STUDIES ON EFFECT OF LYCOPENE ON FREEZABILITY OF HARIANA BULL SPERMATOZOA

THESIS SUBMITTED FOR PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF

MASTER OF VETERINARY SCIENCE

IN

VETERINARY GYNAECOLOGY AND OBSTETRICS

BY

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COLLEGE OF VETERINARY SCIENCE AND ANIMAL HUSBANDRY

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(2021)

CERTIFICATE

This is to certify that the thesis entitled **"STUDIES ON EFFECT OF LYOCOPENE ON FREEZABILITY OF HARIANA BULL SPERMATOZOA"** submitted by **Dr. Avaneesh Kumar Singh,** Enrollment No. **V-2005/18** in partial fulfillment of the requirements for the award of the **Master of Veterinary Science** in **Veterinary Gynaecology and Obstetrics** of the **U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan (DUVASU), Mathura (UP), India,** is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

Dated: 15/05/2021

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We have carefully gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which being presented by him for the award of the Degree of this University.

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Veterinary Gynaecology and Obstetrics

%	Per cent
&	And
*	Significant P<0.05
**	Significant P<0.01
/	Per
$/10^6$	Per million
@	At the rate of
+	Plus or add
<	Less than
>	More than
±	Plus, Minus
≅	Equivalent to
≤	Less than or equal to
≥	More than or equal to
µg	Microgram
µL or µl	Micro liter
µM	Micromole
µm	Micrometer
µm sec^{-1}	Micrometer per second
°	Degree
°C	Degree Centigrade/Celsius
100X	Magnification hundred time
10^6	Million
10X	Magnification ten time
40X	Magnification forty time
A_B	Blank Absorbance
AI	Artificial Insemination
ALH	Amplitude of lateral head displacement
ALP	Alkaline phosphate
ANOVA	Analysis of Variance
A_T	Test Absorbance
ATP	Adenosine Triphosphate

AV	Artificial vagina
BCF	Beat cross frequency
BCMPT	Bovine Cervical Mucus Penetration Test
BQ	Black Quarter Disease
Bull No.	Bull number
Ca^{+2}	Calcium ion
CASA	Computer Assisted Sperm Analysis
CAT	Catalase
CDNB	Chloro dinitro benzene
cm	Centimeter
CMPT	Cervical Mucus Penetration Test
CP	Crude Proteins
CTC	Chlortetracycline
Cu	Copper
DAP	Average path distance
DCL	Curve linear distance
DCP	Digestible crude protein
dl	Deci litre
DMRT	Duncan's Multiple Range Test
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose Nucleic Acid
DPBS	Dulbecco's Phosphate Buffered Saline
DSL	Straight line distance
e.g.	Exempli gratia
ELISA	Enzyme-linked immunosorbent assay
et al.	et alii /alia
etc.	et cetera/And so forth
EYTG	Egg Yolk Tris Glycerol
Fe	Iron
FMD	Foot and Mouth Disease
FR	Frame Rate
gm	Gram
GNC	Groundnut cake
GOI	Government of India
GPx	Glutathione peroxidase

GSH	Reduced Glutathione
GSH-Px	Glutathione peroxidase
GST	Glutathione-S-transferase
H	Hydrogen
H_2O_2	Hydrogen peroxide
H-395	Hariana-395
H-448	Hariana-448
H-580	Hariana-580
H-623	Hariana-623
HAC	Head Activity Parameter
HF	Holstein Friesian
HOS^+	Hypo osmotic swelling reactive
HOST	Hypo osmotic swelling test
hr (s)	Hours
HS	Hemorrhagic Septicemia
Hz	Hertz
i.e.	id est/that is
ILFC	Instructional Livestock Farm Complex
IM	Immotile
IMV	International Minh Viet Joint Stock Company
IU	International Unit
IU/ L	International Unit per liter
IVF	*In-vitro* fertilization
JD	Johne's Disease
KF	Karan Fries
kg	Kilogram
$KH_2PO_4.2H_2O$	Potassium phosphate monobasic
L or l	Liter
Lab	Laboratory
lb	Pound
LCM	Local Circular Motile
LDH	Lactate dehydrogenase
LDL	Low Density Lipoprotein
LIN	Linearity
LM	Local Motile

LN_2	Liquid nitrogen
LPO	Lipid peroxidase
Ltd.	Limited
M	Molar
m	Meter
MDA	Malondialdehyde
mg	Milligram
Mg	Magnesium
min	Minute
Min.	Minimum
ml	Mililiter
mM	Milimole
mm	Millimeter
mm^2	Milimeter square
mm^3	Cubic Millimeter
Mn	Manganese
MOET	Multiple ovulation embryo transfer
mOsm	Milli Osmole
mosmol/litre	Mili osmole per liter
MTT	Methyl thiazol tetrazolium dye
$Na_2HPO_4.2H_2O$	Sodium phosphate dibasic
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
nM	Nano mole
nM/µl	Nano mole per micro liter
no.	Number
NS	Non-significant
O_2	Oxygen
O_2^-	Superoxide anion
OD	Optical Density
OH^-	Hydroxide
OS	Oxidative stress
PBS	Phosphate Buffered Saline
PCM	Progressive Circular motility
PFM	Progressive Fast Motility
pH	Potential of Hydrogen

PM	Progressive motility
PSM	Progressive sperm motility
RBC	Red Blood Cell
RGM	Rashtriya Gokul Mission
ROS	Reactive Oxygen Species
rpm	Revolution per minute
S.E.	Standard Error
S.No.	Serial Number
SDS	Sodium Dodecyl Sulphate
Se	Selenium
sec	Second
SMPT	Semen Mucus Penetration Test
SOD	Superoxide dismutase
STR	Straightness
TALP	Tyrode’s albumin lactate pyruvate
TB	Tuberculosis
TBA	Thio Barbituric Acid
TBARS	Thio Barbituric Acid Reactive Substance
TDN	Total Digestible Nutrient
TM	Total Motility
Tris	Hydroxy methyl amino methane
TWD	Triple Distilled Water
U	Unit
U.P.	Uttar Pradesh
UV	Ultra violet
VAP	Average Path Velocity
VCL	Curvilinear Velocity
vs.	Versus
VSL	Straight Line Velocity
WOB	Wobble
X-XO	Xanthine-xanthine oxidase
Zn	Zinc
α	Alfa

LIST OF TABLES

LIST OF FIGURES

Figure 21.0	Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on curvilinear distance (µm) of Hariana bull spermatozoa (CASA) during freeze-thaw process
Figure 22.0	Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on straight line distance (µm) of Hariana bull spermatozoa (CASA) during freeze-thaw process
Figure 23.0	Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on average path distance (µm) of Hariana bull spermatozoa (CASA) during freeze-thaw process
Figure 24.0	Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on amplitude of lateral head displacement (µm) of Hariana bull spermatozoa (CASA) during freeze-thaw process
Figure 25.0	Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on beat cross frequency (Hz) of Hariana bull spermatozoa (CASA) during freeze-thaw process
Figure 26.0	Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on head activity parameter (Rad) of Hariana bull spermatozoa (CASA) during freeze-thaw process
Figure 27.0	Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on wobble (VAP/VCL) of Hariana bull spermatozoa (CASA) during freeze-thaw process
Figure 28.0	Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on linearity (VSL/VCL) of Hariana bull spermatozoa (CASA) during freeze-thaw process
Figure 29.0	Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on straightness (VSL/VAP) of Hariana bull spermatozoa (CASA) during freeze-thaw process

Figure 30.0	Graphical presentation of effect of different concentrations of lyocopene(Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on distance (mm/hr) travelled by spermatozoa in oestrus mucus as observed through Bovine Cervical Mucus Penetration Test of Hariana bull spermatozoa during post-thaw process
Figure 31.0	Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on superoxide dismutase (SOD) activity (U/ml) in the seminal plasma of Hariana bull semen during post-thaw process
Figure 32.0	Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on glutathione-S transferase (GST) activity (nM/min/ml) in the seminal plasma of Hariana bull semen during post-thaw process
Figure 33.0	Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on Malondialdehyde (MDA) activity (nM/µl) in the seminal plasma of Hariana bull semen during post-thaw process

LIST OF PHOTOGRAPHS

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CONTENTS

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ABSTRACT

The present study was designed to determine the effect of lycopene as an additive in Hariana bull semen for cryopreservation. The study evaluated different seminal attributes (progressive motility, liveability, HOST reactivity, acrosomal integrity, capacitation status and sperm kinematic (CASA) after equilibration (pre-freeze) and thawing. Besides *in vitro* fertility test (BCMPT), antioxidative enzymes (SOD, GST, LPO [MDA]) were also evaluated at post-thaw stage. Eight ejaculates collected from four Hariana bull (8X4 = 32) were evaluated for volume (ml), seminal pH, mass motility (0-5 scale), concentration (millions/ml), progressive motility and live percentage of spermatozoa. Upon evaluation of their parameters in the prescribed range, the semen was used for cryopreservation. Freshly collected semen was extended in egg yolk tris glycerol (EYTG) was divided into four aliquots and grouped as Group I: control (without lycopene), Group II, Group III and Group IV as treatment group with 0.25 mM, 0.5 mM and 1 mM lycopene respectively. In freshly collected semen, significant ($P<0.05$) difference was observed for mean semen volume and mass motility. Semen evaluated at pre-freeze and post-thaw stage showed that supplementation of 0.25 mM lycopene (Group II) in semen significantly ($P<0.01$) increased per cent progressive motility, liveability, HOST reactive sperm, acrosomal integrity, uncapacitated spermatozoa and motion and kinematic parameters at both stages. Further, 0.25 mM lycopene group showed significant ($P<0.01$) increased in vanguard spermatozoa (mm/h) and decreased in antioxidative enzymes (GST and MDA) activities.

Key words: Bull semen / Cryopreservation / Hariana / Lycopene / Antioxidant / capacitation / Vanguard spermatozoa / antioxidative enzymes

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Introduction

CHAPTER-1

INTRODUCTION

As per the latest census (20th Livestock Census, GOI, 2019), the cattle population in the country is 192.49 million showing an increase of 0.8 % over previous Census. The Female Cattle (Cows population) is 145.12 million showing an increase by 18.0 % over the previous census (2012). The Exotic/Crossbred and Indigenous/Non-descript Cattle population in the country is 50.42 million and 142.11 million respectively. The Indigenous/Non-descript female cattle population has increased by 10 % in 2019 as compared to previous census. The Cattle Population of Uttar Pradesh in 2019 is estimated as 18.8 millions showing a deline of 3.93 per cent compare to previous census.

Livestock in general and dairying in particular play a vital role in the Indian economy and also in the socio-economic development of millions of rural households. About 70 million rural households are engaged in dairying, one of every two rural households with women playing a vital role. India ranks first in milk production, accounting for 20 % of world production. The average productivity of in milk cattle and buffalo in the country is about 4.65 Kg/day (2015-16) which is far below as compared to the productivity levels of In-milk animals in dairy developed nations.

For increasing productivity per indigenous and non descript cattle, Government of India under Rashtriya Gokul Mission (RGM) has proposed meassures as (1) propagation of super elite population through MOET & IVF (2) selective breeding among pure bred animals (3) upgrading the graded cattle with pure bred semen and (4) conversion of non-descript cattle to defined breed through upgrading. To achieve these meassures, efficent AI delivery system along with more coverage of AI network has been kept as priority.

Artificial Insemination with frozen semen has been proved to be the best tool worldwide for mass genetic improvement through dissemination of superior germplasm. This objective can be achieved only if the frozen semen used in AI programme conforms to certain prescribed quality standards. Due to the importance of

cryobiology in reproductive technologies, new protocols are being developed and cryo-protective agents tested for enhanced cryo-survival of sperm.

During cooling, cells are exposed to harmful effects including metabolic decoupling, ionic imbalance, activation of proteases, cellular acidosis, deprivation of energy, membrane phase transition, destabilization of the cytoskeleton, and production of free radicals or reactive oxygen species (ROS) whereas during freezing, sperm are predisposed to detrimental effects of ice crystal formation, hyper-osmolarity, alterations in the cell volume, and protein denaturation (Baust et al., 2009).

Seminal oxidative stress may result in lipid peroxidation (LPO), DNA fragmentation, alterations to the cellular communication and enzymatic pathways (Aitken et al., 1989; Aitken and Koppers, 2011), which in turn are correlated with motility loss, alterations of membrane fusion events (Aitken et al., 1989), poor fertilization rates or impaired embryogenesis (Baker and Aitken, 2005; Lewis and Aitken, 2005). Studies have shown that *in vitro* supplementation of hydrophilic or lipophilic antioxidants may have positive effects on critical semen parameters including sperm motility, membrane and DNA integrity (Twigg et al., 1998). Moreover, antioxidants may protect spermatozoa from ROS produced by leukocytes, reduce cryodamage to spermatozoa, block premature sperm maturation and provide an overall stimulation to the male gamete (Sheweita et al., 2005; Bansal and Bilaspuri, 2011).

Plants and animals have a complex system of multiple types of antioxidants such as vitamin C and vitamin E, as well as enzymes, such as catalase (CAT), superoxide dismutase (SOD) and various peroxidases (Hamid et al., 2010). Antioxidants in the form of micronutrients (vitamin E, vitamin C, beta cartone) cannot be manufactured by body and required supplementation in normal diet. As per the source of antioxidants they are classified as (1) natural and (2) synthetic type. Natural antioxidants are either synthesized in human body or are supplemented from natural sources. The natural antioxidants are further classified as (i) enzymatic (eg superoxide dismutase (SOD), catalase (CAT), glutathione peroxidise (GPx)) and (ii) non enzymatic eg minerals (Fe, Mg, Se, Cu, Zn, Mn), vitamins (A, C, E), caretinoids (beta carotene, lycopene, lutein, zeaxanthin), polypenols (curcumin).

Lycopene is a carotenoid (highly unsaturated hydrocarbon) which can be found in tomatoes, watermelon, papaya, red grapefruits, apricots, and guava, and gives their red color (Kong et al., 2010; Gajendragadkar et al., 2014; Mozos et al., 2017). Lycopene from natural plant sources exists predominantly in an all-trans configuration, the most thermodynamically stable form (Zechmeister et al., 1941; Nguyen and Schwartz, 1999). Lycopene is one of the most potent antioxidants (DiMascio et al., 1989; Miller et al., 1996; Mortensen and Skibsted, 1997; Woodall et al., 1997) with a singlet-oxygen quenching ability twice as high as that of β-carotene and 10 times higher than that of α-tocopherol (DiMascio et al., 1989).

The molecular formula of lycopene, is C40H56 (molecular mass as 536.873 g/mole) has an acyclic open chain structure consisting of 13 double-bonds. Two of them are non-conjugated and eleven are conjugated double bonds, thereby building a chromophore responsible for the characteristic ruby color and the antioxidant properties of lycopene (Shi and Maguer, 2000).

Lycopene provides the ability to intervene in reactions initiated by free radicals, like OH− or peroxy radicals (Stahl and Sies, 2003). Due to its polyene structure, providing an electron-rich system, lycopene is an eligible target for electrophilic reagents. Thus, it performs an uttermost reactivity towards oxygen and free radicals (Ronsein et al., 2006).

In view of the paucity of literature on effect of lycopene in Hariana bull semen preservation, the study was designed with following objectives:

OBJECTIVES

- To study the effect of lycopene on physio-morphology properties of cryopreserved Hariana bull spermatozoa.
- To study the antioxidant activity of lycopene on cryopreseved Hariana bull semen.

Review of Literature

CHAPTER-2

REVIEW OF LITERATURE

In order to double farmer's income, several efforts have been made to increase the milk production of cattle and buffalo in India. Widespread use of frozen semen of high genetic merit bulls through artificial insemination is the key component in achieving the goal. A significant progress has been made in refining and perfecting the technique of cryopreservation for maximizing the recovery of spermatozoa. Detailed studies on cooling rates, equilibration timing, freezing protocol, thaw rates and their interdependence have been studied for increasing the viability of spermatozoa thus, maximizing their availability, resulting in optimum prolificacy of germplasm from the selected bulls. Several researchers have tried to improve the frozen semen quality, minimizing the post thaw discard rate and enhancing conception rate by using different additives like enzymes, antioxidants, proteins, vitamins, carbohydrates, amino acids, hormones, etc.

The present study deals with addition of lycopene (antioxidant) to Egg Yolk Tris Glycerol (EYTG) semen extender. Related literature and corroborate reports from India and abroad have been reviewed and predicts the study of Hariana bulls in respect of standard semen characteristics like progressive motility, viability, membrane integrity, acrosomal abnormalities in the fresh, pre-freeze (post-equilibration) and post-thaw stages under the following heads and subheads.

2.1 Cryoprotectants in semen cryopreservation

2.1.1 Egg Yolk

A common ingredient of mammalian semen extender to protect sperm against initial cold shock. It seems to increase the sperm fertilizing ability at ambient temperature (Shannon and Curson, 1983) and appears to prevent sperm cell damage during cooling and freezing (Phillips and Lardy, 1940; De Leeuw et al., 1993).

It is generally used at a concentration of 20% (v/v) in extender and evidences indicate that low-density lipoproteins (LDL) and phospholipid (lecithin) components of the egg yolk are responsible for sperm protection (Moussa et al., 2002; Amirat et al., 2005; Purdy, 2006).

Cold shock is the process whereby the lipid bi-layer of the spermatozoa is altered, due to rapid changes in temperature (Hammerstedt et al., 1990). This rapid change in temperature can be due to many factors during semen processing. Cold shock occurs most frequently when undiluted semen at 30ºC or above is added to a pre-cooled (5ºC) diluent, or allowed to come into contact with pre-cooled glasswares or instruments, and when transferring a small volume of semen in a holding container to a pre-cooled water jacket to cool the semen to 5ºC (Watson, 1981). The effect of cold shock is much more evident in the cells in semen than on cells in diluents containing egg yolk or other lecithin and lipoprotein containing materials (Salisbury and Van Demark, 1961).

Swanson (1949) reported that 10% of egg yolk was sufficient for protection against cold shock; motility was lowered at this level. The reduction in motility was not evident at 20% of egg yolk level.

Singh et al. (2007) reported that egg yolk was able to reduce the heparin binding protein mediated cryoinjury in spermatozoa. One of the mechanisms of protection of spermatozoa from cryoinjury by egg yolk may be due to inhibition of lethal action of heparin binding protein on the spermatozoa.

2.1.2 Glycerol

Pace and Graham (1974) reported that freezing and thawing bovine semen in an egg yolk-based extender without glycerol yielded higher percentages for spermatozoal motility as compared to extenders composed of glycerol alone.

Fahy (1986) used glycerol concentrations between 0.25M (2.25 %) and 1M (9 %) for freezing of semen and reported toxicity beyond this concentration. Miller and Van Demark (1954) while working with glycerol levels ranging from 5 % to 40 % observed that a level of 7 % resulted into optimum survival after freezing. The 7 % level has also been found superior by Jones et al. (1956) in comparison to 10 % and 15 % levels.

Abbas and Andrabi (2002) used glycerol in different concentrations (2, 4, 5, 6, 7, 8, 10, or 12) % in the buffalo semen samples having more than 65 % progressive motility. Mean sperm longitivity was highest for 6, 7, or 8 % glycerol, intermediate for 4, 5, or 10 % and lowest for 2 or 12 %. The plasma membrane integrity of spermatozoa was least protected when frozen in 2 % or 4 % glycerol and concluded

that glycerol concentration of 6 or 7 % may be suitable for cryopreservation of bovine semen.

Furthermore, use of an egg yolk based extender in combination with glycerol resulted in higher percentages of spermatozoa motility than for extenders with the use of either egg yolk or glycerol alone (40, 24, and 3 % respectively). Their results suggest that both the egg yolk and the glycerol fractions of the extender are involved in protecting spermatozoa during cryopreservation and inclusion of both fractions is imperative for obtaining adequate spermatozoa motility post-thaw.

2.2 Antioxidants

Spermatozoa are affected by many factors during cryopreservation, like temperature, change in the environment, osmotic stress, toxic effect of cryoprotectants and formation of ice crystals (Watson, 2000; Li et al., 2010). Oxidative stress is one among them due to imbalance between free radical formation and the antioxidant defense, thus causing the tissue damage (De Lamirande et al., 1997; Watson, 2000). Oxidative stress (OS) refers to a disturbance in the balance between the production of reactive oxygen species (ROS) (free radicals) and the antioxidant defense that helps to counteract or detoxify their harmful effects.

The freezing thawing procedures of cryopreservation are known to produce ROS in sperm samples. Exposure of semen to cold shock and atmospheric oxygen during cryopreservation increases the susceptibility to lipid peroxidation (LPO) due to higher production of ROS (Bucak et al., 2008; Bansal and Bilaspuri, 2009).

Antioxidants such as lycopene, taurine, hypotaurine, vitamin E, vitamin C etc have been added to the extender to increase the resistance of the cells during cryopreservation. A compound such as superoxide dismutase or glutathione also serves as physiological antioxidants and provides resistance to spermatozoa against oxidative stress (Cabrita et al., 2010). It balances the lipid peroxidation with the help of the antioxidants existing in the semen by preventing the excessive production of free radicals (Alvarez et al., 1987; Aruoma et al., 1988; Aurich et al., 1997; Bucak and Tekin, 2007). Thus, provides protective effect to sperm (Aurich et al., 1997; Foote et al., 2002).

Lycopene is a lipophilic carotenoid with both antioxidant and pro-oxidant activities (Young and Lowe, 2001). It is the main carotenoid in tomatoes, also present

in guava, watermelon, pink grapefruit and papaya (Stahl and Sies, 1996). Lycopene is most likely involved in the quenching of singlet molecular oxygen and the trapping of peroxy radicals and thereby contributes to the protection of cells and tissues against the harmful effects of lipid peroxidation (Bucak et al., 2015). It can also act on various other free radicals, including hydroxyl radicals, nitrogen dioxide and hydrogen peroxide (Rao and Agarwal, 1999). Due to its lipophilic effect, lycopene is accumulated in the membranes of cells and lipoproteins and creates a considerable affect within cells (Rao and Agarwal, 1999). The antioxidant activity of lycopene is mostly catalytic (Stahl and Sies, 1996, 2003; Velmurugan et al., 2004).

Lycopene acts by donating its electrons to free oxygen radicals thus quenching and neutralising them before they can damage cells (Kelkel et al., 2011). Thus, lycopene has been attributed a role in preventing diseases caused by oxidative stress, such as certain cancers, cardiovascular diseases (Rao and Agarwal, 1999; Kong et al., 2010; Mordente et al., 2011) and infertility (Palan and Naz, 1996). In effect, oral lycopene supplements have been reported to improve the quality of human (Gupta and Kumar, 2002) and chicken spermatozoa (Mangiagalli et al., 2010). Several studies have demonstrated that sperm motility, membrane integrity (Uysal and Bucak, 2007; Bucak et al., 2015) and DNA integrity (Zini et al., 2010) can be improved by the addition of lycopene to diluents. Akalin et al. (2016) reported that adding lycopene at 0.5 and 2 mM improved sperm quality and reduced oxidative stress parameters in cooled-stored ram semen. Lycopene has been reported to have promising results for the conservation of fowl (Mangiagalli et al., 2010; Rosato et al., 2012) and bull spermatozoa (Tuncer et al., 2014; Tvrda et al., 2016; Tvrda et al., 2017).

2.3 Physico-morphological attributes

Studies on physico-morphological attributes of semen will help in identifying good quality ejaculates. Observation of semen volume, its sperm-cell concentration and motility are necessary to provide a gross estimate of reproductive performance of a bull and more particularly to establish the routines involved in extension and storage (Salisbury et al., 1985). Accurate screening of physico-morphological attributes of the ejaculates allows elimination of bulls with a potential low fertility.

2.3.1 Color and Consistency

The color and appearance of semen depends on the number of sperm cells in a given volume of seminal fluid. The colour of normal semen ejaculate of bulls varies from milky white to creamy white. Abnormal color of semen denotes pathological conditions of genitalia. In pathological conditions the color of semen gets changed like reddish or pinkish color indicates blood or degenerative tissues in the genital tract, yellowish color indicates presence of pus or urine in the semen, and brownish colored semen probably due to blood pigment may be observed in orchitis.

Shukla and Bhattacharya (1949) reported that, the semen of healthy bull is milky white and yellowish milky in color and consistency is either thick or thin. The semen color of Thai swamp buffalo is milky to creamy white (Koonjaenak et al., 2007). The Sahiwal bulls produced thicker semen than KF bulls with no significant variation in color and consistency either between breed or within age group (Ulfina and Raina, 2002). Semen that is uniform, opaque and thick is relatively high and which is clear and translucent is relatively low in sperm concentration. Kodagali (1962) also reported that, thicker semen samples had a higher sperm concentration and thinner semen samples had a lower sperm concentration. Corneo (1946) after observing the seminal traits reported that yellow color is normal characteristics of semen of many bulls and in no way influences the fertility of a bull. Lemon colour in semen of some individuals is due to flavin compounds especially riboflavin pigments.

2.3.2 Ejaculate volume

This is an important parameter of semen evaluation and is not only important for extensive utilization of semen in Artificial Insemination but to some extent, has been positively correlated with fertility (Wiggins et al., 1953). Ejaculate volume varies from breed to breed and within a breed from bull to bull (Rao et al., 1996). Ejaculate volume is probably a breed characteristic, which depends upon the body/scrotal size and weight, reproductive health condition of bulls, exercise, age of bulls, method and frequency of collection, teasing, pooled volume, nutrition, season and management (Nazir, 1988). The semen volume is generally less in young and small sized bulls. Teasing increases the ejaculate volume.

Fiaz et al. (2010) reported that ejaculate volume was significantly lower during summer in HF bulls, whereas, it was higher (3.025 ± 0.03 ml) during wet

summer in Jersey bulls compared to other seasons. In Thai swamp buffaloes average volume of semen varies between 3-4 ml (Koonjaenak et al., 2007). The ejaculate volume of semen in Sahiwal bulls varies from 3.20 to 6.00 ml. Average neat semen volume of Murrah buffalo bull has been reported as 3.67±0.21 ml (Saini et al., 2016).

2.3.3 Seminal pH

The pH is determined by acidic secretion of the prostate gland and alkaline secretion of seminal vesicle. pH of semen is the indication of semen quality. An increase or decrease in pH of semen has been proved to be detrimental to the sperms in terms of motility and fertility (Purdy, 2006).

Shaha et al. (2008) found that the pH of Friesian cross Zebu varied from 6.1 to 6.5. In Hariana bulls the mean pH has been reported as 6.84±0.04 (Sachan, 2013), 6.70±0.06 (Yadav, 2018) and 6.58±0.03 (Rathore, 2019).

2.3.4 Sperm Concentration

It is highly variable semen characteristic which correctly determines the number of spermatozoa per ml of semen. Sperm concentration in semen collection could be considered as an initial indicator of semen quality in semen used for cryopreservation (Belorkar et al., 1988; Shelke and Dhami, 2001). When combined with volume of the ejaculate, this quantity of spermatozoa determines number of females that can be inseminated, each with the optimal number of sperm cells (Hafez & Hafez, 2000). The concentration of the spermatozoa varies with breed, growth and maturity of bull, number of false mount, frequency of collection and reproductive soundness and the size of testes.

The sperm concentration in a bull ranges between 300-2500 millions/ml with an average of 1200 millions/ml (Roberts, 1982). Banerjee and Ganguli (1973) reported the sperm concentration of Zebu bull semen as 1091.8×10^6 per ml which was higher than that of buffalo bull semen (578.5×10^6 per ml).

A lot of variation in the sperm concentration has been observed by different workers (millions/ml) in Hariana bulls are depicted in the table as follows:

Breed	Concentration (millions/ml)	References
Hariana	1262.50 ± 41.69	Sachan (2013)
Hariana	1403.83+95.13	Patel (2014)
Hariana	1479.42+96.32	Yadav (2014)
Hariana	1457.83+70.69	Verma (2015)
Hariana	825.66 ± 27	Yadav (2018)
Hariana	1867.71±72.03	Rathore (2019)

2.3.5 Sperm Motility

It is one of the most reliable parameter to evaluate the fertilizing capacity of bovine spermatozoa (Correa et al., 1997; Zhang et al., 1998; Verberckmoes et al., 2002). However, not only sperm motility, but also the movement characteristics of the sperm can be important and correlated to the *in vivo* fertility in bulls (Januskauskas et al., 2000). Sperm motility is known to be prerequisite for penetration through cervical mucus (Mortimer and Swan, 1999), the cumulus (Tesarik et al., 1990) and zona pellucida (Green, 1988).

Mass motility and individual motility are two methods of assessing sperm motility which are traditionally employed. Salamon and Maxwell (2000) proved that frozen-thawed semen contains only 50 % of motile spermatozoa in comparison with fresh semen. Sperm motility requires adenosine triphosphate (ATP) which is produced by mitochondria (10 %) and by anaerobic glycolysis in the sperm tail (90 %) (Mukai and Okuno, 2004).

2.3.6 Mass Motility

The mass motility of semen is generally assessed by graded estimate of the vigor of swirls and waves formed in undiluted semen under low power of microscope (10x objectives) and expressed on 0 to +5 scale.

Tomar et al. (1966) observed the initial sperm motility in Hariana and Murrah bull semen as +3.5 and +3.8 respectively. Others reported the average mass motility of Hariana bull semen between 3.92±0.10 to 3.88±0.14 (Patel et al., 2015). Mass

motility observed by different workers in Hariana bulls are depicted in the below table as follows:

Breed	Mass motility (0-5 scale)	Reference
Hariana	3.38±0.13 to 3.75±0.16	Sachan (2013)
Hariana	3.88±0.14 to 3.92±0.10	Yadav (2014)
Hariana	3.60±0.05	Verma (2015)
Hariana	3.67±0.08	Yadav (2016)
Hariana	3.83 ±0.08)	Yadav (2017)
Hariana	3.56 ±0.06	Yadav (2018)
Hariana	3.50±0.11	Rathore (2019)

2.3.7 Progressive Motility

Individual motility refers to the percentage of progressively motile sperm in the semen sample. Progressively motile spermatozoa, provides an accurate prediction of semen quality required for fertilization (Lasley, 1951; Tomar et al., 1966). The Progressive motility of sperm is significantly correlated with per cent live sperm, cold shock resistance, freezability and fertility. Oscillatory motility is normally seen in aged sperms, which have exhausted all energy and are on the verge of death. Sperms, which have suffered either thermal shock or osmotic shock due to dilutor not being isotonic, show circular motility. The progressive motility is also reported to decrease with the increase in dilution rate.

Mammalian semen motility is affected by cryopreservation of sperm and enhanced the production of ROS molecules through lipid peroxidation (Chatterjee et al., 2001). Addition of lycopene @ 1.5 mM/liter to the semen extender led to higher sperm motility and progressive motility in comparison with the control ($P < 0.001$) following freezing and thawing (Tvrda et al., 2017).

Bucak et al. (2015) compared the effect of lycopene and resveratrol on post-thaw bull spermatozoa. Lycopene was added at a concentration of 0.001 gm/ml. The result revealed a significant increase in motility, progressive motility compare to control.

Tuncer et al. (2014) studied the cryoprotective effects of Lycopene and Cysteamine in Holstein bull semen. Lycopene in a concentration of 500µg/ml in the tris egg yolk based extender results in a significant increase in motility.

Akalin et al. (2016) treated the ram semen with different concentrations of lycopene (0.5, 1 and 2 mM) and reported that lycopene at 0.5 mM led to higher motility rate (62.50 ± 1.11%), when compared to lycopene at 2 mM dose (49.16 ± 4.72%) at 72 h of liquid storage ($P < 0.05$).

Tvrda et al. (2016) studied antioxidant efficiency of lycopene on oxidative stress in Holstein Friesian bulls spermatozoa. In this study spermatozoa were washed out of fresh bovine semen, suspended in 2.9 % sodium citrate and subjected to lycopene treatment (0.25, 0.5, 1 or 2 mmol/L). The study revealed that supplementation of 0.5–2 mmol/L lycopene resulted in a significantly increased motility and progressive motility in comparison with the control and this process is a dose and time dependent phenomena.

Ren et al. (2018) studied the effect of lycopene in cryopreservation of Cashmere goat sperm. The study revealed a significant effect of lycopene at a concentration of 1.0 mg/mL compare to control for sperm motility. The study further suggests that addition of alpha-lipoic acid at a concentration of 10 µg/mL potentiate the effect of lycopene.

Usually the progressive motility of a good semen sample should range between 50 % to 90 %. Progressive motility as reported by various workers for Hariana bulls are as follows:

Breed	Progressive motility (%)	Reference
Hariana	76.63 ± 1.45 to 79.38 ± 1.19	Sachan (2013)
Hariana	87.83 ±0.58 to 87.92± 0.68	Yadav (2014)
Hariana	77.83 ± 0.64	Verma (2015)
Hariana	86.40 ± 1.25	Yadav (2016)
Hariana	82.97± 0.55	Yadav (2018)
Hariana	83.93±1.07	Rathore (2019)
Hariana	85.89±0.50	Gupta (2020)

2.3.8 Live Sperm Percentage

Live and dead count of spermatozoa could be differentiated by their ability to be stained by eosin-nigrosin dye. Supravital staining is based on the fact that the membrane of dead spermatozoa permits the passage of the red stain (eosin) into the cytoplasm, but the membrane of live spermatozoa does not permit that. Thus, all dead spermatozoa in ejaculate will be coloured, while live spermatozoa will remain colourless. Dead spermatozoa were found to be stained (eosinophilic) and live spermatozoa not stained (non-eosinophilic) by the dye. There is a positive correlation between the motility and the percentage of live spermatozoa (Hafez & Hafez, 2000). Season is one of the most important factors influencing the reproductive performance of breeding bulls and it exerts its effect through macro and micro climatic factors like temperature, humidity, rainfall, photo-period (Mandal et al., 2000). The live percent of spermatozoa in a semen sample is affected by species and bulls within the same species (Saxena and Tripathi, 1978, 1981), ejaculate frequency (Nath et al., 1991), age of bull (Menendez et al., 1978), sex libido, nutritional status (Tripathi and Saxena, 1983) and season (Dhami et al., 1987).

All live spermatozoa are not necessarily motile, so total numbers of live spermatozoa in semen ejaculate are found to be higher than the total motile spermatozoa (Tomar, 1967).

Saxena et al. (1978) reported that, higher non-eosinophilic sperm percentage is an indicator of greater chance of survival in a buffer or in a female genital tract resulting in increased conception rate. A decrease in livability was observed with increased post thaw storage duration.

Bhalde et al. (1991) reported non-eosinophilic percent value of 60.76 ±0.68 and 57.64 ± 0.78 in post-thawed semen after thawing at 35°C for 30 sec and at 5°C for 5 min, respectively. A decrease in livability was observed with increased post thaw storage duration.

Rao et al. (1996) reported that semen of Ongole bulls had 84.60 noneosinophilic sperm percentage which was higher than Jersey with 83 percent and Jersey x Ongole with 83.6 percent.

Paudel (2002) reported the percent live spermatozoa in the fresh ejaculate of crossbred bull semen (HF x Hariana) as 82.8 ± 0.79.

Uysal and Bucak (2007) studied the effects of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen-thawed ram semen and reported that lycopene @ 800 µg and 1600 µg had beneficial effect on liveability.

Bucak et al. (2015) compared the effect of lycopene and resveratrol on post-thaw bull spermatozoa. Lycopene was added at a concentration of 0.001 gm/ml. The result revealed no significant effect of lycopene compared to control. Similarly, Rosato et al. (2012) have reported non significant effect of lycopene.

The various reports for live sperm (Mean ± S.E.) in the fresh ejaculate of Hariana bull by different researchers has been tabulated as under

Breed	Live Sperm (%)	Reference
Hariana	84.75 ± 2.52 to 90.38 ± 0.91	Sachan (2013)
Hariana	94.99 ± 0.48 to 95.17 ± 0.37	Yadav (2014)
Hariana	85.00 ± 1.26	Verma (2015)
Hariana	93.85 ± 0.68	Yadav (2016)
Hariana	87.49±1.09	Rathore (2019)
Hariana	89.92±0.46	Gupta (2020)

2.3.9 Hypo Osmotic Swelling Test (HOST)

Evaluation of functional integrity of the mammalian sperm membrane is of vital importance as it plays a significant role in fertilization as well as in the sperm metabolism because a correct change in the properties of membrane is required for sperm capacitation, acrosome reaction and binding of the spermatozoa to the egg surface for which a biochemically active membrane is required (Jayendran et al., 1984; Chan et al., 1985). The principle of the HOS assay is based on fluid transport across the sperm tail membrane under hypo-osmotic conditions until equilibrium is reached. Due to this influx of fluid, the tail expands and bulges and expresses a characteristic pattern, considered as a hypo-osmotic response, which can readily be identified with phase contrast microscope (Cabrita et al., 1999). Such spermatozoa are denoted as swollen or HOS reactive (HOS +). Spermatozoa with functionally defected membrane do not swell and denoted as HOS negative (Zhu and Liu, 2000). HOST has

a high correlation (0.80 in cattle and 0.93 in buffalo) with fertility (Goswami, 2006). The assessment of the membrane may be a useful indicator of the fertilizing ability of spermatozoa (Barrat et al., 1989; Avery et al., 1990).

Keel and Webster (1990) reported that membrane integrity is important for sperm metabolism, motility, capacitation, acrosome reaction and the binding of spermatozoa to the egg surface.

Takahasi et al. (1990) hypothesized that the ability of the sperms to swell in hypotonic solution indicates its membrane integrity and normal function activity. The appropriate osmotic pressure to form maximum tail curls are reported as 150mOsm for human (Jayendran et al., 1984), buffalo (Shukla and Mishra, 2007) and bull (Kathiravan et al., 2008).

Kumi Diaka (1993) reported that plasma membrane must be intact for normal sperm function and intactness of the plasma membrane is of vital importance and it is tested using HOS tests.

Muino et al. (2007) while working on ten Holstein Friesian bull reported percentage of HOS positive spermatozoa as 40.9 and 30.3 at 0 and 9 h of incubation at 37ºC.

Uysal and Bucak (2007) studied the effects of oxidized glutathione, bovine serum albumin, cysteine and lycopene on the quality of frozen-thawed ram semen and reports higher concentration of lycopene having negative effect.

Ramachandran et al. (2007) reported that tests which evaluate the sperm membrane functional status like acrosomal integrity test, HOST are highly interrelated with the fertility of bulls. HOST is used as preliminary test to estimate the functional integrity of the sperm membrane (Srivastava and Kumar, 2006; Gebreselassie, 2009).

Khan and Ijaz (2008) reported higher ($P< 0.05$) plasma membrane integrity in frozen thawed semen at 50, 100, 150 mOsm/L as compared with 250 mOsm/L solution.

Ijaz et al. (2009) reported HOS positive spermatozoa percentage as (44.5±3.0) in post thawed semen of Nili-Ravi buffalo bull.

Perumal et al. (2013) observed percentage of hypo osmotic swelling positive sperm in Jersey crossbred bulls. They selected 6 bulls with good body condition and

divided them into two groups based on the seminal characters i.e. good and poor freezable bulls and reported the values as 85.78±0.42 and 80.38±0.32 percent respectively.

Rajoriya et al. (2014) observed that season had significant effect on HOST positive spermatozoa as evidenced by significantly ($P<0.01$) higher HOST positive spermatozoa observed in winter season. The higher value of HOST positive indicates higher percentage of membrane integrity of the spermatozoa. The higher percentage of HOST positive spermatozoa in Tharparkar bull semen than crossbred bull semen (Loyi, 2007 and Gebreselassie, 2009) is an indicative of superiority of Tharparkar bull semen over crossbred semen.

Yadav (2017) reported percentage of HOS positive spermatozoa as 72.79±0.85 at pre-freeze and 62.46±0.76 at post thaw stage in EYTG extended Hariana bull semen.

Yadav (2018) reported percentage of HOS positive spermatozoa as 74.21±0.69 at pre-freeze and 63.96±0.52 at post thaw stage in EYTG extended Hariana bull semen.

Ren et al. (2018) studied the effect of lycopene in cryopreservation of Cashmere goat sperm. The study revealed a significant effect of lycopene at a concentration of 0.5 to 4.0 mg/mL compare to control for HOST responsive spermatozoa. The study further suggests that addition of alpha-lipoic acid at a concentration of 10 μg/mL potentiate the effect of lycopene.

2.3.10 Acrosomal Integrity

Acrosomal integrity is another necessary indicator of potential sperm functions. Acrosome, a cap like structure on the head of the spermatozoa covers 60% of the anterior portion of the sperm head. Capacitation and the subsequent acrosome reaction of spermatozoa are the essential steps before fertilization and formation of zygote. The determination of acrosomal integrity of spermatozoa by single Giemsa's staining technique is an essential tool to evaluate fertilizing capacity of the semen. Presence of normal acrosome is important for successful acrosome reaction and ultimately fertilization (Bailey et al., 2000).

Wells et al. (1970) reported that acrosome plays a vital role in ovum fertilization by dispersing the enzymes into the interstitium of the zona cells. The

abnormal acrosomes such as knobbed, ruffled, swollen, denuded, incomplete and shrunken may cause leakage of membrane bound enzymes resulting into poor conception rate. These workers observed an acrosomal damage between 12.7-29.3 percent in crossbred bulls.

Anderson et al. (1990) studied the relationship between three types of acrosomal aberrations of bull spermatozoa and the bull's fertility, and suggested that the acrosome morphology was worth of evaluating during routine morphological analysis of bovine semen.

Fiser and Fairfall (1990) did not find any correlation between motility and acrosomal damage. However Goswami, (2006) reported that acrosome reaction test has a high correlation (0.91 in cattle and 0.77 in buffalo) with fertility. Thundathil et al. (2000) studied the effect of knobbed acrosome defects in bovine sperms on IVF and embryo production. They concluded that knobbed acrosome sperm completely failed to penetrate the zona pellucida. They also concluded that knobbed acrosome defect as uncompansable defect and these defects are considered.

Thundathil et al. (2001) studied flattened or indented acrosome defect and reported that sperm with flattened acrosome had a tendency to undergo spontaneous acrosome reaction. Thus, have a reduced ability to fuse with oolemma. Thus, the ability to penetrate ooplasma and undergo sperm chromatin decondensation was impaired.

Thundathil et al. (2002) reported that spermatozoa with knobbed acrosome defect have impaired plasma membrane function, which predisposes them to premature capacitation and spontaneous acrosome reaction on incubation after thawing.

Yadav (2017) reported value of acrosomal intact spermatozoa as 78.42±0.87 at pre-freeze and 66.58±0.92 at post thaw stage in EYTG extended Harana bull semen.

Tvrda et al. (2017) used lycopene in the concentration of 1.5 mM and found a positive effect of lycopene. The average acrosomal integrity recorded was 85.20±0.42 per cent. Similarly, AI- Sarray et al. (2019) have reported a positive effect of lycopene on acrosomal integrity.

2.4 Chlortetracycline Fluorescence (CTC) Assay

Capacitation is a collective term for the changes which a sperm undergoes when it comes in contact with female genital tract (Rathi et al., 2001). These changes include reorganization of membrane proteins, membrane phospholipid metabolism and reduction in membrane cholesterol levels (Yanagimachi et al., 1994). Capacitation changes the distribution of Ca^{2+} in sperm head, plasma membrane causing change in CTC fluorescence patterns in sperm depending upon the physiological status of sperm (Yanagimachi et al., 1994). On comparing sex sorted spermatozoa with fresh, in vitro capacitated and in vitro acrosome reacted sperm, sex sorted sperms showed a CTC staining pattern similar to that observed after in vitro capacitation. The actin pattern distribution after sperm sorting also tended to be similar to that observed after in vitro capacitation, but this effect was more distinct in bull than in boar spermatozoa.

Cryopreserved sperm cells exhibit a capacitation like behaviour and appear to be in a partially capacitated state due to the cryopreservation induced membrane changes that makes the cell to be more active to their environment after thawing. Capacitation normally creates a state of destabilization with which the sperm cell acquires the fertilizing capacity while remaining susceptible to membrane degeneration and spontaneous acrosomal reaction when fertilization fails (Bailey et al., 2000). Cryopreservation creates a subpopulation of killed and partially or fully capacitated sperm thereby reducing the heterogeneity of the sperm population. This produces a sperm subpopulation with a shortened lifespan in vivo and whose fertilization potential has been severely compromised, reducing the fertility of the semen sample as a whole.

Demonstration of capacitation or capacitation related structural modifications through fluorescent labeling of sperm surface antigens (Byrd, 1981) has been used to demonstrate capacitation. The evaluation of capacitation by changes in the expression and/or distribution of cell surface molecules by staining with chlortetracycline have been used as a method of choice for the semen of bovine. This was first performed with mouse spermatozoa (Ward and Storey, 1984) and was subsequently applied to human (Perry et al., 1995) and bull (Fraser et al., 1993) spermatozoa.

Chlortetracycline is an antibiotic, the fluorescence of which changes when it chelates membrane associated divalent ions (mainly calcium) (Hallet et al., 1972). The technique offer the advantage of measuring directly the percentage of noncapacitated, capacitated and acrosome reacted spermatozoa in the same preparation.

According to Fraser et al. (1993) and Maxwell and Johnson (1997) sperm population can be classified into 3 different categories based on CTC fluorescent patterns:

F-pattern: Uniform fluorescence on the sperm head (uncapacitated, intact spermatozoa)

B-pattern: Fluorescent acrosomal region and fluorescent free post acrosomal area (capacitated sperm)

AR-pattern: Acrosomal and post-acrosomal region are fluorescence -free except a thin fluorescent band at the equatorial region (acrosomal reacted sperm)

Based on flow cytometric analysis of CTC stained sperm, Maxwell and Johnson (1997) observed that, pattern B reflects membrane destabilization and calcium influx to the cell. Thus the CTC pattern B fluorescence induced by cooling and cryopreservation appears to be related to sperm capacitation in the functional sense, as these treatments favour sperm fertilization in vitro. According to Parrish et al. (1999), in bovine sperm an increase in intracellular calcium accompanies heparin-induced capacitation in vitro.

Visconti et al. (1998) speculated that during capacitation, membrane modifications stimulate adenyl cyclase to initiate cyclic adenosine monophosphate mediated tyrosine phosphorylation of sperm proteins. Furthermore, elevated sperm calcium levels are thought to trigger an intracellular signaling cascade that has recently been related to capacitation.

The acrosome reaction seems to be an absolute requirement for fertilization *in vivo.* Only acrosome reacted spermatozoa has the ability to pass through the zona pellucid and later on fuse with the oocyte membrane to form a zygote (Yanagimachi et al., 1994). In capacitated spermatozoa, the acrosome reaction results in loss of the acrosomal cap which can be visualized readily by light microscopy in those species

that have large acrosomes (guinea pig and hamster). However, in most mammals, the acrosome is small and difficult to visualize.

Thus, a number of different staining techniques have been developed for visualization of mammalian sperm acrosome, like use of multiple histochemical stains, labeled lectins and labeled antibodies (Koehler, 1978). CTC fluorescent assay, originally developed for mouse spermatozoa (Saling and Storey, 1979; Ward and Storey, 1984) has also been found to be quiet convenient for bovine spermatozoa.

Cryo-capacitation is the major factor related with reduced longevity and poor survivability of cryopreserved spermatozoa in female reproductive tract (Bailey et al, 2000; Watson 2000) resulting in reduced fertility of frozen-thawed semen. At present it is generally accepted that poor survival of spermatozoa in female reproductive tract is among the most important consequences of sperm cryoinjury caused by cryopreservation. This concept of premature capacitation and reduced longevity of sperm cells in female reproductive tract has led to the routine use of oviductal insemination by laproscopy rather than or even trans cervical insemination in different animals (Bailey et al., 2000).

The capacitation of the mammalian spermatozoa is judged by using chlortetracycline assay (CTC), measurements of CASA motility characteristics and assessment of tyrosine phosphorylation within plasma membrane. The major advantage of CTC is that it not only allows discrimination between acrosome-intact cells and acrosome reacted ones, but also divides acrosome-intact cells into two further, functionally different, categories, i.e. uncapacitated and capacitated.

2.5 Sperm Kinematics

The development of computer assisted semen analysis on the basis of sperm head movements has facilitated the accuracy and dependability of sperm motility evaluation. CASA systems can also analyze sperm concentration, motility, viability (World Health Organization, 2010) and morphology (An et al., 2011). The robust sperm analyzer CASA systems can be used effectively to compare the modifications in spermatozoa motion, velocity and kinematics both prior to freezing and after freeze-thawing of the samples (Amann and Katz, 2004; Liu et al., 2004).

Total motility (TM) is the ratio of motile cells to the total concentration of spermatozoa (Mortimer, 2000). Progressive motility (PM) is the sperm number

expressed as percentage moving with path velocity (VAP) higher than the medium VAP cut-off and with straightness (STR) higher than the standardized level (Mortimer, 2000). Sperm velocity parameters are rapid velocity, medium velocity, slow velocity and static velocity. Average path velocity (VAP, μm sec−1) is the time averaged velocity of a spermatozoa head along their average path. Straight line velocity (VSL, μm sec−1) is the time averaged velocity of spermatozoa head along the straight line between their first and last detected positions, respectively. Curvilinear velocity (VCL, μm sec−1) is the time averaged velocity of spermatozoa head along their actual curvilinear path. Amplitude of lateral displacement of a sperm head (ALH, μm) is the magnitude of lateral displacement of a sperm head about its average path, which can be expressed as a maximum or an average of such displacements. Beat cross frequency (BCF, Hz) is the average rate at which curvilinear path crosses the average path. Straightness (STR, VSL⁄VAP) refers to the linearity of the average path. Linearity (LIN, VSL⁄VCL) refers to the linearity of a curvilinear path, (Mortimer and Swan 1999; Amann and Waberski, 2014).

Several studies have been carried out to compare analyses on manual methods of semen quality with those determined by CASA (Hu et al., 2006; Akashi et al., 2010; Tomlinson et al., 2010; Vested et al., 2011). Liu et al. (1991) and Akashi et al. (2010) have found remarkable correlation between these subjective and objective evaluation methods. Conversely, Spiropoulos (2001) and Vested et al. (2011) reported significant differences among evaluation of sperm parameters in both methods. Some studies have also reported a weaker correlation between ALH and BCF (Ge et al., 2008; Akashi et al., 2010). Different studies revealed correlations of CASA variables (Rapid velocity, VAP, VSL, VCL, ALH, STR, LIN) with *in vitro* fertilization in different species (Ren et al., 2004). Other studies have however reported a positive correlation among CASA variables particularly the VSL and *in vivo* fertility (Kathiravan et al., 2008).

Many factors can influence the accuracy and precision of CASA output values. It is also important to determine the appropriate subgroup limits for each measure of sperm motion in typical samples being evaluated, and to use these boundaries in order to visualize and summarize subpopulations of sperm in each sample analysed, and do not rely on means or medians (Amann and Waberski, 2014). All of the commercial computer assisted sperm analysis (CASA) systems acquire a

frame rate (FR) of 30–60 Hz; a higher FR is required to give the estimation output nearer to the ‘‘real path’’ chiefly for fast nonlinear spermatozoa (Mortimer, 2000; Castellini et al., 2011). The depth of the counting chamber is particularly important during evaluation of the motion and kinematic parameters of spermatozoa (Mortimer, 2000; Lu et al., 2014). Other factors that affect CASA motility output include number of fields, concentration and dilution of semen samples (Anzar et al., 2010; Kumar et al., 2015).

Tuncer et al. (2014), in Holistein bulls used 500µg/ml lycopene and evaluated the sperm velocity parameters. All sperm kinematic parameters in lycopene treated group compared to control having higher values except, BCF. Bucak et al. (2015), in Holistein bulls also reported higher VAP, VSL, VCL, ALH and LIN values as compare to control.

Tvrda et al. (2017) used 1.5 mM lycopene for bull semen and reported higher VAP, VSL, VCL, ALH, BCF, STR and LIN values as compared to control after freezing and thawing.

2.6 Bovine Cervical Mucus Penetration Test (BCMPT)

The *in-vitro* sperm mucus penetration test (SMPT) is a sperm function test which measures the ability of sperm in the semen to swim up into a column of cervical mucus or substitute. If it can be proven to be as good as semen analysis in assessing progressive sperm motility, then it is suitable and cheaper alternative to the present combination of semen analysis and sperm separation procedures. Sperm migration into cervical mucus or any of its substitutes is based on the same principle as the Kremer (1965) test. Fertilizing capacity of spermatozoa has been shown to be strongly related to cervical mucus penetration test (CMPT) in bull (Tas et al., 2007).

Zubair et al. (2013) found a significant positive correlation between progressive motility, morphologically normal spermatozoa, sperm viability and percentage of HOS-positive spermatozoa in HF, Sahiwal and cross-bred (HF X Sahiwal) bulls. Galli et al. (1991) also demonstrated that sperm penetration in mucus was significantly correlated with total motility, sperm morphology and progressive motility, acrosome integrity and concentration.

Cervical mucus is produced by endocervical secretory cells. Its quality and quantity depend on the status of gonadal hormones during the estrous cycle

(Tsiligianni et al., 2001). The main functions of cervical mucus are sperm transportation and its role as a barrier against microbial infections. Cervical mucus consists of 92-95% water, along with carbohydrates, amino acids, lipids, and a mixture of dissolved macromolecules such as proteins and polysaccharides (Schumacher, 1970). Cervical mucus also contains several enzymes such as alkaline phosphatase (ALP), lactate dehydrogenase (LDH), and α -amylase.

The characteristics of cervical mucus play an important role in the success or failure of pregnancy. An indirect evaluation of cervical mucus can be used as an indicator of the current estrous cycle stage or hormonal status in domestic animals. Cervical secretion also has roles in sperm endurance and transportation to the uterine cavity (Kumar et al., 2012).

2.7 Seminal Enzymatic Profile

Seminal plasma is endowed with an array of antioxidants to protect spermatozoa against oxidative stress (Sikka, 1996; Armstrong et al., 1999).

2.7.1 Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) is a well-known enzymatic biological antioxidant that scavenges superoxide and peroxide and thus controls the oxidative stress in mammalian sperm (Fridovich, 1985). SOD protects spermatozoa by catalysing the dismutation of superoxide anions (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen (O_2) (Agarwal et al., 2003). The physiological role of superoxide dismutase is to protect cells from the toxic effects of superoxide anions (O_2^-) generated during enzymatic oxidation of NADPH in neutrophils and mitochondrial electron-transfer reactions. It catalyses a dismutation reaction leading to the formation of H_2O_2 and O_2:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

The H_2O_2 is then destroyed by tissue catalases and peroxidases.

SOD protects spermatozoa against spontaneous O_2 toxicity and lipid peroxidation (Alvarez and Storey, 1992). SOD plays an important role in decreasing LPO and protecting spermatozoa during genito-urinary inflammation (Aitken, 1995). Seminal plasma gives some protection against its constituent antioxidants but such level of protection is not enough for long term storage. Hence, increased oxidative

stress is encountered during the manipulation involved in reproductive techniques, or the antioxidants are removed along with the seminal plasma when the sperms are being prepared for cryopreservation (Strzezek et al., 1999).

The fertility rates of human sperm can be predicted by measuring the SOD activity which is thought to be the major factor protecting human sperm against lipid peroxidation (Alvarez et al., 1987; Zini et al., 1993). SOD activity is positively associated with sperm viability and HOST response in buffaloes. Addition of SOD to bovine semen improved the capacitation status of a sample and addition of vitamin E, as an antioxidant protected the sperm membranes from peroxidation (O'Flaherty et al., 1999).

Bilodeau et al. (2000) reported the level of SOD activity in frozen seminal plasma of Holstein bull as (111.0±9.0) U/mg protein.

Kadirve et al. (2014) reported the SOD activity in a range of 14.34 to 29.34 IU/10^9 spermatozoa in fresh and 6.86 to 19.56 IU/10^9 spermatozoa in frozen thawed buffalo semen.

Tvrda et al. (2016) used different concentrations of lycopene (0.25 mM to 2.0 mM/L) and did not find any significant effect on SOD activities in HF bull seminal plasma. However, Tvrda et al. (2017) using 1.5 mM lycopene find a significant reduction in superoxide production compare to control (42.58±3.47 vs. 100.00±0.00 per cent) in the seminal plasma of adult Simmental- Fleckvieh breeding bulls.

Ren et al. (2018) reported the addition of 1.0 mg/mL lycopene and 10 μg/mL alpha-lipoic acid to bull semen enhanced the activities of SOD, CAT, and GSH-Px, and reduced ROS levels. Compared with lycopene or alpha-lipoic acid alone, the combined supplementation of 1.0 mg/mL + 5.0 μg/mL lycopene + alpha-lipoic acid to the semen cryopreservation extender had greater potential to decrease intracellular ROS, and subsequently elevated thawed-frozen Cashmere goat sperm quality and pregnancy rates.

2.7.2 Glutathione-S-Transferase (GST)

Products generated by reactive oxygen species (ROS) or metabolites of drugs and chemicals that induce stress must be eliminated from a cell to prevent cell damage. Detoxification of products of oxidative stress and electrophilic compounds is carried out by enzymes called glutathione-S-transferase (Kumar et al., 2013). GST is a

part of glutathione cycle that is responsible for detoxification of H_2O_2 (Li, 1975) and prevention of sperm membrane fragility (Aitken et al., 1993).

GST has been reported to play an important role in sperm capacitation, acrosome reaction and fertilization (Kumar et al., 2013). Increase in GST activity was indicative of enhanced antioxidant ability (Perumal et al., 2013). Therefore higher GST 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 (Mohanty et al., 2004).

Addition of antioxidants improves the structural membrane integrity and motility of liquid storage or cryopreserved ram sperm cells. Waheed et al. (2013) reported GST activity in the fresh semen of buffalo as 1.90 ±0.20 nM/min/ml.

2.7.3 Lipid Peroxidation

Lipid peroxidation is an important patho-physiological process occurring in numerous diseases and stress conditions and results in a series of degradative processes affecting the organization and function of cellular components (Aitken et al., 1989). A simple tool to evaluate the effect of lipid peroxidation on the spermatozoan is the assay of sperm malondialdehyde (MDA), a thiobarbituric-acidreactive substance (TBARS) that is a stable lipid peroxidation product (Kasimanickam, 2006). Production of malondialdehyde (MDA) was used as an index of spontaneous lipid peroxidation in spermatozoa (Jones and Mann, 1973). Aitken et al. (1993) reported that the xanthine-xanthine oxidase (X-XO) system was used to generate ROS, and the lipid peroxidation status of spermatozoa was monitored using the generation of malondialdehyde (MDA). In bovine and ram semen, ROS are generated primarily by dead spermatozoa via an aromatic amino acid oxidase catalyzed reaction (Upreti et al., 1994a, b). All aerobic cells have substrates and enzymes to neutralize these potentially toxic ROS but the antioxidant defences of spermatozoa are comparatively weak and such cells are very much prone to oxidative damage (Jones and Mann, 1977).

During cryopreservation, the semen is exposed to cold shock and atmospheric oxygen, which in turn increases their vulnerability to lipid peroxidation (LPO) due to higher production of ROS. In bovine semen the latter induces aging of spermatozoa, thus reducing their life span and affecting the protection of semen for artificial

insemination ultimately leading to poor insemination rates (Alvarez and Storey, 1982). Most studies on semen viability and enzyme leakage from spermatozoa due to ROS production have been conducted on frozen semen samples (Dhami and Kodagali, 1990; Upreti et al., 1996).

Tvrda et al. (2016) used different concentrations of lycopene (0.25 mM to 2.0 mM/L) and did not find any significant effect on MDA activities in HF bull seminal plasma. However, Tvrda et al. (2017) using 1.5 mM lycopene find a significant reduction in lipid peroxidation (20.43±0.56 vs. 7.45±0.81 µmol/g) compare to control in the seminal plasma of adult Simmental- Fleckvieh breeding bulls.

Literature reviewed so far does not reveal any work related to this antioxidant (lycopene) in Hariana bull semen.

Materials and Methods

CHAPTER-3

MATERIALS AND METHODS

3.1 Experimental animals

The present study was conducted on four Hariana bulls of the age group between 5.0 - 9.0 years and weighing around 450-600 kg body weight, reared at Semen Biology Lab, Department of Gynaecology and Obstetrics situated at ILFC, DUVASU, Mathura, (U.P.) India, during the session 2020-2021.

3.1.1 Climatic conditions

Mathura is situated in Northern part of India. It is a semi arid zone, lying at 27.17^0 North-latitude and 77.41^0 East-longitudes and about 287 m above the sea level.

3.1.2 Housing system

The Hariana bulls were housed in individual pans with cemented floor which were separated by Iron partitions that restricted direct physical contact of bulls in adjacent pens. One third of the area was having the roof of corrugated asbestos sheets. In winter season paddy straw was used as bedding on the floors and thick curtains were also hanged to protect the bulls from cold waves in extreme cold. In summer season all the measures were taken to protect the bulls from hot wind during day time. The shed were cleaned once daily in the early morning.

3.1.3 Feeding and management of Hariana bulls

The bulls were fed a uniform balanced ration consisting of wheat straw which was one of the chief and common fodder available. Protein was supplemented from other sources like concentrate mixture, consisting of bran, oil cakes, grains and green fodder like berseem, maize, bajra and jowar etc. was provided in different seasons depending on their availability for nutritional requirements. Clean and fresh drinking water was provided to the bulls adlibidum.

3.1.4 Feeding schedule of Hariana bulls

Each bull was provided with the following:

S. No.	Ingredients	Quantity
1.	Green maize	30 – 40 %
2.	Green jowar	21.15 kg. (30 to 40 %)
3.	Bhoosa (Wheat straw)	4.95 to 5.77 kg.
4.	Concentrate	3.83 kg.
5.	Service ration	1.00 kg additional concentrate per day

Ingredients of concentrate

Ingredients	Percentage
Wheat bran	32
Maize	18
Barley	17
GNC (decorticated)	30
Mineral mixture	02
Salt	01
Total	**100**
DCP	21.64
TDN	76.67

3.1.5 Health care and management

Proper health care schedule was being followed at the farm. Animals were regularly tested for the semen quality and diseases like Brucellosis, Tuberculosis (T.B.) and Johne's disease (J.D.). They were routinely vaccinated against important contagious and infectious diseases like FMD, HS and BQ. Deworming was also done with broad spectrum dewormers. Management including watering, feeding, washing, exercise and timely collection of semen were done in a routine manner to keep animals in healthy and stress free condition.

3.1.6 Sterilization of articles

All the glasswares which were used during the course of study were thoroughly washed with teepol water and rinsed with tap water fallowed by double distilled water. These were dried in the air and sterilized in hot air oven at 180^{o}C for

60 minutes. Buffer solutions, rubber articles (latex lining, rubber cone, outer hard cover etc of A.V), micropipettes, microtips along with their boxes were sterilized in autoclave at 10 lb pressure, 115^{o}C temperature for 20 minutes.

3.1.7 Semen collection

On the day of collection, before collecting semen, the bulls were properly washed and cleaned with fresh borewell water. After that, semen was collected using sterilized artificial vagina (IMV Technologies, France), which is nearly 40cm long and 6.5 cm in diameter whose, smooth rubber lining was lubricated with white paraffin wax. Semen collection was performed before feeding the bulls. Semen was collected twice a week from each bull during early morning hours between 7:00-8:00 am in summer and between 8:30-9:30 am during winters. The temperature of A.V was maintained between 42^{0}C to 45^{0}C. Semen was collected directly into a clean, dry, sterilized 15 ml centrifuge tube graduated at 0.1 ml intervals, attached to the latex cone of the A.V. Immediately after collection, the tube containing semen was properly labelled and placed in the beaker having water at a temperature of 37°C and then was transferred to water bath already maintained at 37^{o} C in the laboratory. Eight ejaculates (n=8) each from four bulls were considered for neat semen evaluation. Thirty-two (n=32) samples were used for freeze-thaw study.

3.2 Evaluation of fresh semen

The semen was evaluated for following parameters.

3.2.1 Colour and Consistency

Colour and consistency of semen sample were observed by direct visualization with naked eyes as yellowish thick, thick creamy, milky white etc. and any other abnormalities in colour or consistency were treated as abnormal and the samples were discarded.

3.2.2 Ejaculate Volume

The volume of semen was directly measured in milliliter (ml) from the graduated centrifuge semen collection tube.

3.2.3 pH Value

pH of semen was measured by using pH paper (B.D.H. 5570 Indicator Paper) having the range of 5.5 - 7.0 and the colour developed was matched with the colour standards provided with the strip.

3.2.4 Mass Motility

Mass motility was evaluated as per method described by Salisbury et al. (1985). It was determined by placing a small drop of semen of uniform size and thickness over a clean, dry and dirt free glass slide held on the thermostage. The semen drop was examined under low power objective (10x) of microscope on a thermostatically controlled warm stage at 37°C. Nazir (1988) reported that motility was rated according to the vigour wave motion on a grade scale of 0 to 5 as given below into following categories.

S. No.	Observation	Mass motility score (0-5 scale)
1.	No motility	0
2.	No wave but sperm movement evident	+1
3.	Slow wave formation	+2
4.	Relatively more wave formation with swirls	+3
5.	Wave with swirls and eddies	+4
6.	Wave with very rapid swirls and eddies	+5

3.2.5 Sperm Concentration

Sperm concentration was estimated using Haemocytometer (Improved Neubauer's chamber) method (Salisbury et al., 1985). Central primary square is divided into 25 (5x5) secondary squares while each secondary square is further divided into 16 (4x4) tertiary squares. Thus central primary square is divided into 400 (25x16) tertiary squares. These total 400 tertiary squares have a total area of $1mm^2$. When a drop is charged under the cover slip in a Neubauer cell counting chamber, the thickness of the film on chamber is 0.1 mm. Thus the total volume of the semen

covering 400 tertiary squares in RBC chamber is $0.1mm^3$. Sperm concentration estimate was made by diluting semen samples 200 times with semen diluting fluid.

Composition of semen diluting fluid

Ingredients	Quantity
Eosin-Y (Water soluble)	0.05 gm
Sodium Chloride	1.0 gm
Distilled water upto	100 ml

Semen was sucked upto 0.5 mark into RBC diluting pipette. The outside of pipette was wiped with tissue paper to wipe off semen sample sticked outside to the tip of the pipette. Then the semen diluting fluid was sucked upto 101 mark on the pipette. Now the pipette was hold in between the hand palms and vortexed horizontally to ensure proper mixing. Haemocytometer along with its coverslip was cleaned, dried and focussed firstly at low power (10x) and then at (40x) power under the microscope. Discard first few drops of diluted semen from the RBC diluting pipette and a small drop is charged in between the coverslip and haemocytometer. After 30 seconds (time to ensure proper settling of spermatozoa) spermatozoa was then counted in five secondary squares of central primary square, namely right and left top, right and left bottom and central squares. Those spermatozoa, which were lying in square itself, on top line and right side line of the square, were also counted. Each secondary square contained 16 tertiary squares so total numbers of spermatozoa in 80 tertiary squares were counted. The average of the counts on both side of haemocytometer chamber was taken for calculating the sperm density and the total number of spermatozoa per ml of neat semen was calculated as follows.

Calculation

Area of 1 tertiary square $= 1/20 \times 1/20\ mm^2$

Volume of 1 tertiary square $= 1/4000\ mm^3$

Number of spermatozoa in 80 tertiary squares $= n$

Volume of 80 tertiary squares $= 1/4000 \times 80mm^3$

Where, $80/4000\ mm^3$ volume has ‘n’ number of spermatozoa.

Therefore, $1mm^3$ $= n/80 \times 400$

$= n \times 50$

Total number of undiluted spermatozoa = $50 \times n \times 200$

Therefore, $1mm^3$ of fresh semen = $10000 \times n$

Fresh semen contains (per ml) = $10000 \times n \times 1000$

= $10 \times n \times 10^6$

Sperm concentration = n x 10 x 10^6 per ml

Here, n = number of sperms counted in five secondary squares.

3.2.6 Progressive Motility

The progressive motility of the spermatozoa was observed under high power phase objective (40x) on a thermostatically controlled stage maintained at 37°C. A small drop of diluted semen was put on a clean grease free slide and was covered with a cover slip. The slide was examined to observe vigorously motile spermatozoa exhibiting progressive path. The progressive motility of spermatozoa was then calculated as given by Salisbury et al. (1985) at post dilution, pre-freeze and post-thaw stages as follows

$$\text{Progressive motility (\%)} = \frac{\text{No. of progressively motile spermatozoa}}{\text{Total number of spermatozoa observed}} \times 100$$

3.2.7 Live and Dead Spermatozoa Count

Method described by Campbell et al. (1953) was followed to estimate the live percentage of the semen sample at 3 different stages viz. post dilution, pre-freeze and post-thaw stage. Dead spermatozoa could be differentiated by their ability to get stained by Eosin dye. The live spermatozoa, which were alive at the time of staining, remain colourless since they were impermeable to Eosin stain. Nigrosin provided a blue-black background.

3.2.7.1 Preparation of stain

Eosin-Nigrosin stain was prepared by the method described by Campbell et al. (1953). The composition of the stain was as follows

Ingredients	**Quantity**
Eosin – Y (Water Soluble)	5 gm
Nigrosin (Water Soluble)	10 gm

These two chemicals were dissolved separately in 100 ml of 2.9% sodium citrate solution. The mixture was boiled for 15 min. Finally stain was prepared by mixing 10% Nigrosin solution and 5% Eosin-Y solution in the ratio of 2:1 in a container. The mixture was then kept for 7 days for maturation and filtered through Whatman's filter paper No. 40. Fresh stain was prepared every 15 days to prevent artifacts and filtered through quality filter paper (Whatman's filter paper no. 40) and stored in a dark and sealed glass bottle. Before use, the staining solution was brought to room temperature. Fresh stain was prepared every 15 days to prevent artifacts.

3.2.7.2 Staining technique

Semen samples were kept at 37°C before analysis. One drop of semen sample was mixed with 2 to 3 drops of Eosin-Nigrosin stain on a clean glass slide at 37°C using warm stage. It was kept for 3 min. A smear was made from the mixture on a clean and grease free glass slide. It was dried in air and examined under the bright field 100x oil immersion objective of phase contrast microscope. Atleast 200 sperms were counted. Sperms that were white (unstained) were classified as live and those showed any pink or red colourations were classified as dead (Photograph 1). Per cent live spermatozoa were calculated as follows:

$$\text{Percent live spermatozoa} = \frac{\text{Live spermatozoa counted}}{\text{Total spermatozoa counted}} \times 100$$

3.3 Cryopreservation of semen

3.3.1 Preparation of Egg Yolk Tris Glycerol (EYTG) Extender

The eggs yolk was recovered from a fresh egg. First the egg was sterilized with rectified spirit and then egg was broken from the narrower end with the help of sterilized forceps and then all the albumin was drained out. The yolk ball was transferred on the Whatman's filter paper no. 40 and rolled in order to clear all the traces of albumen and chalaza. The yolk membrane was broken and clear yolk was poured into a sterilized beaker and allowed to churn by using magnetic bead onto the magnetic stirrer.

The buffer solution was prepared by mixing the Tris and Citric acid in required appropriate proportions. Sterilization of buffer was carried out by autoclaving at 121°C and 15 lbs pressure for 20 minutes. Penicillin-G sodium,

fructose and dihydro streptomycin were added in the buffer. Later on, glycerol @ 7% and egg yolk @ 20% was mixed to the buffer solution. Finally EYTG extender is prepared.

Composition of Egg Yolk Tris Glycerol (EYTG)

S. No.	Ingredients	Quantity
1.	Tris buffer (hydroxymethyl) aminomethane	3.028g
2.	Citric acid monohydrate	1.675g
3.	D-(-)-Fructose	1.250 g
4.	Penicillin -G Sodium	1 lakh unit (50mg)
5.	Dihydro Streptomycin sulphate	100mg
6.	Glycerol	7.00 ml
7.	Egg yolk	20 ml
8.	TDW(Triple Distilled Water)	Upto100 ml

3.3.2 Dilution of semen

Dilution rate was decided based on the percent individual motility rate and sperm concentration. Following collection, the semen was kept at 37^0C in the water bath and evaluated. The neat semen having mass motility of $\geq$ 3.0 (0-5 scale) was selected and subjected to further dilution with EYTG extender. Dilution was such that concentration of diluted semen becomes approximately 80 million sperms per ml.

3.3.3 Preparation and incorporation of lycopene as semen additive

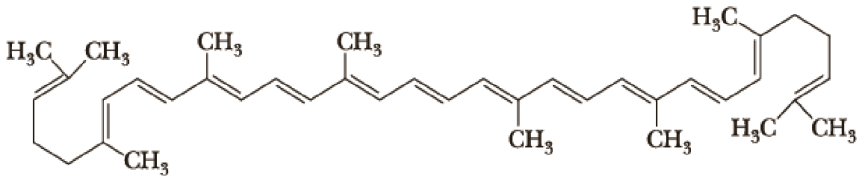
Lycopene and other chemicals used in this study were obtained from Sigma Chemicals (Sigma, St Louis, USA). Lycopene used as semen additive in this study was added at different concentration levels in milli Mole (mM) which was calculated using following formula:

$$\text{Molarity (M)} = \frac{\text{Weight of solute (mg)}}{\text{Volume of solution (ml) x Molecular weight}} \times 1000$$

$$\text{Weight of solute (mg)} = \frac{\text{Molarity x Volume of solution (ml) x Molecular weight}}{1000}$$

Lycopene

Brand	:	Sigma-Aldrich
Empirical Formula	:	$C_{40}H_{56}$
Synonym	:	ψ,ψ-Carotene, 2,6,10,14,19,23,27,31-Octamethyl-dotriaconta-2, 6, 8,10,12,14,16,18,20,22,24,26,30-tridecaene
Molecular weight	:	536.87
Product Number	:	PHR1770
Storage temperature	:	2 - 8 °C
CAS Number	:	502-65-8
Molecular Structure	:	



For the present study a stock solution of 50mM of lycopene was prepared by dissolving 6.7mg of lycopene in 250 µl of DMSO.

The extended semen was dividedin four different test tubes in equal volume (5 ml each). To the first test tube was added 100µl of DMSO (Control) where as in second, third and fourth test tube, lycopene stock (50mM) was added @ of 25µl, 50µl and 100µl making the effective concentration of lycopene as 0.25mM,0.5mM and1mM. To balance the amount of DMSO in treatment groups, additionally 75µl and 50µl DMSO was added in second and third test tubes. Thus, making the amount of DMSO same in control and treatment groups. These diluted samples were than utilized for further processing. The various groups so formed after addition of lycopene are described as under:

Group I : Control (EYTG) + DMSO

Group II : Treatment-1 (EYTG + Lycopene @ 0.25mM)

Group III : Treatment-2 (EYTG + Lycopene @ 0.5mM)

Group IV : Treatment-3 (EYTG + Lycopene @ 1mM)

3.3.4 Printing of straws

After final dilution of semen sample different coloured straws were printed by computerized auto printing through Domino Ink Jet Printer A200 Pinpoint (Domino UK Ltd, Bar Hill, Cambridge, CB3 8TU, England). Printing Performa includes bull no., breed, extender, institutional code and date.

Pink: straws for standard EYTG diluents + DMSO as Control

White: straws for standard EYTG diluent + Lycopene @ 0.25mM as Treatment-1

Green: straws for standard EYTG diluent + Lycopene @ 0.5mM as Treatment-2

Blue: straws for standard EYTG diluent + Lycopene @ 1mM as Treatment-3

3.3.5 Filling and sealing of straws

Diluted semen was aspirated into French Top Bull mini straws (0.25 ml, 135mm length and 2 mm diameter, IMV) of different colours and sealed by automatic straw filling and sealing machine (IMV, France).

3.3.6 Equilibration time

The above filled and sealed straws were kept on freezing racks and placed in cold handling cabinet, which was already maintained at 4°C for 4 hours. After completion of equilibration time, each group were evaluate for the parameters viz. progressive motility, spermatozoa live percentage, sperm motility and kinematics (CASA),HOST response, acrosomal integrity and CTC assay.

3.3.7 Freezing

After completion of equilibration time, the semen filled straws along with freezing racks were kept in liquid nitrogen vapour freezing chamber of biological freezer, where the temperature of semen filled straws reaches from 4^{o}C to -140^{o}C in different temperature gradients within 7 minutes as shown below

Temperature (^{o}C)	Rate of freezing (^{o}C/min)
4 to -10	5
-10 to -100	40
-100 to -140	20

3.3.8 Storage

The semen filled straws were then transferred into goblets with the help of cryo-gloved hand and the goblets were stored in separate canisters in the liquid nitrogen (LN_2) in cryovessel. Cryovessel was always kept full by replenishing liquid nitrogen from time to time.

3.3.9 Thawing of frozen semen

The frozen semen was then thawed, after the storage period of at least 24 hours of freezing, in thawing unit (IMV, France) maintained at 37^0C for45 seconds. The thawed semen samples were subjected to different tests i.e. progressive motility, spermatozoa live percentage, sperm kinematics (CASA), HOST response, acrosomal integrity, CTC Assay, Bovine Cervical Mucus Penitration Test (BCMPT) and antioxidative enzymes (SOD, GST, LPO [MDA]).

3.4 Evaluation of Parameters in Pre-freeze and Post-thaw Stages

3.4.1 Progressive Motility: As per the method stated vide-supra.

3.4.2 Live Spermatozoa: As per the method stated vide-supra.

3.4.3 Hypo-Osmotic Swelling Test (HOST)

The hypo-osmotic swelling test was performed according to the method described by Jayendran et al. (1984) at pre-freeze and post-thaw stage for assessing the functional integrity of the sperm tail membrane. Hypo-osmotic solution of 150 mOsmol/litre was prepared as shown below

Ingredients	Quantity
Sodium citrate	0.735 g
Fructose	1.351 g
Millipore/ double distilled water	upto100 ml

3.4.3.1 Procedure

0.9 ml HOST solution (maintained at 37^0C for 5 min before use) was added to 0.1 ml semen and incubated at 37°C for one hour. Following incubation, a drop (10 µl) of eosin stain was added to the solution to provide background which facilitates the counting. Now a drop of well mixed solution was taken on a clean and dry glass slide and covered with a cover-slip. Sperm tail curling was recorded as an effect of swelling due to influx of water. Sperms with coiled tail were considered as membrane intact sperms and those with straight tail as membrane damaged sperms. A minimum of 200 spermatozoa were counted in different fields (at least 5 different fields) of a given slide on 40x objective under phase contrast microscope for different types of swelling patterns. The total proportion of swollen spermatozoa with coiled tail was calculated by dividing number of reacted cells by total spermatozoa counted in same area and multiplying figure by 100. These spermatozoa were classified in four different classes according to presence of following swelling patterns.

A. No swelling, no membrane reaction

B. Swelling of the tip of the tail

C. Different types of hair pin like swelling or swelling of mid-piece

D. Complete tail coiling

Spermatozoa showing B, C, D type of pattern were considered to be HOST positive (Photograph 2).

$$\text{HOST Reacted Spermatozoa (\%)} = \frac{\text{Number of curled tail spermatozoa}}{\text{Total number of spermatozoa counted}} \times 100$$

3.4.4 Acrosomal Integrity

Method described by Watson (1975) was followed to assess the quality of semen doses which in terms validates the fertilizing capacity and conception rate. Staining was carried out as described by Hancock (1952).

3.4.4.1 Composition of Giemsa stain

Ingredients	Quantity
Giemsa stain	3.8 g
Absolute alcohol (GR grade)	375 ml
Glycerol (AR grade)	125 ml

3.4.4.2 Preparation of Giemsa stain

Giemsa stain was grounded with absolute methanol in pestle and mortar and glycerol was added. Stain mixture was stored at 37^0 C for one week. During this storage period, it was shaken for two minutes each day. After 7 days the stain was filtered in Whatman's filter paper No. 40. The solution can be used for 45 days.

Sorenson's phosphate buffer

It was prepared by mixing 61.2 ml of solution A and 58.8 ml of solution B as per following composition to obtain a pH of 7.0.

Solution A

Ingredients	Quantity
Sodium phosphate dibasic ($Na_2HPO_4.2H_2O$)	11.876 g
Distilled water	1000 ml

Solution B

Ingredients	Quantity
Potassium phosphate monobasic ($KH_2PO_4.2H_2O$)	9.08 g
Distilled water	1000 ml

Fixative

Five percent (5%) formal saline solution was used for fixation of smear.

Preparation of final stain solution

Three milliliter (3.0 ml) of Giemsa stain was diluted with 2.0 ml Sorenson's phospate buffer (pH = 7.0). To this was added distilled water 35.0 ml. The staining solution was stored in refrigerator. Fresh stain was prepared every week.

Staining procedure

A thin smear of diluted semen was prepared on a clean, grease free and dry slide. Smear was air-dried at room temperature for at least 10 minutes. Smear was fixed by immersion in 5 percent formal saline (Campbell et al., 1956) for 30 minutes at 37^0C. Then it was washed in running tap water and air dried for 15-20 minutes. In a staining coplin jar, 2ml Sorenson phosphate buffer, 3ml Giemsa stain and 35 ml double distilled water were allowed to mixed thoroughly. Now the slide wasimmersed in the coplin jar solution for 4 hours in incubator maintained at 37^oC. Stained slides were washed in running tap water and air dried. The dried smears were studied at 100x oil immersion objectives. Atleast 200 spermatozoa were counted for acrosomal status after staining. Stained acrosome appears dark purple color. The acrosome was considered to be normal when the stain was clearly and evenly distributed over the spermatozoa anterior to the equatorial segment (Photograph 3).

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

3.4.5 Evaluation of capacitation status by Chlortetracycline (CTC) assay

Principle

The technique is based on the principle that Chlortetracycline (CTC) binds with membrane calcium, whose distribution appears to change through capacitation, and is visualized by fluorescence microscopy. Thus CTC distribution pattern seems to be related to the capacitation stage as well as status.

Capacitation status and acrosome reaction were assessed using Chlortetracycline (CTC) staining as described by Rathi et al. (2001) with little modification. Briefly sperm at a concentration of (1×10^6 /ml) were suspended in TALP and were centrifuged at 1000rpm for 5 min. The collected sperm pellet was washed 3 times with TALP and final pellet of sperm was resuspended in 0.5 ml of

TALP to serve as the sperm stock solution. CTC stock solution was prepared by dissolving 1 mg of CTC in 1 ml of PBS (pH = 7.4). 250 µl of sperm suspension was mixed with 250 µl of CTC stock solution and was incubated in the incubator in dark at 37^{o}C for 30 minutes. 5 µl of CTC labelled sperm suspension was taken on a clean grease free glass slide and then uniform thin smear was made. Smear was allowed for drying in dark. Slides were observed using Nikon Eclipse TE 2000-S microscope with phase contrast and epifluorescence optics under blue-violet illumination (excitation at 400 - 440 nm and emission at 470 nm by using 40x objective). A total of 200 sperms per slide were observed and different patterns of sperm were evaluated as established in literature. Three different forms of CTC pattern were observed namely,

a. F pattern: Even distribution of fluorescence over the entire head (uncapacitated sperm).

b. B pattern: Fluorescence-free band in the post-acrosomal region (capacitated, acrosome inact sperm), fluorescence in anterior portion of the head.

c. AR pattern: Fluorescence free head except for a thin bright fluorescent band along the equatorial segment (acrosome-reacted cells).

In all cases, fluorescence in the middle piece of the flagellum was observed as well (Photograph 4).

3.4.6 Sperm Motility and Kinematics

These parameters were assessed using Computer Assisted Semen Analyser (CASA), Minitube, Tifenberg, Germany which is supported with AndroVision® software (Version 6.1, Photograph 5). The CASA is equipped with ZeisAxioscope Microscope, Germany with 20x negative phase contrast with blue filter for determination of sperm motility and kinetics. The microscope is equipped with thermostatic stage to maintain a constant temperature of 37 ºC. For motility and kinematics, at least 10 fields were counted having a minimum of 2000 spermatozoa. The inbuilt software of CASA produces two reports; first report is an average of all sperm cells analyzed along with different motion and kinetics and second report is a detailed report of each and individual spermatozoa. The detailed report shows every parameter of spermatozoa along with tracking and sorting.

The CASA is equipped to measure following motion and kinematic parameters of spermatozoa (Photograph 6).

Different motion and kinematic parameters measured by CASA, Androvision, Minitube, Germany

Name of the parameter	Abbreviation	Unit
Total motility	TM	%
Progressive motility	PM	%
Progressive fast motility	PFM	%
Progressive slow motility	PSM	%
Progressive circular motility	PCM	%
Local circular motility	LCM	%
Local motile	LM	%
Immotile	IM	%
Curve linear velocity	VCL	µm/s
Straight line velocity	VSL	µm/s
Average path velocity	VAP	µm/s
Curve linear distance	DCL	µm
Straight line distance	DSL	µm
Average path distance	DAP	µm
Amplitude of lateral head displacement	ALH	µm
Beat cross frequency	BCF	Hz
Linearity	LIN (VSL/VCL)	Unit less
Wobble	WOB (VAP/VCL)	Unit less
Straightness	STR (VSL/VAP)	Unit less

The CASA records following parameters with its setting outlined below.

- Spermatozoa were classified as immotile when **HAC < 0.087**
- Spermatozoa were classified as local motile when **VCL < 48 and VSL < 24**
- Spermatozoa were classified as circular motile when **Radius > 9 and < 90 and Rotation is > 0.70**
- Spermatozoa were classified as slow motile when **VCL < 120**

- Spermatozoa were classified as fast motile when **VCL > 120**
- Spermatozoa are hypomotile when **VCL > 150**
- Spermatozoa are hypermotile when **LIN < 0.5 or 50%**
- Spermatozoa are hypermotile when **ALH > 3**

3.4.7 Bovine Cervical Mucus Penetration Test (BCMPT)

The bovine cervical mucus penetration test was performed as per the method described by Kremer (1965) and Matousek et al. (1989).

Collection of cervical mucus

Cervical mucus was collected from estrus cattle as per the method described by Dabas and Maurya (1988) under asepticconditions.

Evaluation and storage of mucus sample

The collected mucus was selected on the basis of elasticity and the degree of crystallization. Mucus samples showing typical fern pattern were selected for experiment. White side test (Popov, 1969; Pateria and Rawal, 1990) using 5% sodium hydroxide was performed to evaluate the mucus sample for any infection. One ml of mucus was taken in a test tube along with one ml of 5% sodium hydroxide. This was heated up to boiling point and colour of the mixture was observed. Samples developing a yellow colour were considered positive for mild endometritis and discarded. Only clear samples were selected for conducting the experiment.

After evaluation, suitable samples were stored in 2 ml storage vials which were kept at -20°C.

Preparation of the test system

A loading manifold was prepared for loading the capillary tubes with cervical mucus. The loading manifold was prepared from a scalp vein set and a five ml syringe. The needle from one end of the scalp vein set was replaced by a capillary tube and the other end was attached to a five ml plastic disposable syringe. The stored cervical mucus was emptied into a petri dish and was warmed to room temperature. The warmed cervical mucus was then aspirated into the capillary tube by drawing plunger of the syringe. The trailing mucus was cut with scissors leaving a small amount protruding from the filled end of the tube. Capillary tube was then removed

from the loading manifold and one end of the capillary tube was plugged using "Haemoseal". The tubes were then allowed to stand for 5 minutes at 37°C. Only non-heparinized hematocrit capillary tubes were used for the test.

Procedure

Collection of cervical mucus from the cattle in estrus after disinfecting the perineal region with 70% alcohol

⇩

Observation of mucus for typical fern pattern under microscope

⇩

White side test

⇩

Loading of hematocrit non-heparinized round capillary tube with cervical mucus

⇩

Sealing of one end of the capillary tube with Haemoseal

⇩

Placement of open end into the sperm reservoir

⇩

Incubation of test system at 37 °C for 60 min

⇩

Removal, wiping and cleaning of capillary tubes

⇩

Placement of capillary tubes on graduated glass slide under microscope

⇩

Observation of distance travelled by the vanguard spermatozoa - high power microscope

Grading of semen sample

Grading of the semen samples was carried out according to Matousek et al. (1989) as mentioned below:

Grade	Distance travelled
Excellent	more than 30 mm
Good	between 20mm to 30 mm
Medium	between 12 to 20 mm
Poor	between 8 to 12 mm

Sperm penetration distance: sperm penetration distance is the distance travelled in millimeters by the most progressive or vanguard spermatozoa after 60 minutes of incubation (Photograph 7).

3.4.8 Estimation of Enzymes in Seminal Plasma

3.4.8.1 Estimation of Superoxide dismutase (SOD) activity (U/ml)

Seminal plasma superoxide dismutase (SOD) activity was measured using the method as described by Madesh and Balasubramanian (1997) with some modifications.

It involves generation of superoxide by pyrogallol antioxidation and the inhibition of superoxide-dependent reduction of the tetrazolidium dye MTT to its formazan was measured at 570 nm. The reaction was terminated by the addition of dimethyl sulfoxide (DMSO), which helps to solubilize the formazan formed and the color evolved is stable for many hours.

Pyrogallol used as source of superoxide based on the inhibition of MTT formazan formation from the reaction of MTT and superoxide. MTT is a tetrazolidium compound which can be reduced to its coloured formazan by superoxide.

Procedure

The total volume of mixture 3000 µl consisted of 1200 µl PBS, 100 µl seminal plasma, 50 µl of 1.25 mM MTT (1.035 mg/ml) and 150 µl of 1 mM pyrogallol solution (0.126 mg/ml) was added at the end. Sample was replaced with PBS in the

blank. After incubation period of 15 minutes 1500 µl DMSO was added and absorbance was taken in ELISA reader at 570 nm. The percent inhibition by the presence of SOD was calculated from the reduction of the MTT color formation as compared to the MTT formazan formed in the absence of SOD which was taken as 100%. One unit of SOD was defined as the amount of protein required to inhibit the MTT reduction by 50%.

SOD activity was calculated using the following formula

SOD activity (units/ml) = 2 x 100 x A_T/A_B x dilution factor

A_T = absorbance test

A_B = absorbance blank

3.4.8.2 Estimation of Glutathione-S-Transferase (GST) activity (nM/min/ml)

Glutathione -S- transferase was estimated as described by Habig et al. (1974) with some modifications.

Reagents

1) 0.3 M Potassium phosphate buffer (pH 6.5)

a. Potassium dihydrogen orthophosphate (40.84mg/ml distilled water)

b. Dipotassium hydrogen phosphate (52.26mg/ml distilled water)

2) 30 mM reduced glutathione (GSH) (9.21mg/ml distilled water)

3) 30 mM CDNB in 95% ethyl alcohol (6.076 mg/ml alcohol)

Procedure

To 1 ml of phosphate buffer in a test tube, 0.1 ml of CDNB and 0.1 ml seminal plasma/ distilled water was added. 1.7 ml of distilled water was added to make final volume of 2.9 ml.

The reaction mixture was incubated at 37^{o}C for 5 min and reaction was started by adding 0.1 ml of 30 mM reduced glutathione. The absorbance was measured at 60 sec interval for 5 minutes at 340 nm. Reaction mixture without seminal plasma was used as blank.

Calculation

$$\text{GST activity} = \frac{\text{OD of sample}}{\text{EC}} \times \frac{\text{Total volume}}{\text{Volume of seminal plasma}} \times 10^6 \text{ x DF} \times \frac{1}{\text{Time}}$$

Where,

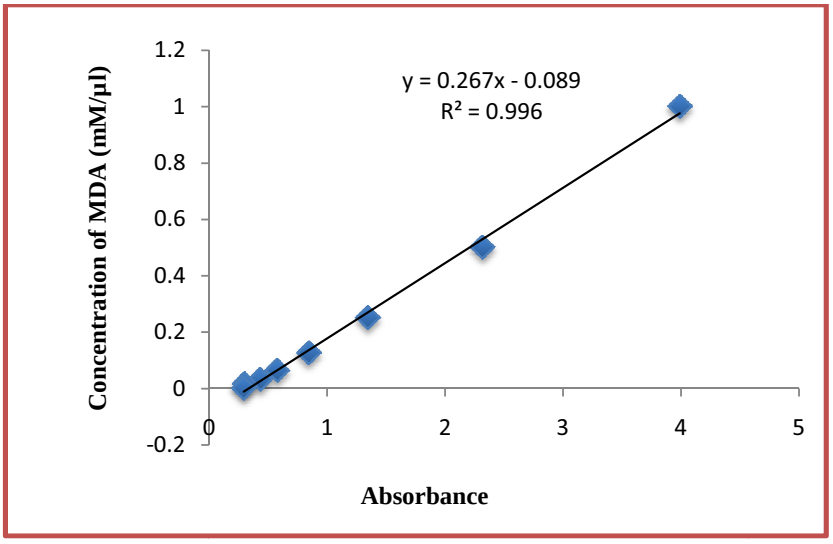
EC = 9600/M/cm

DF = dilution factor

3.4.8.3 Estimation of Lipid peroxidation (MDA) activity (nM/µl)

The MDA assay was done by the TBARS (Thio barbituric acid reactive substance) method of Ohkawa et al. (1979). Thio Barbituric Acid (TBA) forms colored adduct which are quantified at 532 nm.

To 100 µl of seminal plasma was mixed with 1.5 ml of 20% acetic acid (pH3.5), subsequently 500 µl of 8.1% sodium dodecyl sulphate (SDS), 1.5 ml of 0.8% TBA and 900 µl phosphate buffer (pH 7.4) was added and the mixture was vortexed. The reaction mixture was incubated in a boiling water bath for 1 hour. After cooling at room temperature, 5 ml of butanol: pyrimidine mixture (15:1) was added and this reaction mixture was centrifuged at 5000 rpm for 20 min. A clear supernatant obtained after centrifugation was used for measuring the absorbance at 532 nm against reagent blank. A series of known concentration for standard of malondialdehyde (1 nM/ µl) were also run simultaneously and a standard curve was plotted. Against the standard curve, the concentration of MDA was determined.



Standard Curve of MDA

3.5 Statistical analysis

Data were analyzed using SPSS software (version 20). Descriptive statistical characteristics (mean, standard error) were evaluated at first. One-way ANOVA was used for specific statistical evaluations. Means with different small letter superscripts differed significantly within a row except for neat semen (within a column). Figures in parenthesis indicate range.

TECHNICAL PROGRAMME

(Flow Chart)

Ejaculated semen from Hariana bulls (4)

(n=8x4=32)

⇩

Evaluation of Semen

(Colour, Viscosity, Volume, Concentration, Mass motility, PM, Live spermatozoa)

⇩

Extension of fresh semen (Temp 32-34 °C)

Group I	Group II	Group III	Group IV
EYTG + semen + DMSO	EYTG +@ 0.25mM/L lycopene + Semen	EYTG +@ 0.5mM/L lycopene + Semen	EYTG + @ 1.0mM/L lycopene + Semen

⇩

Each sample diluted to

$80x10^6$ cells/ml in Tris-egg yolk-glycerol.

⇩

Samples filled in labeled straws

(0.25ml straw, using filling & sealing machine)

⇩

Cooled samples kept for equilibration (4^oC for 4 h)

⇩

Pre-freeze evaluation of semen

Parameters for evaluation

- Progressive motility (Salisbury et al.,1985)
- Sperm kinematics (CASA, Androvision, Minitube, Germany)
  - Total Motility (%)
  - PM (%)
  - Average path velocity (µm/s)
  - Linear velocity (VSL, µm/s)
  - Curvilinear motion (VCL, µm/s)

- Beat cross frequency (BCF, Hz)
- Path straightness (STR, %)
- Linearity (LIN, %)

- % Live spermatozoa (Campbell et al.,1953)
- Acrosomal integrity (Watson, 1975)
- Hypo osmotic swelling test (HOST). (Jayendran et al.,1984)
- CTC Assay (Rathi et al., 2001)

Transfer in programmable biological freezer

⇩

LN_2vapors freezing

(Freezing rate: 4 to -10^{o}C @ 5^{o}C/min, -10 to -100@ 40^{o}C/min and -100 to -140@ 20^{o}C/min)

⇩

After completion of freezing, straw collected & plunged in LN_2

⇩

After 48 hr of LN_2 preservation

Sample thawed (37^{0}C for 45sec)

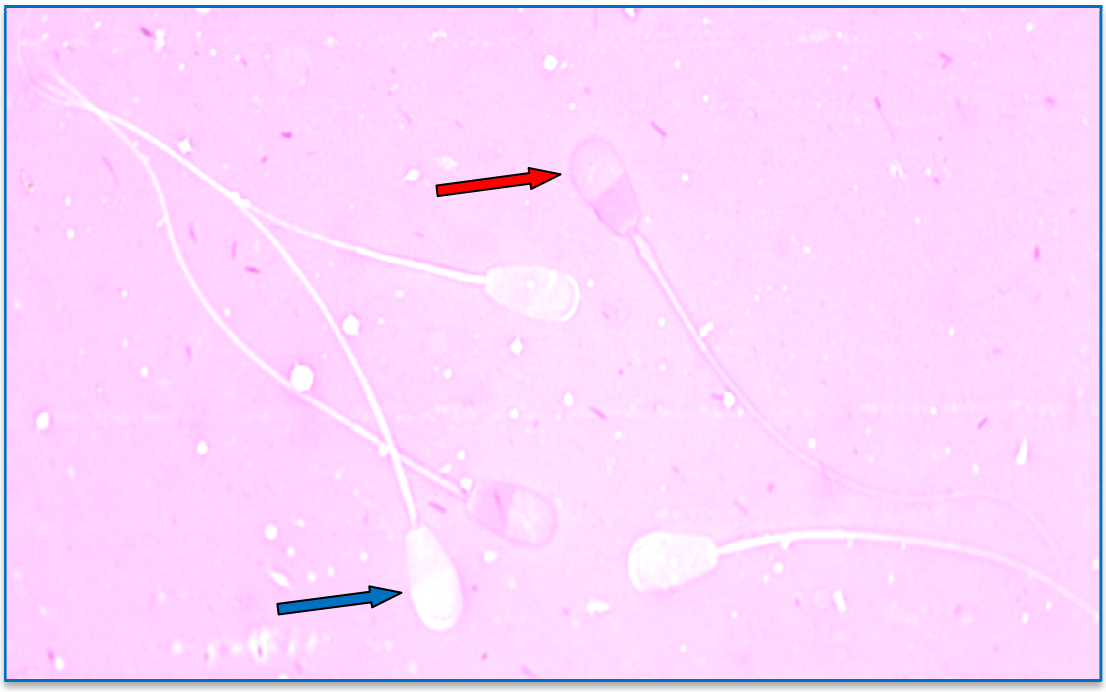
⇩

Post thaw evaluation of semen

Parameters for evaluation

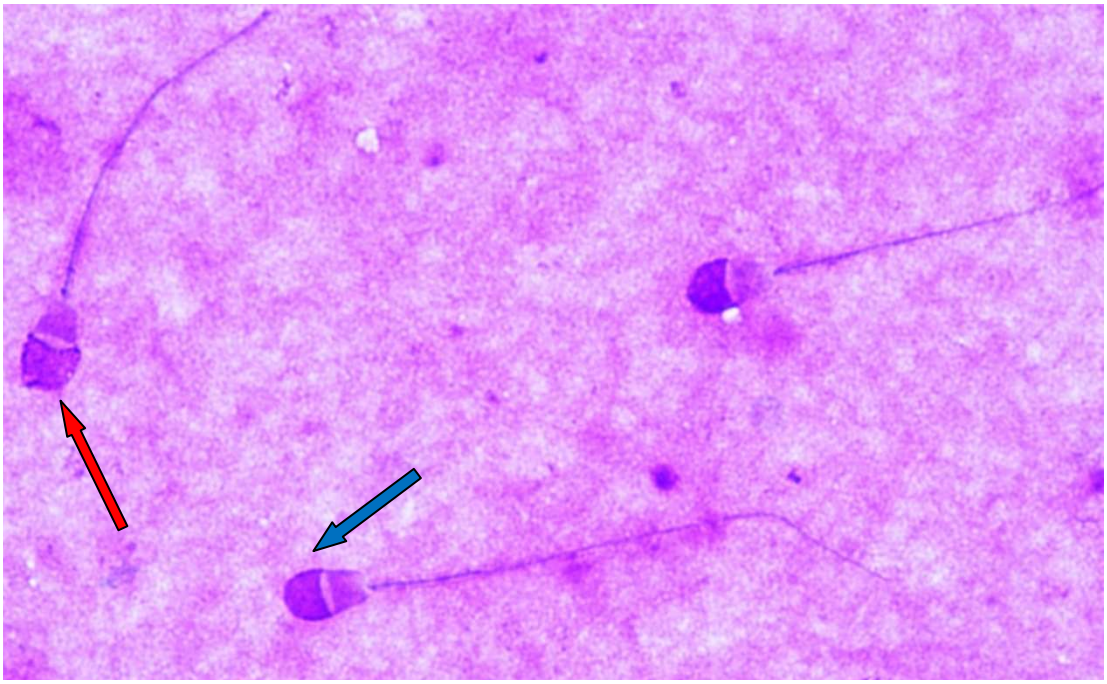
- Progressive motility (Salisbury et al.,1985)
- Sperm kinematics (CASA, Androvision, Minitube, Germany)
  - Total Motility (%)
  - PM (%)
  - Average path velocity (µm/s)
  - Linear velocity (VSL, µm/s)
  - Curvilinear motion (VCL, µm/s)
  - Beat cross frequency (BCF, Hz)
  - Path straightness (STR, %)
  - Linearity (LIN, %)

- % Live spermatozoa (Campbell et al.,1953)
- Acrosomal integrity (Watson, 1975)
- Hypo osmotic swelling test (HOST). (Jayendran et al.,1984)
- CTC Assay (Rathi et al., 2001)
- Oxidative enzymes
    - SOD (Madesh and Balasubramanian, 1997)
    - MDA (Ohkawa et al., 1979)
    - GST (Habig et al., 1974)

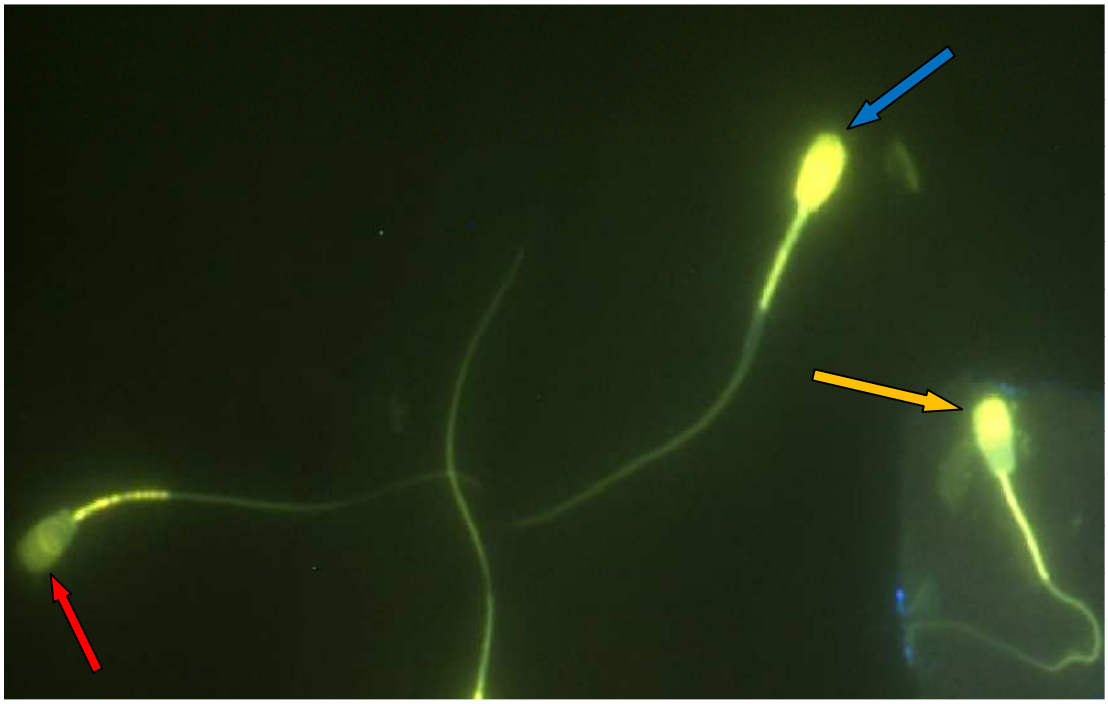


Photograph 1: Live and Dead spermatozoa (Eosin–Nigrosin stain, magnification: 100x, Blue arrow showing live spermatozoa, Red arrow showing dead spermatozoa)

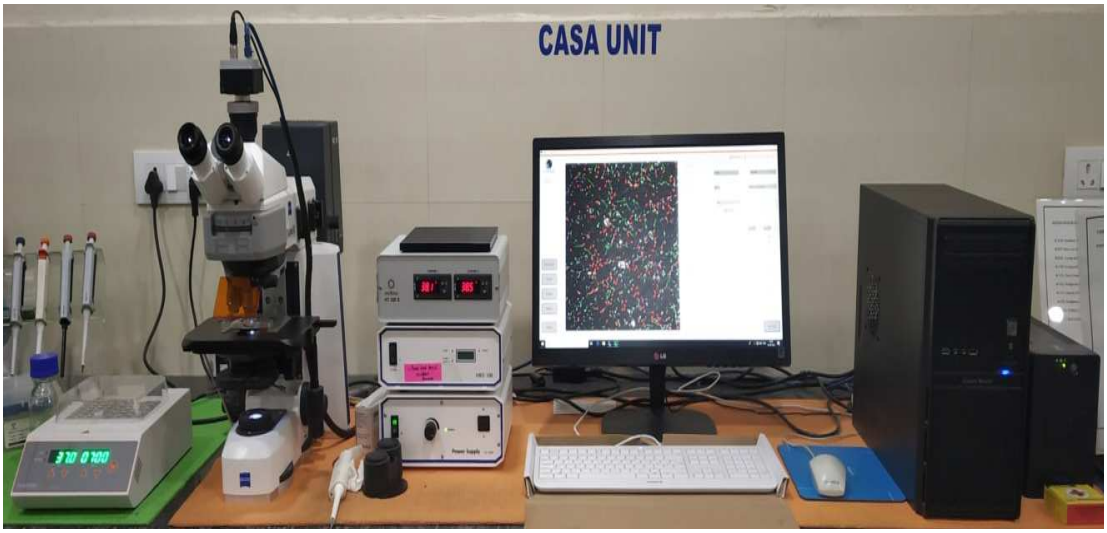
Photograph 2: Hypo osmotic swelling test (HOST) reactive and non-reactive spermatozoa (Hypo osmotic solution 150mOsmol/liter, magnification: 40x, Blue arrow showing various degrees of HOST reactive spermatozoa, Red arrow showing non-reactive spermatozoa)



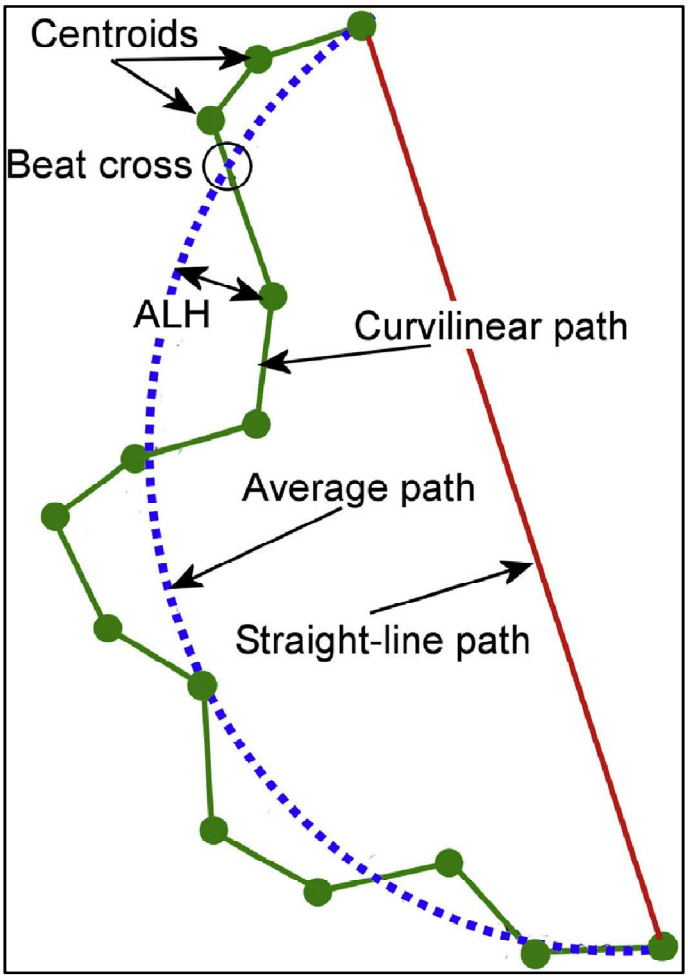
Photograph 3: Acrosomal integrity of spermatozoa (Giemsa staining, magnification: 100x, Blue arrow indicating intact acrosome, Red arrow indicating damaged acrosome)



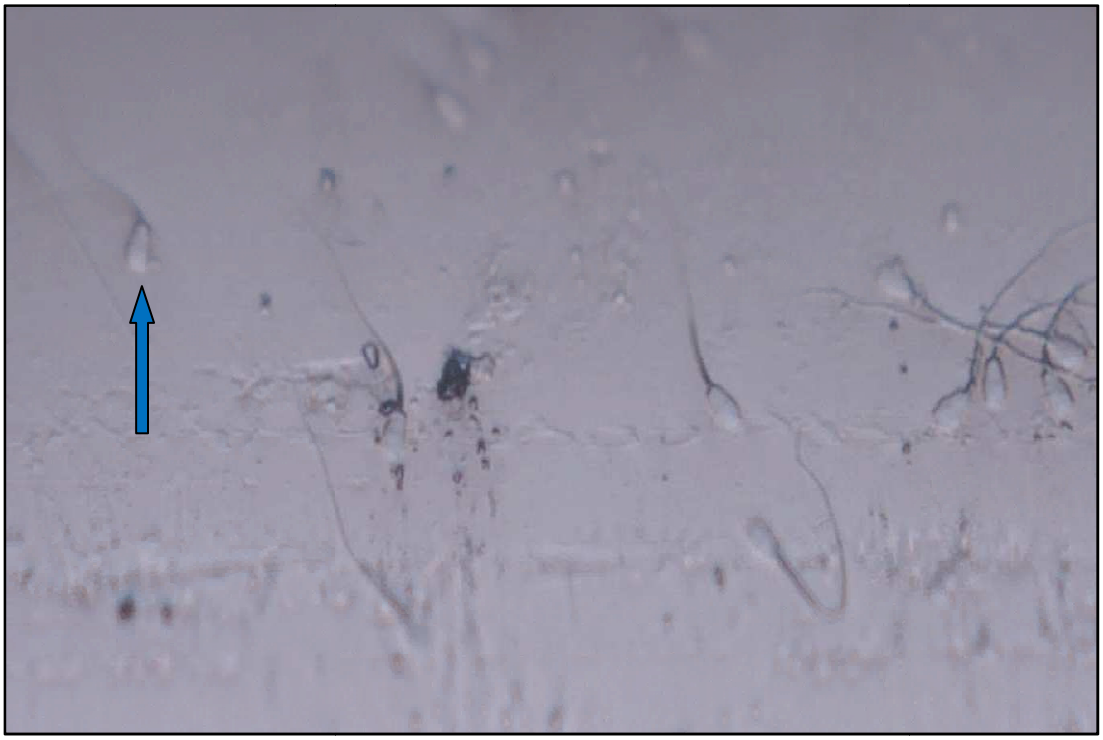
Photograph 4: Chlortetracycline (CTC) assay (Fluorescent microscope, magnification: 40x, Blue arrow showing "F"-pattern, uncapacitated and intact acrosome; Yellow arrow showing "B"-pattern, capacitated and intact acrosome; Red arrow showing "AR" pattern, capacitated and acrosome reacted)



Photograph 5: CASA unit (Minitube, Tifenberg, Germany which is supported with Andro Vision® software, Version 6.1)



Photograph 6: Path velocities and kinematics of spermatozoa for CASA study



Photograph 7: Vanguard spermatozoa (In capillary tube filled with cervical mucus, magnification: 40x, Blue arrow showing Vanguard spermatozoa)

Results

CHAPTER-4

RESULTS

In the present study, four Hariana bulls having their age between 5.0 to 9.0 years and weighing around 450 - 600 kg were used for the collection of semen. Semen was collected twice a week from each bull by means of artificial vagina and was evaluated for the various seminal attributes e.g. volume (ml), seminal pH, mass motility (0-5 scale), concentration (millions / ml), progressive motility, live percent. The ejaculate which appears to be apparently normal, having $\geq$ 3.0 mass motility and $\geq$ 70 % progressive motility were processed further for cryopreservation.

The extended semen was evaluated at different stages of cryopreservation (pre-freeze and post-thaw) for progressive motility, live spermatozoa, membrane integrity (HOST), spermatozoa with intact acrosome (Giemsa), capacitation status of spermatozoa, sperm motility kinematics, *in vitro* fertility test (BCMPT) and antioxidative enzymes (SOD, GST, LPO [MDA]). The entire data has been presented in Table 1.0 to 9.0 and illustrated in Figure 1.0 to 33.0 and described as follows

4.1 Seminal attributes in the freshly collected semen of Hariana bulls

The relevant data has been presented in Table 1.0 and illustrated in Figure 1.0 to 6.0.

1) Ejaculate volume (ml)

The mean semen volume of bull number H-395 was 4.28±0.33 ml. The respective values for bull number H-448, H-580 and H-623 were 4.75±0.20, 4.92±0.30 and 5.30±0.40 ml. The overall mean seminal volume irrespective of the bull was recorded as 4.81±0.16 ml. A significant ($P<0.05$) difference in the mean semen volume was found amongst the bulls, however, bull number H-623 had significantly ($P<0.05$) higher ejaculate volume compared to other bulls (Table 1.0 and Figure 1.0).

2) Seminal pH

The mean seminal pH of bull number H-395 was 6.54±0.04. The respective values for bull number H-448, H-580 and H-623 were 6.52±0.40, 6.61±0.05 and

6.65±0.05. The overall mean seminal pH in the fresh ejaculate of the Hariana bull was 6.58±0.02. The seminal pH did not differ significantly amongst different bulls (Table 1.0 and Figure 2.0).

3) Mass motility (0-5 scale)

The mean mass motility of bull number H-395 was 3.50±0.13. The respective values for bull number H-448, H-580 and H-623 were 4.16±0.19, 3.81±0.19 and 3.88±0.12. The overall mean mass motility of spermatozoa in the fresh ejaculate of Hariana bull was 3.84±0.08 (0-5 scale). A significant ($P<0.05$) difference in the mass motility was found amongst the bulls. Bull number H-448 had significantly ($P<0.05$) higher mass motility compared to other bulls (Table 1.0 and Figure 3.0).

4) Sperm concentration (millions/ml)

The mean sperm concentration of bull number H-395 was 1663.75±124.27 millions/ml. The respective values for bull number H-448, H-580 and H-623 were 1908.75±109.32, 1783.75±68.63 and 1778.75±100.45 millions/ml. The overall mean sperm concentration in the fresh ejaculate of Hariana bull was 1783.75±51.23 millions/ml. The sperm concentration did not differ significantly amongst different bulls (Table 1.0 and Figure 4.0).

5) Progressive motility (%)

The mean percentage of progressively motile spermatozoa in the semen of bull number H-395 was 83.75±1.57. The respective values for bull number H-448, H-580 and H-623 were 86.25±1.57, 82.50±0.94 and 84.38±1.13 per cent. The overall mean percentage of progressively motile spermatozoa in the fresh ejaculate of Hariana bull was 84.22±0.68 per cent. The progressive motility did not differ significantly amongst different bulls (Table 1.0 and Figure 5.0).

6) Live spermatozoa (%)

The mean percentage of live spermatozoa in the semen of bull number H-395 was 90.29±1.04. The respective values for bull number H-448, H-580 and H-623 were 90.96±1.00, 89.76±0.64 and 90.29±0.78. The overall mean percentage of live spermatozoa in the fresh ejaculate of Hariana bull was 90.32±0.42. The live percentage of spermatozoa did not differ significantly amongst different bulls (Table 1.0 and Figure 6.0).

Table 1.0: Seminal attributes in the freshly collected semen of Hariana bulls

(Mean±SE=8)

Bull No.	Semen Volume (ml)	Seminal pH	Sperm Mass Motility (0-5 scale)	Sperm Concentration ($x10^6$/ml)	Sperm Progressive Motility (%)	Live sperm (%)
H-395	4.28 ± 0.33^{b} (3.0 - 5.6)	6.54±0.04 (6.4 - 6.7)	3.50 ± 0.13^{b} (3.0 - 4.0)	1663.75±124.27 (1200.0 - 2220.0)	83.75±1.57 (80.0 - 90.0)	90.29±1.04 (86.3 - 94.0)
H-448	4.75 ± 0.20^{ab} (4.0 - 5.4)	6.52±0.40 (6.4 - 6.7)	4.16 ± 0.19^{a} (3.5 - 5.0)	1908.75±109.32 (1350.0 - 2310.0)	86.25±1.57 (80.0 - 90.0)	90.96±1.00 (86.7 - 94.0)
H-580	4.92 ± 0.30^{ab} (3.4 - 6.0)	6.61±0.05 (6.4 - 6.8)	3.81 ± 0.19^{ab} (3.0 - 4.5)	1783.75±68.63 (1470.0 - 2040.0)	82.50±0.94 (80.0 - 85.0)	89.76±0.64 (87.5 - 92.5)
H-623	5.30 ± 0.40^{a} (3.8 - 7.0)	6.65±0.05 (6.4 - 6.8)	3.88 ± 0.12^{ab} (3.5 - 4.5)	1778.75±100.45 (1230.0 - 2120.0)	84.38±1.13 (80.0 - 90.0)	90.29±0.78 (86.9 - 93.5)
Overall	4.81±0.16 (3.0 - 7.0)	6.58±0.02 (6.4 - 6.8)	3.84±0.08 (3.0 - 5.0)	1783.75±51.23 (1200.0 - 2310.0)	84.22±0.68 (80.0 - 90.0)	90.32±0.42 (86.3 - 94.0)
'F' Value	1.77^{NS}	1.91^{NS}	2.79^{*}	0.95^{NS}	1.38^{NS}	0.31^{NS}

Means with different small letters superscript differed significantly within a column

DMRT was used for comparing means

Figures in parenthesis indicate range

NS : Non-significant

* : Significant (P<0.05)

Table 2.0: Effect of different concentrations of lycopene on per cent progressively motile spermatozoa at pre-freeze and post-thaw stages of Hariana bull semen.

(Mean±SE=32)

Stage	Group I (Control)	Group II (0.25mM)	Group III (0.5mM)	Group IV (1mM)	'F' Value
Pre-freeze	69.84 ± 20.93^{b} (65.0 – 75.0)	72.97 ± 14.11^{a} (70.0 – 75.0)	49.84 ± 19.65^{c} (45.0 – 55.0)	39.53 ± 33.97^{d} (30.0 – 45.0)	484.04**
Post-thaw	43.44 ± 23.21^{b} (40.0 – 55.0)	50.16 ± 29.17^{a} (40.0 – 60.0)	32.97 ± 23.67^{c} (25.0 – 45.0)	23.6 ± 21.8^{d} (20.0 – 30.0)	230.32**

Means with different small letter superscripts differed significantly within a row

DMRT was used for comparing means

Figures in parenthesis indicate range

*: Significant (P<0.05)

** highly significant (P<0.01)

This pattern has been followed in subsequent tables

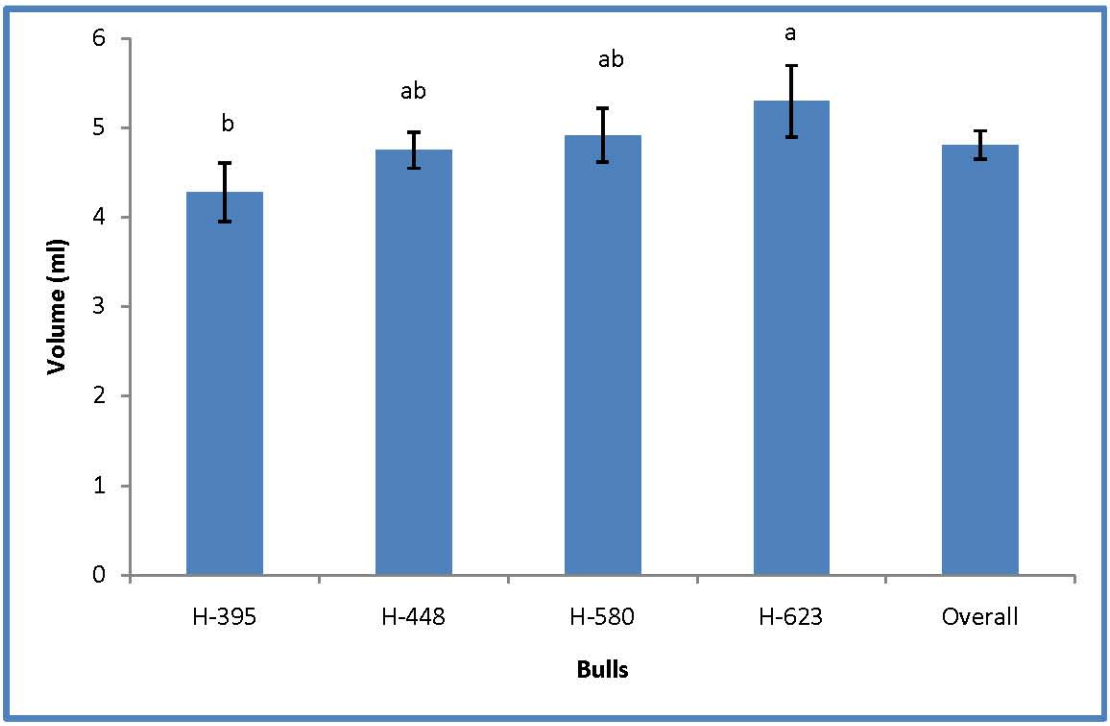


Figure 1.0: Graphical presentation of mean seminal volume (ml) in the freshly collected semen of Hariana bulls

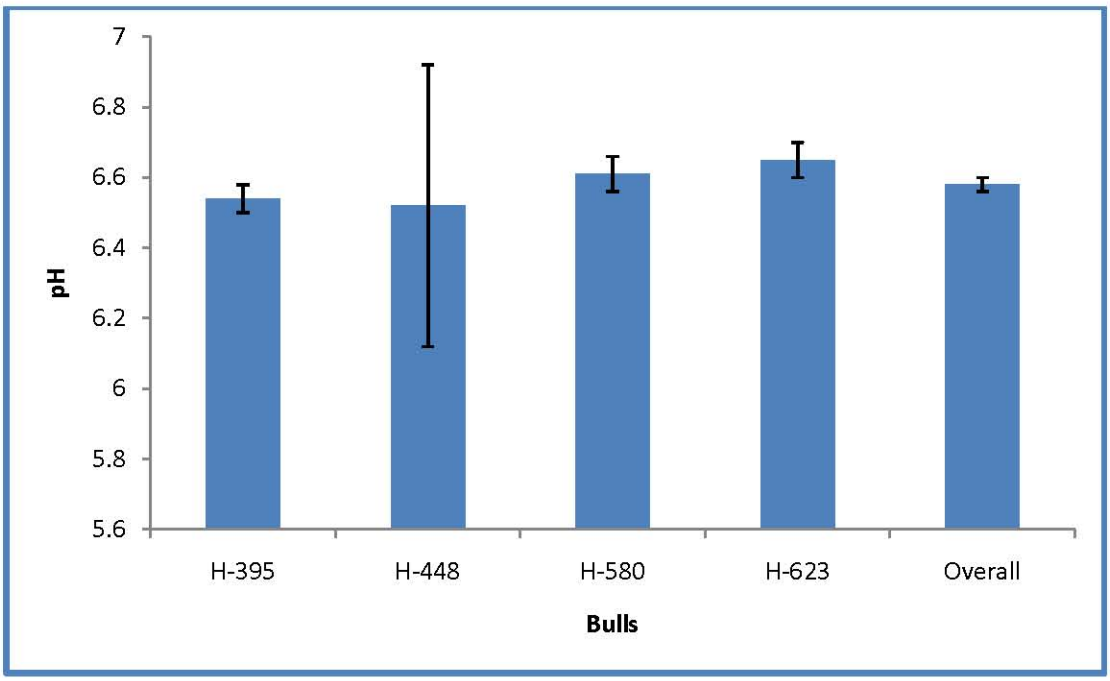


Figure 2.0: Graphical presentation of mean seminal pH in the freshly collected semen of Hariana bulls

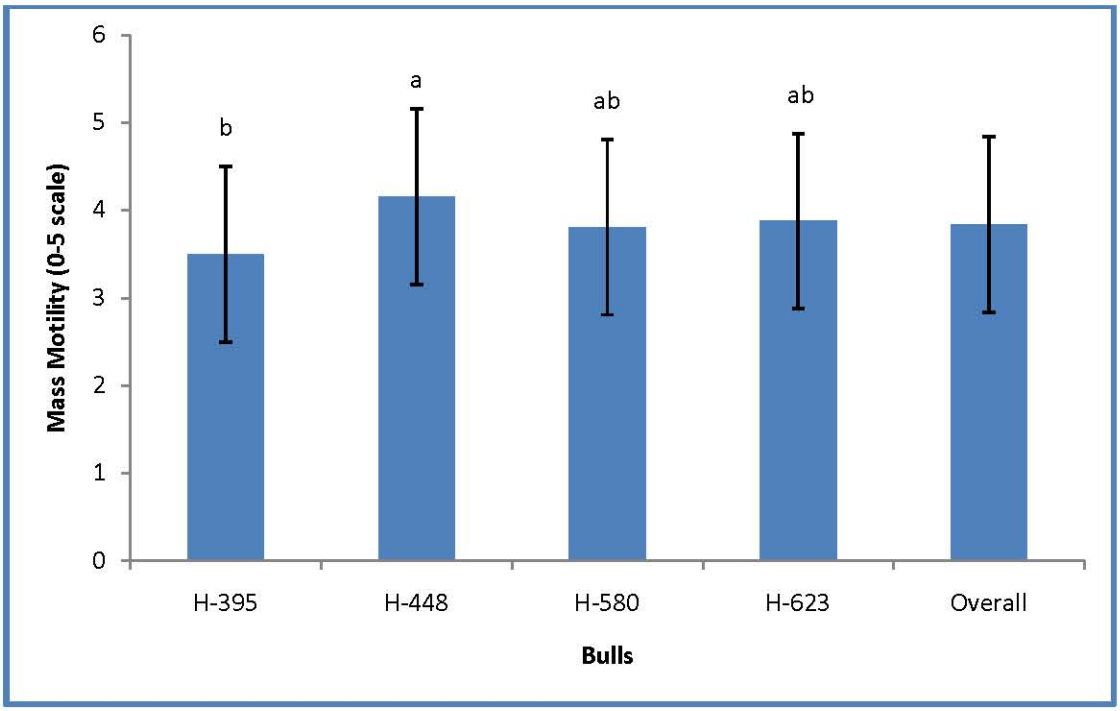


Figure 3.0: Graphical presentation of mean mass motility (0-5 scale) of Spermatozoa in the freshly collected semen of Hariana bulls

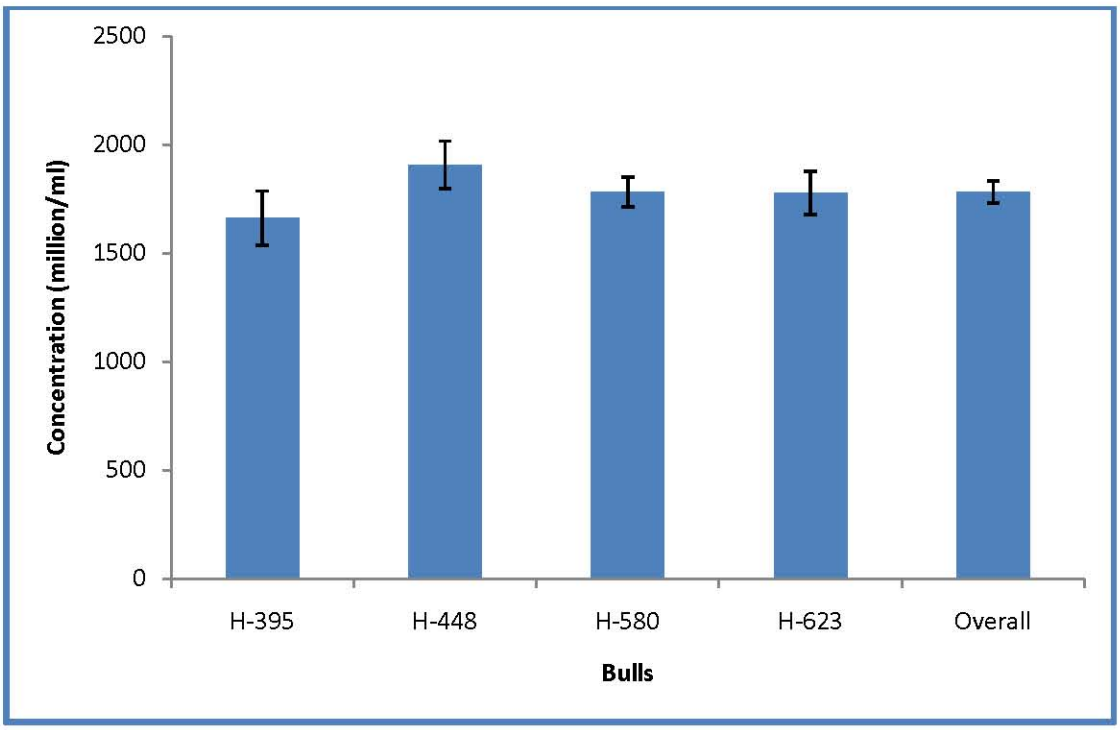


Figure 4.0: Graphical presentation of mean sperm concentration (millions/ml) in the freshly collected semen of Hariana bulls.

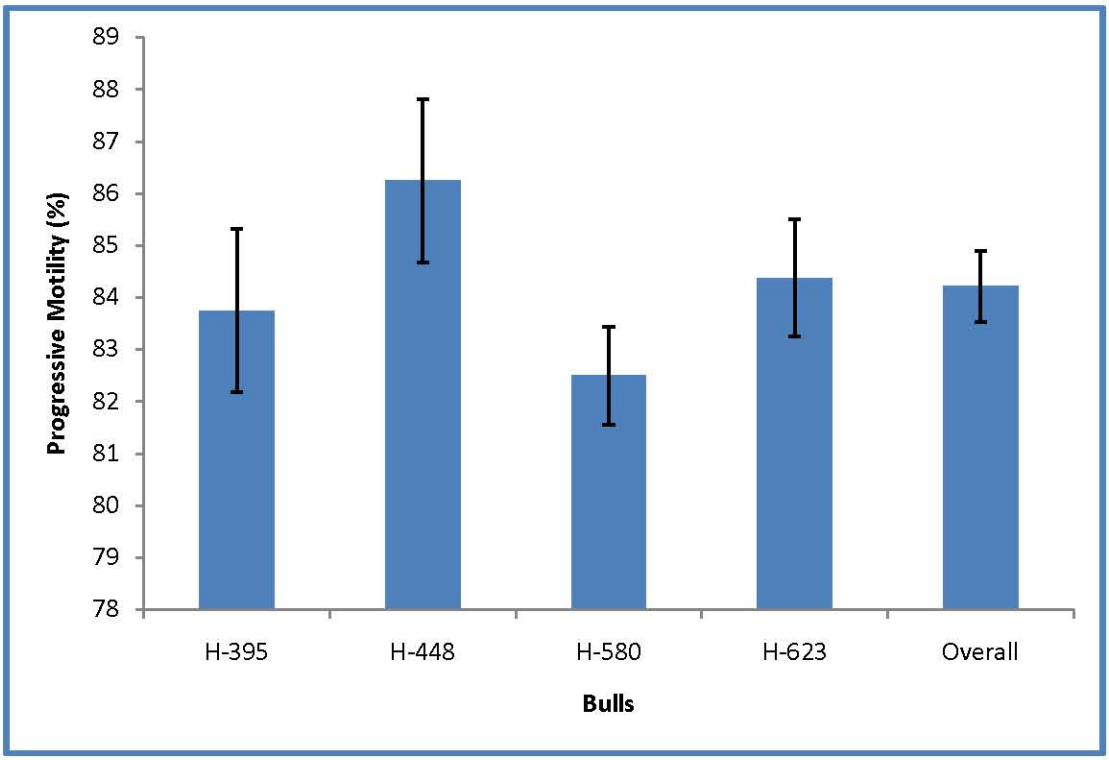


Figure 5.0: Graphical presentation of mean per cent progressive motility of spermatozoa in diluted semen of Hariana bulls

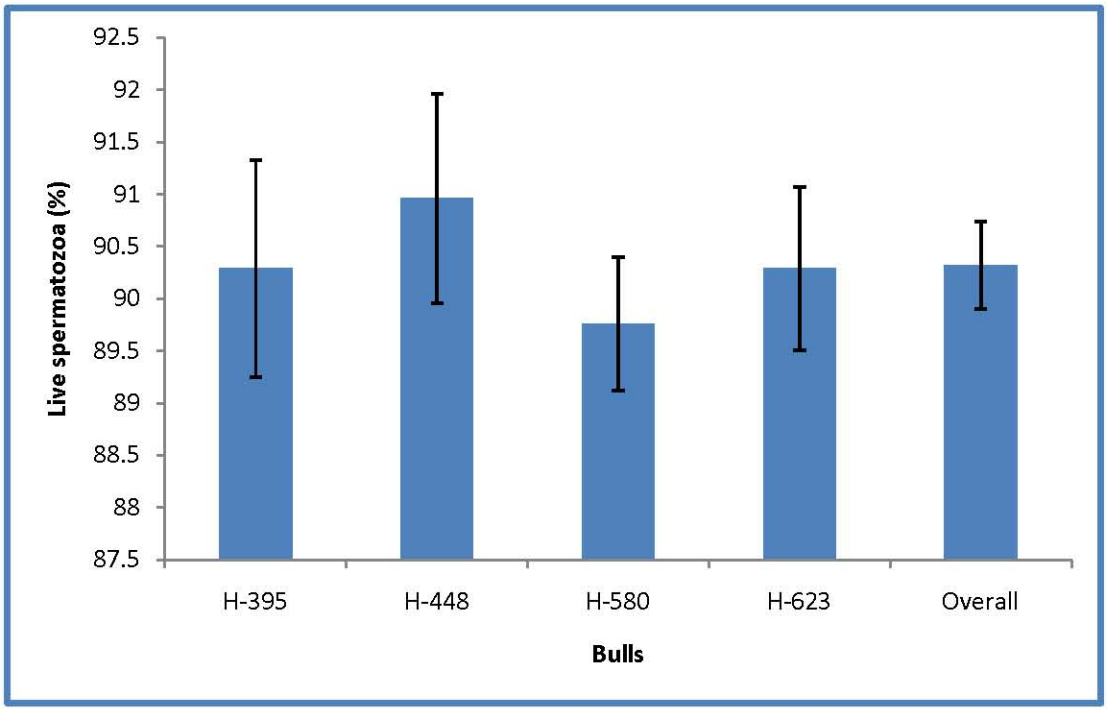


Figure 6.0: Graphical presentation of mean per cent live spermatozoa in the freshly collected semen of Hariana bulls

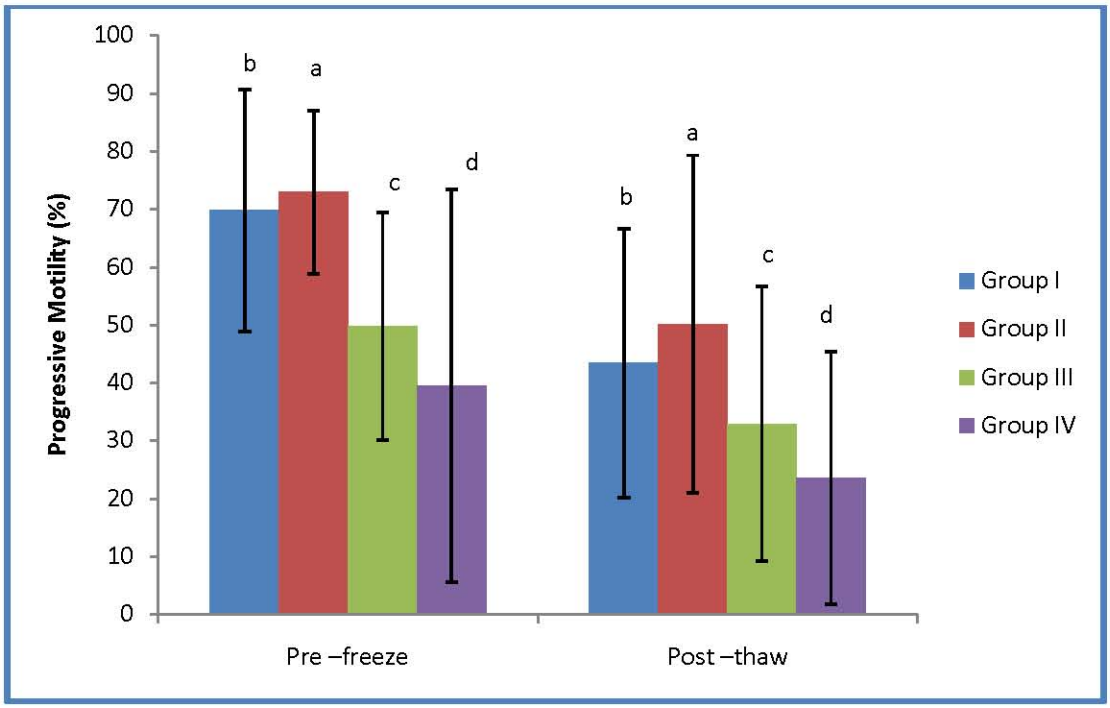


Figure 7.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5Mm and Group IV- 1mM) on per cent progressively motile spermatozoa of Hariana bull semen during freeze-thaw process

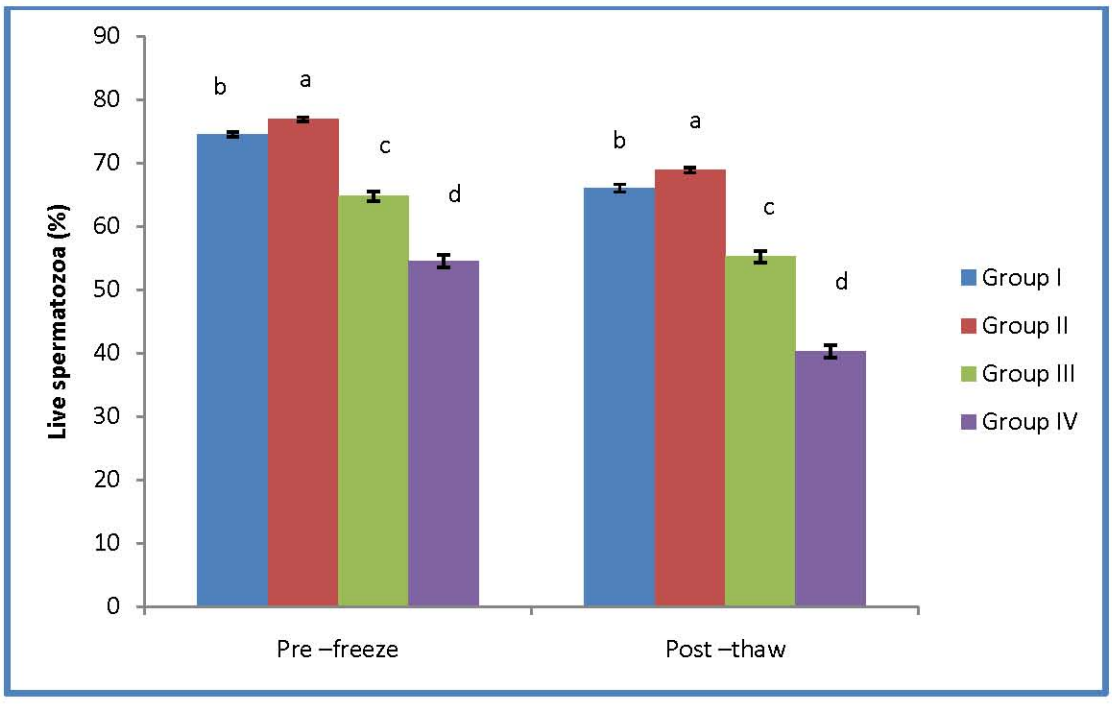


Figure 8.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on per cent live spermatozoa of Hariana bull semen during freeze-thaw process

4.2 Effect of lycopene on semen quality of Hariana bull at pre-freeze and post-thaw stage

After the evaluation of freshly collected semen, samples which fulfil the criteria as stated vide supra, the semen was extended with EYTG. The extended semen was divided and supplemented with lycopene into four different portions to make four groups as Group I (Control), Group II (0.25mM), Group III (0.5mM) and Group IV (1mM). These samples were then processed for freezing in a programmable biological freezer in the presence of LN_2 vapour in a controlled manner. The vapour freezed semen samples were then stored in LN_2 container and after 24 hours of storage in LN_2 were subjected to thawing (37°C for 45 seconds).

The relevant data has been presented in Table 2.0 to 9.0 and Figure 7.0 to 33.0.

(1) Progressively motile spermatozoa (%)

The relevant data has been presented in Table 2.0 and illustrated in Figure 7.0.

At pre-freeze stage, percentage of progressively motile spermatozoa of Hariana bull was found as 69.84±20.93 in group I. The respective values in group II, group III and group IV were 72.97±14.11, 49.84±19.65 and 39.53±33.97 per cent. Per cent individual progressive motility of spermatozoa was highly significant ($P<0.01$) in group II as compared to I, III and IV groups.

At post-thaw stage, percentage of progressively motile spermatozoa of Hariana bull was found as 43.44±23.21 in group I. The respective values in group II, group III and group IV were 50.16±29.17, 32.97±23.67 and 23.6±21.80 percent. Per cent individual progressive motility of spermatozoa was highly significant ($P<0.01$) in group II as compared to I, III and IV groups.

(2) Live spermatozoa (%)

The relevant data has been presented in Table 3.0 and illustrated in Figure 8.0.

At pre-freeze stage, the live sperm percentage of Hariana bull was found as 74.58±0.37 in group I. The respective values in group II, group III and group IV were 76.92±0.37, 64.81±0.73 and 54.59±0.98 per cent. Per cent live spermatozoa were highly significant ($P<0.01$) in group II as compared to I, III and IV groups.

At post-thaw stage, the live sperm percentage of Hariana bull was found as 66.08±0.61 in group I. The respective values in group II, group III and group IV were 68.88±0.39, 55.25±0.94 and 40.31±0.99 per cent. Per cent live spermatozoa were highly significant (P<0.01) in group II as compared to I, III and IV groups.

(3) HOST reactive spermatozoa (%)

The relevant data has been presented in Table 4.0 and illustrated in Figure 9.0.

At pre-freeze stage, percentage of HOST reactive spermatozoa of Hariana bull was found as 70.17±0.66 in group I. The respective values in group II, group III and group IV were 72.06±0.55, 51.50±0.71 and 44.38±0.52 per cent. Percent HOST reactive spermatozoa were highly significant (P<0.01) in group II as compared to I, III and IV groups.

At post-thaw stage, percentage of HOST reactive spermatozoa of Hariana bull was found as 61.55±0.75 in group I. The respective values in group II, group III and group IV were 63.90±0.54, 43.72±0.64 and 34.88±0.93 per cent. Per cent HOST reactive spermatozoa were highly significant (P<0.01) in group II as compared to I, III and IV groups.

(4) Spermatozoa with Intact acrosome (%)

The relevant data has been presented in Table 5.0 and illustrated in Figure 10.0.

At pre-freeze stage, percentage of spermatozoa with intact acrosome in the semen of Hariana bull was found as 70.56±0.68 in group I. The respective values in group II, group III and group IV were 73.36±0.78, 58.81±0.99 and 48.88±0.85 per cent. Percentage of spermatozoa with intact acrosome were highly significant (P<0.01) in group II as compared to I, III and IV groups.

At post-thaw stage, percentage of spermatozoa with intact acrosome in the semen of Hariana bull was found as 63.16±0.78 in group I. The respective values in group II, group III and group IV were 66.04±0.81, 50.00±0.98 and 40.42±0.84 per cent. Percentage of spermatozoa with intact acrosome were highly significant (P<0.01) in group II as compared to I, III and IV groups.

Table 3.0: Effect of different concentrations of lycopene on per cent live spermatozoa at pre-freeze and post-thaw stages of Hariana bull semen

(Mean±SE=32)

Stage	Group I (Control)	Group II (0.25mM)	Group III (0.5mM)	Group IV (1mM)	'F' Value
Pre-freeze	74.58±0.37^{b} (69.4 - 77.9)	76.92±0.37^{a} (72.4 - 79.6)	64.81±0.73^{c} (52.6 - 70.0)	54.59±0.98^{d} (41.9 - 68.6)	237.29**
Post-thaw	66.08±0.61^{b} (54.5 - 70.9)	68.88±0.39^{a} (65.0 - 73.9)	55.25±0.94^{c} (40.8 - 63.2)	40.31±0.99^{d} (28.9 - 50.1)	280.82**

Table 4.0: Effect of different concentrations of lycopene on per cent HOS test responsive spermatozoa at pre-freeze and post-thaw stages of Hariana bull semen

(Mean±SE=32)

Stage	Group I (Control)	Group II (0.25mM)	Group III (0.5mM)	Group IV (1mM)	'F' Value
Pre-freeze	70.17±0.66^{b} (61.5 - 76.6)	72.06±0.55^{a} (64.5 - 76.7)	51.50±0.71^{c} (43.2 - 62.5)	44.38±0.52^{d} (38.9 - 50.5)	495.00**
Post-thaw	61.65±0.75^{b} (48.4 - 67.2)	63.90±0.54^{a} (58.5 - 68.94)	43.72±0.64^{c} (36.9 - 48.7)	34.88±0.93^{d} (23.4 - 47.5)	369.82**

Table 5.0: Effect of different concentrations of lycopene on per cent Spermatozoa with intact acrosome at pre-freeze and post-thaw stages of Hariana bull semen

(Mean±SE=32)

Stage	Group I (Control)	Group II (0.25mM)	Group III (0.5mM)	Group IV (1mM)	'F' Value
Pre-freeze	70.56±0.68^{b} (62.7 - 74.5)	73.36±0.78^{a} (60.7 - 84.4)	58.81±0.99^{c} (46.8 - 70.4)	48.88±0.85^{d} (40.2 - 62.5)	182.93**
Post-thaw	63.16±0.78^{b} (53.2 - 69.0)	66.04±0.81^{a} (51.6 - 76.2)	50.00±0.98^{c} (38.2 - 64.8)	40.42±0.84^{d} (29.5 - 48.8)	193.64**

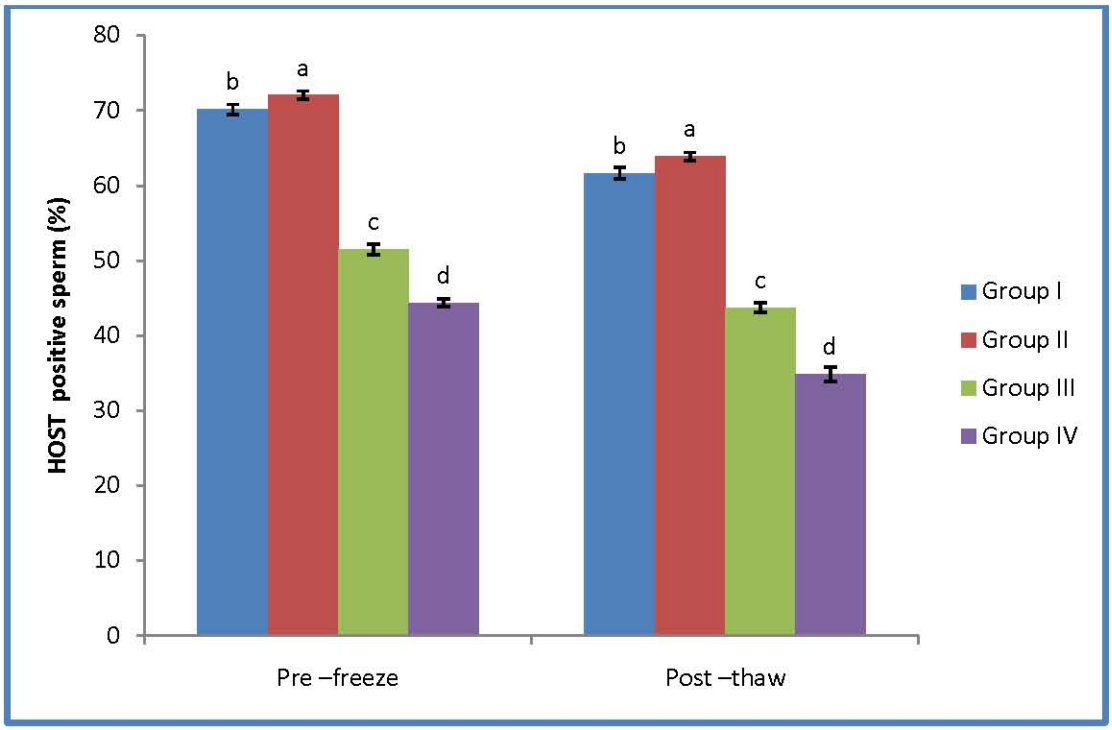


Figure 9.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on per cent HOST reactive spermatozoa of Hariana bull semen during freeze-thaw process

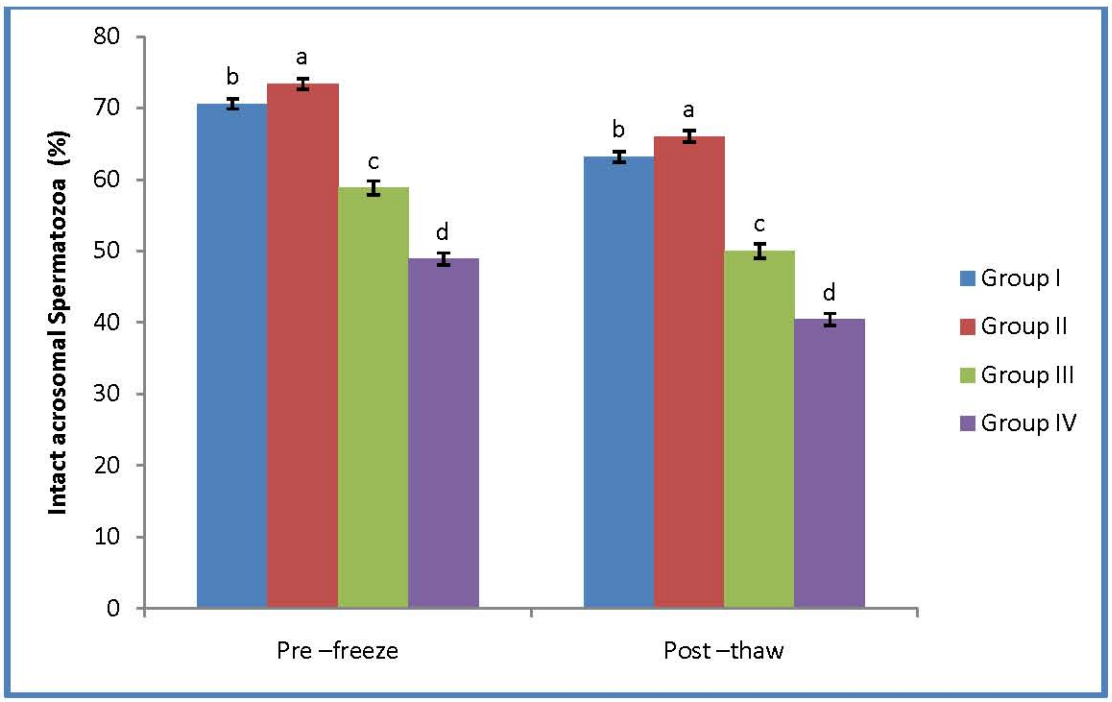


Figure 10.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on per cent spermatozoa with intact acrosome of Hariana bull semen during freeze-thaw process.

4.3 Evaluation of capacitation like changes (CTC Assay)

(1) Uncapacitated Spermatozoa (F-pattern)

The relevant data has been presented in Table 6.0 and illustrated in Figure 11.0.

At pre-freeze stage, the mean percentage of uncapacitated spermatozoa was found as 74.70±0.41 in group I. The respective values in group II, group III and group IV were 78.53±0.32, 71.36±0.44 and 64.20±0.45. Percentage of uncapacitated spermatozoa was found to be highly significant ($P<0.01$) in group II as compared to I, III and IV groups.

At post-thaw stage, the mean percentage of uncapacitated spermatozoa was found as 47.86±0.98 in group I. The respective values in group II, group III and group IV were 56.20±0.87, 49.80±0.76 and 37.70±0.94. Percentage of uncapacitated spermatozoa was found to be highly significant ($P<0.01$) in group II as compared to I, III and IV groups.

(2) Capacitated Spermatozoa (B-pattern)

The relevant data has been presented in Table 6.0 and illustrated in Figure 12.0.

At pre-freeze stage, the mean percentage of capacitated spermatozoa was found as 16.71±0.26 in group I. The respective values in group II, group III and group IV were 13.18±0.50, 18.50±0.48 and 22.24±0.56. Percentage of capacitated spermatozoa was found to be highly significant ($P<0.01$) in group IV as compared to I, II and III groups.

At post-thaw stage, the mean percentage of capacitated spermatozoa was found as 42.55±0.79 in group I. The respective values in group II, group III and group IV were 35.00±0.75, 36.71±0.91 and 44.61±0.90. Percentage of capacitated spermatozoa was found to be highly significant ($P<0.01$) in group I and group IV as compared to II and III groups.

(3) Acrosome reacted Spermatozoa (AR-pattern)

The relevant data has been presented in Table 6.0 and illustrated in Figure 13.0.

At pre-freeze stage, the mean percentage of acrosome reacted spermatozoa was found as 8.59±0.42 in group I. The respective values in group II, group III and group IV were 8.30±0.64, 10.13±0.59 and 13.55±0.48. Percentage of acrosome reacted spermatozoa was found to be highly significant ($P<0.01$) in group IV as compared to I, II and III groups.

At post-thaw stage, the mean percentage of acrosome reacted spermatozoa was found as 9.60±0.58 in group I. The respective values in group II, group III and group IV were 8.79±0.63, 13.49±0.87 and 17.68±0.98. Percentage of acrosome reacted spermatozoa was found to be highly significant ($P<0.01$) in group IV as compared to I, II and III groups.

4.4 Sperm motility and kinematic parameters

The relevant data has been presented in Table 7.0 and illustrated in Figure 14.0 to 29.0.

Total sperm motility was estimated in control (group I) and different lycopene treated groups (group II, III and IV) both during pre-freezing and post-thaw and the results are summarized in Table 7.0 and Figure 14.0. At pre-freeze stage, total sperm motility was significantly decreased in group III and group IV compared to group I and II. Higher total sperm motility was observed in group II. Similar trend in total sperm motility was observed after post-thaw in control and different lycopene treated groups.

Sperm progressive motility was estimated in control (group I) and different Lycopene treated groups (group II, III and IV) both during pre-freezing and post-thaw and the results are illustrated in Table 7.0 and Figure 15.0. Perusal of results revealed that at pre-freeze stage significant reduction in progressive motile spermatozoa in group III and group IV compared to group I and II. Higher progressive sperm motility was observed in group II. Post-thaw semen evaluation showed similar trend for progressive motile sperm in control and different lycopene treated groups.

Fast motility was estimated in control (group I) and different lycopene treated groups (group II, III and IV) both during pre-freezing and post-thaw and the results are illustrated in Table 7.0 and Figure 16.0. Perusal of results revealed that at pre-freeze stage significant reduction in fast motile spermatozoa in group III and group IV compared to group I and II. Higher fast motility was observed in group II. Post-thaw

Table 6.0: Effect of different concentrations of lycopene on per cent spermatozoa showing different patterns of chlortetracycline fluorescent staining at pre-freeze and post-thaw stages of Hariana bull semen

(Mean±SE=32)

CTC staining pattern	Stage	Group I (Control)	Group II (0.25mM)	Group III (0.5mM)	Group IV (1mM)	'F' Value
"F" pattern (Uncapacitated, acrosome intact)	Pre-freeze	74.70±0.41^{b} (69.9 - 79.8)	78.53±0.32^{a} (75.6 - 82.2)	71.36±0.44^{c} (66.5 - 75.4)	64.20±0.45^{d} (60.1 - 69.8)	222.18**
	Post-thaw	47.86±0.98^{b} (38.2 - 58.4)	56.20±0.87^{a} (44.8 - 65.6)	49.80±0.76^{b} (41.6 - 58.2)	37.70±0.94^{c} (30.0 - 46.8)	73.72**
"B" pattern (Capacitated, acrosome intact)	Pre-freeze	16.71±0.26^{c} (12.0 - 18.9)	13.18±0.50^{d} (6.8 - 18.0)	18.50±0.48^{b} (12.0 - 24.6)	22.24±0.56^{a} (13.7 - 26.4)	65.40**
	Post-thaw	42.55±0.79^{a} (34.6 - 49.6)	35.00±0.75^{b} (25.2 - 44.2)	36.71±0.91^{b} (26.8 – 45.0)	44.61±0.90^{a} (35.8 - 56.2)	29.75**
"AR" pattern (Capacitated, acrosome reacted)	Pre-freeze	8.59±0.42^{c} (18.0 - 12.6)	8.30±0.64^{c} (0.4 - 15.4)	10.13±0.59^{b} (3.6 - 18.3)	13.55±0.48^{a} (8.2 - 18.4)	19.94**
	Post-thaw	9.60±0.58^{c} (3.2 - 16.4)	8.79±0.63^{c} (4.5 - 19.2)	13.49±0.87^{b} (4.4 - 22.4)	17.68±0.98^{a} (8.3 - 26.0)	27.32**

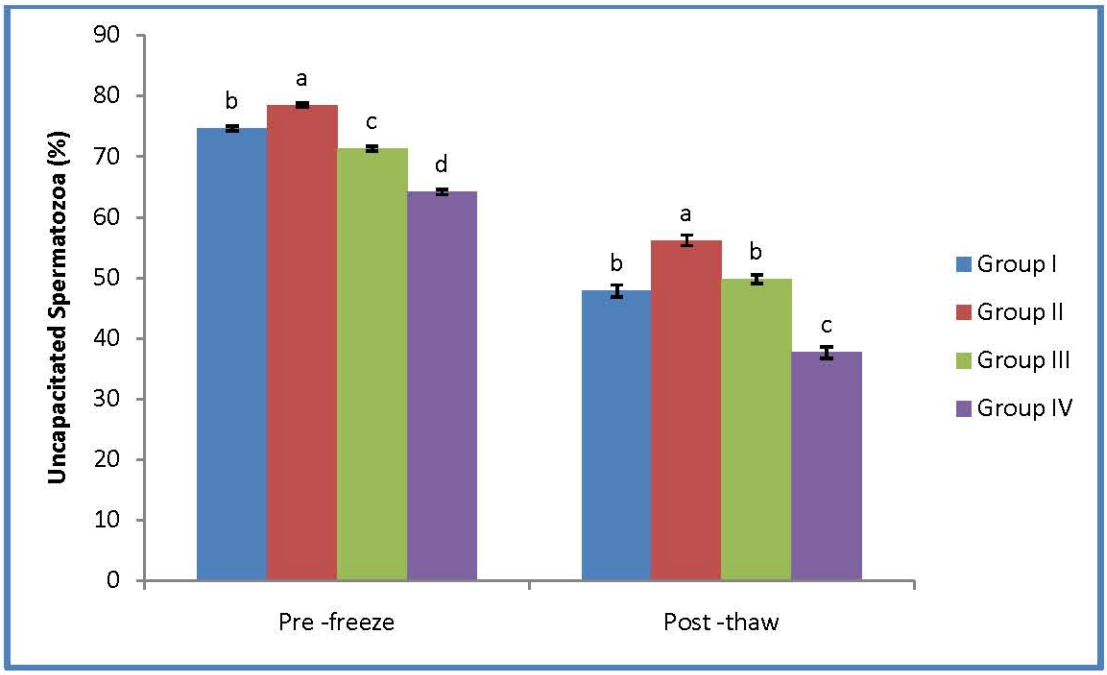


Figure 11.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on per cent spermatozoa showing F-pattern (uncapacitated, acrosome intact) as observed with CTC staining of Hariana bull semen during freeze-thaw process

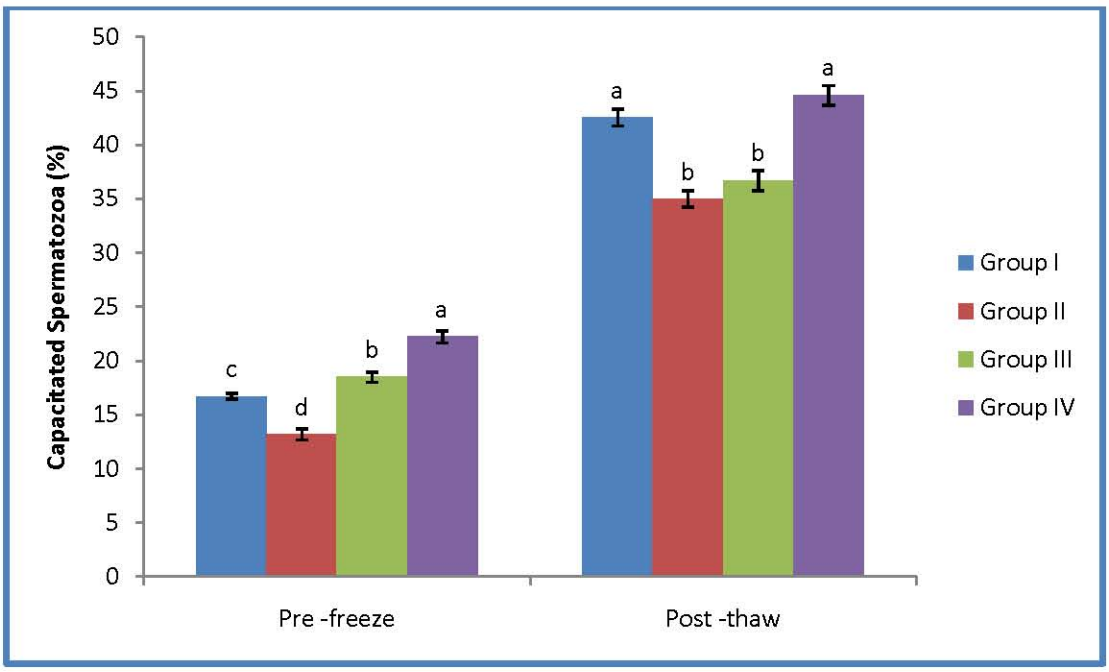


Figure 12.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on per cent spermatozoa showing B-pattern (capacitated, acrosome intact) as observed with CTC staining of Hariana bull semen during freeze-thaw process

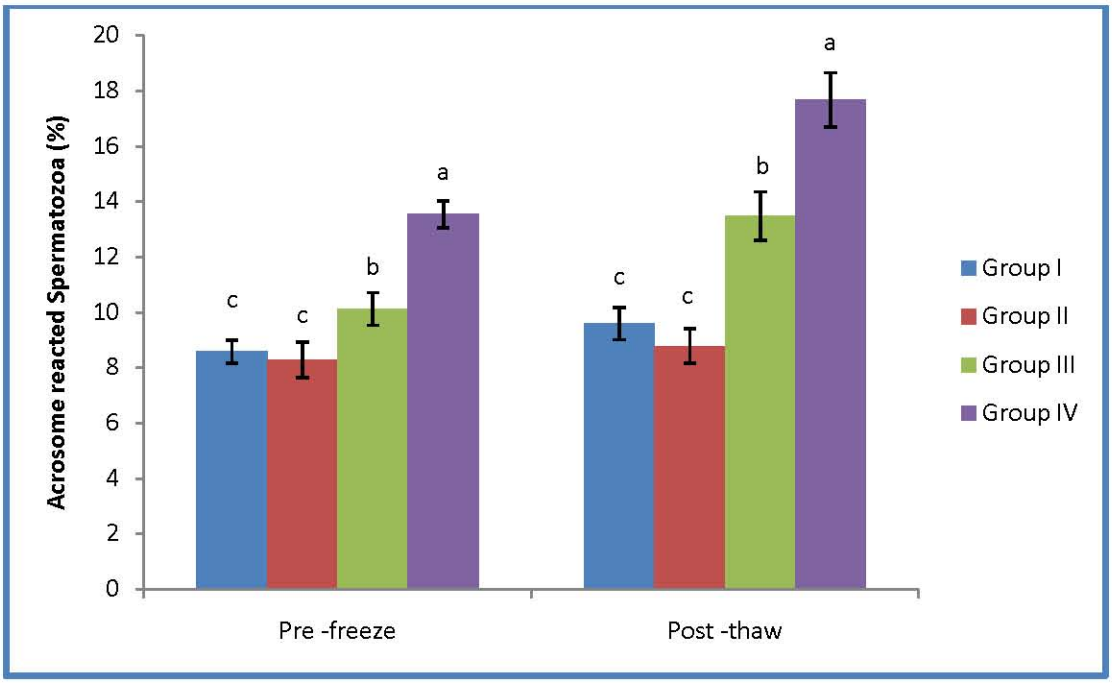


Figure 13.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on per cent spermatozoa showing AR-pattern (capacitated, acrosome reacted) as observed with CTC staining of Hariana bull semen during freeze-thaw process

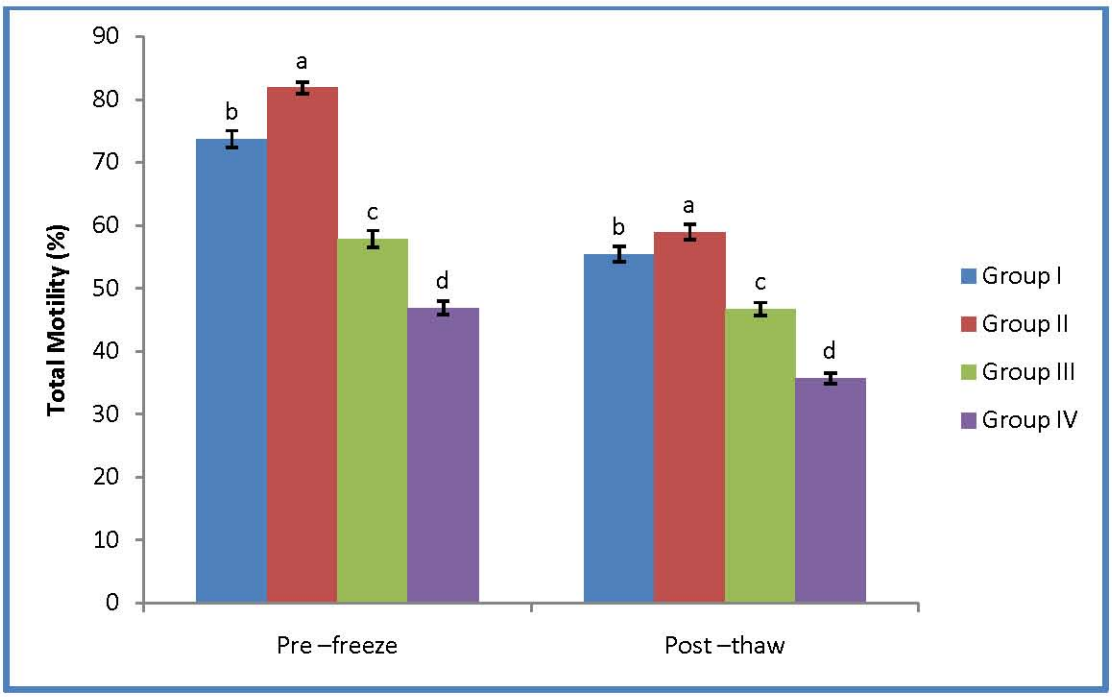


Figure 14.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on per cent total motile spermatozoa (CASA) of Hariana bull semen during freeze-thaw process

Table 7.0: Effect of different concentrations of lycopene on **sperm motility and kinematic parameters as observed through CASA** at pre-freeze and post-thaw stages of Hariana bull semen

(Mean±SE=32)

Parameters	Stage	Group I (Control)	Group II (0.25mM)	Group III (0.5mM)	Group IV (1mM)	'F' Value
Total motility (%)	Pre -freeze	73.7±1.35^{b} (60-84.8)	81.85±0.94^{a} (67.1-92.3)	57.87±1.30^{c} (42.66-69.64)	46.89±1.06^{d} (33.11-55.8)	178.08**
	Post -thaw	55.44±1.18^{b} (45.29-66)	58.95±1.21^{a} (47.88-69.64)	46.74±1.00^{c} (36.86-55.73)	35.70±0.82^{d} (26.89-41.64)	94.39**
Progressive motility (%)	Pre -freeze	66.80±1.41^{b} (54-79.3)	76.22±1.22^{a} (63.05-89.03	49.99±1.36^{c} (35.22-62.6)	38.32±1.02^{d} (22.22-47.56)	179.91**
	Post -thaw	47.18±1.27^{b} (36.47-59.79)	51.39±1.22^{a} (38.94-62.60)	38.29±0.87^{c} (29.21-45.48)	28.11±0.76^{d} (20.48-34.61)	95.71**
Fast motility (%)	Pre -freeze	47.80±1.11^{b} (34-56.26)	55.98±1.70^{a} (37.07-76.06)	34.22±1.15^{c} (23.23-49.11)	26.24±0.90^{d} (14.67-35.03)	114.96**
	Post -thaw	32.00±0.95^{b} (21.01-48)	35.34±1.08^{a} (27.77-49.11)	26.38±0.74^{c} (20.02-35.03)	17.63±0.74^{d} (10.08-25.83)	75.69**
Slow motility (%)	Pre -freeze	18.43±0.84^{a} (11.5-27)	19.56±0.97^{a} (11.67-29.08)	14.83±0.54^{b} (11.02-20.94)	11.78±0.39^{c} (7.48-16.99)	24.08**
	Post -thaw	14.12±0.58^{a} (7.64-20.94)	15.28±0.56^{a} (11.02-21.79)	11.53±0.43^{b} (7.64-16.99)	10.41±0.40^{b} (5.73-14.74)	20.45**
VCL (µm/s)	Pre -freeze	123.06±1.96^{b} (103-138)	142.97±3.96^{a} (105.9-201.3)	95.27±2.68^{c} (71.17-123.7)	77.5±2.08^{d} (49.52-97.58)	108.65**
	Post -thaw	90.58±2.57^{b} (63.56-123.69)	99.10±2.39^{a} (74.87-123.74)	77.08±1.72^{c} (56.47-93.97)	56.32±1.78^{d} (37.76-74.17)	75.42**
VSL (µm/s)	Pre -freeze	42.93±0.80^{b} (36.9-50)	50.80±1.70^{a} (38.09-73.84)	34.31±1.12^{c} (23.24-47.38)	28.80±0.80^{d} (20.05-38.95)	69.36**
	Post -thaw	33.30±1.14^{b} (23.24-47.38)	35.86±0.95^{a} (26.12-47.38)	28.76±0.71^{c} (19.28-38.95)	20.55±0.70^{d} (12.95-27.68)	56.44**
VAP (µm/s)	Pre -freeze	59.88±1.05^{b} (50-68.37)	69.63±1.81^{a} (51.79-95.19)	46.79±1.32^{c} (32.53-61.41)	39.35±1.08^{d} (27.88-49.79)	99.47**
	Post -thaw	45.12±1.28^{b} (32.53-61.41)	48.23±1.13^{a} (37.97-61.41)	39.04±0.92^{c} (28.33-49.05)	28.15±0.80^{d} (19.49-36.40)	71.15**

DCL (µm)	**Pre -freeze**	38.87±1.25^{b} (25-46.85)	43.72±1.06^{a} (29.76-54.35)	31.60±0.91^{c} (24.5-44.05)	26.76±0.77^{d} (15.95-35.11)	55.37**
	Post -thaw	30.19±0.85^{a} (24.93-44.05)	31.70±0.95^{a} (24.00-44.05)	26.53±0.60^{b} (20.66-34.45)	19.77±0.51^{c} (14.48-25.15)	50.36**
DSL (µm)	**Pre -freeze**	10.56±0.34^{b} (6-12.4)	12.26±0.31^{a} (7.97-15.52)	8.85±0.32^{c} (6-14.08)	7.20±0.22^{d} (4.31-9.62)	52.72**
	Post -thaw	8.51±0.34^{b} (6-14.08)	9.22±0.30^{a} (6.00-14.08)	7.20±0.18^{c} (5.63-9.62)	5.32±0.15^{d} (3.67-6.98)	44.79**
DAP (µm)	**Pre -freeze**	17.30±0.60^{b} (11-21.5)	19.43±0.49^{a} (12.93-23.26)	14.18±0.43^{c} (10.25-20.03)	11.63±0.36^{d} (7.07-15.52)	51.64**
	Post -thaw	13.63±0.41^{a} (11-20.03)	14.12±0.43^{a} (11-20.03)	11.50±0.28^{b} (8.42-15.15)	8.55±0.20^{c} (6.30-10.66)	53.75**
ALH (µm)	**Pre -freeze**	1.45±0.03^{b} (1-1.62)	1.65±0.04^{a} (1.22-2.14)	1.10±0.03^{c} (0.82-1.47)	0.93±0.02^{d} (0.56-1.18)	107.92**
	Post -thaw	1.04±0.02^{b} (0.82-1.4)	1.16±0.03^{a} (0.94-1.47)	0.92±0.02^{c} (0.74-1.12)	0.70±0.02^{d} (0.52-0.88)	75.94**
BCF (Hz)	**Pre -freeze**	8.63±0.16^{b} (6-10)	9.64±0.13^{a} (8.22-11.11)	7.50±0.21^{c} (5.61-10.27)	5.88±0.15^{d} (3.61-7.72)	90.69**
	Post -thaw	7.09±0.23^{a} (5.32-10.21)	7.58±0.22^{a} (5-10.27)	5.88±0.14^{b} (4.43-7.72)	4.70±0.13^{c} (3.21-6.52)	47.69**
HAC (Rad)	**Pre -freeze**	0.30±0.00^{b} (0.19-0.35)	0.35±0.00^{a} (0.26-0.46)	0.23±0.00^{c} (0.16-0.32)	0.18±0.00^{d} (0.13-0.23)	98.83**
	Post -thaw	0.21±0.00^{b} (0.16-0.3)	0.23±0.00^{a} (0.19-0.32)	0.19±0.01^{c} (0.14-0.52)	0.13±0.00^{d} (0.09-0.17)	33.16**
WOB (VAP/VSL)	**Pre -freeze**	0.48±0.00^{b} (0.4-0.51)	0.50±0.00^{b} (0.47-0.51)	0.49±0.00^{b} (0.4-0.52)	0.51±0.00^{a} (0.47-0.56)	8.18**
	Post -thaw	0.50±0.0^{a} (0.4-0.54)	0.47±0.00^{b} (0.40-0.51)	0.51±0.00^{a} (0.47-0.54)	0.50±0.00^{a} (0.47-0.54)	8.56**
LIN (VSL/VCL)	**Pre -freeze**	0.34±0.00^{c} (0.3-0.38)	0.36±0.00^{b} (0.32-0.4)	0.36±0.00^{b} (0.3-0.39)	0.37±0.00^{a} (0.33-0.43)	8.89**
	Post -thaw	0.36±0.00^{a} (0.3-0.41)	0.35±0.00^{b} (0.30-0.39)	0.37±0.00^{a} (0.33-0.41)	0.36±0.00^{a} (0.33-0.4)	6.07*
STR (VSL/VAP)	**Pre –freeze**	0.71±0.00^{b} (0.65-0.79)	0.72±0.00^{ab} (0.65-0.8)	0.73±0.00^{ab} (0.65-0.78)	0.73±0.00^{a} (0.67-0.79)	1.92*
	Post -thaw	0.73±0.00 (0.67-0.79)	0.73±0.00 (0.65-0.78)	0.74±0.00 (0.67-0.79)	0.73±0.00 (0.66-0.78)	0.75^{NS}

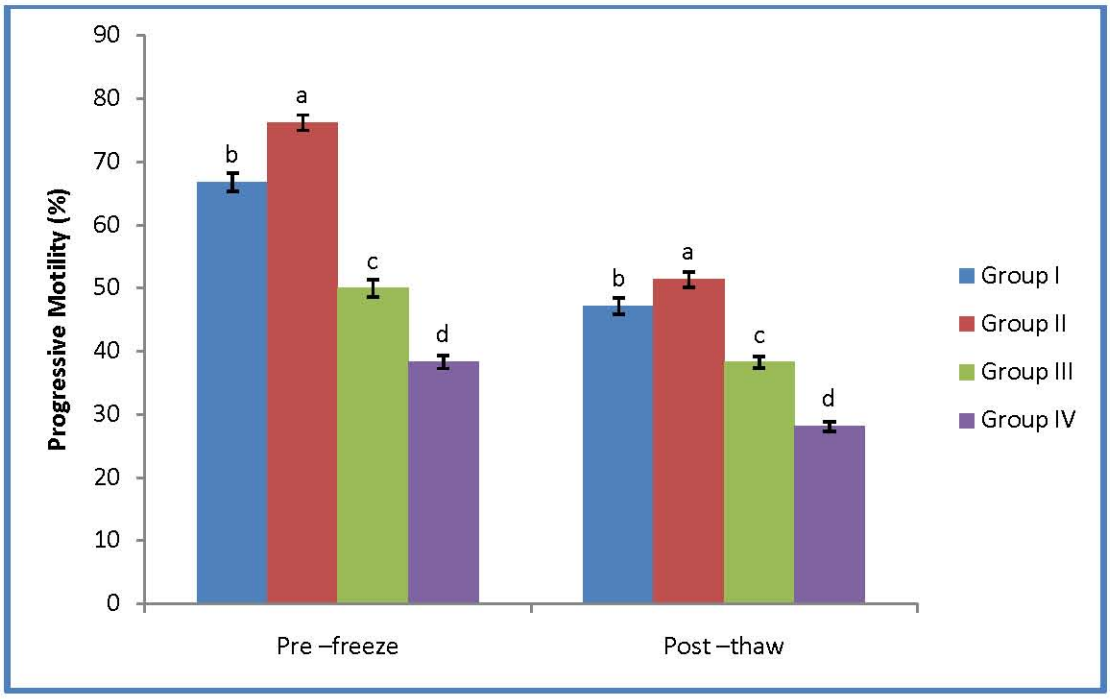


Figure 15.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on per cent progressively motile spermatozoa (CASA) of Hariana bull semen during freeze-thaw process

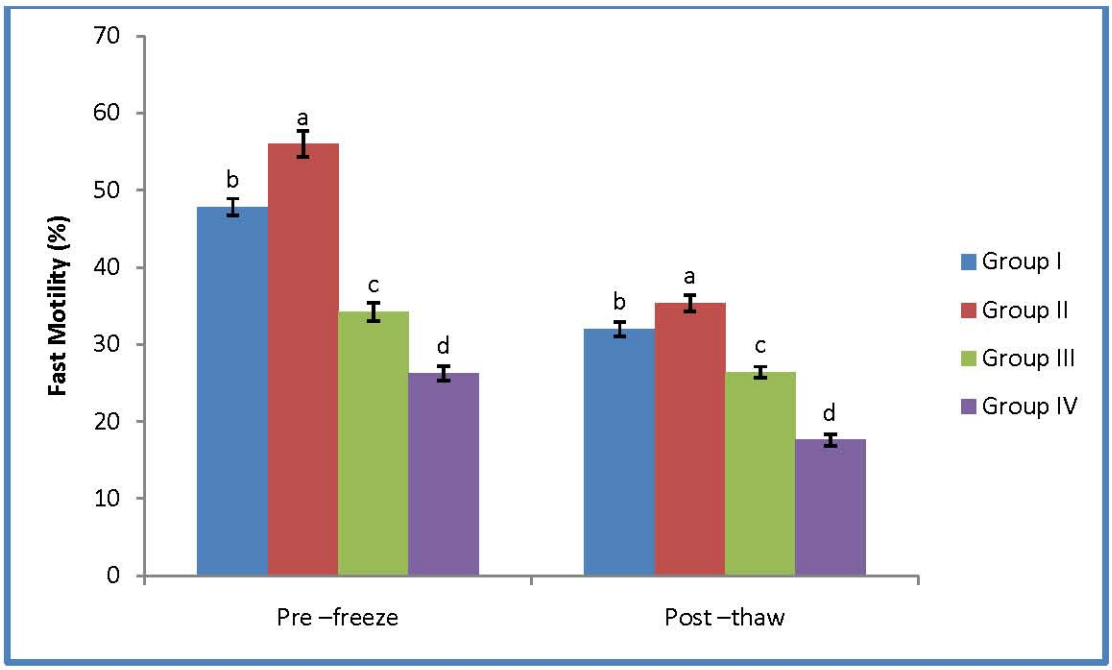


Figure 16.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on per cent fast motile spermatozoa (CASA) of Hariana bull semen during freeze-thaw process

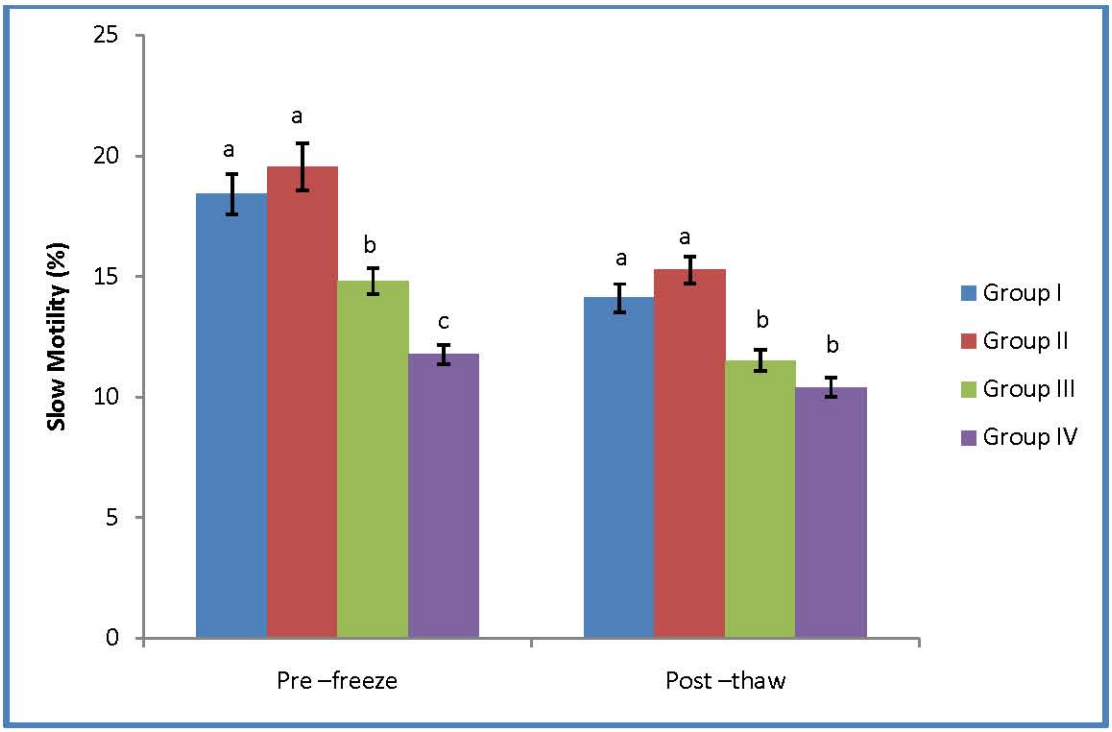


Figure 17.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on per cent slow motile spermatozoa (CASA) of Hariana bull semen during freeze-thaw process

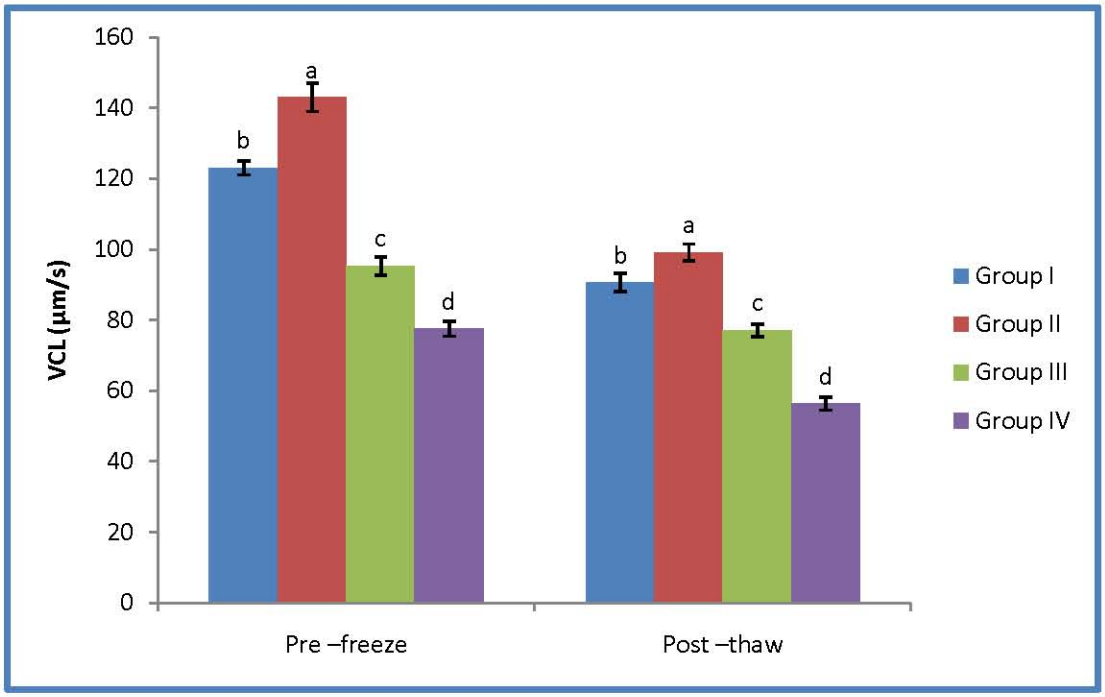


Figure 18.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on curvilinear velocity (µm/s) of Hariana bull spermatozoa (CASA) during freeze-thaw process

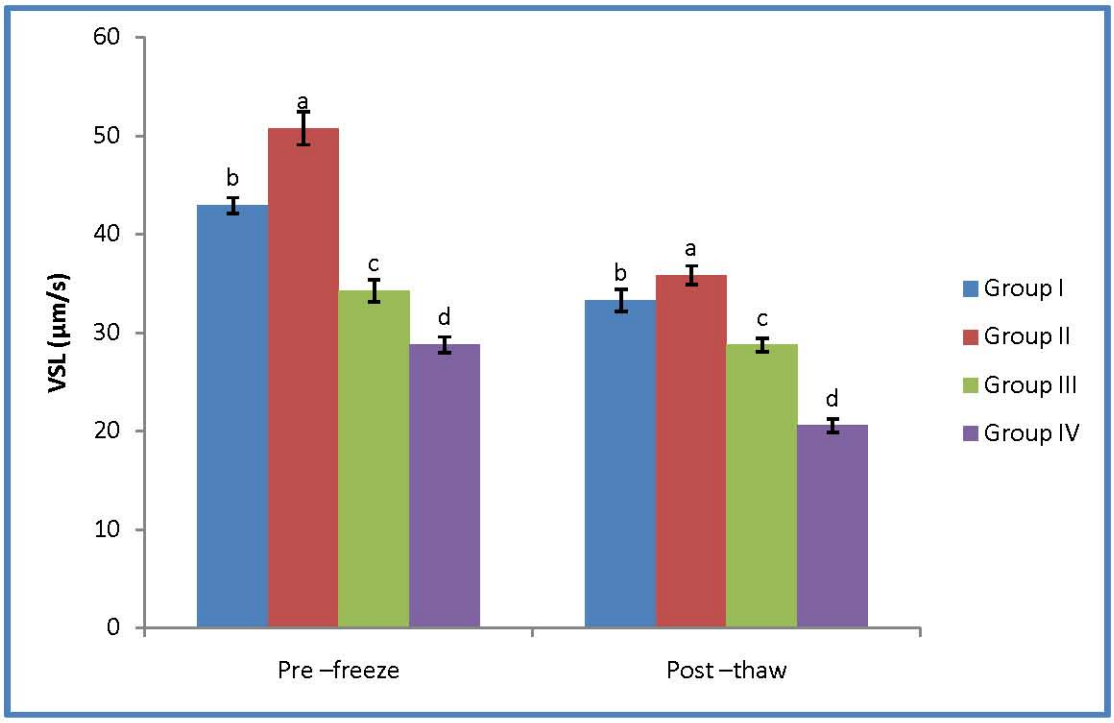


Figure 19.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on straight line velocity (µm/s) of Hariana bull spermatozoa (CASA) during freeze-thaw process

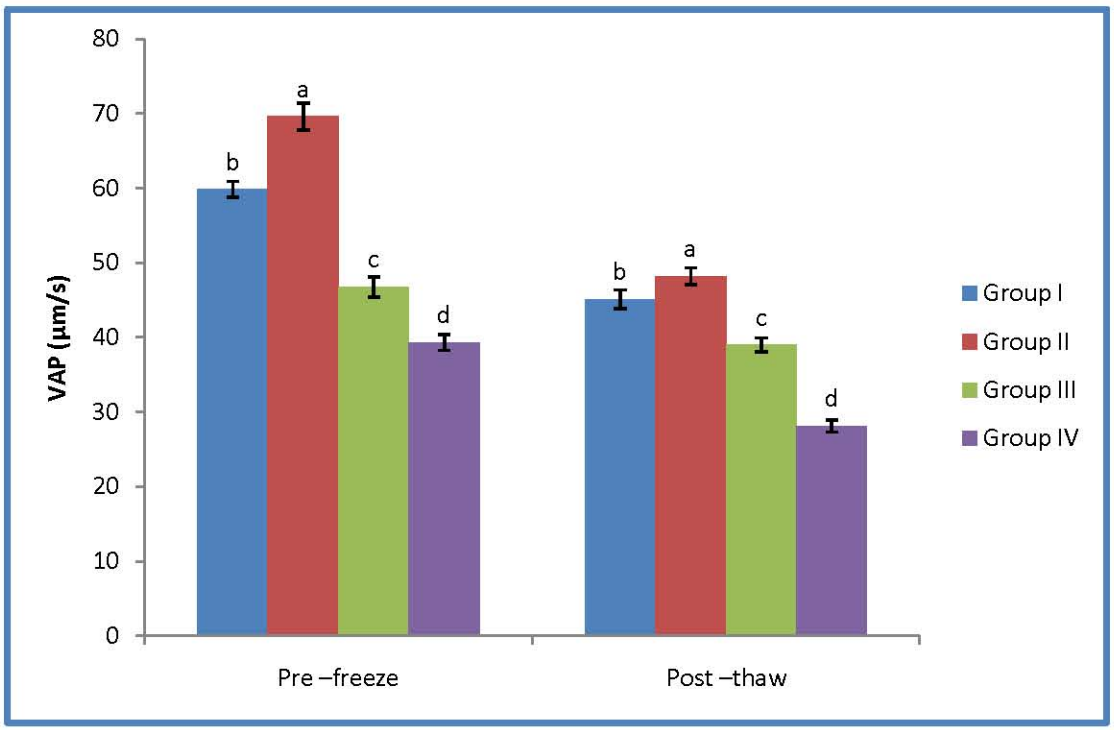


Figure 20.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on average path velocity (µm/s) of Hariana bull spermatozoa (CASA) during freeze-thaw process

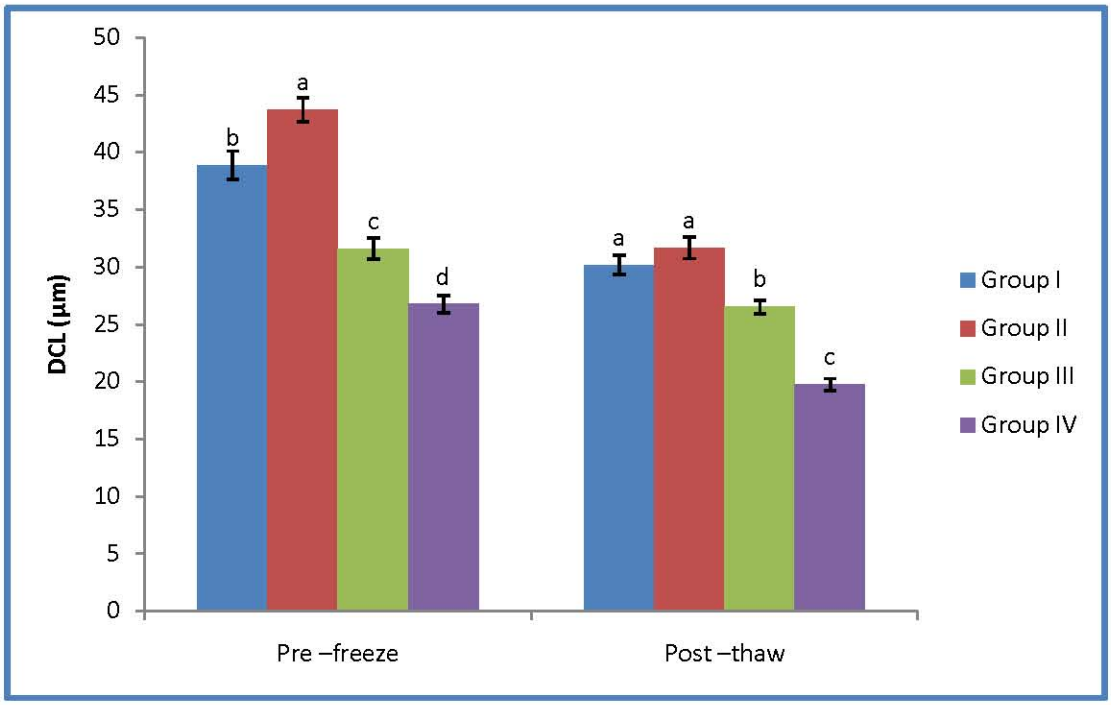


Figure 21.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on curvilinear distance (µm) of Hariana bull spermatozoa (CASA) during freeze-thaw process

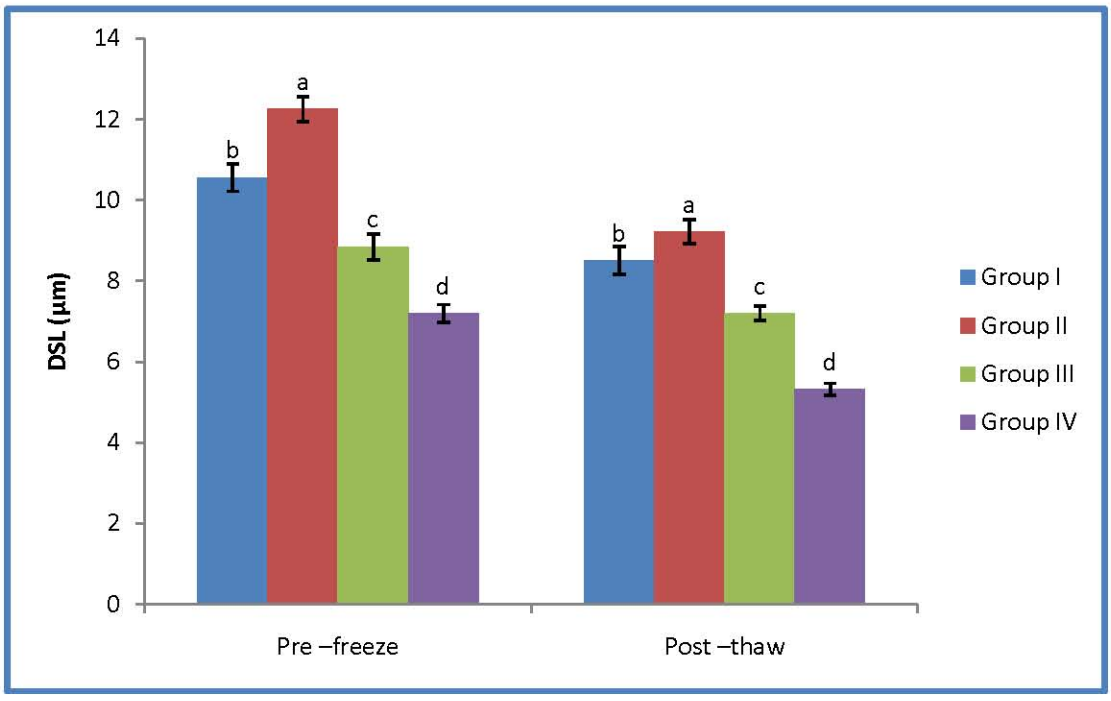


Figure 22.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on straight line distance (µm) of Hariana bull spermatozoa (CASA) during freeze-thaw process

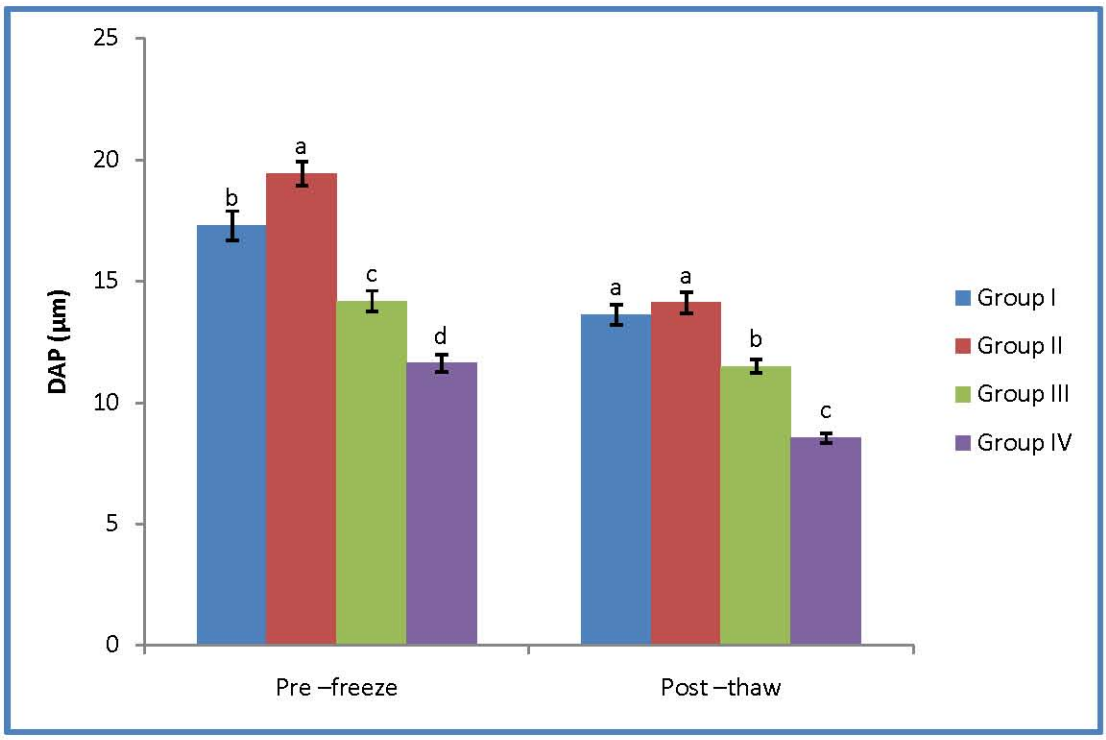


Figure 23.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on average path distance (µm) of Hariana bull spermatozoa (CASA) during freeze-thaw process

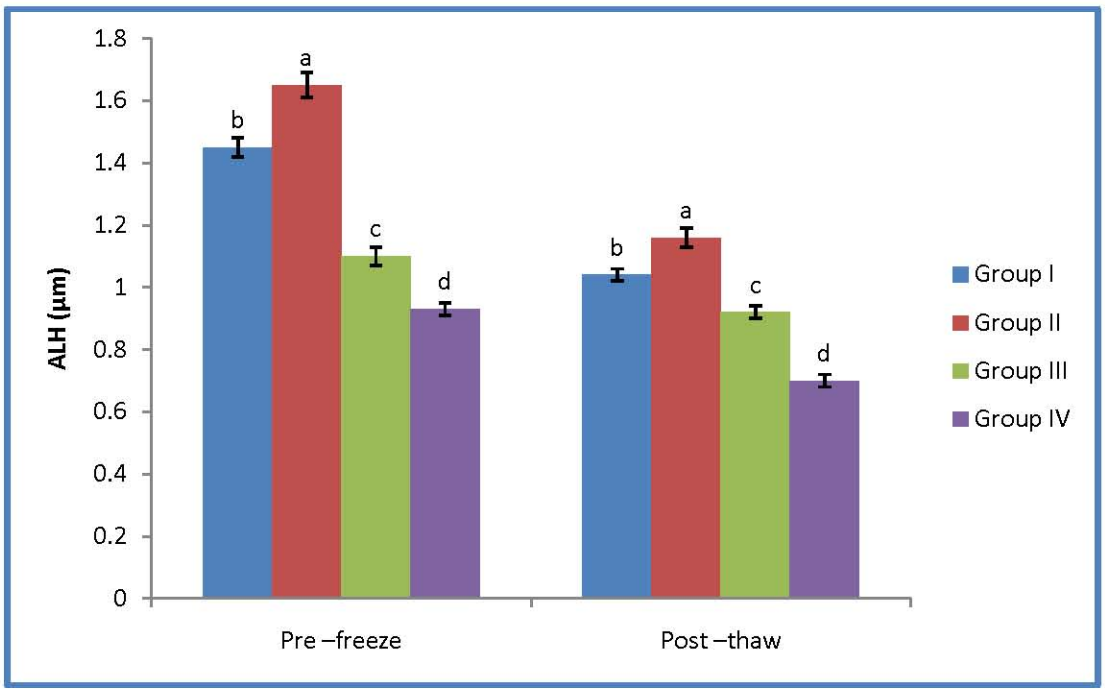


Figure 24.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on amplitude of lateral head displacement (µm) of Hariana bull spermatozoa (CASA) during freeze-thaw process

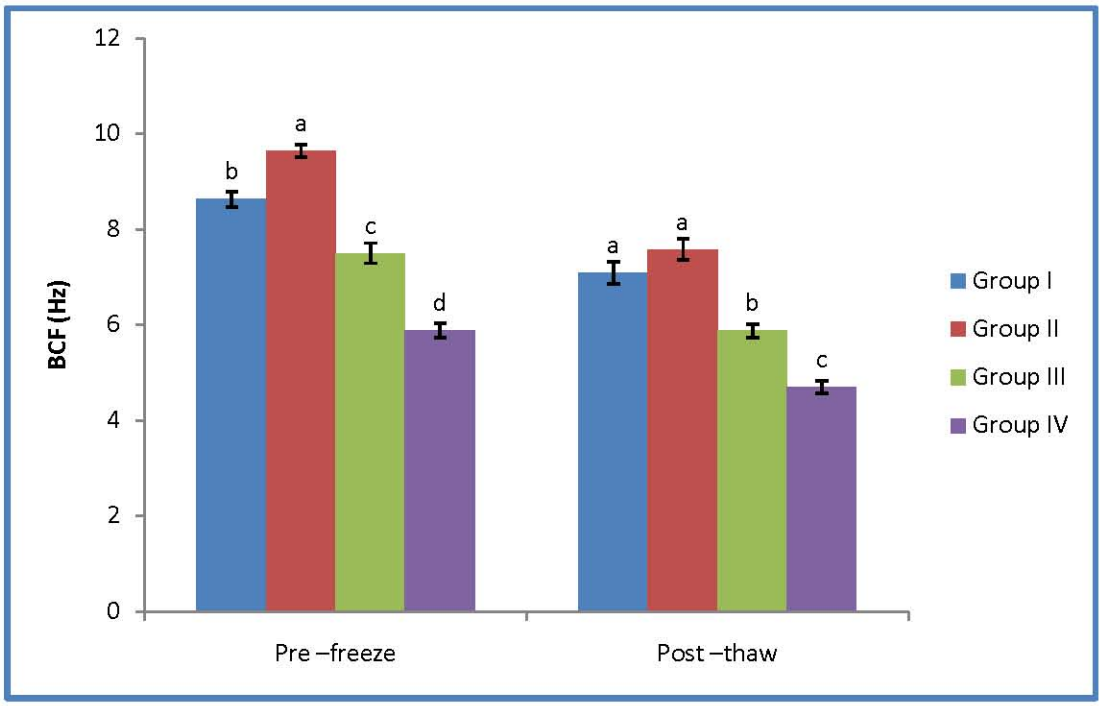


Figure 25.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on beat cross frequency (Hz) of Hariana bull spermatozoa (CASA) during freeze-thaw process

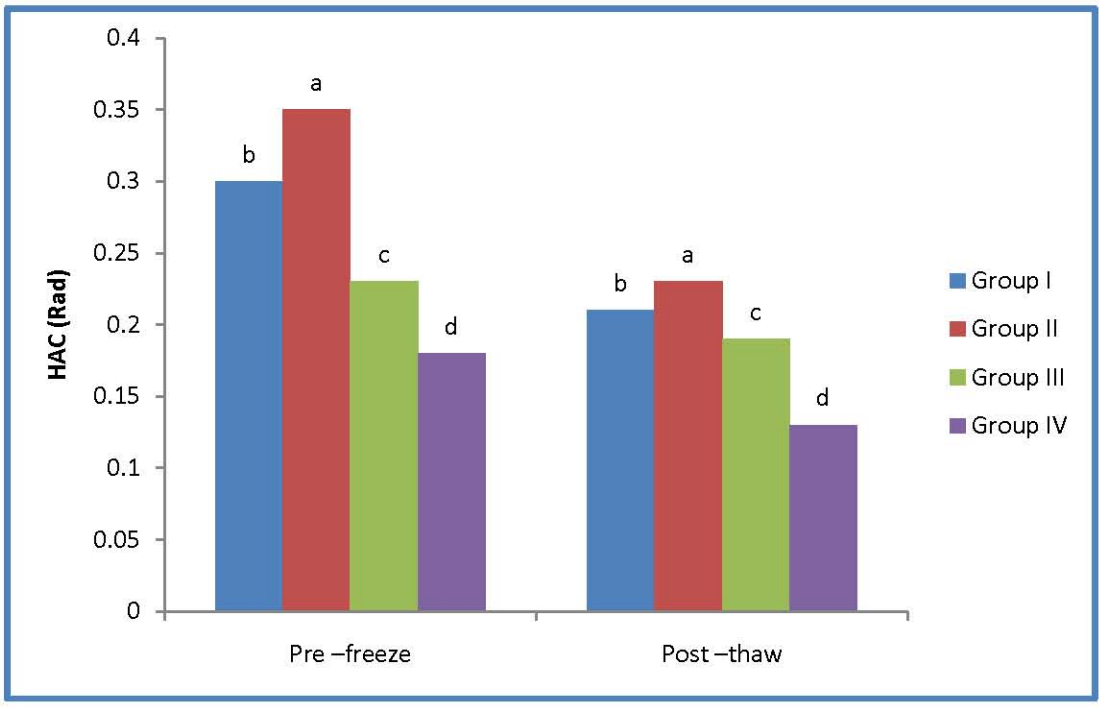


Figure 26.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on head activity parameter (Rad) of Hariana bull spermatozoa (CASA) during freeze-thaw process

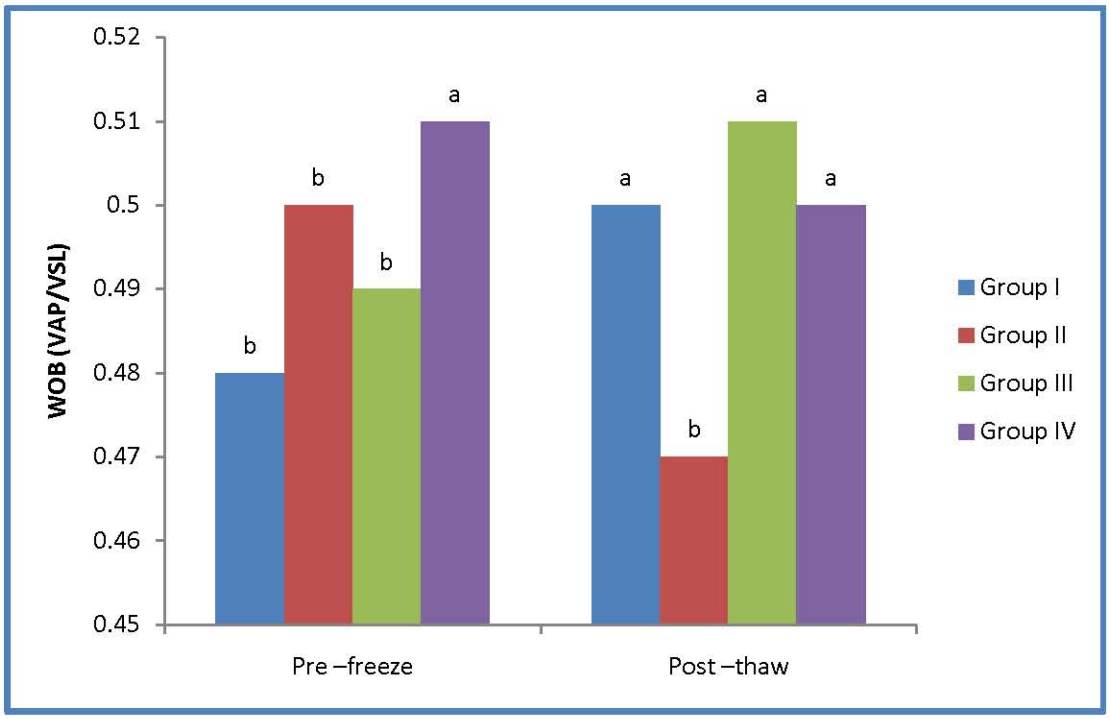


Figure 27.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on wobble (VAP/VCL) of Hariana bull spermatozoa (CASA) during freeze-thaw process

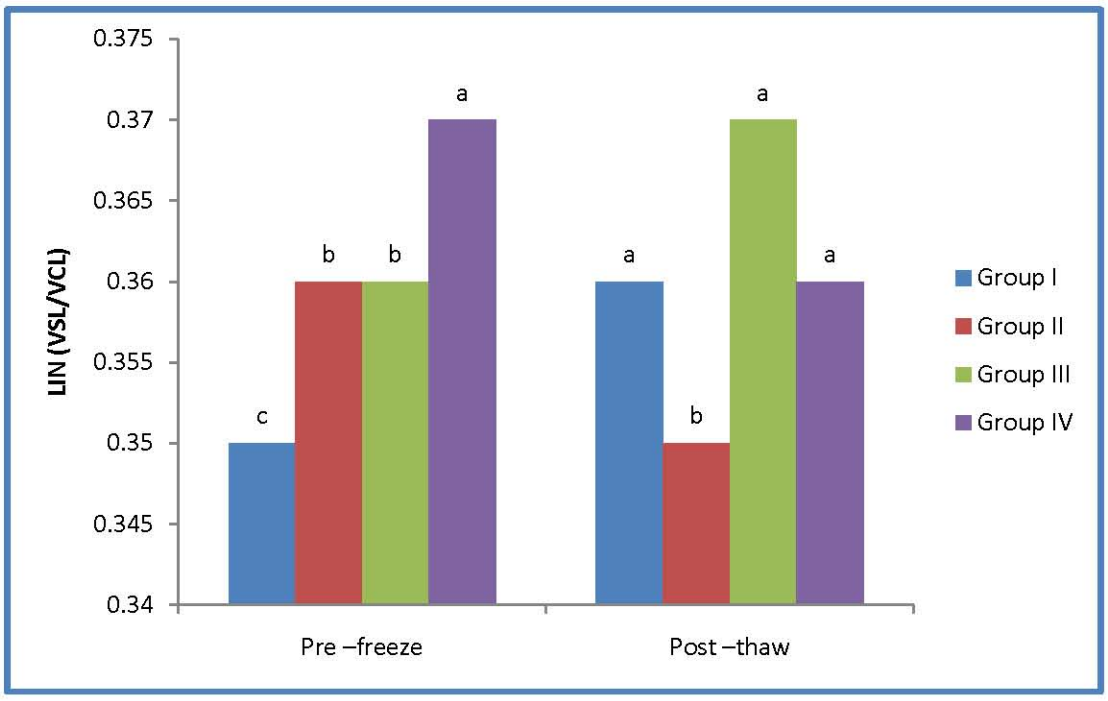


Figure 28.0: Graphical presentation of effect of different concentrations of lyocopene (GroupI- control, GroupII- 0.25mM, GroupIII- 0.5mM and GroupIV- 1mM) on linearity (VSL/VCL) of Hariana bull spermatozoa (CASA) during freeze-thaw process

semen evaluation showed similar trend for fast motile sperm in control and different lycopene treated groups.

Slow motility was estimated in control (group I) and different lycopene treated groups (group II, III and IV) both during pre-freezing and post-thaw and the results are illustrated in Table 7.0 and Figure 17.0. Perusal of results revealed that at pre-freeze stage significant reduction in slow motile spermatozoa in group III and group IV compared to group I and II. However, non-significant difference was observed for slow motile population between group I and II. Post-thaw semen evaluation showed similar trend for slow motile sperm in control and different lycopene treated groups.

Velocity and motion parameters of spermatozoa of different groups after equilibration and post-thaw are illustrated in Table 7.0 and Figure 18.0 to Figure 23.0. Velocity parameters like VCL, VSL and VAP showed similar trend both before freezing and after freezing-thawing. The velocity parameters were highly significant ($P<0.01$) in group II compared to other groups during both the phases. Similar to the velocity parameters, motion parameters (distance) revealed highly significant ($P<0.01$) values in group II compared to other groups. After thawing, DCL and DAP did not show significant variation between group I and group II (Table 7.0).

The kinematic parameters like ALH and HAC showed similar trend like that of velocity and motion parameters at pre-freeze and post-thaw stage freezing-thawing (Table 7.0 and Figure 24 & 26). While, parameters like BCF showed significant reduction in group III and IV and non-significant difference between group I and II after post-thaw (Table 7.0 and Figure 25). Interestingly, WOB and LIN showed similar trend after post thaw and group III and IV did not show any significant difference compared to other groups (Table 7.0 and Figure 27 & 28). STR is the only kinematic parameter which did not show any significant variation between all groups at pre-freeze and post-thaw stage (Table 7.0 and Figure 29).

4.5 *In vitro* fertility test Bovine Cervical Mucus Penetration Test (BCMPT, mm/hr)

The relevant data has been presented in Table 8.0 and illustrated in Figure 30.0.

At post-thaw stage, the mean Vanguard distance (mm/h) was determined as 23.53±0.60 mm in group I. The respective values in group II, group III and group IV were 34.75±0.68, 28.75±0.75 and 22.56±0.47 mm. Vanguard distance were highly significant (P<0.01) in group II as compared to I, III and IV groups. However, no significant difference was observed in Vanguard distance among group I and IV, being highest in group II and lowest in group IV.

4.6 Seminal Plasma Enzymatic Profile

Seminal plasma is a complex biological fluid containing various biochemical enzymes. Seminal plasma enzymes were evaluated at post thaw stage and enzyme activities were measured for SOD (Superoxide dismutase), GST (Glutathione-S-transferase) and MDA (Malondialdehyde) enzymes. The relevant data has been presented in Table 9.0 and illustrated in Figure 31.0 to 33.0.

(1) Superoxide dismutase (SOD) activity (U/ml)

The relevant data has been presented in Table 9.0 and illustrated in Figure 31.0.

At post-thaw stage, the SOD activity (U/ml) of Hariana bull seminal plasma was found as 804.07±20.51 in group I. The respective values in group II, group III and group IV were 770.51±19.12, 782.41±21.09 and 787.91±21.69 (U/ml). No significant (P>0.05) difference was found in SOD activity amongst different groups.

(2) Glutathione-S-Transferase (GST) activity (nM/min/ml)

The relevant data has been presented in Table 9.0 and illustrated in Figure 32.0.

At post-thaw stage, the GST activity (nM/min/ml) of Hariana bull seminal plasma was found as 65.09±1.63 in group I. The respective values in group II, group III and group IV were 46.17±4.72, 59.18±1.85 and 66.99±4.99 (nM/min/ml). GST activity was found to be significantly (P<0.01) lower in group II compare to group I, III and IV. However, no significant (P>0.05) difference was observed in GST activity among group I, group III and group IV.

Table 8.0: Effect of different concentrations of lycopene on distance (mm/hr) traveled by spermatozoa in oestrus mucus as observed through Bovine Cervical Mucus Penetration Test at post-thaw stage of Hariana bull semen

(Mean±SE=32)

Stage	Group I (Control)	Group II (0.25mM)	Group III (0.5mM)	Group IV (1mM)	'F' Value
Post-thaw	23.53 ± 0.60^{c} (18-30)	34.75 ± 0.68^{a} (28-45)	28.75 ± 0.75^{b} (22-40)	22.56 ± 0.47^{c} (18-28)	78.09**

Table 9.0: Effect of different concentrations of lycopene on seminal plasma enzymatic profile (SOD (U/ml); GST (nM/min/ml); MDA (nM/µl)) at post-thaw stage of Hariana bull semen

(Mean±SE=32)

Seminal plasma enzymatic profiles	Stage	Group I (Control)	Group II (0.25mM)	Group III (0.5mM)	Group IV (1mM)	'F' Value
SOD (U/ml)	Post-thaw	804.07±20.51 (652.69-942.47)	770.51±19.12 (643.55-936.56)	782.41±21.09 (643.55-1000.54)	787.91±21.69 (653.22-973.66)	0.456^{NS}
GST (nM/min/ml)	Post-thaw	65.09 ± 1.63^{a} (46.09-74.22)	46.17 ± 4.72^{b} (14.06-117.19)	59.18 ± 1.85^{a} (44.53-76.56)	66.99 ± 4.99^{a} (25.78-103.91)	6.64**
MDA (nM/µl)	Post-thaw	0.13 ± 0.01^{a} (0.03-0.23)	0.07 ± 0.01^{c} (0.03-0.09)	0.09 ± 0.01^{bc} (0.05-0.12)	0.10 ± 0.01^{b} (0.02-0.16)	9.81**

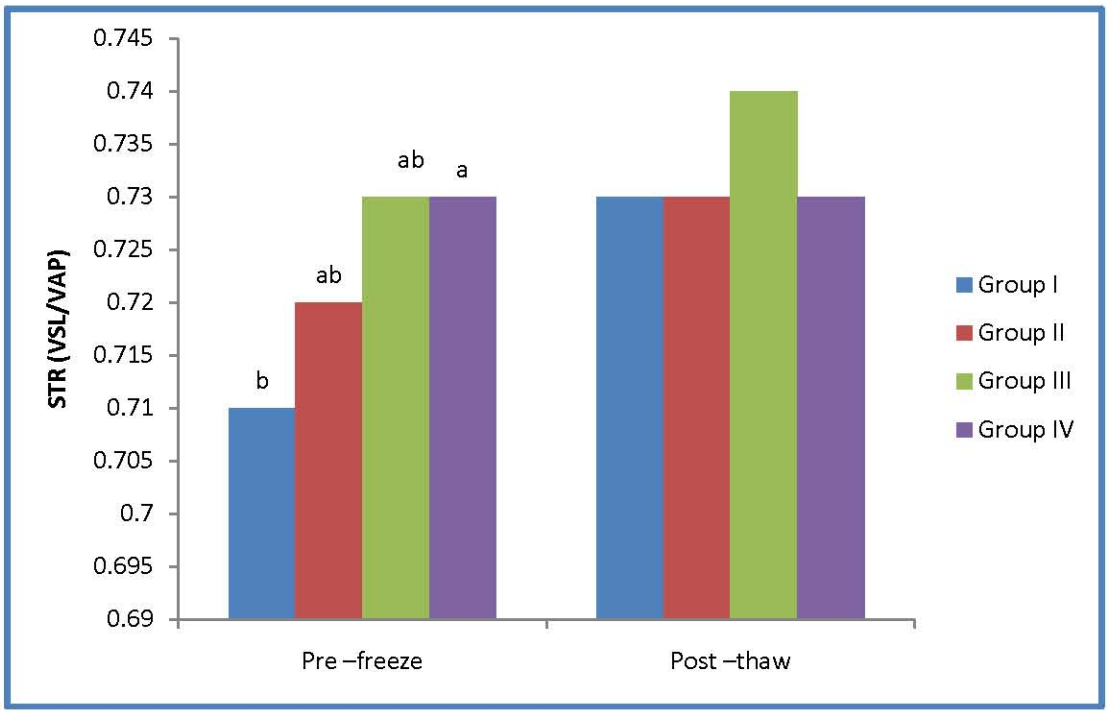


Figure 29.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on straightness (VSL/VAP) of Hariana bull spermatozoa (CASA) during freeze-thaw process

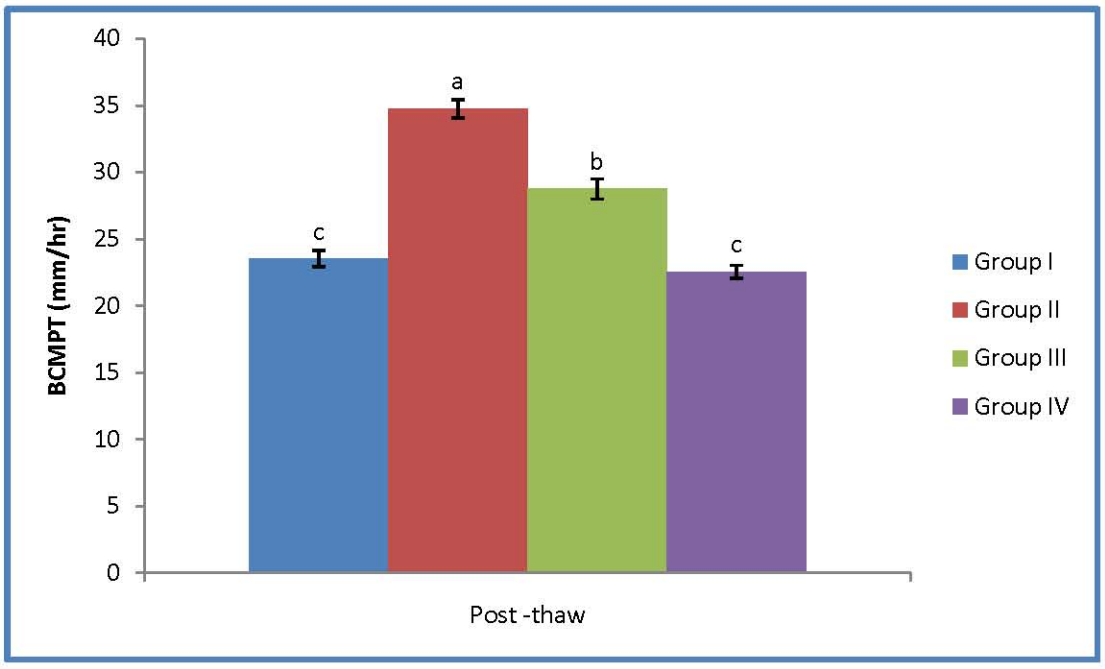


Figure 30.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on distance (mm/hr) travelled by spermatozoa in oestrus mucus as observed through Bovine Cervical Mucus Penetration Test of Hariana bull spermatozoa during post-thaw process

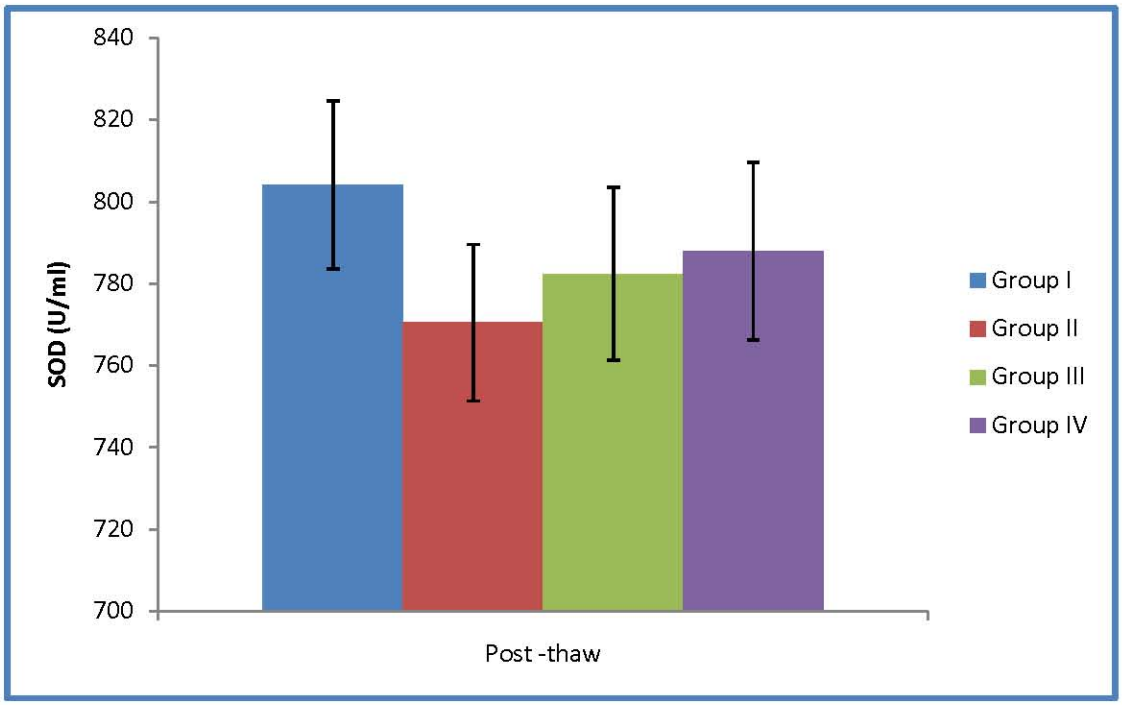


Figure 31.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on superoxide dismutase (SOD) activity (U/ml) in the seminal plasma of Hariana bull semen during post-thaw process

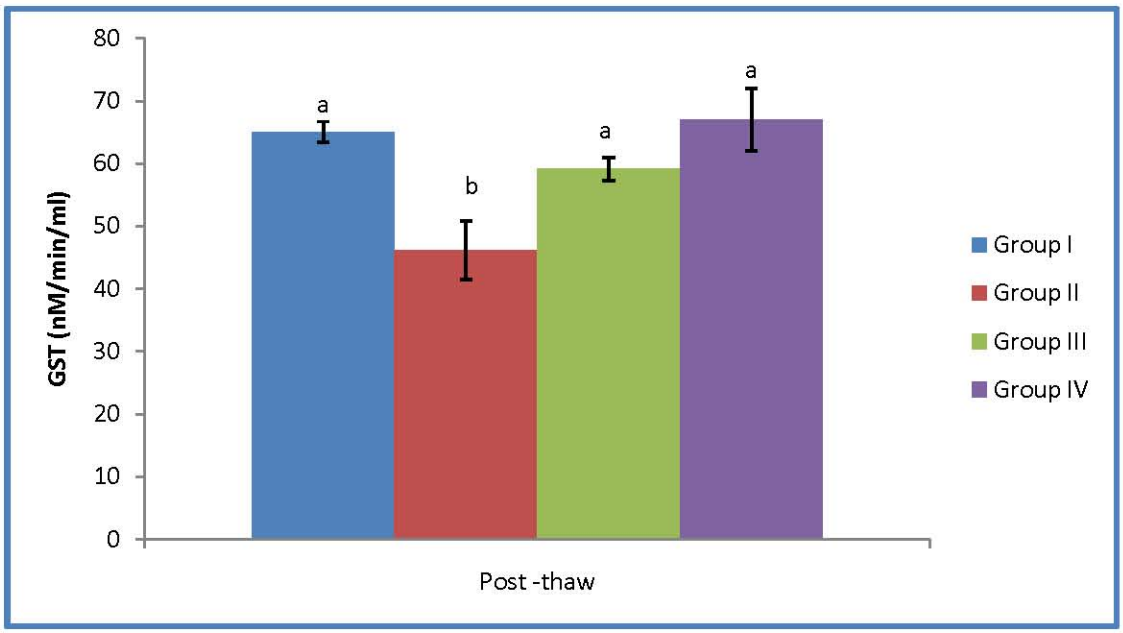


Figure 32.0: Graphical presentation of effect of different concentrations of lyocopene (GroupI- control, GroupII- 0.25mM, GroupIII- 0.5mM and GroupIV- 1mM) on glutathione-S transferase (GST) activity (nM/min/ml) in the seminal plasma of Hariana bull semen during post-thaw process

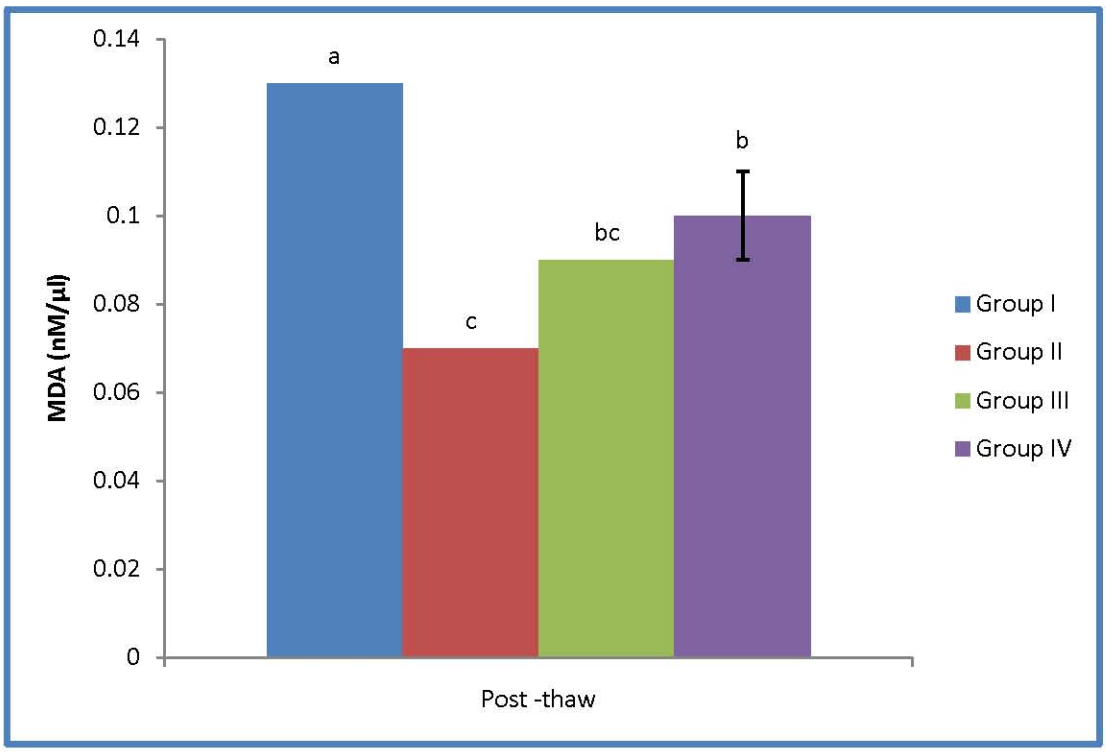


Figure 33.0: Graphical presentation of effect of different concentrations of lyocopene (Group I- control, Group II- 0.25mM, Group III- 0.5mM and Group IV- 1mM) on Malondialdehyde (MDA) activity (nM/µl) in the seminal plasma of Hariana bull semen during post-thaw process

(3) Malondialdehyde (MDA) activity (nM/µl)

The relevant data has been presented in Table 9.0 and illustrated in Figure 33.0.

At post-thaw stage, the level of MDA activity (nM/µl) was found as 0.13±0.00 in group I. The respective values in group II, group III and group IV were 0.07±0.00, 0.09±0.00 and 0.10±0.01 (nM/µl). MDA activity was found to be significantly ($P<0.01$) lower in group II compare to group I, III and IV.

Discussion

CHAPTER-5

DISCUSSION

Lycopene has been used for semen cryopreservation of animal species like Bull, Buck, Ram, Rabbit, Fowl, Turkey and Dog with varying degree of beneficial effect on semen cryopreservation of these species. It was further postulated that Lycopene can be useful upto certain extent for cryopreservation of bull spermatozoa.

In the present study, four Hariana bulls having their age between 5.0 to 9.0 years and weighing around 450 - 600 kg were used for the collection of semen. Semen was collected twice a week from each bull by the means of artificial vagina and was evaluated for the various seminal attributes e.g. volume (ml), seminal pH, mass motility (0-5 scale), concentration (millions / ml), progressive motility, live percent. The ejaculate which seems to be apparently normal, having ≥ 3.0 mass motility, ≥ 70 % progressive motility and concentration ≥ 1200 millions/ml were selected for further cryopreservation.

Ejaculates fulfilling the criteria were subjected to extension with egg-yolk-tris along with supplementation of lycopene in different concentrations. The extended semen was evaluated at pre-freeze and post-thaw stage for progressive motility, live spermatozoa, membrane integrity (HOST), spermatozoa with intact acrosome (Giemsa), capacitation status of spermatozoa, sperm motility & kinematics, *in vitro* fertility test (BCMPT) and antioxidative enzymes (SOD, GST, LPO [MDA]). The major focus of the study was to evaluate the effect of lycopene on various attributes of Hariana bull semen during the freez-thaw process. Following freezing, semen was stored in LN_2 container and was thawed after 24 hours of storage. The thawed semen samples were than evaluated for above cited seminal attributes. The entire results have been discussed as under.

5.0 Seminal attributes in the freshly collected semen of Hariana bulls

1. Ejaculate volume

Ejaculate volume is the most important parameter as the number of breeding doses of A.I. depends on the ejaculate volume and sperm concentration.

In the present study, the ejaculate seminal volume varied significantly ($P<0.05$) among the four Hariana bulls and ranges between 4.28±0.33 ml to 5.30±0.40 ml. Earlier information for this parameter from the same lab revealed a mean volume of 4.38±0.47 ml to 6.58±0.38 ml (Sachan, 2013), 6.38±0.74 ml (Patel, 2014), 5.91± 0.44 ml (Yadav, 2014), 5.65±0.23 ml (Shah, 2016), 5.54±0.21 ml (Yadav, 2017), 5.86±0.17 ml (Yadav, 2018) and 5.12±0.23 ml (Rathore, 2019). Our results are in concurrence with earlier reports and reflect the normal semen volume of this breed of animal. The volume of semen was reported to vary with breed, age, body or scrotal size, frequency of collection, exercise, teasing, season, level of nutrition, reproductive health and method of collection (Hafez & Hafez, 2000). The variation in semen volume in our finding could be because of the any reasons as stated vide supra.

2. pH

pH of the semen regulates the acid balance in the sperms in terms of prostate secretions. The rise in the pH is an indication that the sperms neutralizing capacity in terms of alkali/buffer secretion is poor. The activity of proton channels present in the sperms is also increased and hence an acidification of semen. The change in the pH of semen affects the sperm motility and fertility ability (Purdy, 2006).

In the present study, the overall mean seminal pH of Hariana bull was found as 6.58±0.02 which did not differ significantly amongs the bulls. Earlier studies from the same lab reported a mean seminal pH of 6.84±0.04 (Sachan, 2013), 6.81±0.02 (Patel, 2014), 6.82±0.01 (Yadav, 2014), 6.59±0.03 (Shah, 2016), 6.63±0.03 (Yadav, 2017), 6.55±0.02 (Yadav, 2018) and 6.58±0.03 (Rathore, 2019). Our results are within the reported range indicating normal seminal pH.

3. Mass Motility

The mass motility of spermatozoa is a wholesome effect of sperm concentration and individual motility (Zemjanis, 1970). Sperm motility gives the first hand information regarding the fertilizing capacity of semen. It is positively correlated with keeping quality, freezability and fertility of semen sample as it decides whether to accept or reject the ejaculate sample for further processing and to use in artificial insemination. Sperm motility is crucial in facilitating passage through the cervix and utero-tubal junction and much more importantly in actual penetration of the cumulus cells and zona pellucida of the ovum (Hafez & Hafez, 2000).

In the present study, semen mass motility (0-5 scale) varied significantly ($P<0.05$) among the four Hariana bulls and ranged between 3.50±0.13 to 4.16±0.19. The overall mass motility of semen samples was reported as 3.84±0.08. Earlier studies from the same lab reported a mean mass motility of Hariana bulls as 3.38±0.13 to 3.75±0.16 (Sachan, 2013), 3.88±0.14 to 3.92±0.10 (Patel, 2014; Yadav, 2014), 3.79 ±0.09 (Shah, 2016), 3.83 ±0.08 (Yadav, 2017), 3.56 ±0.06 (Yadav, 2018) and 3.50±0.11 (Rathore, 2019). Our results are in concurrence with the earlier reports of the same laboratory.

The semen motility is affected by improper handling procedure, contaminated glasswares (presence of soap residue), chemicals present over the fingers, cold or hot test tubes, glass slides, microscope stage, rapid drying or cooling of glass slides, prolongation during collection and examination period etc. In the present study, the motility was accessed keeping in mind of these mentioned effects.

4. Sperm Concentration

Accurate determination of the number of spermatozoa per millilitre of semen is extremely important because it is highly variable semen characteristics. When combined with the volume of the ejaculate this quality of spermatozoa determines how many female can be inseminated each with optimal number of sperm cells (Hafez & Hafez, 2000).

The average sperm concentration in present study was in the range of 1663.75±124.27 to 1908.75±109.32 millions/ml which did not differ significantly amongs the bulls. The overall sperm concentration was reported as 1783.75±51.23 millions/ml. Earlier reports from the same lab revealed the average concentration as 1262.50±41.69 to 1517.50±195.05 millions/ml (Sachan, 2013), 1403.67±95.13 to 1479.42±96.32 millions/ml (Patel, 2014; Yadav, 2014), 1269.75±81.97 millions/ml and 1324.17±63.25 millions/ml in winter and summer seasons respectively (Singh, 2017), 825.66±27.82 millions/ml (Yadav, 2018), 1867.71±72.03 millions/ml (Rathore, 2019). In 1966, Tomar et al. reported the sperm concentration in Hariana bulls as 1150±53.44 millions/ml whereas Tomar and Kanaujia (1970) reported it as 1185±65.11 millions/ml. Banerjee and Ganguli (1973) reported sperm concentration in Zebu bull as 1091.8 millions/ml whereas Roberts (1982) suggested a range between 300-2500 millions/ml with an average of 1200 millions/ml. Tomar and Gupta (1984)

further reported the effect of season on the sperm concentration of Hariana bulls which ranges from 822.70±39.90 to 1193.30±51.20 millions/ml. Our result for sperm concentration is within the reported range.

Sperm concentration also varies with managemental practices such as restraint before semen collection, frequency of semen collection (Singh and Sharma, 2001), age of bull (Rao and Rao, 1975) etc. In the present study, all these factors have been taken into consideration and has been kept as nearly same for all the bulls except for age. The higher value could be due to this effect.

5. Progressive Motility

The ejaculated semen was diluted immediately after collection to observe the progressive motility. Sperm progressive motility is an important criterion of semen quality (Lasley, 1951) and is an important determinant of success rate of the fertilization and ability of spermatozoa to withstand the stress of cryopreservation process.

In the present study, the percentage of progressive motile spermatozoa of Hariana bulls was in the range of 82.50±0.94 to 86.25±1.57 which did not differ significantly amongs the bulls. The overall percentage of progressively motile spermatozoa was reported as 84.22±0.68. Earlier reported values of progressive motility from the same lab were 87.83±0.58 to 87.92±068 (Patel, 2014; Yadav, 2014), 87.50±1.15 (Shah, 2016), 85.00±0.95 (Yadav, 2017), 82.81±0.55 (Yadav, 2018) and 83.93±1.07 (Rathore, 2019). Our results are in concurrence with these reported values and are considered to be in normal range. No significant difference in progressive motile spermatozoa were observed amongst differed bull, hence all bull behaves similar for this parameter.

6. Live Spermatozoa

The percentage of live spermatozoa determines the quality of ejaculate. When normal semen has been handled properly and staining is carried out correctly, the percentage of sperm staining alive is highly correlated with individual progressive motility, but the percentage of motility is usually lower than the percentage of live spermatozoa as many of the live sperm may not have motility (Hafez & Hafez, 2000). Viability of spermatozoa is significantly and positively correlated with initial motility, post-thaw motility and fertility of spermatozoa (Foote et al., 2002).

In the present study, the percentage of live spermatozoa of Hariana bulls was in the range of 89.76±0.64 to 90.96±1.00 which did not differ significantly amongst the bulls. The overall percentage of live spermatozoa was reported as 90.32±0.42. Sachan (2013) reported a range 84.75±2.52 to 90.38±0.91 percent. Patel (2014) and Yadav (2014) reported a range of 94.99±0.48 to 95.17±0.37 percent. Shah (2016) reported the mean percentage of live spermatozoa as 92.65±0.85. Yadav (2017) reported the mean percentage of live spermatozoa as 93.00±0.57. Yadav (2018) reported the mean percentage of live spermatozoa as 87.97±0.54. Present findings lie within the range reported by Sachan (2013). Comparing these results with the result of progressive motility clearly indicates that some of the live spermatozoa were not motile.

Semen samples containing initially less than 70 per cent live spermatozoa are of questionable fertilizing capacity and are not good for freezing whereas samples containing more than 70 per cent live spermatozoa showed no difference in fertilizing capacity. Pant et al. (2003) reported that semen with more than 30 per cent initial dead spermatozoa is not good for preservation. Keeping these reports in consideration, in the present study all semen samples which were having >70 per cent live sperm were subjected to cryopreservation.

5.1 Effect of lycopene on semen quality of Hariana bull at pre-freeze and post-thaw stages of cryopreservation

After the evaluation of freshly collected semen, samples that qualify the criteria were further processed. The ejaculated semen was divided into four portions. A portion of fresh semen was extended with EYTG and considering as Group I (Control), while the other three portions were extended with EYTG along with different doses of lycopene i.e. 0.25mM, 0.5mM and 1mM and were considered as group II, group III and group IV respectively. These samples were then evaluated for various seminal attributes at pre-freeze and post-thaw stages. The obtained results are discussed as below.

1. Progressive motility

Individual progressive motility is considered as one of the important criteria in accessing the semen quality as it is indicative of fertilization rate.

In the present study at pre-freeze stage, lycopene in the concentration of 0.25 mM was found to be highly significant ($P<0.01$) as compare to the control and other lycopene treated groups. The same trend was observed at post-thaw stage.

At post-thaw stage, the percentage of progressive motile spermatozoa in 0.25 mM was found to be 50.16±29.17 per cent which was significantly ($P<0.01$) higher as compared to control as well as other treatment groups (group III & IV). Thus in the present study 0.25 mM lycopene was found to be effective compared to the other two doses used. Further, our results suggest that on increasing the concentration of lycopene, a negative impact on progressive motility was observed.

Tvrda et al. (2017) used lycopene in the concentration of 1.5 mM for bull semen and found a positive effect of lycopene. The average post-thaw motility recorded was 43.01±0.91 per cent. Similarly, others (Bucak et al., 2015 and Ren et al., 2018) have reported a positive effect of lycopene on PM when used in $1X10^{-3}$ g ml^{-1} ($\cong$ 1.86 mM) concentration.

Our results are in accordance with the result of the above reports where a positive effect of lycopene in cryopreservation of bull spermatozoa is reported. Further, to our observation, Uysal and Bucak (2007) also find a decline in motility following the increase in concentration of lycopene.

2. Live Spermatozoa

Liveability is one of the major factor for assessment of semen quality. During cryopreservation, the spermatozoa are exposed to an exogenous diluting media as well as to low and very low temperature. Death might occur due to release of toxic substances, ultra low exposure, enzymatic leakage, medium of preservation, degree of sperm permeability, aging effect of sperm and individual variation. Reduction in the viable sperm percentage may be due to disruption of sperm plasma membrane leading to reduced mobility and fertilizing ability (Maxwell and Watson, 1996).

In the present study at pre-freeze stage, lycopene at the concentration of 0.25 mM was found to result in significantly ($P<0.01$) higer motility as compared to the control and other lycopene treated groups. The same trend was observed at post-thaw stage.

At post-thaw stage, the percentage of live spermatozoa in 0.25 mM was found to be 68.88±0.39 per cent which was significantly (P<0.01) higher as compared to control as well as other treatment groups (group III & IV). Thus in the present study 0.25 mM lycopene was found to be effective compared to the other two doses used. Further, our results suggest that on increasing the concentration of lycopene, a negative impact on liveability was observed.

Bucak et al. (2015) used lycopene in the concentration of $1X10^{-3}$ g ml^{-1} (≅ 1.86 mM) and reported a non significant (P>0.05) effect of lycopene with respective values as 31.7±3.9 and 36.2±6.4 per cent (control vs. treated group). Similarly, for turkey and rabbit spermatozoa, Rosato et al. (2012) did not find any effect of lycopene.

Our results are in accordance with the result of Uysal and Bucak (2007) who also report a decline in liveability of spermatozoa following the increase in concentration of lycopene.

3. HOST

The HOST highlights the permeability of sperm membrane to Hypo-osmotic solution and the projection of higher value is a valid indication of intact membrane and sample with higher value is regarded as potent for establishing pregnancy.

In the present study at pre-freeze stage, lycopene at the concentration of 0.25 mM was found to result in significantly (P<0.01) higher HOST responsive spermatozoa as compared to the control and other lycopene treated groups. The same trend was observed at post-thaw stage.

At post-thaw stage, the percentage of HOST responsive spermatozoa in 0.25 mM was found to be 63.90±0.54 per cent which was significantly (P<0.01) higher HOST responsive spermatozoa as compared to control as well as other treatment groups (group III & IV). Thus in the present study 0.25 mM lycopene was found to be effective compared to the other two doses used. Further, our results suggest that on increasing the concentration of lycopene, a negative impact on percentage of HOST responsive spermatozoa was observed.

Ren et al. (2018) used lycopene in Cashmere goat semen in concentration ranging between 0.5 to 4 mg/ml (≅ 0.93 to 7.45 mM) and reported significant

(P<0.05) effect of lycopene in all the used concentration. However, for turkey and rabbit spermatozoa, Rosato et al. (2012) find a non significant (P>0.05) effect of lycopene.

Our results are in agreement with the positive effect of lycopene as reported in other species. Further, our results are in accordance with the result of Uysal and Bucak (2007) who reported a decline in HOST responsive spermatozoa following the increase in concentration of lycopene. Moreover, Kumar et al. (2015) opined that antioxidant additives exhibited cryoprotective activity on sperm in moderate doses, however, increasing doses of antioxidant additives result in a hypertonic property of extender which impairs sperm functions.

4. Acrosomal Integrity

The acrosome reaction is a process which takes place in living spermatozoa in response to natural inducers. Acrosome serves as the limiting structure of fertilization which regulates the spermatozoa fusion with the oocyte and mediates its penetration into the oocyte. Any damage to acrosome and acrosome structures causes loss of functional competence of acrosome and ultimately a failure of fertilization (Wells et al., 1970).

In the present study at pre-freeze stage, lycopene at the concentration of 0.25 mM was found to result in significantly (P<0.01) higher as compared to the control and other lycopene treated groups. The same trend was observed at post-thaw stage.

At post-thaw stage, the percentage of intact acrosome in 0.25 mM was found to be 66.04±0.81 per cent which was significantly (P<0.01) higher as compared to control as well as other treatment groups (group III & IV). Thus in the present study 0.25 mM lycopene was found to be effective compared to the other two doses used. Further, our results suggest that on increasing the concentration of lycopene, a negative impact on acrosomal integrity was observed.

Tvrda et al. (2017) used lyco pene in the concentration of 1.5 mM for bull semen and found a positive effect of lycopene. The per cent acrosomal integrity recorded was 85.20±0.42. Similarly, AI- Sarray et al. (2019) have reported a positive effect of lycopene on acrosomal integrity. Whereas in other studies, (Uysal and Bucak, 2007; Rosato et al., 2012 and Bucak et al., 2015) no significant (P>0.05) effect of lycopene was observed.

Freeze-thaw process induces the generation of free radicals which target the acrosome to bring out acrosome damage as well as mediate acrosomal exocytosis causing preterm acrosome reaction. Various acrosomal defects have been linked to cause reduced fertility (Anderson et al., 1990; Thundathil et al., 2000; 2001; 2002).

5.2 Evaluation of capacitation status

The presence of functional acrosome is required for capacitation and acrosome reaction that are critical steps in the process of fertilization (Thomas et al., 1997). A significant positive correlation exists between the percentage of intact acrosome and fertility of frozen bovine spermatozoa (Saacke and White, 1972). The LDLs (lecithin) interacts with seminal plasma proteins, decrease the efflux of cholesterol and phospholipids from the spermatozoa membrane and prevent premature capacitation and subsequent acrosome reaction.

The ejaculated spermatozoa are fertilization incompetent and acquire the ability to fertilize the oocyte only after a maturation process in the female genital tract known as “capacitation”. The process of capacitation prepares the spermatozoa to undergo acrosome reaction without which there would be no fertilization. Hence, capacitation is the key event in reproduction. Bailey et al. (2000) reported that capacitation-like changes occurs in cryopreserved spermatozoa of bovine, porcine, equine and hamster and it decreases the functional life of spermatozoa leading to reduced fertility.

In the present study, capacitation status was determined using CTC technique. We observed a higher percentage of uncapacitated sperm at 0.25 mM concentration of lycopene. The effect was significantly superior compared to other treated groups (concentration of lycopene as 0.5 mM & 1 mM) as well as the control. Contrarily, the capacitated & acrosomal reacted spermatozoa was significantly ($P<0.01$) reduced in 0.25 mM. The trend in pre-freeze stage was replicated in post-thaw stage for uncapacitated and capacitated spermatozoa; however, acrosomal reacted spermatozoa were less in 0.25 mM lycopene group & in control sample.

An overall view of capacitation study based on our finding revealed that 0.25 mM lycopene control the cryopreservation induced capacitation of Hariana bull semen.

Perusal of available literature did not reveal any report where capacitation study was conducted with the use of lycopene; however, correlating with finding of intact acrosome (reported vide supra) carried by other worker, it can be presumed that capacitation will have followed the same action.

5.3 Effect of lycopene on kinematic parameters of Hariana bull spermatozoa

Sperm motion and velocity parameters are the indication of sperm motility and its kinetic parameters which will allow the sperm to move in the female genital tract to bring fertilization. Although, these parameters are evaluated using a high frame rate and resolution camera along with an integrated software, it gives accurate and precise assessment of sperm movement and its ability to reach the oocyte. Various conflicting reports are available in the literature regarding the sperm motion and kinetic values in different species of animals before freezing and after freezing-thawing. Medium as well as extender used in the semen during freezing also affects the sperm kinematics and therefore, to obtain consistent values standardization of CASA parameters is highly essential.

In present study, all the kinematics values were increased in group II (0.25 mM) compared to group I (control) and other lycopene treated groups (group III and group IV). Improvement in kinematic parameters indicates the effect of lycopene in regulation of sperm motility and velocities.

In earlier studies, different workers have used lycopene in different concentrations in different species of animals and have reported difference in motion and velocity parameters. Tvrda et al. (2017) used bull semen supplemented with 1.5 mM lycopene and reported higher VAP, VSL, VCL, ALH, BCF, STR and LIN values as compared to control after freezing and thawing. However, the kinematic and path velocities reported values were different compared to the values of present study and this may be due to the concentration of lycopene used or may be the different CASA set up or may be the extender along with different breed of bull used. In another study, Tuncer et al. (2014), in his studies on Holistein bulls used 500µg/ml lycopene and evaluated the sperm velocity parameters. All sperm kinematic parameters in lycopene treated group compared to control were having higher values except, BCF. Bucak et al. (2015), in his studies on Holistein bulls also reported higher VAP, VSL, VCL, ALH and LIN values as compared to control. Altogether, these studies indicated

the role of lycopene in increasing the kinematic and velocity parameters after freezing-thawing in lycopene added groups and may be the values of the parameters were different but all of them were higher compared to the control.

Freezing-thawing spermatozoa suffers from serious setbacks like temperature variations, cold shock, osmotic changes, pH alterations, oxidative stress due to free radicals, cryo-capacitation and apoptosis like changes. Freezing-thawing also affects the antioxidant enzyme and defense system of spermatozoa and lowers the metabolic activities of spermatozoa. Along with this, there is reduction in viability and motility of the spermatozoa due to lowering of mitochondrial activity. All these factors result in poor survival and fertilizing competence of freezing-thawing spermatozoa. Studies have shown that the kinematic and motion parameters of the sperm cells are also greatly affected and results in reduction in migration of spermatozoa through cervical mucus. It has also been established in many studies that lowering of kinematic parameters are highly correlated with poor fertilizing ability of frozen-thawed spermatozoa. In the study, viability, membrane integrity, acrosomal integrity and progressive sperm motility were decreased after freezing-thawing in all groups but these values were higher in group II indicating the protective effects of lycopene. It was also noted that, higher Vanguard distance in group II indicating that motion and velocity parameters may be responsible for higher Vanguard distance and membrane protection potential of lycopene. Increase in VCL is an indication that spermatozoa were hyperactive; increased LIN indicated the faster and precise movement of the spermatozoa towards the oocyte; increased ALH was an indication that spermatozoa head was laterally shifted indicating hyperactivity; increased VSL indicated that the spermatozoa were active enough to move in straight line; increased in VAP was an indication that the average path velocity of the spermatozoa was increased so that the sperm may be able to reach the site of oocyte with greater motion potential; increased BCF was an indication of higher flagella beating and more faster movement of the spermatozoa; and increase in STR was an indication of straight movement of spermatozoa which is highly essential for the spermatozoa to reach to site of fertilization. Lycopene addition in semen extender at 0.25 mM concentration increased all the kinematic and motion parameters of spermatozoa which are indicators of better fertilizing competence of spermatozoa.

Average path velocity (VAP) indicates the average path covered and is correlated with directional movement of the sperm; Velocity of straight line (VSL) indicates the sperm capacity for the forward progression and it is positively correlated with travel time in the female genital tract. The degree of sperm movement away from the natural straight path is known as curve linear velocity (VCL); both VCL and VSL affect the movement of spermatozoa in the female genital tract. Studies have shown that, spermatozoa having higher VSL are more capable of crossing the mucosal barriers and to reach to the site of fertilization. It was interesting to obtain higher values of these parameters in the present study in Group II indicating the effect of lycopene on sperm kinematic parameters. Wobble (WOB %) and beat cross frequency (BCF %) indicates the oscillation of sperm trajectory about its averaged path and are the indicators of the spermatozoa having higher flagellar beating potential to cross the mucosal barriers.

Results of the present study indicated the effective cryoprotection ability of lycopene on sperm functional attributes and in specific, sperm kinematic parameters. The marked improvements in velocity and motion parameters in lycopene treated group compared to control is an indication that lycopene favors the sperm movement as well as protects the system which are associated with sperm movement in the female body. The lowering in velocity parameters after freezing-thawing may impair sperm movement; however, addition of lycopene has provided beneficial effect on sperm from the cryo-injuries during freezing-thawing.

5.4 Effect of lycopene on *in vitro* fertility test of Hariana bull spermatozoa.

1. Effect of lycopene on BCMPT of Hariana bull spermatozoa

The *in-vitro* sperm mucus penetration test (SMPT) is a sperm function test which measures the ability of sperm in the semen to swim up into a column of cervical mucus or substitute. Fertilizing capacity of spermatozoa has been shown to be strongly related to cervical mucus penetration test (CMPT) in bull (Tas et al., 2007).

In our study at post-thaw stage, maximum penetration distance was found in the group II and III having 0.25 mM and 0.5 mM lycopene compared to group I (control) and group IV having 1 mM lycopene.

Different researchers have reported CMPT results ranging from 14 to 58.7 mm (Okuda et al., 1988; Matousek et al., 1989; Galli et al., 1991; Kumar et al., 2001).

David et al. (1979) observed a stronger correlation between sperm motility and cervical mucus penetration by spermatozoa whereas as other (Okuda et al., 1988; Matousek et al., 1989; Galli et al., 1991; Verberckmoes et al., 2002) did not found any positive correlation between spermatozoon migration ability and fertility. This may possibly be explained by the fact that evaluation of the vanguard spermatozoon alone would not represent all spermatozoa in the ejaculate.

The rate of sperm penetration and bull fertility is not correlated (Matousek et al., 1989) but the contradictory results of homologous mucus penetration test is significantly correlated with sperm concentration, progressive motile spermatozoa, mean velocity of progression and lateral head displacement of progressive motile spermatozoa as demonstrated in human and cattle (Murase and Braum,1990). During this study, spermatozoa having high curve linear velocity, average path velocity, amplitude of lateral head displacement were found significantly higher in 0.25 mM lycopene added group II and group indicating that higher hyper activation of spermatozoa, results in increased *in-vitro* fertilization.

Perusal of available literature did not reveal any report where cervical penetration test was conducted with the use of lycopene.

5.5 Effect of lycopene on seminal plasma enzymatic profile

1. Superoxide dismutase (SOD) activity (U/ml)

Superoxide dismutase (SOD) is a known enzymatic antioxidant that scavenges superoxide and peroxide and thus controls the oxidative stress in mammalian sperm (Fridovich, 1985). SOD protects spermatozoa by catalysing the dismutation of superoxide anions (O_2^-) to hydrogen peroxide (H_2O_2) and oxygen (O_2) (Agarwal et al., 2003). The physiological role of superoxide dismutase is to protect cells from the toxic effects of superoxide anions (O_2^-) generated during enzymatic oxidation of NADPH in neutrophils and mitochondrial electron-transfer reactions.

In the present study, we could not find any significant ($P>0.05$) effect of supplementation of lycopene (0.25 mM to 1 mM) on SOD activity (U/ml) at post thaw stages compared to the control sample. The SOD activity in the post thaw seminal plasma was 804.07±20.51 U/ml in group I (control) whereas in different treatment groups (group II to group IV) the respective values were in the range of 770.51±19.12 to 787.91±21.69 U/ml.

Tvrda et al. (2016), did not find any significant effect of supplementation of lycopene in the bull semen on SOD activity, which is in confirmation to our results.

2. Glutathione-S-Transferase (GST) activity (nM/min/ml)

GST is a part of glutathione cycle that is responsible for detoxification of H_2O_2 (Li, 1975) and prevention of sperm membrane fragility (Aitken et al., 1993). Increase in GST activity is indicative of enhanced antioxidant ability (Perumal et al., 2013). Therefore higher GST values in the semen reflect a strong sperm membrane and limited spontaneous lipid peroxidation that destroys the structure of the lipid matrix (Mohanty et al., 2004).

In the present study, we observed higly significant ($P<0.01$) effect of lycopene at concentration of 0.25 mM in protection of sperm cell at post-thow stage. In post-thaw semen samples, the GST activity was observed as 65.09±1.63 nM/min/ml in group I (control) whereas in lycopene treated groups (group II to group IV), the respective value were in the range of 46.17±4.72 to 66.99±4.99 nM/min/ml.

Perusal of available literature did not reveal any report where Glutathione-S-Transferase (GST) activity study was conducted in bull seminal plasma with the use of lycopene.

3. Malondialdehyde (MDA) activity (nM/µl)

Under stress conditions, a cell undergoes lipid peroxidation and loses its function (Aitken et al., 1989). The activity of lipid peroxidation can be evaluated by measuring MDA level (Kasimanickam et al., 2006). Jones and Mann (1973) used MDA as an index of spontaneous lipid peroxidation of sperm cells. Thus, lower MDA activity reflects less lipid peroxidation.

In the present study, we found highly significant ($P<0.01$) effect of lycopene in controlling lipid peroxidation at post-thaw stage. Lycopene supplementation in semen @ 0.25 mM showed significantly lower values of MDA activity compare to control or other treatment groups.

Tvrda et al. (2016), did not find any significant effect of MDA in bull semen supplemented with lycopene which is contrary to our results.

Summary and Conclusions

CHAPTER-6

SUMMARY AND CONCLUSIONS

The present study was conducted on four Hariana bulls reared at Semen Biology Lab, Department of Gynaecology and Obstetrics situated at ILFC, DUVASU, Mathura, (U.P), India, during the session 2020-2021.

The present study was aimed with following objectives

- To study the effect of lycopene on physio-morphology properties of cryopreserved Hariana bull spermatozoa.
- To study the antioxidant activity of lycopene on cryopreseved Hariana bull semen.

Eight ejaculates from each bull were collected by means of artificial vagina and were evaluated for freshly collected semen attributes. The data are summarized as under.

6.0 Seminal attributes in the freshly collected semen of Hariana bull

The freshly collected semen was evaluated for volume (ml), pH, mass motility (0-5 scale), concentration (millions/ml), progressively motile spermatozoa (%) and live spermatozoa (%).

1) The overall mean seminal volume irrespective of the bull was recorded as 4.81±0.16 ml. The mean seminal volume differs significantly ($P<0.05$) amongst different bulls. The seminal volume of bull number H-623 was significantly ($P<0.05$) higher as compared to other bulls.
2) The overall mean seminal pH in the freshly collected semen of the Hariana bull was 6.58±0.02. The seminal pH did not differ significantly ($P>0.05$) amongst different bulls.
3) The overall mean mass motility of spermatozoa in the freshly collected semen of Hariana bull was 3.84±0.08 (0-5 scale). The mass motility differs significantly ($P<0.05$) amongst different bulls. The mass motility of bull number H-448 was significantly ($P<0.05$) higher as compared to other bulls.

4) The overall mean sperm concentration in the freshly collected semen of Hariana bull was 1783.75±51.23 millions/ml. The sperm concentration did not differ significantly (P>0.05) amongst different bulls.

5) The overall mean percentage of progressively motile spermatozoa in the freshly collected semen of Hariana bull was 84.22±0.68. The progressive motility did not differ significantly (P>0.05) amongst different bulls.

6) The overall mean percentage of live spermatozoa in the freshly collected semen of Hariana bull was 90.32±0.42. The live percentage of spermatozoa did not differ significantly (P>0.05) amongst different bulls.

All the seminal characters of different Hariana bulls were within normal range. The samples were processed for cryopreservation.

6.1 Effect of lycopene on different seminal attributes at pre-freeze and post-thaw stages of Hariana bull semen

After the evaluation of freshly collected semen, samples which fulfils the criteria as stated vide supra were processed for cryopreservation. Extended semen was divided into four parts in equal volume (5 ml each). To the first test tube was added 100 µl of DMSO (Control) where as in second, third and fourth test tube, lycopene stock (50mM) was added @ of 25 µl, 50 µl and 100 µl making the effective concentration of lycopene as 0.25mM, 0.5mM and 1mM. To balance the amount of DMSO in treatment groups, additionally 75 µl and 50 µl DMSO was added in second and third test tubes. Thus making the amount of DMSO same in control and treatment groups same. These diluted sample was than utilized for further processing. These samples were processed for cryopreservation and were evaluated for progressive motility, live spermatozoa, membrane integrity (HOST), spermatozoa with intact acrosome (Giemsa), capacitation status of spermatozoa, sperm motility kinematics, *in vitro* fertility test (BCMPT) and antioxidative enzymes (SOD, GST, LPO [MDA]) at pre-freeze and post-thaw stages of cryopreservation.

1) At both pre-freeze and post-thaw stages, per cent progressive motility of spermatozoa was significantly (P<0.01) higher in group II (0.25 mM) as compared to I (control), III (0.5 mM) and IV (1 mM) groups. At higher concentration (0.5 mM & 1 mM) of lycopene, a negative influence of lycopene was observed

compared to control. The post-thaw per cent progressive motility in treatment group II was 50.16±29.17.

2) At both pre-freeze and post-thaw stages, per cent live spermatozoa were significantly (P<0.01) higher in group II (0.25 mM) as compared to I (control), III (0.5 mM) and IV (1 mM) groups. At higher concentration (0.5 mM & 1 mM) of lycopene, a negative influence of lycopene was observed compared to control. The post-thaw per cent live spermatozoa in treatment group II was 68.88±0.39.

3) At both pre-freeze and post-thaw stages, per cent HOST reactive spermatozoa was significantly (P<0.01) higher in group II (0.25 mM) as compared to I (control), III (0.5 mM) and IV (1 mM) groups. At higher concentration (0.5 mM & 1 mM) of lycopene, a negative influence of lycopene was observed compared to control. The post-thaw per cent HOST reactive spermatozoa in treatment group II was 63.90±0.54.

4) At both pre-freeze and post-thaw stages, per cent intact acrosome was significantly (P<0.01) higher in group II (0.25 mM) as compared to I (control), III (0.5 mM) and IV (1 mM) groups. At higher concentration (0.5 mM & 1 mM) of lycopene, a negative influence of lycopene was observed compared to control. The post-thaw per cent intact acrosome in treatment group II was 66.04±0.81.

5) At both pre-freeze and post-thaw stages, per cent uncapacitated spermatozoa was significantly (P<0.01) higher in group II (0.25 mM) compare to group I, III & IV. On the other hand there was a significantly (P<0.01) reduction in capacitated and acrosomal reacted spermatozoa in group II (0.25 mM) compare to other groups. At post-thaw stage, the per cent uncapacitated spermatozoa was 56.20±0.87 where as capacitated & acrosome reacted spermatozoa was 35.00±0.75 & 8.79±0.63 in treatment group II.

6) At both pre-freeze and post-thaw stages, all the motion and kinematic parameters was significantly (P<0.01) higher in group II (0.25 mM) as compared to I (control), III (0.5 mM) and IV (1 mM) groups, except LIN, WOB and STR .

7) At post-thaw stage mean distance of vanguard spermatozoa was significantly (P<0.01) higher in group II as compared to group I, III and IV. The recorded mean distance travelled by spermatozoa was 34.75±0.68 mm/hr in treatment group II.

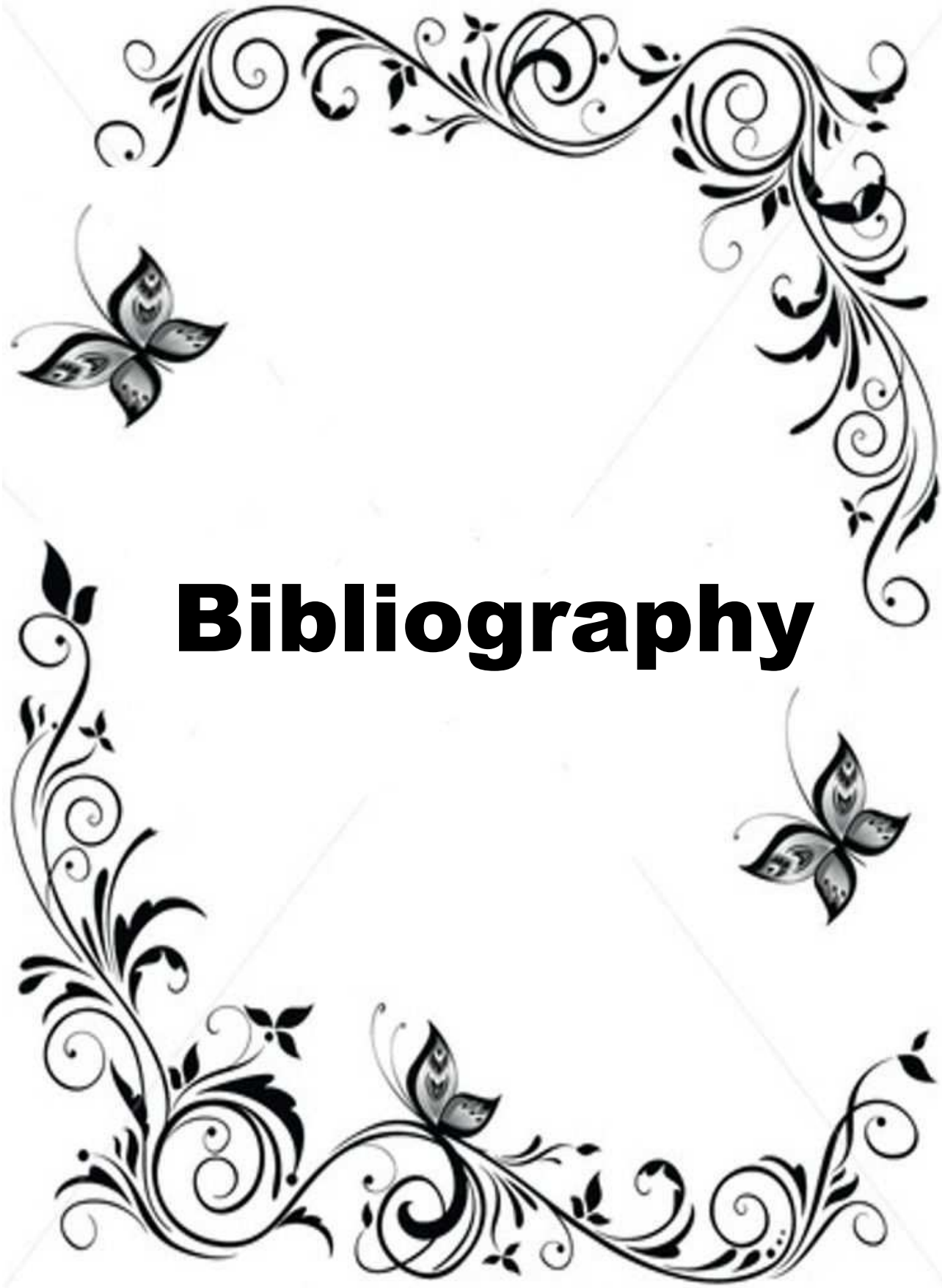
8) At post-thaw stage, the SOD activity (U/ml) did not differ significantly ($P>0.05$) between group I (control) and other lycopene treated groups (group II, III and IV).

9) At post-thaw stage, the GST activity (nM/min/ml) was significantly ($P<0.01$) decreased in group II as compared to group I, III and IV. The mean value of GST was 46.17±4.72 nM/min/ml in treatment group II.

10) At post-thaw stage, the MDA activity (nM/µl) was significantly ($P<0.01$) decreased in group II as compared to group I, III and IV. The mean value of MDA was 0.07±0.01 nM/ µl in treatment group II.

CONCLUSIONS

- Addition of lycopene @ 0.25 mM to egg yolk based extender improved the freezability of Hariana bull spermatozoa in terms of motility, viability, membrane integrity, acrosome integrity, capacitation status, sperm motion & kinematics.
- Further, lycopene @ 0.25 mM effectively control the oxidative stress of semen and improves the quality of spermatozoa based on cervical mucus penetration test.
- Lycopene at lower dose (≤0.25 mM) improves parmeters under study however, at higher concentration (≥0.5 mM) was found to have an inhibitory effect.



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UNDERTAKING OF COPY RIGHT

I, **Dr. Avaneesh Kumar Singh,** Enrollment No. **V-2005/18,** undertake that I give copy right to the DUVASU, Mathura of my thesis entitled, **"Studies on effect of lycopene on freezability of Hariana bull spermatozoa".**

I also undertake that patent, if any, arising out of research work conducted during the programme shall be filed by me only with due permission of the competent authority of U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalya Evam Go-Anusandhan Sansthan, DUVASU, Mathura (U.P.).

Avaneesh

(Signature of Student)