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# EFFECT OF TREHALOSE ON QUALITY OF CRYOPRESERVED BANAS KANKREJ BULL SEMEN

## Thesis submitted to

**FACULTY OF VETERINARY SCIENCE SARDARKRUSHINAGAR DANTIWADA AGRICULTURAL UNIVERSITY**

*In partial fulfillment of the requirements*
*For the award of the degree of*

**MASTER OF VETERINARY SCIENCE**

(ANIMAL REPRODUCTION, GYNAECOLOGY AND OBSTETRICS)

**BY**

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*CERTIFICATE*

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# *D E C L A R A T I O N*

This is to declare that the whole of the research work reported in this thesis for partial fulfillment of the requirement for the degree of **"MASTER OF VETERINARY SCIENCE"** in **ANIMAL REPRODUCTION, GYNAECOLOGY AND OBSTETRICS** by the undersigned is the result of investigations done by me under the direct guidance and supervision of **Dr. H. C. NAKHASHI,** Associate Professor, Department of Veterinary Gynaecology and Obstetrics, College of Veterinary Science and Animal Husbandry, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar and that no part of the work has been submitted for any other degree so far.

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# "EFFECT OF TREHALOSE ON QUALITY OF CRYOPRESERVED BANAS KANKREJ BULL SEMEN"

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## ABSTRACT

The present investigation on the "Effect of Trehalose on Quality of Cryopreserved Banas Kankrej Bull Semen" was undertaken at Dama semen production Unit, Banas dairy, Palanpur for a period of 12 weeks. Total three Banas Kankrej bulls aged between 4 to 5 years were used and semen was collected using artificial vagina method once weekly for twelve weeks from each of the bulls. The semen was evaluated for physical characteristics and enzymatic profile at post-dilution, post-equilibration and frozen thawed stages of cryopreservation using different Trehalose concentrations viz. 50mM Trehalose, 100mM Trehalose, 150mM Trehalose and control without Trehalose.

Upon evaluation of 36 semen ejaculates (12 ejaculates from each bull), individual motility, sperm viability, sperm abnormality, osmotic resistance test (HOST) and acrosomal integrity were found to be 88.75 ± 0.25, 89.69 ± 0.32, 3.08 ± 0.15, 86.80 ± 0.24 and 90.72 ± 0.25 per cent, respectively. And the levels of oxidative stress parameters viz. lipid peroxidation and glutathione reductase were 49.96 ± 0.06 µmol/ml and 30.14 ± 0.06 U/L, respectively.

Correlation matrix revealed that all physical characteristics of semen had significant ($P < 0.05$) positive correlations among each other except the sperm abnormality which had inverse

correlations. Malondialdehyde (µmol/ml) as well as glutathione reductase (U/L) had significant positive correlations with individual motility, sperm viability and acrosomal integrity.

At post-dilution, post-equilibration and post-thaw stages of cryopreservation, 100mM Trehalose group had individual motility as 84.58 ± 0.31, 75.00 ± 0.40 and 64.16 ± 0.52; sperm viability as 85.66 ± 0.46, 81.41 ± 0.28 and 71.41 ± 0.31; HOST reactive sperm as 83.25 ± 0.32, 78.16 ± 0.42 and 73.91 ± 0.35 and acrosomal integrity as 91.08 ± 0.41, 85.41 ± 0.39 and 80.91 ± 0.43 per cent, respectively and were significantly ($P < 0.05$) higher as compared to that of the 50mM Trehalose, 150mM Trehalose and control groups.

Mean per cent sperm abnormality in 100mM Trehalose group were 4.67 ± 0.30, 6.34 ± 0.30 and 5.75 ± 0.35 at post-dilution, post-equilibration and post-thaw stages of cryopreservation, respectively and was significantly ($P < 0.05$) lower as compared to that of the 50mM Trehalose, 150mM Trehalose and control groups.

Mean Malondialdehyde (MDA) levels in 100mM Trehalose group were 43.92 ± 0.05, 34.20 ± 0.09 and 20.06 ± 0.13 µmol/ml at post-dilution, post-equilibration and post-thaw stages of cryopreservation, respectively and were significantly ($P < 0.05$) lower as compared to that of the 50mM Trehalose, 150mM Trehalose and control groups.

Mean glutathione reductase levels in 100mM Trehalose group were 55.83 ± 0.13, 64.00 ± 0.15 and 84.00 ± 0.16 U/L at post-dilution, post-equilibration and post-thaw stages of cryopreservation, respectively and was significantly ($P < 0.05$) higher as compared to that of the 50mM Trehalose, 150mM Trehalose and control groups.

Cryopreservation led to a significant ($P < 0.05$) decrease in semen quality parameters viz. individual motility, sperm viability, sperm abnormality, osmotic resistance test (HOST) and acrosomal integrity. Cryopreservation also led to a significant ($P < 0.05$) increase in Malondialdehyde (MDA) values and significant ($P < 0.05$) decrease in glutathione reductase (GSH) levels.

Conclusively, supplementation of 100mM Trehalose in the semen extender for cryopreservation was most appropriate concentration in view to preserve the semen quality in Banas Kankrej bull.

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# ABBREVIATION

| Abbreviation | Full form |
|---|---|
| < | Less than |
| > | Greater than |
| * | Significant at 5 percent |
| ± | Plus or Minus |
| °C | Degree Celsius |
| % | Percentage |
| Fig. | Figure |
| gm | gram |
| kg | kilogram |
| i.e. | that means |
| µg/ml | micro gram per milliliter |
| µmol/l | micro mol per liter |
| mg % | milligram percent |
| mg/dl | milligram per deciliter |
| ml | milliliter |
| NS | Non-Significant |
| $p < 0.05$ | Significant at 5 per cent level |
| $p < 0.01$ | Significant at 1 per cent level |
| mg/ml | milligram per milliliter |
| A.I. | Artificial Insemination |
| GLDB | Gujarat Livestock Development Board |
| GOI | Government of India |

| | |
|---|---|
| IS-4 | Integrated System-4 |
| IMV | Instrument deMedicine Veterinarian |
| MSP | Minimum Standard Protocol |
| RH | Relative Humidity |
| df | degree of freedom |
| S.E. | Standard Error |
| CRD | Completely Randomized Design |
| ANOVA | Analysis of Variance |
| r | correlation factor |
| *et al.* | and associates |
| LPO | Lipid Peroxidation |
| HOST | Hypoosmotic Swelling Test |
| K x J | Kankrej X Jersey |
| K x HF | Kankrej X Holstein Friesian |
| GSH | Glutathione |
| EDTA | Ethylene Diamine Tetra Acetic Acid |
| S | Sahiwal bull |
| mM | Milli Mole |
| µmol/l | Micro Mole Per Liter |
| MDA | Malondialdehyde |
| BHT | Butylated Hydroxyl Toluene |
| HBP | Heparin Binding Protein |
| Hr | hour |
| IU/L | International Unit per Liter |
| U/L | Unit per Liter |
| $H_2O_2$ | Hydrogen Peroxide |

| | |
|---|---|
| nmol/$10^8$ | Nanomole per $10^8$ cells |
| SS | Sum of Squares |
| MSS | Mean Sum of Squares |
| SEM | Standard Error of Mean |
| MSP | Minimum Standard Protocol |
| viz. | namely |
| PDS | Post Diluted Stage |
| PES | Post Equilibrated Stage |
| PTS | Post Thawed Stage |
| TFYG | Tris Fructose Yolk Glycerol |
| UV | Ultraviolet |
| DTNB | 5, 5'-dithio-bis-2-nitrobenzoic acid |
| TCA | Trichloroacetic Acid |
| v/v | Volume by Volume |
| g | Gravitation |
| HF | Holstein Friesian |
| S.E. | Standard Error/s |
| p.s.i. | pound pressure per square inch |
| OD | Optical Density |
| w/v | Weight by Volume |
| TBA | Thiobarbituric Acid |
| mOsM | Milli Osmoles |
| rpm | Rotations Per Minute |

# CHAPTER 1: INTRODUCTION

India is predominantly an agricultural country with about 70% of its population dependent on income from agriculture. Livestock rearing is done by majority of rural household, majority of landless and marginal farmers which provides employment and supplementary income. Cattle and buffaloes are kept for milk production, for various farm operations, village transport, irrigation, and production of manure in addition to agriculture.

According to 18$^{th}$ Livestock Census, 2007, India had about 200 million cattle and 104 million buffalo's population. Livestock sector contributes for about 25% of the gross agricultural output, milk alone provides around 63% of the total livestock output. India had 28 recognized breeds of cattle and 7 recognized breeds of buffaloes (FAO, 2009). Buffaloes contribute maximum milk production followed by indigenous cattle, according to 17$^{th}$ livestock census, 2003.

Gujarat is the cradle of India's White Revolution in dairy industry and top state for milk production in the country. Gujarat state is well known for different breeds of cattle viz. Dangi, Gir, Kankrej and crossbred. But Kankrej is very high prized fast, powerful draft cattle of Gujarat and also called as "Banas Cattle" and have fair milk production. Banas Kankrej cattle that had been developed from Kankrej breed at Livestock Research Station, SDAU, has proved to be superior to crossbreds with respect to milk production and disease resistance (Annual progress Report-2009, LRS, SDAU, Gujarat). Many farmers in state maintain 2-3 female cattle, but cannot maintain sires of superior germplasm.

To meet this requirement sires of superior germplasm are maintained at institute farms or units for the purpose of providing good quality semen which have facilities of deep freezing. Artificial insemination helps in disseminating

the frozen semen to rural areas for improvement of native cattle. The main goal of research in the laboratory is evaluation of semen, either fresh or frozen-thawed, to predict its fertility. Some of the semen characteristics such as sperm motility, viability, concentration, etc. have been found to be significantly correlated with freezability and/or fertility of bovine semen and hence are currently being used as routine tests for the assessment of semen quality (Bhoite *et al.* 2005).

The enhancement of artificial insemination is a viable tool in genetic improvement programs. However, the biggest problem in exploiting cryopreserved semen is damage to sperm membrane structures during freezing and thawing, which leads to fewer viable and motile cells post-thawing. An intact and functional plasma membrane is a key component of the cell and must be maintained during freezing conditions if the cell is to be kept alive. Therefore, cryoprotectants are included in cryopreservation extender to reduce the damaging effects of the freezing process (Badr *et al.* 2010).

Various effects were observed when different sugars that are not capable of diffusing across a plasma membrane, such as lactose, sucrose, raffinose, trehalose, or dextrans are added to the diluent. The sugars create an osmotic pressure, inducing cell dehydration and results in lower incidence of intracellular ice formation. These sugars also interact with the phospholipids in the plasma membrane, reorganizing the membrane which results in sperm that is better suited to surviving the cryopreservation process (Uysal *et al.* 2007). Unlike, the simple sugars glucose and fructose, trehalose a disaccharide acts primarily as cryoprotectants. More recently, trehalose has been included in bull sperm cryopreservation. Addition of trehalose to bull semen extenders is known to provide a modest improvement in fertility when used in combination with glycerol.

Trehalose, is a non-reducing disaccharide in which two glucose molecules are linked together in a 1, 1-glycosidic linkage (α-d-glucopyranosyl-l, 1-α-d-glucopyranoside), commonly found in high concentrations in many organisms

such as yeast and fungal spores capable of surviving complete dehydration. Supplementation of semen extenders with trehalose is well known to improve the motility and viability of cryopreserved mammalian sperm cells. Trehalose probably plays a key role in preventing deleterious alteration to the membrane during reduced-water states and the action of trehalose appears to be connected with its ability to replace water at the membrane/solution interface. In addition, trehalose have several functions in sperm extender, including providing energy substrate for the sperm cell during incubation, maintaining the osmotic pressure of the diluent, acting as a cryoprotectants and increase sperm in membrane fluidity, rendering the spermatozoa capable of enduring freeze- thawing damage. Moreover, trehalose presumably involves a stabilization of certain cell proteins and/or lipid in membranes during stresses such as cryopreservation, heat, desiccation or oxidative stress. When trehalose was added in hypertonic conditions, it showed a synergic effect with glycerol used as a cryoprotectant in order to avoid intracellular ice crystal formation (Badr *et al*. 2010).

Sperm cells have a high content of unsaturated fatty acids in their membranes and they lack a significant cytoplasmic component containing antioxidants. Therefore, sperm cells are highly susceptible to lipid peroxidation (LPO) by $O_2$ and $H_2O_2$. Semen contains appreciable amounts of antioxidants that balance lipid peroxidation and prevent excessive peroxide formation (Muzafer *et al*. 2012).

Cryopreservation of spermatozoa is associated with an oxidative stress induced by free radicals. The freezing process produces physical and chemical stress on the sperm membrane which in turn reduces sperm viability and fertilizing ability. In recent years, antioxidants have been used to protect spermatozoa from the deleterious effects of cryopreservation and free radicals are eliminated by antioxidants (Umut *et al*. 2012).

Glutathione, a naturally occurring tri-peptide in semen plays an important role in scavenging reactive oxygen intermediates and other radicals with the help of the glutathione reductase/peroxidase cycle. Glutathione has many

important functions in the cellular physiology and metabolism including the protection of the cell from oxidative stress, synthesis of protein as well as DNA and gamete cell fertilization. Glutathione can influence cell metabolism through detoxication and by preventing the formation of free radicals in spermatozoa (Serpil *et al*. 2009).

Hence, this semen extender additive required to be scanned for their efficiency before a package of practice on these aspects can be suggested for Kankrej bull semen. In case, the standard semen extender additive has been evolved for Kankrej bull semen, it might help not only in extending preservability of semen for this species but also give way for fastest utilization of Kankrej bull semen towards getting improvement.

Therefore, purpose for the proposed study was to determine the effect of trehalose and the definite protocol on Banas Kankrej bull semen with the following objectives:

## OBJECTIVES:

1. To study the effect of trehalose on physical characteristics of Banas Kankrej bull semen during cryopreservation.
2. To study the oxidative stress following addition of trehalose on Banas Kankrej bull semen during cryopreservation.
3. To evolve the suitable trehalose concentration on the basis of post thawed seminal characteristics.

# CHAPTER 2: REVIEW OF LITERATURE

The present investigation involved the work on physical characteristics and enzymatic analysis of different trehalose concentrations at post-dilution, post-equilibration and frozen-thawed stages of bull semen. The scientific literature cited for the topic has been reviewed and documented under the following heads and subheads.

## 2.1. EVALUATION OF FRESH SEMEN PHYSICAL CHARACTERISITICS

2.1.1. Individual Motility

2.1.2. Sperm Viability

2.1.3. Sperm Abnormality

2.1.4. Osmotic resistance test (HOST)

2.1.5. Acrosomal Integrity

## 2.2. EFFECT OF TREHALOSE AS A SEMEN EXTENDER ADDITIVE

2.2.1. Individual Motility

2.2.2. Sperm Viability

2.2.3. Sperm Abnormality

2.2.4. Osmotic resistance test (HOST)

2.2.5. Acrosomal Integrity

## 2.3. EVALUATION OF BIOCHEMICAL PARAMETERS OF SEMEN EXTENDER ADDITIVE

2.3.1. Lipid peroxidation

2.3.2. Glutathione reductase

## 2.1. EVALUATION OF FRESH SEMEN PHYSICAL CHARACTERISITICS

### 2.1.1. Individual Motility

Individual motility is a routine test carried out to assess semen quality immediately after collection. Shelke and Dhami (2001) and Bhoite *et al.* (2005) reported that initial sperm motility is an important attribute for acceptance or rejection of the ejaculate for further processing and use in AI, and it is positively correlated with keeping quality, freezability and fertility of that sample.

Patel *et al.* (1989) reported the individual motility as 85.44 ± 1.56 per cent in halfbred bulls (K x J; K x HF). Singh and Pangawkar (1990) recorded the initial motility as 79.41 ± 0.38, 76.57 ± 0.90, 79.74 ± 0.64, 76.77 ± 1.04 and 67.49 ± 0.90 per cent in Holstein-Friesian, Jersey, Holstein-Friesian x Sahiwal, Karan-Swiss x Friesian and Holstein-Friesian x Red Dane x Sahiwal bulls, respectively.

Sharma *et al.* (1992) recorded the individual motility as 76 per cent in crossbred bulls. Dhami and Sahni (1994) recorded the individual motility as 75.86 ± 0.48 per cent in HF bulls. Nath *et al.* (1996) reported the individual motility as 72.52 ± 2.03 per cent in Jersey bulls.

Anzam *et al.* (1998) observed a highly significant difference between fresh and post-thaw sperm motility in Sahiwal bulls. Prasad *et al.* (1999) reported the individual motility as 65.00 ± 3.76 and 53.75 ± 4.24 per cent in halfbred and cross breed bulls, respectively. Tomar *et al.* (2000) observed the sperm motility as 77.14 ± 0.01 per cent in fresh semen of HF bulls. This value significantly ($P < 0.01$) declined at post-dilution and post-thaw stages being 60.02 ± 0.01 and 43.72 ± 0.02 per cent, respectively.

Shelke and Dhami (2001) reported the individual motility as 67.87 ± 2.69 per cent in Gir bulls. All semen traits were highly significantly and positively

interrelated among each other (r= 0.277 to 0.925), except abnormal sperm which had negative correlations. Singh *et al.* (2002) reported the mean sperm motility in buffalo bulls as 60.35 ± 1.61 and 43.92 ± 1.75 per cent at post-dilution and post-thaw stages, respectively.

Mathur *et al.* (2002) reported the individual motility as 52.48 ± 0.78 per cent in Frieswal bulls. Dhami *et al.* (2003) reported the individual motility as 72.31 ± 3.19, 65.83 ± 7.96, 64.50 ± 4.12, 55.83 ± 8.28 and 58.00 ± 3.51 per cent in Gir, Jersey, HF, Crossbred and Jaffarabadi buffalo bulls, respectively.

Kumar (2004) reported the mean progressive motility in Jersey breed bulls as 75.42 ± 0.55 per cent in neat semen, 74.31 ± 0.41, 71.06 ± 0.65 and 47.58 ± 1.08 per cent at post-dilution, post-equilibration and post-thaw stages, respectively.

Rana and Dhami (2004) reported that the sperm motility in Gir bulls revealed significant negative correlation with sperm concentration and positive correlation with mass activity and freezability, but in Jaffarabadi bulls it had significant positive correlation with live sperm, sperm concentration and freezability.

Mandal *et al.* (2005) reported the individual motility as 52.71 ± 1.31 per cent in Sahiwal bulls. Raval (2006) reported the individual motility as 81.20 ± 0.98 per cent in triplebred (HF × J × Kankrej) bulls during autumn season. Sori *et al.* (2006) reported the individual motility as 69.36 ± 1.76 per cent in Ethiopian indigenous (Horro) bulls.

Srivastava and Kumar (2006) reported the individual motility as 82.19 ± 3.64 and 68.13 ± 6.24 per cent in HF and crossbred bulls, respectively. Shaha *et al.* (2008) reported the individual motility as 56.6 to 76 per cent in four mixed cattle breeds (Sahiwal × Zebu, Sindhi × Zebu, Jersey × Zebu and Holstein-Friesian × Zebu).

Perumal *et al.* (2009) reported the mean individual motility in good and poor freezable bulls as 77.13 ± 0.60 and 75.33 ± 0.77) per cent in Jersey crossbred bulls. Patel (2011) found the average individual motility as 74.00 ± 0.67, 72.50 ± 0.83 and 72.00 ± 1.11 per cent in Mehsana buffalo bulls, respectively. Patel (2012) reported that the average individual motility as 86.15 ± 0.30 per cent which ranged between 80 to 92 per cent in Kankrej bulls.

Sharma (2012) found that the initial progressive motility had significant ($P < 0.01$) positive correlation with HOST and acrosomal integrity in Jersey x local hill cattle crossbred bulls. Ray and Ghosh (2013) recorded the progressive sperm motility in fresh semen of Sahiwal bulls varying from 65 to 85 per cent with the overall mean of 76.73 ± 0.43 per cent. Progressively motile sperm had significant ($P < 0.01$) positive correlation with HOST positive sperm.

Srivastava and Kumar (2014) recorded the individual motility as 82.2 ± 3.6 and 78.1 ± 6.3 per cent in Holstein Friesian (HF) and crossbred bulls, respectively.

### 2.1.2. Sperm Viability

A different staining method for differentiating live and dead bull spermatozoa was proposed by Evans and Maxwell (1987) using eosin as the sperm staining substance and nigrosin as the background stain. This mixture gave a very uniform preparation with stained (dead) cells clearly distinguishable from the live or unstained cells.

Singh and Pangawkar (1990) reported the mean live sperm per cent to be 86.75 ± 0.41, 82.97 ± 0.90, 86.40 ± 0.48, 82.12 ± 0.92 and 74.53 ± 1.04 in Holstein-Friesian, Jersey, Holstein-Friesian x Sahiwal, Karan-Swiss x Friesian and Holstein-Friesian x Red Dane x Sahiwal bulls, respectively.

Sharma *et al.* (1992) reported live sperm per cent to be 78 in crossbred bulls. Nath *et al.* (1996) reported the live sperm per cent to be 82.83 ± 1.17 in Jersey bulls. Dhami *et al.* (1998) reported the mean live sperm per cent to be

87.44 ± 0.71, 87.78 ± 0.72 and 86.83 ± 0.48 in Friesian bulls during hot (Mar-Jun), humid (Jul-Oct) and cold (Nov-Feb) seasons, respectively.

Prasad *et al.* (1999) reported that live sperm per cent to be 65.00 ± 3.76 and 53.75 ± 4.24 in halfbred and cross bred bulls, respectively. Kanwal *et al.* (2000) reported that the percentage of dead spermatozoa was significantly higher ($P < 0.01$) in the semen of crossbred bulls as compared to that of the Nili-Ravi buffalo bulls.

Singh *et al.* (2000) documented the mean value of live sperm in Jersey, Sahiwal and halfbred bull semen as 82.50 ± 0.04, 85.75 ± 0.04 and 83.40 ± 0.04 per cent, respectively. Tomar *et al.* (2000) observed that the live sperm as 73.69 ± 0.12 per cent in fresh semen of HF bulls. This value significantly ($P < 0.01$) declined at post-dilution and post-thaw stages being 55.36 ± 0.12 and 37.02 ± 0.03 per cent, respectively.

Rana (2001) reported the mean values of live sperm as 71.85 ± 1.49 per cent in Gir bulls. Dhami *et al.* (2003) reported the live sperm count to be 80.81 ± 1.29, 78.17 ± 3.66, 79.40 ± 2.88, 82.50 ± 4.92 and 78.20 ± 1.84 per cent in Gir, Jersey, HF, Crossbred and Jaffarabadi buffalo bulls, respectively.

Kumar (2004) recorded the mean live sperm in Jersey breed bulls as 81.39 ± 0.88 per cent in neat semen, 80.53 ± 0.76, 76.77 ± 0.68 and 53.16 ± 1.19 per cent at post-dilution, post-equilibration and post-thaw stages, respectively. The live sperm had significant ($P < 0.01$) positive correlation with progressively motile spermatozoa.

Rana and Dhami (2004) reported that the live sperm per cent was negatively correlated with abnormal sperm in Gir bulls and positively with freezability in Jaffarabadi bulls.

Bhoite *et al.* (2005) reported the mean values of live spermatozoa as 76.45 ± 0.64, 76.89 ± 0.38 and 76.04 ± 0.40, 77.81 ± 0.36, 77.02 ± 0.27 and 77.31 ± 0.26, 71.21 ± 0.59, 70.96 ± 0.55 and 71.27 ± 0.46 and 78.88 ± 0.55, 78.04 ±

0.45 and 78.55 ± 0.47 per cent in crosses of Gir bulls: IFG (50% Holstein-Friesian + 50% Gir Interse), IFJG (50% Holstein-Friesian + 25% Jersey + 25% Gir Interse), IJFG (50% Jersey + 25% Holstein-Friesian + 25% Gir Interse) and IBFG (50% Brown Swiss + 25% Holstein-Friesian + 25% Gir Interse) during summer, rainy and winter seasons, respectively.

Mandal *et al*. (2005) reported live sperm per cent to be 70.40 ± 3.9 in Sahiwal bulls. Raval (2006) reported the mean live sperm to be 88.89 ± 0.83 per cent in triplebred (HF × J × Kankrej) bulls during autumn season. The live sperm had significant ($P < 0.01$) positive correlation with initial motility.

Johar *et al*. (2006) reported the mean live sperm as 76.00 ± 1.90 and 58.64 ± 6.01 per cent in Jersey and its crossbred bulls, respectively. Sori *et al*. (2006) reported the sperm viability as 81.47 ± 1.20 per cent in Ethiopian indigenous (Horro) bulls.

Srivastava and Kumar (2006) reported the live sperm as 87.38 ± 11.46 and 84.13 ± 2.31 in HF and crossbred bulls, respectively. Shaha *et al*. (2008) reported a range of 18.4 to 24.7 per cent of dead sperm in semen of four cow bulls (Sahiwal × Zebu, Sindhi × Zebu, Jersey × Zebu and Holstein-Friesian × Zebu) of mixed breeds.

Perumal *et al*. (2009) reported the live sperm in good and poor freezable bulls as 89.95 ± 0.49 per cent 85.22 ± 0.28 per cent in Jersey crossbred bulls. Rajoria *et al*. (2011) reported the live sperm as 88.23 ± 1.82 and 88.60 ± 1.71 per cent in Tharaparkar bulls during winter and summer seasons, respectively.

Patel (2012) reported the per cent live sperm to be 90.58 ± 0.20 with a range of 81 to 94 per cent in Kankrej bulls. Sharma (2012) found that the sperm viability had significant ($P < 0.01$) positive correlation with progressive motility, HOST and acrosomal integrity in Jersey x local hill cattle crossbred bulls.

Desai (2013) observed the live sperm as 90.27 ± 0.44 and 90.15 ± 0.52, 90.10 ± 0.45 and 90.27 ± 0.46 per cent in Kankrej and cross-bred bulls during the post rainy and winter seasons, respectively.

Ray and Ghosh (2013) recorded the live sperm in fresh semen of Sahiwal bulls varying from 79 to 88 per cent with the overall mean of 83.37 ± 0.34 per cent. The live sperm had significant ($P < 0.01$) positive correlation with intact acrosome sperm and HOST positive sperm and negative correlation with abnormal sperm. Srivastava and Kumar (2014) reported the live sperm as 87.4 ± 1.4 and 84.1 ± 2.3 per cent in Holstein Friesian (HF) and crossbred bulls, respectively.

### 2.1.3. Sperm Abnormality

The fertility of bull depends upon morphologically normal spermatozoa present in the ejaculate. The fertility is hardly affected if the abnormal spermatozoa do not exceed 15 to 20 % (Saxena, 2000).

Patel *et al.* (1989) reported the abnormal sperm per cent to be 5.76 ± 0.37 in halfbred bulls (Kankrej x J; Kankrej x HF). Singh and Pangawkar (1990) reported the total sperm abnormalities as 10.01 ± 0.14, 10.45 ± 0.20, 8.48 ± 0.15, 8.82 ± 0.16 and 15.80 ± 0.23 per cent in breeds of Holstein-Friesian, Jersey, Holstein-Friesian x Sahiwal, Karan-Swiss x Friesian and Holstein-Friesian x Red Dane x Sahiwal bulls, respectively.

Salah *et al.* (1992) reported the sperm abnormalities as 17.79 ± 0.61, 8.89 ± 0.81 and 11.11 ± 0.91 per cent in Holstein bulls during hot dry, cold humid and warm humid seasons, respectively. Dhami and Sahni (1994) recorded the abnormal sperm per cent to be 8.86 ± 0.38 in HF bulls.

Rao *et al.* (1996) recorded the overall mean per cent sperm abnormality of head, mid-piece, tail and protoplasmic droplets as 2.18 ± 0.17, 3.16 ± 0.34, 1.73 ± 0.86 and 1.48 ± 0.92 in Jersey and its crosses bulls, respectively. Dhami *et al.* (1998) documented the mean abnormal sperm as 9.94 ± 0.63, 9.00 ± 0.52 and

8.86 ± 0.38 per cent in Friesian bulls during hot, humid and cold seasons, respectively.

Baburao *et al.* (1999) reported overall mean value of head, mid piece, tail and total sperm abnormalities in fresh semen of Punganur bulls as 4.93 ± 0.19, 2.03 ± 0.13, 5.27 ± 0.24 and 12.23 ± 0.24 per cent, respectively. Whereas in Ongole bulls, the average sperm abnormalities of sperm tail, head and protoplasmic droplets were 5.72 ± 0.42, 24.5 ± 0.29 and 5.31 ± 0.26 per cent, respectively.

Prasad *et al.* (1999) reported the abnormal sperm per cent to be 14.13 ± 1.41 and 17.38 ± 1.04 in halfbred and crossbred bulls, respectively. Sansone *et al.* (2000) reported that abnormalities were higher with sperm heads being 5.78 ± 2.1 per cent, while middle piece abnormalities were less than 1 per cent and abnormal tails varied from 3.92 ± 1.0 to 5.7 ± 0.4 per cent, in Nili–Ravi buffaloes.

Tomar *et al.* (2000) observed the abnormal sperm as 1.54 ± 0.01 per cent in fresh semen of HF bulls. This value significantly ($P < 0.01$) increased at post-dilution and post-thaw stages being 3.12 ± 0.01 and 4.59 ± 0.01 per cent, respectively. Rana (2001) reported the mean values of total abnormalities as 22.50 ± 1.40 per cent in Gir bulls, whereas, head, mid-piece and tail abnormalities were 4.35 ± 0.47, 2.42 ± 0.38 and 15.65 ± 1.18 per cent, respectively.

Shelke and Dhami (2001) recorded the mean values of head, midpiece, tail and total sperm abnormalities as 1.78 ± 0.34, 4.39 ± 0.47, 9.48 ± 0.77 and 15.54 ± 1.09 per cent in Gir bulls, respectively. All semen traits were highly significantly and positively interrelated among each other (r= 0.277 to 0.925), except abnormal sperm which had negative correlations.

Andrabi *et al.* (2002) reported the mean values of total sperm abnormality as 21.50 ± 9.1, 16.70 ± 7.6, 35.60 ± 11.5, 31.12 ± 7.0 and 21.71 ± 6.96 per cent in crossbred (Friesian × Sahiwal) bulls, comprising abnormal head as 4.00 ± 3.9,

4.0 ± 2.7, 5.71 ± 3.1, 11.42 ± 6.4 and 10.71 ± 1.00, abnormal mid-piece as 6.29 ± 1.86, 3.14 ± 3.5, 23.80 ± 13.3, 11.86 ± 3.9 and 7.42 ± 6.1, abnormal tail as 12.14 ± 9.22, 9.50 ± 5.96, 6.10 ± 3.5, 7.86 ± 4.8 and 3.57 ± 2.37 per cent during the month January, February, October, November and December, respectively.

Dhami *et al.* (2003) documented the mean values of abnormal sperm as 13.12 ± 1.48, 24.50 ± 4.78, 14.10 ± 2.04, 17.83 ± 4.74 and 16.30 ± 3.93 per cent in Gir, Jersey, HF, Crossbred and Jaffarabadi buffalo bulls, respectively.

Jain *et al.* (2004) reported the mean values of sperm head, mid-piece, tail and total abnormalities in Karan-Fries bulls as 4.73, 4.59, 4.96 and 14.28 per cent, respectively. Rana and Dhami (2004) reported that the ejaculate volume had significant ($P < 0.05$) positive correlation with abnormal sperm per cent whereas, inverse correlation with live sperm count in Gir bulls. Mandal *et al.* (2005) reported the abnormal sperm as 18.40 ± 3.03 per cent of which head, mid-piece and tail abnormalities were 4.50 ± 1.37, 6.80 ± 1.82 and 7.10 ± 1.26 per cent in Sahiwal bulls, respectively.

Raval (2006) recorded the mean abnormal sperm as 7.45 ± 0.42 per cent in triplebred (HF × J × Kankrej) bulls during autumn season. The mean per cent sperm abnormalities with head, mid-piece and tail in fresh semen were 2.33 ± 0.11, 1.37 ± 0.11 and 3.78 ± 0.21 per cent, respectively. The abnormal spermatozoa had significant ($P < 0.01$) negative correlation with initial motility and live sperm.

Dhanju *et al.* (2006) reported the mean sperm abnormalities varying between 15.44 ± 4.35 to 54.77 ± 7.2 per cent in five crossbred bulls. Sori *et al.* (2006) reported total morphological abnormalities to be 5.90 ± 0.50 per cent of which head, mid-piece and tail abnormalities were 1.81 ± 0.20, 2.60 ± 0.35 and 1.50 ± 0.18 per cent in Ethiopian indigenous (Horro) bulls.

Srivastava and Kumar (2006) recorded the total abnormal sperm were 10.06 ± 1.57 and 12.63 ± 2.34 per cent in HF and crossbred bulls, respectively. Rajoria *et al.* (2011) reported the average sperm abnormalities were 9.47 ± 0.43

and 9.30 ± 0.35 per cent in Tharaparkar bulls during winter and summer seasons, respectively.

Talluri *et al.* (2011) reported the mean total abnormal sperm percent to be 8.08 ± 0.10 in Ongole bulls. The mean per cent of head, mid piece and tail abnormalities were 2.34 ± 0.06, 6.96 ± 0.90 and 4.72 ± 0.10 per cent, respectively.

Patel (2012) reported the abnormal sperm had a range of 3 to 9 per cent with the mean value 4.24 ± 0.03 in Kankrej bulls. Desai (2013) observed the sperm abnormal count as 9.08 ± 0.25 and 9.79 ± 0.20, 8.85 ± 0.26 and 9.69 ± 0.24 per cent in Kankrej and cross-bred bulls during the post rainy and winter seasons, respectively.

Ray and Ghosh (2013) recorded the abnormal sperm in fresh semen of Sahiwal bulls varying from 7 to 14 per cent with the overall mean of 9.97 ± 0.28 per cent. The abnormal sperm had significant ($P < 0.01$) negative correlation with HOST positive sperm and intact acrosome sperm. Srivastava and Kumar (2014) reported the total abnormal sperm as 10.1 ± 1.5 and 12.6 ± 2.3 per cent in Holstein Friesian (HF) and crossbred bulls, respectively.

### 2.1.4. Osmotic resistance test (HOST)

The hypo-osmotic swelling test (HOST) has been proved to be a good tool for evaluating the membrane integrity of spermatozoa of various domestic animals including cattle. The functionally active spermatozoa exposed to a hypo osmotic stress swell due to influx of water and subsequently increase in volume to establish the equilibrium between the cytosol and the extra cellular milieu, spermatozoa with compromised or inactive membranes are unable to regulate water influx and remain not swollen. Thus, hypo osmotic swelling test may be useful in assessing changes in the sperm membrane functional integrity during freezing and thawing procedures (Revell and Mrode, 1994).

Salah *et al.* (1992) reported the mean HOST reactive sperm were 58.93 ± 0.72, 59.33 ± 1.01, 58.63 ± 1.01 and 58.94 ± 0.46 per cent in Holstein bulls

during summer (Hot-Dry, June-October), winter (Warm-Humid, November-February), spring (Warm-Humid, March-May) and pooled seasons, respectively.

Prasad *et al.* (1999) carried out hypo-osmotic swelling test (HOST) using fresh and freeze thawed semen in crossbred bulls and observed that the percentage of coiling of tail was strongly correlated to mass movement, progressive motility, live sperm count, total intact acrosome and sperm concentration.

Kumar (2004) reported the mean HOST reactive sperm in Jersey breed bulls as 66.42 ± 1.12 per cent in neat semen, 67.47 ± 0.05, 63.31 ± 1.11 and 40.78 ± 0.93 per cent at post-dilution, post-equilibration and post-thaw stages, respectively. The HOST reactive sperm had significant ($P < 0.01$) positive correlation with progressively motile spermatozoa.

Rana and Dhami (2004) recorded the HOST positive sperm per cent as 84.80 ± 0.89 and 83.50 ± 1.24 in Gir and Jaffarabadi bulls, respectively. Kumar *et al.* (2006) reported the mean HOST positive sperm per cent as 67.60 ± 2.53 in crossbred cattle (*Bos indicus* × *Bos taurus*).

Srivastava and Kumar (2006) reported the mean HOST positive sperm as 49.38 ± 2.80 and 42.06 ± 3.57 per cent in HF and crossbred bulls, respectively. The HOST positive sperm was significantly ($P < 0.01$) positively correlated with initial progressive motility, live sperm count and intact acrosome and negatively correlated with total sperm abnormalities in both the breeds.

Lodhi *et al.* (2008) observed the mean HOST positive sperm as 82.25 per cent both in Nili-Ravi and Sahiwal bulls. Statistical analysis of the data revealed a significant ($P < 0.05$) positive correlation of HOST positive sperm with progressive motility, sperm viability and morphologically normal spermatozoa in both species.

Perumal *et al.* (2009) reported the HOST positive sperm in good and poor freezable bulls as 85.78 ± 0.42 and 80.38 ± 0.32 per cent in Jersey crossbred

bulls. Serpil *et al.* (2009) reported the mean values of HOST reactive sperm as 74.59 ± 2.20 per cent in neat semen and 46.59 ± 1.62 per cent in frozen-thawed semen in exotic bulls.

Vera-Munoz *et al.* (2009) recorded the mean HOST reactive sperm as 68.1 per cent in raw semen and 48.8 per cent in frozen-thawed semen in cross-bred bulls indicating the damage to the plasma membrane by the cryopreservation process.

Martins *et al.* (2011) reported the mean HOST reactive sperm as 60.3 per cent of in raw semen and 30.8 per cent in frozen-thawed semen in Nellore bulls indicating the damage to the plasma membrane by the cryopreservation process. Rajoria *et al.* (2011) reported the HOST reactive sperm an as 81.43 ± 1.49 and 78.20 ± 1.94 in Tharaparkar bulls during winter and summer seasons.

Sharma (2012) found that the HOST positive sperm had significant ($P < 0.05$) positive correlation with acrosomal integrity in Jersey x local hill cattle crossbred bulls. Desai (2013) observed the HOST positive sperm per cent as 77.52 ± 0.38 and 73.31 ± 0.48, 78.04 ± 0.33 and 72.79 ± 0.41 per cent in Kankrej and cross-bred bulls during the post rainy and winter seasons, respectively.

Ray and Ghosh (2013) recorded the HOST positive sperm in fresh semen of Sahiwal bulls varying from 74 to 84 per cent with the overall mean of 79.00 ± 0.55 per cent. The HOST positive sperm had significant ($P < 0.05$) positive correlation with progressive sperm motility and live sperm and negative correlation with abnormal sperm.

Srivastava and Kumar (2014) recorded the HOST positive sperm as 69.4 ± 2.8 and 64.1 ± 2.3 per cent in Holstein Friesian (HF) and crossbred bulls, respectively.

### 2.1.5. Acrosomal Integrity

The presence of an acrosomal cap is important in the fertilization process and has been documented to be highly related with fertility of the bull semen. The acrosomal damage might occur during dilution, cooling, freezing and thawing processes. The assessment of semen quality for evaluating the fertility of a particular male or the evaluation of semen preservation procedures requires assessment of both the viability and the acrosomal status of spermatozoa in either fresh or preserved semen.

Pathak *et al.* (1989) reported the acrosomal integrity in HF x S x Hariana bulls to be 79.23 ± 3.41 per cent. The various acrosomal abnormalities included ruffled, swollen, incomplete, damaged and knobbed acrosomes were 0.23 ± 0.06, 3.29 ± 0.67, 5.35 ± 0.94, 11.34 ± 1.82 and 0.52 ± 0.10 per cent, respectively.

Sharma *et al.* (1992) recorded the per cent intact acrosome in fresh (74.05 ± 1.15) and post-thaw semen (69.40 ± 1.29) in HF x J x Hariana bulls along with the incidence of knobbed, swollen, denuded, incomplete and ruffled acrosomes.

Singh *et al.* (1992) found the mean sperm acrosomal abnormalities as 6.41 ± 0.42 and 7.38 ± 0.48 per cent in Hariana and Friesian x Hariana bulls, respectively. Nath *et al.* (1996) reported the acrosomal abnormality was 8.75 ± 0.67 per cent in Jersey bulls. Anzam *et al.* (1998) reported the post-thaw swollen sperm and normal acrosome as 23.4 ± 9.5 and 26.4 ± 10.4 per cent in Sahiwal bulls, respectively.

Prasad *et al.* (1999) reported the intact acrosome were 81.00 ± 1.58 and 80.29 ± 2.22 per cent in halfbred and cross breed bulls, respectively. Singh *et al.* (1999) reported the post-thaw acrosomal integrity as 54.50 ± 1.38 per cent in HF bulls. Rasul *et al.* (2001) documented that the per cent normal acrosome during cryopreservation of buffalo spermatozoa remained higher after dilution, cooling or equilibration (73.2 ± 2.4) than after freezing and thawing (61.8 ± 2.4), respectively.

Kumar (2004) reported the mean acrosomal integrity in Jersey breed bulls as 87.28 ± 0.94 per cent in neat semen, 86.17 ± 0.94, 82.47 ± 1.05 and 67.92 ± 0.93 per cent at post-dilution, post-equilibration and post-thaw stages, respectively. Rana and Dhami (2004) recorded the mean values of intact acrosomes as well as swollen, ruffled and denuded acrosomes in fresh Gir bulls semen as 84.80 ± 0.90, 2.12 ± 0.31, 2.22 ± 0.33, 10.70 ± 0.83 percent, respectively. The corresponding values were 74.55 ± 1.34, 3.21 ± 0.34, 7.81 ± 0.85 and 18.55 ± 1.45 per cent at post-thaw stage.

Raval (2006) reported the mean value of intact acrosome and damaged acrosome as 92.33 ± 0.39 and 7.67 ± 0.33 per cent in triplebred (HF × J × Kankrej) bulls during autumn season, respectively. The overall mean per cent of sperm with swollen, ruffled, denuded and detached abnormalities in fresh semen were 2.25 ± 0.11, 2.03 ± 0.14, 2.00 ± 0.16 and 2.55 ± 0.17, respectively.

Sori *et al*. (2006) reported the normal acrosome as 98.23 ± 0.33 per cent in Ethiopian indigenous (Horro) bulls. Srivastava and Kumar (2006) reported the intact acrosome were 89.94 ± 1.57 and 87.38 ± 2.34 per cent in HF and crossbred bulls, respectively.

Perumal *et al.* (2009) reported the mean acrosomal integrity in good and poor freezable bulls as 6.46 ± 0.90 and 12.73 ± 1.12 per cent in Jersey crossbred bulls. Giri *et al*. (2011) reported the per cent of intact acrosome semen to be 77.75 ± 0.75 in Jersey bulls. Rajoria *et al*., (2011) found the intact acrosome per cent to be 90.77 ± 1.68 and 91.47 ± 1.61 per cent in Tharaparkar bulls during winter and summer seasons, respectively.

Patel (2012) reported the mean intact acrosome as 81.17 ± 0.11 percent which ranged between 79 to 88 percent in Kankrej bulls. Sharma (2012) found that the acrosomal integrity had significant ($P < 0.05$) negative correlation with morphological abnormalities in Jersey x local hill cattle crossbred bulls.

Desai (2013) observed the acrosomal integrity per cent as 85.77 ± 0.48 and 84.98 ± 0.56, 85.56 ± 0.50 and 85.61 ± 0.52 per cent in Kankrej and cross-

bred bulls during the post rainy and winter seasons, respectively. Ray and Ghosh (2013) recorded the intact acrosome in fresh semen of Sahiwal bulls varying from 70 to 88 per cent with the overall mean of 82.70 ± 0.39 per cent. The intact acrosome had significant ($P < 0.01$) positive correlation with live sperm and negative correlation with abnormal sperm.

Srivastava and Kumar (2014) reported the intact acrosome as 89.9 ± 1.0 and 87.4 ± 2.3 per cent in Holstein Friesian (HF) and crossbred bulls, respectively.

## 2.2. EFFECT OF TREHALOSE AS A SEMEN EXTENDER ADDITIVE

Trehalose, a disaccharide, is a non-permeating cryoprotectant that has a protective role against osmotic stress. Trehalose decreases amount of cell injury by ice crystallization and hence protects sperm plasma membrane during freezing and thawing. In addition, supplementation of trehalose in semen diluents has been reported to improve the viability and motility of cryopreserved bull spermatozoa (Uysal *et al.* 2007; Hu *et al.* 2010).

Cryopreserved bovine spermatozoa, when used for artificial insemination, generally produced lower conception rates than fresh spermatozoa for which post thaw evaluation is one of the important factors in semen processing laboratories as motility, sperm viability, acrosome integrity and membrane stability all of which contribute to fertility.

### 2.2.1. Individual Motility

Uysal *et al.* (2007) studied the effect of various antioxidants on the quality of frozen thawed bull semen and assessed post-thaw sperm motility for 50mM trehalose. They reported that addition of 50mM trehalose to bull semen significantly ($P < 0.001$) improved post-thaw sperm motility as 45.00 ± 2.0 per cent over other groups.

Badr *et al.* (2010) studied the effect of trehalose on cryopreservation of buffalo spermatozoa and assessed post-dilution motility and post-thaw motility for different trehalose concentrations. They reported that the addition of 100mM trehalose to buffalo bull semen significantly ($P < 0.05$) improved post-dilution motility and post-thaw motility as 82.50 ± 1.45 and 61.25 ± 1.25 per cent in comparison with that of the 76.25 ± 2.40 and 41.25 ± 4.32 per cent in control group.

Hu *et al.* (2010) studied the effect of various trehalose supplementation on semen quality of frozen thawed bovine semen and assessed post-thaw sperm motility for different trehalose concentrations. They reported that addition of 100mM trehalose to the bovine semen extender showed significantly ($P < 0.05$) greater post-thaw sperm motility as 46.61 ± 1.62 per cent in comparison with that of the 36.88 ± 1.53 per cent in control group.

Reddy *et al.* (2010) studied the effect of adding trehalose to a tris-based egg yolk extender on buffalo sperm quality following cryopreservation and assessed post-thaw sperm motility for 100mM trehalose. They reported that addition of 100mM trehalose in buffalo semen significantly ($P < 0.05$) improved post-thaw sperm motility as 41.67 ± 1.67 per cent in comparison with that of the 31.67 ± 1.67 per cent in control group.

Chhillar *et al.* (2012) studied the effect of trehalose supplementation on functional competence of cryopreserved Karan-Fries semen and assessed post-thaw sperm motility for 100 mM trehalose. They reported that addition of 100mM trehalose in Karan Fries semen extender significantly ($P < 0.05$) increased post-thaw sperm motility as 47.00 ± 1.15 per cent in comparison with that of the 36.00 ± 3.05 per cent in control group.

Kumar *et al.* (2012) studied the effect of supplementation of trehalose in extender on immune-localization of tyrosine phosphoproteins in buffalo (Murrah) and cattle (Karan Fries) during cryopreservation and assessed post thawed sperm motility for 100mM trehalose. They recorded that addition of

100mM trehalose in semen extender significantly increased motility in Karan Fries cattle and Murrah buffalo bulls.

### 2.1.2. Sperm viability

Uysal *et al.* (2007) studied the effect of various antioxidants on the quality of frozen thawed bull semen and assessed post-thaw sperm viability for 50mM trehalose. They reported that addition of 50mM trehalose to bull semen significantly ($P < 0.05$) improved post-thaw sperm viability as 59.6 ± 2.6 per cent over other groups.

Badr *et al.* (2010) studied the effect of trehalose on cryopreservation of buffalo spermatozoa and assessed post-thaw sperm viability index for different trehalose concentrations. They reported that the addition of 100mM trehalose to buffalo bull semen significantly ($P < 0.05$) improved post-thaw sperm viability index up to 172.25 ± 5.58 in comparison with that of the 89.13 ± 15.57 in control group.

Reddy *et al.* (2010) studied the effect of adding trehalose to a tris-based egg yolk extender on buffalo sperm quality following cryopreservation and assessed post-thaw sperm viability for 100mM trehalose. They reported that addition of 100mM trehalose in buffalo semen significantly ($P < 0.05$) improved post-thaw sperm viability as 71.00 per cent in comparison with that of the 61.00 per cent in control group.

Chhillar *et al.* (2012) studied the effect of trehalose supplementation on functional competence of cryopreserved Karan-Fries semen and assessed post-thaw sperm viability for 100mM trehalose. They reported that addition of 100mM trehalose in Karan Fries semen extender significantly ($P < 0.05$) improved post-thaw sperm viability as 52.00 ± 1.15 per cent in comparison with that of the 38.66 ± 1.2 per cent in control group.

Kumar *et al.* (2012) studied the effect of supplementation of trehalose in extender on immune-localization of tyrosine phosphoproteins in buffalo (Murrah) and cattle (Karan Fries) cryopreserved spermatozoa assessed sperm

viability for 100mM trehalose. They recorded that addition of 100mM trehalose in semen extender significantly improved sperm viability in Karan Fries cattle and Murrah buffalo bulls.

### 2.1.3. Sperm Abnormality

Uysal *et al.* (2007) studied the effect of various antioxidants on the quality of frozen thawed bull semen and assessed post-thaw total sperm abnormality for 50mM trehalose. They reported that addition of 50mM trehalose to bull semen significantly ($P < 0.05$) decreased post-thaw total abnormality as 10.5 ± 2.1 per cent over other groups.

### 2.1.4. Osmotic resistance test (HOST)

Uysal *et al.* (2007) studied the effect of various antioxidants on the quality of frozen thawed bull semen and assessed post-thaw HOST for 50mM trehalose. They reported that addition of 50mM trehalose to bull semen significantly ($P < 0.05$) improved post-thaw HOST as 50.1 ± 2.2 per cent over other groups.

Hu *et al.* (2010) studied the effect of trehalose supplementation on semen quality of frozen thawed bovine semen and assessed post-thaw plasma membrane integrity for different trehalose concentrations. They reported that addition of 100mM trehalose to the bovine semen extender showed significantly ($P < 0.05$) greater post-thaw plasma membrane integrity as 44.33 ± 1.71 per cent in comparison with that of the 36.32 ± 2.11 per cent in control group.

Reddy *et al.* (2010) studied the effect of adding trehalose to a tris-based egg yolk extender on buffalo sperm quality following cryopreservation and assessed post-thaw plasma membrane integrity for 100mM trehalose. They reported that addition of 100mM trehalose in buffalo semen significantly ($P < 0.05$) improved post-thaw plasma membrane integrity as 53.15 per cent in comparison with that of the 40.05 per cent in control group.

Chhillar *et al.* (2012) studied the effect of trehalose supplementation on functional competence of cryopreserved Karan-Fries semen and assessed post-thaw plasma membrane integrity for 100mM trehalose. They reported that addition of 100mM trehalose in Karan Fries semen extender significantly ($P < 0.05$) improved post-thaw plasma membrane integrity 50.0 ± 1.52 per cent in comparison with that of the 37.667 ± 3.38 per cent in control group.

Kumar *et al.* (2012) studied the effect of supplementation of trehalose in extender on immune-localization of tyrosine phosphoproteins in buffalo (Murrah) and cattle (Karan Fries) cryopreserved spermatozoa assessed post-thaw plasma membrane integrity for 100mM trehalose. They recorded that addition of 100mM trehalose in semen extender significantly improved post-thaw plasma membrane integrity in Karan Fries cattle and Murrah buffalo bulls.

### 2.1.5. Acrosomal Integrity

Uysal *et al.* (2007) studied the effect of various antioxidants on the quality of frozen thawed bull semen and assessed post-thaw acrosomal damage for 50mM trehalose. They reported that addition of 50mM trehalose to bull semen significantly ($P < 0.05$) improved post-thaw acrosomal integrity 9.7 ± 2.0 per cent in comparison with that of the 10.0 ± 1.3 per cent in control group.

Badr *et al.* (2010) studied the effect of trehalose on cryopreservation of buffalo spermatozoa and assessed post-thaw acrosomal intergrity for different trehalose concentrations. They reported that the addition of 100mM trehalose to buffalo bull semen significantly ($P < 0.05$) improved post-thaw acrosomal integrity up to 10.25 ± 2.39 per cent in comparison with that of the 23.5 ± 1.85 per cent in control group.

Hu *et al.* (2010) studied the effect of trehalose supplementation on semen quality of frozen thawed bovine semen and assessed post-thaw acrosomal membrane integrity for different trehalose concentrations. They reported that addition of 100mM trehalose to the bovine semen extender showed significantly

(P < 0.05) greater post-thaw acrosomal membrane integrity 64.78 ± 1.35 per cent in comparison with 53.40 ± 1.85 per cent in control group.

## 2.3. EVALUATION OF BIOCHEMICAL PARAMETERS OF SEMEN EXTENDER ADDITIVE

The seminal enzymes play a crucial role in fertilization and are affected due to cryopreservation. The assessments of levels of certain enzymes in the seminal plasma have been very important in judging the preservability and fertilizing capacity of spermatozoa.

### 2.3.1. Lipid peroxidation (LPO)

The bovine spermatozoa are prone to membrane damages due to high content of unsaturated fatty acids in the sperm membrane and lack of significant antioxidant system in cytoplasmic component. This makes bovine spermatozoa particularly susceptible to lipid peroxidation (LPO) in presence of reactive oxygen species leading to changes in membrane fluidity which finally leads to decreased fertilizing capacity (Chillar *et al.* 2012). Lipid peroxidation occurring in both the seminal plasma and spermatozoa was estimated by Malondialdehyde (MDA) concentration.

Serpil *et al.* (2009) recorded the MDA levels between 1.37 ± 0.21 to 2.43 ± 0.25 µmol/l in post-thawed bovine bull semen supplemented with antioxidants and these levels did not differ with that of the control diluent.

Badr *et al.* (2010) studied the effect of trehalose on cryopreservation of buffalo spermatozoa and assessed post-thaw lipid peroxidation for different trehalose concentrations. They reported that addition of 100mM trehalose to the buffalo bull semen significantly (P < 0.05) decreased lipid peroxidation up to 9.18 ± 1.46 nmol/ml in post-thaw semen in comparison with that of the 21.57 ± 1.45 nmol/ml in control group.

Muzafer *et al.* (2012) reported that the MDA concentration was significantly ($P < 0.05$) lower in diluents containing Vit. E (0.12 ± 0.01 µmol/ ml) and BHT (0.19 ± 0.02 µmol/ ml) compared to the control diluents (0.60 ± 0.06 µmol/ ml) of post-thaw bovine bull semen.

Karunakaran *et al.* (2012) recorded the MDA level to be 3.28 ± 0.11 µmol/ml in $H_2O_2$ and 1.95 ± 0.11 µmol/ml in HBP treated group, respectively. These levels were significantly higher than that of the control (2.85 ± 0.12 µmol/ml) at 60 min incubation period of frozen thawed bovine bull semen.

Umut *et al.* (2012) reported that addition of various cryoprotectants showed decreased levels of lipid peroxidation in frozen thawed bovine bull semen as compared with that of the other groups. Chhillar *et al.* (2012) reported that the presence of 100mM trehalose in Karan-Fries semen extender during cryopreservation significantly ($P < 0.05$) decreased lipid peroxidation 1.49 ± 0.14 nmol MDA/$10^8$ cells in comparison with that of the 2.61 ± 0.33 nmol MDA/$10^8$ cells in control group.

### 2.3.2. Glutathione Reductase (GSH)

The higher glutathione reductase (GSH) value in the seminal plasma might be a factor in making the sperm membrane more resistant to the spontaneous lipid peroxidation that destroys the structure of the lipid matrix which is associated with the loss of motility.

Mohanty and Ansari (2004) reported the mean values of glutathione reductase as 1.85 ± 0.21 and 0.91 ± 0.15 mmol/l with a significant difference ($P < 0.05$) in the seminal plasma of good and poor freezable bovine bull semen, respectively. Serpil *et al.* (2009) recorded the GSH levels of 1.39 ± 0.24 to 1.19 ± 0.14 µmol/l in post thawed bovine bull semen supplemented with antioxidants and these levels did not differ with that of the control diluent.

Badr *et al.* (2010) studied the effect of trehalose on cryopreservation of buffalo spermatozoa and assessed post-thaw glutathione reductase for different

trehalose concentrations. They reported that addition of 100mM trehalose to the buffalo bull semen significantly ($P < 0.05$) increased the level of glutathione reductase (GSH) up to 110.64 ± 5.39 U/L in comparison with that of the 62.01 ± 5.74 U/L in control group.

Hu *et al.* (2010) studied the effect of trehalose supplementation on semen quality of frozen thawed bovine bull semen and assessed post-thaw glutathione reductase (GSH) for different trehalose concentrations. They reported that addition of 100mM trehalose to the bovine bull semen extender showed significantly ($P < 0.05$) greater level of glutathione reductase up to 40.81 ± 2.97 U/L in comparison with that of the 19.57 ± 2.36 U/L in control group.

Umut *et al.* (2012) reported that addition of various cryoprotectants showed increased levels of glutathione reductase in frozen thawed bovine bull semen as compared with that of the other groups.

# CHAPTER 3: MATERIALS AND METHODS

The present research work was carried out on "Effect of trehalose on quality of cryopreserved Banas Kankrej bull semen" at Department of Gynaecology and Obstetrics, College of Veterinary Science and Animal Husbandry, SDAU, Sardarkrushinagar in collaboration with Dama semen production Unit, Banas dairy, Palanpur. The study was carried out over a period of 12 weeks from December, 2013 to February, 2014.

## 3.1. LOCATION OF RESEARCH

Geographically, Dama is situated at 24°- 10′ North latitude and 72°- 10′ East latitude, at an altitude of 154.52 meter above means sea level. The climate is harsh and the ambient summer temperature goes up to 45°C while winter temperature runs down to 5°C. The rains were reported to be highly irregular. The data regarding the temperature and humidity over the period of the study from December, 2013 to February, 2014 were as below.

a. Maximum Temperature ranged between 27$^{o}$C to 30$^{o}$C.
b. Minimum Temperature ranged between 19$^{o}$C to 22$^{o}$C.
c. Average Temperature recorded was 21$^{o}$C.
d. Relative Humidity below 50%.

## 3.2. EXPERIMENTAL ANIMALS USED

Total, three Banas Kankrej bulls aged between 4 to 5 years of Dama semen production Unit, Banas dairy, Palanpur were used for the study. All bulls were in good health, under uniform veterinary care and maintained in identical sanitary conditions. Before including bulls for the investigation, they were properly examined and screened for their normal external and internal reproductive organs, respectively. According to aforesaid examinations all the bulls had normal libido and sexual behaviour.

The details of three bulls used for the investigation were as below.

| Bull No. | Name | Tag No. | Birth date | Age (in months) as on December, 2013 |
|---|---|---|---|---|
| 1 | K-26 | 7790/1711 | 10/02/2009 | 58 |
| 2 | K-28 | 7790/8892 | 28/09/2009 | 52 |
| 3 | K-30 | 7790/1378 | 15/01/2009 | 59 |

## 3.3. MANAGEMENT OF BREEDING BULLS

Bulls utilized in this study were maintained at Dama semen production Unit under the following identical managemental and nutritional practices:

### 3.3.1. Feeding

As per the Minimum Standard Protocol (MSP) decided by Government of India, each bull was fed @ 0.8 kg concentrate, 1.6 kg dry fodder and 4.16 kg of green fodder per 100 kg body weight. They were also feed with 60 gm of mineral mixture supplement per day.

### 3.3.2. Watering

Bulls were provided with clean ample drinking water throughout the day by automatic watering system. Bathing and grooming were carried out daily before semen collection as a routine practice.

### 3.3.3. Hoof Trimming

Hoof trimming was carried out to maintain the health and normal structure of hoof so as to prevent them from slipping and self injury.

### 3.3.4. Housing

Individual bull sheds were provided to each bull to maintain optimum health, bedding and sanitary condition.

### 3.3.5. Health management

All the clinical parameters like temperature, pulse, respiration, etc. were recorded regularly. Clipping of prepucial hairs was carried out at regular interval and deworming was done regularly before and after monsoon.

### 3.3.6. Vaccination

All the bulls were vaccinated yearly for Hemorrhagic Septicemia and twice yearly for Foot and Mouth Disease. The experimental animals were screened annually for Tuberculosis, Johne's disease, Brucellosis, Camphylobacteriosis and Trichomoniasis by Western Regional Disease Diagnostic Laboratory (WRDDL), Pune.

## 3.4. STERILIZATION OF EQUIPMENTS

The glass wares used throughout the study were thoroughly cleaned, dried, covered with aluminium foil and sterilized in hot air oven at 160$^{o}$ C for 1 hour. The artificial vagina was autoclaved at 121$^{o}$ C and 15 p.s.i. pressure for 20 minutes and stored overnight in an incubator at 45$^{o}$C. Thermo-sensitive rubber wares were packed and sealed in specific polythene bags and sterilized in Ethylene Oxide gas sterilizer. Thermo-resistant rubber wares were sterilized by autoclaving at 5p.s.i. for 10 minutes (MSP guideline, GOI).

## 3.5. SEMEN ADDITIVE AND OTHER CHEMICALS USED

The additive used in this study such as Trehalose (GRM110) and other chemicals were obtained from Himedia Chemicals (Chiti-Chem Corporation, Baroda) and they were added at different concentration levels in milli Mole (mM) which was calculated using following formula:

***milli Mole*** = *Molarity x Litre x Mol. Wt.*

Where, *Mol. Wt. of Trehalose* = **378.33**

## 3.6. COLLECTION OF SEMEN

The semen collection was done once in a week during 7.00 to 8.00 am in the morning. The bulls of the same species and breed were used as dummy for collection. The semen was collected using artificial vagina (Danish Model) maintained at 41$^{o}$C. Immediately after collection, semen collection tubes were placed in water bath at 37$^{o}$C. Total 36 ejaculates (12 ejaculates from each bull) were collected for the present study.

## 3.7. PREPARATION OF BUFFER

Tris-Fructose Egg Yolk Citrate Glycerol (TFYG) buffer was prepared for dilution of semen during the study as described by Foote (1980).

**Composition of TFYG Buffer (100ml)**

| | |
|---|---|
| Tris powder | : 2.422 g |
| Citric acid | : 1.36 g |
| D (+) Fructose | : 1.0 g |
| Glycerol | : 6.4 ml |
| Egg Yolk | : 20 ml |
| Penicillin G Sodium | : 1000 IU/ml |
| Streptomycin Sulphate | : 1000 µg/ml |
| Double Glass Water | : 73.6 ml |
| pH adjusted to | : 6.8 |

## 3.8. DILUTION OF SEMEN ADDITIVE

After dilution and evaluation of semen ejaculates with >70% initial progressive motility, were divided in four equal aliquots. Subsequently, Aliquot-1 was diluted with 50mM Trehalose, Aliquot-2 was diluted with 100mM Trehalose, Aliquot-3 was diluted 150mM Trehalose and Aliquot-4 was without any additive which served as control. The dilution rate was calculated keeping in view the final sperm concentration of 80 x $10^6$ sperms per ml of diluted semen. After the final dilution, one third of the each semen aliquot was evaluated for post-dilution individual motility under phase contrast microscope (magnification 10x20), sperm viability, sperm abnormality, osmotic resistance test (HOST) and acrosomal integrity under phase contrast microscope (magnification 10x100, oil immersion of "Olympus").

## 3.9. PROCESSING OF SEMEN

After dilution, remaining two third of the each semen aliquots were filled, sealed and printed in French Mini Straw of orange colour (0.25 ml capacity) using IS-4, IMV-France. Straws were then arranged in Freezing Rack and were transferred to cold handling cabinet already maintained at $4^0$C kept for 3 hours of equilibration period.

After equilibration, half of the straws were collected and semen was evaluated for post-equilibration individual motility, sperm viability, sperm abnormality, osmotic resistance test (HOST) and acrosomal integrity.

Remaining straws were frozen by static vapour freezing method using Programmable Bio Freezer (IMV, France) step by step up to -$140^0$C. After achieving -$140^0$C temperature, all the straws were submerged in Liquid Nitrogen at -$196^0$C for storage.

After cryopreservation period of 24 hrs, straws were thawed at $37^0$C for 30 seconds in a water bath to evaluate post-thaw individual motility, sperm

viability, sperm abnormality, osmotic resistance test (HOST) and acrosomal integrity.

## 3.10. EVALUATION OF SEMEN

The semen samples were evaluated immediately after collection for individual motility, sperm viability, sperm abnormality, osmotic resistance test (HOST) and acrosomal integrity.

### 3.10.1. Individual Motility

The Individual motility was subjectively evaluated using the standard method described by Serpil *et al.* (2009). The Individual motility of freshly diluted semen was assessed after covering the one drop of semen with cover glass, under phase contrast microscope (magnification 10x20 of "Olympus") with a warm stage maintained at 37˚C. The semen was extended at 37˚C with TFYG diluent to the obtained volume of ejaculates. The individual motility estimations were performed in 3 different fields and their means was recorded as per cent progressive motility.

The same procedure was adopted for estimating the per cent individual motility at all three stages namely, post-dilution (34°C), post-equilibration (4°C), post-thaw (37°C).

### 3.10.2. Sperm Viability

The sperm viability was calculated using Eosin-Nigrosin stain as per the method described by Evans and Maxwell (1987).

### 3.10.2a. Preparation of Eosin - Nigrosin Stain

| | |
|---|---|
| Eosin – Y | - 1.67 g |
| Nigrosin | - 10.00 g |
| Sodium citrate | - 2.90 g |
| Double glass distilled water | - 100 ml |

The semen smears were prepared by mixing one drop of sperm sample with two drops of stain on a warm slide and spreading the stain with a second slide. The slides are then air dried and sperm viability was evaluated by counting 200 cells under phase contrast microscope (magnification 10x100, oil immersion of "Olympus"). Sperm displaying partial or complete purple staining were considered nonviable; only sperm showing strict exclusion of stain were counted as viable.

The same procedure was adopted for estimating the per cent sperm viability at all three stages namely, post-dilution (34°C), post-equilibration (4°C), post-thaw (37°C).

### 3.10.3. Sperm Abnormality

The sperm abnormality was calculated using eosin-nigrosin stain used as per the method described by Evans and Maxwell (1987).

The Eosin-Nigrosin Solution was prepared similarly as described for the sperm viability and the smear were prepared in the similar fashion to count the sperm abnormalities by counting 200 cells under phase contrast microscope (magnification 10x100, oil immersion of "Olympus"). The per cent total sperm abnormality (acrosomal integrity, detached heads, abnormal mid-pieces and tail defects) were counted as abnormal spermatozoa.

The same procedure was adopted for estimating the per cent sperm abnormality at all three stages namely, post-dilution (34°C), post-equilibration (4°C), post-thaw (37°C).

### 3.10.4. Acrosomal Integrity

The acrosomal integrity is one of the essential laboratory tests to assess the potential fertility of the semen. The acrosomal integrity is affected during different steps of semen processing and thawing. The acrosomal integrity of spermatozoa was evaluated by means of modified giemsa staining technique as described by Manokaran *et al.* (2010).

### 3.10.4a. Preparation of Giemsa Stock Solution

Giemsa powder (Himedia, Chiti-Chem, Baroda) 1g was dissolved with 66 ml methanol and 60 ml glycerol, mixed properly. This mixture was stored at room temperature, in air tight container for a week for proper ripening and then filtered. During the ripening/maturation process the mixture was shaken vigorously and regularly atleast once in a day.

### 3.10.4b. Preparation of Giemsa Working Solution

| | |
|---|---|
| Giemsa stock solution | - 2.0 ml |
| Absolute Methanol | - 20.0 ml |
| Double glass distilled water | - 23.0 ml |

Thin semen smears were prepared on clean grease-free glass slides at different steps of processing / preservation and air dried quickly. The slides after drying were immediately immersed in the Giemsa working solution for 5-6 hours using a staining jar at room temperature. The slides were then removed and washed gently under running tap water. The slides were then air dried and assessed by counting a total of 200 spermatozoa under phase contrast microscope (magnification 10x100, oil immersion of “Olympus”). The per cent intact or damaged acrosomes were counted in different fields.

The same procedure was adopted for estimating the per cent acrosomal integrity at all three stages namely, post-dilution (34°C), post-equilibration (4°C), post-thaw (37°C).

### 3.10.5. Osmotic Resistance Test (HOST)

The osmotic resistance test (HOST) was done to evaluate the functional integrity of the sperm membrane, based on curled and swollen tails as per the method described by Revell and Mrode (1994).

### 3.10.5a. Preparation of Hypo-Osmotic Solution

| | |
|---|---|
| Fructose | - 1.351 g |
| Sodium citrate | - 0.735 g |

Double glass distilled water - 100 ml

The Rose Bengal Solution and Control Solution were prepared as described by Sharma *et al.* (2012).

### 3.10.5b. Preparation of Rose Bengal Solution

Rose Bengal - 3g
Commercial Formalin (37%) - 1ml
Double Glass Distilled water - 100ml

### 3.10.5c. Preparation of Control Solution

Sodium chloride - 9.01g
Double Glass Distilled water - 1000ml

Add 0.1 ml of the semen containing the spermatozoa with 1 ml of 150 mOsM hypo-osmotic solution. And 0.1 ml of same semen in another test tube was set by mixing 1 ml of 300 mOsM control solution. The solutions were then incubated at 37°C for one hour. After incubation, one drop of semen sample mixed with one drop Rose Bengal and was spread with second slide. Two hundred sperm were counted under phase contrast microscope (magnification 10x100, oil immersion of “Olympus”).

The proportion of swollen and curled spermatozoa in the control sample was subtracted from the proportion of swollen and curled spermatozoa in hypo-osmotic solution. The resultant figure was considered as per cent HOST reactive sperm.

The same procedure was adopted for estimating the per cent functional integrity of the sperm membrane at all three stages namely, post-dilution (34°C), post-equilibration (4°C), post-thaw (37°C).

## 3.11. BIOCHEMICAL ASSAYS

The freshly collection semen samples were centrifuged at 5000 rpm for 10 min. immediately and obtained seminal plasma was assessed for the lipid

peroxidation and glutathione reductase activities. The seminal plasma separated from processed semen straws at different stages of cryopreservation by centrifugation at 5000 rpm for 10 min. and stored at -20° C before being assayed. The seminal plasma samples were thawed before analysing the lipid peroxidation and glutathione reductase activities.

### 3.11.1 Lipid Peroxidation (LPO)

Membrane peroxidative damage in seminal plasma was determined in terms of malondialdehyde (MDA) by using the method of Placer *et al*. (1966).

### 3.11.1a. Preparation of Trichloroacetic Acid Solution

| | |
|---|---|
| Trichloroacetic acid | - 20g |
| Double Glass distilled water | - 100ml |

### 3.11.1b. Preparation of Thiobarbituric Acid Solution

| | |
|---|---|
| Thiobarbituric acid | - 0.67g |
| Double Glass distilled water | - 100ml |

Briefly, 1ml of seminal plasma was mixed with 1 ml of 20% TCA (w/v) to precipitate protein. After thorough mixing, the reaction mixture was centrifuged at 1000 x g for ten minutes. 1 ml of supernatant was incubated with 1 ml of 0.67 % TBA (w/v) in boiling water bath for ten minutes. After cooling, the absorbance was recorded at 532 nm on a UV spectrophotometer ("VARAN" 50 Bio, UV Spectrophotometer). Blank was made by adding all the reagents except the seminal plasma.

### Calculation

The calculation was done by using the molar extinction coefficient (ε) of MDA-TBA complex at 532 nm, *i.e.*, 1.56 x $10^5$ $M^{-1}cm^{-1}$. The values of MDA were expressed as **µmol / ml**.

$$\textbf{µmol / ml} = \frac{\text{(OD of test sample) x } 10^6 \text{ x Vol. of sample in ml}}{\varepsilon \text{ x } 10^5 \text{ x Vol. of test sample in ml}}$$

$\varepsilon = 1.56 \text{ x } 10^5 \ M^{-1}cm^{-1}$

### 3.11.2. Glutathione Reductase (GSH)

The GSH content of sperm was measured using the method of Sedlak and Lindsay (1968).

#### 3.11.2a. Preparation of Trichloroacetic Acid Solution

| | |
|---|---|
| Trichloroacetic acid | - 50g |
| Double Glass distilled water | - 100ml |

#### 3.11.2b. Preparation of 5, 5'-dithio-bis-2-nitrobenzoic acid Solution

| | |
|---|---|
| 5, 5'-dithio-bis-2-nitrobenzoic acid | - 0.397g |
| Double Glass distilled water | - 100ml |

Briefly, 1ml of seminal plasma was mixed with 1ml of 50% TCA (w/v) to precipitate protein. After thorough mixing, the reaction mixture was centrifuged at 1000 x g for 5 min. 0.5 mL of supernatant was incubated with 2.0 mL of Tris-EDTA buffer, and 0.1 mL of 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) at room temperature for 5 min. The absorbance was recorded at 412 nm on a UV spectrophotometer. Blank was made by adding all the reagents expect the seminal plasma.

#### Calculation

The calculation was done by using an extinction coefficient (ε) of DTNB at 412 nm, *i.e.*, 14.15 $mM^{-1}cm^{-1}$. The values of GSH were expressed as **U / L**.

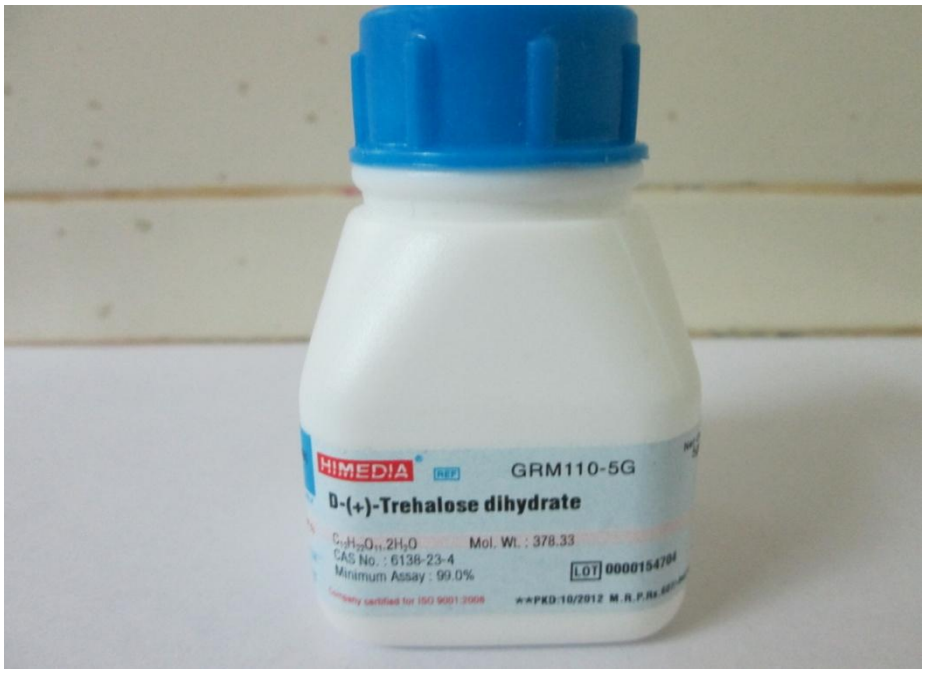
$$\mathbf{U / L} = \frac{(\text{OD of test sample} - \text{OD of blank}) \times \text{D.F.}}{\varepsilon \times \text{Volume of sample in ml}}$$

D.F. = Dilution Factor

ε = 14.15 $mM^{-1}cm^{-1}$

## 3.12. STATISTICAL ANALYSIS

The results was statistically analyzed with multiple range test and expressed as Mean ± SE. The data on semen characteristics were analyzed using Completely Randomized Design (CRD). For the analysis of the effect of semen additive, 12 values of observations were used. Two factors (4 additive x 3 stages of semen preservation) factorial CRD was used for analyzing the effect of additive on cryopreserved bull semen. The means were analyzed by analysis of variance as per the procedure described by Snedecor and Cochran (1994). Differences with values of $P < 0.05$ were considered to be statistically significant.




- **Semen Additive used during Research**

- **Semen Evaluation During Research**

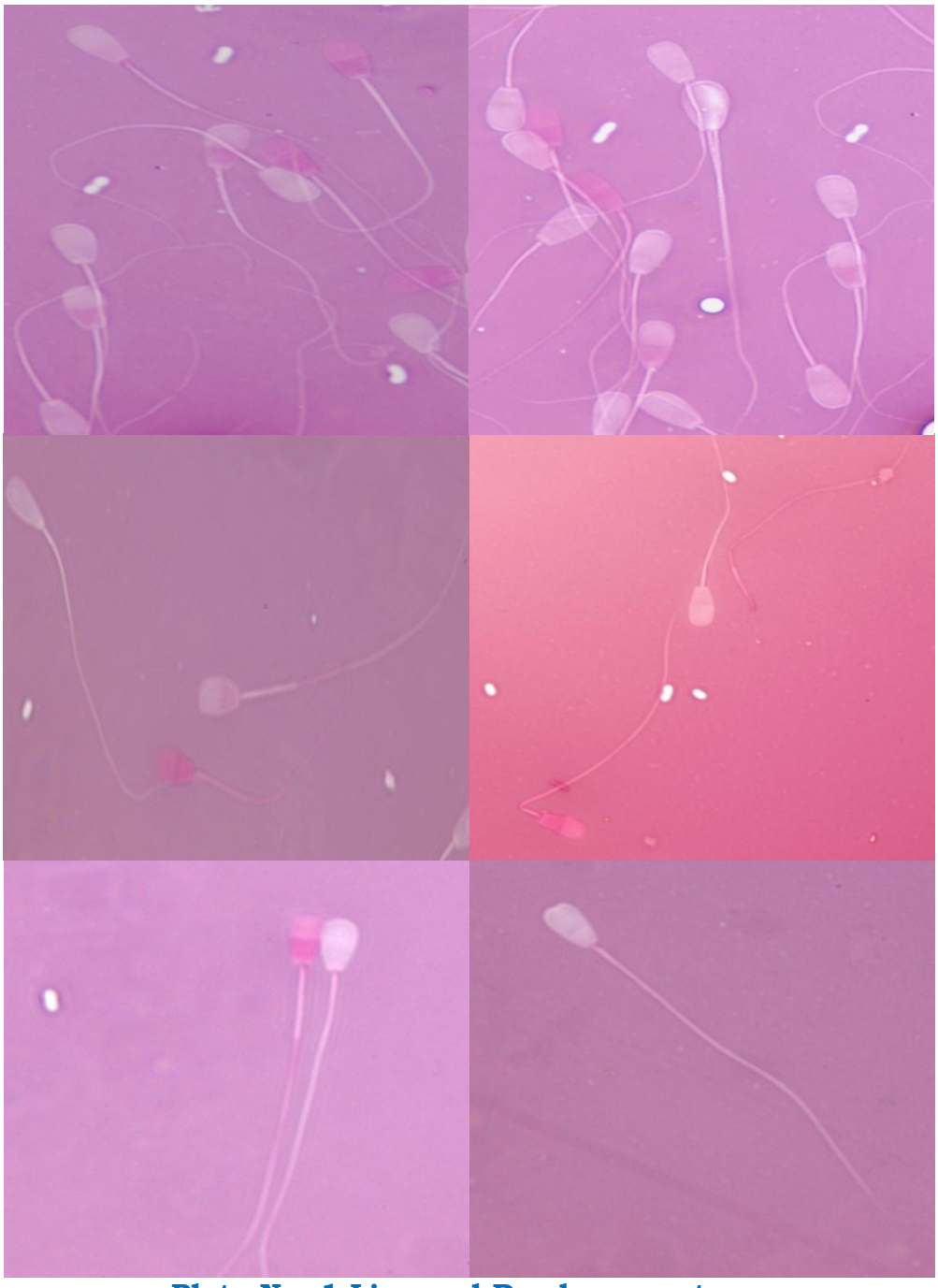


**Plate No. 1 Live and Dead spermatozoa**

**(magnification 10 x 100)**

A. **Live sperm** – White Colour B. **Dead sperm** – Pink Colour

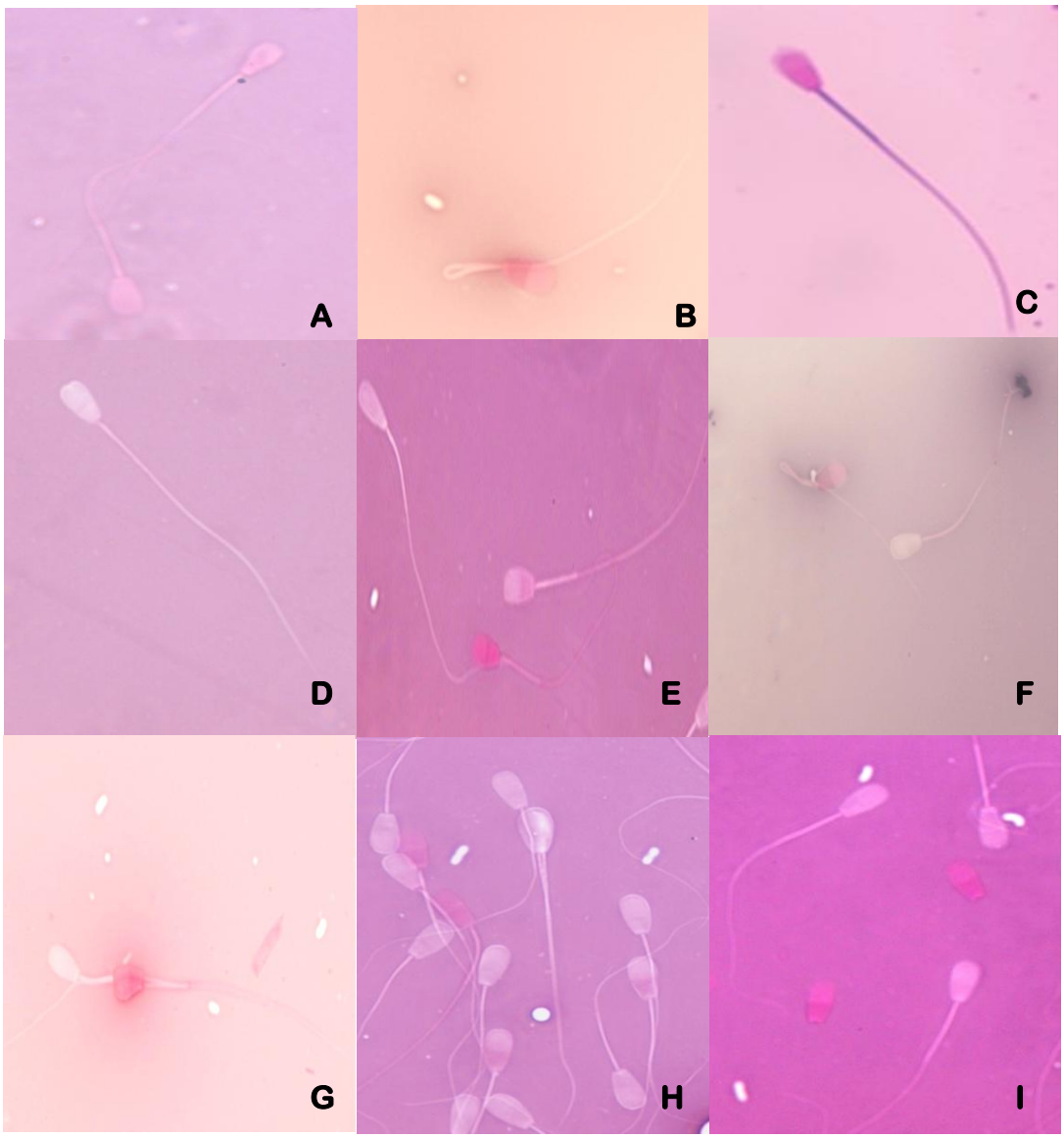



**Plate No. 2 Head Abnormalities of Spermatozoa**
**(magnification 10 x 100)**

A. Pear shaped Head

B. Abaxial Head

C. Ruffled Head

D. Knobbed Head

E. Elongated Head

F. Swollen Head

G. Double Head

H. Macrocephalic

I. Detached Head

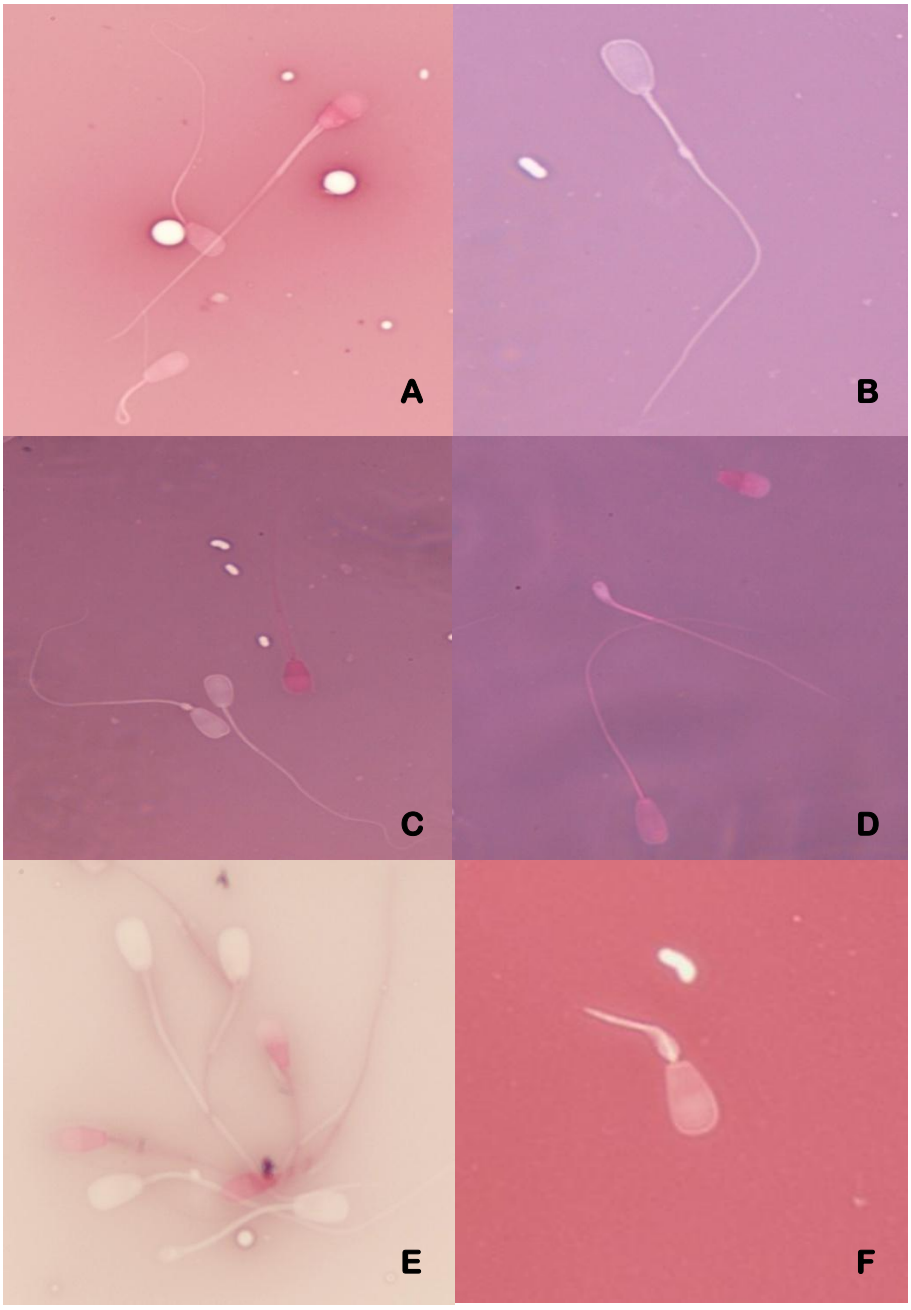



**Plate No. 3 Mid-piece Abnormalities of Spermatozoa (magnification 10 x 100)**

A. Double Mid-piece B. Distal droplet

C. Proximal droplet D. Coiled Mid-piece

E. Broken Mid-piece F. Swollen Mid-piece

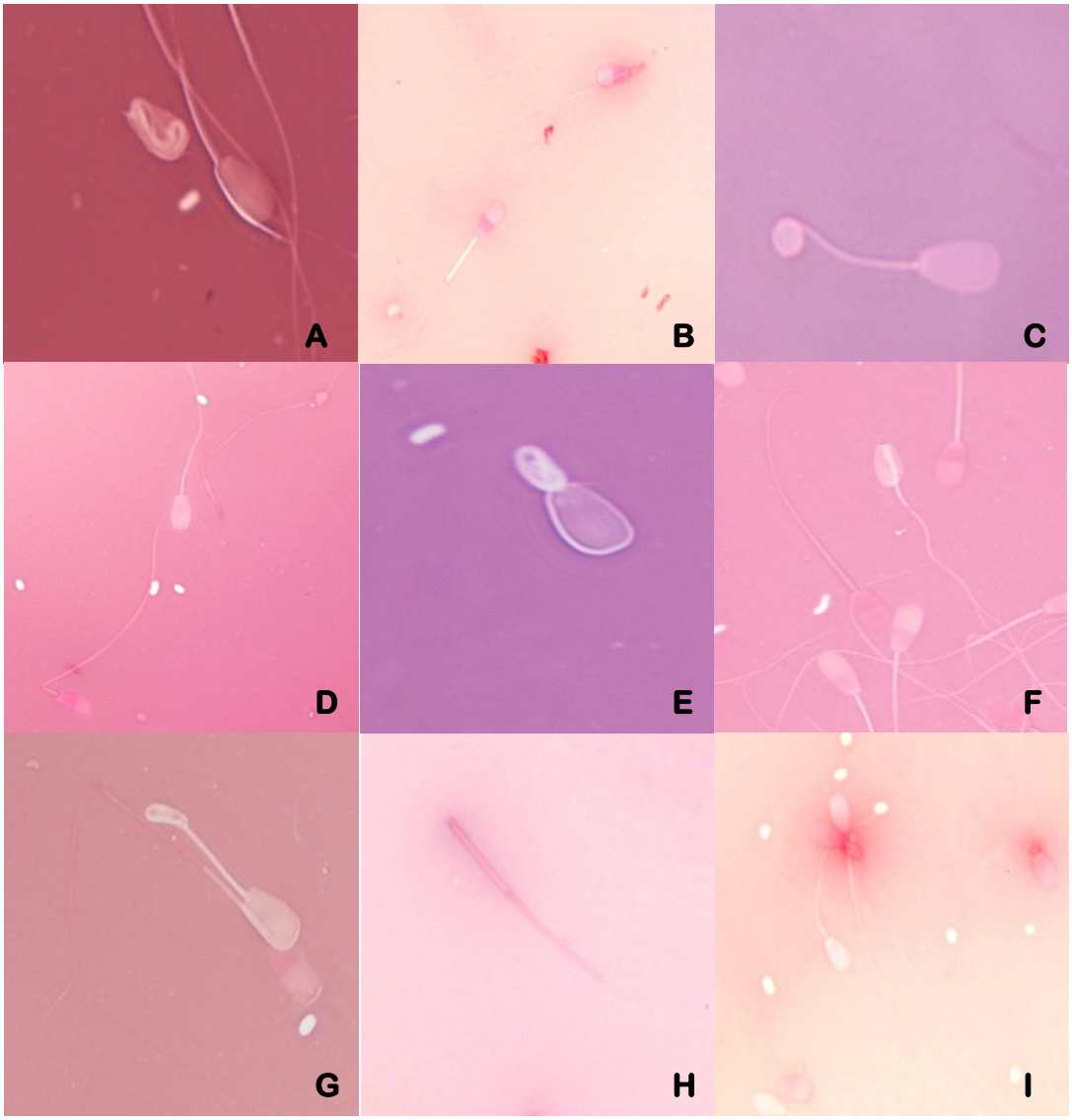



**Plate No. 4 Tail Abnormalities of Spermatozoa**

**(magnification 10 x 100)**

A. Coiled Tail over Head

B. Bend Tail

C. Tightly Coiled Tail

D. Partial Broken Tail

E. Dag Defect

F. Broken Neck

G. Coiled Tail

H. Broken Tail

I. Stumped Tail

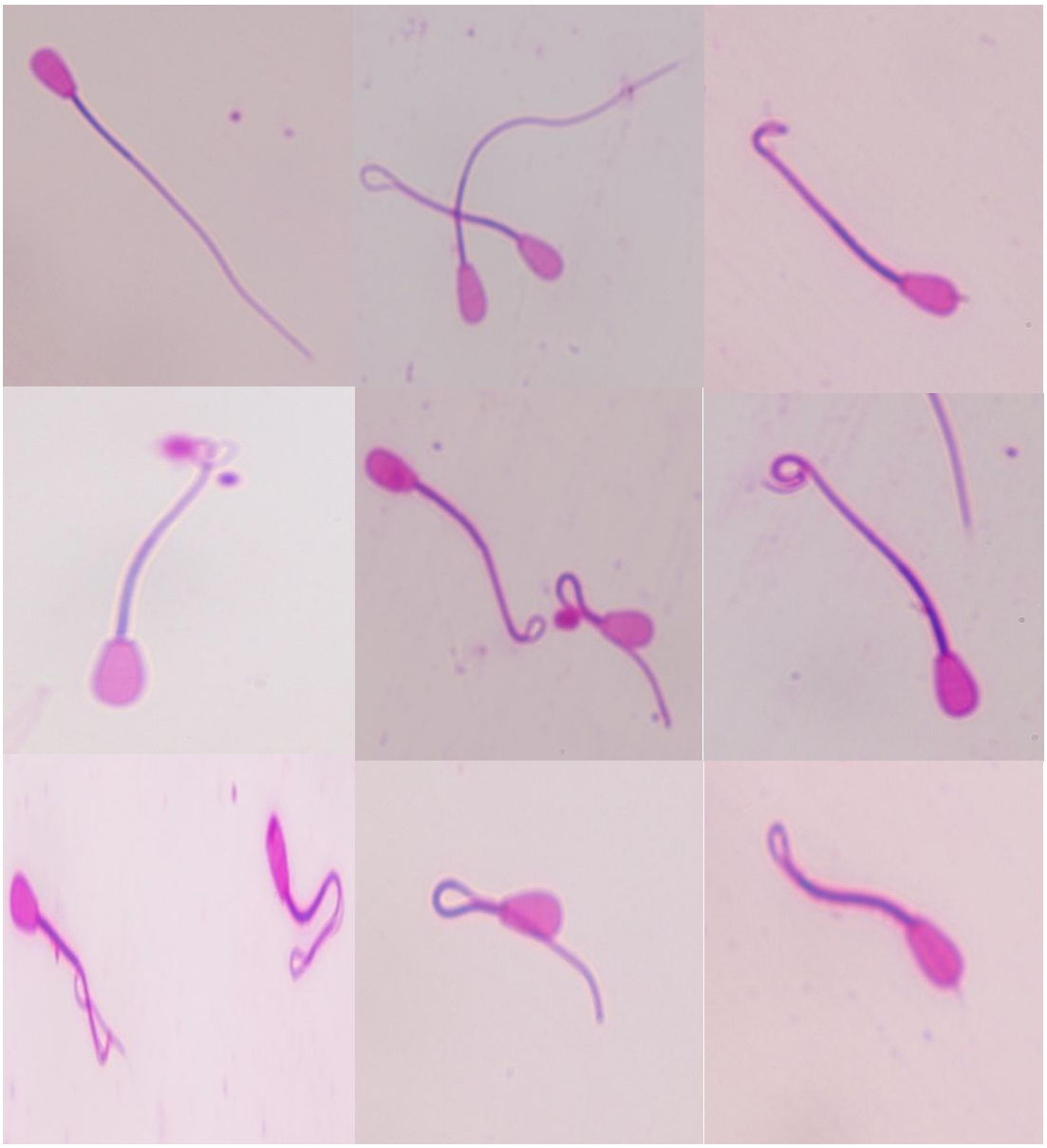


**Plate No 5. HOST of spermatozoa**

**(magnification 10 x 100)**

A. **HOST non reactive** – Non-swollen tail

B. **HOST reactive** – Swollen tail

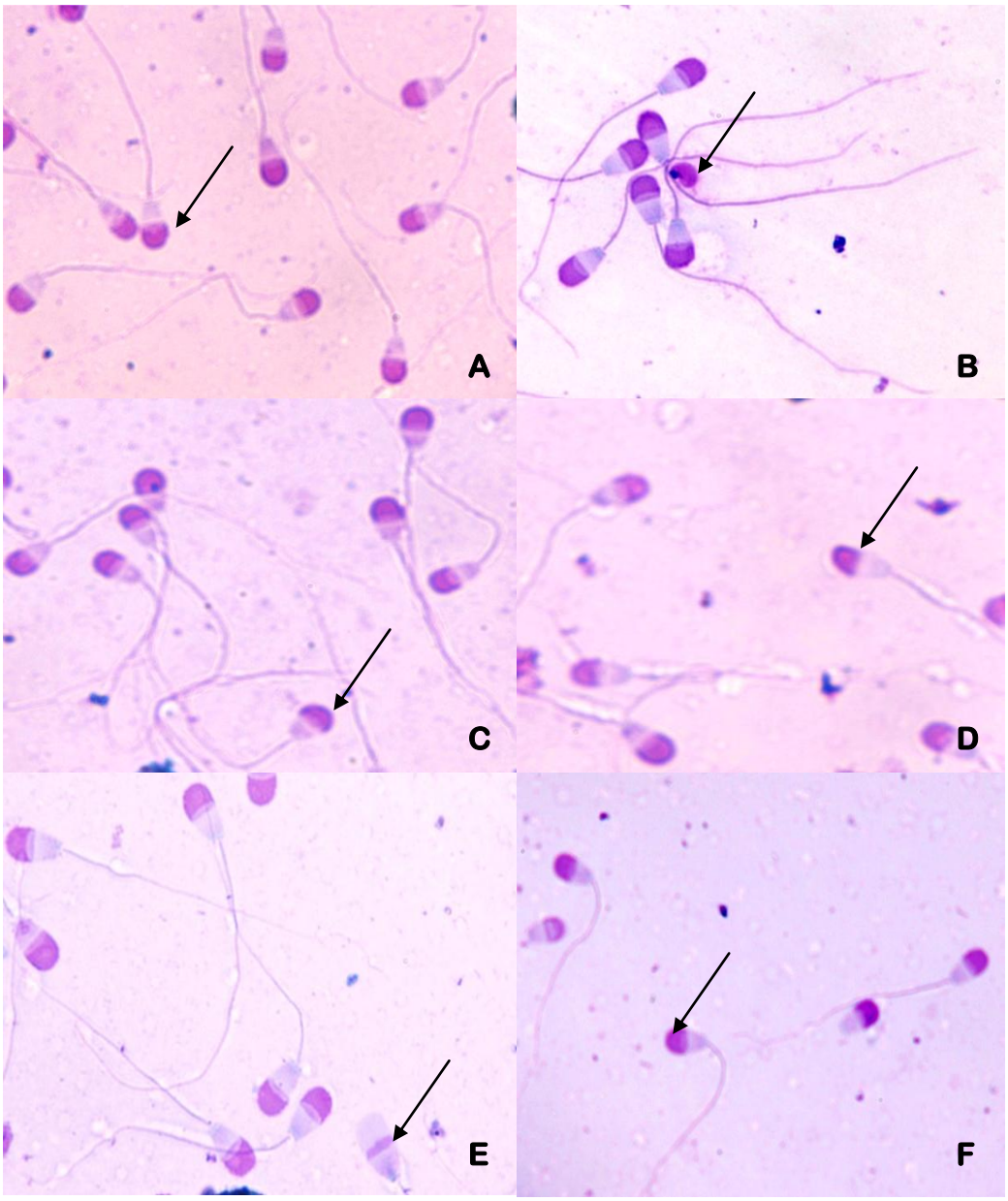



**Plate No. 6 Acrosomes of spermatozoa**

**(magnification 10 x 100)**

A & F. Intact acrosome

C. Swollen acrosome

B. Detached acrosome

D. Ruffled acrosome

E. Denuded acrosome

# CHAPTER 4: OBSERVATIONS AND RESULTS

The present investigation showed the following observations on physical characteristics and enzymatic analysis of semen at different trehalose concentrations during post-dilution, post-equilibration and frozen thawed stages of cryopreservation.

## 4.1. EVALUATION OF PHYSICAL CHARACTERISTICS OF FRESH SEMEN

The detailed findings of the present investigation obtained from three Banas Kankrej bull semen ejaculates have been documented under following subheadings:

### 4.1.1. Individual Motility

The overall mean per cent individual motility was 88.75 ± 0.25 with a range of 86 to 92 per cent (Table 1). The overall mean individual motility per cent did not differ significantly among the bulls (Table 3, Figure 1). The individual motility had highly significant ($P < 0.05$) positive correlation with sperm viability ($r= 0.839$, $n= 36$), acrosomal integrity ($r= 0.976$, $n= 36$) and lipid peroxidation ($r= 0.890$, $n= 36$) whereas, inverse correlation was observed with sperm abnormality ($r= -0.890$, $n= 36$). It also had significant ($P < 0.05$) positive correlation with HOST ($r= 0.631$, $n= 36$) and glutathione reductase ($r= 0.563$, $n= 36$) (Table 5).

### 4.1.2. Sperm Viability

The overall mean per cent sperm viability was 89.69 ± 0.32 with a range of 86 to 92 per cent (Table 1). The overall mean sperm viability per cent did not differ significantly among the bulls (Table 3, Figure 1). The sperm viability had highly significant ($P < 0.05$) positive correlation with glutathione reductase ($r= 0.921$, $n= 36$). It also had significant ($P < 0.05$) positive correlation with

acrosomal integrity (r= 0.704, n= 36) and lipid peroxidation (r= 0.5, n= 36) whereas, inverse correlation with sperm abnormality (r= -0.5, n= 36) (Table 5).

### 4.1.3. Sperm Abnormality

The overall mean per cent sperm abnormality was 3.08 ± 0.15 with a range of 2 to 5 per cent (Table 1). The overall mean sperm abnormality per cent did not differ significantly among the bulls (Table 3, Figure 1). The sperm abnormality had highly significant (P < 0.05) inverse correlation with individual motility (r= -0.890, n= 36), acrosomal integrity (r= -0.967, n= 36) and HOST (r= -0.915, n= 36). It also had significant (P < 0.05) inverse correlation with sperm viability (r= -0.5, n= 36) (Table 5).

### 4.1.4. Osmotic Resistance Test (HOST)

The overall mean per cent HOST reactive sperm was 86.80 ± 0.24 with a range of 85 to 90 per cent (Table 1). The overall mean HOST positive sperm did not differ significantly among the bulls (Table 3, Figure 1). The HOST positive sperm had highly significant (P < 0.05) positive correlation with lipid peroxidation (r= 0.915, n= 36) and acrosomal integrity (r= 0.782, n= 36) whereas, inverse correlation with sperm abnormality (r= -0.915, n= 36). It also had significant (P < 0.05) positive correlation with individual motility (r= 0.631, n= 36) (Table 5).

### 4.1.5. Acrosomal Integrity

The overall mean per cent acrosomal integrity was 90.72 ± 0.25 with a range of 88 to 93 per cent (Table 1). The overall mean acrosomal integrity did not differ significantly among bulls (Table 3, Figure 1). The acrosomal integrity had highly significant (P < 0.05) positive correlation with individual motility (r= 0.976, n= 36), HOST (r= 0.782, n= 36) and lipid peroxidation (r= 0.967, n= 36) whereas, inverse correlation with sperm abnormality (r= -0.967, n= 36). It also had significant (P < 0.05) positive correlation with sperm viability (r= 0.704, n= 36) (Table 5).

## 4.2. EVALUATION OF BIOCHEMICAL PARAMETERS OF FRESH SEMEN

The detailed findings of the present investigation obtained from three Banas Kankrej bull semen ejaculates have been documented under following subheadings:

### 4.2.1. Lipid peroxidation (LPO)

The overall mean Malondialdehyde (MDA) of semen was 49.96 ± 0.06 µmol/ml with a range of 49.12 to 50.65 µmol/ml (Table 2). The overall mean Malondialdehyde (MDA) did not differ significantly among the bulls (Table 4, Figure 2). The Malondialdehyde (MDA) values had highly significant ($P < 0.05$) positive correlation with individual motility (r= 0.890, n= 36), HOST (r= 0.915, n= 36) and acrosomal integrity (r= 0.967, n= 36). It also had significant ($P < 0.05$) positive correlation with sperm viability (r= 0.5, n= 36) (Table 5).

### 4.2.2. Glutathione reductase (GSH)

The overall mean glutathione reductase (GSH) of semen was 30.14 ± 0.06 U/L with a range of 29.24 to 30.87 U/L (Table 2). The overall mean glutathione reductase did not differ significantly among the bulls (Table 4, Figure 2). The glutathione reductase had highly significant ($P < 0.05$) positive correlation with sperm viability (r= 0.921, n= 36). It also had significant ($P < 0.05$) positive correlation with individual motility (r= 0.563, n= 36) (Table 5).

## 4.3. EFFECT OF TREHALOSE AS A SEMEN EXTENDER ADDITIVE

In order to increase semen volume and eliminate variability between the evaluated samples, the semen ejaculates were pooled and divided into four equal aliquots. The semen samples under investigation were evaluated at post-dilution, post-equilibration and post-thaw stages of freezing process for individual motility, sperm viability, sperm abnormality, osmotic resistance test and acrosomal integrity.

### 4.3.1. Individual Motility

The overall mean per cent individual motility, using different concentrations of Trehalose in different groups are tabulated (Table 6). The overall mean individual motility per cent in 100mM Trehalose group was significantly ($P < 0.05$) higher in post-dilution, post-equilibration and post-thaw stages of cryopreservation as compared to that of the 50mM Trehalose, 150mM Trehalose and control groups (Table 7, Figure 3).

### 4.3.2. Sperm Viability

The overall mean per cent sperm viability, using different concentrations of Trehalose in different groups are tabulated (Table 8). The overall mean sperm viability per cent in 100mM Trehalose group was significantly ($P < 0.05$) higher in post-dilution, post-equilibration and post-thaw stages of cryopreservation as compared to that of the 50mM Trehalose, 150mM Trehalose and control groups (Table 9, Figure 4).

### 4.3.3. Sperm Abnormality

The overall mean per cent sperm abnormalities, using different concentrations of Trehalose in different groups are tabulated (Table 10). The overall mean sperm abnormality per cent in 100mM Trehalose group was significantly ($P < 0.05$) lower in post-dilution, post-equilibration and post-thaw stages of cryopreservation as compared to that of the 50mM Trehalose, 150mM Trehalose and control groups (Table 11, Figure 5).

### 4.3.4. Osmotic Resistance Test (HOST)

The overall mean per cent HOST reactive sperm, using different concentrations of Trehalose in different groups are tabulated (Table 12). The overall mean HOST reactive sperm per cent in 100mM Trehalose group was significantly ($P < 0.05$) higher in post-dilution, post-equilibration and post-thaw stages of cryopreservation as compared to that of the 50mM Trehalose, 150mM Trehalose and control groups (Table 13, Figure 6).

### 4.3.5. Acrosomal Integrity

The overall mean per cent acrosomal integrity, using different concentrations of Trehalose in different groups are tabulated (Table 14). The overall mean acrosomal integrity per cent in 100mM Trehalose group was significantly ($P < 0.05$) higher in post-dilution, post-equilibration and post-thaw stages of cryopreservation as compared to that of the 50mM Trehalose, 150mM Trehalose and control groups (Table 15, Figure 7).

## 4.4. EVALUATION OF BIOCHEMICAL PARAMETERS OF SEMEN EXTENDER ADDITIVE

### 4.4.1. Lipid Peroxidation (LPO)

The overall mean Malondialdehyde (MDA) values using different concentrations of Trehalose in different groups are tabulated (Table 16). The overall mean Malondialdehyde (MDA) value in 100mM Trehalose group was significantly ($P < 0.05$) lower in post-dilution, post-equilibration and post-thaw stages of cryopreservation as compared to that of the 50mM Trehalose, 150mM Trehalose and control groups (Table 17, Figure 8).

### 4.4.2. Glutathione Reductase (GSH)

The overall mean glutathione reductase (GSH) using different concentrations of Trehalose in different groups are tabulated (Table 18). The overall mean glutathione reductase (GSH) value in 100mM Trehalose group was significantly ($P < 0.05$) higher in post-dilution, post-equilibration and post-thaw stages of cryopreservation as compared to that of the 50mM Trehalose, 150mM Trehalose and control groups (Table 19, Figure 9).

## 4.5. COMPARISON OF FRESH SEMEN PARAMETERS WITH FROZEN THAWED SEMEN

The overall mean per cent individual motility, sperm viability, sperm abnormality, osmotic resistance test (HOST) and acrosomal integrity of fresh semen were 88.75 ± 0.25, 89.69 ± 0.32, 3.08 ± 0.15, 86.80 ± 0.24 and 90.72 ±

0.25 respectively, whereas, corresponding values in 100mM Trehalose group at post-thaw stage of cryopreservation were 64.16 ± 0.52, 71.41 ± 0.31, 5.75 ± 0.35, 73.91 ± 0.35 and 80.91 ± 0.43 respectively, which was significantly ($P < 0.05$) higher as compared to that of 50mM Trehalose, 150mM Trehalose and control groups (Table 20, Table 21, Fig. 10).

In present investigation, the overall mean Malondialdehyde (MDA) and glutathione reductase (GSH) of fresh semen were 49.96 ± 0.06 µmol/ml and 30.14 ± 0.06 U/L respectively, whereas corresponding values in 100mM Trehalose group at post-thaw stage of cryopreservation were 20.06 ± 0.13 µmol/ml and 84.00 ± 0.16 U/L respectively, which significantly ($P < 0.05$) differed as compared to that of 50mM Trehalose, 150mM Trehalose and control groups (Table 22, Table 23, Fig. 11).

**Table 1. PHYSICAL CHARACTERISTICS OF FRESH SEMEN OF BANAS KANKREJ BULLS**

**(MEAN ± SE)**

| Bull No. | Individual Motility | Sperm Viability | Sperm Abnormality | Osmotic Resistance test | Acrosomal Integrity |
|---|---|---|---|---|---|
| **K26 (n=12)** | 89.50 ± 0.33 | 90.25 ± 0.27 | 2.91 ± 0.22 | 87.25 ± 0.51 | 91.25 ± 0.42 |
| **K28 (n=12)** | 89.00 ± 0.24 | 90.25 ± 0.44 | 3.16 ± 0.32 | 86.41 ± 0.35 | 90.58 ± 0.35 |
| **K=30 (n=12)** | 88.58 ± 0.37 | 89.41 ± 0.37 | 3.16 ± 0.24 | 86.75 ± 0.39 | 90.34 ± 0.49 |
| **Average (n=36)** | 89.02 ± 0.19 | 89.97 ± 0.21 | 3.08 ± 0.15 | 86.80 ± 0.24 | 90.72 0.25 |

- Means with different superscripts within column differ significantly at ($P < 0.05$) level.

**Table 2. ANOVA : PHYSICAL CHARACTERISTICS OF FRESH SEMEN OF BANAS KANKREJ BULLS**

| **Source of Variation** | **Individual Motility** | | | | |
|---|---|---|---|---|---|
| | **Df** | **SS** | **MS** | **Cal. F** | **Tab. F** |
| Between Bulls | 2 | 5.05 | 2.52 | 1.99 $^{NS}$ | 3.14 |
| Error | 33 | 41.91 | 1.27 | | |
| **Source of Variation** | **Sperm Viability** | | | | |
| | **Df** | **SS** | **MS** | **Cal. F** | **Tab. F** |
| Between Bulls | 2 | 5.55 | 2.77 | 1.65 $^{NS}$ | 3.14 |
| Error | 33 | 55.41 | 1.67 | | |
| **Source of Variation** | **Sperm Abnormality** | | | | |
| | **Df** | **SS** | **MS** | **Cal. F** | **Tab. F** |
| Between Bulls | 2 | 0.50 | 0.25 | 0.29 $^{NS}$ | 3.14 |
| Error | 33 | 28.25 | 0.85 | | |
| **Source of Variation** | **Osmotic Resistance test** | | | | |
| | **Df** | **SS** | **MS** | **Cal. F** | **Tab. F** |
| Between Bulls | 2 | 4.22 | 2.11 | 0.97 $^{NS}$ | 3.14 |
| Error | 33 | 71.41 | 2.16 | | |
| **Source of Variation** | **Acrosomal Integrity** | | | | |
| | **Df** | **SS** | **MS** | **Cal. F** | **Tab. F** |
| Between Bulls | 2 | 5.39 | 2.69 | 1.20 $^{NS}$ | 3.14 |
| Error | 33 | 73.83 | 2.23 | | |

**Note:** * - Significant at (P < 0.05) level, **NS** – Non Significant at (P < 0.05) level.

**Table 3. BIOCHEMICAL PARAMETERS OF FRESH SEMEN OF BANAS KANKREJ BULLS**

**(MEAN ± SE)**

| Bull No. | Lipid Peroxidation | Glutathione Reductase |
|---|---|---|
| **K26 (n=12)** | 50.03 ± 0.07 | 30.16 ± 0.12 |
| **K28 (n=12)** | 49.93 ± 0.14 | 30.25 ± 0.10 |
| **K=30 (n=12)** | 49.93 ± 0.12 | 30.02 ± 0.12 |
| **Average (n=36)** | 49.96 ± 0.06 | 30.14 ± 0.06 |

- Means with different superscripts within column differ significantly at ($P < 0.05$) level.

**Table 4. ANOVA : BIOCHEMICAL PARAMETERS OF FRESH SEMEN OF BANAS KANKREJ BULLS**

| Source of Variation | Lipid Peroxidation | | | | |
|---|---|---|---|---|---|
| | **df** | **SS** | **MS** | **Cal. F** | **Tab. F** |
| Between Bulls | 2 | 0.08 | 0.04 | $0.25^{NS}$ | 3.14 |
| Error | 33 | 5.33 | 0.16 | | |
| **Source of Variation** | **Glutathione Reductase** | | | | |
| | **df** | **SS** | **MS** | **Cal. F** | **Tab. F** |
| Between Bulls | 2 | 0.30 | 0.15 | $0.90^{NS}$ | 3.14 |
| Error | 33 | 5.52 | 0.16 | | |

**Note:** * - Significant at (P < 0.05) level, **NS** – Non Significant at (P < 0.05) level.

**Table 5. CORRELATION BETWEEN PHYSICAL CHARACTERISTICS OF FRESH SEMEN OF BANAS KANKREJ BULLS**

| Parameters | Individual Motility | Sperm Viability | Sperm Abnormality | Acrosomal Integrity | HOST | LPO | GSH |
|---|---|---|---|---|---|---|---|
| **Individual Motility** | 1 | | | | | | |
| **Sperm Viability** | 0.839* | 1 | | | | | |
| **Sperm Abnormality** | -0.890* | -0.5* | 1 | | | | |
| **Acrosomal Integrity** | 0.976* | 0.704* | -0.967* | 1 | | | |
| **HOST** | 0.631* | 0.109 | -0.915* | 0.782* | 1 | | |
| **LPO** | 0.890* | 0.5* | -0.105 | 0.967* | 0.915* | 1 | |
| **GSH** | 0.563* | 0.921* | -0.124 | 0.372 | -0.285 | 0.124 | 1 |

**Note:** * - Significant at ($P < 0.05$) level.

**TABLE 6. INDIVIDUAL MOTILITY PER CENT IN DIFFERENT GROUPS OF ADDITIVE AT VARIOUS STAGES OF CRYOPRESERVATION (MEAN ± S.E.)**

| Semen Additive Concentration | Post-Dilution Stage (PDS) | Post-Equilibration Stage (PES) | Post-Thaw Stage (PTS) | Overall Mean |
|---|---|---|---|---|
| Trehalose 50mM (n=36) | 80.08 ± 0.39[b] | 68.33 ± 0.35[b] | 58.00 ± 0.42[b] | 68.65 ± 0.15 |
| Trehalose 100mM(n=36) | 84.58 ± 0.31[c] | 75.00 ± 0.40[c] | 64.16 ± 0.52[c] | 74.54 ± 0.04 |
| Trehalose 150mM (n=36) | 79.41 ± 0.31[b] | 68.58 ± 0.31[b] | 57.58 ± 0.35[b] | 68.40 ± 0.12 |
| Control (n=36) | 77.91 ± 0.46[a] | 60.91 ± 0.45[a] | 53.91 ± 0.57[a] | 64.05 ± 0.20 |
| Overall Mean (n=144) | 80.50± 0.40 | 68.20± 0.75 | 58.41± 0.58 | 68.42 ± 0.62 |

- Means with different superscripts within column differ significantly at ($P < 0.05$) level.

**Table 7. ANOVA : INDIVIDUAL MOTILITY IN DIFFERENT GROUPS OF ADDITIVE AT VARIOUS STAGES OF CRYOPRESERVATION**

| Source of Variation | Df | SS | MS | Cal. F | Tab. F |
|---|---|---|---|---|---|
| Between Additives(T) | 3 | 1943.63 | 647.87 | 312.49* | 2.67 |
| Between Stages(S) | 2 | 11754.16 | 5877.08 | 2834.74* | 3.06 |
| T x S | 6 | 196.27 | 32.71 | 15.78* | 2.16 |
| Error | 132 | 273.67 | 2.07 | | |
| Total | 143 | 14167.75 | | | |

**Note:** * - Significant at (P < 0.05) level, **NS** – Non Significant at (P < 0.05) level.

**Table 8. SPERM VIABILITY PER CENT IN DIFFERENT GROUPS OF ADDITIVE AT VARIOUS STAGES OF CRYOPRESERVATION (MEAN ± S.E.)**

| Semen Additive Concentration | Post-Dilution Stage (PDS) | Post-Equilibration Stage (PES) | Post-Thaw Stage (PTS) | Overall Mean |
|---|---|---|---|---|
| Trehalose 50mM (n=36) | 81.16 ± 0.29$^{b}$ | 74.08 ± 0.25$^{b}$ | 65.58 ± 0.35$^{b}$ | 73.50 ± 0.11 |
| Trehalose 100mM (n=36) | 85.66 ± 0.46$^{c}$ | 81.41 ± 0.28$^{c}$ | 71.41 ± 0.31$^{c}$ | 79.00 ± 0.50 |
| Trehalose 150mM (n=36) | 81.41 ± 0.31$^{b}$ | 73.83 ± 0.32$^{b}$ | 65.83 ± 0.40$^{b}$ | 73.36 ± 0.33 |
| Control (n=36) | 76.08 ± 0.35$^{a}$ | 69.41 ± 0.33$^{a}$ | 60.33 ± 0.45$^{a}$ | 68.60 ± 0.01 |
| Overall Mean (n=144) | 81.08 ± 0.52 | 74.68 ± 0.64 | 65.79 ± 0.60 | 73.40 ± 0.45 |

- Means with different superscripts within column differ significantly at ($P < 0.05$) level.

**Table 9. ANOVA : SPERM VIABILITY IN DIFFERENT GROUPS OF ADDITIVE AT VARIOUS STAGES OF CRYOPRESERVATION**

| Source of Variation | Df | SS | MS | Cal. F | Tab. F |
|---|---|---|---|---|---|
| Between Additives(T) | 3 | 2140.18 | 713.39 | 478.61* | 2.67 |
| Between Stages(S) | 2 | 5662.04 | 2831.02 | 1899.33* | 3.06 |
| T x S | 6 | 40.96 | 6.82 | 4.578* | 2.16 |
| Error | 132 | 196.75 | 1.49 | | |
| Total | 143 | 8039.93 | | | |

**Note:** * - Significant at (P < 0.05) level, **NS** – Non Significant at (P < 0.05) level.

**Table 10. SPERM ABNORMALITY PER CENT IN DIFFERENT GROUPS OF ADDITIVE AT VARIOUS STAGES OF CRYOPRESERVATION (MEAN ± S.E.)**

| Semen Additive Concentration | Post-Dilution Stage (PDS) | Post-Equilibration Stage (PES) | Post-Thaw Stage (PTS) | Overall Mean |
|---|---|---|---|---|
| Trehalose 50mM (n=36) | 7.50 ± 0.39$^{c}$ | 8.08 ± 0.22$^{b}$ | 8.58 ± 0.31$^{b}$ | 7.30 ± 0.17 |
| Trehalose 100mM (n=36) | 4.67 ± 0.30$^{a}$ | 6.34 ± 0.30$^{a}$ | 5.75 ± 0.35$^{a}$ | 6.06 ± 0.02 |
| Trehalose 150mM (n=36) | 7.75 ± 0.32$^{c}$ | 9.08 ± 0.22$^{c}$ | 9.58 ± 0.31$^{c}$ | 8.06 ± 0.80 |
| Control (n=36) | 6.50 ± 0.33$^{b}$ | 7.75 ± 0.27$^{b}$ | 9.67 ± 0.35$^{c}$ | 8.12 ± 0.40 |
| Overall Mean (n=144) | 6.60 ± 0.24 | 7.81 ± 0.19 | 8.39 ± 0.28 | 7.23 ± 0.50 |

- Means with different superscripts within column differ significantly at ($P < 0.05$) level.

**Table 11. ANOVA : SPERM ABNORMALITY IN DIFFERENT GROUPS OF ADDITIVE AT VARIOUS STAGES OF CRYOPRESERVATION**

| Source of Variation | Df | SS | MS | Cal. F | Tab. F |
|---|---|---|---|---|---|
| **Between Additives(T)** | 3 | 168.97 | 56.32 | 35.74* | 2.67 |
| **Between Stages(S)** | 2 | 189.84 | 94.92 | 60.23* | 3.06 |
| **T x S** | 6 | 51.15 | 8.52 | 5.41* | 2.16 |
| **Error** | 132 | 208 | 1.57 | | |
| **Total** | 143 | 617.97 | | | |

**Note:** * - Significant at (P < 0.05) level, **NS** – Non Significant at (P < 0.05) level.

**Table 12. OSMOTIC RESISTANCE TEST PER CENT IN DIFFERENT GROUPS OF ADDITIVE AT VARIOUS STAGES OF CRYOPRESERVATION (MEAN ± S.E.)**

| Semen Additive Concentration | Post-Dilution Stage (PDS) | Post-Equilibration Stage (PES) | Post-Thaw Stage (PTS) | Overall Mean |
|---|---|---|---|---|
| Trehalose 50mM (n=36) | 81.25 ± 0.32[b] | 74.66 ± 0.37[b] | 67.25 ± 0.32[a] | 74.30 ± 0.08 |
| Trehalose 100mM (n=36) | 83.25 ± 0.32[c] | 78.16 ± 0.42[c] | 73.91 ± 0.35[c] | 78.04 ± 0.40 |
| Trehalose 150mM (n=36) | 79.83 ± 0.30[a] | 74.25 ± 0.35[b] | 69.91 ± 0.22[b] | 74.33 ± 0.33 |
| Control (n=36) | 79.33 ± 0.41[a] | 72.41 ± 0.67[a] | 66.50 ± 0.55[a] | 72.25 ± 0.55 |
| Overall Mean (n=144) | 80.91 ± 0.27 | 74.87 ± 0.38 | 69.39 ± 0.46 | 74.85 ± 0.21 |

- Means with different superscripts within column differ significantly at ($P < 0.05$) level.

**Table 13. ANOVA : OSMOTIC RESISTANCE TEST IN DIFFERENT GROUPS OF ADDITIVE AT VARIOUS STAGES OF CRYOPRESERVATION**

| Source of Variation | Df | SS | MS | Cal. F | Tab. F |
|---|---|---|---|---|---|
| Between Additives(T) | 3 | 626.24 | 208.74 | 105.40* | 2.67 |
| Between Stages(S) | 2 | 3188.04 | 1594.02 | 804.88* | 3.06 |
| T x S | 6 | 96.73 | 16.12 | 8.14* | 2.16 |
| Error | 132 | 261.41 | 1.98 | | |
| Total | 143 | 4172.43 | | | |

**Note:** * - Significant at (P < 0.05) level, **NS** – Non Significant at (P < 0.05) level.

**Table 14. ACROSOMAL INTEGRITY PER CENT IN DIFFERENT GROUPS OF ADDITIVE AT VARIOUS STAGES OF CRYOPRESERVATION (MEAN ± S.E.)**

| Semen Additive Concentration | Post-Dilution Stage (PDS) | Post-Equilibration Stage (PES) | Post-Thaw Stage (PTS) | Overall Mean |
|---|---|---|---|---|
| Trehalose 50mM (n=36) | 87.25 ± 0.46$^{b}$ | 81.91 ± 0.43$^{b}$ | 72.41 ± 0.37$^{b}$ | 80.30 ± 0.22 |
| Trehalose 100mM (n=36) | 91.08 ± 0.41$^{c}$ | 85.41 ± 0.39$^{c}$ | 80.91 ± 0.43$^{c}$ | 85.40 ± 0.40 |
| Trehalose 150mM (n=36) | 87.58 ± 0.33$^{b}$ | 82.00 ± 0.38$^{b}$ | 72.91 ± 0.39$^{b}$ | 80.56 ± 0.27 |
| Control (n=36) | 81.75 ± 0.32$^{a}$ | 78.50 ± 0.33$^{a}$ | 68.83 ± 0.38$^{a}$ | 76.25 ± 0.11 |
| Overall Mean (n=144) | 86.91 ± 0.52 | 81.95 ± 0.40 | 73.77 ± 0.67 | 80.50 ± 0.38 |

- Means with different superscripts within column differ significantly at ($P < 0.05$) level.

**Table 15. ANOVA : ACROSOMAL INTEGRITY IN DIFFERENT GROUPS OF ADDITIVE AT VARIOUS STAGES OF CRYOPRESERVATION**

| Source of Variation | Df | SS | MS | Cal. F | Tab. F |
|---|---|---|---|---|---|
| Between Additives(T) | 3 | 1613.07 | 537.69 | 289.00* | 2.67 |
| Between Stages(S) | 2 | 4230.93 | 2115.46 | 1137.05* | 3.06 |
| T x S | 6 | 145.40 | 24.23 | 13.02* | 2.16 |
| Error | 132 | 245.58 | 1.86 | | |
| Total | 143 | 6234.99 | | | |

**Note:** * - Significant at (P < 0.05) level, **NS** – Non Significant at (P < 0.05) level.

**Table 16. LIPID PEROXIDATION (µmol/ml) IN DIFFERENT GROUPS OF ADDITIVE AT VARIOUS STAGES OF CRYOPRESERVATION (MEAN ± S.E.)**

| Semen Additive Concentration | Post-Dilution Stage (PDS) | Post-Equilibration Stage (PES) | Post-Thaw Stage (PTS) | Overall Mean |
|---|---|---|---|---|
| Trehalose 50mM (n=36) | 57.77 ± 0.11$^{c}$ | 40.11 ± 0.06$^{c}$ | 32.15 ± 0.09$^{c}$ | 43.22 ± 0.12 |
| Trehalose 100mM (n=36) | 43.92 ± 0.05$^{a}$ | 34.20 ± 0.09$^{a}$ | 20.06 ± 0.13$^{a}$ | 32.43 ± 0.30 |
| Trehalose 150mM (n=36) | 50.06 ± 0.08$^{b}$ | 39.01 ± 0.10$^{b}$ | 28.04 ± 0.08$^{b}$ | 38.16 ± 0.88 |
| Control (n=36) | 58.01 ± 0.08$^{c}$ | 40.10 ± 0.07$^{c}$ | 31.98 ± 0.07$^{c}$ | 43.10 ± 0.26 |
| Overall Mean (n=144) | 52.44 ± 0.85 | 38.35 ± 0.35 | 28.06 ± 0.71 | 38.92 ± 0.70 |

- Means with different superscripts within column differ significantly at ($P < 0.05$) level.

**Table 17. ANOVA: LIPID PEROXIDATION IN DIFFERENT GROUPS OF ADDITIVE AT VARIOUS STAGES OF CRYOPRESERVATION**

| Source of Variation | Df | SS | MS | Cal. F | Tab. F |
|---|---|---|---|---|---|
| **Between Additives(T)** | 3 | 2722.71 | 907.57 | 8589.83* | 2.67 |
| **Between Stages(S)** | 2 | 14386.81 | 7193.40 | 68083.01* | 3.06 |
| **T x S** | 6 | 365.90 | 60.98 | 577.18* | 2.16 |
| **Error** | 132 | 13.94 | 0.10 | | |
| **Total** | 143 | 17489.36 | | | |

**Note:** * - Significant at (P < 0.05) level, **NS** – Non Significant at (P < 0.05) level.

**Table 18. GLUTATHIONE REDUCTASE (U/L) IN DIFFERENT GROUPS OF ADDITIVE AT VARIOUS STAGES OF CRYOPRESERVATION (MEAN ± S.E.)**

| Semen Additive Concentration | Post-Dilution Stage (PDS) | Post-Equilibration Stage (PES) | Post-Thaw Stage (PTS) | Overall Mean |
|---|---|---|---|---|
| Trehalose 50mM (n=36) | 38.12 ± 0.18[a] | 50.24 ± 0.07[b] | 62.15 ± 0.08[b] | 50.15 ± 0.02 |
| Trehalose 100mM (n=36) | 55.83 ± 0.13[c] | 64.00 ± 0.15[c] | 84.00 ± 0.16[c] | 67.74 ± 0.20 |
| Trehalose 150mM (n=36) | 42.11 ± 0.07[b] | 50.18 ± 0.07[b] | 57.07 ± 0.06[a] | 49.60 ± 0.18 |
| Control (n=36) | 38.35 ± 0.13[a] | 49.14 ± 0.07[a] | 62.04 ± 0.07[b] | 49.62 ± 0.22 |
| Overall Mean (n=144) | 43.60 ± 1.05 | 53.39 ± 0.89 | 66.31 ± 1.51 | 54.33 ± 1.01 |

- Means with different superscripts within column differ significantly at ($P < 0.05$) level.

**Table 19. ANOVA : GLUTATHIONE REDUCTASE IN DIFFERENT GROUPS OF ADDITIVE AT VARIOUS STAGES OF CRYOPRESERVATION**

| Source of Variation | Df | SS | MS | Cal. F | Tab. F |
|---|---|---|---|---|---|
| Between Additives(T) | 3 | 8760.63 | 2920.21 | 18450.60* | 2.67 |
| Between Stages(S) | 2 | 12458.23 | 6229.11 | 39357.03* | 3.06 |
| T x S | 6 | 768.56 | 128.09 | 809.34* | 2.16 |
| Error | 132 | 20.89 | 0.15 | | |
| Total | 143 | 22008.33 | | | |

**Note:** * - Significant at (P < 0.05) level, **NS** – Non Significant at (P < 0.05) level.

**TABLE 20. COMPARISON OF FRESH SEMEN PARAMETERS WITH FROZEN THAWED SEMEN (MEAN ± SE) (n= 36)**

| Semen Additive Concentration | Individual Motility | Sperm Viability | Sperm Abnormality | Osmotic Resistance test | Acrosomal Integrity |
|---|---|---|---|---|---|
| Overall (Fresh) | 89.02 ± 0.19$^{d}$ | 89.97 ± 0.21$^{d}$ | 3.08 ± 0.15$^{a}$ | 86.80 ± 0.24$^{d}$ | 90.73 ±0.25$^{d}$ |
| Trehalose 50mM | 58.00 ± 0.42$^{b}$ | 65.58± 0.35$^{b}$ | 8.58 ± 0.31$^{c}$ | 67.25 ± 0.32$^{a}$ | 72.41 ± 0.37$^{b}$ |
| Trehalose 100mM | 64.16 ± 0.52$^{c}$ | 71.41± 0.31$^{c}$ | 5.75 ± 0.35$^{b}$ | 73.91 ± 0.35$^{c}$ | 80.91 ± 0.43$^{c}$ |
| Trehalose 150mM | 57.58 ± 0.35$^{b}$ | 65.83± 0.40$^{b}$ | 9.58 ± 0.31$^{d}$ | 69.91 ± 0.22$^{b}$ | 72.91 ± 0.39$^{b}$ |
| Control | 53.91 ± 0.57$^{a}$ | 60.33± 0.45$^{a}$ | 9.67 ± 0.35$^{d}$ | 66.50 ± 0.55$^{a}$ | 68.83 ± 0.38$^{a}$ |

- Means with different superscripts within column differ significantly at ($P < 0.05$) level.

**Table 21. ANOVA : COMPARISON OF FRESH SEMEN PARAMETERS WITH FROZEN THAWED SEMEN**

| Source of Variation | df | SS | MS | Cal. F | Tab. F |
|---|---|---|---|---|---|
| **Between Parameters(P)** | 4 | 198294.94 | 49573.74 | 25561.45* | 2.40 |
| **Between Additives(A)** | 4 | 15191.37 | 3797.84 | 1958.26* | 2.40 |
| **P x A** | 16 | 8669.04 | 541.81 | 279.37* | 1.68 |
| **Error** | 275 | 533.34 | 1.93 | | |
| **Total** | 299 | 222688.67 | | | |

**Note:** * - Significant at (P < 0.05) level, **NS** – Non Significant at (P < 0.05) level.

**Table 22. COMPARISON OF FRESH SEMEN BIOCHEMICAL PARAMETERS WITH FROZEN THAWED SEMEN (MEAN ± SE) (n= 36)**

| Semen Additive Concentration | Lipid Peroxidation | Glutathione Reductase |
|---|---|---|
| Overall (Fresh) | $49.96 \pm 0.06^{d}$ | $30.14 \pm 0.06^{a}$ |
| Trehalose 50mM (n=36) | $32.15 \pm 0.09^{c}$ | $62.15 \pm 0.08^{c}$ |
| Trehalose 100mM(n=36) | $20.06 \pm 0.13^{a}$ | $84.00 \pm 0.16^{d}$ |
| Trehalose 150mM (n=36) | $28.04 \pm 0.08^{b}$ | $57.07 \pm 0.06^{b}$ |
| Control (n=36) | $31.98 \pm 0.07^{c}$ | $62.04 \pm 0.07^{c}$ |

- Means with different superscripts within column differ significantly at ($P < 0.05$) level.

**Table 23. ANOVA : COMPARISON OF FRESH SEMEN BIOCHEMICAL PARAMETERS WITH FROZEN THAWED SEMEN**

| Source of Variation | df | SS | MS | Cal. F | Tab. F |
|---|---|---|---|---|---|
| **Between Parameters(P)** | 1 | 21338.93 | 21338.93 | 149226.90* | 3.92 |
| **Between Additives(A)** | 4 | 2045.45 | 511.36 | 3576.06* | 2.45 |
| **P x A** | 4 | 21395.73 | 5348.93 | 37406.01* | 2.45 |
| **Error** | 110 | 15.72963 | 0.14 | | |
| **Total** | 119 | 44795.85 | | | |

**Note:** *- Significant at (P < 0.05) level, **NS**- Non Significant at (P < 0.05) level.

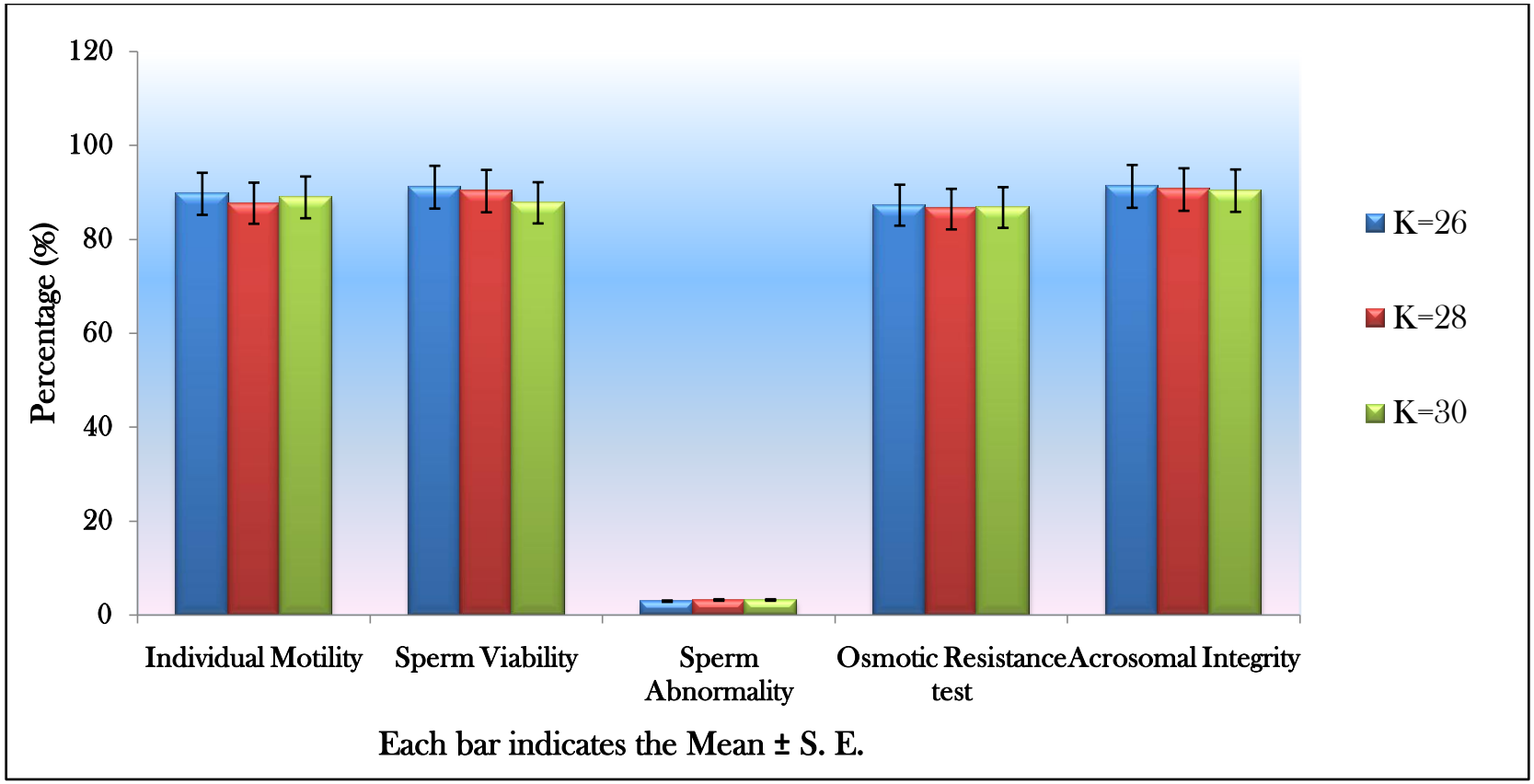



**Figure 1. GRAPHICAL REPRESENTATION OF PHYSICAL CHARACTERISTICS OF FRESH SEMEN OF BANAS KANKREJ BULLS (MEAN ± SE)**

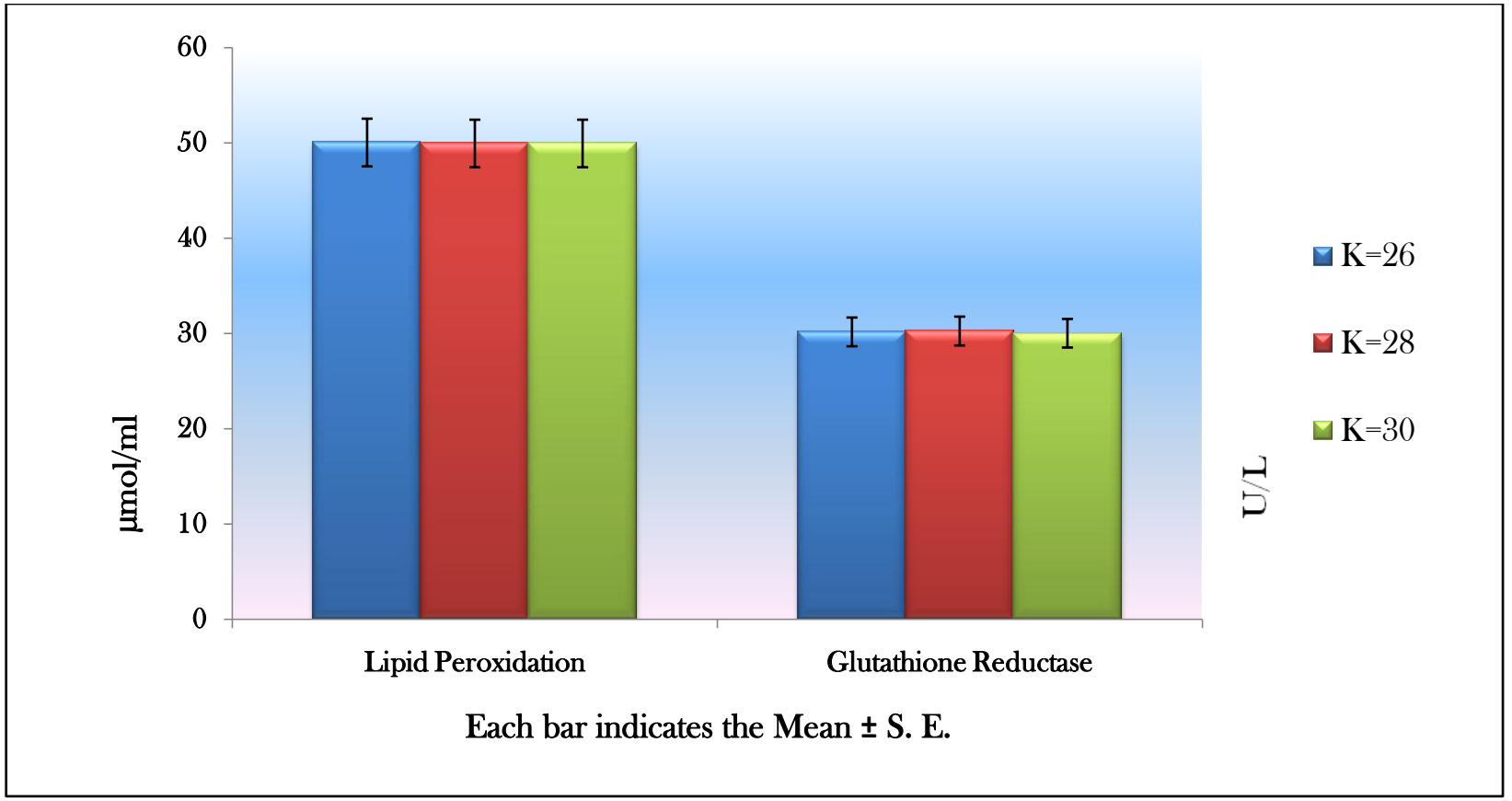



**Figure 2. GRAPHICAL REPRESENTATION OF BIOCHEMICAL PARAMETERS OF FRESH SEMEN OF BANAS KANKREJ BULLS**

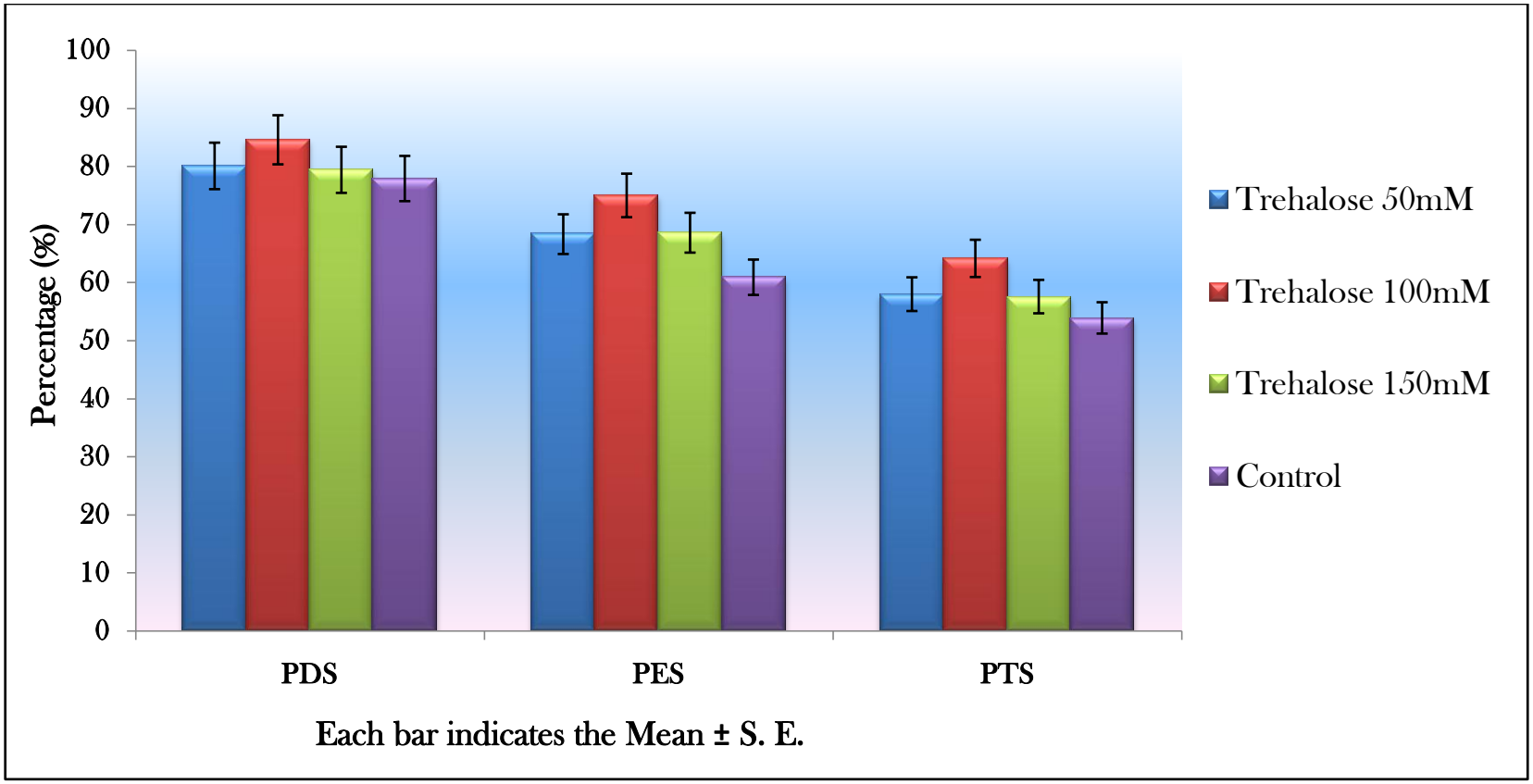



**Figure 3. GRAPHICAL REPRESENTATION OF PER CENT INDIVIDUAL MOTILITY IN DIFFERENT GROUPS AT VARIOUS STAGES OF CRYOPRESERVATION**

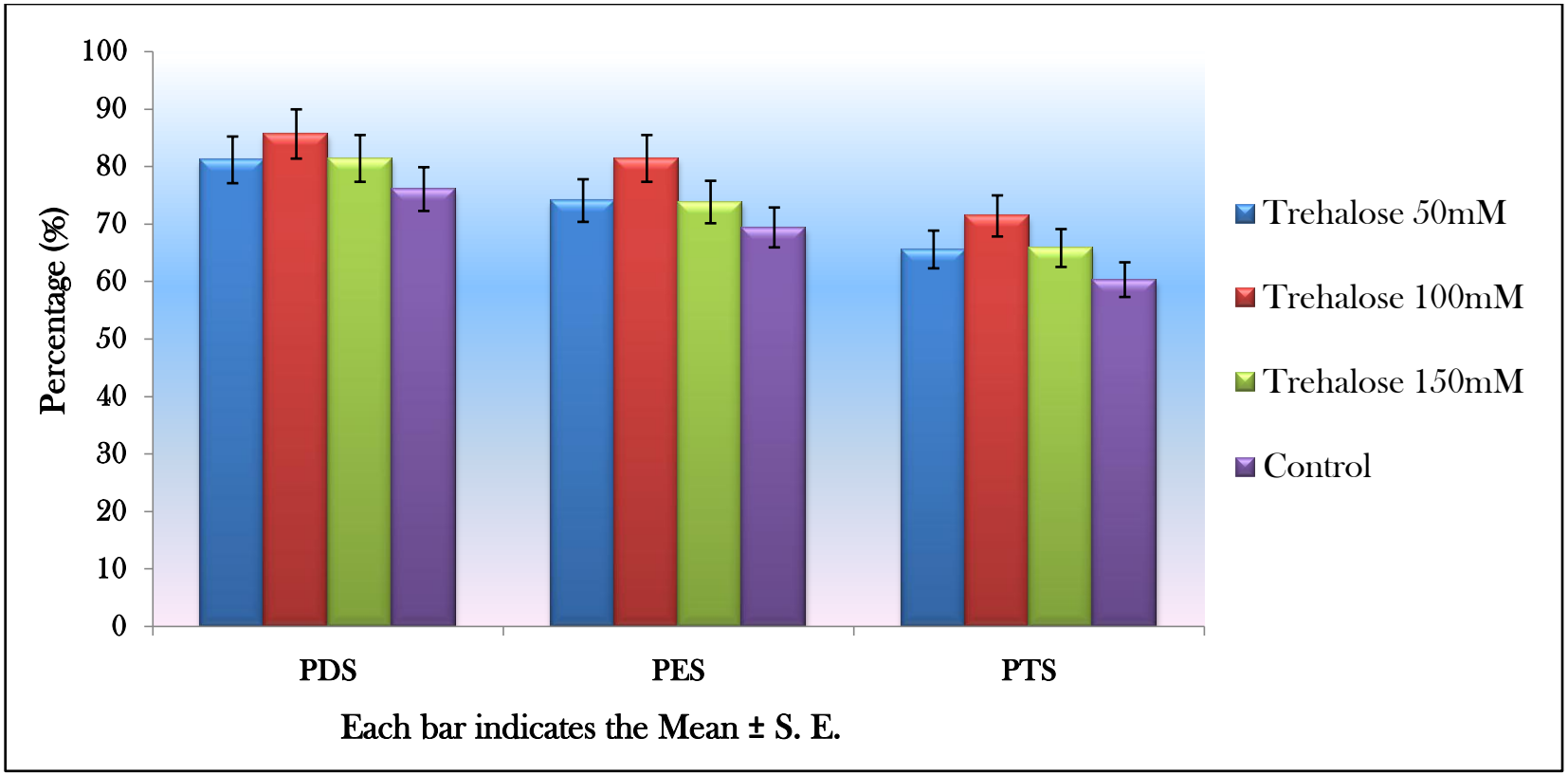



**Figure 4. GRAPHICAL REPRESENTATION OF PER CENT SPERM VIABILITY IN DIFFERENT GROUPS OF ADDITIVE AT VARIOUS STAGES OF CRYOPRESERVATION**

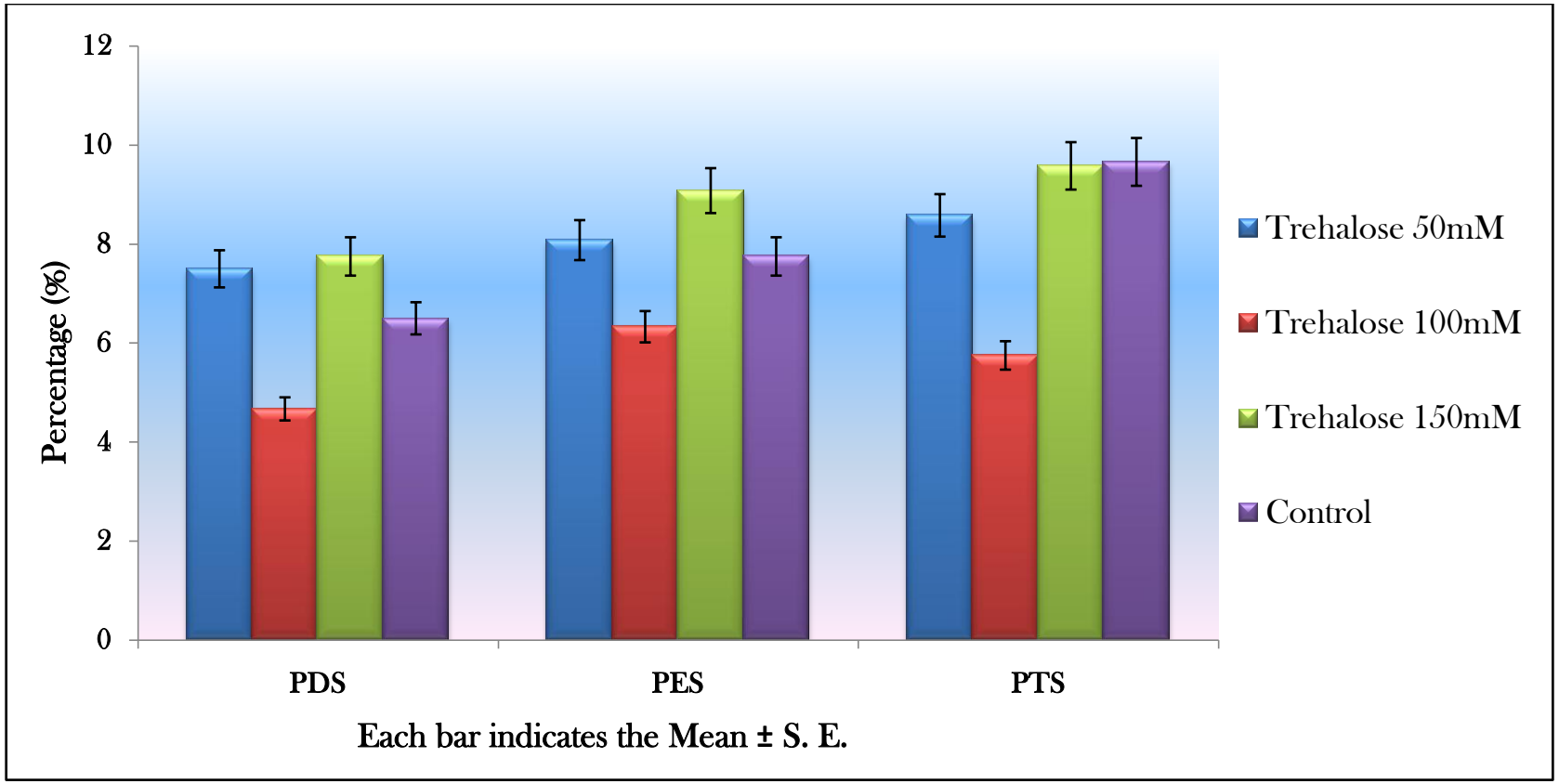



**Figure 5. GRAPHICAL REPRESENTATION OF PER CENT SPERM ABNORMALITY IN DIFFERENT GROUPS OF ADDITIVE AT VARIOUS STAGES OF CRYOPRESERVATION**

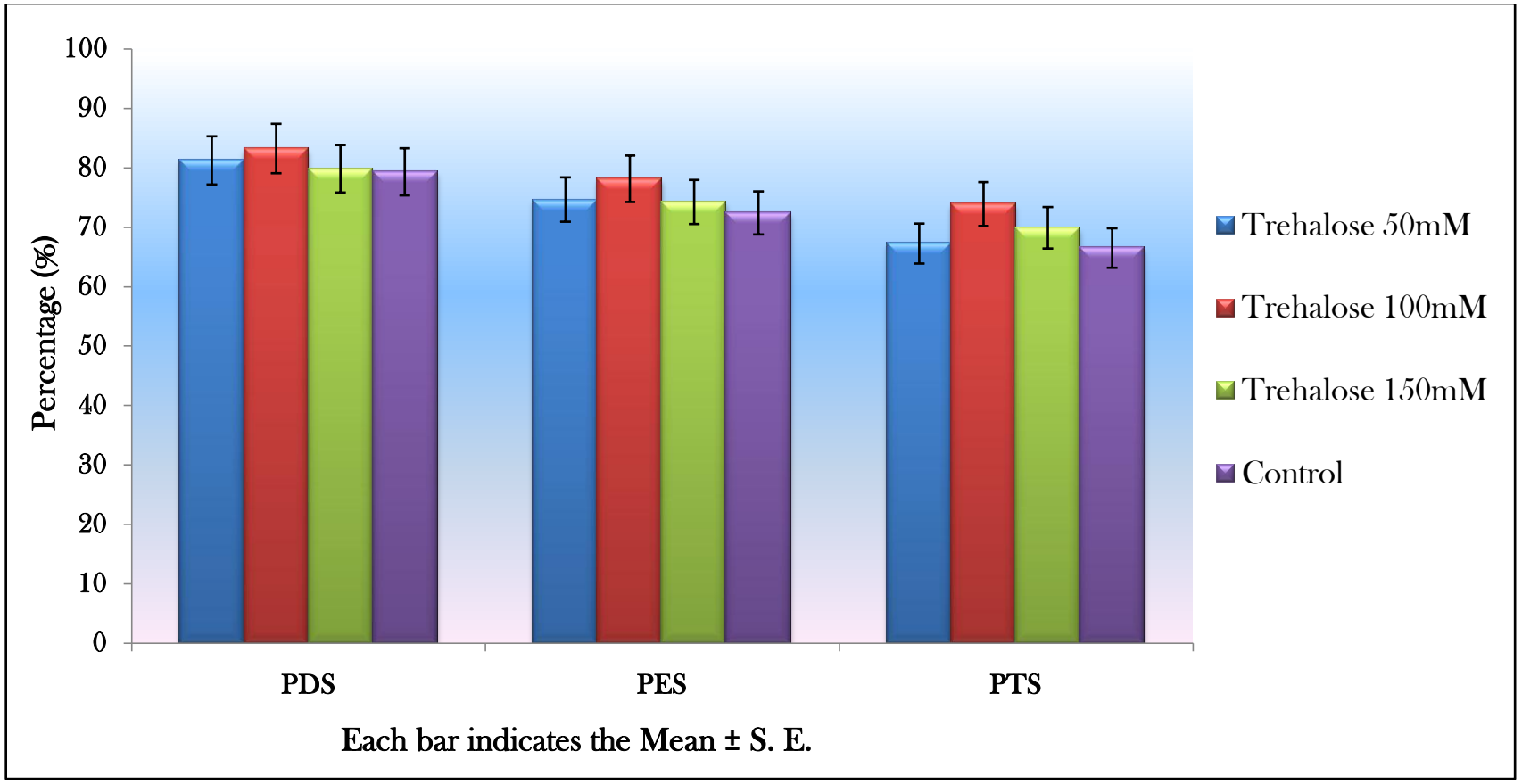



**Figure 6. GRAPHICAL REPRESENTATION OF PER CENT OSMOTIC RESISTANCE TEST IN DIFFERENT GROUPS OF ADDITIVE AT VARIOUS STAGES OF CRYOPRESERVATION**

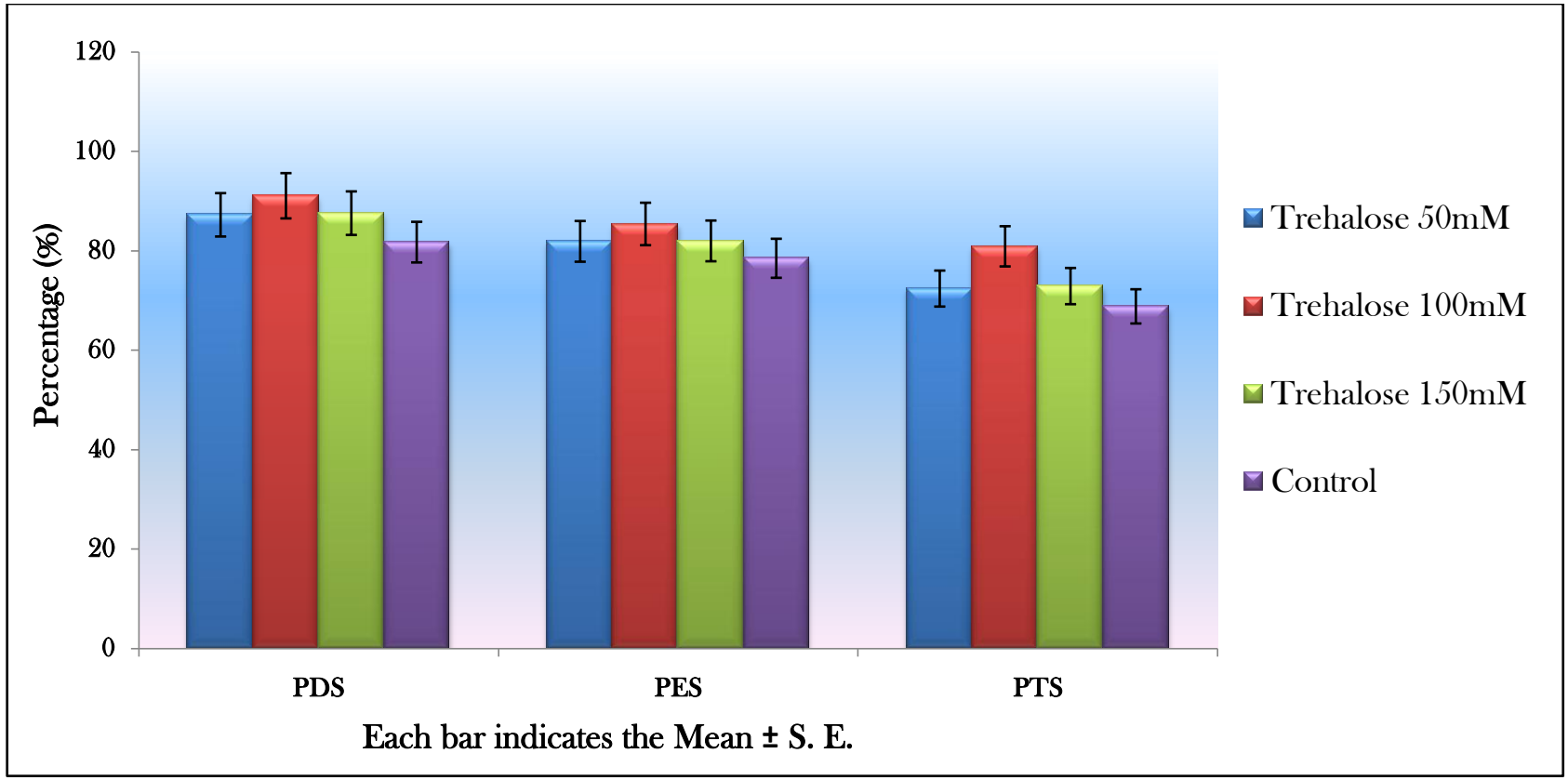



**Figure 7. GRAPHICAL REPRESENTATION OF PER CENT ACROSOMAL INTEGRITY IN DIFFERENT GROUPS OF ADDITIVE AT VARIOUS STAGES OF CRYOPRESERVATION**

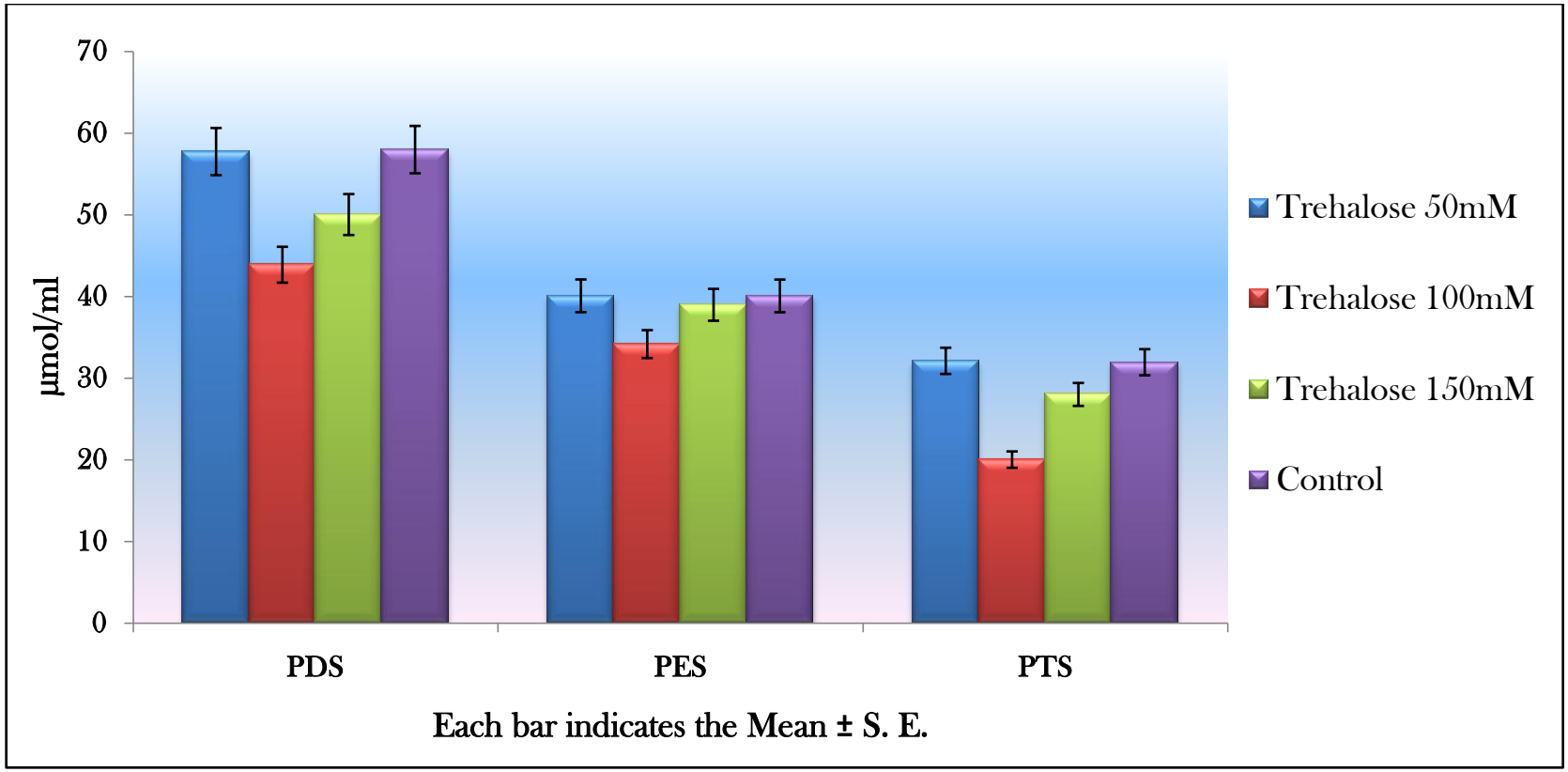



**Figure 8. GRAPHICAL REPRESENTATION OF LIPID PEROXIDATION(µmol/ml) IN DIFFERENT GROUPS OF ADDITIVE AT VARIOUS STAGES OF CRYOPRESERVATION**

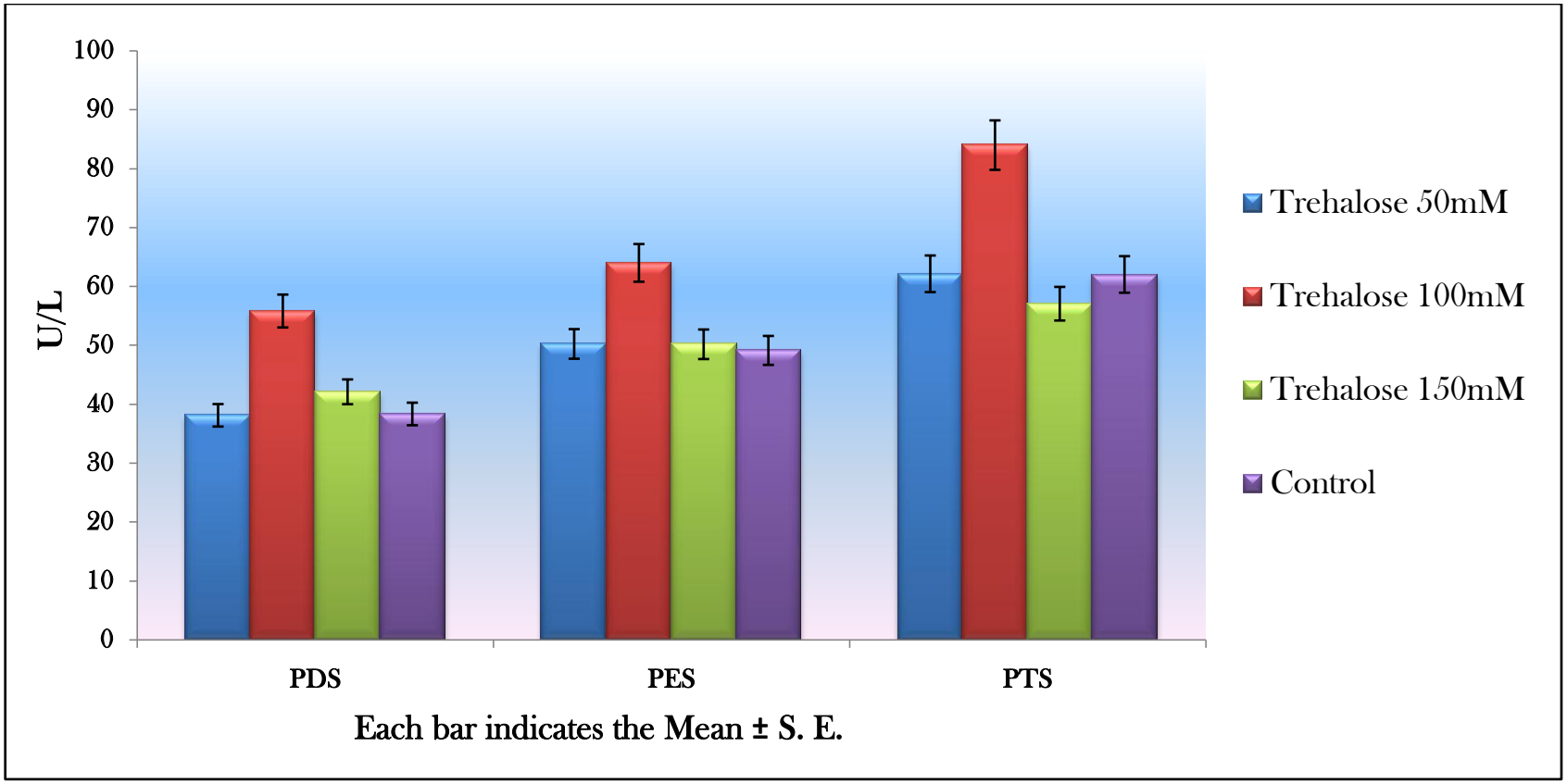



**Figure 9. GRAPHICAL REPRESENTATION OF GLUTATHIONE REDUCTASE (U/L) IN DIFFERENT GROUPS OF ADDITIVE AT VARIOUS STAGES OF CRYOPRESERVATION**

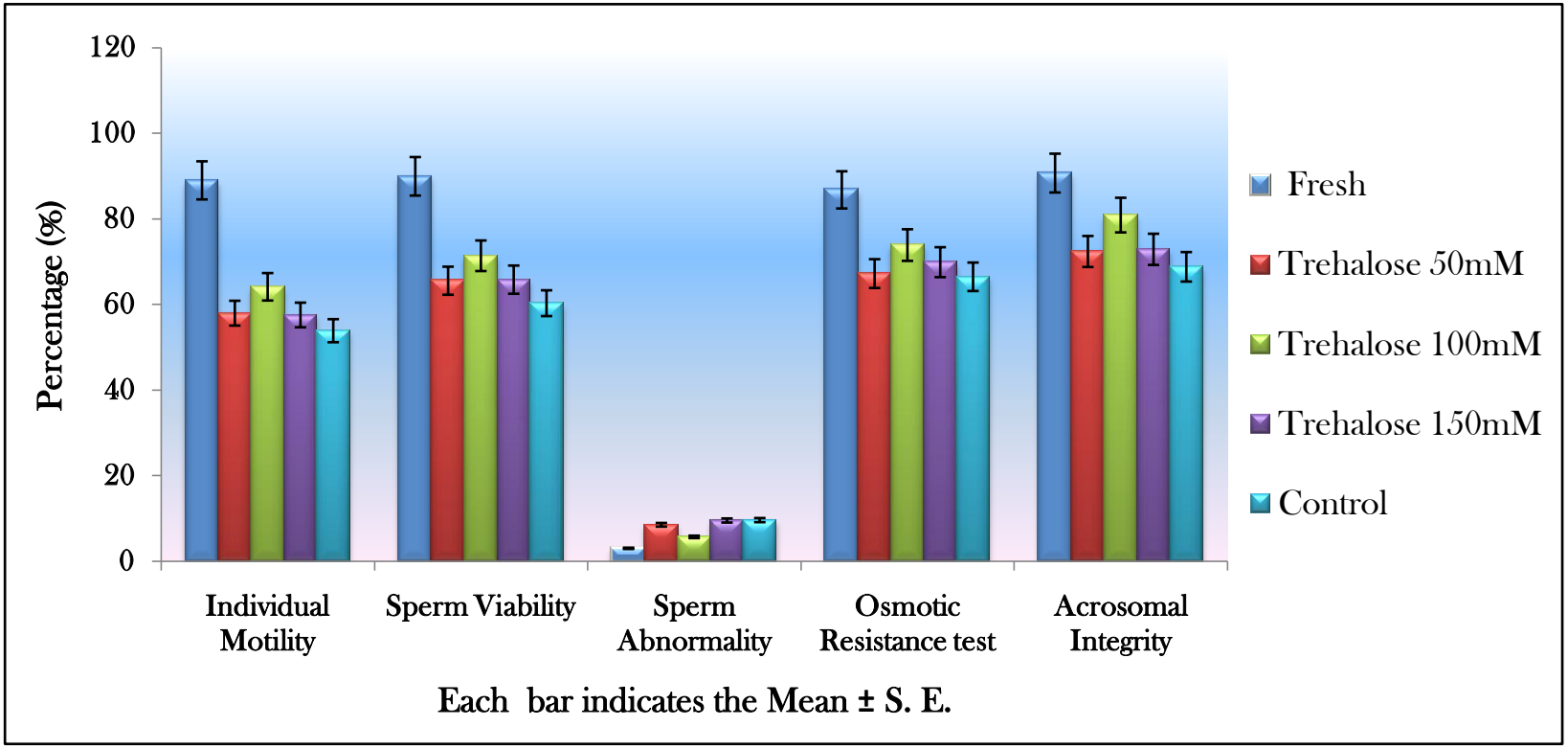



**Figure 10. GRAPHICAL REPRESENTATION OFCOMPARISION OF FRESH SEMEN PARAMETERS WITH FROZEN THAWED SEMEN**

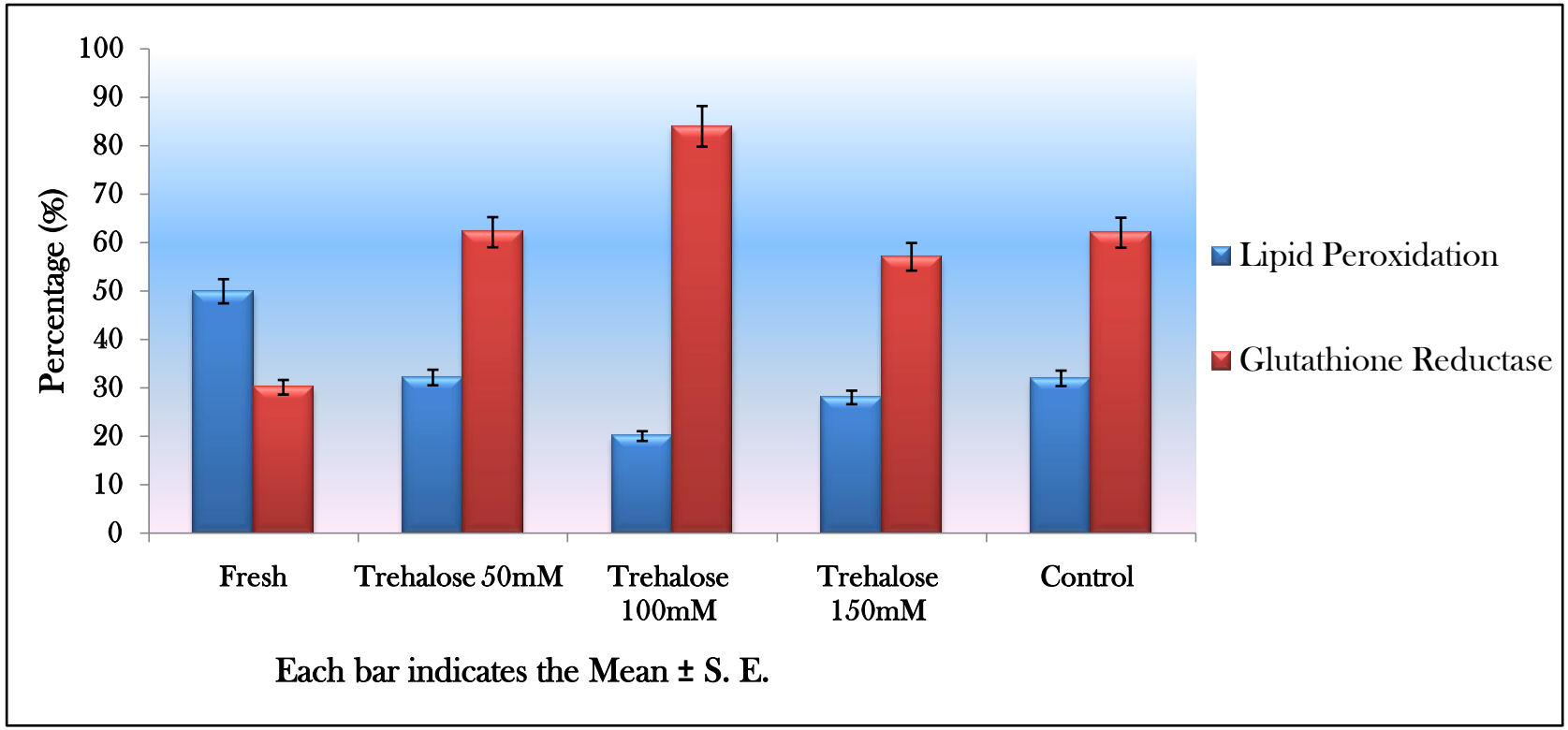



**Figure 11. GRAPHICAL REPRESENTATION OF COMPARISON OF FRESH SEMEN BIOCHEMICAL PARAMETERS WITH FROZEN THAWED SEMEN**

# CHAPTER 5: DISCUSSION

Trehalose was added as semen diluent additive at different concentrations in semen aliquots to work out the freezability and enzymatic alterations in different groups. The results obtained during the investigation have been discussed under different headings.

## 5.1. EVALUATION OF PHYSICAL CHARACTERISTICS OF FRESH SEMEN

### 5.1.1. Individual Motility

The mean individual motility recorded was 88.75 ± 0.25 per cent which ranged between 86 to 92 per cent. There was no significant statistical difference in individual motility per cent among the three bulls.

The present findings corroborate Patel (2012) who has reported the mean individual motility as 86.15 ± 0.30 per cent in Kankrej bulls. Whilst, a lower but variable mean per cent individual motility in Gir bulls as compared to that of the Banas Kankrej bull have been reported viz. 67.87 ± 2.69 (Shelke and Dhami, 2001) and 72.31 ± 3.19 (Dhami *et al*. 2003).

Similarly, Mandal *et al*. (2005) and Ray and Ghosh, (2013) have reported the lower mean per cent individual motility being 52.71 ± 1.31 and 76.73 ± 0.43 per cent in Sahiwal bulls as compared to that of the Banas Kankrej bulls.

Whereas, a variable per cent individual motility in exotic bulls has been reported by Srivastava and Kumar, (2014); Srivastava and Kumar, (2006); Kumar, (2004); Dhami *et al*. (2003); Mathur *et al*. (2002); Tomar *et al*. (2000); Nath *et al*. (1996); Dhami and Sahni, (1994) and Singh and Pangawkar, (1990). Screening through the literature on crossbred bulls of different breeds revealed a variable mean per cent individual motility with more than 80 per cent as 81.20

± 0.98 in triplebred (HF × J × Kankrej) bulls (Raval, 2006) and 85.44 ± 1.56 in halfbred bulls (Patel *et al.* 1989).

Different authors have reported below 80 per cent and variable individual motility among the crossbred bulls of different breeds (Srivastava and Kumar, 2014; Perumal *et al.* 2009; Srivastava and Kumar, 2006; Shaha *et al.* 2008; Sharma *et al.* 1992; Dhami *et al.* 2003; Prasad *et al.* 1999; Singh and Pangawkar, 1990) which were lower than that of the Banas Kankrej bull in the present study.

The variation observed in per cent individual motility reported by different researchers for the different breeds of cattle might be due to the differences of breed, age of bull, season, viscosity of diluents, increasing concentration of spermatozoa, pH etc.

Higher individual motility per cent in Banas Kankrej bull recorded in the present study might be due to factors like breed, age of bull and frequency of collection. However, like as in present study. Patel and Siddiquee (2013) have also reported higher individual motility count in the Kankrej bull semen in comparison with other breeds of Indian cattle.

Correlation studies revealed that the individual motility had significant ($P < 0.05$) positive correlation with sperm viability, HOST reactive sperm per cent, acrosomal integrity, lipid peroxidation and glutathione reductase and an inverse correlation with sperm abnormality which corroborate the findings of Shelke and Dhami (2001) in Gir bulls, Ray and Ghosh (2013) in Sahiwal bulls and Sharma (2012) in Jersey x local hill cattle crossbred bulls.

### 5.1.2. Sperm Viability

In the present study, sperm viability ranged between 86 to 92 per cent. The overall mean value of sperm viability was 89.69 ± 0.32 per cent with no significant difference among bulls.

The present findings are in agreement with Patel (2012) and Desai (2013) who have reported the mean sperm viability per cent in Kankrej bulls as 90.58 ± 0.20 and 90.15 ± 0.52, respectively. Similarly, Rajoria *et al.* (2011) has also reported higher mean viable sperm per cent (88.23 ± 1.82) in Tharaparkar bulls.

In comparison with Banas Kankrej breed, a lower and variable mean sperm viability has been reported by Singh *et al.* (2000); Ray and Ghosh (2013) and Mandal *et al.* (2005) in Sahiwal bulls being 85.75 ± 0.04, 83.37 ± 0.34 and 70.40 ± 3.9 per cent, respectively. Similarly in Gir bulls, a variation in sperm viability also exists i.e. 80.81 ± 1.29 per cent (Dhami *et al.* 2003) and 71.85 ± 1.49 per cent (Rana, 2001).

Screening the literature has revealed a higher than 85 per cent mean sperm viability among the crossbred bulls of different breeds viz. 90.27 ± 0.46 in cross-bred bulls (Desai, 2013); 88.89 ± 0.83 in triplebred (HF × J × Kankrej) bulls (Raval, 2006); 89.95 ± 0.49 and 85.22 ± 0.28 in good freezable and poor freezable Jersey crossbred bulls (Perumal *et al.* 2009) which is closely similar to that of the Banas Kankrej bull in the present study. Whereas, different authors have reported sperm viability below 85 per cent among the crossbred bulls (Sharma *et al.* 1992; Prasad *et al.* 1999; Bhoite *et al.* 2005; Johar *et al.* 2006 and Shaha *et al.* 2008) which is lower than that of the Kankrej bull in the present study.

Documentation for per cent sperm viability in HF bulls (Singh and Pangawkar, 1990; Dhami *et al.* 1998; Tomar *et al.* 2000; Dhami *et al.* 2003 and Srivastava and Kumar, 2006) have shown variable findings with as low as 73.69 ± 0.12 (Tomar *et al.* 2000) and highest as 87.38 ± 11.46 (Srivastava and Kumar, 2006). Whereas, sperm viability per cent in Jersey bulls among the various authors (Singh and Pangawkar, 1990; Nath *et al.* 1996; Singh *et al.* 2000; Dhami *et al.* 2003; Kumar, 2004 and Johar *et al.* 2006) were as low as 76.00 ± 1.90 (Johar *et al.* 2006) and highest as 82.97 ± 0.90 (Singh and Pangawkar, 1990).

The variation in sperm viability observed in different reports of different breeds of cattle might be due to environmental affliction, seasonal variations, temperature shock, state of maturation, frequency of collection, etc.

In the present study, higher sperm viability observed in Kankrej bull might be due to various factors like age of bull, season and frequency of collection. However, Patel and Siddiquee (2013) have also reported higher live sperm count in Kankrej bull in comparison with other breeds of Indian cattle.

Correlation studies revealed that the sperm viability had highly significant ($P < 0.05$) positive correlation with acrosomal integrity, lipid peroxidation and glutathione reductase whereas, inverse correlation with sperm abnormality which is in harmony with the findings of Rana and Dhami (2004) in Gir bulls, Ray and Ghosh (2013) in Sahiwal bulls, Sharma (2012) in Jersey x local hill cattle crossbred bulls, Raval (2006) in triplebred (HF × J × Kankrej) bulls and Kumar (2004) in Jersey bulls.

### 5.1.3. Sperm Abnormality

The mean sperm abnormality observed between 2 to 5 per cent with mean value of 3.08 ± 0.15 per cent which did not differ significantly among bulls. The present findings corroborate (Patel, 2012) who has reported the 4.24 ± 0.03 per cent sperm abnormality in Kankrej bulls, whereas, comparatively higher 9.79 ± 0.20 per cent sperm abnormality has been reported in Kankrej bulls (Desai, 2013).

In comparison with Banas Kankrej breed, a higher but variable mean abnormal sperm has been reported in Sahiwal bulls by Mandal *et al.* (2005) and Ray and Ghosh (2013) being 18.40 ± 3.03 and 9.97 ± 0.28 per cent, respectively.

Similarly, higher per cent sperm abnormality have also been recorded in Gir bulls (Rana, 2001; Shelke and Dhami, 2001 and Dhami *et al.* 2003); in Ongole bulls (Baburao *et al.* 1999 and Talluri *et al.* 2011); in Punganur and Tharaparkar bulls (Baburao *et al.* 1999 and Rajoria *et al.* 2011).

Whilst, in HF bulls a variable sperm abnormality as low as 1.54 ± 0.01 (Tomar *et al.* 2000) and as high as 14.10 ± 2.04 per cent (Dhami *et al.* 2003) have been reported. Similarly, reports for the Jersey bulls revealed higher sperm abnormality as 10.45 ± 0.20 per cent (Singh and Pangawkar, 1990) to 24.50 ± 4.78 (Dhami *et al.* 2003).

Similar variation in mean per cent abnormal sperm for crossbred bulls of different breeds have also been reported (Desai, 2013; Raval, 2006; Dhami *et al.* 2003; Rao *et al.* 1996; Patel *et al.* 1989; Jain *et al.* 2004; Singh and Pangawkar, 1990; Rao *et al.* 1996; Prasad *et al.* 1999; Srivastava and Kumar; 2006; Andrabi *et al.* 2002; Dhanju *et al.*, 2006 and Srivastava and Kumar, 2014) with higher upto 54.77 ± 7.2 (Dhanju *et al.*, 2006) and lower as 7.45 ± 0.42 (Raval, 2006).

A wide variation in abnormal sperm count has been attributed to factors like species, breed, season, hereditary, environmental affliction, testicular affection, medication, etc.

In the present study, the overall sperm abnormality comprised the head, mid-piece and tail abnormalities. The sperm abnormality recorded lower for the Banas Kankrej bull in present study may be due to various factors like age of bull, season and good andrological condition etc. However, like as that of the present study Patel and Siddiquee (2013) have also reported lower abnormal sperm count in Kankrej bull semen.

Correlation studies revealed that the sperm abnormality had highly significant ($P < 0.05$) inverse correlation with individual motility, sperm viability, HOST and acrosomal integrity which corroborate the findings of Rana and Dhami (2004) in Gir bulls, Raval (2006) in triplebred (HF × J × Kankrej) bulls, Ray and Ghosh (2013) in Sahiwal bulls.

### 5.1.4. Osmotic Resistance Test (HOST)

The hypo osmotic swelling ability of spermatozoa has been reported as sign of membrane integrity and normal functional activity which is not only essential for the maintenance of sperm motility but also for the induction for

acrosome reaction and possibly by other event related to fertility (Lodhi *et al.* 2008).

The HOST reactive sperm per cent recorded was 86.80 ± 0.24 and ranged between 85 to 90 per cent. As evident from result there was no significant difference among the bulls.

Values observed in the present study are higher than that of the Desai (2013) who has reported the HOST reactive sperm as 73.31 ± 0.48 per cent in Kankrej bulls.

HOST reactive sperm percent observed for the Banas Kankrej bull in the present study corroborate the reports of other indigenous cow bulls wherein, a higher than 80 per cent HOST reactive sperm has been reported viz. 84.80 ± 0.89 in Gir bulls (Rana and Dhami, 2004); 81.43 ± 1.49 in Tharaparkar bulls (Rajoria *et al.* 2011); 82.25 and 74 to 84 by Lodhi *et al.* (2008) and Ray and Ghosh, (2013) in Sahiwal bulls. However, Martins *et al.* (2011) have reported a lower HOST reactive sperm per cent as 60.3 in Nellore bulls.

Findings in the present study corroborate with the Perumal *et al.* (2009) who have reported 85.78 ± 0.42 and 80.38 ± 0.32 HOST reactive sperm per cent in good and poor freezable Jersey bulls.

In contrast to the findings of Banas Kankrej bull comparatively lower HOST reactive sperm per cent has been documented Viz. 59.33 ± 1.01 (Salah *et al.*1992); 49.38 ± 2.80 (Srivastava and Kumar, 2006); 74.59 ± 2.20 (Serpil *et al.* 2009) and 69.4 ± 2.8 (Srivastava and Kumar, 2014) in Holstein bulls. Similarly, lower HOST reactive sperm per cent has also been reported in Jersey bulls (Kumar, 2004) and in crossbred bulls (Kumar *et al.* 2006; Srivastava and Kumar, 2006; Vera-Munoz *et al.* 2009; Srivastava and Kumar, 2014).

Correlation studies revealed that the HOST reactive sperm per cent had highly significant ($P < 0.05$) positive correlation with individual motility, acrosomal integrity and lipid peroxidation whereas, inverse correlation with sperm abnormality which akin with reports of Ray and Ghosh (2013) in Sahiwal

bulls, Sharma (2012) in Jersey x local hill cattle crossbred bulls, Prasad *et al.* (1999) in crossbred bulls, Kumar (2004) in Jersey bulls, Srivastava and Kumar (2006) in HF and crossbred bulls and Lodhi *et al.* (2008) in Nilli-Ravi and Sahiwal bulls.

### 5.1.5. Acrosomal Integrity

Intact apical ridge of acrosome has been necessary for fertilizing capacity of spermatozoa and for functional efficiency of acrosome. The presence of an acrosomal cap has been important in the fertilization process and highly related with fertility of frozen semen. However, some spermatozoa could be highly motile but not fertile, owing to the acrosomal damage (Perumal *et al*. 2009).

The acrosomal integrity per cent in Banas Kankrej bull was recorded as 90.72 ± 0.25 which ranged between 88 to 93 per cent. As evident from results, there was no significant difference among the bulls under investigation.

The present findings akin with Patel (2012) and Desai (2013) who have reported the acrosomal integrity in Kankrej bulls as 79 to 88 and 84.98 ± 0.56 per cent, respectively.

Similarly, the present findings corroborate the reports on per cent acrosomal integrity in other indigenous cow bulls viz. 90.77 ± 1.68 and 91.47 ± 1.61 in Tharaparkar bulls (Rajoria *et al*. 2011); around 93 in Hariana bulls (Singh *et al*. 1992) and 84.80 ± 0.90 in Gir bulls semen (Rana and Dhami, 2004).Whilst in other indigenous cow bulls a lower per cent acrosomal integrity has been reported as 82.70 ± 0.39 per cent in Sahiwal bulls (Ray and Ghosh, 2013) as compared to that of the Banas Kankrej bulls.

The present findings also corroborate the reports on per cent acrosomal integrity in Holstein Friesian (HF) bulls (Srivastava and Kumar, 2006; Srivastava and Kumar, 2014), in Jersey bulls (Nath *et al*. 1996; Kumar, 2004), in triple bred bulls (Raval, 2006), in crossbred bulls (Srivastava and Kumar, 2006; Desai, 2013; Srivastava and Kumar, 2014) and in Friesian x Hariana bulls (Singh *et al*. 1992).

In comparison with the present findings in Banas Kankrej breed, a lower but variable acrosomal integrity per cent were reported in halfbred and cross breed bulls (Prasad *et al*. 1999), in HF x BS x Hariana bulls (Pathak *et al*.1989), in HF x J x Hariana bulls (Sharma *et al*. 1992).

Correlation studies revealed that the acrosomal integrity had highly significant ($P < 0.05$) positive correlation with individual motility, sperm viability, HOST and lipid peroxidation whereas, inverse correlation with sperm abnormality which were in proximity with reports of Ray and Ghosh (2013) in Sahiwal bulls and Sharma (2012) in Jersey x local hill cattle crossbred bulls.

## 5.2. EFFECT OF TREHALOSE AS A SEMEN EXTENDER ADDITIVE

Various steps of cryopreservation such as processing, freezing and thawing exert physiological as well as chemical stress on the sperm membrane, associated with an oxidative stress induced by free radical. Sperm cells have a high content of unsaturated fatty acids in their membrane but lack in a significant cytoplasmic component containing antioxidants. Hence, they are highly susceptible to lipid peroxidation by oxygen free radicals and $H_2O_2$ (Karunakaran *et al*. 2012).

Trehalose was used as semen diluent additive at different concentrations to evaluate their effect on individual motility, sperm viability, sperm abnormality, osmotic resistance test and acrosomal integrity at post-dilution, post-equilibration and post-thaw stages of cryopreservation.

### 5.2.1 Individual Motility

Cryopreservation causes irreversible damage to sperm organelles and changes in membrane fluidity and enzymatic activity associated with a reduction in sperm motility. Better sperm motility of cryopreserved bull sperm can be achieved by addition of certain additives having antioxidant property (Serpil *et al*. 2009).

The overall mean individual motility per cent of 100mM Trehalose group were 84.58 ± 0.31, 75.00 ± 0.40 and 64.16 ± 0.52 at post-dilution, post-equilibration and post-thaw stages of cryopreservation, respectively. And it was significantly ($P < 0.05$) higher as compared to 50mM Trehalose, 150mM Trehalose and control groups at all the stages of cryopreservation.

The present findings were in accordance with Badr *et al.* (2010) who have reported individual motility as 82.50 ± 1.45 and 61.25 ± 1.25 per cent in buffalo bulls at post-dilution and post-thaw stages of cryopreservation, respectively. Whilst using 100mM Trehalose, lower individual motility per cent than that of the present findings in Banas Kankrej bulls, have been reported in Karan-Fries bulls (Chhillar *et al.* 2012), in bovine bulls (Hu *et al.* 2010) and in buffalo bulls (Reddy *et al.* 2010) at post-thaw stage of cryopreservation.

Contrary to the present findings, Uysal *et al.* (2007) have reported improved individual motility per cent using 50mM Trehalose in bovine bulls in comparison with the control groups at post-thaw stage of cryopreservation. Our findings support the Hu *et al.* (2010) who have reported that the extender supplemented with 100 mM Trehalose resulted in the greater sperm motility. But in his study, both Trehalose 50 and 100 mM groups had significantly ($P < 0.05$) higher motility as compared to control group.

Supplementation of 100mM Trehalose to the freezing extender led to significantly ($P < 0.05$) higher post-thaw sperm motility compared to sperm cryopreserved in their absence (Chhillar *et al.* 2012). Post-thaw semen evaluation clearly indicated that the addition of 100mM Trehalose significantly ($P < 0.05$) improved the sperm motility compared to control group (Reddy *et al.* 2010).

The axosome and associated dense fibers of the middle pieces in sperm are covered by mitochondria that generate energy from intracellular stores of ATP. These are responsible for sperm motility. The additives might have displayed cryoprotective effect on the functional integrity of mitochondria that

is responsible for the generation of energy from intracellular stores of ATP led to improved post-thaw sperm motility (Reddy *et al.* 2010).

### 5.2.2. Sperm Viability

During cryopreservation the sperms are exposed to exogenous diluting media as well as to low and ultra low freezing temperature. Death might occur due to release of toxic substances, ultra low exposure, enzymatic leakage, medium of preservation, aging effect of sperm and individual variation.

The overall mean per cent sperm viability of 100mM Trehalose group were 85.66 ± 0.46, 81.41± 0.28 and 71.41± 0.31 at post-dilution, post-equilibration and post-thaw stages of cryopreservation, respectively. Wherein, it was significantly ($P < 0.05$) higher as compared to 50mM Trehalose, 150mM Trehalose and control groups at all the stages of cryopreservation.

The present findings were in proximity with Badr *et al.* (2010) who have reported the mean sperm viability with viability index of 172.25 ± 5.58 in buffalo bulls and Reddy *et al.* (2010) as 71.00 per cent in buffalo bulls.

Whereas, lower per cent sperm viability than that of the Banas Kankrej bulls using 100mM Trehalose has been reported in Karan-Fries bulls (Chhillar *et al.* 2012) at post-thaw stages of cryopreservation. However, Uysal *et al.* (2007) could find improved per cent sperm viability using 50mM Trehalose in bovine bulls in comparison with the control groups at post-thaw stage of cryopreservation.

Our findings support the Hu *et al.* (2010) who have reported that the extender supplemented with 100 mM trehalose resulted in the greatest sperm viability. But in his study, both Trehalose 50 and 100mM groups had significantly ($P < 0.05$) higher viability as compared to control group.

Chhillar *et al.* (2012) reported a significant ($P < 0.05$) increase was observed in sperm viability upon addition of 100mM Trehalose to the freezing extender prior to cryopreservation as compared in their absence in the extender.

Post-thaw semen evaluation clearly indicated that the addition of 100mM Trehalose significantly ($P < 0.05$) improved the sperm viability compared to control group (Reddy *et al.* 2010). Present findings corroborate the view of Uysal *et al*. (2007) who have stated that the addition of Trehalose might have protective action rendering better viability per cent in respective groups.

### 5.2.3. Sperm Abnormality

The overall mean per cent sperm abnormality per cent of 100mM Trehalose group were 2.08 ± 0.25, 3.50 ± 0.15 and 5.00 ± 0.21 at post-dilution, post-equilibration and post-thaw stages of cryopreservation, respectively. Wherein, it was significantly ($P < 0.05$) lower as compared to that of 50mM Trehalose, 150mM Trehalose and control groups at all the stages of cryopreservation.

Present findings for the Banas Kankrej bulls corroborate the effect of Trehalose reported by Uysal *et al.* (2007) who have reported a significantly lower sperm abnormality per cent using Trehalose additive in bovine bulls in comparison with the control groups at post-thaw stage of cryopreservation. Although, the beneficial effect was with lesser 50mM concentration of Trehalose as compared to that of the 100mM in the present study.

### 5.2.4. Osmotic Resistance Test

The overall mean HOST reactive sperm per cent with 100mM Trehalose group were 83.25 ± 0.32, 78.16 ± 0.42 and 73.91 ± 0.35 at post-dilution, post-equilibration and post-thaw stages of cryopreservation, respectively. Wherein, it was significantly ($P < 0.05$) higher as compared to 50mM Trehalose, 150mM Trehalose and control groups at all the stages of cryopreservation.

On reviewing the various reports, a lower HOST reactive sperm per cent was revealed as 53.15 in buffalo bulls (Reddy *et al.* 2010), 50.0 ± 1.52 in Karan-Fries bulls (Chhillar *et al.* 2012) and 44.33 ± 1.71 in bovine bulls (Hu *et al.* 2010) at post-thaw stages of cryopreservation. Whereas, Uysal *et al.* (2007)

have recorded improved post-thaw HOST reactive sperm per cent in 50mM Trehalose group in bovine bulls in comparison with the control group.

The present findings were in close agreement with Hu *et al.* (2010) who have reported that the extender supplemented with 100 mM Trehalose resulted in the greatest plasma membrane integrity. But in his study, both Trehalose 50 and 100 mM groups had significantly ($P < 0.05$) greater plasma membrane integrity as compared to control and other treatment groups. Our findings corroborate with Reddy *et al.* (2010) and Chhillar *et al.* (2012) who have also reported significantly ($P < 0.05$) improved post-thaw plasma membrane integrity using 100mM Trehalose.

The exact mechanism by which Trehalose affect the sperm membrane is unknown, but it is theorized that it penetrate into the plasma membrane of the spermatozoa and form hydrogen bonds with the polar head groups of the phospholipids (Kumar *et al.* 2012). The Trehalose entering into the sperm membrane limits the amount of dehydration that can occur and consequently inhibits the physical damage to cell volume changes associated with freezing and thawing.

### 5.2.5. Acrosomal Integrity

The physiologic acrosome reaction is a well-coordinated process that can occur only in a living spermatozoan in response to natural inducers. In contrast, loss of acrosomal content can occur with the breakdown of the membranes during cell death, cryopreservation and during addition of oxidants which shows similar acrosomal changes (Perumal *et al.* 2009).

The overall mean acrosomal integrity per cent with 100mM Trehalose group were 91.08 ± 0.41, 85.41 ± 0.39 and 80.91 ± 0.43 at post-dilution, post-equilibration and post-thaw stages of cryopreservation, respectively. Wherein, acrosomal integrity per cent of 100mM Trehalose group was significantly ($P < 0.05$) higher as compared to 50mM Trehalose, 150mM Trehalose and control groups at all the stages of cryopreservation.

Acrosomal integrity per cent of 100mM Trehalose group has been reported as 64.78 ± 1.35 in bovine bulls (Hu *et al.* 2010) and 10.25± 2.39 in buffalo bulls (Badr *et al.* 2010) at post-thaw stage of cryopreservation. Our Observation for the acrosomal integrity intimates with Hu *et al.* (2010) who have reported that the extender supplemented with 100 mM Trehalose resulted in the greatest acrosomal membrane integrity in bovine bulls. But in his study, both Trehalose 50 and 100mM groups had significantly ($P < 0.05$) higher acrosomal membrane integrity as compared to control and other treatment groups. Whereas, Uysal *et al.* (2007) have found it to be better with 50mM Trehalose supplementation in bovine bulls during post-thaw stage of cryopreservation.

Trehalose as semen diluent additive had been reported to improve post thaw motility and normal acrosome. It has also been reported to reduce enzyme leakage and consistently improved fertility. The mode of action as suggested might be due to limited production of lipid peroxides (Kumar *et al.* 2012).

## 5.3. EVALUATION OF BIOCHEMICAL PARAMETERS OF SEMEN EXTENDER ADDITIVE

### 5.3.1. Lipid Peroxidation (LPO)

Sperm cells have a high content of unsaturated fatty acids in their membranes but lack in significant cytoplasmic component containing antioxidants. Therefore, sperm cells are highly susceptible to lipid peroxidation (LPO) by free radicals such as hydrogen peroxide, superoxide anion, and hydroxyl radical, which lead to the structural damage of sperm membranes during the freezing-thawing process (Serpil *et al.* 2009).

In present investigation, the overall mean Malondialdehyde (MDA) of fresh semen was 49.96 ± 0.06 µmol/ml with a range of 49.12 to 50.65 µmol/ml. The overall mean Malondialdehyde (MDA) did not differ significantly among bulls. The Malondialdehyde (MDA) had significant ($P < 0.05$) positive correlation with sperm viability, individual motility, HOST and acrosomal integrity.

The overall mean Malondialdehyde (MDA) values of 100mM Trehalose group were 43.92 ± 0.05, 34.20 ± 0.09 and 20.06 ± 0.13 µmol/ml at post-dilution, post-equilibration and post-thaw stages of cryopreservation, respectively. Wherein, it was significantly ($P < 0.05$) lower as compared to 50mM Trehalose, 150mM Trehalose and control groups at all the stages of cryopreservation. These findings are in accordance with, Badr *et al.* (2010) who have reported that the addition of 100 mM Trehalose to the freezing extender resulted in decreased Malondialdehyde (MDA) values in buffalo bulls and also with that of the similar findings in Karan-Fries bulls (Chhillar *et al.* 2012).

During preservation or cryopreservation the semen is exposed to cold shock at atmospheric oxygen which in turn increases the susceptibility to lipid peroxidation due to higher production of reactive oxygen species (Perumal *et al.* 2009). The free radicals are known to be involved in lipid peroxidation as well as DNA and sperm membrane damages which may lead to decreased sperm motility or cell death. Therefore, in the present study addition of Trehalose in semen might be a beneficial factor in avoiding the process of damage and reduce generation of ROS which would otherwise have negatively affected the spermatozoa (Uysal *et al.* 2007).

### 5.3.2. Glutathione Reductase (GSH)

It is well known that GSH plays a critical role in protecting mammalian cells from oxidative damages. The elevation of GSH activity was indicative of improved antioxidant capacity (Serpil *et al.* 2009).

The overall mean glutathione reductase of fresh semen was 30.14 ± 0.06 U/L with a range of 29.24 to 30.87 U/L. The overall mean glutathione reductase did not differ significantly among bulls. The glutathione reductase had significant ($P < 0.05$) positive correlation with individual motility and sperm viability.

In present study, glutathione reductase level in 100mM Trehalose group was significantly ($P < 0.05$) higher than that of the 50mM Trehalose, 150mM

Trehalose and control groups at post-dilution, post-equilibration and post-thaw stages of cryopreservation, respectively.

Present findings are in harmony with Badr *et al.* (2010) and Hu *et al.* (2010) who have reported that the addition of 100 mM Trehalose to the freezing extender resulted in increase glutathione reductase levels in buffalo and bovine bulls, respectively.

The GSH sustained ability in the maintenance of sperm membrane integrity and individual motility. So, higher the GSH values in the semen might be a factor in making the sperm membrane more resistant to the spontaneous lipid peroxidation that destroys the structure of the lipid matrix and is associated with the loss of motility (Perumal *et al.* 2009). Trapping of the free radicals by Trehalose, thereby alleviating GSH consumption by the enzymatic antioxidant defenses might be implicated in higher GSH levels observed in the present study.

## 5.4. COMPARISON OF FRESH SEMEN PARAMETERS WITH FROZEN THAWED SEMEN

The semen quality parameters like sperm motility, sperm viability, plasma-membrane integrity, acrosomal integrity in fresh and cryopreserved semen were assessed. Cryopreservation led to a significant ($P < 0.05$) decrease in these semen quality parameters. Supplementation of Trehalose to freezing extender led to significantly ($P < 0.05$) higher post-thaw sperm motility compared to semen cryopreserved in its absence in the extender.

The overall mean per cent individual motility, sperm viability, sperm abnormality, osmotic resistance test (HOST) and acrosomal integrity of fresh semen were 88.75 ± 0.25, 89.69 ± 0.32, 3.08 ± 0.15, 86.80 ± 0.24 and 90.72 ± 0.25, respectively. Whereas, corresponding values in 100mM Trehalose group at post-thaw stage of cryopreservation were 64.16 ± 0.52, 71.41 ± 0.31, 5.75 ± 0.35, 73.91 ± 0.35 and 80.91 ± 0.43 respectively. These aforementioned values for

100mM Trehalose group were significantly ($P < 0.05$) better as compared to that of the 50mM Trehalose, 150mM Trehalose and control groups.

These findings are in accordance with Chhillar *et al.* (2012) in Karan-Fries bulls who have reported a significant ($P < 0.05$) decrease in post-thaw motility, sperm viability and membrane integrity in frozen thawed semen as compared to fresh semen.

Similarly, Badr *et al.* (2010) in buffalo bulls, Hu *et al.* (2010) in bovine bulls and Kumar *et al.* (2012) in buffalo (Murrah) and cattle (Karan Fries) bulls have also reported that the post thaw individual motility and sperm viability were significantly ($P < 0.05$) decreased, whereas per cent sperm abnormality significantly ($P < 0.05$) increased in frozen thawed semen as compared to fresh semen.

The semen oxidative stress parameters like lipid peroxidation, glutathione reductase in fresh and cryopreserved semen were assessed. Cryopreservation led to a significant ($P < 0.05$) increase in Malondialdehyde (MDA) values and significantly ($P < 0.05$) decrease in glutathione reductase (GSH) levels. Supplementation of Trehalose to freezing extender led to significantly ($P < 0.05$) lower post-thaw Malondialdehyde (MDA) values and significantly ($P < 0.05$) higher post-thaw glutathione reductase (GSH) compared to semen cryopreserved in its absence in the extender.

In present investigation, the overall mean Malondialdehyde (MDA) and glutathione reductase (GSH) of fresh semen were 49.96 ± 0.06 µmol/ml and 30.14 ± 0.06 U/L respectively. Whereas, corresponding values in 100mM Trehalose group at post-thaw stage of cryopreservation were 20.06 ± 0.13 µmol/ml and 84.00 ± 0.16 U/L respectively. These aforementioned values for 100mM group were significantly ($P < 0.05$) better as compared to that of 50mM Trehalose, 150mM Trehalose and control groups.

The present investigation was in accordance with Chhillar *et al.* (2012) in Karan-Fries bulls who have reported that the post-thaw lipid peroxidation was

significantly ($P < 0.05$) higher as compared to fresh but upon supplementation of 100mM Trehalose to freezing extender lipid peroxidation was significantly ($P < 0.05$) decreased.

Similarly, Badr *et al.* (2010) in buffalo bulls and Hu *et al.* (2010) in bovine bulls have shown that supplementation of 50mM and 100mM Trehalose in egg yolk based extender improves sperm quality and oxidative stress parameters in frozen thawed bovine semen.

Trehalose has a protective action related to the osmotic effect and to specific interactions with membrane phospholipids, which renders the media hypertonic, thereby minimizing the degree of sperm cell injury during the freeze-thaw process (Kumar *et al.* 2012). The functional integrity of sperm acrosomal membrane and plasma membrane associated with sperm motility can be expected to have been destroyed by high doses of Trehalose.

In the present study, the highest protective effects of Trehalose were at the concentration of 100mM, and a much reduced extent at 150mM. The latter concentration resulted in a high osmolarity of the extender was in itself deleterious to the sperm cells. When Trehalose concentration was 150mM, the percent sperm motility, intact-acrosomal membrane, and intact-plasma membrane sperm of frozen-thawed bovine semen were decreased. Hu *et al.* (2010) shown in his research that antioxidant additives exhibited cryoprotective activity on certain sperm parameters in moderate doses, but increasing doses of antioxidant additives would result in a hypertonic property of extender and impair sperm function viz. sperm motility, membrane integrity and fertility.

The extender supplemented with 100mM trehalose resulted in the highest sperm motility, acrosomal membrane integrity, and plasma membrane integrity in this study. There was an increase in the levels of GSH and decrease in LPO values in Trehalose supplemented groups during the cryopreservation of semen in the present study.

## CHAPTER 6 : SUMMARY AND CONCLUSIONS

Trehalose, acts as disaccharide and non-permeating cryoprotectant which causes dehydration of spermatozoa due to the osmosis of water. Due to this mild dehydration, spermatozoa have less intracellular water which results in reduced intracellular ice crystal formation. Semen diluted in Tris-Fructose Egg Yolk Citrate Glycerol based semen extender along with Trehalose as additive, might be one of the causes for improved post-thaw sperm viability and intact acrosomal integrity of frozen-thawed spermatozoa.

**The following conclusions were drawn from the findings of present investigation:**

1. Individual motility, sperm viability, plasma membrane integrity, acrosomal integrity and abnormal sperm count were better than other cattle breeds in fresh Banas Kankrej bull semen.
2. Correlation matrix revealed that all the physical characteristics have significantly ($P < 0.05$) positive correlations among each other, except sperm abnormality which had inverse correlations.
3. Individual motility, sperm viability, plasma membrane integrity, acrosomal integrity and abnormal sperm count were significantly better using extender with 100mM Trehalose concentration during cryopreservation of Banas Kankrej bull semen.
4. There was a significant increase in GSH and a significant decrease in MDA levels at all stages of cryopreservation in extender supplemented with 100mM Trehalose.
5. Correlation matrix revelaed that the biochemical parameters have significantly positive correlations with individual motility, sperm viability and acrosomal integrity.

6. Supplementation of 100mM Trehalose in the semen extender for cryopreservation is most appropriate concentration in view to preserve the semen quality in Banas Kankrej bull.

To conclude with, the extender supplemented with 100mM Trehalose could reduce the oxidative stress provoked by frozen thawed and improves bovine sperm motility, acrosomal membrane integrity, and plasma membrane integrity after freeze-thaw. The optimum Trehalose concentration had been determined to be 100mM in Kankrej bull semen. Increasing the doses of Trehalose to the extender decreases levels of GSH and increase LPO values in cryopreserved semen.

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