{Cc} \pm 1.25$	$72.05^{Ab} \pm 1.51$	$73.54^{Ab} \pm 1.49$	$73.88^{Ab} \pm 1.52$
Equilibrated		$66.35^{Ab} \pm 1.23$	$68.58^{Ab} \pm 0.96$	$68.97^{Ab} \pm 1.05$
Post thaw		$59.53^{Aa} \pm 0.87$	$62.64^{Ba} \pm 0.93$	$62.38^{ABa} \pm 1.00$

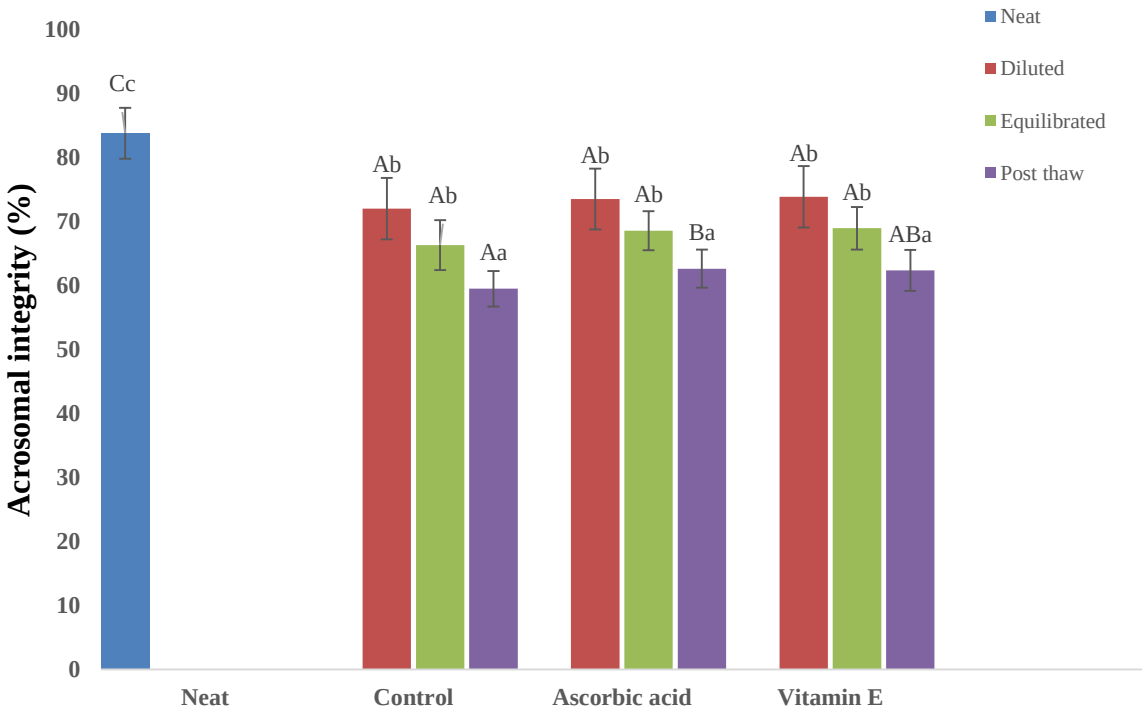
- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar **capital** and **small** letter superscripts within the **row** and **column,** respectively, differ significantly (P<0.05).

As evident from the Table (4.13), the per cent acrosomal integrity in neat semen was 83.82 ± 1.25. Its values following dilution, equilibration and thawing were 72.05 ± 1.51, 66.35 ± 1.23 and 59.53 ± 0.87, respectively in control aliquot; 73.54 ± 1.49, 68.58 ± 0.96 and 62.64 ± 0.93, respectively in ascorbic acid added aliquot; 73.88 ± 1.52, 68.97 ± 1.05 and 62.38 ± 1.00, respectively in vitamin E added aliquot.

Viewing the Table (4.13), it was observed that the highest value of acrosomal integrity (%) was observed in the neat semen, which declined after dilution and further declined in equilibrated and post thaw semen samples, respectively. This trend of decline was observed in all the 3 aliquots of the group *i.e.* in control, ascorbic acid and vitamin E supplemented aliquots.

Comparing the Table (4.13), acrosomal integrity (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, ascorbic acid and vitamin E supplemented aliquots, a significant (P<0.05) difference was observed within and between all the aliquots at all the stages. It was also observed that the acrosomal integrity (%) of post thaw semen sample differed significantly (P<0.05) within all aliquots *i.e.* control, ascorbic acid and vitamin E supplemented aliquots from diluted and equilibrated semen. Whereas, within all the aliquots, the diluted semen differed non-significantly (P<0.05) from the equilibrated semen.

Perusal of Table (4.13), it was observed that the acrosomal integrity (%) between different stage *i.e.* diluted, equilibrated and post thaw stages, all the aliquots *i.e.* control, ascorbic acid and vitamin E supplemented aliquots differed non-significantly (P<0.05) at diluted and equilibrated stage. Between the post thaw stage of all the aliquots the ascorbic acid supplemented semen differed significantly (P<0.05) from control. Moreover, vitamin E added semen differed non-significantly from control as well as ascorbic supplemented aliquots at post thaw stage.



- Means with dissimilar **capital** and **small** letter superscripts between different aliquots and within same stage, respectively, differ significantly (P<0.05).

Figure 11: Bar diagram showing acrosomal integrity (%) in neat, diluted, equilibrated and post thaw semen

4.2.7 Sperm abnormalities

Table 4.14: Sperm abnormalities (%) in neat, diluted, equilibrated and post-thaw semen.

Stage	Sperm abnormalities (%)			
	Neat semen	Control	Ascorbic acid	Vitamin E
Diluted	$3.73^{Aa} \pm 0.41$	$6.62^{Bb} \pm 0.41$	$5.99^{Bb} \pm 0.34$	$6.34^{Bb} \pm 0.34$
Equilibrated		$9.36^{Bc} \pm 0.59$	$8.85^{Bc} \pm 0.45$	$8.85^{Bc} \pm 0.59$
Post thaw		$13.42^{Bd} \pm 0.64$	$12.25^{Bd} \pm 0.50$	$12.39^{Bd} \pm 0.42$

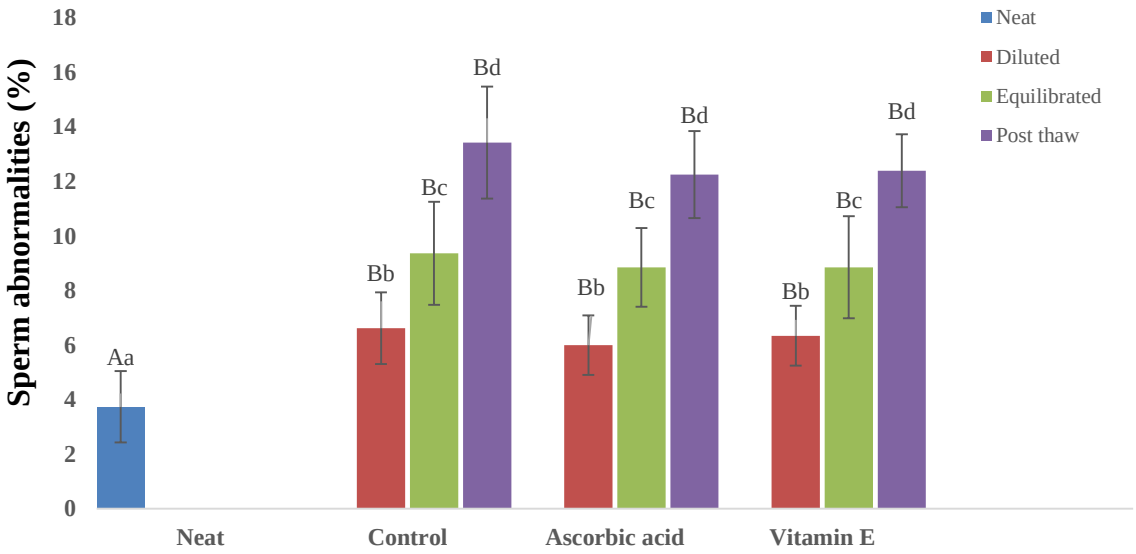
- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar **capital** and **small** letter superscripts within the **row** and **column,** respectively, differ significantly ($P<0.05$).

As evident from the Table (4.14), the per cent sperm abnormalities in neat semen was 3.73 ± 0.41. Its values following dilution, equilibration and thawing were 6.62 ± 0.41, 9.36 ± 0.59 and 13.42 ± 0.64, respectively in control aliquot; 5.99 ± 0.34, 8.85 ± 0.45 and 12.25 ± 0.50, respectively in ascorbic acid added aliquot; 6.34 ± 0.34, 8.85 ± 0.59, 12.39 ± 0.42, respectively in vitamin E added aliquot.

Viewing the Table (4.14), it was observed that the lowest value of sperm abnormalities (%) was observed in the neat semen which inclined after dilution and further inclined in equilibrated and post thaw semen samples, respectively. This trend of incline was observed in all the 3 aliquots of the group *i.e.* in control, ascorbic acid and vitamin E supplemented aliquots.

Comparing the Table (4.14), sperm abnormalities (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, ascorbic acid and vitamin E supplemented aliquots, a significant ($P<0.05$) difference was observed within and between all the aliquots at all the stages. It was also observed that the sperm abnormalities (%) of all the stages *i.e.* diluted, equilibrated and post thaw stages differed significantly ($P<0.05$) within all aliquots *i.e.* control, ascorbic acid and vitamin E supplemented aliquots.

Perusal of Table (4.14), it was observed that the sperm abnormalities (%) between all the different stages *i.e.* diluted, equilibrated, post thaw stages and between all the aliquots *i.e.* control, ascorbic acid and vitamin E supplemented aliquots differed non-significantly.



- Means with dissimilar **capital** and **small** letter superscripts between different aliquots and within same stage, respectively, differ significantly (P<0.05).

Figure 12: Bar diagram showing sperm abnormalities (%) in neat, diluted, equilibrated and post thaw semen

4.2.8 Hypo-osmotic swelling test (HOST)

Table 4.15: Hypo-osmotic swelling test (HOST) positive (%) in neat, diluted, equilibrated and post-thaw semen

Stage	HOST (%)			
	Neat semen	Control	Ascorbic acid	Vitamin E
Diluted	$81.94^{Cc} \pm 0.95$	$73.14^{Ab} \pm 1.23$	$74.97^{Ab} \pm 1.02$	$74.55^{Ab} \pm 1.13$
Equilibrated		$68.36^{Ab} \pm 1.39$	$70.69^{Ab} \pm 1.45$	$70.45^{Ab} \pm 1.47$
Post thaw		$58.75^{Aa} \pm 0.59$	$61.67^{Ba} \pm 0.52$	$60.27^{ABa} \pm 0.62$

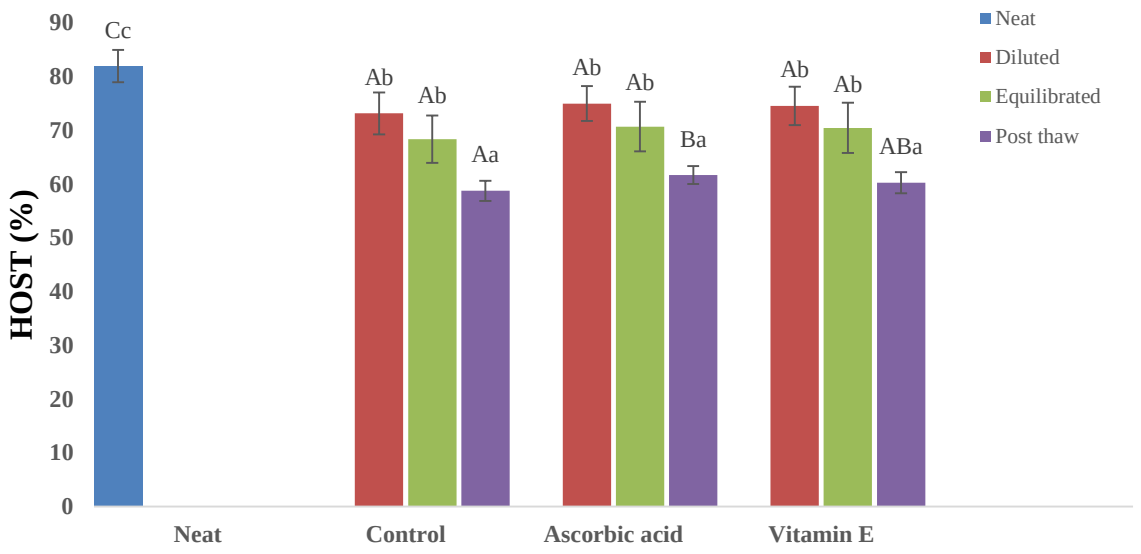
- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar **capital** and **small** letter superscripts within the **row** and **column,** respectively, differ significantly (P<0.05).

As evident from the Table (4.15), the per cent HOST positive in neat semen was 81.94 ± 0.95. Its values following dilution, equilibration and thawing were 73.14 ± 1.23, 68.36 ± 1.39 and 58.75 ± 0.59, respectively in control aliquot; 74.97 ± 1.02, 70.69 ± 1.45 and 61.67 ± 0.52, respectively in ascorbic acid added aliquot; 74.55 ± 1.13, 70.45 ± 1.47 and 60.27 ± 0.62, respectively in vitamin E added aliquot.

Viewing the Table (4.15), it was observed that the highest value of HOST positive (%) was observed in the neat semen, which declined after dilution and further declined in equilibrated and post thaw semen samples, respectively. This trend of decline was observed in all the 3 aliquots of the group *i.e.* in control, ascorbic acid and vitamin E supplemented aliquots.

Comparing the Table (4.15), HOST positive (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, ascorbic acid and vitamin E supplemented aliquots, a significant ($P<0.05$) difference was observed within and between all the aliquots at all the stages. It was also observed that the HOST positive (%) of post thaw semen sample differed significantly ($P<0.05$) within all aliquots *i.e.* control, ascorbic acid and vitamin E supplemented semen from diluted and equilibrated semen. Whereas, within all the aliquots, the diluted semen differed non-significantly ($P<0.05$) from the equilibrated semen.

Perusal of Table (4.15), it was observed that the HOST positive (%) between different stage *i.e.* diluted, equilibrated and post thaw stages, all the aliquots *i.e.* control, ascorbic acid and vitamin E supplemented semen differed non-significantly ($P<0.05$) at diluted and equilibrated stage. Between the post thaw stage of all the aliquots the ascorbic acid added semen differed significantly ($P<0.05$) from control. Moreover, vitamin E supplemented aliquots differed non-significantly from control as well as ascorbic acid added semen sample at post thaw stage.



- Means with dissimilar **capital** and **small** letter superscripts between different aliquots and within same stage, respectively, differ significantly (P<0.05).

Figure 13: Bar diagram showing hypo-osmotic swelling test (HOST) positive (%) in neat, diluted, equilibrated and post thaw semen

4.2.9 Lipid peroxidation (LPO)

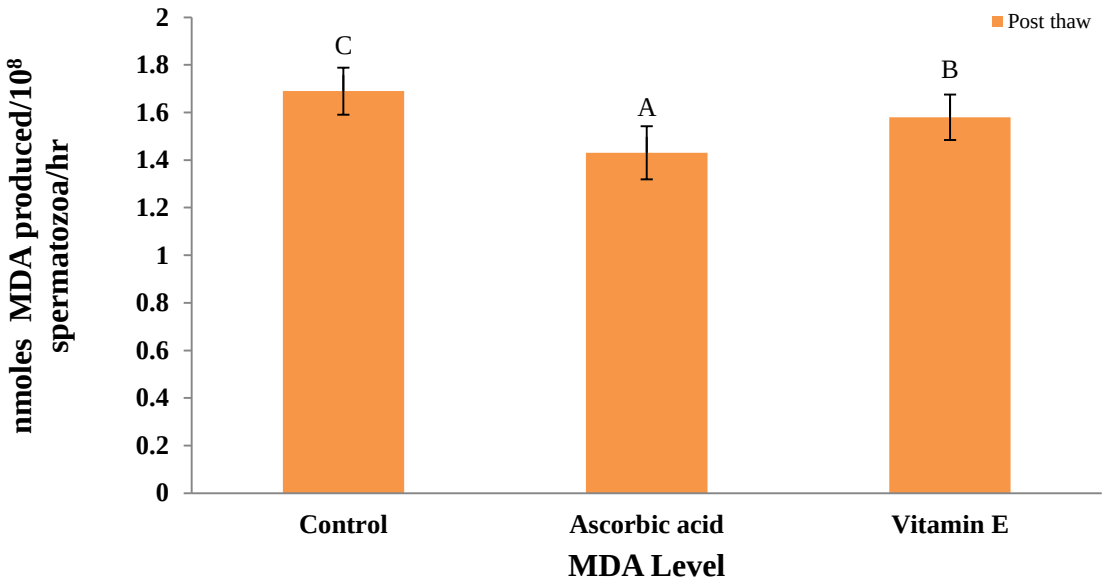
Table 4.16: Lipid peroxidation (LPO) measured as level of MDA in post thaw semen

Stage	MDA level (nmoles of MDA produced/ 10^8 spermatozoa/hr)		
	Control	Ascorbic acid	Vitamin E
Post thaw	$1.69^{C} \pm 0.03$	$1.43^{A} \pm 0.03$	$1.58^{B} \pm 0.03$

- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar letter superscripts in the **row** differ significantly (P<0.05).

As evident from the Table (4.16), the mean value of MDA level (nmoles of MDA produced/ 10^8 spermatozoa/hr) in post thaw semen was 1.69 ± 0.03 in control aliquot, 1.43 ± 0.03 in ascorbic acid added aliquot and 1.58 ± 0.03 in vitamin E added aliquot.

Comparing the Table (4.16), it was observed that the MDA level between the post thaw semen with or without additives *i.e.* control, ascorbic acid added and vitamin E supplemented aliquots all differed significantly (P<0.05).



- Means with dissimilar letter superscripts in the bar differ significantly (P<0.05).

Figure 14: Bar diagram showing lipid peroxidation (LPO) measured as level of MDA in post thaw semen

4.2.10 Catalase (CAT)

Table 4.17: Catalase (CAT) level in post thaw semen

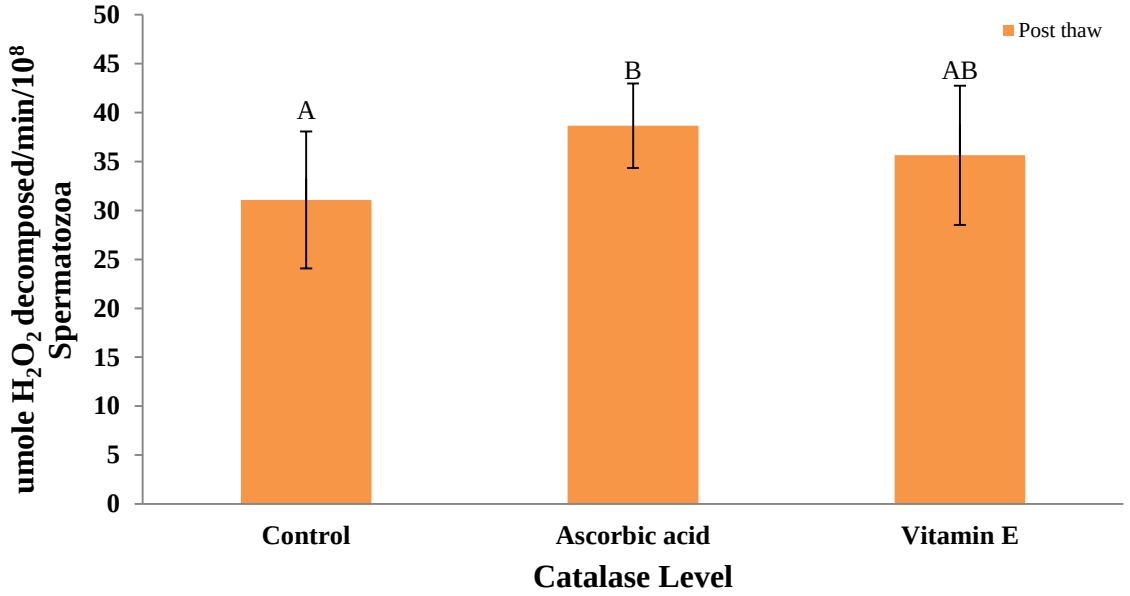
Stage	Catalase (µmol H_2O_2 decomposed/min/10^8 spermatozoa)		
	Control	Ascorbic acid	Vitamin E
Post thaw	$31.08^{A} \pm 2.21$	$38.67^{B} \pm 1.36$	$35.65^{AB} \pm 2.24$

- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar letter superscripts in the **row** differ significantly (P<0.05).

As evident from the Table (4.17), the mean value of catalase (µmol H_2O_2 decomposed/min/10^8 spermatozoa) in post thaw semen was 31.08 ± 2.21 in control aliquot, 38.67 ± 1.36 in ascorbic acid added aliquot and 35.65 ± 2.24 in vitamin E added aliquot.

Comparing the Table (4.17), it was observed that the catalase level between the post thaw semen with or without additive *i.e.* ascorbic acid supplemented semen differed significantly (P<0.05) from control and there was no significant difference between

ascorbic acid added and vitamin E supplemented aliquots. Moreover, vitamin E added differed non-significantly from control semen sample.



- Means with dissimilar letter superscripts in the bar differ significantly (P<0.05).

Figure 15: Bar diagram showing catalase (CAT) levels in post thaw semen

4.2.11 Superoxide dismutase (SOD)

Table 4.18: Superoxide dismutase (SOD) level in post thaw semen.

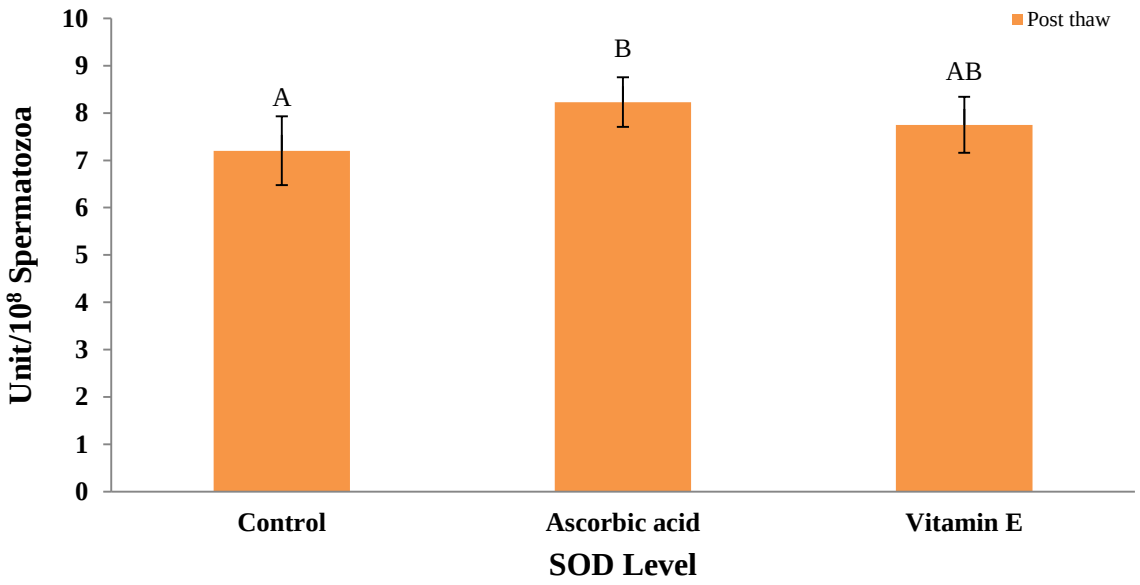
Stage	Superoxide dismutase (Unit/ 10^8 spermatozoa)		
	Control	Ascorbic acid	Vitamin E
Post thaw	$7.20^{A} \pm 0.23$	$8.23^{B} \pm 0.16$	$7.75^{AB} \pm 0.18$

- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar letter superscripts in the **row** differ significantly (P<0.05).

As evident from the Table (4.18), the mean value of SOD level (Unit/ 10^8 spermatozoa) in post thaw semen was 7.20 ± 0.23 in control aliquot, 8.23 ± 0.16 in ascorbic acid added aliquot and 7.75 ± 0.18 in vitamin E added aliquot.

Comparing the Table (4.18), it was observed that the SOD level between the post thaw semen with or without additive *i.e.* ascorbic acid added supplemented semen significantly (P<0.05) from control and there was no significant difference between

ascorbic acid added and vitamin E supplemented aliquots. Moreover, vitamin E added differed non-significantly from control semen sample.



- Means with dissimilar letter superscripts in the bar differ significantly (P<0.05).

Figure 16: Bar diagram showing superoxide dismutase (SOD) level in post thaw semen

4.3 Evaluation of Semen in Group III

4.3.1 Ejaculate volume

Perusal of Table (4.19), it was observed that mean volume of ejaculated semen in cattle bulls was 5.02 ± 0.44 ml with a range of 2.5 to 7.5 ml.

4.3.2 Mass activity (0-5)

Viewing the Table (4.19), it was observed that mean mass activity (0-5 scale) of ejaculated semen in cattle bulls was 3.68 ± 0.44 with a range of 3.4 to 3.8.

4.3.3 Sperm concentration (10^6/ml)

As evident from Table (4.19), it was observed that mean sperm concentration (millions/ml) of ejaculated semen in cattle bulls was 966.1 ± 45.4 millions/ml with a range of 713 to 1178 millions/ml.

Table 4.19: Seminal attributes in neat semen

Parameter	Mean ± S.E	Range
Volume (ml)	5.02 ± 0.44	2.5 to 7.5
Mass activity (0-5)	3.68 ± 0.44	3.4 to 3.8
Sperm concentration (million/ml)	966.1 ± 45.4	713 to 1178

- Values are given as mean ± S.E of 10 animals unless and otherwise stated.

4.3.4 Progressive motility

Table 4.20: Progressive motility (%) in neat, diluted, equilibrated and post-thaw semen

Stage	Progressive motility (%)			
	Neat semen	**Control**	**Trehalose**	**BHT**
Diluted	$84.38^{Cd} \pm 0.35$	$69.36^{Ac} \pm 0.46$	$72.18^{Bc} \pm 0.55$	$71.63^{Bc} \pm 0.61$
Equilibrated		$63.21^{Ab} \pm 0.45$	$65.31^{Bb} \pm 0.41$	$64.25^{ABb} \pm 0.46$
Post thaw		$48.97^{Aa} \pm 1.11$	$48.55^{Aa} \pm 2.83$	$51.36^{Aa} \pm 1.15$

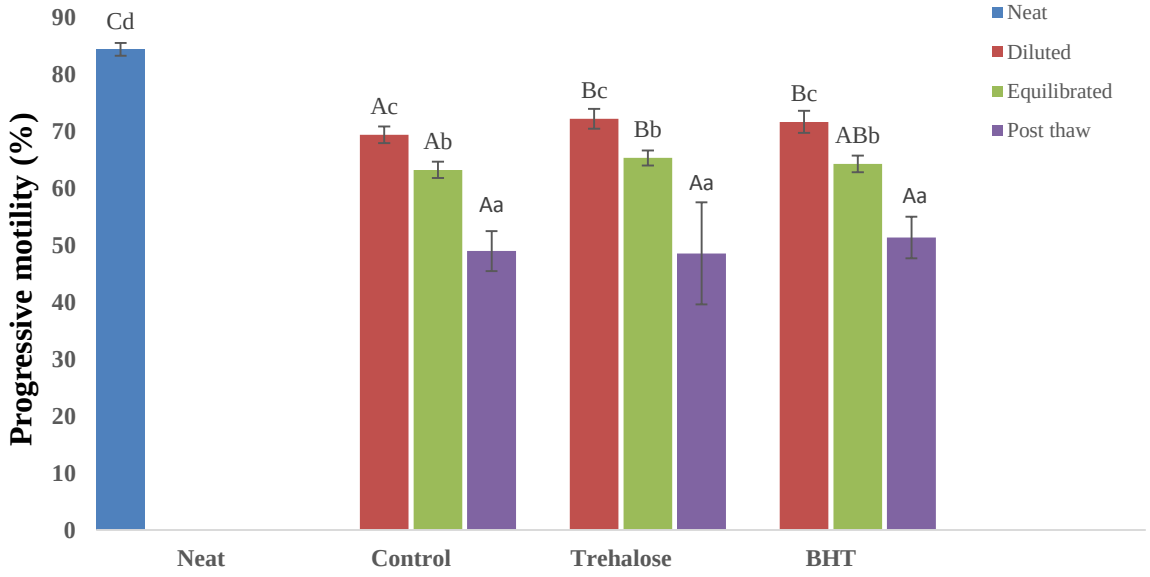
- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar **capital** and **small** letter superscripts within the **row** and **column,** respectively, differ significantly ($P<0.05$).

As evident from the Table (4.20), the per cent progressive motility in neat semen was 84.38 ± 0.35. Its values following dilution, equilibration and thawing semen were 69.36 ± 0.46, 63.21 ± 0.45 and 48.97 ± 1.11, respectively in control aliquot; 72.18 ± 0.55, 65.31 ± 0.41 and 48.55 ± 2.83, respectively in trehalose added aliquot; 71.63 ± 0.61, 64.25 ± 0.46 and 51.36 ± 1.15, respectively in BHT added aliquot.

Viewing the Table (4.20), it was observed that the highest value of progressive motility (%) was observed in the neat semen, which declined after dilution and further declined in equilibrated and post thaw semen samples, respectively. This trend of decline was observed in all the 3 aliquots of the group *i.e.* in control, trehalose and BHT supplemented aliquots.

Comparing the Table (4.20), progressive motility (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, trehalose and BHT supplemented aliquots, a significant (P<0.05) difference was observed within and between all the aliquots at all the stages. It was also observed that the progressive motility (%) of all the stages *i.e.* diluted, equilibrated and post thaw stages differed significantly (P<0.05) within all aliquots *i.e.* control, trehalose and BHT supplemented aliquots.

Perusal of Table (4.20), it was observed that the progressive motility (%) between different stage *i.e.* diluted, equilibrated and post thaw stages, the trehalose and BHT supplemented aliquots differed significantly (P<0.05) from control semen sample at diluted stage, whereas between trehalose and BHT added aliquots there was no significant (P<0.05) difference at diluted stage. Among equilibrated stage the trehalose added semen sample differed significantly (P<0.05) from control, whereas there was no significant difference between trehalose and BHT added semen samples and also between control and BHT added semen sample at equilibration stage. Between the post thaw stages of all the aliquots *i.e.* control, trehalose and BHT supplemented semen differed non-significantly.



- Means with dissimilar **capital** and **small** letter superscripts between different aliquots and within same stage, respectively, differ significantly (P<0.05).

Figure 17: Bar diagram showing progressive motility (%) in neat, diluted, equilibrated and post thaw semen

4.3.5 Live sperm

Table 4.21: Live sperm (%) in neat, diluted, equilibrated and post-thaw semen

Stage	Live sperm (%)			
	Neat semen	**Control**	**Trehalose**	**BHT**
Diluted	$88.00^{Cd} \pm 0.66$	$74.93^{Ac} \pm 0.62$	$78.48^{Bc} \pm 0.59$	$77.66^{Bc} \pm 0.53$
Equilibrated		$68.65^{Ab} \pm 0.59$	$71.44^{Bb} \pm 0.56$	$70.53^{Bb} \pm 0.45$
Post thaw		$57.17^{Aa} \pm 1.31$	$58.11^{Aa} \pm 2.98$	$59.52^{Aa} \pm 1.60$

- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar **capital** and **small** letter superscripts within the **row** and **column,** respectively, differ significantly ($P<0.05$).

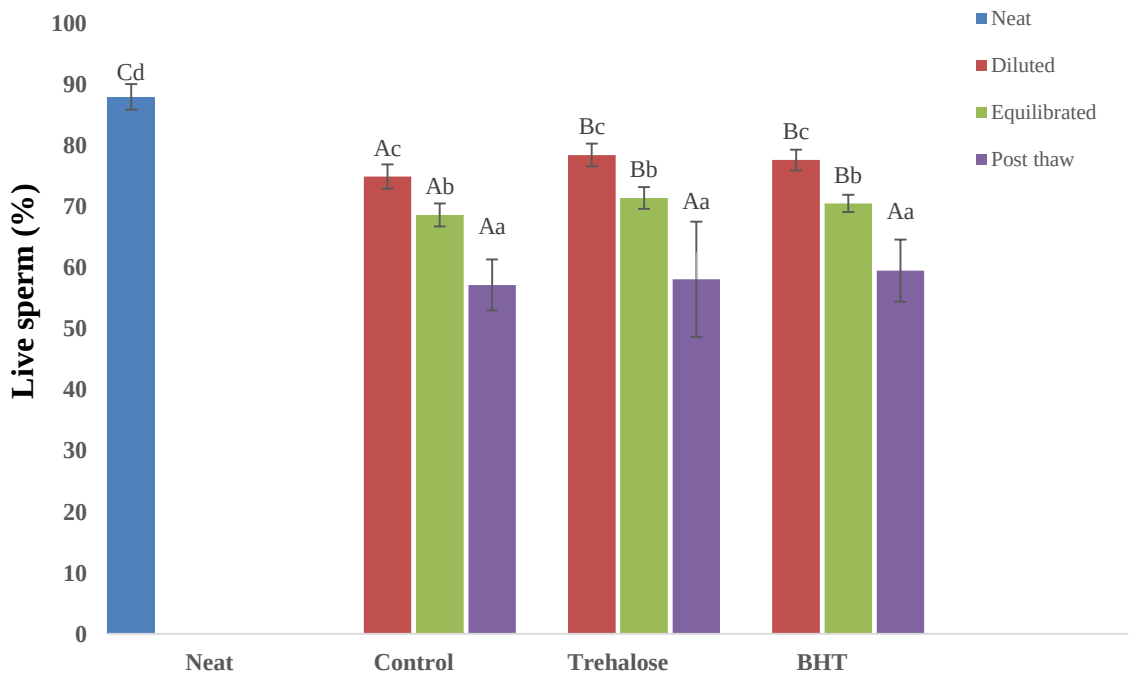
As evident from the Table (4.21), the per cent live sperm of neat semen was 88.00 ± 0.66. Its values following dilution, equilibration and thawing were 74.93 ± 0.62, 68.65 ± 0.59 and 57.17 ± 1.31, respectively in control aliquot; 78.48 ± 0.59, 71.44 ± 0.56 and 58.11 ± 2.98, respectively in trehalose added aliquot; 77.66 ± 0.53, 70.53 ± 0.45 and 59.52 ± 1.60, respectively in BHT added aliquot.

Viewing the Table (4.21), it was observed that the highest value of live sperm (%) was observed in the neat semen, which declined after dilution and further declined in equilibrated and post thaw semen samples, respectively. This trend of decline was observed in all the 3 aliquots of the group *i.e.* in control, trehalose and BHT supplemented aliquots.

Comparing the Table (4.21), live sperm (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, trehalose and BHT supplemented aliquots, a significant ($P<0.05$) difference was observed within and between all the aliquots at all the stages. It was also observed that the live sperm (%) of all the stages *i.e.* diluted, equilibrated and post thaw stages differed significantly ($P<0.05$) within all aliquots *i.e.* control, trehalose and BHT supplemented semen.

Perusal of Table (4.21), it was observed that the progressive motility (%) between different stage *i.e.* diluted, equilibrated and post thaw stages, the trehalose and BHT

supplemented aliquots differed significantly (P<0.05) from control semen sample at diluted stage, whereas between trehalose and BHT added aliquots there was no significant (P<0.05) difference at diluted stage. Among equilibrated stage the trehalose and BHT supplemented aliquots differed significantly (P<0.05) from control, whereas there was no significant difference between trehalose and BHT added semen samples at equilibration stage. Between the post thaw stages of all the aliquots *i.e.* control, trehalose and BHT added aliquots differed non-significantly.



- Means with dissimilar **capital** and **small** letter superscripts between different aliquots and within same stage, respectively, differ significantly (P<0.05).

Figure 18: Bar diagram showing live sperm (%) in neat, diluted, equilibrated and post thaw semen

4.3.6 Acrosomal integrity

Table 4.22: Acrosomal integrity (%) in neat, diluted, equilibrated and post-thaw semen

Stage	Acrosomal integrity (%)			
	Neat semen	Control	Trehalose	BHT
Diluted	$82.74^{Cd} \pm 0.28$	$70.81^{Ac} \pm 0.56$	$72.91^{Bc} \pm 0.53$	$72.01^{ABc} \pm 0.45$
Equilibrated		$63.65^{Ab} \pm 0.19$	$66.1^{Bb} \pm 0.48$	$65.46^{Bb} \pm 0.36$
Post thaw		$52.93^{Aa} \pm 1.39$	$53.82^{Aa} \pm 2.93$	$56.11^{Aa} \pm 1.49$

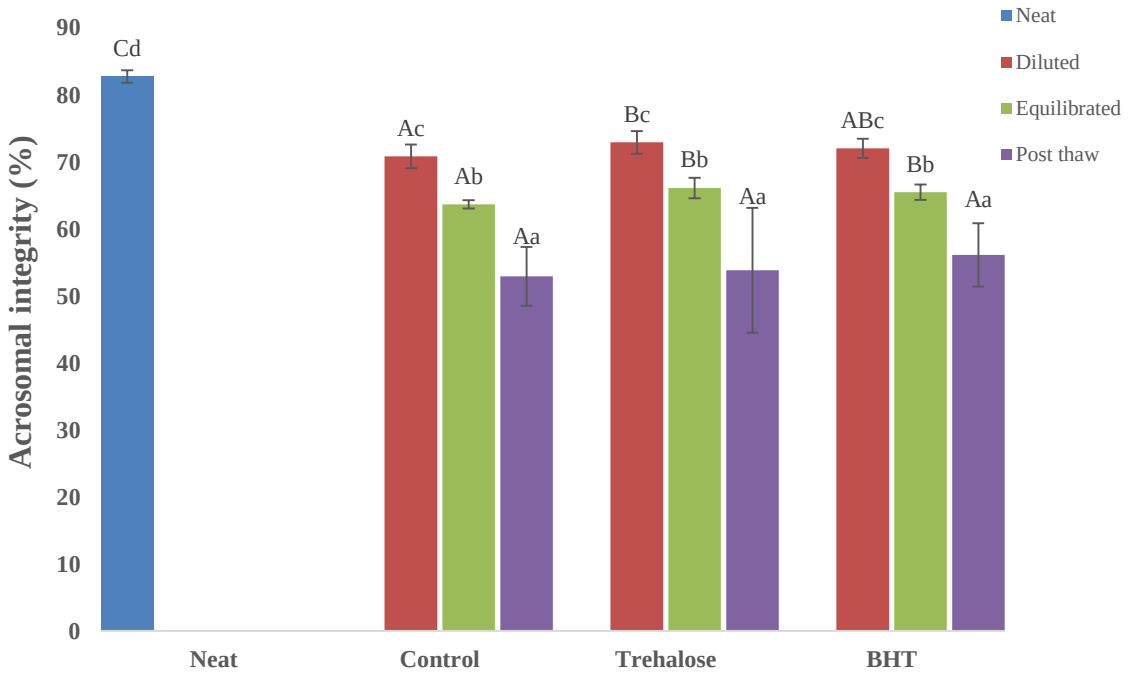
- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar **capital** and **small** letter superscripts within the **row** and **column,** respectively, differ significantly (P<0.05).

As evident from the Table (4.22), the per cent acrosomal integrity in neat semen was 82.74 ± 0.28. Its values following dilution, equilibration and thawing were 70.81 ± 0.56, 63.65 ± 0.19 and 52.93 ± 1.39, respectively in control aliquot; 72.91 ± 0.53, 66.1 ± 0.48 and 53.82 ± 2.93, respectively in trehalose added aliquot; 72.01 ± 0.45, 65.46 ± 0.36 and 56.11 ± 1.49, respectively in BHT added aliquot.

Viewing the Table (4.22), it was observed that the highest value of acrosomal integrity (%) was observed in the neat semen, which declined after dilution and further declined in equilibrated and post thaw semen samples, respectively. This trend of decline was observed in all the 3 aliquots of the group *i.e.* in control, trehalose and BHT added group.

Comparing the Table (4.22), acrosomal integrity (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, trehalose and BHT supplemented aliquots, a significant (P<0.05) difference was observed within and between all the aliquots at all the stages. It was also observed that the acrosomal integrity (%) of all the stages *i.e.* diluted, equilibrated and post thaw stages differed significantly (P<0.05) within all aliquots *i.e.* control, trehalose and BHT added semen.

Perusal of Table (4.22), it was observed that the acrosomal integrity (%) between different stage *i.e.* diluted, equilibrated and post thaw stages, the trehalose supplemented aliquots differed significantly (P<0.05) from control semen sample at diluted stage, whereas between trehalose and BHT added aliquots there was no significant (P<0.05) difference and also there was no significant difference between control and BHT added semen samples at diluted stage. Among equilibrated stage the trehalose and BHT supplemented aliquots differed significantly (P<0.05) from control, whereas there was no significant difference between trehalose and BHT added semen at equilibration stage. Between the post thaw stages of all the aliquots *i.e.* control, trehalose and BHT supplemented semen differed non-significantly.



- Means with dissimilar **capital** and **small** letter superscripts between different aliquots and within same stage, respectively, differ significantly (P<0.05).

Figure 19: Bar diagram showing acrosomal integrity (%) in neat, diluted, equilibrated and post thaw semen

4.3.7 Sperm abnormalities

Table 4.23: Sperm abnormalities (%) in neat, diluted, equilibrated and post-thaw semen

Stage	Sperm abnormalities (%)			
	Neat semen	**Control**	**Trehalose**	**BHT**
Diluted	$5.11^{Aa} \pm 0.12$	$8.27^{Cb} \pm 0.27$	$7.45^{Bb} \pm 0.19$	$7.72^{BCb} \pm 0.12$
Equilibrated		$11.93^{Cc} \pm 0.27$	$11.02^{Bc} \pm 0.27$	$11.30^{BCc} \pm 0.23$
Post thaw		$15.05^{Bd} \pm 0.38$	$14.82^{Bd} \pm 0.89$	$14.41^{Bd} \pm 0.44$

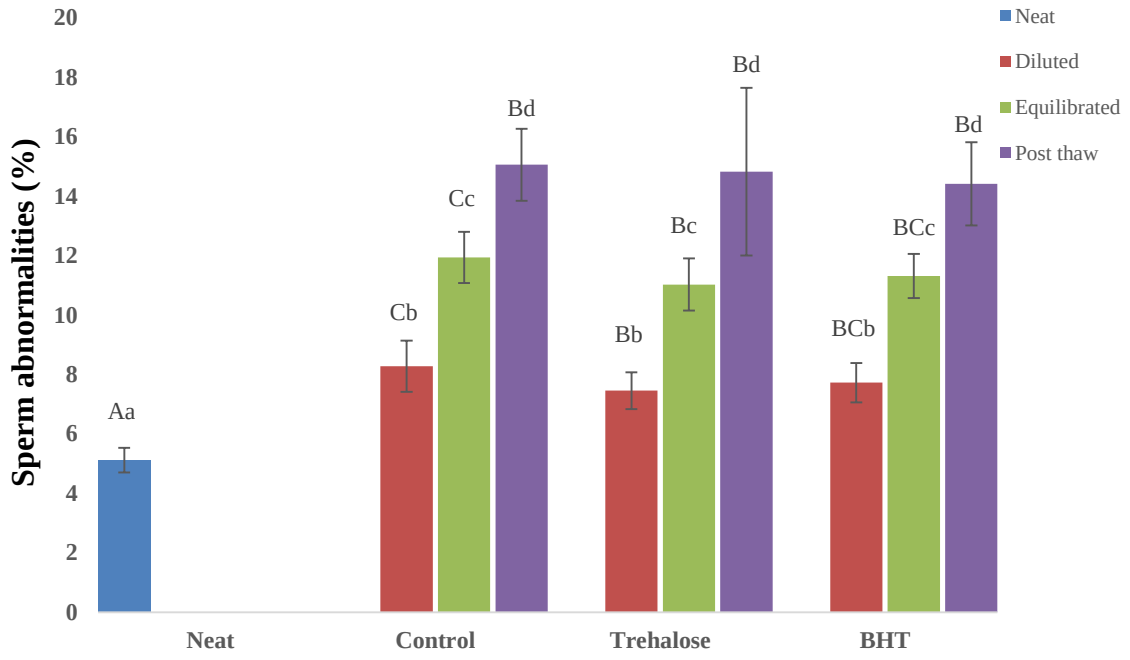
- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar **capital** and **small** letter superscripts within the **row** and **column,** respectively, differ significantly (P<0.05).

As evident from the Table (4.23), the per cent sperm abnormalities in neat semen was 5.11 ± 0.12. Its values following dilution, equilibration and thawing were 8.27 ± 0.27, 11.93 ± 0.27 and 15.05 ± 0.38, respectively in control aliquot; 7.45 ± 0.19, 11.02 ± 0.27 and 14.82 ± 0.89, respectively in trehalose added aliquot; 7.72 ± 0.12, 11.30 ± 0.23 and 14.41 ± 0.44, respectively in BHT added aliquot.

Viewing the Table (4.23), it was observed that the lowest value of sperm abnormalities (%) was observed in the neat semen which increased after dilution and further inclined in equilibrated and post thaw semen samples, respectively. This trend of increasing was observed in all the 3 aliquots of the group *i.e.* in control, trehalose and BHT supplemented aliquots.

Comparing the Table (4.23), sperm abnormalities (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, trehalose and BHT supplemented aliquots, a significant (P<0.05) difference was observed within and between all the aliquots at all the stages. It was also observed that the sperm abnormalities (%) of all the stages *i.e.* diluted, equilibrated and post thaw stages differed significantly (P<0.05) within all aliquots *i.e.* control, trehalose and BHT added semen.

Perusal of Table (4.23), it was observed that the sperm abnormalities (%) between different stage *i.e.* diluted, equilibrated and post thaw stages, the trehalose added semen differed significantly (P<0.05) from control semen sample at diluted stage, whereas between trehalose and BHT added aliquots there was no significant (P<0.05) difference and also there was no significant difference between control and BHT supplemented aliquots at diluted stage. Among equilibrated stage the trehalose supplemented semen sample differed significantly (P<0.05) from control, whereas there was no significant difference between trehalose and BHT and also there was no significant difference between control and BHT added semen samples at equilibration stage. Between the post thaw stages of all the aliquots *i.e.* control, trehalose and BHT added aliquot differed non-significantly.



- Means with dissimilar **capital** and **small** letter superscripts between different aliquots and within same stage, respectively, differ significantly (P<0.05).

Figure 20: Bar diagram showing sperm abnormalities (%) in neat, diluted, equilibrated and post thaw semen

4.3.8 Hypo-osmotic swelling test (HOST)

Table 4.24: Hypo-osmotic swelling test (HOST) positive (%) in neat, diluted, equilibrated and post-thaw semen

Stage	HOST (%)			
	Neat semen	Control	Trehalose	BHT
Diluted	$82.25^{Cd} \pm 0.41$	$70.52^{Ac} \pm 0.72$	$72.90^{Bc} \pm 0.75$	$72.6^{Bb} \pm 1.51$
Equilibrated		$64.48^{Ab} \pm 0.58$	$67.46^{Bb} \pm 0.77$	$67.04^{Bb} \pm 0.71$
Post thaw		$51.85^{Aa} \pm 2.35$	$49.86^{Aa} \pm 4.44$	$53.61^{Aa} \pm 2.25$

- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar **capital** and **small** letter superscripts within the **row** and **column,** respectively, differ significantly (P<0.05).

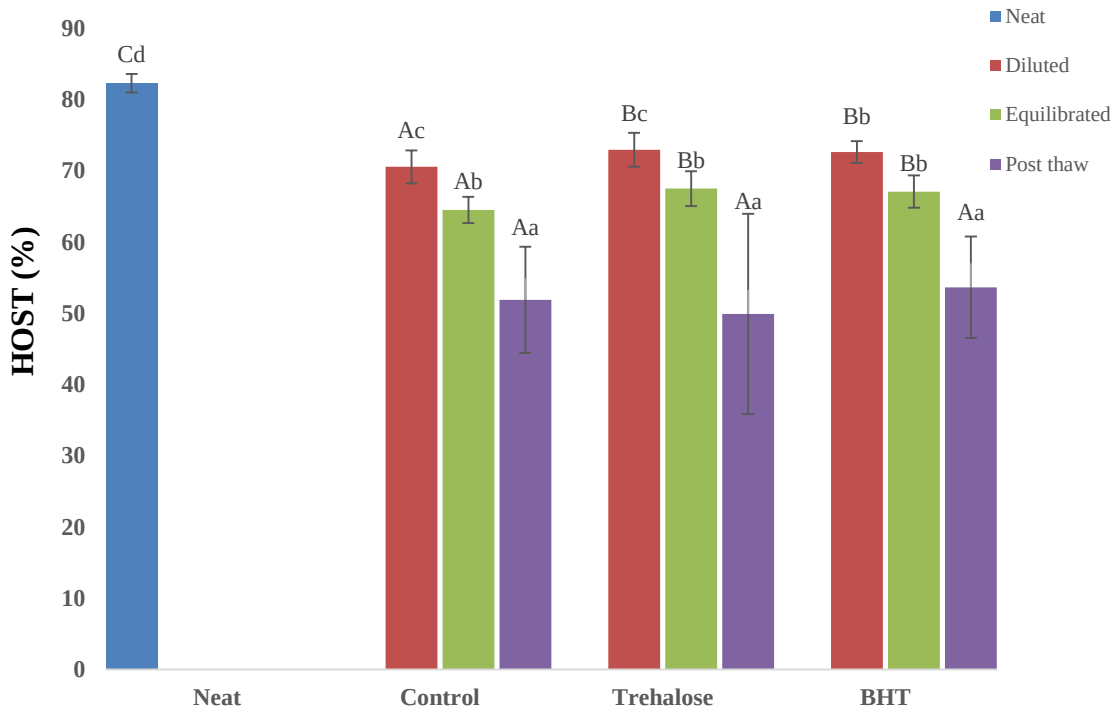
As evident from the Table (4.24), the per cent HOST positive in neat semen was 82.25 ± 0.41. Its values following dilution, equilibration and thawing semen were 70.52 ± 0.72, 64.48 ± 0.58 and 51.85 ± 2.35, respectively in control aliquot; 72.90 ± 0.75, 67.46 ± 0.77 and 49.86 ± 4.44, respectively in trehalose added aliquot; 72.6 ± 1.51, 67.04 ± 0.71 and 53.61 ± 2.25, respectively in BHT added aliquot.

Viewing the Table (4.24), it was observed that the highest value of HOST positive (%) was observed in the neat semen, which declined after dilution and further declined in equilibrated and post thaw semen samples, respectively. This trend of decline was observed in all the 3 aliquots of the group *i.e.* in control, trehalose and BHT supplemented aliquots.

Comparing the Table (4.24), HOST positive (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, trehalose and BHT supplemented aliquots, a significant (P<0.05) difference was observed within and between all the aliquots at all the stages. It was also observed that the HOST positive (%) of post thaw semen sample differed significantly (P<0.05) within all aliquots *i.e.* control, trehalose and BHT added semen from diluted and equilibrated semen. Whereas, within control and trehalose the diluted semen differed significantly (P<0.05) from the

equilibrated semen. Moreover, within BHT, the diluted semen differed non-significantly (P<0.05) from the equilibrated semen.

Perusal of Table (4.24), it was observed that the HOST positive (%) between different stage *i.e.* diluted, equilibrated and post thaw stages, the trehalose and BHT added aliquots differed significantly (P<0.05) from control semen sample at diluted stage, whereas between trehalose and BHT added aliquots there was no significant (P<0.05) difference at diluted stage. Among equilibrated stage the trehalose and BHT supplemented aliquots differed significantly (P<0.05) from control, whereas there was no significant difference between trehalose and BHT added aliquots at equilibration stage. Between the post thaw stages of all the aliquots *i.e.* control, trehalose and BHT supplemented semen differed non-significantly.



- Means with dissimilar **capital** and **small** letter superscripts between different aliquots and within same stage, respectively, differ significantly (P<0.05).

Figure 21: Bar diagram showing hypo-osmotic swelling test (HOST) positive (%) in neat, diluted, equilibrated and post thaw semen

4.3.9 Lipid peroxidation (LPO)

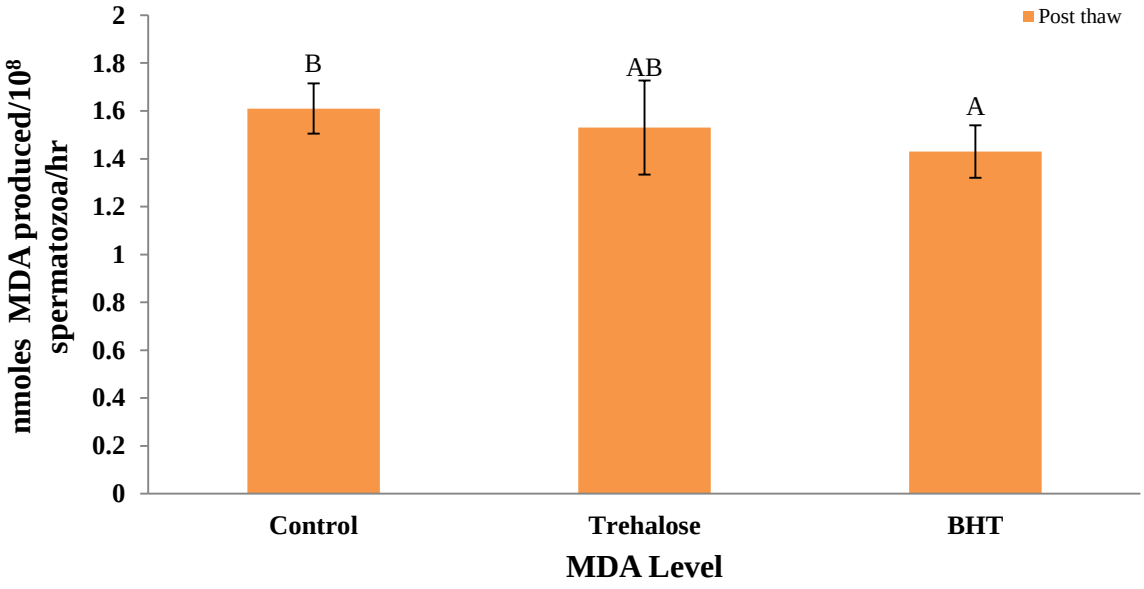
Table 4.25: Lipid peroxidation (LPO) measured as level of MDA in post thaw semen

Stage	MDA level (nmoles of MDA produced/ 10^8 spermatozoa/hr)		
	Control	Trehalose	BHT
Post thaw	$1.61^{B} \pm 0.03$	$1.53^{AB} \pm 0.06$	$1.43^{A} \pm 0.03$

- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar letter superscripts in the **row** differ significantly (P<0.05).

As evident from the Table (4.25), the mean value of MDA level (nmoles of MDA produced/ 10^8 spermatozoa/hr) in post thaw semen was 1.61 ± 0.03 in control aliquot, 1.53 ± 0.06 in trehalose added aliquot and 1.43 ± 0.03 in BHT added aliquot.

Comparing the Table (4.25), it was observed that the MDA level between the post thaw semen with or without additives *i.e.* BHT supplemented semen differ significantly (P<0.05) from control. Whereas, between trehalose and BHT added aliquots there was no significant difference and also between control and trehalose supplemented aliquots.



- Means with dissimilar letter superscripts in the bar differ significantly (P<0.05).

Figure 22: Bar diagram showing lipid peroxidation (LPO) measured as level of MDA in post thaw semen.

4.3.10 Catalase (CAT)

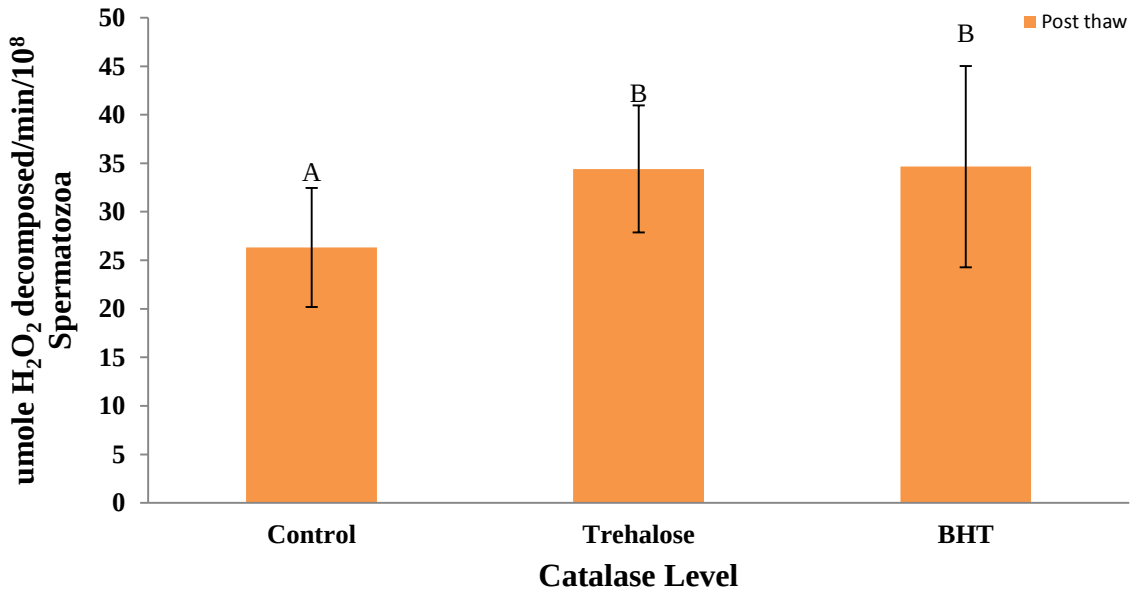
Table 4.26: Catalase (CAT) level in post thaw semen

Stage	Catalase (µmol H_2O_2 decomosed/min/10^8 spermatozoa)		
	Control	Trehalose	BHT
Post thaw	$26.31^{A} \pm 1.93$	$34.41^{B} \pm 2.06$	$34.65^{B} \pm 3.27$

- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar letter superscripts in the **row** differ significantly (P<0.05).

As evident from the Table (4.26), the mean value of catalase (µmol H_2O_2 decomposed/min/10^8 spermatozoa) in post thaw semen was 26.31 ± 1.93 in control aliquots, 34.41 ± 2.06 in trehalose added aliquots and 34.65 ± 3.27 in BHT added aliquots.

Comparing the Table (4.26), it was observed that the catalase level between the post thaw semen with or without additive *i.e.* both trehalose and BHT supplemented aliquots differ significantly (P<0.05) from control. Whereas, between trehalose and BHT added aliquots there was no significant difference in post thaw semen.



- Means with dissimilar letter superscripts in the bar differ significantly (P<0.05).

Figure 23: **Bar diagram showing catalase (CAT) level in post thaw semen**

4.3.11 Superoxide dismutase (SOD)

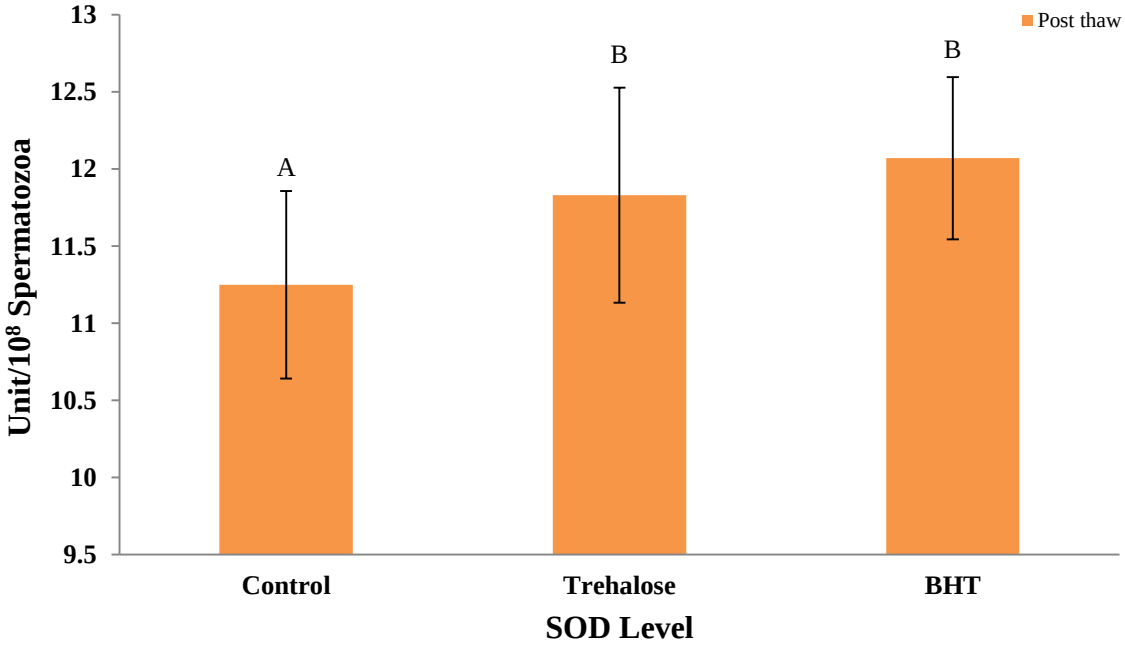
Table 4.27: Superoxide dismutase (SOD) level in post thaw semen

Stage	Superoxide dismutase (Unit/ 10^8 spermatozoa)		
	Control	Trehalose	BHT
Post thaw	$11.25^{A} \pm 0.19$	$11.83^{B} \pm 0.22$	$12.07^{B} \pm 0.16$

- Values are given as mean ± SE of 10 animals unless and otherwise stated.
- Means with dissimilar letter superscripts in the **row** differ significantly (P<0.05).

As evident from the Table (4.27), the mean value of SOD level (Unit/ 10^8 spermatozoa) in post thaw semen was 11.25 ± 0.19 in control aliquot, 11.83 ± 0.22 in trehalose added aliquot and 12.07 ± 0.16 in BHT added aliquot.

Comparing the Table (4.27), it was observed that the SOD levels between the post thaw semen with or without additive *i.e*. both trehalose and BHT supplemented aliquots significantly (P<0.05) from control. Whereas, between trehalose and BHT added aliquots there was no significant difference in post thaw semen.



- Means with dissimilar letter superscripts in the bar differ significantly (P<0.05).

Figure 24: Bar diagram showing superoxide dismutase (SOD) level in post thaw semen

Chapter-V

Discussion

CHAPTER-V

DISCUSSION

In the present investigation three experimental group were designed namely group I (amino acid group), group II (vitamin group) and group III (miscellaneous group). Tris-Egg yolk-Citric acid-Fructose-Glycerol (TEYCAFG) dilutor was used as control in all the groups. In group I TEYCAFG plus cysteine and TEYCAFG plus taurine were used, in group II TEYCAFG plus ascorbic acid, TEYCAFG plus vitamin E were used and in group III TEYCAFG plus trehalose and TEYCAFG plus BHT were used. The basic composition and percentage of egg yolk, glycerol, rate of freezing and thawing for all different dilutors used were same except the addition of antioxidant used *i.e.* cysteine, taurine, ascorbic acid, vitamin E, trehalose and BHT. Therefore any variation in physio-chemical parameters (progressive motility, live sperm, acrosomal integrity, sperm abnormalities), *in-vitro* fertility test (HOST) and oxidative stress (LPO, CAT, SOD) will reflect change due to addition of these antioxidant.

5.1 Evaluation of Semen in Group I

5.1.1 Ejaculated volume (ml)

The ejaculated volume of semen varies widely *i.e.* ranging from 3 to 11 ml as reported by various scientists (Bhakat *et al.,* 2014; Bhoite *et al.,* 2008; Sarder, 2007; Javed *et al.,* 2000).

The higher volume of semen have been reported as 11.25± 0.06, 9.8 ± 0.10 and 9.3± 0.01 ml in Holstein Friesian, Sahiwal and Local bull, respectively (Hossain *et al.,* 2012); 7.10± 0.14 ml in Holstein Friesian bull (Baloch *et al.,* 2019); 6.20± 0.42 ml in Gir bull (Chaudhary *et al.,* 2017); 5.50± 0.04 ml in Sahiwal bull (Rehman *et al.,* 2016); 5.48 ± 1.83 in Holstein Friesian bull (Mathevon *et al.,* 1998); 5.38± 0.20 and 5.04± 0.34 ml in two Hariana bulls (Pal *et al.,* 2020b); 5.3± 0.25 ml in Gir bull (Sannat *et al.,* 2015). Whereas, the lowest volume of semen is reported as 3.02± 0.03 in Jersey bull (Fiaz *et al.,* 2010).

In the present investigation the overall mean ejaculate volume (Table 4.1) was recorded as 5.46± 1.62 ml in bulls which was in agreement reported by Rehman *et al.,* 2016 (5.50± 0.04 ml), Mathevon *et al.,* 1998 (5.48± 1.83 ml), Pal *et al.,* 2020b (5.38± 0.20) and Sannat *et al.,* 2015 (5.3±0.25 ml).

The variation in ejaculated volume reported by various scientists may be due to the differences in age, breed, reproductive health status, frequency of collection, nutrition, seasonal variation, management and additionally due to ability or skill of semen collector or attendant and temperature of AV (Javed *et al.,* 2000).

5.1.2 Mass activity (0-5 scale)

The mass activity is the rough estimation to assess motility of spermatozoa and thus it is very essential criteria for the evaluation of fresh semen**.**

The mass activity on 0-5 scale is reported as 4.0± 0.4 in HF bull (Srivastava and Kumar, 2014); 3.8± 0.3 in HF crossbred bull (Munsi *et al.,* 2007); 3.72± 0.02 in Kankrej bull (Patel and Siddiquee, 2013); 3.69± 0.09 and 3.63± 0.08 in two Hariana bull (Pal *et al.,* 2020b); 3.6± 0.05 in HF bull (Baloch *et al.,* 2019); 3.44± 0.09 in Gir bull (Chaudhary *et al.,* 2017); 2.39± 0.03 in crossbred bull (Matharoo, 2015); 1.92± 0.07 in HF bull (Fiaz *et al.,* 2010).

In our present study the mass activity (0-5 scale) in bull (Table 4.1) was 3.65± 0.11 which was in accordance with as reported by Munsi *et al.,* 2017 (3.8± 0.3), Patel and Siddiquee, 2013 (3.72± 0.02), Pal *et al.,* 2020b (3.69± 0.09 and 3.63± 0.08) and Baloch *et al.,* 2019 (3.6± 0.05).

The variation in mass activity is due to improper handling procedure, contaminated glass wares, chemicals, cold or hot test tubes, glass slides, microscope stage, rapid drying or cooling of glass slides, prolongation during collection and examination period etc and mass activity varies from individual to individual because it is a subjective test (Pal *et al.,* 2020a).

5.1.3 Sperm concentration

Sperm concentration refers to the number of sperm per unit volume (ml) of semen and when it is combined with ejaculated volume, it determine how many female can be inseminated with optimal number of sperm cells (Hafez, 1987).

The sperm concentration has been reported by various scientists as 1560.0± 59.00 $x10^6$ in Jersey bull (Rehman *et al.,* 2016); 1410.0± 70.0 $x10^6$ in HF crossbred bull (Munsi *et al.,* 2007); 1078.1± 32.56 $x10^6$ in Frieswal bull (Chauhan *et al.,* 2017); 968.1± 111.3 $x10^6$ and 917.5± 127.9 $x10^6$ in crossbred and HF bull, respectively (Srivastava and Kumar, 2014); 846.30± 54.82 $x10^6$ in Surti buffalo (Chaudhary *et al.,* 2017).

In the present study the sperm concentration (10^6) in bull (Table 4.1) was 806.76± 65.29 $x10^6$ Our finding was lower than reported by Rehman *et al.,* 2016, Munsi *et al.,* 2007, Chauhan *et al.,* 2017 and Srivastava and Kumar, 2014.

The possible reason for variation in sperm concentration may be due to some factors like breed, age, seasonal variation, period of sexual rest, number of false mount, successive ejaculates and collection method etc.

5.1.4 Progressive motility

Sperm motility gives the first hand information regarding the fertilizing capacity of semen and is crucial in facilitating passage through the cervix and utero-tubal junction and much more important in actual penetration of the cumulus cells and zona pellucida of the ovum (Hafez, 1987).

The per cent motility in neat semen is reported as 89.02± 0.19 in Kankrej bull (Shaikh *et al.,* 2016); 86.15± 0.30 in Kankrej bull (Patel and Siddiquee, 2013); 85.12± 0.51 in Sahiwal and 84.61± 16 in HF bull (Khan *et al.,* 2018); 85.1± 0.3 in bovine bull (Li *et al.,* 2016); 83.66± 2.27 in Karan fries bull (Kumar *et al.,* 2013); 83.5± 2.4 in HF bull (Uysal *et al.,* 2007); 82.2± 3.6 in HF bull (Srivastava and Kumar); 77± 1.7 in HF bull (Khalil *et al.,* 2018); 74.58± 1.75 in HF bull (Baloch *et al.,* 2019); 70.95± 0.7 in HF bull (Fiaz *et al.,* 2010).

In the present research trial the per cent progressive motility of neat semen (Table 4.2) was 84.19± 0.37. This finding was in agreement with findings of Khan *et al.,* 2018 (85.12± 0.51 and 84.61± 16), Li *et al.,* 2016 (85.1± 0.3), Kumar *et al.,* 2013 (83.66± 2.27) and Uysal *et al.,* 2007 (83.5± 2.4).

The per cent motility in diluted semen is reported as 85.50± 0.92 in Gir bull (Chaturvedi *et al.,* 2020); 80.59± 0.43 in Kankrej bull (Patel and Siddiquee, 2013); 77.3± 2.3 in buffalo bull (Rasul *et al.,* 2001); 73.92± 2.15 in crossbred bull (Singh *et al.,* 2017); 71± 0.8 in HF bull (Khalil *et al.,* 2018); 70.33± 0.23 in buffalo bull (Surabh *et al.,* 2021); 70.12± 0.51 in buffalo bull (Hegazy *et al.,* 2020); 69.46± 0.63 in Hariana bull (Patel *et al.,* 2015); 67.70± 1.12 in buffalo bull (Sandeep *et al.,* 2015); 67.0± 1.52 in crossbred bull (Singh *et al.,* 2020); 58.47± 4.00 in crossbred bull (Khumran *et al.,* 2017).

In the present study the per cent progressive motility of diluted semen (Table 4.2) was 69.01± 0.29 and this finding was in agreement with Surabh *et al.,* 2021 (70.33± 0.23), Hegazy *et al.,* 2020 (70.12± 0.51) and Patel *et al.,* 2015 (69.46± 0.63).

The per cent progressive motility of equilibrated semen is reported as 78.86± 0.76 in Jersey bull (Singh and Sharma, 2018); 74.73± 0.58 in Kankrej bull (Patel and Siddiquee, 2013); 74.58± 5.63 in buffalo bull (Lone *et al.,* 2018); 76.70±1.12 in buffalo bull (Sandeep *et al.,* 2015); 65.37± 0.84 in buffalo bull (Varghese *et al.,* 2015); 64.28± 2.96 in crossbred bull (Singh *et al.,* 2017); 63.7 in crossbred bull (Hossain *et al.,* 2012); 63.54± 0.60 in Hariana bull (Patel *et al.,* 2015); 63.00± 0.84 in buffalo bull (Hegazy *et al.,* 2020); 62.1± 2.5 in HF bull (Shoae and Zamiri, 2008); 54.5± 1.5 in buffalo bull (Sakr *et al.,* 2021); 40.54± 0.51 in HF bull (Khan *et al.,* 2021b).

In present investigation the per cent progressive motility of equilibrated semen (Table 4.2) was 62.31± 0.54 which was in accordance to finding reported by Hossain *et al.,* 2012 (63.7), Patel *et al.,* 2015 (63.54± 0.60), Hegazy *et al.,* 2020 (63.00± 0.84) and Shoae and Zamiri, 2008 (62.1± 2.5).

The higher per cent progressive motility of post thaw semen is reported as 76.54± 0.55 in Sahiwal bull (Khan *et al.,* 2018); 67.60± 0.47 in crossbred bull (Matharoo, 2015); 56.83± 0.34 in Kankrej bull (Patel and Siddiquee, 2013); 50.8± 2.7 in HF bull (Khalil *et*

al., 2018); 49.7± 2.5 in buffalo bull (Mughal *et al.,* 2013); 49.6±1.1 in cattle bull (Sariozhan *et al.,* 2009a); 49.53± 2.00 in crossbred bull (Singh *et al.,* 2017); 49.17± 0.57 in Hariana bull (Patel *et al.,* 2015); 48.76± 1.69 in Gir bull (Chaturvedi *et al.,* 2020); 48.33±1.06 in buffalo bull (Wafa *et al.,* 2021); 48.0± 2.49 in crossbred bull (Singh *et al.,* 2020); 46.63± 2.40 in HF bull (Buyukleblebici *et al.,* 2014) and the lowest as 14.7± 1.4 in bovine bull (Li *et al.,* 2016).

In present research experiment the per cent progressive motility of post thaw semen (Table 4.2) was 47.72± 0.79 which was in agreement with the findings of Chaturvedi *et al.,* 2020 (48.76± 1.69), Wafa *et al.,* 2021 (48.33±1.06), Singh *et al.,* 2020 (48.0± 2.49) and Buyukleblebici *et al.,* 2014 (46.63± 2.40). Though it was lower than cited by Khan *et al.,* 2018 and Matharoo, 2015 and higher than Li *et al.,* 2016.

The decrease in sperm motility is observed in disease condition, change of environment/season and temperature variations, and it swings between breeds, individuals, age groups and the evaluation technique employed.

The variation in sperm motility in post thaw semen occur due to cryopreservation which causes the formation of ice crystal in mitochondria and axoneme or the biochemical oxidative stress resulting in irreversible damage to sperm structure, changes in membrane fluidity and enzymatic activity (Alvarez and Storey, 1983).

5.1.5 Live Sperm

The per cent live sperm in neat semen is reported as 90.58± 0.20 in bull (Patel and Siddiquee, 2013); 89.97± 0.21 in Kankrej bull (Shaikh *et al.,* 2016); 89.53± 0.59, 88.90± 0.47, 88.73± 0.59 and 88.70± 0.59 in Jersey, Red sindhi, crossbred and Haryana bull, respectively (Mishra *et al.,* 2013); 89.45± 4.70 in bull (Lone *et al.,* 2018); 88.43± 0.70 in buffalo bull (Kadirvel *et al.,* 2014); 87.4± 1.4 in HF bull (Srivastava and Kumar, 2014); 87.13± 1.49 in Hariana bull (Pal *et al.,* 2020b) 78.58± 0.28 in crossbred bull (Bhoite *et al.,* 2008); 76.50± 1.30 in Gir bull (Chaudhary *et al.,* 2017); 70.40± 3.91 in Sahiwal bull (Mandal *et al.,* 2005).

In present study the per cent live sperm in neat semen (Table 4.3) was 90.10± 0.67 and this finding was in agreement reported by Patel and Siddiquee, 2013 (90.58± 0.20), Shaikh *et al.,* 2016 (89.97± 0.21), Mishra *et al.,* 2013 (89.53± 0.59) and Lone *et al.,* 2018 (89.45± 4.70).

The per cent live sperm in diluted semen is reported as 82.79± 0.29 in buffalo bull (Saurabh *et al.,* 2021); 82.53± 0.70 in buffalo bull (Varghese *et al.,* 2015); 81.56± 2.30 in crossbred bull (Singh *et al.,* 2017); 80.59± 0.56 in Hariana bull (Patel *et al.,* 2015); 80.53± 0.70 in Jersey bull (Singh and Sharma, 2018); 74.4± 1.82 in crossbred bull (Singh *et al.,* 2020); 73.0± 1.3 in HF bull (Abdel-Khalek *et al.,* 2008); 65.12± 1.44 in buffalo bull (Hegazy *et al.,* 2020); 59.50± 2.37 in crossbred bull (Khumran *et al.,* 2017).

In our study the per cent live sperm in diluted semen (Table 4.3) was 78.73 ±1.46 which was in agreement as reported by Patel *et al.,* 2015 (80.59± 0.56) and Singh and Sharma, 2018 (80.53± 0.70).

The per cent live sperm in equilibrated semen is reported as 76.77± 0.68 in Jersey bull (Singh and Sharma, 2018); 72.75± 0.99 in buffalo bull (Varghese *et al.,* 2015); 72.50± 2.63 in crossbred bull (Singh *et al.,* 2017); 70.77± 1.10 in buffalo bull (Sandeep *et al.,* 2015); 70.5± 1.1 in HF bull (Shoae and Zamiri, 2008); 69.92± 1.74 in Gir bull (Chaudhary *et al.,* 2017); 59.75± 1.95 in buffalo bull (Hegazy *et al.,* 2020).

In the present experiment the per cent live sperm in equilibrated semen (Table 4.3) was 71.22± 1.40 which was in agreement with the findings of Varghese *et al.,* 2015 (72.75± 0.99), Singh *et al.,* 2017 (72.50± 2.63), Sandeep *et al.,* 2015 (70.77± 1.10) and Shoae and Zamiri, 2008 (70.5± 1.1).

The higher per cent live sperm of post thaw semen is reported as 78.70± 0.21 in buffalo bull (Patel *et al.,* 2016); 64.27± 0.41 in buffalo bull (Saurabh *et al.,* 2021); 61.3± 1.8 in HF bull (Motemani *et al.,* 2017); 60.33± 0.45 in Kankrej bull (Shaikh *et al.,* 2016); 60± 1 in HF bull (Asadpour *et al.,* 2011); 59.3± 3.7 in Zebu cattle (Batool *et al.,* 2012); 59.14± 2.18 in crossbred bull (Singh *et al.,* 2017); 58.25± 6.03 in Korean jeju black bull (Oh *et al.,* 2012); 58.22± 0.24 in Kankrej bull (Patel and Siddiquee, 2013); 58.00± 1.21 in Sahiwal bull (Baber *et al.,* 2016); 57.9± 3.3 in buffalo bull (Mughal *et al.,* 2013); 56.5±

2.1 in buffalo bull (Kumar *et al.,* 2018a); 56.24± 0.01 in crossbred bull (Rao *et al.,* 2017) and lower as 39.36± 0.90 in buffalo full (Sandeep *et al.,* 2015).

In the present research trial the per cent live sperm in post thaw semen (Table 4.3) was 59.59± 0.69 which was in accordance to the findings of Shaikh *et al.,* 2016 (60.33± 0.45), Asadpour *et al.,* 2011 (60± 1), Batool *et al.,* 2012 (59.3± 3.7), Singh *et al.,* 2017 (59.14± 2.18), Oh *et al.,* 2012 (58.25± 6.03), Patel and Siddiquee, 2013 (58.22± 0.24) and Baber *et al.,* 2016 (58.00± 1.21).

The variation in percentage of stained spermatozoa is dependent on various factors like duration, type of diluents used, stains integrity and technique employed by various scientists.

The reasons for varying live sperm count during cryopreservation may be due to bio-physical damage to the spermatozoa due to formation of ice-crystals in the extracellular and intracellular surroundings and expanding solute concentration that results in the production of ROS (Mazur, 1984) and sperm susceptible for freezing and thawing temperature, ROS production and lipid peroxidation (Aitken *et al*., 1989).

5.1.6 Acrosomal integrity

The estimation of acrosomal integrity is main consideration for assurance of semen quality as enzymes vital for fertilization are present in the acrosomal cap.

The per cent acrosomal integrity of neat semen is reported as 95.59± 0.35 in Gir bull (Dhami *et al.,* 2017); 94.50± 0.40 in Gir bull (Chaudhary *et al.,* 2017); 93.5± 0.5 in HF bull (Munsi *et al.,* 2007); 90.72± 0.25 in Kankrej bull (Shaikh *et al.,* 2016); 89.9± 1.2 in HF bull (Srivastava and Kumar, 2014); 84.80± 0.89 and 83.50± 1.24 in Gir and Jaffarabady bull, respectively (Rana and Dhami, 2004); 82.20± 0.47, 81.73±0.69 and 81.00± 0.69 in HF, Jersey, and Haryana bull, respectively (Mishra *et al.,* 2013); 81.71± 0.11 in Kankrej bull (Patel and Siddiquee, 2013); 79.91± 0.51 in Sahiwal bull, 71.50± 0.53 in HF bull and 62.58± 0.67 in crossbred bull (Khan *et al.,* 2018).

In present experimental trial the per cent acrosomal integrity of neat semen (Table 4.4) was 84.39± 1.25. This finding was in agreement with the findings of Rana and Dhami, 2004 (84.80± 0.89 and 83.50± 1.24) and Mishra *et al.,* 2013 (82.20± 0.47).

The per cent acrosomal integrity in diluted semen is reported as 92.85± 0.34 in buffalo bull (Varghese *et al.,* 2015); 90.13± 0.18 in Murrah buffalo (Saurabh *et al.,* 2021); 86.17± 0.94 in Jersey bull (Singh and Sharma, 2018); 85.7± 0.01 in crossbred bull (Rao *et al.,* 2017); 80.17± 3.62 in buffalo bull (Lone *et al.,* 2018); 80.16± 0.50 in Bhadawari bull (Mittal *et al.,* 2014); 79.7± 1.52 in crossbred bull (Singh *et al.,* 2020); 78.6± 1.4 in buffalo bull (Sakr *et al.,* 2021); 75.13± 0.42 in Hariana bull (Patel and Siddiquee, 2013); 73.2± 2.4 in buffalo bull (Rasul *et al.,* 2001).

In present research trial the per cent acrosomal integrity in diluted semen (Table 4.4) was 74.44± 1.43 and this finding was in agreement reported by Patel and Siddiquee, 2013 (75.13± 0.42) and Rasul *et al.,* 2001 (73.2± 2.4).

The per cent acrosomal integrity in equilibrated semen is reported as 90.50± 0.45 in Gir bull (Chaudhary *et al.,* 2017); 89.70± 0.39 in buffalo bull (Varghese *et al.,* 2015); 82.47± 1.05 and 74.59± 0.50 in Jersey and Bhadawari bull, respectively (Mittal *et al.,* 2014); 70.0± 1.1 in HF bull (Shoae and Zamiri, 2008); 68.41± 0.32, 63.20± 0.31 and 59.29± 0.26 in Jersey, HF and Nili-ravi bull, respectively (Khan *et al.,* 2021b); 68.16± 0.80 in Kankrej bull (Patel and Siddiquee, 2013) 63.14± 4.03 in Hariana bull (Kumar *et al.,* 2019).

In our present study the per cent acrosomal integrity in equilibrated semen (Table 4.4) was 67.33±1.14 which was in accordance with the findings reported by Patel and Siddiquee, 2013 (68.16± 0.80) and Khan *et al.,* 2021b (68.41± 0.32).

The higher per cent acrosomal integrity of post thaw semen is reported as 82.43± 0.32 in buffalo bull (Varghese *et al.,* 2015); 70.5± 0.8 and 67.7± 1.2 in HF and crossbred bull, respectively (Srivastava and Kumar, 2014); 69.41± 0.54 in Bhadawari bull (Mittal *et al.,* 2014); 60.0± 2.6 in buffalo bull (Kumar *et al.,* 2018a); 56.24± 0.01 in crossbred bull (Rao *et al.,* 2017); 53.65± 0.58 in Kankrej bull (Patel and Siddiquee, 2013); 52.3± 1.58 in

bovine bull (Hu *et al.*, 2010a); 40.83± 1.56 in Sahiwal x HF cross (Khan *et al.,* 2021a) and the lower as 35.83± 0.56 in crossbred bull (Khan *et al.,* 2018).

In the present investigation the per cent acrosomal integrity in post thaw semen (Table 4.4) was 59.53± 0.49 which was in agreement with the finding of Kumar *et al.,* 2018a (60.0± 2.6). Our finding was higher than the findings of Rao *et al.,* 2017, Patel and Siddiquee, 2013, Hu *et al.,* 2010a, Khan *et al.,* 2021a and Khan *et al.,* 2018 but lower than the findings of Srivastava and Kumar, 2014 and Mittal *et al.,* 2014.

These variations reported in acrosomal integrity could be due to inherent quality of particular breed/bull, age, physio-pathological status, stain, staining technique & duration of staining used etc by different workers.

The variation or decrease in results of acrosomal integrity occurs in post thaw semen is because of cryopreservation, as it induces various degrees of acrosomal damage i.e. primary, secondary or tertiary damage which may be due to the loss of plasma membrane and loss of plasmalemma over entire acrosome during freezing and thawing.

5.1.7 Sperm abnormalities

The per cent sperm abnormalities in neat semen has been reported by various scientist as 3.08± 0.15 in Kankrej bull (Shaikh *et al.,* 2016); 3.50± 0.42 and 3.75± 0.45 in two Hariana bull (Pal *et al.,* 2020b); 4.24± 0.03 in Kankrej bull (Patel and Siddiquee, 2013); 5.25± 0.36 in Gir bull (Chaudhary *et al.,* 2017); 5.40± 0.6 in HF bull (Uysal *et al.,* 2007); 6.5± 1.0 in HF bull (Munsi *et al.,* 2007); 10.1± 1.5 in HF and 12.6± 2.3 in crossbred bull (Srivastava and Kumar, 2014); 18.04± 3.03 in Sahiwal bull (Mandal *et al.,* 2005).

In the present study the per cent sperm abnormalities in neat semen (Table 4.5) was 3.30± 0.30 which was in agreement with the findings of Shaikh *et al.,* 2016 (3.08± 0.15), Pal *et al.,* 2020b (3.50± 0.42 and 3.75± 0.45) and Patel and Siddiquee, 2013 (4.24± 0.03).

The per cent sperm abnormalities in diluted semen is reported as 4.91± 0.14 in Bhadawari bull (Mittal *et al.,* 2014); 4.93± 0.25 in buffalo bull (Varghese *et al.,* 2015);

6.13± 0.07 in Kankrej bull (Patel and Siddiquee, 2013); 6.99± 0.55 in HF bull (Al-Badrany *et al.,* 2020); 7.20± 0.68 in crossbred bull (Singh *et al.,* 2020); 7.28± 0.02 in crossbred bull (Rao *et al.,* 2017); 8.08±0.18 in Murrah buffalo (Saurabh *et al.,* 2021); 20± 1.2 in HF bull (Khalil *et al.,* 2018); 28.75± 1.54 in buffalo bull (Hegazy *et al.,* 2020).

In the present research experiment the per cent sperm abnormalities in diluted semen (Table 4.5) was 6.22± 0.38. This finding was in agreement with the findings reported by Patel and Siddiquee, 2013 (6.13± 0.07), Al-Badrany *et al.,* 2020 (6.99± 0.55), Singh *et al.,* 2020 (7.20± 0.68) and Rao *et al.,* 2017 (7.28± 0.02).

The per cent sperm abnormalities in equilibrated semen is reported as 6.35± 0.27 in buffalo bull (Varghese *et al.,* 2015); 7.26± 0.42 in Gir bull (Chaudhary *et al.,* 2017); 7.56± 0.15 in Kankrej bull (Patel and Siddiquee, 2013); 9.55± 0.47 in HF bull (Al-Badrany *et al.,* 2020); 15.31± 0.77 in HF bull (Dheerib *et al.,* 2020); 24± 0.5 in HF bull (Khalil *et al.,* 2018).

In our present study the per cent sperm abnormalities in equilibrated semen (Table 4.5) was 8.42± 0.65 which was in agreement with the findings of Chaudhary *et al.,* 2017 (7.26± 0.42), Patel and Siddiquee, 2013 (7.56± 0.15) and Al-Badrany *et al.,* 2020 (9.55± 0.47).

The per cent sperm abnormalities in post thaw semen reported by various scientists are 7.75± 0.71 in Bhadawari bull (Mittal *et al.,* 2014); 8.83± 0.57 in HF bull (Tuncer *et al.,* 2010); 11.00± 1.45 in buffalo bull (Gamal *et al.,* 2016); 11.06± 0.34 in Gir bull (Chaudhary *et al.,* 2017); 11.13± 0.77 in HF bull (Buyukleblebici *et al.,* 2014); 13.52± 0.35 in Kankrej bull (Patel and Siddiquee, 2013); 14.67± 0.45 in HF bull (Al-Badrany *et al.,* 2020); 15.0± 1.1 in cattle bull (Sariozkan *et al.,* 2009a); 16.8± 0.61 in crossbred bull (Singh *et al.,* 2020); 18.36± 0.04 in crossbred bull (Rao *et al.,* 2017); 29± 1.5 in HF bull (Khalil *et al.,* 2018); 35.75± 1.5 in buffalo bull (Wafa *et al.,* 2021).

In present investigation the per cent sperm abnormalities in post thaw semen (Table 4.5) was 12.31± 0.59 and this was in accordance to the findings of Gamal *et al.,* 2016 (11.00± 1.45), Chaudhary *et al.,* 2017 (11.06± 0.34), Buyukleblebici *et al.,* 2014 (11.13± 0.77) and Patel and Siddiquee, 2013 (13.52± 0.35).

The semen from most males contains some abnormal spermatozoa which are not associated with lower fertility rates until the proportion of abnormal sperm exceeds 20 percent (Hafez, 1987). The variation in sperm abnormality may be due to method of collection, temperature shock and technique employed.

5.1.8 Hypo-osmotic swelling test (HOST)

The per cent HOST positive sperm in neat semen has been reported by various scientists as 84.36± 0.66 in Jersey, 83.90± 0.51 in crossbred, 83.53± 0.66 in Haryana and 81.16± 0.51 in Red sindhi bull (Mishra *et al.,* 2017); 84.00± 2.08 in buffalo bull (Reddy *et al.,* 2010); 83.80± 3.3 in HF bull (Uysal *et al.,* 2007); 82.25± 0.43 in Sahiwal, 81.66± 0.48 in HF and 80.62 in crossbred bull (Khan *et al.,* 2018); 80.30± 1.90 in Gir bull (Dhami *et al.,* 2017); 77.37± 5.38 in buffalo bull (Lone *et al.,* 2018); 76.92± 1.27 in Gir bull (Chaudhary *et al.,* 2017); 76.00± 3.05 in Karan fries (Kumar *et al.,* 2013); 66.42± 1.12 in Jersey bull (Singh and Sharma, 2018); 58.37 in HF bull (Baloch *et al.,* 2019).

In the present experiment the per cent HOST positive sperm in neat semen (Table 4.6) was 82.20± 1.00 and this finding was in agreement with the findings of Mishra *et al.,* 2017 (83.90± 0.51 and 83.53± 0.66), Uysal *et al.,* 2007 (83.80± 3.3) and Khan *et al.,* 2018 (82.25± 0.43 and 81.66± 0.48).

The per cent HOST positive sperm in diluted semen is reported as 82.72± 0.91 in buffalo bull (Varghese *et al.,* 2015); 80.2± 3.9 in buffalo bull (Rasul *et al.,* 2001); 78.11± 0.58 in Hariana bull (Patel *et al.,* 2015); 77.67± 1.81 in crossbred bull (Singh *et al.,* 2017); 71.0± 1.10 in crossbred bull (Singh *et al.,* 2020); 70.6± 1.1 in buffalo bull (Sakr *et al.,* 2021); 67.47± 0.05 in Jersey bull (Singh and Sharma, 2018); 50.67± 0.27 in Murrah buffalo (Saurabh *et al.,* 2021).

In the present research trial the per cent HOST positive sperm in diluted semen (Table 4.6) was 74.06± 0.97. Our finding was in normal range as reported by various scientists *i.e.* 50-83 percent. It was higher than the findings of Singh *et al.,* 2020 (71.0± 1.10), Sakr *et al.,* 2021 (70.6± 1.1), Singh and Sharma, 2018 (67.47± 0.05) and Saurabh *et al.,* 2021 (50.67± 0.27) but lower than the findings of Varghese *et al.,* 2015 (82.72±

0.91), Rasul *et al.,* 2001(80.2± 3.9), Patel *et al.,* 2015 (78.11± 0.58) and Singh *et al.,* 2017 (77.67± 1.81).

The per cent HOST positive sperm in equilibrated semen is reported as 77.04± 0.56 in Surti bull and 71.70± 1.44 in Gir bull (Chaudhary *et al.,* 2017); 72.46± 0.97 in Hariana bull (Patel *et al.,* 2015); 70.91± 5.92 in buffalo bull (Lone *et al.,* 2018); 69.92± 2.30 in crossbred bull (Singh *et al.,* 2017); 69.20± 0.31 in HF bull and 66.95± 0.38 (Khan *et al.,* 2021b); 63.31± 1.11 in Jersey bull (Singh and Sharma, 2018); 60.4± 5.6 in buffalo bull (Rasul *et al.,* 2001); 58.0± 1.7 in buffalo bull (Sakr *et al.,* 2021).

In present investigation the per cent HOST positive sperm in equilibrated semen (Table 4.6) was 68.83± 1.32 which was in agreement with the findings of Singh *et al.,* 2017 (69.92± 2.30) and Khan *et al.,* 2021b (69.20± 0.31 and 66.95± 0.38).

The higher per cent HOST positive sperm in post thaw semen is reported as 78.58± 0.45 in Sahiwal bull (Khan *et al.,* 2018); 64.00± 2.08 in buffalo bull (Gamal *et al.,* 2016); 60.56± 2.93 in crossbred bull (Singh *et al.,* 2017); 60.5± 1.6 in HF bull (Motemani *et al.,* 2017); 59.6± 3.4 in buffalo bull (Mughal *et al.,* 2013); 58.50± 1.87 in Maduran bull (Ratnani *et al.,* 2017); 57.34± 0.74 in Hariana bull (Patel *et al.,* 2015); 56.83± 0.90 in buffalo bull (Wafa *et al.,* 2021); 56.24± 3.49 in buffalo bull (Wadood *et al.,* 2015); 52.55± 3.38 in buffalo bull (Wadood *et al.,* 2016); 51.90± 9.99 in Korean jeju black bull (Oh *et al.,* 2012) and lower as 28.67± 1.29 in Gir bull; 21.8± 0.5 in HF and 21.0± 0.6 in crossbred bull (Srivastava and Kumar, 2014).

In present study the per cent HOST positive sperm in post thaw semen (Table 4.6) was 57.61± 0.93 which was in accordance with the findings of Ratnani *et al.,* 2017 (58.50± 1.87), Patel *et al.,* 2015 (57.34± 0.74), Wafa *et al.,* 2021 (56.83± 0.90) and Wadood *et al.,* 2015 (56.24± 3.49).

The variation in HOST may be due to spontaneous lipid peroxidation of the membranes of mammalian sperm destroy the structure of the lipid matrix, as a result of the invasion by reactive oxygen species (ROS). These attacks then ultimately lead to the impairment of sperm function (sperm motility, functional membrane integrity and

fertility), and damage to the sperm DNA, through oxidative stress and the production of cytotoxic aldehydes (Aitken *et al.,* 1993).

5.1.9 Lipid peroxidation

The MDA level (nmoles of MDA produced/10^8 spermatozoa/hr) of post thaw semen is reported by various researchers as 0.50± 0.27 µm/ml-10^9 cell/ml in Brown Swiss bull (Buyukleblebici *et al.,* 2016); 0.60±0.06 µmol/ml in crossbred bull (Muzafer *et al.,* 2012); 0.7± 0.2 nmol/ml in cattle bull (Sariozkan *et al.,* 2009a); 1.02± 0.38 nmol/ml in HF bull (Al-Dahan *et al.,* 2020); 1.44± 0.08 nmol/ml in HF bull (Tuncer *et al.,* 2010); 196.83± 4.89 MDA/µg protein/ml in buffalo bull (Bansal and Cheema, 2016); 2.01± 0.26 nmol/ml in HF bull (Buyukleblebici *et al.,* 2014); 2.61± 0.33 nmol/10^8 cells in Karan fries (Chhillar *et al.,* 2012); 3.49± 0.19 nmol/10^8 in crossbred bull (Singh *et al.,* 2020) and 7.2± 0.6 nmol/ml in HF bull (Motemani *et al.,* 2017).

In present research experiment the MDA level (nmoles of MDA produced/10^8 spermatozoa/hr) in post thaw semen (Table 4.7) was 1.86± 0.02 which was in agreement with findings reported by Tuncer *et al.,* 2010 and Bansal and Cheema, 2016. Our finding was lower than the findings of Buyukleblebici *et al.,* 2014, Chhillar *et al.,* 2012. Singh *et al.,* 2020 and Motemani *et al.,* 2017 whereas, higher than the findings reported by Sariozkan *et al.,* 2009a and Al-Dahan *et al.,* 2020.

The finding of all other scientist could not be compared with our findings due to disparity in method of evaluation and units of measurement.

MDA is one of the final products of polyunsaturated fatty acids (PUFA) peroxidation in the sperm cells. The osmotic stress during the sperm dilution, cooling, cryoprotectant exposure, freezing, and thawing processes resulted in oxidative stress that induced high concentrations of MDA (Ansari *et al.,* 2019). The production of ROS in sperm is a natural process; however, overproduction has a deleterious impact on cellular membranes, resulting in dysfunction of cell organelles leading to death (Partyka *et al.,* 2012). Sperm cells are known to have high levels of polyunsaturated fatty acids in the plasma membrane that makes sperm highly susceptible to LPO (Parks and Hammerstedt, 1985).

5.1.10 Catalase

The catalase activity (µmol H_2O_2 decomposed/ min/ 10^8 spermatozoa) in post thaw semen reported by various scientists as 84829.5± 72368.8 U/ml in HF bull (Varela *et al.,* 2019); 3.89± 0.65 U/ml in HF bull (Hu *et al.,* 2011); 24.25± 0.30 µmol H_2O_2 decomposed/ min/ 10^8 spermatozoa in buffalo bull (Sharma *et al.,* 2016); 2.02± 0.15 U/ml in HF bull (Hu *et al.,* 2010a); 2.01± 0.12 U/ml in HF bull (Hu *et al.,* 2010b); 18.9± 4.94 µm/ml- 10^9 cell/ml in HF bull (Tasdemir *et al.,* 2014); 1.9± 0.11 U/ml in Qinchuan bull (Zhao *et al.,* 2015); 1.18± 0.36 µmol/10^8 spermatozoa in crossbred bull (Singh *et al.,* 2020).

In the present investigation the catalase activity (µmol H_2O_2 decomposed/ min/ 10^8 spermatozoa) in post thaw semen (Table 4.8) was 39.61± 0.62. Our finding was higher than the findings reported by Sharma *et al.,* 2016 and Singh *et al.,* 2020.

The finding of all other scientist could not be compared with our findings due to disparity in method of evaluation and units of measurement.

Catalase is one of the enzymes present in all living organisms which is considered as the major defender of the sperm membranes against ROS and LPO (Bilodeau *et al.,* 2001). In living organism it is presented to oxygen, which disintegrates destructive peroxides and convert them into water and oxygen. The decrease in the catalase activity may be due to over consumption of catalase enzyme to decompose harmful peroxide and free radicals (Chelikani *et al.,* 2004).

5.1.11 Superoxide dismutase

The superoxide dismutase (Unit/10^8 spermatozoa) in post thaw semen is reported as 272.6± 31.1 IU/10^8 spermatozoa in buffalo bull (Kumar *et al.,* 2018a); 2.31± 0.76 nmol ml^{-1} in Brown swiss bull (Sariozkan *et al.,* 2015); 1.58± 0.08 U/ml in HF bull (Hu *et al.,* 2010b); 1.57± 0.11 U/ml in HF bull (Hu *et al.,* 2010a); 11.78± 0.88 U/10^9 spermatozoa in buffalo bull (Kadirvel *et al.,* 2014); 1144.8± 826.6 U/ml in HF bull (Varela *et al.,* 2019); 96± 0.94 IU/10^9/min in buffalo bull (Bansal and Cheema, 2016);

7.2± 1.8 U/g protein in HF bull (Sariozkan *et al.,* 2009b); 5.5± 1.7% inhibition in HF bull (Ashrafi *et al.,* 2013).

In present research experiment the superoxide dismutase activity in post thaw semen (Table 4.9) was 11.41± 0.19 Unit/10^8 spermatozoa.

The finding of all other scientist could not be compared with our findings due to disparity in method of evaluation and units of measurement.

Superoxide ion is free radical which is change over to oxygen and hydrogen peroxide by the dismutation activity of antioxidant enzyme superoxide dismutase (Tariq *et al.,* 2015). The decrease in superoxide activity may be due to neutralization of superoxide during cryopreservation (Bilodeau *et al.,* 2000).

5.2 Effect of Antioxidant Additives in Group I

5.2.1 Cysteine

The per cent progressive motility in diluted semen is reported as 80.62± 0.65 and 81.75± 0.79 with 0.5 and 1.0 mg/ml of cysteine, respectively in buffalo bull (Varghese *et al.,* 2015); 73.75± 0.52 in Hariana bull with 5mM of cysteine (Pal *et al.,* 2020a). The per cent progressive motility in equilibrated semen is reported as 68.37± 0.81 and 72.62± 0.69 with 0.5 and 1.0 mg/ml of cysteine, respectively in buffalo bull (Varghese *et al.,* 2015); 68.38± 1.16 in Hariana bull with 5mM of cysteine (Pal *et al.,* 2020a); 52.57± 3.40 and 53.85± 3.42 with 2 and 4mM cysteine, respectively in HF bull (Ali and Banana, 2020). The per cent progressive motility in post thaw semen is reported by various scientists as 65.002± 2.47 in cattle bull with 5mM cysteine (El-Sheshtawy *et al.,* 2008); 62.75± 1.37 in Haraian bull with 5mM cysteine (Pal *et al.,* 2020a); 52.5± 5.00 in HF bull with 5mM cysteine (Tasdemir *et al.,* 2014); 52.80± 1.44 and 50.50± 1.73 with 5mM and 10mM cysteine, respectively in Sahiwal x HF bull (Khan *et al.,* 2021a); 49.00± 3.10 in HF bull with 5mM cysteine (Buyukleblebici *et al.,* 2014); 48.61± 4.60 in HF bull with 5mM cysteine (Tuncer *et al.,* 2010).

In present investigation the per cent value of progressive motility in diluted and equilibrated semen (Table 4.2) was 70.06± 0.25 and 63.7± 0.53, respectively which was lower than the findings of Varghese et al., 2015 and Pal et al., 2020a.

The per cent progressive motility in post thaw semen (Table 4.2) was 50.1± 1.03 and this finding was in agreement with the findings of Khan *et al.,* 2021a (52.80± 1.44 and 50.50± 1.73) and Buyukleblebici *et al.,* 2014 (49.00± 3.10).

The per cent value of live sperm in diluted semen is reported as 86.37± 0.86 in Hariana bull with 5mM cysteine (Pal *et al.,* 2020a); 82.75± 0.76 and 84.27± 0.66 with 0.5 and 1.0 mg/ml of cysteine, respectively in buffalo bull (Varghese *et al.,* 2015). In equilibrated semen the per cent live sperm is reported as 93.50± 0.77 and 93.65± 0.80 with 2 and 4mM cysteine, respectively in HF bull (Ali and Banana, 2020); 81.88± 0.66 in Hariana bull with 5mM cysteine (Pal *et al.,* 2020a); 72.75± 0.99 and 70.62± 0.8 with 0.5 and 1.0 mg/ml of cysteine, respectively in buffalo bull (Varghese *et al.,* 2015) and in post thaw semen the per cent value of live sperm is reported as 75.88± 0.35 in Hariana bull with 5mM cysteine (Pal *et al.,* 2020a); 65.70± 2.16 in buffalo bull with 5mM cysteine (Wadood *et al.,* 2015); 64.67± 0.49 in Jersey bull with 10mM cysteine (Bhardwaz *et al.,* 2016) and 52.66± 1.44 in Sahiwal x HF cross bull with 5mM cysteine (Khan *et al.,* 2021a).

In present research trial the per cent live sperm in diluted and equilibrated semen (Table 4.3) was 79.52± 0.48 and 74.28± 0.78, respectively and these finding were lower than the findings of Pal *et al.,* 2020a.

The per cent live sperm in post thaw semen (Table 4.3) was 62.18± 0.95 which was lower than the findings of Pal *et al.,* 2020a and Wadood *et al.,* 2015 whereas, greater than the finding of Khan *et al.,* 2021a.

The per cent value of acrosomal integrity in diluted semen was reported as 93.08± 0.32 and 93.28± 0.33 in buffalo bull with 0.5 and 1.0 mg/ml of cysteine, respectively (Varghese *et al.,* 2015); 83.75± 0.88 in Hariana bull with 5mM cysteine (Pal *et al.,* 2020a), in equilibrated semen the per cent acrosomal integrity is reported as 94.85± 0.55 and 94.85± 0.57 in HF bull with 2 and 4mM cysteine, respectively (Ali and Banana,

2020); 90.58± 0.34 and 90.90± 0.35 in buffalo bull with 0.5 and 1.0 mg/ml of cysteine, respectively (Varghese *et al.*, 2015); 78.50± 0.90 in Hariana bull with 5mM cysteine (Pal *et al.,* 2020a) and in post thaw semen the per cent acrosomal integrity was reported as 84.40± 0.18 and 85.73± 0.18 in buffalo bull with 0.5 and 1.0 mg/ml of cysteine, respectively (Varghese *et al.*, 2015); 73.13± 0.69 in Hariana bull with 5mM cysteine (Pal *et al.,* 2020a); 69.60± 1.33 in cattle bull with 5mM cysteine (El-Sheshtawy *et al.,* 2008); 53.20± 3.78 in buffalo bull with 5mM (Wadood *et al.,* 2015); 43.41± 1.60 in Sahiwal x HF cross with 5mM cysteine (Khan *et al.,* 2021a).

In our present study the per cent acrosomal integrity in diluted and equilibrated semen (Table 4.4) was 77.32± 1.11 and 70.18± 1.00 respectively and these findings were lower than the finding of Pal *et al.,* 2020a.

The per cent acrosomal integrity in post thaw semen (Table 4.4) was 62.63± 0.86 which was lower than the findings of Pal *et al.,* 2020a and El-Sheshtawy *et al.,* 2008 whereas, higher than findings of Wadood *et al.,* 2015 and Khan *et al.,* 2021a.

The per cent sperm abnormalities in diluted semen is reported as 4.68± 0.26 and 4.53± 0.25 in buffalo semen with 0.5 and 1.0 mg/ml of cysteine, respectively (Varghese *et al.,* 2015), in equilibrated semen the per cent sperm abnormalities were reported as 5.73± 0.28 and 5.45± 0.25 in buffalo semen with 0.5 and 1.0 mg/ml of cysteine, respectively (Varghese *et al.,* 2015) and in post thaw semen the per cent sperm abnormalities reported by various scientists as 8.15± 0.26 and 7.10± 0.26 in buffalo bull with 0.5 and 1.0 mg/ml of cysteine, respectively (Varghese *et al.,* 2015); 9.8± 0.80 in HF bull with 5mM cysteine (Tasdemir *et al.,* 2014); 9.11± 0.60 in HF bull with 2mM cysteine (Sariozkan *et al.,* 2009a); 11.05± 0.28 in Jersey bull with 15mM cysteine (Bhardwaz *et al.,* 2016); 12.25± 1.21 in HF bull with 5mM cysteine (Buyukleblebici *et al.,* 2014); 25.75± 1.07 in buffalo bull with 1mM cysteine (Wafa *et al.,* 2021).

In the proposed study the per cent sperm abnormalities in diluted and equilibrated semen (Table 4.5) was 5.40± 0.44 and 7.78± 0.62, respectively.

The per cent sperm abnormalities in post thaw semen (Table 4.5) was 11.62± 0.54 which was higher than the finding of Tasdemir *et al.,* 2014 (9.8± 0.80) and lower than the finding of Buyukleblebici *et al.,* 2014 (12.25± 1.21).

The per cent HOST positive sperm in diluted semen is reported as 83.40± 0.70 and 85.85± 0.67 in buffalo semen with 0.5 and 1.0 mg/ml of cysteine, respectively (Varghese *et al.,* 2015); 82.75± 0.98 in Hariana bull with 5mM cysteine (Pal *et al.,* 2020a), in equilibrated semen the per cent HOST positive sperm is reported as 92.57± 0.64 and 91.71± 0.64 in HF bull with 2 and 4mM cysteine, respectively (Ali and Banana, 2020); 76.75± 0.59 in Hariana bull with 5mM (Pal *et al.,* 2020a); 74.17± 0.93 and 78.12± 0.79 in buffalo semen with 0.5 and 1.0 mg/ml of cysteine, respectively (Varghese *et al.,* 2015) and in post thaw semen the per cent HOST positive was reported as 72.88± 0.39 in Hariana bull with 5mM cysteine (Pal *et al.,* 2020a); 72.80± 2.48 in cattle bull with 5mM cysteine (El-Sheshtawy *et al.,* 2008); 63.55± 2.00 in buffalo bull with 5mM cysteine (Topraggaleh *et al.,* 2013); 56.27± 3.07 in buffalo bull with 5mM cysteine (Wadood *et al.,* 2015); 45.56± 1.77 in Sahiwal x HF cross bull with 5mM cysteine (Khan *et al.,* 2021a); 37.75± 2.38 in HF bull with 5mM cysteine (Buyukleblebici *et al.,* 2014).

In the present investigation the per cent HOST positive sperm in diluted and equilibrated semen (Table 4.6) was 76.76± 0.97 and 70.9± 1.51 which was lower than the finding of Pal *et al.,* 2020a.

The per cent HOST positive sperm in post thaw semen (Table 4.6) was 59.94± 1.09 which was lower than the findings of Pal *et al.,* 2020a, El-Sheshtawy *et al.,* 2008, Topraggaleh *et al.,* 2013 whereas, higher than the findings of Wadood *et al.,* 2015, Khan *et al.,* 2021a and Buyukleblebici *et al.,* 2014.

The MDA level (nmoles of MDA produced/10^8 spermatozoa/hr) in post thaw semen reported by various scientists are 0.44± 0.28 µm/ml-10^9 cell/ml in HF bull with 5mM cysteine (Tasdemir *et al.,* 2014); 1.06± 0.12 mmol/ml in HF bull with 5mM cysteine (Al-Dahan *et al.,* 2020); 2.30± 0.11 nmol/ml in HF bull with 5mM (Tuncer *et al.,* 2010); 29.61± 3.99 nm in buffalo bull with 5mM cysteine (Wadood *et al.,* 2015); 3.21± 0.27 nmol/ml in HF bull with 5mM cysteine (Buyukleblebici *et al.,* 2014).

In present research trial the MDA level (nmoles of MDA produced/10^8 spermatozoa/hr) in post thaw semen (Table 4.7) was 1.64± 0.03 which was lower than the findings by Tuncer *et al.,* 2010, Wadood *et al.,* 2015 and Buyukleblebici *et al.,* 2014.

The catalase activity (µmol H_2O_2 decomposed/ min/ 10^8 spermatozoa) in post thaw semen is reported as 842.40± 90.42 kU/L in ram with 5mM semen (Bucak *et al.,* 2008); 373.2± 12.1 kU/L in Angora goat with 5mM cysteine (Atessahin *et al.,* 2008); 15.7± 4.73 µm/ml-10^9 cell/ml in HF bull with 5mM cysteine (Tasdemir *et al.,* 2014); 11.0± 2.6 kU/g in HF bull with 2mM cysteine (Sariozkan *et al.,* 2009a).

In the present experiment the catalase activity (µmol H_2O_2 decomposed/ min/ 10^8 spermatozoa) in post thaw semen (Table 4.8) was 40.52± 0.75 which could not be compared with the finding of other scientist due to disparity in units of measurement.

The SOD activity (Unit/10^8 spermatozoa) in post thaw semen is reported as 21.4± 2.9 U/g in HF bull with 2mM cysteine (Sariozkan *et al.,* 2009a).

In present investigation the SOD activity (Unit/10^8 spermatozoa) in post thaw (Table 4.9) semen was 11.63± 0.12 which could not be compared with the finding of other scientist due to disparity in units of measurement.

The addition of cysteine as an antioxidants in extender leads to improve percent progressive motility, live sperm, acrosomal integrity, reduce sperm abnormality, improves structural and functional integrity of membrane sperm cells and it also reduces the level of lipid peroxidation as compared to control, though the difference was non-significant (Andreea and Stela, 2010) and Holt (1997) also stated that cysteine is one of the additives that have been used in freezing extender of human, boar, goat, bull to improve post-thaw sperm parameters.

5.2.2 Taurine

The per cent value of progressive motility in diluted semen is reported as 85.43± 1.48 in Gir bull with 4mg/ml taurine (Chaturvedi *et al.,* 2020); 81.75± 0.79 and 81.00± 0.82 with 4 and 6 mg/ml taurine, respectively in buffalo bull (Varghese *et al.,* 2015); 75.9± 1.5 in buffalo bull with 50mM taurine (Sakr *et al.,* 2021); 72.75± 1.06 and 78.00±

0.70 in buffalo bull with 20 and 40 ng/ml taurine, respectively (Hegazy *et al.,* 2020), in equilibrated semen the per cent progressive motility was reported as 91.67± 0.88 in buffalo bull with 50mM taurine (El-Seadawy *et al.,* 2021); 73.25± 0.81 and 71.12± 1.02 in buffalo bull with 4 and 6 mg/ml taurine, respectively (Varghese *et al.,* 2015); 59.0± 1.4 in buffalo bull 50mM taurine (Sakr *et al.,* 2021) and in post thaw semen the per cent value of progressive motility was reported as 61.66± 2.02 in buffalo bull with 50mM taurine (Singh *et al.,* 2012); 56.90± 0.19 in buffalo bull with 50mM taurine (Beheshti *et al.,* 2013); 59.33± 5.2 in Buffalo bull and 55.66± 4.25 in Gir bull with 50mM taurine (Kumar *et al.,* 2013); 51.98± 2.87 in buffalo bull and 48.53± 2.75 in Gir bull with 4mg/ml taurine (Chaturvedi *et al.,* 2020); 51± 1.52 in Karan fries with 50mM taurine (Chhillar *et al.,* 2012); 50.50± 0.56 in Sahiwal bull with 20mM taurine (Baber et *al.,* 2016); 50.00± 0.62 in buffalo bull with 4 mg/ml taurine, respectively (Varghese *et al.,* 2015); 43.3± 2.4 in HF bull with 2mM taurine (Sariozkan *et al.,* 2009a).

In the present research trial the per cent progressive motility in diluted semen (Table 4.2) was 70.66± 0.32 which was lower than the finding of 2015, Sakr *et al.,* 2021.

The per cent progressive motility in equilibrated semen (Table 4.2) was 64.16± 0.58 which was lower than the finding of El-Seadawy *et al.,* 2021 and higher than the finding of Sakr *et al.,* 2021.

The per cent progressive motility in post thaw semen (Table 4.2) was 50.7± 1.09 which was in agreement with the finding of Chhillar *et al.,* 2012 (51± 1.52).

The per cent live sperm in diluted semen is reported as 86.17± 0.65 and 84.80± 0.88 in buffalo bull with 4 and 6 mg/ml taurine, respectively (Varghese *et al.,* 2015); 69.75± 1.62 and 76.75± 0.97 in buffalo bull with 20 and 40 ng/ml taurine, respectively (Hegazy *et al.,* 2020), in equilibrated semen the per cent live sperm were reported as 94.00± 1.53 in buffalo bull with 50mM taurine (El-Seadawy *et al.,* 2021); 79.37± 0.88 and 76.32± 1.14 in buffalo bull with 4 and 6 mg/ml taurine, respectively (Varghese *et al.,* 2015); 63.87± 1.63 and 72.12± 1.10 in buffalo bull with 20 and 40 ng/ml taurine, respectively (Hegazy *et al.,* 2020) and in post thaw semen the per cent live sperm is reported as 74.00± 0.82 in buffalo bull with 50mM taurine (El-Seadawy *et al.,* 2021);

73.98± 0.62 in buffalo bull with 50mM taurine (Beheshti *et al.,* 2013); 63.80± 1.50 in Korean jeju black bull with 20mM taurine (Oh *et al.,* 2012); 62.75± 1.23 in Sahiwal bull with 20mM taurine (Baber *et al.,* 2016); 59.00± 1.73 in Karan fries bull with 50mM taurine (Chhillar *et al.*, 2012).

In our present study the per cent live sperm in diluted, equilibrated and post thaw semen (Table 4.3) was 80.27± 0.95, 73.63± 1.03 and 62.61± 0.98 respectively.

The per cent values of acrosomal integrity in diluted semen is reported as 92.23± 0.39 and 90.55± 0.44 in buffalo bull with 4 and 6 mg/ml taurine, respectively (Varghese *et al.,* 2015); 79.4± 1.7 and 78.2± 0.9 in buffalo bull with 50mM and 25mM taurine, respectively (Sakr *et al.,* 2021), in equilibrated semen the per cent acrosomal integrity was reported as 90.67± 1.45 in buffalo bull with 50mM taurine (El-Seadawy *et al.,* 2021); 88.53± 0.46 and 86.28± 0.45 in buffalo bull with 4 and 6 mg/ml taurine, respectively (Varghese *et al.,* 2015); 74.6± 1.8, 72.6± 2.31 and 72.8± 2.1 with 50, 75 and 100mM taurine, respectively (Sakr *et al.,* 2021) and in post thaw semen the per cent acrosomal integrity was reported as 80.75± 0.48 in buffalo bull with 50mM taurine (El-Seadawy *et al.,* 2021); 67.57± 0.33 in buffalo bull with 50mM taurine (Beheshti *et al.,* 2013); 67.3± 2.8, 64.3± 3.1, 63.7± 3.4 and 62.2± 3.8 in buffalo bull with 50, 25, 75 and 100mM taurine, respectively (Sakr *et al.,* 2021); 65.00± 0.79, 61.00± 0.79 and 58.25± 0.73 in Sahiwal bull with 20, 40 and 60mM taurine, respectively (Baber *et al.,* 2016).

In the present investigation the per cent acrosomal integrity in diluted semen (4.4) was 77.18± 0.82, 71.26± 1.15 and 63.86± 0.84, respectively which was in agreement with Sakr *et al.,* 2021 at each stage.

The per cent sperm abnormalities in diluted semen is reported as 4.60± 0.24 and 4.93± 0.20 in buffalo bull with 4 and 6 mg/ml taurine, respectively (Varghese *et al.,* 2015); 23.12± 1.00 and 16.87± 0.71 in buffalo bull with 20 and 40 ng/ml taurine, respectively (Hegazy *et al.,* 2020), in equilibrated semen the per cent sperm abnormalities is reported as 5.00± 1.15 in buffalo bull with 50mM taurine (El-Seadawy *et al.,* 2021); 5.80± 0.28 and 6.38± 0.29 in buffalo bull with 4 and 6 mg/ml taurine, respectively (Varghese *et al.,* 2015); 7.84± 0.74, 5.75± 0.32 and 7.62± 1.23 in Mithun bull with 25, 50

and 100mM taurine, respectively (Perumal *et al.,* 2013); 29.75± 1.19 and 20.87± 0.66 in buffalo bull with 20 and 40 ng/ml taurine, respectively (Hegazy *et al.,* 2020) and in post thaw semen the per cent sperm abnormalities was reported as 7.75± 0.85 in buffalo bull with 50mM taurine (El-Seadawy *et al.,* 2021); 7.90± 0.29 and 8.90± 0.31 in buffalo bull with 4 and 6 mg/ml taurine, respectively (Varghese *et al.,* 2015); 14.0±0.7 in HF bull with 2mM taurine (Sariozkan *et al.,* 2009a); 35.50± 1.34 and 21.37± 0.82 in buffalo bull with 20 and 40 ng/ml taurine, respectively (Hegazy *et al.,* 2020).

In the present investigation the per cent sperm abnormalities in diluted semen (Table 4.5) was 5.67± 0.48 whereas, 7.62± 0.63 at equilibrated stage which was in agreement with the findings of Perumal *et al.,* 2013 (7.84± 0.74 and 7.62± 1.23).

In post thaw semen the sperm abnormalities (Table 4.5) was 11.34± 0.38 which was higher than the findings of El-Seadawy et al., 2021.

The per cent values of HOST positive sperm in diluted semen was reported as 86.25± 0.64 and 85.63± 0.86 in buffalo bull with 4 and 6 mg/ml taurine, respectively (Varghese *et al.,* 2015); 79.4± 1.3 in buffalo bull with 50mM taurine (Sakr *et al.,* 2021), in equilibrated semen the per cent HOST positive sperm was reported as 83.00± 2.08 in buffalo bull with 50mM taurine (El-Seadawy *et al.,* 2021); 78.08± 0.95 and 75.03± 1.08 in buffalo bull with 4 and 6 mg/ml taurine, respectively (Varghese *et al.,* 2015); 68.6± 1.5 and 69.2± 1.5 in buffalo bull with 50 and 25mM taurine, respectively (Sakr *et al.,* 2021) and in post thaw semen the per cent HOST positive sperm was reported as 61.66± 2.03 in buffalo bull and 56.00± 2.08 in Gir cattle with 50mM taurine, respectively (Kumar *et al.,* 2013); 60.50± 6.86 in Korean jeju black bull with 20mM taurine (Oh *et al.,* 2012); 58.00± 0.63 in Sahiwal bull with 20mM taurine (Baber *et al.,* 2016); 54± 1.15 in Karan fries bull with 50mM taurine (Chhillar *et al.,* 2012); 46.6± 1.9 in HF bull with 2mM taurine (Sariozkan *et al.,* 2009a).

In the present research trial the per cent HOST positive sperm in diluted semen (Table 4.6) was 75.28± 1.19 which was lower than the finding of Sakr et al., 2021.

In equilibrated semen the per cent HOST positive sperm (Table 4.6) was 70.25± 1.58. Our finding was higher compared with the finding of Sakr *et al.,* 2021 (68.6± 1.5).

In post thaw semen the per cent HOST positive sperm (Table 4.6) was 59.65± 1.53 which are in accordance with the findings of Kumar *et al.,* 2013 (56.00± 2.08) and Chhillar *et al.,* 2012 (54± 1.15).

The MDA level (nmoles of MDA produce/ 10^8spermatozoa/hr) in post thaw semen is reported as 15.95± 0.74 µmol/ml in Gir bull with 50mM taurine (Chikhaliya *et al.,* 2018); 11.2± 0.9 nmol/ml in ram with 50mM taurine (Bucak *et al.,* 2007); 9.80± 0.51 nmol/ml in Angora goat with 50mM taurine (Atessahin *et al.,* 2008); 1.5± 0.3 nmol/ml in HF bull with 2mM taurine (Sariozkan *et al.,* 2009a); 1.49± 0.14 nmol MDA/ 10^8 cells in Karan fries with 50mM taurine (Chhillar *et al.,* 2012).

In present research experiment the MDA level (nmoles of MDA produce/ 10^8spermatozoa/hr) in post thaw semen (Table 4.7) was 1.62± 0.02 which was in accordance with the finding of Chhillar *et al.,* 2012.

The catalase activity (µmol H_2O_2 decomposed/ min/ 10^8spermatozoa) in post thaw semen is reported as 1037.5± 68.2 kU/L in ram with 50mM taurine (Bucak *et al.,* 2008); 866.7± 129.5 kU/L in Angora goat with 50mM taurine (Atessahin *et al.,* 2008); 35.1± 8.1 kU/g in HF bull with 2mM taurine (Sariozkan *et al.,* 2009a).

In our present experimental trial the catalase activity (µmol H_2O_2 decomposed/ min/ 10^8spermatozoa) in post thaw semen (Table 4.8) was 44.29± 1.59. Our finding could not be compared with the findings of other scientist due to disparity in the units of measurement.

The SOD level (Unit/10^8Spermatozoa) in post thaw semen is reported as 2.1± 0.4 U/g in HF bull with 2mM (Sariozkan *et al.,* 2009a).

In the present study the SOD level (Unit/10^8Spermatozoa) in post thaw semen (Table 4.9) was 11.95± 0.03 which could not be compared with the findings of other scientist due to disparity in the units of measurement.

The taurine is a sulfonic amino acid and it act as a non-enzymatic scavenger that plays a major role in the defence of spermatozoa against ROS in case of exposure to aerobic condition and the freezing-thawing process (Reddy *et al.,* 2010).

5.3 Evaluation of Semen in Group II

5.3.1 Ejaculated volume (ml)

In the present investigation the overall mean ejaculate volume (Table 4.10) was recorded as 5.79± 0.51 ml in bulls which was in agreement reported by Rehman *et al.,* 2016 (5.50± 0.04 ml), Mathevon *et al.,* 1998 (5.48± 1.83 ml), Pal *et al.,* 2020b (5.38± 0.20) and Sannat *et al.,* 2015 (5.3±0.25 ml).

The variation in ejaculated volume reported by various scientists may be due to the differences in age, breed, reproductive health status, frequency of collection, nutrition, seasonal variation and management and additionally due to ability or skill of semen collector or attendant and temperature of AV (Javed *et al.*, 2000).

5.3.2 Mass activity (0-5 scale)

In our present study the mass activity (0-5 scale) in bull (Table 4.10) was 3.64± 0.42 which was in accordance reported by Munsi *et al.,* 2017 (3.8± 0.3), Patel and Siddiquee, 2013 (3.72± 0.02), Pal *et al.,* 2020b (3.69± 0.09 and 3.63± 0.08) and Baloch *et al.,* 2019 (3.6± 0.05). Our finding was lower as reported by the Srivastava and Kumar, 2014.

The variation in mass activity is due to improper handling procedure, contaminated glass wares, chemicals, cold or hot test tubes, glass slides, microscope stage, rapid drying or cooling of glass slides, prolongation during collection and examination period etc and mass activity varies from individual to individual because it is a subjective test (Pal *et al.*, 2020a)

5.3.3 Sperm concentration

Sperm concentration refers to the number of sperm per unit volume (ml) of semen and when it is combined with ejaculated volume, it determine how many female can be inseminated with optimal number of sperm cells (Hafez, 1987).

In the present research trial the sperm concentration (10^6) in bull (Table 4.10) was 877.56± 67.53 x10^6 which was in agreement as reported by Chaudhary *et al.*, 2017

(846.30± 54.82 x10^6). Our finding was lower than reported by Rehman *et al.,* 2016, Munsi *et al.,* 2007, Chauhan *et al.,* 2017 and Srivastava and Kumar, 2014.

The possible reason for variation in sperm concentration may be due to some factors like breed, age, seasonal variation, period of sexual rest, number of false mount, successive ejaculates and collection method etc.

5.3.4 Progressive motility

In the present research trial the per cent progressive motility of neat semen (Table 4.11) was 84.33± 0.36. This finding was in agreement with findings of Khan *et al.,* 2018 (85.12± 0.51 and 84.61± 16), Li *et al.,* 2016 (85.1± 0.3), Kumar *et al.,* 2013 (83.66± 2.27) and Uysal *et al.,* 2007 (83.5± 2.4).

In the present study the per cent progressive motility of diluted semen (Table 4.11) was 68.7± 0.28 and this finding was in agreement with Patel *et al.,* 2015 (69.46± 0.63), Sandeep *et al.,* 2015 (67.70± 1.12) and Singh *et al.,* 2020 (67.0± 1.52).

In present investigation the per cent progressive motility of equilibrated semen (Table 4.11) was 63.12± 0.49 which was in accordance to findings reported by Singh *et al.,* 2017 (64.28± 2.96), Hossain *et al.,* 2012 (63.7), Patel *et al.,* 2015 (63.54± 0.60), Hegazy *et al.,* 2020 (63.00± 0.84) and Shoae and Zamiri, 2008 (62.1± 2.5).

In present research experiment the per cent progressive motility of post thaw semen (Table 4.11) was 48.97± 0.56 which was in agreement with the findings of Mughal *et al.,* 2013 (49.7± 2.5), Sariozkan *et al.,* 2009a (49.6±1.1), Singh *et al.,* 2017 (49.53± 2.00), Patel *et al.,* 2015 (49.17± 0.57), Chaturvedi *et al.,* 2020 (48.76± 1.69), Wafa *et al.,* 2021 (48.33±1.06) and Singh *et al.,* 2020 (48.0± 2.49).

The decrease in sperm motility is observed in disease condition, change of environment/season and temperature variations, and it swings between breeds, individuals, age groups and the evaluation technique employed.

The variation in sperm motility in post thaw semen occur due to cryopreservation which causes the formation of ice crystal in mitochondria and axoneme or the

biochemical oxidative stress resulting in irreversible damage to sperm structure, changes in membrane fluidity and enzymatic activity (Alvarez and Storey, 1983).

5.3.5 Live Sperm

In present study the per cent live sperm in neat semen (Table 4.12) was 89.03± 0.88 and this finding was in agreement reported by Patel and Siddiquee, 2013 (90.58± 0.20), Shaikh *et al.,* 2016 (89.97± 0.21), Mishra *et al.,* 2013 (89.53± 0.59, 88.73± 0.59 and 88.70± 0.59), Lone *et al.,* 2018 (89.45± 4.70) and Kadirvel *et al.,* 2014 (88.43± 0.70).

In our present study the per cent live sperm in diluted semen (Table 4.12) was 76.84±1.23 which was lower than the findings of Singh *et al.,* 2017, Patel *et al.,* 2015 and Singh and Sharma, 2018 whereas higher than the findings of Singh *et al.,* 2020, Abdel-Khalek *et al.,* 2008 and Khumran *et al.,* 2017.

In the present experiment the per cent live sperm in equilibrated semen (Table 4.12) was 70.16± 0.82 which was in agreement with the findings of Shoae and Zamiri, 2008 (70.5± 1.1) and Chaudhary *et al.,* 2017 (69.92± 1.74).

In the present research trial the per cent live sperm in post thaw semen (Table 4.12) was 57.88± 1.34 which was in accordance to the findings of Oh *et al.,* 2012 (58.25± 6.03), Patel and Siddiquee, 2013 (58.22± 0.24), Baber *et al.,* 2016 (58.00± 1.21) and Rao *et al.,* 2017 (56.24± 0.01).

The variation in percentage of stained spermatozoa is dependent on various factors like duration, type of diluents used, stain integrity and technique employed by various scientists.

The reasons for varying live sperm count during cryopreservation may be due to bio-physical damage to the spermatozoa due to formation of ice-crystals in the extracellular and intracellular surroundings and expanding solute concentration that results in the production of ROS (Mazur, 1984) and sperm susceptible for freezing and thawing temperature, ROS production and lipid peroxidation (Aitken *et al*., 1989).

5.3.6 Acrosomal integrity

In present experimental trial the per cent acrosomal integrity of neat semen (Table 4.13) was 83.82± 1.25. This finding was in agreement with the findings of Rana and Dhami, 2004 (84.80± 0.89 and 83.50± 1.24) and Mishra *et al.,* 2013 (82.20± 0.47).

In present research trial the per cent acrosomal integrity in diluted semen (Table 4.13) was 72.05± 1.51 and this finding was in agreement reported by Rasul *et al.,* 2001 (73.2± 2.4).

In our present study the per cent acrosomal integrity in equilibrated semen (Table 4.13) was 66.35±1.23 which was lower than the findings of Chaudhary *et al.,* 2017 (90.50± 0.45), Shoae and Zamiri, 2008 (70.0± 1.1), Patel and Siddiquee, 2013 (68.16± 0.80) whereas, higher than Kumar *et al.,* 2019 (63.14± 4.03).

In the present investigation the per cent acrosomal integrity in post thaw semen (Table 4.13) was 59.53± 0.87 which was in agreement with the finding of Kumar *et al.,* 2018a (60.0± 2.6). Our finding was higher than the findings of Rao *et al.,* 2017 (56.24± 0.01), Patel and Siddiquee, 2013 (53.65± 0.58), Hu *et al.,* 2010a (52.3± 1.58), Khan *et al.,* 2021a (40.83± 1.56) and Khan *et al.,* 2018 (35.83± 0.56) but lower than the findings of Srivastava and Kumar, 2014 (70.5± 0.08).

These variations reported in acrosomal integrity could be due to inherent quality of particular breed/bull, age, physio-pathological status, stain, staining technique & duration of staining used etc. by different workers.

The variation or decrease in results of acrosomal integrity occurs in post thaw semen is because of cryopreservation, as it induces various degrees of acrosomal damage i.e. primary, secondary or tertiary damage which may be due to the loss of plasma membrane and loss of plasmalemma over entire acrosome.

5.3.7 Sperm abnormalities

In the present study the per cent sperm abnormalities in neat semen (Table 4.14) was 3.73± 0.41 which was in agreement with the findings of Shaikh *et al.,* 2016 (3.08± 0.15) and (Pal *et al.,* 2020b (3.75± 0.45).

In the present research experiment the per cent sperm abnormalities in diluted semen (Table 4.14) was 6.62± 0.41. This finding was in agreement with the findings reported by Patel and Siddiquee, 2013 (6.13± 0.07) and Al-Badrany *et al.,* 2020 (6.99± 0.55).

In our present study the per cent sperm abnormalities in equilibrated semen (Table 4.14) was 9.36± 0.59 which was in agreement with the finding of Al-Badrany *et al.,* 2020 (9.55± 0.47).

In present investigation the per cent sperm abnormalities in post thaw semen (Table 4.14) was 13.42± 0.64 and this was in accordance to the findings of Patel and Siddiquee, 2013 (13.52± 0.35) and Al-Badrany *et al.,* 2020 (14.67± 0.45).

The semen from most males contains some abnormally formed spermatozoa which are not associated with lower fertility rates until the proportion of abnormal sperm exceeds 20 percent (Hafez, 1987). The variation in sperm abnormality may be due to method of collection, temperature shock and technique employed etc.

5.3.8 Hypo-osmotic swelling test (HOST)

In the present experiment the per cent HOST positive sperm in neat semen (Table 4.15) was 81.94± 0.95 and this finding was in agreement with the findings of Khan *et al.,* 2018 (82.25± 0.43 and 81.66± 0.48), Mishra *et al.,* 2017 (81.16± 0.51) and Dhami *et al.,* 2017 (80.30± 1.90).

In the present research trial the per cent HOST positive sperm in diluted semen (Table 4.15) was 73.14± 1.23. Our finding was in normal range as reported by various scientists and was higher than the findings of Singh *et al.,* 2020 (71.0± 1.10) and Singh and Sharma, 2018 (67.47± 0.05) but lower than the findings of Rasul *et al.,* 2001 (80.2± 3.9), Patel *et al.,* 2015 (78.11± 0.58) and Singh *et al.,* 2017 (77.67± 1.81).

In present investigation the per cent HOST positive sperm in equilibrated semen (Table 4.15) was 68.36± 1.39 which was in agreement with the findings of Singh *et al.,* 2017 (69.92± 2.30) and Khan *et al.,* 2021b (69.20± 0.31).

In present study the per cent HOS positive sperm in post thaw semen (Table 4.15) was 58.75± 0.59 which was in accordance with the findings of Ratnani *et al.,* 2017 (58.50± 1.87) and Patel *et al.,* 2015 (57.34± 0.74).

The variation in HOST may be due to spontaneous lipid peroxidation of the membranes of mammalian sperm destroy the structure of the lipid matrix, as a result of the invasion by reactive oxygen species (ROS). These attacks then ultimately lead to the impairment of sperm function (sperm motility, functional membrane integrity and fertility), and damage to the sperm DNA, through oxidative stress and the production of cytotoxic aldehydes (Aitken *et al.,* 1993).

5.3.9 Lipid peroxidation

In present research experiment the MDA level (nmoles of MDA produced/10^8 spermatozoa/hr) in post thaw semen (Table 4.16) was 1.69± 0.03 which was in agreement with finding reported by Tuncer *et al.,* 2010 (1.44± 0.08). Our finding was lower than the findings of Buyukleblebici *et al.,* 2014 (2.01± 0.26), Chhillar *et al.,* 2012 (2.67± 0.35). Singh *et al.,* 2020 (3.49± 0.19) and Motemani *et al.,* 2017 (7.2± 0.6) whereas, higher than the findings reported by Sariozkan *et al.,* 2009a (0.7± 0.2) and Al-Dahan *et al.,* 2020 (1.02± 0.38).

MDA is one of the final products of polyunsaturated fatty acids peroxidation in the sperm cells. The osmotic stress during the sperm dilution, cooling, cryoprotectant exposure, freezing, and thawing processes resulted in oxidative stress that induced high concentrations of MDA (Ansari *et al.,* 2019). The production of ROS in sperm is a natural process; however, overproduction has a deleterious impact on cellular membranes, resulting in dysfunction of cell organelles leading to death (Partyka *et al.,* 2012). Sperm cells are known to have high levels of polyunsaturated fatty acids in the plasma membrane that makes sperm highly susceptible to LPO (Parks and Hammerstedt, 1985).

5.3.10 Catalase

In the present investigation the catalase activity (µmol H_2O_2 decomposed/ min/ 10^8 spermatozoa) in post thaw semen (Table 4.17) was 31.08± 2.21. Our finding was

higher than findings reported by Sharma *et al.,* 2016 (24.25± 0.30) and Singh *et al.,* 2020 (1.18± 0.36).

Catalase is one of the enzymes present in all living organisms which is considered as the major defender of the sperm membranes against ROS and LPO (Bilodeau *et al.,* 2001). In living organism it is presented to oxygen, which disintegrates destructive peroxides and convert them into water and oxygen. The decrease in the catalase activity may be due to over consumption of catalase enzyme to decompose harmful peroxide and free radicals (Chelikani *et al.,* 2004).

5.3.11 Superoxide dismutase

In present research experiment the superoxide dismutase activity (Unit/10^8 spermatozoa) in post thaw semen (Table 4.18) was 7.20± 0.23.

Superoxide ion is free radical which is change over to oxygen and hydrogen peroxide by the dismutation activity of antioxidant enzyme superoxide dismutase (Tariq *et al.,* 2015). The decrease in superoxide activity may be due to neutralization of superoxide during cryopreservation (Bilodeau *et al.,* 2000).

5.4 Effect of Antioxidant Additives in Group II

5.4.1 Ascorbic acid

The per cent values of progressive motility in diluted semen is reported as 74.14± 0.39 in Jersey bull with 0.02% ascorbic acid (Singh and Sharma, 2018); 72.31± 0.25 in Murrah buffalo with 0.5mg/ml ascorbic acid (Saurabh *et al.,* 2021); 70.28± 0.97 in Bhadawari bull with 5mM ascorbic acid (Mittal *et al.,* 2014); 68.0± 1.33 in crossbred bull with 5mM ascorbic acid (Singh *et al.,* 2020), in equilibrated semen the per cent progressive motility was reported as 78.05± 0.02 in cattle bull with 5mM ascorbic acid (Rao *et al.,* 2013); 73.33± 1.07 in buffalo bull with 2.5mM ascorbic acid (Sandeep *et al.,* 2015); 71.61± 0.52 in Jersey bull with 0.02% ascorbic acid (Singh and Sharma, 2018); 64.88± 0.92 in Bhadawari bull with 5mM ascorbic acid (Mittal *et al.,* 2014) and in post thaw semen the per cent progressive motility was reported as 59.72± 0.75 in Bhadawari bull with 5mM ascorbic acid (Mittal *et al.,* 2014); 58.77± 0.40 in Murrah bull with

0.5mg/ml ascorbic acid (Saurabh *et al.,* 2021); 57.83± 0.52 in buffalo bull with 0.2mg/ml ascorbic acid (Patel *et al.,* 2016); 51.28± 0.90 in Jersey bull with 0.02% ascorbic acid (Singh and Sharma, 2018); 50.0± 2.11 in crossbred bull with 5mM ascorbic acid (Singh *et al.,* 2020); 46.88± 0.01 in cattle bull with 5mM ascorbic acid (Rao *et al.,* 2013); 43.33± 7.90 in Nellore bull with 2.5mM ascorbic acid (Pinto *et al.,* 2020); 36.7± 3.3 in Zebu cattle with 2.5mM ascorbic acid (Batool *et al.,* 2012).

In present investigation the per cent progressive motility in diluted semen (Table 4.11) was 70.39± 0.31 which is in agreement with the finding of in Mittal *et al.,* 2014 (70.28± 0.97), the per cent progressive motility in equilibrated semen was 64.51± 0.63 which was similar to the finding of Mittal *et al.,* 2014 (64.88± 0.92) and the per cent progressive motility in post thaw semen was 51.47± 0.77 which was in accordance with the findings of Singh and Sharma, 2018 (51.28± 0.90) and Singh *et al.,* 2020 (50.0± 2.11).

The per cent values of live sperm in diluted semen is reported as 83.79± 0.21 in Murrah buffalo with 0.5mg/ml ascorbic acid (Saurabh *et al.,* 2021); 83.19± 0.42 in Bhadawari bull with 5mM ascorbic acid (Mittal *et al.,* 2014); 74.31± 0.41 in Jersey bull with 0.02% ascorbic acid (Singh and Sharma, 2018); 72.4± 1.69 in crossbred bull with 5mM ascorbic acid (Singh *et al.,* 2020), in equilibrated semen the per cent live sperm was reported as 78.28± 0.48 in Bhadawari bull with 5mM ascorbic acid (Mittal *et al.,* 2014); 76.21± 1.01 in buffalo bull with 2.5mM ascorbic acid (Sandeep *et al.,* 2015); 71.06± 0.65 in Jersey bull with 0.02% ascorbic acid (Singh and Sharma, 2018) and in post thaw semen the per cent live sperm was reported as 81.73± 0.22 in buffalo bull with 0.2mg/ml ascorbic acid (Patel *et al.,* 2016); 72.97± 0.41 in Bhadawari bull with 5mM ascorbic acid (Mittal *et al.,* 2014); 71.27± 0.49 in Murrah buffalo with 0.5mg/ml (Saurabh *et al.,* 2021); 62.73± 2.80 in Sahiwal bull with 4mg/ml ascorbic acid (Sohail *et al.,* 2015); 56.7± 1.5 in Zebu cattle with 2.5mM ascorbic acid (Batool *et al.,* 2012); 55.4± 0.88 in bull with 5mM ascorbic acid (Singh *et al.,* 2020); 47.58± 1.08 in Jersey bull with 0.02 ascorbic acid (Singh and Sharma, 2018).

In present research trial the per cent live sperm in diluted semen (Table 4.12) was 78.46± 1.12 which was lower than the findings of Saurabh *et al.,* 2021 (83.79± 0.21)

whereas, higher than by Singh and Sharma, 2018 (74.31± 0.41) and Singh *et al.,* 2020 (72.4± 1.69), the per cent live sperm in equilibrated semen was 72.35± 1.08 which was in agreement with finding of Singh and Sharma, 2018 (71.06± 0.65) and the per cent live sperm in post thaw semen was 62.57± 0.95 which was in agreement with the finding of Sohail *et al.,* 2015 (62.73± 2.80).

The per cent acrosomal integrity values in diluted semen is reported as 90.31± 0.20 in Murrah buffalo with 0.5mg/ml (Saurabh *et al.,* 2021); 84.50± 0.41 in Bhadawari bull with 5mM ascorbic acid (Mittal *et al.,* 2014); 84.47± 0.86 in Jersey bull with 0.02% ascorbic acid (Singh and Sharma, 2018); 78.7± 1.78 in crossbred bull with 5mM ascorbic acid (Singh *et al.,* 2020), in equilibrated semen the per cent acrosomal integrity was reported as 84.47± 0.86 in Jersey bull with 0.02% ascorbic acid (Singh and Sharma, 2018); 79.22± 0.51 in Bhadawari bull with 5mM ascorbic acid (Mittal *et al.,* 2014); 76.50± 2.13 in buffalo bull with 2.5mM ascorbic acid (Sandeep *et al.,* 2015) and in post thaw semen the per cent acrosomal integrity was reported as 81.42± 0.44 in Murrah bull with 0.5mg/ml ascorbic acid (Saurabh *et al.,* 2021); 74.00± 0.49 in Bhadawari bull with 5mM ascorbic acid (Mittal *et al.,* 2014); 69.97± 0.56 in Jersey bull with 0.02% ascorbic acid (Singh and Sharma, 2018); 64.3± 2.42 in crossbred bull with 5mM ascorbic acid (Singh *et al.,* 2020); 50.68± 0.81 in buffalo bull with 2.5mM ascorbic acid (Sandeep *et al.,* 2015); 41.44± 16.82 in Nellore bull with 2.5mM ascorbic acid (Pinto *et al.,* 2020).

In our present study the per cent acrosomal integrity in diluted semen (Table 4.13) was 73.54± 1.49 was lower than the findings of Saurabh *et al.,* 2021 (90.31± 0.20), Mittal *et al.,* 2014 (84.50± 0.41), Singh and Sharma, 2018 (84.47± 0.86) and Singh *et al.,* 2020 (78.7± 1.78), the per cent acrosomal integrity in equilibrated semen was 68.58± 0.96 which was lower than the findings of Singh and Sharma, 2018 (84.47± 0.86), Mittal *et al.,* 2014 (79.22± 0.51) and Sandeep *et al.,* 2015 (76.50± 2.13) and the per cent acrosomal integrity in post thaw semen was 62.64± 0.93 which was lower than the findings of Saurabh *et al.,* 2021 (81.42± 0.44), Mittal *et al.,* 2014 (74.00± 0.49), Singh and Sharma, 2018 (69.97± 0.56) and Singh *et al.,* 2020 (64.3± 2.42) whereas, higher than the findings of Sandeep *et al.,* 2015 (50.68± 0.81) and Pinto *et al.,* 2020 (41.44± 16.82).

The per cent sperm abnormalities in diluted semen was reported as 4.06± 0.09 in Bhadawari bull with 5mM ascorbic acid (Mittal *et al.,* 2014); 6.8± 0.83 in crossbred bull with 5mM (Singh *et al.,* 2020); 8.04± 0.10 in Murrah bull with 0.5mg/ml ascorbic acid (Saurabh *et al.,* 2021), in equilibrated semen the per cent sperm abnormalities is reported as 3.97± 0.11 in Bhadawari bull with 5mM ascorbic acid (Mittal *et al.,* 2014) and in post thaw semen the per cent sperm abnormalities was reported as 5.23± 0.18 in buffalo bull with 0.2mg/ml ascorbic acid (Patel *et al.,* 2016); 6.22± 0.11 in Bhadawari bull with 5mM ascorbic acid (Mittal *et al.,* 2014); 6.5± 0.63 in Nellore bull with 2.5mM ascorbic acid (Pinto *et al.,* 2020); 12.83± 0.22 in Murrah bull with 0.5mg/ml ascorbic acid (Saurabh *et al.,* 2021); 13.2± 0.79 in crossbred bull with 5mM ascorbic acid (Singh *et al.,* 2020).

In the proposed study the per cent sperm abnormalities in diluted semen (Table 4.14) was 5.99± 0.34. Our finding was in agreement with the findings reported by Mittal *et al.,* 2014 (4.06± 0.09) and Singh *et al.,* 2020 (6.8± 0.83), the per cent sperm abnormalities in equilibrated semen was 8.85± 0.45 which was higher than the finding of Mittal *et al.,* 2014 and the per cent sperm abnormalities in post thaw semen was 12.25± 0.50 which was in accordance with the finding of Saurabh *et al.,* 2021 (12.83± 0.22).

The per cent HOST positive sperm in diluted semen was reported by various scientists are as 71.5± 1.69 in crossbred bull with 5mM ascorbic acid (Singh *et al.,* 2020); 69.17± 0.92 in Jersey bull with 0.02% ascorbic acid (Singh and Sharma, 2018); 52.63± 0.24 in Murrah bull with 0.5mg/ml ascorbic acid (Saurabh *et al.,* 2021), in equilibrated semen the per cent HOST positive sperm was reported as 64.58± 0.98 in Jersey bull with 0.02% ascorbic acid (Singh and Sharma, 2018) and in post thaw semen the per cent HOST positive sperm is reported as 60.10± 3.35 in Sahiwal bull with 3.0mg/ml ascorbic acid (Sohail *et al.,* 2015); 49.4± 0.87 in crossbred bull with 5mM ascorbic acid (Singh *et al.,* 2020); 45.56± 0.99 in Jersey bull with 0.02% ascorbic acid (Singh and Sharma, 2018); 40.46± 0.39 in Murrah bull with 0.5mg/ml ascorbic acid (Saurabh *et al.,* 2021).

In present investigation the per cent the HOST positive in diluted semen (Table 4.15) was 74.97± 1.02 which was higher than the findings of Singh *et al.,* 2020 (71.5± 1.69), Singh and Sharma, 2018 (69.17± 0.92) and Saurabh *et al.,* 2021 (52.63± 0.24), the per cent the HOST positive in equilibrated semen was 70.69± 1.45 which was higher than

the findings reported by Singh and Sharma, 2018 (64.58± 0.98) and the per cent the HOST positive in post thaw semen was 61.67± 0.52 which was comparable with the finding of Sohail *et al.,* 2015 (60.10± 3.35).

The MDA level (nmoles of MDA produced/10^8 spermatozoa/hr) in post thaw semen is reported as 1.70± 0.04 nmol/ 10^8spermatozoa in crossbred bull with 5mM ascorbic acid (Singh *et al.,* 2020); 521.16± 8.23 ng/120 million sperm in buffalo bull with 2.5mM ascorbic acid (Sandeep *et al.,* 2015); 5.47± 0.33 nmol/10^9 in HF bull with 1mM ascorbic acid (Asadpour *et al.,* 2011); 712.1± 49.1 nmol/10^9 sperms in Swedish bull with 10mM ascorbic acid (Paudel *et al.,* 2010).

In the present research trial the MDA level (nmoles of MDA produced/10^8 spermatozoa/hr) in post thaw semen (Table 4.16) was 1.43± 0.03 which was in agreement with findings of Singh *et al.,* 2020 (1.70± 0.04) whereas, lower than the findings of Asadpour *et al.,* 2011 (5.47± 0.33) and Paudel *et al.,* 2010 (712.1± 49.1).

The catalase activity (µmol H_2O_2 decomposed/min/10^8 spermatozoa) in post thaw semen is reported as 3.87± 0.36 U/ml in bovine bull with 4.5mg/ml ascorbic acid (Hu *et al.,* 2010a); 1.31± 0.73 µmol/10^8 spermatozoa in crossbred bull with 5mM ascorbic acid (Singh *et al.,* 2020).

In present experiment the catalase activity (µmol H_2O_2 decomposed/min/10^8 spermatozoa) in post thaw semen (Table 4.17) was 38.67± 1.36 which higher than the finding of Singh *et al.,* 2020 (1.31± 0.73).

The SOD level (Unit/ 10^8 spermatozoa) in post thaw semen is reported as 1.36± 0.13 U/ml in bovine bull with 2.5mg/ml (Hu *et al.,* 2010a); 1.31± 0.05 µmol H_2O_2 decomposed/min/ 10^8 spermatozoa in crossbred bull with 5mM ascorbic acid (Singh *et al.,* 2020).

In present investigation the SOD level (Unit/ 10^8 spermatozoa) in post thaw semen (Table 4.18) was 8.23± 0.16 which was higher than the findings of Singh *et al.,* 2020 (1.31± 0.05).

The addition of ascorbic acid in dilutor will overcome the harmful effects of the oxidant factors that may be due to inhibition of lipid peroxidation damage by the effect of this antioxidant (Azawi and Hussein, 2013). Lampiao *et al*. (2012) stated that it reacts with OH, O_2 and H_2O_2 in the extracellular fluid, thus protecting sperm viability and motility.

5.4.2 Vitamin E

The per cent progressive motility in diluted semen is reported as 87.38± 1.00 in Hariana bull with 2.5mM vitamin E (Pal *et al.,* 2020a); 66.59± 0.99 in Bhadawari bull with 5mM vitamin E (Mittal *et al.,* 2014), in equilibrated semen the per cent progressive motility was reported as 83.13± 0.74 in Hariana bull with 2.5mM vitamin E (Pal *et al.,* 2020a); 81.09± 0.01 in bull with 1mg/ml vitamin E (Rao *et al.,* 2013); 79.5± 1.5 in Bali bull with 0.4g/100ml (Lukman *et al.,* 2014); 61.13± 0.94 in Bhadawari bull with 5mM vitamin E (Mittal *et al.,* 2014) and in post thaw semen the per cent progressive motility is reported as 77.63± 0.18 in Hariana bull with 2.5mM vitamin E (Pal *et al.,* 2020a); 75.9± 1.67 in HF bull with 4.8mM vitamin E (Motemani *et al.,* 2017); 56.75± 0.75 in Bhadawari bull with 5mM vitamin E (Mittal *et al.,* 2014); 51.90± 1.60 in Sahiwal x HF cross with 1.2mM vitamin E (Khan *et al.,* 2021a); 50.69± 0.02 in cattle bull with 1mg/ml vitamin E (Rao *et al,.* 2013); 50± 5 in HF bull with 0.1mM vitamin E (Asadpour *et al.,* 2011); 40.91 in Simmental bull with 0.4g/100ml vitamin E (Haris *et al.,* 2020); 40.0± 0.00 in Zebu cattle with 2.5mM vitamin E (Batool *et al.,* 2012).

In present research trial the per cent progressive motility in diluted semen (Table 4.11) was 69.68± 0.23. Our finding was lower than the finding of Pal *et al.,* 2020a (87.38± 1.00) whereas, higher than Mittal *et al.,* 2014 (66.59± 0.99), the per cent progressive motility in equilibrated semen 64.27± 0.50 which was lower than the findings of Pal *et al.,* 2020a (83.13± 0.74), Rao *et al.,* 2013 (81.09± 0.01), Lukman *et al.,* 2014 (79.5± 1.5) whereas, higher than Mittal *et al.,* 2014 (61.13± 0.94) and the per cent progressive motility in post thaw semen was 51.13± 0.77 which was similar to the findings of Khan *et al.,* 2021a (51.90± 1.60), Rao *et al,.* 2013 (50.69± 0.02) and Asadpour *et al.,* 2011 (50± 5).

The per cent live sperm in diluted semen is reported as 87.38± 1.00 in Hariana bull with 2.5mM vitamin E (Pal *et al.,* 2020a); 82.06± 0.38 in Bhadawari bull with 5mM vitamin E (Mittal *et al.,* 2014), in equilibrated semen the per cent live sperm was reported as 94.4± 2.35 in Bali bull with 0.4g/100ml vitamin E (Lukman *et al.,* 2014); 83.13± 0.74 in Hariana bull with 2.5mM vitamin E (Pal *et al.,* 2020a); 76.75± 0.46 in Bhadawari bull with 5mM vitamin E (Mittal *et al.,* 2014) and in post thaw semen the per cent live sperm was reported as 78.2± 1.8 in HF bull with 4.8mM vitamin E (Motemani *et al.,* 2017); 77.63± 0.18 in Hariana bull with 2.5mM vitamin E (Pal *et al.,* 2020a); 71.25± 0.46 in Bhadawari bull with 5mM vitamin E (Mittal *et al.,* 2014); 62.66± 1.60 in Sahiwal x HF cross with 4.8mM vitamin E (Khan *et al.,* 2021a); 61.08± 1.01 in crossbred bull with 0.3mg/ml vitamin E (Muzafer *et al.,* 2012); 59.3± 1.5 in Zebu cattle with 2.5mM vitamin E (Batool *et al.,* 2012).

In our present study the per cent live sperm in diluted semen (Table 4.12) was 77.97± 1.24 which was lower than the findings of Pal *et al.,* 2020a (87.38± 1.00) and Mittal *et al.,* 2014 (82.06± 0.38), the per cent live sperm in equilibrated semen was 72.81± 0.94 which was lower compare to findings of Lukman *et al.,* 2014 (94.4± 2.35), Pal *et al.,* 2020a (83.13± 0.74) and Mittal *et al.,* 2014 (76.75± 0.46) and the per cent live sperm in post thaw semen was 62.12± 0.82 which was in accordance to the findings reported by Khan *et al.,* 2021a (62.66± 1.60) and Muzafer *et al.,* 2012 (61.08± 1.01).

The per cent acrosomal integrity in diluted semen was reported as 84.75± 0.79 in Hariana bull with 2.5mM vitamin E (Pal *et al.,* 2020a); 81.75± 0.47 in Bhadawari bull with 5mM vitamin E (Mittal *et al.,* 2014), in equilibrated semen the per cent acrosomal integrity was reported as 79.75± 1.03 in Hariana bull with 2.5mM vitamin E (Pal *et al.,* 2020a); 76.91± 0.51 in Bhadawari bull with 5mM vitamin E (Mittal *et al.,* 2014) and in post thaw semen the per cent acrosomal integrity reported by various researcher as 84.72± 0.48 in crossbred bull with 0.3mg/ml vitamin E (Muzafer *et al.,* 2012); 74.75± 0.50 in Hariana bull with 2.5mM vitamin E (Pal *et al.,* 2020a); 71.59± 0.48 in Bhadawari bull with 5mM vitamin E (Mittal *et al.,* 2014); 62.3± 2.0 and 62.3± 1.7 in buffalo bull with 1.0mg/ml and 1.5mg/ml vitamin E, respectively (Kumar *et al.,* 2018a); 52.00± 2.15 in Sahiwal x HF cross with 4.8mM vitamin E (Khan *et al.,* 2021a).

In present investigation the per cent acrosomal integrity in diluted semen (Table 4.13) was 73.88± 1.52 which was lower than the findings of Pal *et al.,* 2020a (84.75± 0.79) and Mittal *et al.,* 2014 (81.75± 0.47), the per cent acrosomal integrity in equilibrated semen was 68.97± 1.05. Our finding was lower than the findings of Pal *et al.,* 2020a (79.75± 1.03) and Mittal *et al.,* 2014 (76.91± 0.51) and the per cent acrosomal integrity in post thaw semen was 62.38± 1.00 which was similar to the findings of Kumar *et al.,* 2018a (62.3± 2.0 and 62.3± 1.7) but lower than the findings of Muzafer *et al.,* 2012 (84.72± 0.48), Pal *et al.,* 2020a (74.75± 0.50) and Mittal *et al.,* 2014 (71.59± 0.48).

The per cent sperm abnormalities in diluted semen is reported as 4.28± 0.10 in Bhadawari bull with 5mM vitamin E (Mittal *et al.,* 2014), in equilibrated semen the per cent sperm abnormalities was reported as 4.44± 0.16 in Bhadawari bull with 5mM vitamin E (Mittal *et al.,* 2014); 13.16± 0.64 in HF bull with 0.8mM vitamin E (Dheerib *et al.,* 2020) and in post thaw semen the per cent sperm abnormalities was reported as 4.75± 0.75 in HF bull with 2mM vitamin E (Hassan and Eidan, 2021); 6.84± 0.12 in Bhadawari bull with 5mM vitamin E (Mittal *et al.,* 2014); 9.59 in Simmental bull with 0.4g/100ml vitamin E (Haris *et al.,* 2020); 15.14± 0.46 in crossbred bull with 0.3mg/ml vitamin E (Muzafer *et al.,* 2012); 15.57± 0.72 in HF bull with 0.8mM vitamin E (Dheerib *et al.,* 2020).

In our present study the per cent sperm abnormalities in diluted semen (Table 4.14) was 6.34± 0.34 which was higher than the finding of Mittal *et al.,* 2014 (4.28± 0.10), the per cent sperm abnormalities in equilibrated semen was 8.85± 0.59 which was higher than the finding of Mittal *et al.,* 2014 (4.44± 0.16) whereas lower than Dheerib *et al.,* 2020 (13.16± 0.64) and the per cent sperm abnormalities in post thaw semen was 12.39± 0.42 which was higher than the finding of Hassan and Eidan, 2021 (4.75± 0.75), Mittal *et al.,* 2014 (6.84± 0.12) and Haris *et al.,* 2020 (9.59) whereas lower than Muzafer *et al.,* 2012 (15.14± 0.46) and Dheerib *et al.,* 2020 (15.57± 0.72).

The per cent value of HOST positive sperm in diluted semen is reported as 84.25± 0.98 in Hariana bull with 2.5mM vitamin E (Pal *et al.,* 2020a), in equilibrated semen the per cent HOST positive sperm was reported as 78.50± 0.79 in Hariana bull with 2.5mM vitamin E (Pal *et al.,* 2020a) and in post thaw semen the per cent HOST positive sperm

was reported by various scientists are 74.25± 0.49 in Hariana bull with 2.5mM vitamin E (Pal *et al.,* 2020a); 61.7± 1.6 in HF bull with 1.2mM vitamin E (Motemani *et al.,* 2017); 53.12± 1.77 in Sahiwal x HF cross with 4.8mM vitamin E (Khan *et al.,* 2021a); 48.12± 0.97 in crossbred bull with 0.3mg/ml vitamin E (Muzafer *et al.,* 2012); 32.0± 2.2 in buffalo bull with 2mg/ml vitamin E (Kumar *et al.,* 2018a).

In the present research trial the per cent HOST positive sperm in diluted semen (Table 4.15) was 74.55± 1.13 which was lower than the finding of Pal *et al.,* 2020a (84.25± 0.98), the per cent HOST positive sperm in equilibrated semen was 70.45± 1.47. Our finding was lower as compare to Pal *et al.,* 2020a (78.50± 0.79) and the per cent HOST positive sperm in post thaw semen was 60.27± 0.62 which was in accordance with the finding of Motemani *et al.,* 2017 (61.7± 1.6) whereas, lower than the finding of Pal *et al.,* 2020a (74.25± 0.49).

The MDA level (nmoles of MDA produced/10^8 spermatozoa/hr) of post thaw semen is reported by various researchers as 6.1± 0.6 nmol/ml in HF bull with 4.8mM vitamin E (Motemani *et al.*, 2017); 5.45± 1.42 nmol/ 10^9 cell in HF bull with 0.2mM vitamin E (Asadpour *et al.*, 2011); 324.4± 29.1 IU/ 10^9 in buffalo bull with 1mg/ml vitamin E (Kumar *et al.,* 2018a); 0.12± 0.01 µmol/ml in crossbred bull with 0.3mg/ml vitamin E (Muzafer *et al.*, 2012).

In the present research trial the MDA level (nmoles of MDA produced/10^8 spermatozoa/hr) in post thaw semen (Table 4.16) was 1.58± 0.03. Our finding was lower than the findings of Motemani *et al.,* 2017 (6.1± 0.6) and Asadpour *et al.,* 2011 (5.45± 1.42).

The catalase activity (µmol H_2O_2 decomposed/ min/ 10^8 spermatozoa) in post thaw semen is reported as 6.08± 1.07, 5.97± 0.86, 4.26± 0.71 and 4.03± 0.59 U/ml in cattle bull with 1.5, 1, 0.5, 2mg/ml vitamin E, respectively by (Hu *et al.,* 2011).

In our present the catalase activity (µmol H_2O_2 decomposed/ min/ 10^8 spermatozoa) in post thaw semen (Table 4.17) was 35.65± 2.24 which cannot be compared due to disparity in the measurement units.

The superoxide dismutase (Unit/10^8 spermatozoa) in post thaw semen is reported as 1.84± 0.19 U/ml in cattle bull with 1.5mg/ml vitamin E (Hu *et al.*, 2011); 0.12± 0.00 U/mg in black Bengal buck with 1mg/ml (Tudu *et al.*, 2021).

In the present experimental trial the superoxide dismutase activity (Unit/10^8 spermatozoa) in post thaw semen (Table 4.18) was 7.75± 0.18 which cannot be compared due to disparity in the measurement units.

The addition of various degree of vitamin E to semen extender prior to freezing causes huge improvement in sperm quality parameters such as motility and viability of sperms than control group (Beheshti *et al.*, 2011a).

5.5 Evaluation of Semen in Group III

5.5.1 Ejaculated volume (ml)

In the present investigation the overall mean ejaculate volume (Table 4.19) was recorded as 5.02± 0.44 ml in bulls which was in agreement reported by Rehman *et al.*, 2016 (5.50± 0.04 ml), Mathevon *et al.*, 1998 (5.48± 1.83 ml), Pal *et al.*, 2020b (5.38± 0.20) and Sannat *et al.*, 2015 (5.3±0.25 ml).

The variation in ejaculated volume reported by various scientists may be due to the differences in age, breed, reproductive health status, frequency of collection, nutrition, seasonal variation and management and additionally due to ability or skill of semen collector or attendant and temperature of AV (Javed *et al.*, 2000).

5.5.2 Mass activity (0-5 scale)

In our present study the mass activity (0-5 scale) in bull (Table 4.19) was 3.68± 0.44 which was in accordance reported by Munsi *et al.*, 2017 (3.8± 0.3), Patel and Siddiquee, 2013 (3.72± 0.02), Pal *et al.*, 2020b (3.69± 0.09 and 3.63± 0.08) and Baloch *et al.*, 2019 (3.6± 0.05). Our finding was lower as reported by the Srivastava and Kumar, 2014 (4.0± 0.4).

The variation in mass activity is due to improper handling procedure, contaminated glass wares, chemicals, cold or hot test tubes, glass slides, microscope

stage, rapid drying or cooling of glass slides, prolongation during collection and examination period etc and mass activity varies from individual to individual because it is a subjective test.

5.5.3 Sperm concentration (10^6)

In the present study the sperm concentration (10^6) in bull (Table 4.19) was 966.1± 45.4 x10^6 which was in agreement reported by Srivastava and Kumar, 2014 (968.1± 111.3 x10^6). Our finding was lower than reported by Rehman *et al.,* 2016 (1560.0± 59.00 x10^6), Munsi *et al.,* 2007 (1410.0± 70.0 x10^6) and Chauhan *et al.,* 2017 (1078.1± 32.56 x10^6).

The possible reason for variation in sperm concentration may be due to some factors like breed, age, seasonal variation, period of sexual rest, number of false mount, successive ejaculates and collection method etc.

5.5.4 Progressive motility

In the present research trial the per cent progressive motility of neat semen (Table 4.20) was 84.38± 0.35. This finding was in agreement with findings of Khan *et al.,* 2018 (85.12± 0.51 and 84.61± 16), Li *et al.,* 2016 (85.1± 0.3), Kumar *et al.,* 2013 (83.66± 2.27) and Uysal *et al.,* 2007 (83.5± 2.4).

In the present study the per cent progressive motility of diluted semen (Table 4.20) was 69.36± 0.46 and this finding was in agreement with Surabh *et al.,* 2021 (70.33± 0.23), Hegazy *et al.,* 2020 (70.12± 0.51) and Patel *et al.,* 2015 (69.46± 0.63).

In present investigation the per cent progressive motility of equilibrated semen (Table 4.20) was 63.21± 0.45 which was in accordance to findings reported by Singh *et al.,* 2017 (64.28± 2.96), Hossain *et al.,* 2012 (63.7), Patel *et al.,* 2015 (63.54± 0.60), Hegazy *et al.,* 2020 (63.00± 0.84) and Shoae and Zamiri, 2008 (62.1± 2.5).

In present research experiment the per cent progressive motility of post thaw semen (Table 4.20) was 48.97± 1.11 which was in agreement with the findings of Mughal *et al.,* 2013 (49.7± 2.5), Sariozkan *et al.,* 2009a (49.6±1.1), Singh *et al.,* 2017

(49.53± 2.00), Patel *et al.,* 2015 (49.17± 0.57), Chaturvedi *et al.,* 2020 (48.76± 1.69), Wafa *et al.,* 2021 (48.33±1.06) and Singh *et al.,* 2020 (48.0± 2.49).

The decrease in sperm motility is observed in disease condition, change of environment/season and temperature variations, and it swings between breeds, individuals, age groups and the evaluation technique employed.

The variation in sperm motility in post thaw semen occur due to cryopreservation which causes the formation of ice crystal in mitochondria and axoneme or the biochemical oxidative stress resulting in irreversible damage to sperm structure, changes in membrane fluidity and enzymatic activity (Alvarez and Storey, 1983).

5.5.5 Live Sperm

In present study the per cent live sperm in neat semen (Table 4.21) was 88.00± 0.66 and this finding was in agreement reported by Shaikh *et al.,* 2016 (89.97± 0.21), Mishra *et al.,* 2013 (89.53± 0.59, 88.73± 0.59 and 88.70± 0.59), Lone *et al.,* 2018 (89.45± 4.70), Kadirvel *et al.,* 2014 (88.43± 0.70), Srivastava and Kumar, 2014 (87.4± 1.4) and Pal *et al.,* 2020b (87.13± 1.49).

In our present study the per cent live sperm in diluted semen (Table 4.21) was 74.93 ±0.62 which was in agreement as reported by Singh *et al.,* 2020 (74.4± 1.82) and Abdel-Khalek *et al.,* 2008 (73.0± 1.3).

In the present experiment the per cent live sperm in equilibrated semen (Table 4.21) was 68.65± 0.59 which was in agreement with the findings of Shoae and Zamiri, 2008 (70.5± 1.1) and Chaudhary *et al.,* 2017 (69.92± 1.74).

In the present research trial the per cent live sperm in post thaw semen (Table 4.21) was 57.17± 1.31 which was in accordance to the findings of Oh *et al.,* 2012 (58.25± 6.03), Patel and Siddiquee, 2013 (58.22± 0.24), Baber *et al.,* 2016 (58.00± 1.21), Mughal *et al.,* 2013 (57.9± 3.3), Kumar *et al.,* 2018a (56.5± 2.1) and Rao *et al.,* 2017 (56.24± 0.01).

The variation in percentage of stained spermatozoa is dependent on various factors like duration, type of diluents used, stain integrity and technique employed by various scientists.

The reasons for varying live sperm count during cryopreservation may be due to bio-physical damage to the spermatozoa due to formation of ice-crystals in the extracellular and intracellular surroundings and expanding solute concentration that results in the production of ROS (Mazur, 1984) and sperm susceptible for freezing and thawing temperature, ROS production and lipid peroxidation (Aitken *et al*., 1989).

5.5.6 Acrosomal integrity

In present experimental trial the per cent acrosomal integrity of neat semen (Table 4.22) was 82.74± 0.28. This finding was in agreement with the findings of Rana and Dhami, 2004 (83.50± 1.24), Mishra *et al.,* 2013 (82.20± 0.47, 81.73±0.69 and 81.00± 0.69) and Patel and Siddiquee, 2013 (81.71± 0.11).

In present research trial the per cent acrosomal integrity in diluted semen (Table 4.22) was 70.81± 0.56 and this finding was lower than the findings of Varghese *et al.,* 2015 (92.85± 0.34), Saurabh *et al.,* 2021 (90.13± 0.18), Singh and Sharma, 2018 (86.17± 0.94), Rao *et al.,* 2017 (85.7± 0.01), Lone *et al.,* 2018 (80.17± 3.62), Mittal *et al.,* 2014 (80.16± 0.50), Singh *et al.,* 2020 (79.7± 1.52), Sakr *et al.,* 2021 (78.6± 1.4), Patel and Siddiquee, 2013 (75.13± 0.42) and Rasul *et al.,* 2001 (73.2± 2.4).

In our present study the per cent acrosomal integrity in equilibrated semen (Table 4.22) was 63.65±0.19 which was in agreement with findings reported by Khan *et al.,* 2021b (63.20± 0.31) and Kumar *et al.,* 2019 (63.14± 4.03) whereas, lower than the findings of Chaudhary *et al.,* 2017 (90.50± 0.45), Varghese *et al.,* 2015 (89.70± 0.39), Mittal *et al.,* 2014 (74.59± 0.50), Shoae and Zamiri, 2008 (70.0± 1.1) and Patel and Siddiquee, 2013 (68.16± 0.80).

In the present investigation the per cent acrosomal integrity in post thaw semen (Table 4.22) was 52.93± 1.39 which was in agreement with the findings of Patel and Siddiquee, 2013 (53.65± 0.58) and Hu *et al.,* 2010a (52.3± 1.58).

These variations reported in acrosomal integrity could be due to inherent quality of particular breed/bull, age, physio-pathological status, stain, staining technique & duration of staining used etc by different workers.

The variation or decrease in results of acrosomal integrity occurs in post thaw semen is because of cryopreservation, as it induces various degrees of acrosomal damage i.e. primary, secondary or tertiary damage which may be due to the loss of plasma membrane and loss of plasmalemma over entire acrosome.

5.5.7 Sperm abnormalities

In the present study the per cent sperm abnormalities in neat semen (Table 4.23) was 5.11± 0.12 which was in agreement with the findings of Patel and Siddiquee, 2013 (4.24± 0.03), Chaudhary *et al.,* 2017 (5.25± 0.36), Uysal *et al.,* 2007 (5.40± 0.6) and Munsi *et al.,* 2007 (6.5± 1.0).

In the present research experiment the per cent sperm abnormalities in diluted semen (Table 4.23) was 8.27± 0.27. This finding was in agreement with the findings reported by Singh *et al.,* 2020 (7.20± 0.68), Rao *et al.,* 2017 (7.28± 0.02) and Saurabh *et al.,* 2021 (8.08±0.18).

In our present study the per cent sperm abnormalities in equilibrated semen (Table 4.23) was 11.93± 0.27 which was lower than the findings of Dheerib *et al.,* 2020 (15.31± 0.77) and Khalil *et al.,* 2018 (24± 0.5) whereas, higher than the findings of Varghese *et al.,* 2015 (6.35± 0.27), Chaudhary *et al.,* 2017 (7.26± 0.42), Patel and Siddiquee, 2013 (7.56± 0.15) and Al-Badrany *et al.,* 2020 (9.55± 0.47).

In present investigation the per cent sperm abnormalities in post thaw semen (Table 4.23) was 15.05± 0.38 and this was in accordance to the findings of Al-Badrany *et al.,* 2020 (14.67± 0.45), Sariozkan *et al.,* 2009a (15.0± 1.1) and Singh *et al.,* 2020 (16.8± 0.61).

The semen from most males contains some abnormally formed spermatozoa which are not associated with lower fertility rates until the proportion of abnormal sperm

exceeds 20 percent (Hafez, 1987). The variation in sperm abnormality may be due to method of collection, temperature shock and technique employed.

5.5.8 Hypo-osmotic swelling test (HOST)

In the present experiment the per cent HOST positive sperm in neat semen (Table 4.24) was 82.25± 0.41 and this finding was in agreement with the findings of Mishra *et al.,* 2017 (83.90± 0.51, 83.53± 0.66 and 82.20± 1.00), Uysal *et al.,* 2007 (83.80± 3.3) and Khan *et al.,* 2018 (82.25± 0.43 and 81.66± 0.48).

In the present research trial the per cent HOST positive sperm in diluted semen (Table 4.24) was 70.52± 0.72. Our finding was in accordance to the findings reported by Singh *et al.,* 2020 (71.0± 1.10) and Sakr *et al.,* 2021 (70.6± 1.1).

In present investigation the per cent HOST positive sperm in equilibrated semen (Table 4.24) was 64.48± 0.58 which was in agreement with the finding of Singh and Sharma, 2018 (63.31± 1.11) whereas, higher than Rasul *et al.,* 2001 (60.4± 5.6) and Sakr *et al.,* 2021 (58.0± 1.7).

In present study the per cent HOST positive sperm in post thaw semen (Table 4.24) was 51.85± 2.35 which was in accordance with the findings of Wadood *et al.,* 2016 (52.55± 3.38) and Oh *et al.,* 2012 (51.90± 9.99).

The variation in HOST may be due to spontaneous lipid peroxidation of the membranes of mammalian sperm destroy the structure of the lipid matrix, as a result of the invasion by reactive oxygen species (ROS). These attacks then ultimately lead to the impairment of sperm function (sperm motility, functional membrane integrity and fertility), and damage to the sperm DNA, through oxidative stress and the production of cytotoxic aldehydes (Aitken *et al.,* 1993).

5.5.9 Lipid peroxidation

In present research experiment the MDA level (nmoles of MDA produced/10^8 spermatozoa/hr) in post thaw semen (Table 4.25) was 1.61± 0.03 which was in agreement with findings reported by Tuncer *et al.,* 2010 (1.44± 0.08) and Bansal and Cheema, 2016

(196.83± 4.89). Our finding was lower than the findings of Buyukleblebici *et al.*, 2014 (2.01± 0.26), Chhillar *et al.*, 2012 (2.61± 0.33), Singh *et al.*, 2020 (3.49± 0.19) and Motemani *et al.*, 2017 (7.2± 0.6) whereas, higher than the findings reported by Buyukleblebici *et al.*, 2016 (0.50± 0.27), Muzafer *et al.*, 2012 (0.60±0.06), Sariozkan *et al.*, 2009a (0.7± 0.2) and Al-Dahan *et al.*, 2020 (1.02± 0.38).

MDA is one of the final products of polyunsaturated fatty acids peroxidation in the sperm cells. The osmotic stress during the sperm dilution, cooling, cryoprotectant exposure, freezing, and thawing processes resulted in oxidative stress that induced high concentrations of MDA (Ansari *et al.*, 2019). The production of ROS in sperm is a natural process; however, overproduction has a deleterious impact on cellular membranes, resulting in dysfunction of cell organelles leading to death (Partyka *et al.*, 2012). Sperm cells are known to have high levels of polyunsaturated fatty acids in the plasma membrane that makes sperm highly susceptible to LPO (Parks and Hammerstedt, 1985).

5.5.10 Catalase

In the present investigation the catalase activity (µmol H_2O_2 decomposed/ min/ 10^8 spermatozoa) in post thaw semen (Table 4.26) was 26.31± 1.93. Our finding was higher than findings reported by Hu *et al.*, 2011 (3.89± 0.65), Sharma *et al.*, 2016 (24.25± 0.30), Hu *et al.*, 2010a (2.02± 0.15), Hu *et al.*, 2010b (2.01± 0.12), Tasdemir *et al.*, 2014 (18.9± 4.94), Zhao *et al.*, 2015 (1.9± 0.11) and Singh *et al.*, 2020 (1.18± 0.36) whereas, lower than Varela *et al.*, 2019 (84829.5± 72368.8).

Catalase is one of the enzymes present in all living organisms which is considered as the major defender of the sperm membranes against ROS and LPO (Bilodeau *et al.*, 2001). In living organism it is presented to oxygen, which disintegrates destructive peroxides and convert them into water and oxygen. The decrease in the catalase activity may be due to over consumption of catalase enzyme to decompose harmful peroxide and free radicals (Chelikani *et al.*, 2004).

5.5.11 Superoxide dismutase

In present research experiment the superoxide dismutase activity (Unit/10^8 spermatozoa) in post thaw semen (Table 4.27) was 11.25± 0.19 which was in agreement with the findings by Kadirvel *et al.,* 2014 (11.78± 0.88) and Varela *et al.,* 2019 (1144.8± 826.6).

Superoxide ion is free radical which is change over to oxygen and hydrogen peroxide by the dismutation activity of antioxidant enzyme superoxide dismutase (Tariq *et al.,* 2015). The decrease in superoxide activity may be due to neutralization of superoxide during cryopreservation (Bilodeau *et al.,* 2000).

5.6 Effect of Antioxidant Additives in Group III

5.6.1 Trehalose

The per cent values of progressive motility in diluted semen was reported as 70.83± 0.83 in HF bull with 100mM trehalsoe (Al-Badrany *et al.,* 2020); 55.67± 1.61 in HF bull with 100mM trehalose (Al-Badrany *et al.,* 2017), in equilibrated semen the per cent progressive motility was reported as 66.38± 0.88 in HF bull with 100mM trehalose (Al-Badrany *et al.,* 2020); 51.33± 1.3 in HF bull with 100mM trehalose (Al-Badrany *et al.,* 2017) and in post thaw semen the per cent progressive motility was reported as 65.55± 0.89 in HF bull (Al-Badrany *et al.,* 2020); 64.16± 0.52 in Kankrej bull with 100mM trehalose (Shaikh *et al.,* 2016); 54.67± 1.03 in HF bull with 100mM trehalose (Al-Badrany *et al.,* 2017); 51.66± 7.27 in buffalo bull with 100mM trehalose (Badr *et al.,* 2014); 49.00± 3.10 in HF bull with 25mM trehalose (Buyukleblebici *et al.,* 2014); 47.0± 1.67 in Karan fries bull with 100mM trehalose (Kumar *et al.,* 2013); 47± 1.15 in Karan Fries bull with 100mM trehalsoe (Chhillar *et al.,* 2012); 46.61± 1.62 in cattle bull with 100mM trehalose (Hu *et al.,* 2010b); 41.67± 1.67 in buffalo bull with 100mM trehalose (Reddy *et al.,* 2010).

In present investigation the per cent progressive motility in diluted semen (Table 4.20) was 72.18± 0.55 which was in accordance with the finding of Al-Badrany *et al.,* 2020 (70.83± 0.83) whereas, higher than Al-Badrany *et al.,* 2017 (55.67± 1.61) ,the per cent progressive motility in equilibrated semen was 65.31± 0.41. Our finding was

comparable with the finding of Al-Badrany *et al.,* 2020 (66.38± 0.88) whereas, higher than Al-Badrany *et al.,* 2017 (51.33± 1.3) and the per cent progressive motility in post thaw semen was 48.55± 2.83 which was in agreement with the findings of Buyukleblebici *et al.,* 2014 (49.00± 3.10), Kumar *et al.,* 2013 (47.0± 1.67) and Chhillar *et al.,* 2012 (47± 1.15).

The per cent live sperm in diluted semen is reported as 14.15± 5.36 dead sperm in HF bull with 100mM trehalose (Al-Badrany *et al.,* 2020); 26.44± 1.09 dead sperm in HF bull with 100mM trehalose (Al-Badrany *et al.,* 2017), in equilibrated semen the per cent live sperm was reported as 21.80± 0.33 dead sperm in HF bull with 100mM trehalose (Al-Badrany *et al.,* 2020); 28.62± 1.33 dead sperm in HF bull with 100mM trehalose (Al-Badrany *et al.,* 2017) and in post thaw semen the per cent live sperm was reported as 68.40± 3.14 in buffalo bull with 4.9g/100ml trehalose (Gamal *et al.,* 2016); 65.58± 0.35 in Kankrej bull with 100mM trehalose (Shaikh *et al.,* 2016); 52.00± 1.15 in Karan fries with 100mM trehalose (Chhillar *et al.,* 2012).

In present research trial the per cent live sperm in diluted semen (Table 4.21) was 78.48± 0.59 which was lower than the findings of Al-Badrany *et al.,* 2020 (14.15± 5.36) whereas, higher than Al-Badrany *et al.,* 2017 (26.44± 1.09), the per cent live sperm in equilibrated semen was 71.44± 0.56. Our finding was in agreement with Al-Badrany *et al.,* 2017 (28.62± 1.33) whereas, lower than Al-Badrany *et al.,* 2020 (21.80± 0.33) and the per cent live sperm in post thaw semen was 58.11± 2.98 which was lower than the findings of Gamal *et al.,* 2016 (68.40± 3.14) and Shaikh *et al.,* 2016 (65.58± 0.35) whereas, higher than Chhillar *et al.,* 2012 (52.00± 1.15).

The per cent acrosomal integrity in diluted semen with trehalose has not been reported, in equilibrated semen the per cent acrosomal integrity was reported as 78.00± 1.10 in cattle bull with 100mM trehalose (El-Sheshtamy *et al.,* 2015) and in post thaw semen the per cent acrosomal integrity is reported as 80.9± 0.43 in Kankrej bull with 100mM trehalose (Shaikh *et al.,* 2016); 64.78± 1.35 in cattle bull with 100mM trehalose (Hu *et al.,* 2010b); 49.00± 1.87 in cattle bull with 100mM trehalose (El-Sheshtamy *et al.,* 2015); 48.2± 8.70 in HF bull with 25mM trehalose (Ozturk *et al.,* 2017); 14.33± 2.40 in buffalo bull with 100mM trehalose (Badr *et al.,* 2014).

In our present study the per cent acrosomal integrity in diluted semen (Table 4.22) was 72.91± 0.53, the per cent acrosomal integrity in equilibrated semen was 66.1± 0.48 which was lower than the finding of El-Sheshtamy *et al.,* 2015 (78.00± 1.10) and the per cent acrosomal integrity in post thaw semen was 53.82± 2.93 which was lower than the findings of Shaikh *et al.,* 2016 (80.9± 0.43) and Hu *et al.,* 2010b (64.78± 1.35) whereas, higher than El-Sheshtamy *et al.,* 2015 (49.00± 1.87), Ozturk *et al.,* 2017 (48.2± 8.70) and Badr *et al.,* 2014 (14.33± 2.40).

The per cent sperm abnormalities in diluted semen was reported as 4.22± 0.32 in HF bull with 100mM trehalsoe (Al-Badrany *et al.,* 2020); 7.97± 0.65 in HF bull with 100mM trehalose (Al-Badrany *et al.,* 2017), in equilibrated semen the per cent sperm abnormalities was reported as 5.77± 0.27 in HF bull with 100mM trehalose (Al-Badrany *et al.,* 2020); 9.10± 0.78 in HF bull with 100mM (Al-Badrany *et al.,* 2017) and in post thaw semen per cent sperm abnormalities was reported as 5.00± 0.21 in Kankrej bull with 100mM trehalose (Shaikh *et al.,* 2016); 10.04± 0.31 in HF bull with 100mM trehalose (Al-Badrany *et al.,* 2020); 10.43± 0.89 in HF bull with 100mM trehalose (Al-Badrany *et al.,* 2017); 16.30± 0.90 in buffalo bull with 4.9g/100ml trehalose (Gamal *et al.,* 2016); 39.13± 3.17 in HF bull with 25mM trehalose (Buyukleblebici *et al.,* 2014).

In the proposed study the per cent sperm abnormalities in diluted semen (Table 4.23) was 7.45± 0.19 which was similar with the finding of Al-Badrany *et al.,* 2017 (7.97± 0.65) whereas, higher than Al-Badrany *et al.,* 2020 (4.22± 0.32), the per cent sperm abnormalities in equilibrated semen was 11.02± 0.27 which was higher than the findings of Al-Badrany *et al.,* 2020 (5.77± 0.27) and Al-Badrany *et al.,* 2017 (9.10± 0.78) and the per cent sperm abnormalities in post thaw semen (Table 4.23) was 14.82± 0.89 which was higher than Shaikh *et al.,* 2016 (5.00± 0.21), Al-Badrany *et al.,* 2020 (10.04± 0.31) and Al-Badrany *et al.,* 2017 (10.43± 0.89) whereas, lower than Gamal *et al.,* 2016 (16.30± 0.90) and Buyukleblebici *et al.,* 2014 (39.13± 3.17).

The per cent HOST positive sperm in diluted semen with trehalose has not been reported, in equilibrated semen the per cent HOST positive was reported as 83.40± 2.69 in cattle bull with 100mM trehalose (El-Sheshtamy *et al.,* 2015) and in post thaw semen the per cent HOST positive sperm was reported as 60.20± 1.83 in cattle bull with 100mM

trehalose (El-Sheshtamy *et al.,* 2015); 52.33± 2.70 in Karan fries bull with 100mM trehalose (Kumar *et al.,* 2013); 50± 1.52 in Karan fries bull with 100mM trehalose (Chhillar *et al.,* 2012); 44.33± 1.71 *et al.,* in cattle bull with 100mM trehalose (Hu *et al.,* 2010b).

In present investigation the per cent HOST positive sperm in diluted semen (Table 4.24) was 72.90± 0.75, the per cent HOST positive sperm in equilibrated semen was 67.46± 0.71 which is lower than the finding of El-Sheshtamy *et al.,* 2015 (83.40± 2.69) and the per cent HOST positive sperm in post thaw semen (Table 4.24) was 49.86± 4.44 which in accordance with the finding of Chhillar *et al.,* 2012 (50± 1.52) whereas higher than Hu *et al.,* 2010b (44.33± 1.71).

The MDA level (nmoles of MDA produced/10^8 spermatozoa/hr) of post thaw semen is reported by various researchers as 20.60± 0.13 µmol/ml in Kankrej bull with 100mM trehalose (Shaikh *et al.,* 2016); 19.82± 8.96 µM x 10^9 in HF bull with 25mM trehalose (Ozturk *et al.,* 2017); 11.33± 1.85 nmol/ml in buffalo bull with 100mM trehalose (Badr *et al.,* 2014); 2.27± 0.23 nmol/ml in HF bull with 25mM trehalose (Buyukleblebici *et al.,* 2014); 1.48 MDA/10^8 cells in buffalo bull with 100mM trehalose (Singh *et al.,* 2014).

In present research trial the MDA level (nmoles of MDA produced/10^8 spermatozoa/hr) in post thaw semen (Table 4.25) was 1.53± 0.06 which was comparable with the findings of Singh *et al.,* 2014 (1.48) whereas lower than Badr *et al.,* 2014 (11.33± 1.85) and Buyukleblebici *et al.,* 2014 (2.27± 0.23).

The catalase activity (µmol H_2O_2 decomposed/ min/ 10^8 spermatozoa) in post thaw semen is reported as 18875± 41432.0 U/ml in HF bull with 100mM trehalose (Varela *et al.,* 2019); 3.17± 0.56 U/ml in cattle bull with 100mM trehalose (Hu *et al.,* 2010b); 1.92± 0.19 U/ml in Nili-ravi bull with 60mM trehalose (Iqbal *et al.,* 2016).

In the present experiment the catalase activity (µmol H_2O_2 decomposed/ min/ 10^8 spermatozoa) in post thaw semen (Table 4.26) was 34.41± 2.06. Our findings could not be compared with findings of other scientists due to disparity in the units of measurement.

The superoxide dismutase (Unit/10^8 spermatozoa) in post thaw semen is reported as 3324± 3233.5 U/ml in HF bull with 100mM trehalose (Varela *et al.,* 2019); 48.33± 6.02 U/l in buffalo bull with 100mM trehalose (Badr *et al.,* 2014); 49.96± 0.85 U/ml in Nili-ravi bull with 60mM trehalose (Iqbal *et al.,* 2016); 1.38± 0.09 U/ml in cattle bull with 100mM trehalose (Hu *et al.,* 2010b).

In present investigation the superoxide dismutase activity (Unit/10^8 spermatozoa) in post thaw semen (Table 4.27) was 11.83± 0.22. Our findings could not be compared with findings of other scientists due to disparity in the units of measurement.

The scientists have revealed that the mechanisms of trehalose has an antioxidant action (Reddy *et al.,* 2010) as it decrease the rate of cell injury by reducing formation of ice crystallization (Molinia *et al*., 1994).

5.6.2 Butylated hydroxytoluene (BHT)

The per cent progressive motility in diluted semen was reported as 77.50± 0.70 in Hariana bull with 1mM BHT (Patel *et al.,* 2015); 77.33± 2.24 in crossbred bull with 1mM BHT (Singh *et al.,* 2017); 61.07± 4.22 in crossbred bull with 1mM BHT (Khumran *et al.,* 2017), in equilibrated semen the per cent progressive motility was reported as 71.92± 0.65 in Hariana bull with 1mM BHT (Patel *et al.,* 2015); 68.47± 2.58 in crossbred bull with 1mM BHT (Singh *et al.,* 2017); 60.8± 2.4 in HF bull with 1mM BHT (Shoae and Zamiri, 2008) and in post thaw semen the per cent progressive motility is reported as 59.13± 0.60 in Hariana bull with 1mM BHT (Patel *et al.,* 2015); 58.2± 2.6 in buffalo bull with 1mM BHT (Ijaz *et al.,* 2009); 56.58± 1.38 in crossbred bull with 1mM BHT (Singh *et al.,* 2017); 47.25± 4.66 in buffalo bull with 1mM BHT (Mostafa *et al.,* 2019); 44.2± 1.3 in HF bull with 1mM BHT (Shoae and Zamiri, 2008); 41± 1 in HF bull with 1mM BHT (Asadpour *et al.,* 2012).

In present research trial the per cent progressive motility in diluted semen (Table 4.20) was 71.63± 0.61. Our finding was lower than Patel *et al.,* 2015 (77.50± 0.70) and Singh *et al.,* 2017 (77.33± 2.24) whereas, higher than Khumran *et al.,* 2017 (61.07± 4.22), the per cent progressive motility in equilibrated semen was 64.25± 0.46 which was lower than Patel *et al.,* 2015 (71.92± 0.65) and Singh *et al.,* 2017 (68.47± 2.58) whereas,

higher Shoae and Zamiri, 2008 (60.8± 2.4) and the per cent progressive motility in post thaw semen was 51.36± 1.15 which was lower than the findings reported by Patel *et al.,* 2015 (59.13± 0.60), Ijaz *et al.,* 2009 (58.2± 2.6) and Singh *et al.,* 2017 (56.58± 1.38) whereas, higher than Mostafa *et al.,* 2019 (47.25± 4.66), Shoae and Zamiri, 2008 (44.2± 1.3) and Asadpour *et al.,* 2012 (41± 1).

The per cent live sperm in diluted semen is reported as 88.10± 0.64 in Hariana bull with 1mM BHT (Patel *et al.,* 2015); 86.58± 2.34 in crossbred bull with 1mM BHT (Singh *et al.,* 2017); 67.83± 1.26 in crossbred bull with 1mM BHT (Khumran *et al.,* 2017), in equilibrated semen the per cent live sperm is reported as 82.44± 0.64 in Hariana bull with 1mM BHT (Patel *et al.,* 2015); 81.33± 2.77 in crossbred bull with 1mM BHT (Singh *et al.,* 2017); 71.5± 1.0 in HF bull with 1mM BHT (Shoae and Zamiri, 2008) and in post thaw semen the per cent live sperm is reported as 70.72± 0.78 in Hariana bull with 1mM BHT (Patel *et al.,* 2015); 69.22± 2.99 in crossbred bull with 1mM BHT (Singh *et al.,* 2017); 63.1± 2.9 in buffalo bull with 1mM BHT (Ijaz *et al.,* 2009); 55.43± 5.50 in buffalo bull with 1mM BHT (Mostafa *et al.,* 2019); 48.9± 0.9 in HF bull with 1mM BHT (Shoae and Zamiri, 2008); 37± 2 in HF bull with 1mM BHT (Asadpour *et al.,* 2012).

In our present study the per cent live sperm in diluted semen (Table 4.21) was 77.66± 0.53 which was lower than the findings of Patel *et al.,* 2015 (88.10± 0.64) and Singh *et al.,* 2017 (86.58± 2.34) whereas, higher than Khumran *et al.,* 2017 (67.83± 1.26), the per cent live sperm in equilibrated semen was 70.53± 0.45 which was in agreement with finding reported by Shoae and Zamiri, 2008 (71.5± 1.0) whereas, lower than Patel *et al.,* 2015 (82.44± 0.64) and Singh *et al.,* 2017 (81.33± 2.77) and the per cent live sperm in post thaw semen was 59.52± 1.60 which was lower than the findings reported by Patel *et al.,* 2015 (70.72± 0.78), Singh *et al.,* 2017 (69.22± 2.99) and Ijaz *et al.,* 2009 (63.1± 2.9) whereas, higher than Mostafa *et al.,* 2019 (55.43± 5.50), Shoae and Zamiri, 2008 (48.9± 0.9) and Asadpour *et al.,* 2012 (37± 2).

The per cent acrosomal integrity in diluted semen was reported as 4.50± 0.94 abnormal acrosome in crossbred bull with 1mM BHT (Khumran *et al.,* 2017), in equilibrated semen the per cent acrosomal integrity is reported as 70.8± 0.9 in HF bull with 1mM BHT (Shoae and Zamiri, 2008) and in post thaw semen the per cent acrosomal

integrity is reported as 63.28± 6.28 in buffalo bull with1mM BHT (Mostafa *et al.*, 2019); 48.7± 0.9 in HF bull with 1mM BHT (Shoae and Zamiri, 2008); 27.8± 2.9 in buffalo bull with 1mM BHT (Ijaz *et al.,* 2009).

In present investigation the per cent acrosomal integrity in diluted semen (Table 4.22) was 72.01± 0.45 which was lower than the finding of Khumran *et al.,* 2017 (4.50± 0.94), the per cent acrosomal integrity in equilibrated semen was 65.46± 0.36 which was lower than the finding of Shoae and Zamiri, 2008 (70.8± 0.9) and the per cent acrosomal integrity in post thaw semen was 56.11± 1.49 which was lower as compare to Mostafa *et al.,* 2019 (63.28± 6.28) whereas, higher than Shoae and Zamiri, 2008 (48.7± 0.9) and Ijaz *et al.,* 2009 (27.8± 2.9).

The per cent sperm abnormalities in diluted semen is reported as 94.50± 0.54 normal sperm in crossbred bull with 1mM BHT (Khumran *et al.,* 2017); 10.14± 1.02 in Frieswal bull with 2.5mM BHT (Kumar *et al.,* 2018b), in equilibrated semen the per cent sperm abnormalities is reported as 12.45± 0.88 in Frieswal bull with 2.5mM BHT (Kumar *et al.,* 2018b) and in post thaw semen the per cent sperm abnormalities is reported as 14.45± 0.26 in Frieswal bull with 2.5mM BHT (Kumar *et al.,* 2018b); 15.69± 0.49 in crossbred bull with 2mM BHT (Muzafer *et al.,* 2012); 74.81± 7.50 normal sperm in buffalo bull with 1mM BHT (Mostafa *et al.,* 2019).

In our present study the per cent sperm abnormalities in diluted semen (Table 4.23) was 7.72± 0.12 which was comparable with the finding of Khumran *et al.,* 2017 (94.50± 0.54 normal spermatozoa), the per cent sperm abnormalities in equilibrated semen was 11.30± 0.23 which was in accordance with the finding of Kumar *et al.,* 2018b (12.45± 0.88) and the per cent sperm abnormalities in post thaw semen was 14.41± 0.44 which was in agreement with the findings of Kumar *et al.,* 2018b (14.45± 0.26) and Muzafer *et al.,* 2012 (15.69± 0.49) whereas, lower than Mostafa *et al.,* 2019 (74.81± 7.50 normal sperm).

The per cent HOST positive sperm values in diluted semen is reported as 84.92± 0.92 in Hariana bull with 1mM BHT (Patel *et al.,* 2015); 83.36± 1.95 in crossbred bull with 1mM BHT (Singh *et al.,* 2017), in equilibrated semen the per cent HOST positive

sperm is reported as 79.51± 0.69 in Hariana bull with 1mM BHT (Patel *et al.,* 2015); 76.61± 2.20 in crossbred bull with 1mM BHT (Singh *et al.,* 2017) and in post thaw semen the per cent HOST positive sperm is reported as 72.24± 7.25 in buffalo bull with 1mM BHT (Mostafa *et al.,* 2019); 70.31± 3.27 in crossbred bull with 1mM BHT (Singh *et al.,* 2017); 67.92± 0.73 in Hariana bull with 1mM BHT (Patel *et al.,* 2015); 51.3± 3.9 in buffalo bull with 1mM BHT (Ijaz *et al.,* 2009); 50.62± 2.16 in buffalo bull with 1.75mM BHT (Wadood *et al.,* 2016); 49.21± 0.89 in crossbred bull with 2mM BHT (Muzafer *et al.,* 2012).

In the present research trial the per cent HOST positive sperm in diluted semen (Table 4.24) was 72.6± 1.51 which was lower than the findings reported by Patel *et al.,* 2015 (84.92± 0.92) and Singh *et al.,* 2017 (83.36± 1.95), the per cent HOST positive sperm in equilibrated semen was 67.04± 0.71. Our finding was lower than the findings reported by Patel *et al.,* 2015 (79.51± 0.69) and Singh *et al.,* 2017 (76.61± 2.20) and the per cent HOST positive sperm in post thaw semen was 53.61± 2.25 which was lower than findings reported by Mostafa *et al.,* 2019 (72.24± 7.25), Singh *et al.,* 2017 (70.31± 3.27) and Patel *et al.,* 2015 (67.92± 0.73) whereas, higher than Ijaz *et al.,* 2009 (51.3± 3.9), Wadood *et al.,* 2016 (50.62± 2.16) and Muzafer *et al.,* 2012 (49.21± 0.89).

The MDA level (nmoles of MDA produced/10^8 spermatozoa/hr) of post thaw semen is reported by various researchers as 10.61± 2.34 nM/ml in buffalo bull with 1.75mM BHT (Wadood *et al.,* 2016); 5.4± 0.17 nmol/dL in HF bull with 1mM BHT (Asadpour *et al.,* 2012); 1.26± 0.07 µmol/mL in Frieswal bull with 5mM BHT (Kumar *et al.,* 2018b); 0.19± 0.02 µmol/ml in crossbred bull with 2mM BHT (Muzafer *et al.,* 2012).

In present research experiment the MDA level (nmoles of MDA produced/10^8 spermatozoa/hr) in post thaw semen (Table 4.25) was 1.43± 0.03. Our finding was lower than reported by Wadood *et al.,* 2016 (10.61± 2.34) and Asadpour *et al.,* 2012 (5.4± 0.17).

The catalase activity (µmol H_2O_2 decomposed/ min/ 10^8 spermatozoa) in post thaw semen is reported as 0.004±0.0, 0.02±0.0 and 0.005±0.0 U/mg in Frieswal bull with 2.5, 5 and 10mM BHT, respectively (Kumar *et al.,* 2018b).

In our present study the catalase activity (µmol H_2O_2 decomposed/ min/ 10^8 spermatozoa) in post thaw semen (Table 4.26) was 34.65± 3.27. Our finding could not be compared with other scientists due to disparity in the units of measurement.

The superoxide dismutase (Unit/10^8 spermatozoa) in post thaw semen is reported as 38.82± 0.94, 43.16± 1.24 and 39.61± 1.14 U/mL in Frieswal bull with 2.5, 5 and 10mM BHT, respectively (Kumar *et al.*, 2018b); 0.16± 0.00 U/mg in black Bengal goat with 1mM BHT (Tudu *et al.*, 2021).

In present experimental trial the superoxide dismutase activity (Unit/10^8 spermatozoa) in post thaw semen (Table 4.27) was 12.07± 0.16 which could not be compared with other scientists due to disparity in the units of measurement.

The addition of BHT has a brilliant antioxidant capacity and it behave as an artificial analogue of vitamin E. It mainly acts as a terminating agent which reduces autoxidation by converting peroxy radicals to hydroperoxides (Fujisawa *et al*., 2004).

Chapter-VI

Summary and Conclusions

CHAPTER-VI

SUMMARY AND CONCLUSIONS

Cryopreservation of semen is the most stressful process that has a harmful effect on the normal physiology of sperm, causing damage and modifications that eventually lead to the death of the sperm, thereby reducing freeze-thawed quality parameters. In this process, the mammalian spermatozoa have to tolerate various types of stresses caused by ice crystal formation, chemical toxicity, cold shock and oxidative stress which alter lipid and protein configuration which leads to generation of ROS, decreases motility and viability, causes sub-lethal damage to mitochondria, cytoplasm, membrane and spermatozoa tail and intensify sperm DNA fragmentation, thus leading to a lower post-thawed quality and fertility leading to a decline in freeze-thawed sperm quality. Therefore the present study investigation has been designed with following objectives:

1. To study the effect of cryopreservation on different physio-morphological characteristics of the semen.
2. To ascertain the effects of different antioxidants (Ascorbic acid, Vitamin-E, Cysteine, Taurine, Butylated hydroxy toulene and Trehalose) on cryopreservation of semen.
3. To evaluate the oxidative stress to the spermatozoa.

Semen samples (n=10) from mature cattle bull stationed at Central Artificial Breeding Station (CABS) Frozen semen processing laboratory, Department of Animal Husbandary, Hakkal, U.T. of Jammu & Kashmir were taken to evaluate the effect of additives on the cryopreservation of the semen. The experiment was carried out at Central Artificial Breeding Station (CABS), Frozen semen processing laboratory, Departmentof Animal Husbandary, Hakkal, U.T. of Jammu & Kashmir, Division of V.G.O. and Division of V.P.T., F.V.Sc & A.H., SKUAST- J, R.S.PURA Jammu.

Semen collection from donor bulls was done twice a week (Tuesday and Saturday) with the help of artificial vagina (AV) on live dummy animals between 8.30 to 9.30 am in winters and 7:30 to 8:30 am in summers. After collection the semen sample was extended with Tris-Egg yolk-Citric acid-Fructose-Glycerol (TEYCAFG) and was divided into 3

aliquots in each group. The complete experiment was divided into 3 groups (Group I, II, III). TEYCAFG dilutor was used as control in all the groups, TEYCAFG plus cysteine (5mM), TEYCAFG plus taurine (50mM) in group I, TEYCAFG plus ascorbic acid (0.5mg/ml), TEYCAFG plus vitamin E (4mg/ml) in group II and TEYCAFG plus trehalose (100mM), TEYCAFG plus BHT (1mM) in group III.

The semen evaluation was done in neat, diluted, equilibrated and post thawed samples for physio-morphological and lab fertility test whereas, oxidative stress parameters were evaluated in post thaw semen.

6.1 Evaluation of Semen Group I

6.1.1 Evaluation in neat semen

In table 4.1 our finding related to seminal attributes in neat semen are provided. It was observed that in neat semen mean ejaculate volume was 5.46 ± 1.62, Mass activity was 3.65 ± 0.11 and sperm concentration 806.76 ± 65.92 (10^6 /ml) which were in the normal range.

6.1.2 Progressive motility

In Table 4.2, our findings related to progressive motility of neat, diluted, equilibrated and post thaw semen are present. It was observed that the progressive motility (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, cysteine and taurine added aliquots, a significant ($P<0.05$) difference was observed within and between all the aliquots at all the stages. It was also observed that the progressive motility (%) of post thaw semen sample differed significantly ($P<0.05$) within all aliquots *i.e.* control, cysteine and taurine supplemented aliquots from diluted and equilibrated semen. Whereas, within all the aliquots, the diluted semen differed non-significantly from the equilibrated semen. Viewing table it was observed the progressive motility (%) between different stage *i.e.* diluted, equilibrated and post thaw stages, the cysteine and taurine added semen differed significantly ($P<0.05$) from control semen sample at diluted stage, whereas between cysteine and taurine added aliquots there was no significant difference at diluted stage. Among equilibrated stage the taurine supplemented aliquots differed significantly ($P<0.05$) from control, whereas there was no significant

difference between cysteine and taurine added semen samples and also between control and cysteine supplemented semen at equilibration stage. Between the post thaw stage of all the aliquots the taurine added semen differed significantly (P<0.05) from control, whereas non-significantly from cysteine supplemented aliquots. Moreover, between control and cysteine supplemented semen there was no significant difference at post thaw stage.

6.1.3 Live sperm

In Table 4.3, our findings related to live sperm of neat, diluted, equilibrated and post thaw semen are given. It was observed the live sperm (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, cysteine and taurine added aliquots, a significant (P<0.05) difference was observed within and between all the aliquots at all the stages. It was also observed that the live sperm (%) of all the stages *i.e.* diluted, equilibrated and post thaw stages differed significantly (P<0.05) within all aliquots *i.e.* control, cysteine and taurine supplemented semen. Comparing table it was observed that the live sperm (%) between different stages i.e. diluted, equilibrated and post thaw stages, the control, cysteine and taurine supplemented aliquots were non-significant (P<0.05) at diluted stage and equilibrated stage. Between the post thaw stage of all the aliquots the taurine added semen differed significantly (P<0.05) from control, whereas non-significantly from cysteine supplemented aliquots. Moreover, between control and cysteine supplemented semen sample there was no significant difference at post thaw stage.

6.1.4 Acrosomal integrity

In Table 4.4, our findings related to acrosomal integrity of neat, diluted, equilibrated and post thaw semen are stated. It was observed that the acrosomal integrity (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, cysteine and taurine added aliquots, a significant (P<0.05) difference was observed within and between all the aliquots at all stages. It was also observed that the acrosomal integrity (%) of all the stages *i.e.* diluted, equilibrated and post thaw stages differed significantly (P<0.05) within all aliquots *i.e.* control, cysteine and taurine supplemented aliquots. Analyzing table it was observed that the acrosomal integrity (%) between different stages i.e. diluted, equilibrated and post thaw stages, the control, cysteine

and taurine added semen were non-significant (P<0.05) at diluted stage. Among equilibrated stage the taurine supplemented semen differed significantly (P<0.05) from control, whereas there was no significant difference between cysteine and taurine supplemented aliquots and also between control and cysteine added semen sample at equilibration stage. Between the post thaw stage of all the aliquots the cysteine and taurine added semen differed significantly (P<0.05) from control. Moreover, between cysteine and taurine supplemented aliquots there was no significant difference at post thaw stage.

6.1.5 Sperm abnormalities

In Table 4.5, our findings related to sperm abnormalities of neat, diluted, equilibrated and post thaw semen are present. It was observed that the sperm abnormalities (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, cysteine and taurine added aliquots, a significant (P<0.05) difference was observed within and between the all the aliquots at all stages. It was observed that the sperm abnormalities (%) of post thaw semen sample differed significantly (P<0.05) within all the aliquots *i.e.* control cysteine and taurine added semen from diluted and equilibrated semen. Whereas, within control semen sample, the diluted semen differed significantly (P<0.05) from the equilibrated semen. Moreover, within cysteine and taurine added aliquots, the diluted semen was non-significant from the equilibrated semen. Viewing table it was observed that the sperm abnormalities (%) between all the different stages *i.e.* diluted, equilibrated, post thaw stages and between all the aliquots *i.e*. control, cysteine, taurine supplemented aliquots differed non-significantly.

6.1.6 Hypo-osmotic swelling test (HOST)

In Table 4.6, our findings related to HOST of neat, diluted, equilibrated and post thaw semen are present. It was observed that the HOST positive (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, cysteine and taurine added aliquots, a significant (P<0.05) difference was observed within and between all the aliquots at all the stages. It was also observed that the HOST positive (%) of post thaw semen sample differed significantly (P<0.05) within all aliquots *i.e.* control, cysteine and taurine supplemented semen from diluted and equilibrated semen. Whereas,

within all the aliquots, the diluted semen differ non-significantly (P<0.05) from the equilibrated semen. Comparing table it was observed that the HOST positive (%) between all the different stages *i.e.* diluted, equilibrated, post thaw stages and between all the aliquots *i.e.* control, cysteine, taurine supplemented aliquots differed non-significantly.

6.1.7 Lipid peroxidation

In Table 4.7, our findings related to lipid peroxidation of post thaw semen is given. It was observed that the MDA level between the post thaw semen sample with or without additives *i.e.* both cysteine and taurine added aliquots differed significantly (P<0.05) from control. Whereas, cysteine added differed non-significantly from taurine added semen sample.

6.1.8 Catalase

In Table 4.8, our findings related to catalase level of post thaw semen is given. It was observed that the catalase level between the post thaw semen with or without additive *i.e*. taurine supplemented semen differ significantly (P<0.05) from control and cysteine supplemented aliquots. Whereas cysteine added differed non-significantly from control semen sample.

6.1.9 Superoxide dismutase

In Table 4.9, our findings related to SOD level of post thaw semen is given. It was observed that the SOD level between the post thaw semen sample with or without additive *i.e.* taurine supplemented semen differ significantly (P<0.05) from the control semen sample. Whereas, cysteine added differed non-significantly from both control and taurine supplemented aliquots.

6.2 Evaluation of Semen Group II

6.2.1 Evaluation in neat semen

In table 4.10 our finding related to seminal attributes in neat semen are provided. It was observed that in neat semen mean ejaculate volume was 5.79 ± 0.51, Mass activity

was 3.64 ± 0.42 and sperm concentration 877.56 ± 67.53 (10^6 /ml) which were in the normal range.

6.2.2 Progressive motility

In Table 4.11, our findings related to progressive motility of neat, diluted, equilibrated and post thaw semen are present. It was observed that the progressive motility (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, ascorbic acid and vitamin E added aliquots, a significant ($P<0.05$) difference was observed within and between all the aliquots at all the stages. It was also observed that the progressive motility (%) of all the stages *i.e.* diluted, equilibrated and post thaw stages differed significantly ($P<0.05$) within all aliquots *i.e.* control, ascorbic acid and vitamin E added semen. Comparing table it was observed that the progressive motility (%) between different stage *i.e.* diluted, equilibrated and post thaw stages, the ascorbic acid and vitamin E supplemented aliquots differed significantly ($P<0.05$) from control semen sample at diluted stage, whereas between ascorbic acid and vitamin E added aliquots there was no significant ($P<0.05$) difference at diluted stage. Among equilibrated stage, all the aliquots *i.e.* control, ascorbic acid and vitamin E added supplemented semen differed non-significantly. Between the post thaw stage of all the aliquots the ascorbic acid and vitamin E added semen differed significantly ($P<0.05$) from control semen. Moreover, between ascorbic acid and vitamin E supplemented aliquots there was no significant difference at post thaw stage.

6.2.3 Live sperm

In Table 4.12, our findings related to live sperm of neat, diluted, equilibrated and post thaw semen are given. It was observed that the live sperm (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, ascorbic acid and vitamin E added aliquots, a significant ($P<0.05$) difference was observed within and between all the aliquots at all the stages. It was also observed that the live sperm (%) of post thaw semen sample differed significantly ($P<0.05$) within all aliquots *i.e.* control, ascorbic acid and vitamin E added semen from diluted and equilibrated semen. Whereas, within control and ascorbic acid the diluted semen differed significantly ($P<0.05$) from the

equilibrated semen. Moreover, within vitamin E, the diluted semen differed non-significantly (P<0.05) from the equilibrated semen. Viewing table it was observed that the live sperm (%) between different stage *i.e.* diluted, equilibrated and post thaw stages, all the aliquots *i.e.* control, ascorbic acid and vitamin E supplemented semen differed non-significantly (P<0.05) at diluted and equilibrated stage. Between the post thaw stage of all the aliquots the ascorbic acid and vitamin E added semen differed significantly (P<0.05) from control. Moreover, ascorbic acid added semen differed non-significantly from vitamin E supplemented aliquots at post thaw stage.

6.2.4 Acrosomal integrity

In Table 4.13, our findings related to acrosomal integrity of neat, diluted, equilibrated and post thaw semen are stated. It was observed that the acrosomal integrity (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, ascorbic acid and vitamin E added aliquots, a significant (P<0.05) difference was observed within and between all the aliquots at all the stages. It was also observed that the acrosomal integrity (%) of post thaw semen sample differed significantly (P<0.05) within all aliquots *i.e.* control, ascorbic acid and vitamin E added semen from diluted and equilibrated semen. Whereas, within all the aliquots, the diluted semen differed non-significantly (P<0.05) from the equilibrated semen. Analyzing table it was observed that the acrosomal integrity (%) between different stage *i.e.* diluted, equilibrated and post thaw stages, all the aliquots *i.e.* control, ascorbic acid and vitamin E supplemented aliquots differed non-significantly (P<0.05) at diluted and equilibrated stage. Between the post thaw stage of all the aliquots the ascorbic acid added semen differed significantly (P<0.05) from control. Moreover, vitamin E added semen differed non-significantly from control as well as ascorbic added semen sample at post thaw stage.

6.2.5 Sperm abnormalities

In Table 4.14, our findings related to sperm abnormalities of neat, diluted, equilibrated and post thaw semen are present. It was observed that the sperm abnormalities (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, ascorbic acid and vitamin E added aliquots, a significant (P<0.05) difference

was observed within and between all the aliquots at all the stages. It was also observed that the sperm abnormalities (%) of all the stages *i.e.* diluted, equilibrated and post thaw stages differed significantly (P<0.05) within all aliquots *i.e.* control, ascorbic acid and vitamin E supplemented semen. Comparing table it was observed that the sperm abnormalities (%) between all the different stages *i.e.* diluted, equilibrated, post thaw stages and between all the aliquots *i.e*. control, ascorbic acid and vitamin E supplemented aliquots differed non-significantly.

6.2.6 Hypo-osmotic swelling test (HOST)

In Table 4.15, our findings related to HOST of neat, diluted, equilibrated and post thaw semen are present. It was observed that the HOST positive (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, ascorbic acid and vitamin E added aliquots, a significant (P<0.05) difference was observed within and between all the aliquots at all the stages. It was also observed that the HOST positive (%) of post thaw semen sample differed significantly (P<0.05) within all aliquots *i.e.* control, ascorbic acid and vitamin E supplemented semen from diluted and equilibrated semen. Whereas, within all the aliquots, the diluted semen differed non-significantly (P<0.05) from the equilibrated semen. Comparing table it was observed that the HOST positive (%) between different stage *i.e.* diluted, equilibrated and post thaw stages, all the aliquots *i.e.* control, ascorbic acid and vitamin E added semen differed non-significantly (P<0.05) at diluted and equilibrated stage. Between the post thaw stage of all the aliquots the ascorbic acid added semen differed significantly (P<0.05) from control. Moreover, vitamin E added semen differed non-significantly from control as well as ascorbic acid supplemented aliquots at post thaw stage.

6.2.7 Lipid peroxidation

In Table 4.16, our findings related to lipid peroxidation of post thaw semen is given. It was observed that the MDA level between the post thaw semen with or without additives *i.e.* control, ascorbic acid and vitamin E supplemented aliquots all differed significantly (P<0.05).

6.2.8 Catalase

In Table 4.17, our findings related to catalase level of post thaw semen is given. It was observed that the catalase level between the post thaw semen with or without additive *i.e*. ascorbic acid added aliquot differed significantly (P<0.05) from control and there was no significant difference between ascorbic acid added and vitamin E supplemented aliquots. Moreover, vitamin E added differed non-significantly from control semen sample.

6.2.9 Superoxide dismutase

In Table 4.18, our findings related to SOD level of post thaw semen is given. It was observed that the SOD level between the post thaw semen with or without additive *i.e*. ascorbic acid added semen differed significantly (P<0.05) from control and there was no significant difference between ascorbic acid added and vitamin E supplemented aliquots. Moreover, vitamin E added differed non-significantly from control semen sample.

6.3 Evaluation of Semen Group III

6.3.1 Evaluation in neat semen

In table 4.19 our finding related to seminal attributes in neat semen are provided. It was observed that in neat semen mean ejaculate volume was 5.02 ± 0.44, Mass activity was 3.68 ± 0.44 and sperm concentration 966.1 ± 45.4 (10^6 /ml) which were in the normal range.

6.3.2 Progressive motility

In Table 4.20, our findings related to progressive motility of neat, diluted, equilibrated and post thaw semen are present. It was observed that the progressive motility (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, trehalose and BHT added aliquots, a significant (P<0.05) difference was observed within and between all the aliquots at all the stages. It was also observed that the progressive motility (%) of all the stages *i.e.* diluted, equilibrated and post thaw stages differed significantly (P<0.05) within all aliquots *i.e.* control, trehalose and BHT added semen. Viewing table it was observed that the progressive motility (%) between different stage *i.e.* diluted, equilibrated and post thaw stages, the trehalose and BHT added semen

differed significantly (P<0.05) from control semen sample at diluted stage, whereas between trehalose and BHT added aliquots there was no significant (P<0.05) difference at diluted stage. Among equilibrated stage the trehalose supplemented semen differed significantly (P<0.05) from control, whereas there was no significant difference between trehalose and BHT supplemented aliquots and also between control and BHT added semen at equilibration stage. Between the post thaw stages of all the aliquots *i.e.* control, trehalose and BHT supplemented semen differed non-significantly.

6.3.3 Live sperm

In Table 4.21, our findings related to live sperm of neat, diluted, equilibrated and post thaw semen are given. It was observed the live sperm (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, trehalose and BHT added aliquots, a significant (P<0.05) difference was observed within and between all the aliquots at all the stages. It was also observed that the live sperm (%) of all the stages *i.e.* diluted, equilibrated and post thaw stages differed significantly (P<0.05) within all aliquots *i.e.* control, trehalose and BHT added semen. Analyzing table it was observed that the progressive motility (%) between different stage *i.e.* diluted, equilibrated and post thaw stages, the trehalose and BHT supplemented aliquots differed significantly (P<0.05) from control semen sample at diluted stage, whereas between trehalose and BHT added aliquots there was no significant (P<0.05) difference at diluted stage. Among equilibrated stage the trehalose and BHT supplemented aliquots differed significantly (P<0.05) from control, whereas there was no significant difference between trehalose and BHT supplemented semen at equilibration stage. Between the post thaw stages of all the aliquots *i.e.* control, trehalose and BHT added semen differed non-significantly.

6.3.4 Acrosomal integrity

In Table 4.22, our findings related to acrosomal integrity of neat, diluted, equilibrated and post thaw semen are stated. It was observed that the acrosomal integrity (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, trehalose and BHT added aliquots, a significant (P<0.05) difference was observed within and between all the aliquots at all the stages. It was also observed that the

acrosomal integrity (%) of all the stages *i.e.* diluted, equilibrated and post thaw stages differed significantly (P<0.05) within all aliquots *i.e.* control, trehalose and BHT added semen. Viewing table it was observed that the acrosomal integrity (%) between different stage *i.e.* diluted, equilibrated and post thaw stages, the trehalose supplemented semen differed significantly (P<0.05) from control semen sample at diluted stage, whereas between trehalose and BHT added aliquots there was no significant (P<0.05) difference and also there was no significant difference between control and BHT added aliquots at diluted stage. Among equilibrated stage the trehalose and BHT supplemented aliquots differed significantly (P<0.05) from control, whereas there was no significant difference between trehalose and BHT supplemented aliquots at equilibration stage. Between the post thaw stages of all the aliquots *i.e.* control, trehalose and BHT added semen differed non-significantly.

6.3.5 Sperm abnormalities

In Table 4.23, our findings related to sperm abnormalities of neat, diluted, equilibrated and post thaw semen are present. It was observed that the sperm abnormalities (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, trehalose and BHT added aliquots, a significant (P<0.05) difference was observed within and between all the aliquots at all the stages. It was also observed that the sperm abnormalities (%) of all the stages *i.e.* diluted, equilibrated and post thaw stages differed significantly (P<0.05) within all aliquots *i.e.* control, trehalose added semen. Comparing table it was observed that the sperm abnormalities (%) between different stage *i.e.* diluted, equilibrated and post thaw stages, the trehalose and BHT added semen differed significantly (P<0.05) from control semen sample at diluted stage, whereas between trehalose and BHT added aliquots there was no significant (P<0.05) difference and also there was no significant difference between control and BHT added semen at diluted stage. Among equilibrated stage the trehalose supplemented semen sample differed significantly (P<0.05) from control, whereas there was no significant difference between trehalose and BHT supplemented aliquots and also there was no significant difference between control and BHT added semen samples at equilibration stage. Between the post thaw stages of all the aliquots *i.e.* control, trehalose and BHT supplemented semen differed non-significantly.

6.3.6 Hypo-osmotic swelling test (HOST)

In Table 4.24, our findings related to HOST of neat, diluted, equilibrated and post thaw semen are present. It was observed that the HOST positive (%) of neat semen with diluted, equilibrated and post thaw semen with or without additives *i.e.* control, trehalose and BHT added aliquots, a significant ($P<0.05$) difference was observed within and between all the aliquots at all the stages. It was also observed that the HOST positive (%) of post thaw semen sample differed significantly ($P<0.05$) within all aliquots *i.e.* control, trehalose and BHT added semen from diluted and equilibrated semen. Whereas, within control and trehalose the diluted semen differed significantly ($P<0.05$) from the equilibrated semen. Moreover, within BHT, the diluted semen differed non-significantly ($P<0.05$) from the equilibrated semen. Viewing table it was observed that the HOST positive (%) between different stage *i.e.* diluted, equilibrated and post thaw stages, the trehalose and BHT supplemented aliquots differed significantly ($P<0.05$) from control semen sample at diluted stage, whereas between trehalose and BHT added aliquots there was no significant ($P<0.05$) difference at diluted stage. Among equilibrated stage the trehalose and BHT supplemented aliquots differed significantly ($P<0.05$) from control, whereas there was no significant difference between BHT added aliquots at equilibration stage. Between the post thaw stages of all the aliquots *i.e.* control, trehalose and BHT supplemented semen differed non-significantly.

6.3.7 Lipid peroxidation

In Table 4.25, our findings related to lipid peroxidation of post thaw semen is given. It was observed that the MDA level between the post thaw semen with or without additives *i.e.* BHT added semen significantly ($P<0.05$) differ from control semen sample. Whereas, between trehalose and BHT supplemented aliquots there was no significant difference and also between control and trehalose supplemented semen sample.

6.3.8 Catalase

In Table 4.26, our findings related to catalase level of post thaw semen is given. It was observed that the catalase level between the post thaw semen with or without additive *i.e*. both trehalose and BHT supplemented aliquots significantly ($P<0.05$) differ from

control semen sample. Whereas, between trehalose and BHT added aliquots there was no significant difference in post thaw semen.

6.3.9 Superoxide dismutase

In Table 4.27, our findings related to SOD level of post thaw semen is given. It was observed that the SOD levels between the post thaw semen with or without additive *i.e*. both trehalose and BHT supplemented aliquots significantly ($P<0.05$) differ from control semen sample. Whereas, between trehalose and BHT added aliquots there was no significant difference in post thaw semen.

6.4 Conclusions

- **Group I-** The taurine added additive show promising effect on cryopreserved semen than cysteine though the increase was non-significant.
- **Group II-** The addition of ascorbic acid improved the quality of post-thaw semen than vitamin E though the increase was non-significant.
- **Group III-** The addition of BHT improved the quality of post-thaw semen than trehalose though the increase was non-significant.

6.5 Suggestion

In future more research with other concentrations and with some combinations may be tried for better results.

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Title of Master's Thesis	:	"Studies on role of some non-enzymatic anti-oxidant additives on the cryopreservation of bovine semen"
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CERTIFICATE-IV

Certified that all the necessary corrections as suggested by the External Examiner/ Evaluator and the Advisory Committee have been duly incorporated in the thesis entitled **"Studies on role of some non-enzymatic anti-oxidant additives on the cryopreservation of bovine semen"** submitted by **Mohammad Shaheem Butt,** Registration No. **J-19-MV-584.**

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Place: R.S.Pura
Date: 20/12/2021

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