EFFECT OF PARTIAL DEOXYGENATION OF SEMEN DILUTOR WITH OXYRASE ON FREEZABILITY OF BULL SPERMATOZOA

Thesis

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Veterinary Science
(Veterinary Gynaecology and Obstetrics)

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Dedicated To...

My lovely Wife and Daughters for their patient during the whole time of two good years of my absence

Bihawa Salumu Ndwangira (Wife)

Neema Athanas Ngou (Mtoto)

Upendo Athanas Ngou (Mtoto)

Also to my parents, relatives, Supervisor and all who devoted their time and resources to make my career successful

भा.कृ.अनु.प.–भारतीय पशु चिकित्सा अनुसंधान संस्थान
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Dated:28th June, 2019

Certificate

This is to be certified that the research work embodied in this thesis entitled **"Effect of partial deoxygenation of semen dilutor with Oxyrase on freezability of bull spermatozoa"** *submitted by* **Dr. Athanas Alex Ngou, Roll No. M-5915,** *for the award of* **Master of Veterinary Science** *Degree in* **Veterinary Gynaecology and Obstetrics** *at ICAR-Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.*

It is further certified that **Dr. Athanas Alex Ngou, Roll No. M-5915,** *has worked for more than 21 months in the Institute and has put in more than 150 days attendance under me from the date of registration for the* **Master of Veterinary Science** *Degree in this Deemed University, as required under the relevant ordinance.*

S.Ghosh.

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Chairman
Advisory Committee

Certificate

We the undersigned members of Advisory Committee of **Dr. Athanas Alex Ngou, Roll No. M-5915,** *a candidate for the degree of* **Master of Veterinary Science** *with the major discipline in* **Veterinary Gynaecology and Obstetrics,** *agree that the thesis entitled* **"Effect of partial deoxygenation of semen dilutor with Oxyrase on freezability of bull spermatozoa"** *may be submitted in partial fulfilment of the requirement for the degree.*

We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented for the award of **Master of Veterinary Science** *Degree of this Institute.*

It is further certified that the candidate has completed all the prescribed requirements governing the award of **Master of Veterinary Science** *Degree of the Deemed University, ICAR-Indian Veterinary Research Institute, Izatnagar.*

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Place: ICAR-IVRI, Izatnagar

(Athanas Alex Ngou)

ABBREVIATIONS

%	:	per cent
µL	:	microliter
µmol	:	Micromole
$^{\cdot}O^{2-}$	:	Superoxide anion
AI	:	Artificial Insemination
ATP	:	Adenosine triphosphate
AV	:	Artificial vagina
C	:	Celsius
cm	:	centimeter
DNA	:	Deoxyribonucleic acid
DO	:	Dissolved Oxygen
DPX	:	Distyrene, a plasticizer, and xylene
FRAP	:	Ferric reducing ability of plasma
g	:	relative centrifugal force
GPx	:	Glutathione peroxidase
GR	:	Glutathione reductase
GSH	:	Glutathione
h	:	hour
H_2O_2	:	Hydrogen peroxide
HCl	:	Hydrochloric acid
HEPES	:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LPO	:	Lipid peroxidation
LN_2	:	Liquid Nitrogen
M	:	Molarity
MDA	:	Malondialdehyde
min	:	minutes
mL	:	millilitre
mm	:	millimeter
MSP	:	Minimum Standard Protocol
N	:	Normality

NADP	:	Nicotinamide adenosine di phosphate
nm	:	Nanometer
nmole	:	Nanomole
OD	:	Optical density
$OH^{\cdot}$	:	Hydroxyl radical
$OHCl^{\cdot}$	:	Hypochlorite radical
OS	:	Oxidative stress
ppm	:	Parts per million
PS	:	Phosphatidylserine
PUFA	:	Polyunsaturated fatty acids
ROS	:	Reactive Oxygen Species
rpm	:	revolutions per minute
SD	:	Standard deviation
SEM	:	Standard error of mean
SOD	:	Superoxide dismutase
TAC	:	Total antioxidant capacity
TB	:	Trypan Blue
TBA	:	Thiobarbituric acid
TCA	:	Trichloro acetic acid
TPTZ	:	2, 4, 6-tripyridyl-*s*- triazine
Tris	:	Tris (hydroxyl methyl) amino methane
TYG	:	Tris-Yolk-Glycerol

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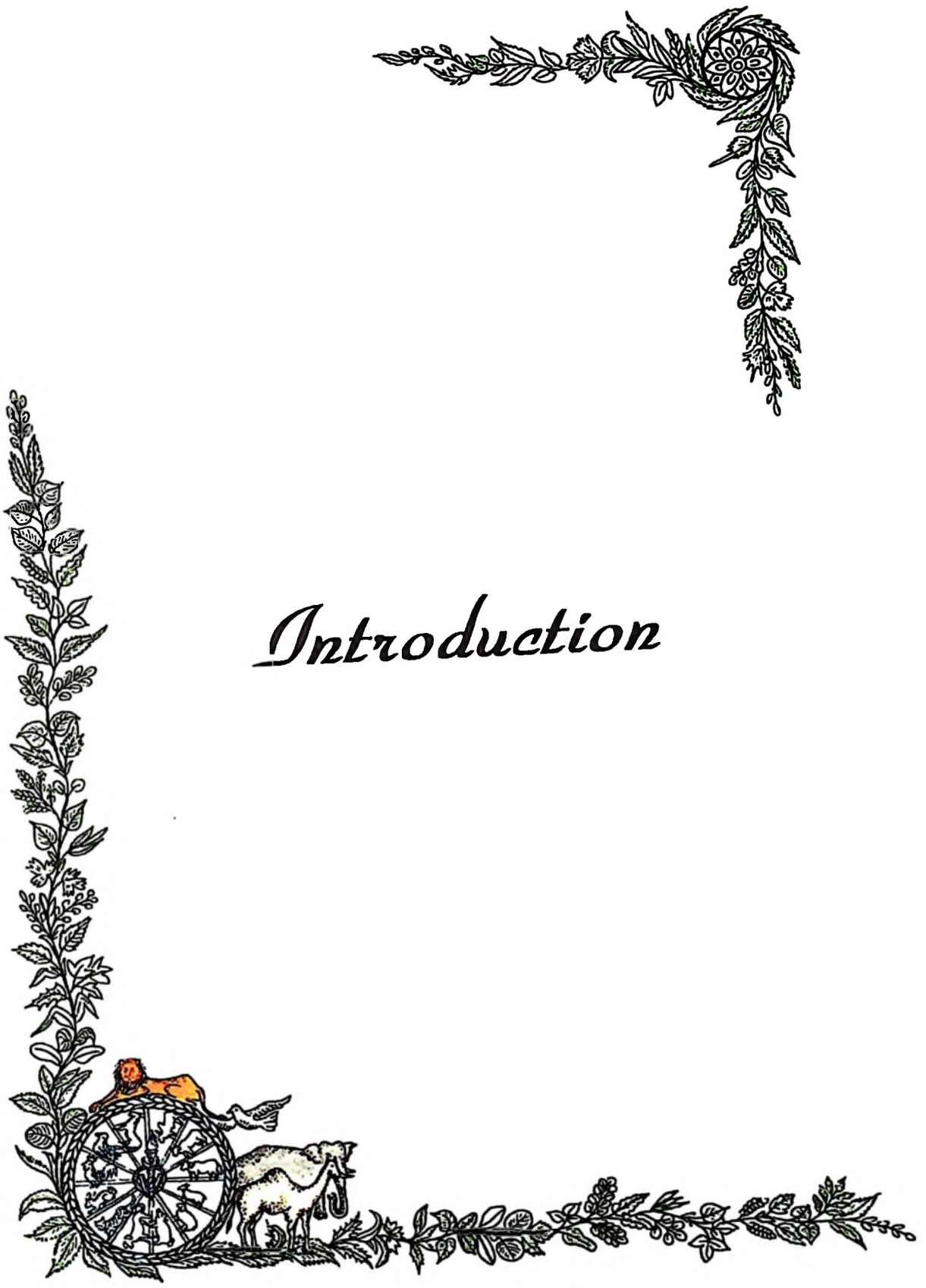

Introduction

1 INTRODUCTION

In the present-day, artificial insemination (AI) is the simplest and largely used animal reproductive technology to enable extensive use and spread of germ-plasm from genetically improved sires. The convenience of AI can wholly be achieved when there is a vast use of frozen germ-plasm, as it allows imprecise storage and delivery.

Cryopreservation of semen is accompanied with solidification of water which is usually lethal to the spermatozoa. Paradoxically freezing is the only way which preserves spermatozoa for long periods of time in a viable state (Mazur, 1984). Discovery of glycerol as a cryoprotectant by Polge *et al.*, (1949) allowed semen to be stored for a long time in liquid nitrogen *i.e.* at -196°C. Various studies have shown the effects of cryopreservation in spermatozoa. Watson (2000) reported that about 40-50% of spermatozoa which do not survive the cryopreservation process even when the standardized protocols are followed. Similarly, Ghosh *et al.* (2007) reported the deleterious effects occurring to spermatozoa after freezing. That is why, there is poor recovery rate of viable spermatozoa at the post-thaw stage even when glycerol is used at recommended levels.

In most of the species, the use of frozen-thawed semen is facing number of bottlenecks that limit its use and wide applicability. Temperature stress (cold shock), osmotic stress (ice crystal formation and recrystallization) and oxidative stress (during cooling, freezing and thawing) prevent broad success and widespread standardization of cryopreservation protocols (MacLeod, 1943; Gao and Critser, 2000; Martorana *et al.*, 2014). The response of

spermatozoa to stress does vary among species, among individuals and among ejaculates. This reflect the level of spermatozoa sensitivity to the freeze-thaw process (Mayers, 2012).

The freezing process is preceded by the process of cooling of semen from body temperature to 4-5°C which is an important step in the cryopreservation process for most of the biophysical sense for spermatozoa survival upon thawing. Cooling process exposes spermatozoa to osmotic fluctuation and membrane changes that eventually lead to a state of oxidative stress (OS) and finally up-regulation of reactive oxygen species (ROS) (Koppers *et al.*, 2008; Salazar *et al.*, 2011; Martorana *et al.*, 2014).

The susceptibility of the spermatozoa mitochondria to OS is greatly detrimental to post-thaw spermatozoa survival because the integrity of these organelles is crucial to ATP production, Ca^{2+} homeostasis and other cell functions (Mazur, 1984). Successful low-temperature survival requires sperm to pass through numerous stressful events that are encountered. These events are: volume excursions, mechanical stresses, centrifugation, osmotic damage and cryopreservative toxicity (Mazur, 1984 and Mazur *et al.*, 2000). Oxidative by-products are believed to be the major contributing factor to sperm cell death and poor fertility of frozen sperm (Bansal and Bilaspuri, 2010).

The process of freeze-thaw results in injuries as it causes phase changes between intra and extra-cellular water. The most crucial point where most of the cryoinjuries occur is when the spermatozoa are passing the temperature between -15°C and -60°C during freezing (Gao and Critser, 2000). Cells and extracellular medium remain unfrozen and supercooled at -5°C. At temperatures between -5°C and -15°C, ice is formed in the surrounding medium, but the intracellular contents remain unfrozen and super-cooled. Because the chemical potential of water is higher in supercooled (intracellular) than in frozen (extracellular) state, water flows out of the cell and freezes externally. The cooling rate determines what occurs thereafter as it is the one which determines the extent of injury to the spermatozoa (Mazur, 1984).

Upon exposing the spermatozoa to low temperatures, cellular production of ROS increases and these results in amplified vulnerability to DNA, membrane, and mitochondrial oxidative attack from metabolic by-products compared to fresh spermatozoa (Chatterjee *et*

al., 2001; Druart *et al.*, 2009; Ortega *et al.*, 2010). Plasma membranes, particularly high in polyunsaturated fatty acids (PUFA), are extremely sensitive to peroxidation by ROS and these reactive molecules are produced by membrane exposure to oxygen (Griveau and Lannou, 1997). Consequently, peroxidation results in leaky membranes and eventually a total loss of motility (Alvarez and Storey, 1985 and Bailey and Buhr, 1994). A likely explanation for the loss of motility could be due to the proximity of the damage in sperm flagella to energy-generating mitochondria.

The mitochondria are the main source of ROS production (Koppers *et al.*, 2008), which are implicated in many pathological processes in spermatozoa cells (Pena *et al.*, 2009). Further, mitochondria are more likely to sustain damage from oxidative attack because mitochondrial DNA (mtDNA) is not protected by protamines and mtDNA may not be transcriptionally and translationally functional in ejaculated sperm, unlike the nuclear DNA. Regardless of the efforts so far attained on improving the spermatozoa defects following cryopreservation, still, the survival, acrosome integrity, motility and fertilizing ability of frozen-thawed mammalian spermatozoa are reduced (Ozkavukcu *et al.*, 2008; Yeste *et al.*, 2015).

Without neglecting the fact that O_2 has a vital role in ATP production, there is evidence that stallion sperm utilizes O_2 primarily by oxidative phosphorylation (Gibb *et al.*, 2014). Also, ROS production by the mitochondria is dependent on the O_2 availability in the extender during cooling as well as at freeze-thaw. The rate of lipid peroxidation (LPO) in spermatozoa and the rate of consequent motility loss are linear functions of the partial pressure of O_2 in the medium (Alvarez and Storey, 1985). Oxidative stress can be mitigated in two ways; first is reduction of generated ROS and second is reduction of the sources of ROS production (Amidi *et al.*, 2016). Antioxidants *i.e.* enzymatic (Superoxide dismutase, Catalase, Glutathione peroxidise and Glutathione reductase) or non enzymatic (Vitamin E, vitamin C, Zinc taurine, Hypotaurine, Glutathione and Selenium) and herbs (geistein, resveratrol and *Rhodiola sacra* extract) are the neutralizers of generated ROS (Asadpour,*et al.*, 2012; Amidi *et al.*, 2016). On the other hand, reduction of ROS production is achieved through lowering O_2 tension (partial deoxygenation) of the semen dilutor by either Freeze-Pump-Thaw cycling method (Pande *et al.*, 2015, Balamurugan *et al.*, 2017), mechanical method - Vacuum pump (Balamurugan *et al.*, 2017) or nitrogen gassing (Mustapha, 2017; Bhutia, 2018, Kumar *et al.*, 2018 and Amin

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et al., 2018). The other way is by removing the dead or damaged spermatozoa from the ejaculate by using anti-ubiquitin nanoparticles (Omer Din, 2017) or by swim-up or sephadex techniques (Ramamoorthy, 2018).

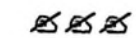
Oxyrase, the product containing an extract of bacterial electron transport system that in the presence of a suitable hydrogen donor substrate (*i.e.* lactate) can decrease O_2 levels in solutions to effectively low levels (Alder, 1990 and Kressin *et al.*, 1997). This enzyme act as oxygen scavenger obtained from *E. coli* membrane preparation. There are few studies on incorporation of Oxyrase in semen freezing extender as reported earlier (Koshimoto *et al.*, 2000; Mazur *et al.*, 2000; Dong *et al.*, 2010). Recent reports on incorporation of Oxyrase in the semen freezing extender improved post-thaw semen qualities of stallion (Darr *et al.*, 2016; London *et al.*, 2017).

So far there is no report on the use of Oxyrase and its effect on semen qualities of cattle bulls. The current study aimed at elucidating the levels of Oxyrase that would reduce dissolved oxygen (DO) in the Tris Egg Yolk dilutor and its effect on semen quality parameters at post dilution, pre-freeze and post-thaw period.

1.1. Objectives of the study

The present study intended at evaluating the effectiveness of the semen dilutor after partial deoxygenation using Oxyrase on semen quality parameters with the objectives:

- To standardize the levels of Oxyrase in semen dilutor
- To evaluate the effect of Oxyrase on semen freezability



Review of Literature

2 REVIEW OF LITERATURE

The whole process of semen cryopreservation exposes spermatozoon to oxidative stress and the mitochondrion is the organelle which is highly affected. Mitochondria need oxygen for energy production but the regulation of the use of oxygen is a problem. The susceptibility of the spermatozoa mitochondria to oxidative stress is greatly detrimental to post-thaw spermatozoa survival because the integrity of these organelles is crucial to ATP production, Ca^{2+} homeostasis and other cell functions. The current study intends to reduce the availability of oxygen in the extender used for semen extension. This will make less oxygen available for the spermatozoa and hence less oxidative stress.

2.0. Semen parameters

2.1. Colour and consistency

The colour and consistency of bull semen is influenced by concentration of spermatozoa, characteristics of breed, frequency of collection and agro climatic conditions (Bhakat *et al.*, 2014). Normally, bull semen is either creamy or milky in consistency depending on spermatozoa concentration. Sometimes the semen is yellowish appearance in colour which is due to the presence of riboflavin (Salisbury *et al.*, 1978).

2.2. Volume

Variation in volume of semen/ejaculate from breed to breed and from bull to bull, is influenced by several factors like body weight, scrotal circumference, age, pre-coital stimulation and frequency of collection (Gopalakrishna and Rao, 1978; Rao, B. and Rao A., 1978). Furthermore, the season during which the ejaculate is collected do contribute to the fluctuations

in volume (Swain and Singh, 2004; Bhoite *et al.*, 2008 and Rajoriya *et al.* 2015). Usually, the ejaculate volumes do ranges from 1-15 mL, with the mean of 6.29±0.28mL to 7.0±0.22mL (Patel *et al.*, 1989; Singh and Pangawkar, 1990). The average volumes reported in Sahiwal breed by various workers are 4.59±0.15 mL, 5.50±0.04 mL and 9.8 mL (Ahmad *et al.*, 2003; Hossain *et al.*, 2012; Rehman *et al.*, 2016).

2.3. Mass motility

This is mostly used parameter in assessing semen quality due to its simplicity (Mishra and Tyagi, 2006). It is highly correlated with concentration of spermatozoa, initial progressive motility as well as live spermatozoa counts (Rajoriya *et al.*, 2013). In addition, mass motility is affected by several factors like climate (Javed *et al.*, 2000), frequency of collection (Sayed and Oloufa, 1957) and age of the bull (Collins *et al.*, 1951).The mean mass motility was reported to be 2.6±0.04 with the range of 0.5-5. Higher values were recorded during spring (3.38±0.03) and low values (2.05±0.02) during summer (Ahmad *et al.*, 2003).

2.4. Concentration of spermatozoa

In order to cryopreserve the semen, spermatozoa concentration has to be elucidated first before deciding the level of dilution (Belorkar *et al.*, 1987 and Januskauskas *et al.*, 1996). The differences in spermatozoa concentration of an ejaculate may be due to various reasons like restraint before collection (Collins, 1951), season of the year and semen collection frequency (Gupta *et al.*, 1978; Singh and Prabhu, 1983). Furthermore, concentration is higher during spring (1.04±0.01 billion/mL) and low during summer (0.90±0.01 billion/mL) (Ahmad *et al.*, 2003).

2.5. Initial progressive motility

The capacity of mammalian spermatozoa to move is gained when it is passing through the epididymis during maturation phase of spermatogenesis. By the time when spermatozoa reach the cauda epididymis for storage they already attained the capacity to move and fertilize the oocyte (Bedford, 1975; Mann and Lutwak-Mann, 1981). Factors reported to affect initial progressive motility are age of the bull, temperature, frequency of collection and sexual

excitement before collection (Prasad *et al.*, 1999 and Javed *et al.*, 2000). The initial progressive motility of Sahiwal bulls have been reported to be 65.14±0.021% to 76.50±3.55% (Ahmad *et al.*, 2003 and Rehman *et al.*, 2016). In our lab records, the average initial progressive motility of Sahiwa bulls is more than 80% at fresh stage for the ejaculates utilized for freezing.

2.5.1. Effect of Oxyrase on spermatozoa motility

Previous study reported a significant improvement of motility of mouse semen when Oxyrase is incorporated in the dilutor and this is due to the anaerobiosis which induces motility (Koshimoto *et al.*, 2000). It has also been observed in our lab that, initial progressive motility is higher in semen extended with partially deoxygenised dilutor in crossbreed and buffalo bulls (Pande *et al.*, 2015, Mustapha, 2017; Amin *et al.*, 2018; Bhutia, 2018; Kumar *et al.*, 2018). Also, Oxyrase do confer protection to spermatozoa at both unfrozen and frozen samples containing cryoprotectants. Though, others reported that Oxyrase tend to lower the progressive motility of fresh extended semen at ambient temperature as well as frozen thawed stallion semen (Darr *et al.*, 2016 and London *et al.*, 2017). However, there is no report so far in bovine semen.

2.6. Viability

There is a straight and positive correlation between the fertility of a bull and the percentage of live spermatozoa in the semen (Swanson and Bearden, 1951). The percentage of live spermatozoa in crossbred (Holstein-Friesian × Brown Swiss × Jersey × Hariana) bull semen ranges from 80.25±1.31% to 85.34±1.26% (Pande *et al.*, 2015 and Kumar *et al.*, 2018). Sahiwal bulls have been reported to have per cent live spermatozoa of 81±2.54% (Rehman *et al.*, 2016).

2.6.1. Effect of Oxyrase on spermatozoa viability

In equine, spermatozoa viability was reported to be low after freezing when low concentration of Oxyrase was used, while higher concentration of Oxyrase preserved 34.9±3% which was higher than the control (Darr *et al.*, 2016 and London *et al.*, 2017). In Rhesus monkey, Oxyrase reported to have benefit to semen with low cryoresistance (Dong *et al.*,

2010). There is no report found on effects of Oxyrase on percentage of live spermatozoa in bovines.

2.7. Morphology and abnormalities

The grouping of mammalian spermatozoa abnormalities based on the origin of the abnormality, can be primary and secondary (Blom, 1950 & 1968). Those abnormalities occurring during spermatogenesis are termed as primary abnormalities. While those occurring during maturing in the epididymis and transit through the ductal system and preparations are called secondary abnormalities. On the other hand the major and minor spermatozoa abnormalities are based on the fertility effect (Blom, 1983).

The decrease in number of morphologically normal spermatozoa in ejaculate may result into low fertility (Chandler *et al.*, 1988; Gravance *et al.*, 1997 and Gravance *et al.*, 1998). The morphological abnormalities of the head include: microcephalic, macrocephalic, pyriform, elongated-narrow, short broad and double. The mid-piece abnormalities include: double, swollen, coiled, kinked and abaxial. Tail abnormalities include: double, kinked and coiled all these are classified to be primary abnormalities of the tail. While others like free heads, midpieces and tail, protoplasmic droplets (proximal or distal), bent mid-piece and detached head/capitis are referred as secondary spermatozoa abnormalities (Roberts, 1982). The major and minor spermatozoa abnormalities also referred as compensable and non compensable. This indicates the desired fertility could be achieved if the number of spermatozoa per insemination is raised to compensate the abnormal one (Saacke *et al.*, 2000; Walters *et al.*, 2005).

2.7.1. Effect of Oxyrase on Morphology

So far, the studies conducted on reducing the levels of oxygen from the extender have shown no effects on the morphology of bovine spermatozoa by degassing (Mustapha, 2017). The like findings were reported in equine semen which incorporated Oxyrase in the dilutor (Darr *et al.*, 2016; London *et al.*, 2017).

2.8. Acrosomal integrity

Acrosome is an organelle located at the head of the spermatozoa filled with hydrolytic enzymes which are necessary during the time of oocyte penetration through the zona pellucida and oocyte plasma membrane fusion (Qura and Toshimori, 1990; Flesh and Gedella, 2000). Structural intactness of this organelle is vital in assuring that optimal fertility will be attained (Kumar and Atreja, 2012; Srivastava *et al.*, 2012). Reaction of the acrosome before fertilization may either be physiological process of maturation before fertilization or it could mean damage in the spermatozoa as a result of cryopreservation which sometimes referred as false acrosomal reaction (Sukardi *et al.*, 1997 and Nizanski, *et al.*, 2012).

2.8.1. Effect of Oxyrase on acrosomal integrity

Using degassing method to remove oxygen from the dilutor, increases spermatozoa acrosome integrity of crossbred bull semen by 5% i.e. from 62.12% to 67.92% (Pande *et al.*, 2015, Mustapha, 2017; Amin *et al.*, 2018; Bhutia, 2018 and Kumar *et al.*, 2018,). There is no report on effects of Oxyrase on acrosomal integrity when Oxyrase in incorporated in the bovine semen dilutor.

2.9. Membrane integrity

Functional spermatozoa membrane is necessary for the fertilizing ability of spermatozoa. It is also required during processes like sperm capacitation, acrosome reaction, and binding of the spermatozoon to the egg surface to be accomplished (Selvaraju *et al.*, 2008). The hypo-osmotic swelling (HOS) test evaluates the functional integrity of the spermatozoa plasma membrane and also serves as a useful indicator of fertility potential of spermatozoa. Influx of the fluid due to hypo-osmotic stress causes the spermatozoa tail to coil and balloon or "swell" in a way to achieve a minimum surface-to-volume ratio (Drevius and Eriksson, 1966 and Jeyendran *et al.*, 1984). Spermatozoa with intact membrane take up water apparently without a significant enlargement of their area, thus forcing the flexible motor apparatus of the tail to bent and coil (Drevius and Eriksson, 1966; Kanno *et al.*, 2016). The tail of the spermatozoa appears to be susceptible to hypo-osmotic stress, and based on the spermatozoa vigor, different patterns of tail swelling are visible with phase-contrast microscopy. A higher percentage of swollen sperm indicates the presence of sperm having a functional and intact plasma membrane.

The HOST is not only an indicator of the chemical integrity of the plasma membrane, but also of its physical integrity (a broken membrane will not allow swelling to occur) therefore it is not necessary to perform the viability stain if the HOST is performed (Sofikitis *et al.*, 1992). This test is useful for detection of damage during cryopreservation, toxic effect of drugs, chemical and to assess quality of sperm (Sharma *et al.*, 2004).

The percentage of HOST reactive spermatozoa of crossbred bull semen extended with degasified dilutor is 76.80 ± 1.29% (Mean ± SEM) at fresh stage and 55.61±2.70 to 61.12 ± 1.41% at post thaw stage (Pande *et al.* 2015; Karunakaran and Devanathan, 2016).

2.9.1. Effect of Oxyrase on membrane integrity

The use of Oxyrase has been reported to increase spermatozoa membrane damage in equine (London *et al.*, 2017). However, there is no report on the effect of Oxyrase on bull spermatozoa membrane integrity so far available to the best of our knowledge.

2.10. Cholesterol in the semen

The cell plasma membrane structure is impermeable and cohesive membrane, which is due to the presence of cholesterol as an important structural component (White, 1993). Cholesterol also plays other crucial functions like membrane stability, facilitation of membrane morphological characters and allowing cell to cell interactions. Other functions are influencing the membrane phase transition and offering suitable micro-environments (chemical/physical) for membrane associated protein (Crockett, 1998 and Therien *et al.*, 1998). The decrease of membrane fluidity at temperatures above the phase transition and the increase of membrane fluidity at temperatures below the phase transition are influenced by cholesterol too (Crockett, 1998).

The study by Drobnis *et al.*, (1993) indicated the increase in ratio of cholesterol to phospholipids broadens the membrane phase transition and reduces membrane leakage. Furthermore, the presence of sufficient cholesterol on the membrane lipid is protected from transitioning into the crystalline gel state at temperatures below the phase which results into the prevention of lateral deviation of the phospholipids (Darin-Bennett and White, 1977).

The influx of cholesterol tends to reduce the rate of spontaneous spermatozoa acrosomal reaction and fertilization inhibition (via inhibition or delaying capacitation) in bovines (Davis,

1981). While membrane cholesterol efflux results into spermatozoa capacitation which predisposes spermatozoa to penetrate ova at a higher rate than those spermatozoa without reduced cholesterol levels (Ehrenwald *et al.*, 1988; Leahy and Gadella, 2015; Rajoriya *et al.*, 2013 and Rajoriya *et al.*, 2016). Under *in vitro* conditions, cholesterol efflux reported to increase the disorientation of phospholipid packing and resulting into increased membrane permeability (Cross, 1998) and greater lateral movements of integral membrane proteins (Fayrer-Hosken *et al.*, 1987).

Cholesterol is removed during capacitation which makes the acrosomal membrane unstable and ready to fuse with outer acrosomal membrane finally resulting in acrosome reaction (Nolan *et al.*, 1992; Leahy and Gadella, 2015). In non- capacitated state, the distribution of cholesterol is over the entire head of the spermatozoa, once acrosomal reaction occurs. Also cholesterol redistributes from equatorial to the apical region of the spermatozoa head. Acrosomal reaction is enhanced by calcium influx and mediated by increase in membrane fluidity (Fayrer-Hosken *et al.*, 1987). The process of semen cryopreservation found to significantly reduce the cholesterol content and increase the phospholipids and trigylcerol (Kadirvel *et al.*, 2009; Talukdar, 2016).

The means on how the loss of spermatozoa cholesterol during cryopreservasion occurs is not well clear, though most of losses are due to slow diffusion from the cell and transport to the medium have been studied in rat and bovine spermatozoa (Darin-Bennett *et al.*, 1973: Cormier *et al.*, 1997). The loss of cholesterol results into peroxidative damage to the lipids of the membrane which triggers premature capacitation of bull spermatozoa (Cormier and Bailey, 2003). While, cholesterol efflux after desulfation is thought to increase membrane fluidity and may stand for an integral part of the inherent regulatory property of spermatozoa undergoing capacitation-like changes during cryopreservation.

The inhibition of spontaneous acrosome reaction can be achieved by 1 µg/mL concentration of cholesterol. In human, Rathi *et al.*, (2001) reported cholesterol to be present in spermatozoa plasma membranes (13 nmol/10^8 cells in human spermatozoa) and seminal plasma at the rate of 250µg/mL with wide variations among individuals and ejaculates. Cholesterol

content in the spermatozoa of crossbred bull at fresh stage was 21.95±0.97 µg/100 million spermatozoa (Srivastava *et al.*, 2015). In buffalo bull cholesterol content at fresh and frozen thawed spermatozoa were 9.92 ± 1.6 and 5.38±1.85 µg/50x10^6 spermatozoa, respectively (Kadirvel *et al.*, 2009). Further, total cholesterol in seminal plasma of crossbred bulls was 45.93 ± 12.58 mg% which was higher in comparison to 31.87 ± 2.39 mg % of Gir bulls at fresh stage (Dhami *et al.*, 2003).

To the best of our knowledge, there was no reference which could be traced on the effect of Oxyrase on seminal plasma cholesterol at three different stages of cryopreservation (fresh, pre-freeze and post-thaw).

2.11. Phospholipids in spermatozoa

Phospholipids are important structural component of the spermatozoa plasma membrane. The spermatozoa plasma membrane fluidity, motility and viability are chiefly determined by its lipids composition because of the role it plays in spermatozoa physiology and metabolism (Ahluwalia and Holman, 1969; Miller *et al.*, 2004). A significant proportion of the phospholipids are in the form of plasmalogens, phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin are the major components and quite distinct group of aldehydogenic phosphatidyl lipids that contain one fatty acid esterified with glycerol and an unsaturated ester with a long carbon chain (C-20, C-24). The hydrolysis of a plasmalogen yields a fatty aldehyde and a fatty acid.

The phospholipids of bull spermatozoa are composed of 50% phosphatidyl choline and around 10% phosphatidyl ethanolamine (Parks *et al.*, 1987). Phosphatidylserine phospholipid is present in the mid-piece of the spermatozoa but after capacitation it is translocated to other portions like acrosomal region but never to the equatorial area (Kotwicka *et al.*, 2002). There are other phospholipids like sphingomyelin which influences the rate of spermatozoa capacitation via slowing down the loss of sterols, while, exogenous sphingomyelinase accelerates capacitation hence promoting the loss of sterols (Cross, 1989).

The study on model membranes showed the importance of cholesterol in organizing phospholipids as it was found to have capacity of inducing condensation effect of the

phospholipids resulting in increase in thickness and the order of the bilayer finally transforming a membrane 'ripple phase' (liquid-disordered phase) into a 'liquid-ordered phase' (De Meyer and Smit, 2009). Cholesterol induced membrane condensation reduces membrane permeability to water in model membranes and in erythrocytes (Muller *et al.*, 2008).

2.11.1. Effect of Oxyrase on plasma membrane phospholipids

There is no report that could be traced on the effect of Oxyrase on plasma membrane phospholipids.

2.12. Cholesterol: Phospholipids ratio

The ratio of cholesterol to phospholipids and amount of polyunsaturated fatty acid chains composing the phospholipids determines the overall fluidity of a membrane (Amann and Pickett, 1987). The study by Drobnis *et al.*, (1993) in model membranes reported the increased ratio of cholesterol to phospholipids broadens the membrane phase transition while reduces membrane leakage and membrane phase transition. Further, the capacitation process in spermatozoa is accompanied by change in the lipid composition of the plasma membrane which results into cholesterol: phospholipids ratio decrease (Davis, 1981). These changes appear to be a reversible phenomenon influencing the fluidity and ionic permeability of the plasma membrane (Naseer *et al.*, 2014). The spermatozoa of species with inherently higher cholesterol: phospholipids ratio are as follows; human (0.99), rabbit (*Oryctolagus cuniculus*) (0.88) which appear to withstand cryopreservation better. While those with lower ratios, including the bull (*Bos indicus*) (0.45), rat (*Rattus norvegicus*) (0.58), ram (*Ovis aries*) (0.24) and boar (*Sus scrofa domesticus*) (0.23). Spermatozoa sensitivity to cold shock is determined by membrane phospholipids composition and membrane cholesterol to phospholipids ratio (Watson, 1981; Davis, 1981).

2.12.1. Effect of Oxyrase

So far no report could be traced out on effect of Oxyrase on C: P ratio in Sahiwal Spermatozoa.

2.13. Oxidative stress

Oxidative stress denotes a state linked with an increased rate of cellular damage induced by reactive oxygen species (ROS) (Sofikitis *et al.*, 1995 and Chatterjee *et al.*, 2001). For the proper function of the spermatozoa, the amount of ROS produced has to be at the amount that spermatozoa antioxidant defences can deal with them. The cause of the pro-oxidant–antioxidant shift may be due to an increase in ROS production or decrease in antioxidant capacity or possibly a combination of the two. Oxidative stress induced by the production of ROS *in vitro* lead to reduction in sperm motility, viability, ionophore-induced acrosome reaction and sperm–oocyte fusion (MacLeod, 1943). Hydrogen peroxide appears to be the primary ROS . responsible for these changes and membrane lipid peroxidation is believed to be an important mechanism of action (Alverez and Storey, 1993; Aitken *et al.*, 2010; Totane *et al.*, 2010; Aitken and Curry, 2011). Other ROS reported are superoxide anion and nitric acid (Aiteken *et al.*, 2010).

The lipid peroxidation cascade is initiated when ROS attack polyunsaturated fatty acids (PUFAs) in the sperm cell membrane (Aitken *et al.*, 1995; Aitken and Roman, 2008; Totane *et al.*, 2010). Spermatozoa are particularly susceptible to oxidative attack because they contain high concentrations of PUFAs and being terminally differentiated cells, have limited repair mechanisms. As a consequence of lipid peroxidation, the plasma membrane loses the fluidity and integrity it requires for participation in the membrane fusion actions associated with fertilization. In addition to membrane effects, lipid peroxidation can also damage DNA. Peroxidation of DNA can lead to chromatin cross-linking, base changes, and DNA strand breaks. Several researchers (Bailey *et al.*, 2000; Linfor and Mayers, 2002; Baumber *et al.*, 2003; Aitken and Curry, 2011) have reported DNA damage in spermatozoa associated with membrane lipid peroxidation and oxidative stress.

2.13.1. Reactive oxygen species (ROS)

2.13.1.1. Sources of ROS

Spermatozoa and seminal plasma have several mechanisms that generate ROS, (Mayers, 2012) also there are a number of enzymes in it which scavenges these ROS to prevent cellular

damages. Spermatozoa losses many of these enzymes during the process of spermatogenesis which makes them more vulnerable to ROS and the cryopreservation process further aggravate the problem (Baker and Aitken, 2005; Aitken and Curry, 2011). There are two major sources of ROS which are spermatozoa and seminal plasma *i.e.* leukocytes (Padron *et al.*, 1997). It is believed that they have the same mechanism of ROS generation i.e. an NADPH oxdase (NOX) located in or near the cell membrane (Bedard and Krause, 2007; Pourova *et al.*, 2010).

Leukocytes are not very usually found in the mammalian ejaculates and even if there are, their number is very less in most of the species semen (Ford *et al.*, 1997). Mitochondria of spermatozoa are both the main source and target of ROS in the spermatozoa (Koppers *et al.*, 2008). Also ROS are concerned in many of the pathologic processes in spermatozoa (Pena *et al.*, 2009). Mitochondria are more likely to sustain damage from oxidative attack because mitochondrial DNA (mtDNA) is not protected by protamines like nuclear DNA and it may not be transcriptionally and translationally functional in ejaculated sperm (Darr *et al.*, 2016). In bovine semen, ROS are primarily generated by dead spermatozoa via cytosolic L-amino acid oxidases catalysed reaction (Sariozkan *et al.*, 2009).

The reported values in seminal plasma of different species range from 1338-9700 RLU (relative light unity) in human, 100 – 250 units oh H_2O_2 in crossbred bull semen (Moein *et al.*, 2007; Amin *et al.*, 2018 and Kumar *et al.*, 2018).

2.13.1.2. Physiological and pathological role of ROS

Normal spermatozoa functions such as capacitation, acrosome reaction and preservation of fertilizing ability do require optimal amount of ROS. Nevertheless, increase in ROS production causes oxidative stress which further damages the spermatozoa (Sofikitis *et al.*, 1995). The pro-oxidant-antioxidant shift may be either/ and due to an increase in ROS production, decrease in antioxidant capacity. Oxidative stress reduces sperm motility, viability, ionophore-induced acrosome reaction and sperm oocyte fusion whereby hydrogen peroxide appears to be the primary ROS responsible for damages via lipid peroxidation (Alvarez and Storey, 1983; Barbas and Mascarenhas, 2009; Tatone *et al.*, 2010; Aitken and Curry, 2011).

2.13.2. Lipid peroxidation (LPO)

The damage that the ROS inflicted to the spermatozoa is lipid peroxidation of the plasma membrane (Chatterjee and Gagnon, 2001). The increase in the levels of LPO in frozen-thawed semen in comparison to its counterpart fresh semen is clear (Mustapha, 2017). There are two main types of LPO: (I) non enzymatic membrane LPO, and (II) enzymatic (NADPH and ADP dependent) LPO. The enzymatic reaction involves NADPH-cytochrome P-450 reductase and proceeds via an ADP-Fe_3+O^{2-}(perferryl) complex. In spermatozoa, production of malondialdehyde (MDA) an end product of LPO induced by ferrous ion promoters. Also, lipid peroxidation can contribute directly to specific sub lethal effects; like chromatin cross-linking, base changes and DNA strand breaks (Twigg *et al.*, 1998 and Barratt *et al.*, 2010).

Formation of MDA can be assayed by the thiobarbituric acid (TBA) reaction as a tool for LPO measurement *in vitro* and *in vivo* systems (Suleiman *et al.*, 1996). In the year 1997, Beorlegui *et al.*, reported MDA values for cattle bull spermatozoa ranging from 0.34±0.18 to 4.95±0.31µM/mL in frozen thawed semen sample while Selvaraju *et al.*, (2008) reported a bit higher values in buffalo bulls which is 8.00±0.31 µM/ml at immediate post thaw and 9.36±0.36 µM/mL i.e. 120 min of post thaw incubation. The high level of MDA concentration in buffalo sperm in comparison with bull sperm might be due to the fact that the sperm membrane of buffalo is richer in PUFAs (Nair *et al.*, 2006).

2.13.2.1. Detection of ROS and LPO

Spectrophotometric analysis as proposed by Hayashi *et al.*, (2007), is the method used in estimation of ROS. During cryopreservation, semen is exposed to cold shock and atmospheric oxygen, which make them vulnerable to LPO due to higher production of reactive oxygen species. The level of ROS is expressed as unit of H_2O_2 *i.e.* one unit correspond to 1 mg/L H_2O_2.

When LPO of the plasma membrane occurs Malondialdehyde (MDA) is produced as one of the by-products and thiobarbituric acid (TBA). MDA assay is used to measure its levels both *in vitro* and *in vivo* (Suleiman *et al.*, 1996).

2.13.2.2. Effect of Oxyrase

According to London *et al.*, (2017), there was no significant difference found on LPO when Oxyrase was used i.e. treated and non-treated groups in Coconut and Egg Yolk extenders in equine semen. This was previously found to be the same by Darr *et al.*, (2016), where the levels of ROS in fresh extended semen of control and treated samples were the same. At post thaw stage there was five folds increase in ROS in treated and control samples, but there was no clear finding that Oxyrase was able to reduce ROS (Darr *et al.*, 2016).

2.13.3. Total antioxidant capacity (TAC)

The new way of determination of total antioxidant status was developed by Miller *et al.* (1993) and designated as total antioxidant capacity. The advantage of this test is to measure the antioxidant capacity of all antioxidants (thus termed as TAC) in a biological sample and not just of a single compound. The TAC do varies among bulls but not between ejaculates among bulls. Also there is an inverse relation between TAC and LPO potential, which indicates that this assay might be valuable for the evaluation of bull sperm quality and fertility (Gürler *et al.*, 2015).

The tests for "total antioxidant power" which are currently in use have do measured the ability of plasma membrane to withstand the oxidative effects of reactive species purposefully generated in the reaction mixture. The depletion of antioxidants is denoted by a change in signal, such as rate of oxygen utilization (Wayner *et al.*, 1987). The "antioxidant power" is measured by using ferric reducing ability of plasma (FRAP), (Benzie and Strain 1996), as novel method for assessing "antioxidant power." Under low pH the reduction reaction of converting ferric to ferrous ion results into the formation of a colored ferrous-tripyridyltriazine complex. The FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration and the absorbance changes are linear over a wide concentration range with antioxidant mixtures in seminal plasma.

TAC (trolox equivalents micromol/L) was 389.7±187.6 (mean±SD) from eight consecutive seminal plasma samples of nine bulls (Gürler *et al.* 2015). The report by Kumar

et al., (2018) where FRAP Assay was used, TAC (nmol/L) in 4ppm group to be 887.72±26.72 and 883.42±42.82 at pre-freeze and post thaw stage, respectively. Also they reported for 8 ppm and control group where the findings for 8 ppm were less than that of 4 ppm but greater than the control group at pre-freeze and post-thaw stages. There are no reports so far observed indicating the effect of Oxyrase on spermatozoa qualities in bull semen.

2.13.4. Oxidative stress mitigation strategies

Spermatozoa are living under aerobic conditions like other living cells and they constantly face oxygen paradox. Oxygen is required for life, but oxidative metabolism of biological molecules can be potentially toxic. This is due to the formation of reactive oxygen species (ROS) which have impact on cell function and viability (Mayers, 2012). Studies have shown that ROS do play an important role in sperm function. The imbalance in its production or degradation may result into adverse effect to the function of the spermatozoa in combination with other factors like osmotic imbalance and ice crystals formation (Sariozkan *et al.*, 2015). Cryopreservation poses insults into the spermatozoa which finally results into ROS production, DNA damage, aneuploidy and chromosomal fragmentation (Mayers, 2012; Aitken *et al.*, 2017).

Spermatozoa have got a limited ability to store antioxidant enzymes. The spermatozoa plasma membrane is rich in unsaturated fatty acids makes them vulnerable to oxidative stress and peroxidative attack by ROS, especially the superoxide ion and hydrogen peroxide (Baker *et al.*, 2005). The spermatozoa shape and morphology has been modified and streamlined by losing most of the cytoplasm during spermiogenesis. Together with this, most of the intrinsic cytoplasmic enzyme scavenger system is also lost, and this makes sperm vulnerable to oxidative attack (Bilodeau *et al.*, 2000). Furthermore, spermatozoa are transcriptionally and translationally silent, as they are unable to modify their function based on the surrounding (O'Flaherty, 2006).

Seminal plasma and spermatozoa posses some enzymes and low molecular weight antioxidants that scavenge ROS and reduce its lethal effects (du Plessis *et al.*, 2014). In response the damages brought by ROS to the spermatozoa, initiatives have been taken to prevent its production and finally reduce the damaged to spermatozoa at post-thaw stage.

Basically there are two ways of OS mitigation, first is through reduction of generated ROS and second is the reduction of sources of ROS production (Amidi *et al.*, 2016). Reduction

of generated ROS is achieved through the addition of antioxidants in diet or in extender. Enzymatic antioxidants they include; Superoxide dismutase (SOD), Catalase, Glutathione peroxidase (GPx) and Glutathione reductase (GR). While, non-enzymatic antioxidants include: Vitamin E and Vitamin C, Zinc, Taurine, Hypotaurine, Glutathione and selenium. So far herbs reported to be used are geistein, resveratrol and *Rhodiola sarca* extract in man, ram and boar semen, respectively (Asadpour *et al.*, 2012; Amidi *et al.*, 2016).

Reduction of ROS production is achieved through lowering O_2 tension (partial deoxygenation) of the semen dilutor by either Freeze-Pump-Thaw cycling method (Pande *et al.*, 2015, Balamurugan *et al.*, 2017), mechanical method - Vacuum pump (Balamurugan *et al.*, 2017) and nitrogen gassing (Mustapha, 2017; Kumar *et al.*, 2018; Amin *et al.*, 2018; Bhutia, 2018).

Removal of dead or damaged spermatozoa from the ejaculate by using anti-ubiquitin nanoparticles (Omer Din, 2017) or swim-up or sephadex techniques (Ramamoorthy, 2018). Also, incorporation of CO_2 in the liquid semen lowers spermatozoa metabolism hence, there will be less oxygen metabolism and finally low ROS are produced (Van Demark and Sharma, 1957).

Another approach to reduce the ROS production is via reduction of O_2 level in the semen dilutor through addition of an *Escherichia coli* membrane fraction (Oxyrase) which has been reported to be used in mouse (Koshimoto *et al.*, 2000; Mazur *et al.*, 2000), rhesus monkey (Dong *et al.*, 2010), and equine (Darr *et al.*, 2016 and London *et al.*, 2017).

2.14 Oxyrase

Oxyrase is an *Escherichia coli* (*E. coli*) membrane derivative with the bacteria's electron transport system. Oxyrase can reduce oxygen concentrations in solutions in the presence of cells with a hydrogen donor substrate (Adler, 1990 and Dong *et al.*, 2009). Oxyrase is found to have a capacity to create artificial anaerobic environment as reported to be used in industrial food preservation (Kressin *et al.*, 1997 and Dong *et al.*, 2008), antibiotic testing (Dong *et al.*, 2005 and Hernandez *et al.*, 2007). Oxyrase has also been used in cryopreservation of mammalian spermatozoa; in mouse (Katkov *et al.*, 1998; Koshimoto *et al.*, 2000; Mazur

et al., 2000), rhesus monkey (Dong *et al.*, 2010) and equine (Darr *et al.*, 2016; London *et al.*, 2017).

Oxyrase contains a penicillin-binding protein that may inactivate penicillin and related antibiotics according to the company's package insert. The fact that in most of the semen extenders like INRA96 cryopreservation medium contains: sodium penicillin, gentamicin sulfate and amphotericin B. These antibiotics cover a broad spectrum of bacteria, due to this there were no bacterial contamination when Oxyrase was incorporated in the semen dilutor (Dong *et al.*, 2010). In the presence of suitable hydrogen donor substrate (i.e. lactate) Oxyrase can reduce O_2 in the solution to effectively low levels (Alder, 1990 and Kressin *et al.*, 1997) more so it has been seen to have no cytotoxic effect to the spermatozoa (Darr *et al.*, 2016).

2.15 Semen bacterial load

Contamination of the semen with bacteria could be originated from the animal itself or from the environment during semen collection, processing and storage. It has been observed that the lowest bacterial load of bovine fresh semen is 5.0cfu/mL (Almquist *et al.*, 1949) while the highest is $444x10^4$cfu/mL (Ronald and Prabhakar, 2001). While the bacterial load of frozen-thaw semen was reported to be $1.77\pm0.13 \times 10^3$cfu/mL in Sahiwal and $0.85\pm0.09 \times 10^3$cfu/mL in Crossbred bulls (Ankesh, 2017). Due to this, most of the semen extenders i.e. TYG, INRA96 contains antibiotics like sodium penicillin, streptomycin sulfate, gentamicin sulfate, and amphotericin B. These antibiotics cover a broad spectrum of action to minimize or eliminate the bacterial contaminants in the semen used for artificial insemination (Dar *et al.*, 2016). As per Minimum Standard Protocol (MSP) of cryopreserved bovine semen, maximum permissible bacterial load should be 5000 cfu/mL.

2.15.1 Effect of Oxyrase on semen bacterial Load

So far there is no report on the effect of Oxyrase on bacterial load of bovine semen. There is one report in Rhesus monkey which indicated no bacterial contamination in the treatment group (Dong *et al.*, 2010).

Materials and Methods

3 MATERIALS AND METHODS

3.1. Study area

This study was conducted at the Germ-Plasm Centre (GPC), Division of Animal Reproduction, Indian Veterinary Research Institute, Izatnagar, Bareilly (UP). The location of the institute is 564 feet altitude above the sea level at latitude of 28° North and longitude of 79° East. The particular place is in subtropical climate experiencing the extreme temperature (hot and cold) with the relative humidity ranging from 15 to 85% throughout the year.

3.2. Sterilization of articles

All the glassware used for semen collection and processing were washed thoroughly with soap water (exceptions, if any, as indicated in the body of experiments), rinsed twice in double distilled water and then dipped in 10 per cent HCl for 12 h. Thereafter, glassware were washed vigorously with tap water, rinsed twice in double distilled water, dried and sterilized in a hot air oven at 160°C for 1h. Buffer solutions (20min), rubber articles, artificial vagina were autoclaved at 10 lb pressure at 110°C for 10 min while straws, polyvinyl alcohol and other polyethylene articles were exposed to ultraviolet rays for one hour before use.

3.3. Experimental animals

Four healthy Sahiwal breeding bulls maintained at GPC were utilized for the study. These bulls were maintained in the same daily managerial conditions during the entire course of the study.

3.4. Preparation of semen dilutor

At GPC laboratory the dilutor used for semen processing is Tris-EggYolk-Glycerol (TYG) and the same was used in the present study. Tris buffer was prepared and autoclaved a day before semen collection. Other ingredients like antibiotics, egg yolk (10%) and glycerol (7%) were added into Tris buffer and mixed with the help of a magnetic stirrer on the day of semen collection.

3.5. Standardization of Oxyrase in semen dilutor

After the preparation of the TYG extender as per standard protocol, it was kept in a water bath at constant temperature (35°C) till when the temperature of the extender reached 35°C. Thereafter, 40ml of the extender was put into 50ml capacity test tube followed by addition of a specific amount of Oxyrase, thoroughly mixed and left to incubate while levels of DO were determined after every two minutes. Precaution was taken to prevent the environmental oxygen by covering the test tube with parafilm. Also the turbidity of the solution (extender) and the incubation temperature had to be kept constant, this is because they are negatively correlated to dissolved oxygen levels.

Test Tube I: Extender with 0.125 IU/ml of Oxyrase

Test Tube II: Extender with 0.25 IU/ml of Oxyrase

Test Tube III: Extender with 0.5 IU/ml of Oxyrase

Test Tube IV: Extender with 1.0 IU/ml of Oxyrase

Test Tube V: Extender with 2.0 IU/ml of Oxyrase

From the above standardization, the desired level of DO was 4ppm. The appropriate concentration of Oxyrase which was able to reduce the DO level in the extender to around 4ppm for a particular period of time and suits with the semen processing procedures was chosen among the five. Measurement of DO levels was accomplished by using DO meter (Century, CD 501, India).

3.6. Collection of ejaculates

The routine semen collection from the bulls under the study was done twice a week, two times a day (collected at interval of 15-30 minutes) during morning hours, between 8:00 AM to 9:00 AM by using artificial vagina (AV) as per the standard procedure. Immediately after collection, tubes containing the ejaculated semen were placed in the water bath at 35°C.

3.7. Selection of ejaculates

A total of 24 ejaculates were selected for the study. Based on initial progressive motility, ejaculates were divided into two groups *i.e.* poor ejaculates (having motility of 55 to 65%) and good ejaculates (having motility of ≥ 70%). The initial progressive motility was assessed by using light microscope.

3.8. Semen extension

Before dilution of the selected ejaculates, concentration was determined by Accucell (BOVINE Photometer n* 1445, IMV technologies, L'Aigla, France). Then, each ejaculate was divided into two aliquots and extended separately *i.e* control (dilutor without Oxyrase) and treatment (dilutor with Oxyrase). However, the extender of the treatment group was first mixed with Oxyrase and incubated for 5min at 35°C before being used for semen dilution. The final extension rate was adjusted accordingly to obtain a concentration of 80 million progressive motile spermatozoa per mL of extended semen.

3.9. Evaluation of semen at fresh stage

From the selected ejaculates, a representative sample of neat semen was taken for bacterial load evaluation and a representative sample for the estimation of seminal plasma cholesterol and plasma membrane phospholipids.

3.9.1. Separation and storage of seminal plasma and spermatozoal pellets

From each experimental group, a representative semen sample (1 mL) were taken into 2 mL micro centrifuge tube and centrifuged at 4000 g for 20 minutes at 5°C in a refrigerated centrifuge (Sigma). The supernatant and pellet were separated and stored in labelled micro centrifuge tubes at -20°C till their use for the estimation of cholesterol from supernatant and

phospholipids from the pellet (at fresh stage). The same was done at pre freeze and post thaw stages for evaluation of cholesterol, phospholipids and oxidative stress status. Spermatozoa pellet was utilized for phospholipids and LPO estimation and the supernatant for cholesterol, TAC and ROS.

3.9.2. Cholesterol assay

Cholesterol content in seminal plasma was estimated using AUTOSPAN(R) Liquid Gold Cholesterol kit supplied by ARKRAY Healthcare Pv. Ltd. This assay was performed at three different stages: fresh, pre-freeze and post-thaw.

Method

In a test tube marked 'blank' 1000 µl of reagent 1 (Annexure) whereas in another tube marked 'standard' 1000 µl of reagent 1 and 10 µl of reagent 2 (Annexure) was added. In third tube marked 'test' 1000 µl of reagent 1 was mixed with 10 µl of seminal plasma sample. These tubes were incubated at 37° C for 10 min and O.D. was taken at 505 nm in spectrophotometer (Eppendorf BioSpectrometer kinetic, Device #: 6136GN903757). The formula below was applied for calculating the cholesterol content of spermatozoa:

$$\text{Cholesterol concentration} = \frac{\text{O.D (Test)}}{\text{O.D (Standard)}} \times 200 \text{ mg/dL}$$

3.9.3. Phospholipids assay

The technique for estimation of Phospholipids in the sperm lipid extract was adopted from the one described by Bartlett *et al.*, (1959) as follows:

Reagents

- **Standard phosphorus solution (8µg/5ml):** Prepared by dissolving 3.5 mg KH_2PO_4 in 10 mL of 10 N H_2SO_4 in 100 mL double distilled water.
- **Fiske-Subba Row reagent** (Sigma Aldrich, USA): 1.2 g of sodium metabisulfite, 20 mg of 1-amino-2-nephthol-4-sulphonic acid, and 120 mg of anhydrous sodium sulfite in 10 mL of double distilled water.
- **Percholoric acid (70%)** (Sigma Aldrich, USA).

Procedure

A total of 100 million washed spermatozoa were taken in a 10 ml vial. The sperm pellet was extracted with 20 volume of chloroform: Methanol (1:1, V/V) solution, vertexed for 20 sec and centrifuged at 800g for 5min. The spermatozoa was evaporated to dryness by flowing Nitrogen gas at low pressure and kept at -20°C. For the estimation of phospholipids content, 0.5 mL of chloroform and 1 mL of perchloric acid (70%) were added to each tube containing 0.5 ml lipid extract, and the mixture was digested in a sand bath at 150 °C to 160 °C until it became clear. The samples were then removed and cooled to room temperature. Subsequently, 6 mL of double distilled water and 0.8 mL of ammonium molybdate (2.5%) were added, followed by 0.2 mL of Fiske-Subba Row reagent. The samples were heated for 7 minutes in a boiling water bath, cooled to room temperature and absorbance was taken at 660 nm in spectrometer (Eppendorf BioSpectrometer kinetic, Device #: 6136GN903757). The standard phosphorus solution and a blank with 0.5 choloroform were simultaneously run in the same manner.

Calculation

Calculation was done by employing the following formula and results were expressed as µg phospholipid/100 million of spermatozoa.

$$\text{Phospholipid concentration } (\mu g/100 \times 10^6 \text{ spermatozoa}) = \frac{\text{O.D (Test)} \times \text{Conc. of Standard } (\mu g) \times 25}{\text{O.D (Standard)}}$$

3.9.4. Standard plate count (SPC) for bacterial load evaluation

Processed samples for bacterial load were collected under aseptic condition. Fresh samples of semen for bacterial counting were used within half an hour from the time of collection while post-thaw semen samples were utilised after 24h of freezing. Standard plate count was done by spread plate technique in duplicate plates as per Cruickshank *et al.*, (1975). Ten folds serial dilution from 1:10 to 1:1000 were made for all the semen samples for standardization of SPC. Subsequently 1:10 to 1:100 dilutions were used for bacterial count from fresh and post-thaw semen samples. The normal physiological saline solution was used as routine diluent

for all the samples. The inoculated nutrient agar plates were allowed to dry for 10 minutes before incubation at 37°C for 24h. The bacterial count (CFU/mL) of each sample was calculated by multiplying the dilution factor with number of colonies in plate.

3.10. Measurement of DO (ppm) levels

The DO levels of extended semen were accomplished by using DO meter (Century, CD 501, India). Dissolved oxygen levels at pre-freeze and post-thaw stage were carried out using a representative samples from each group *i.e.* control and treatment.

3.11. Evaluation at post-dilution stage

Immediately after each semen aliquots being extended with respective dilutor and kept in water bath at 35°C individual progressive motility was evaluated. Also samples for parameters like plasma membrane integrity and acrosomal integrity were assessed.

3.11.1. Individual progressive motility

The individual progressive motility of the diluted semen from each group (control and treatment) was evaluated by placing a small (5-10μL) drop of diluted semen on a pre-warmed glass slide (37°C) and allowing it to spread uniformly under the cover slip (18x18 mm). Microscopic field in the slide was chosen randomly and spermatozoa showing progressive motility (forward progressive) at 37°C were counted at 20X using light microscope fitted with a thermo-stage. At least ten widely spaced fields were examined to provide an estimate of percentage of progressive motile spermatozoa.

3.11.2. Acrosomal integrity

The acrosomal integrity (percentage of normal acrosome) based on acrosome damage was studied in Giemsa stained smears according to method described by Watson (1975).

Procedure

- A smear from a drop of fresh semen was prepared on a clean, grease free glass slide and air dried.
- The smear was then fixed in Hancock's fixative for 15 min.

- Fixed smear was then washed in slow running water for 15 min.
- This was followed by rinsing of the smear with distilled water and air dried.
- After drying the smear was stained in Giemsa working solution for 90 min.
- Slides containing smears were then removed from the stain solution and rinsed quickly in distilled water, air dried and mounted in DPX.

The smear was examined under oil immersion objective (100X) of the microscope to assess acrosomal integrity. At least 200 spermatozoa were counted from each slide for the estimation of per cent intact acrosome. The abnormal acrosome was the one manifesting with marked swelling, knobbing, ruffling, or incomplete contour and denudation.

3.11.3. Plasma membrane intergrity

The integrity of the plasma membrane was evaluated by using Hypo-osmotic swelling test (HOS test). The HOS test was performed as per the method described by Prasad *et al.*, (1999) to assess the biochemical integrity of plasma membrane of sperm. The osmolality of hypo-osmotic solution was 150mOsm/L.

Procedure

- 1.0 mL of hypo-osmotic solution was taken into a glass sugar tube.
- 0.1 mL of semen was added and mixed well. The sperm suspension was incubated in water bath at 37°C for 60 min.
- After incubation a drop of eosin-Y solution was added (to increase the visibility). Then a small drop of the suspension from the bottom of the tube was placed on clean, grease free glass slide and covered with cover slip.
- The slides were examined under the high power magnification (40x) of a phase contrast microscope (Motic, China).
- A minimum of 200 spermatozoa were counted to record different types of tail swelling pattern.

➢ Similar procedure was followed for the semen samples at pre-freeze and post- thaw stage of both control and treatment groups.

Spermatozoa were classified as per cent HOS responsive according to the presence of the tail swelling patterns (Prasad *et al.*, 1999) as mentioned below.

- Pattern A: No swelling, complete loss of membrane integrity.
- Pattern B: Swelling at the tip of the tail.
- Pattern C: Different types of hair-pin like swelling.
- Pattern D: Complete tail swelling.

The sperm cells displaying the swelling pattern B, C and D were considered positive for the HOST.

3.12. Filling and sealing of straws

French mini straws (0.25 mL) were used for filling the extended semen. The filling and sealing of the semen straws was accomplished by using automatic filling, sealing and printing machine (IS-4, IMV Technologies, France). This was done within 5 minutes from the time of extension especially for the group using Oxyrase incorporated extender.

3.13. Equilibration of straws

Soon after filling and sealing, straws were spread evenly on the straw racks and kept at 4°C for 4 h for equilibration in the cold handling cabinet.

3.14. Evaluation at pre-freeze stage

Parameters like Individual progressive motility, plasma membrane integrity, acrosomal integrity assessment, seminal plasma cholesterol and plasma membrane phospholipids were determined as described before. In addition to that, evaluation of Oxidative stress status (Reactive Oxygen species, Lipid peroxidation and Total Antioxidant Capacity) was conducted at this stage. Also DO levels were determined at this stage.

3.14.1. Lipid peroxidation (LPO)

Lipid peroxidation level of sperm pellet was measured by determining the malonaldehyde (MDA) production, using thiobarbituric acid (TBA) as per described by Buege and Aust (1978) and modified by Suleiman *et al.* (1996).

Procedure

The spermatozoa pellet obtained after separation of by centrifugation as described earlier was suspended in PBS (pH 7.2) of variable volume to obtain a concentration of 20×10^6/ mL. Lipid peroxide levels were measured in spermatozoa after the addition of 2 ml of TBA-TCA reagent (15% w/v TCA, 0.375% w/v TBA and 0.25 N HCL) to 1 mL of sperm suspension. The mixture was treated in a boiling water bath for 1 hour. After cooling, the suspension was centrifuged at 3000 rpm for 10 min. The supernatant was then separated, and absorbance was measured at 535 nm in spectrometer (Eppendorf BioSpectrometer kinetic, Device #: 6136GN903757). The MDA concentration was determined by the specific absorbance coefficient ($1.56 \times 10^5 \mu mol/cm^3$)

$$\text{MDA produced } (\mu mol/mL) = \frac{OD\times10^6\times \text{Total volume (3 mL)}}{1.56\times10^5\times \text{Test volume (1mL)}}$$

$$= \frac{OD\times30}{1.56}$$

3.14.2. Total antioxidant capacity (TAC)

Total antioxidant activity in seminal plasma was measured using ferric reducing/ antioxidant power (FRAP) assay described by Benzie and Strain (1996).

Principle

At low pH, reduction of ferric tripyridyl triazine (Fe-TPTZ) complex to ferrous form (which gives an intense blue colour) is monitored by measuring the change in absorbance at 593 nm. The change in absorbance is directly related to the combined or total reducing power of electron donating antioxidants present in reaction mixture.

Preparation of reagents

a) Acetate buffer 300 mM pH 3.6

b) TPTZ (2, 4, 6-tripyridyl-*s*- triazine) (M.W. 312.34) 10 mM in 40mM HCl (M.W. 36.46)

c) $FeCl_3$. $6H_2O$ (M.W. 270.30) 20 mM

The working FRAP reagent was prepared by mixing a b & c in the ratio of 10:1:1 at the time of use.

Standard: Ascorbic Acid (M.W. 176.13) 1M

Procedure

100 µl of seminal plasma (sample) was mixed with 3 mL of working FRAP reagent and absorbance (593nm) in spectrometer (Eppendorf BioSpectrometer kinetic, Device #: 6136GN903757) was measured at 0 min after vortexing. Thereafter, samples were placed at 37°C in water bath and absorption was again measured after 4 min. Ascorbic acid standards (1M) was processed in the same way as the seminal plasma sample. Three mL of working FRAP solution was used as blank.

Calculation:

$$\text{FRAP value of Sample } (\mu\text{mol/mL}) = \frac{\text{Change in absorbance of sample from0 to 4 minute}}{\text{Change in absorbance of standard from0 to 4 minute}} \times \text{FRAP value of standard(1M)}$$

Note: FRAP value of 1M Ascobic acid is 2000

3.14.3. Reactive oxygen species (ROS)

Estimation of ROS was done using a spectrophotometer as described by Hayashi *et al.,* (2007). The sample contained 5µL of seminal plasma, 140 µL of 0.1M sodium acetate buffer (pH 4.8) and 100 µL of the mixed solution prepared from R_1 and R_2 at the ratio of 1:25. The absorbance was measured at 505 nm for 2 minutes at 15 seconds interval using a spectrophotometric plate reader. R_1 solution contained 100 µg/mL of N, N diethyl para-

phenylendiamine (DEPPD) sulphate in 0.1M sodium acetate buffer while R_2 solution was prepared by dissolving ferrous sulphate in 0.1M sodium acetate buffer to attain a final concentration of 4.37μM.

Ten different concentrations of hydrogen peroxide solution (50, 100, 150, 200, 250, 300, 350, 400, 450 and 500 mg/L) were used as standard. A calibration curve for the standard solutions was developed by calculating slopes (absorbance increase at 505 nm/min×1000) and the level of ROS was expressed as unit of H_2O_2. One unit corresponded to 1 mg/L H_2O_2.

3.15. Evaluation at post-thaw stage

Straws were thawed in water at 37°C in a breadbox for 30s and then wiped with tissue paper, cutting was done by using straw cutter and the content was decanted into a sugar tube kept in water bath at 37°C.

Immediately, the decanted semen was used for evaluation of desired parameters like individual progressive motility, sperm plasma membrane integrity, acrosomal assessment, seminal plasma cholesterol, plasma membrane phospholipids, reactive oxygen species, lipid peroxidation, total antioxidant capacity and bacterial load as described earlier.

3.16. Statistical analysis

All experimental data are presented as Mean ± SE. For analyzing the effect of methods and effect of treatments within methods for the different parameters the following general linear model was used.

$$y_{ijk} = \mu + M_i + T_j(M_i) + e_{ij}$$

Where:

y_{ijk} = k-th observed value of the response variable for i-th method for the j-th treatment

μ = General mean effect

M_i = Effect of i-th method

$T_j(M_i)$ = Effect of the j-th treatment within i-th method

e_{ij} = error term

(Methods stand for stages of freezing i.e. fresh or post-dilution, pre-freeze and post-thaw; treatments stand for Oxyrase treated and non-treated groups)

The multiple comparisons between methods and treatment within methods for different parameters were done by using Tukey test. The analysis was done by SAS 9.3 software.

Results

4 RESULTS

A total of 24 ejaculates from four Sahiwal bulls were utilized for the study. At fresh stage, spermatozoa concentration and initial progressive motility (%) were evaluated for selection of semen ejaculates. Other parameters like bacterial load (at fresh and post thaw), plasma membrane cholesterol and plasma membrane phospholipids (at pre-freeze and post thaw) were evaluated. All the selected ejaculates were categorised into two broad groups i.e. good and poor on the basis of initial progressive motility. Each ejaculate was further split into two aliquots and extended with dilutor without (control) and with Oxyrase (treatment). Individual progressive motility, spermatozoa plasma membrane integrity and acrosomal integrity were evaluated at post dilution, pre-freeze and post-thaw. Lastly, oxidative stress status: Lipid peroxidation (LPO), reactive oxygen species (ROS) and Total antioxidant capacity (TAC) were evaluated at pre-freeze and post thaw stages.

4.1. Standardization of concentration of Oxyrase in semen dilutor

The levels of dissolved oxygen (ppm) in semen dilutor with time (Minutes) at different concentrations of Oxyrare (IU/mL) are shown in Table 1 and graphically represented in Fig. 1a-e. From the results of DO (ppm) with time, 0.125 IU/mL concentration of Oxyrase was selected for being incorporated in the semen dilutor for this study.

Table 1: Time (min) required to obtain various levels of dissolved oxygen (ppm) in semen dilutor at different concentrations of Oxyrase (IU/mL) when incubated at 35°C.

Time (Min)	DO Level (ppm)				
	0.125 IU/ml	0.25 IU/ml	0.5 IU/mL	1.0 IU/mL	2.0 IU/mL
0	11.82±0.15	11.69±0.20	11.80±0.19	11.89±0.18	11.74±0.04
2	11.03±0.03	10.83±0.14	11.35±0.16	9.94±0.15	9.59±0.06
4	10.56±0.08	9.68±0.15	8.20±0.18	7.89±0.17	7.06±0.04
6	9.54±0.05	7.33±0.14	5.35±0.11	5.26±0.13	4.39±0.07
8	8.75±0.11	6.51±0.22	4.45±0.09	3.76±0.15	2.89±0.04
10	7.71±0.08	4.89±0.15	3.35±0.09	2.59±0.10	0.87±0.06
12	6.42±0.24	4.02±0.14	2.30±0.18	1.16±0.13	0.35±0.03
14	5.96±0.09	2.86±0.17	1.50±0.10	0.41±0.10	0.10±0.01
16	4.34±0.41	1.71±0.15	0.75±0.16	0.20±0.01	0.03±0.03
18	3.82±0.03	1.16±0.13	0.55±0.14	0.09±0.02	0.00
20	3.40±0.07	0.76±0.13	0.37±0.09	0.03±0.03	0.00
22	2.90±0.02	0.48±0.12	0.19±0.09	0.00	0.00
24	2.31±0.09	0.25±0.05	0.07±0.03	0.00	0.00
26	1.88±0.06	0.12±0.02	0.00	0.00	0.00
28	1.48±0.05	0.00	0.00	0.00	0.00
30	1.36±0.03	0.00	0.00	0.00	0.00

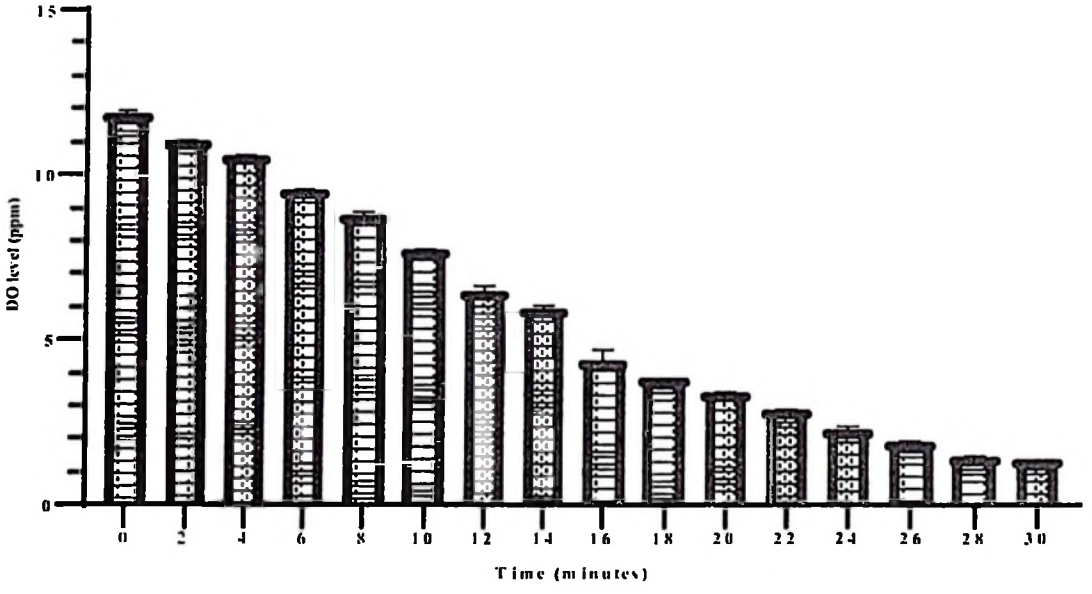


Fig. 1a: Time (min) required to obtain various levels of dissolved oxygen (ppm) in semen dilutor at 0.125 IU/ml of Oxyrase

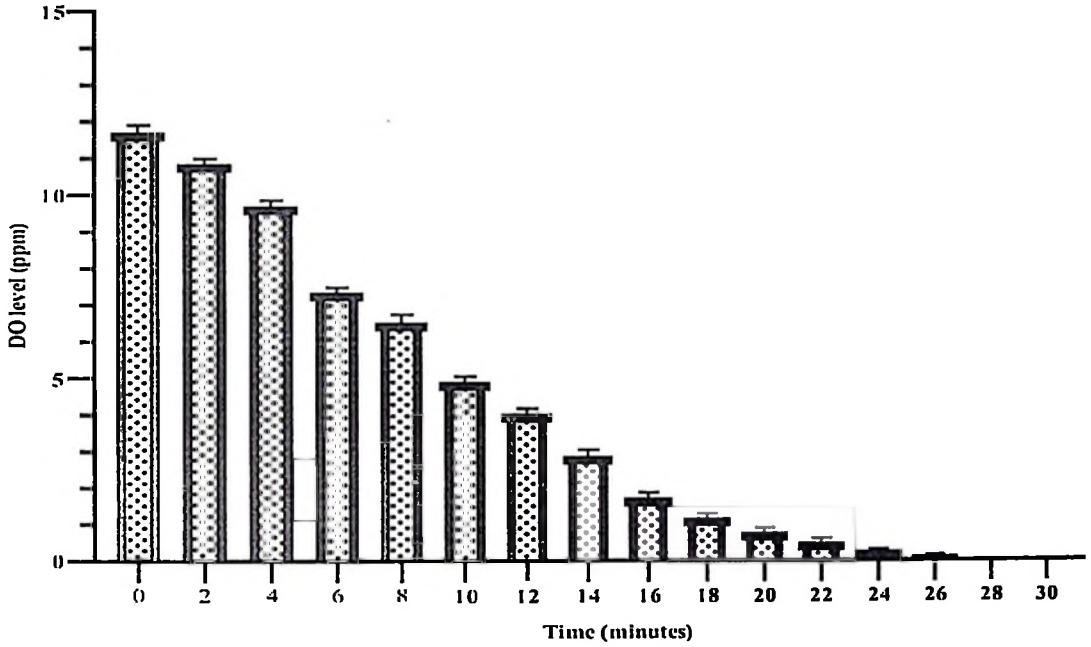


Fig. 1b: Time (min) required to obtain various levels of dissolved oxygen (ppm) in semen dilutor at 0.25 IU/ml of Oxyrase

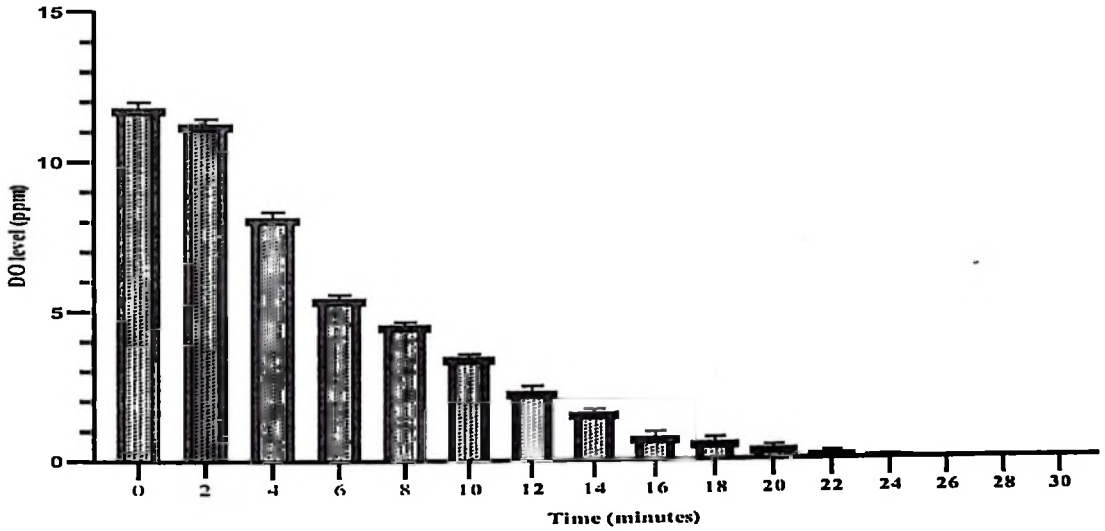


Fig. 1c: Time (min) required to obtain various levels of dissolved oxygen (ppm) in semen dilutor at 0.5 IU/ml of Oxyrase

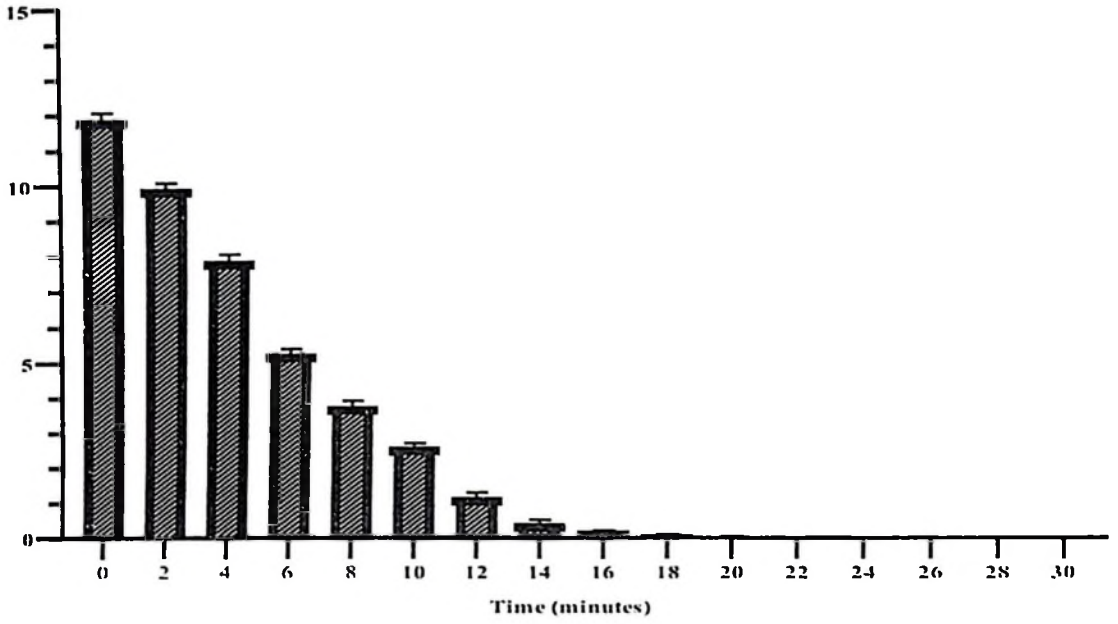


Fig. 1d: Time (min) required to obtain various levels of dissolved oxygen (ppm) in semen dilutor at 1 IU/ml of Oxyrase

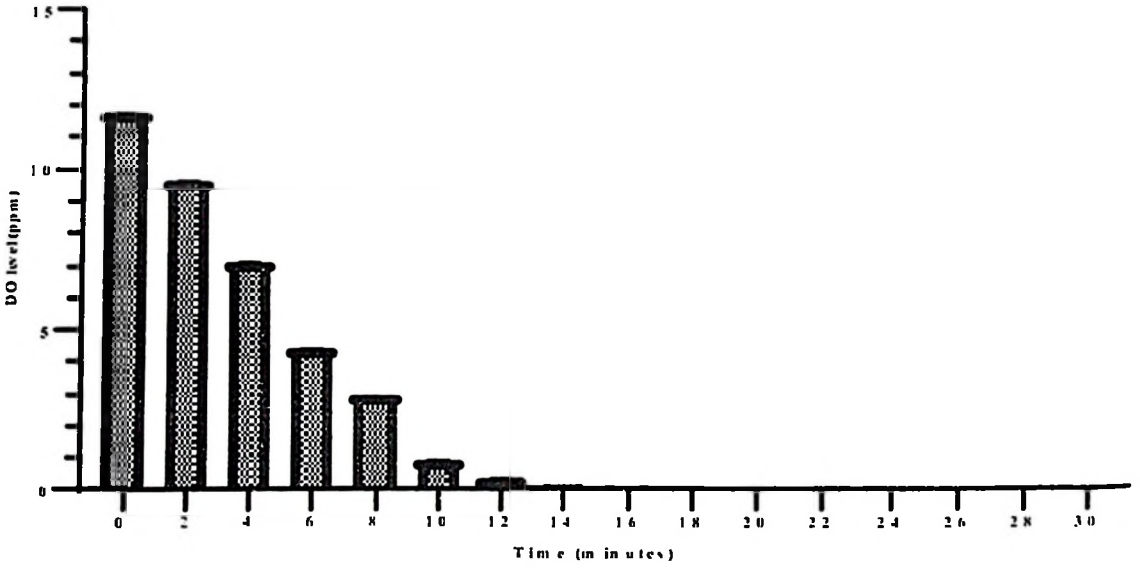


Fig. 1e: Time (min) required to obtain various levels of dissolved oxygen (ppm) in semen dilutor at 2 IU/ml of Oxyrase

Plate 1.a: DO meter

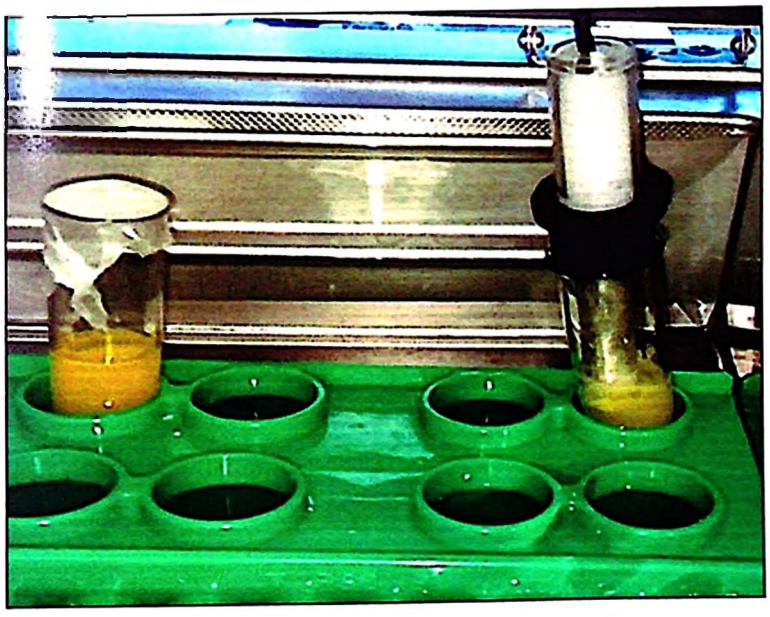


Plate 1.b: Measurement of DO in semen dilutor

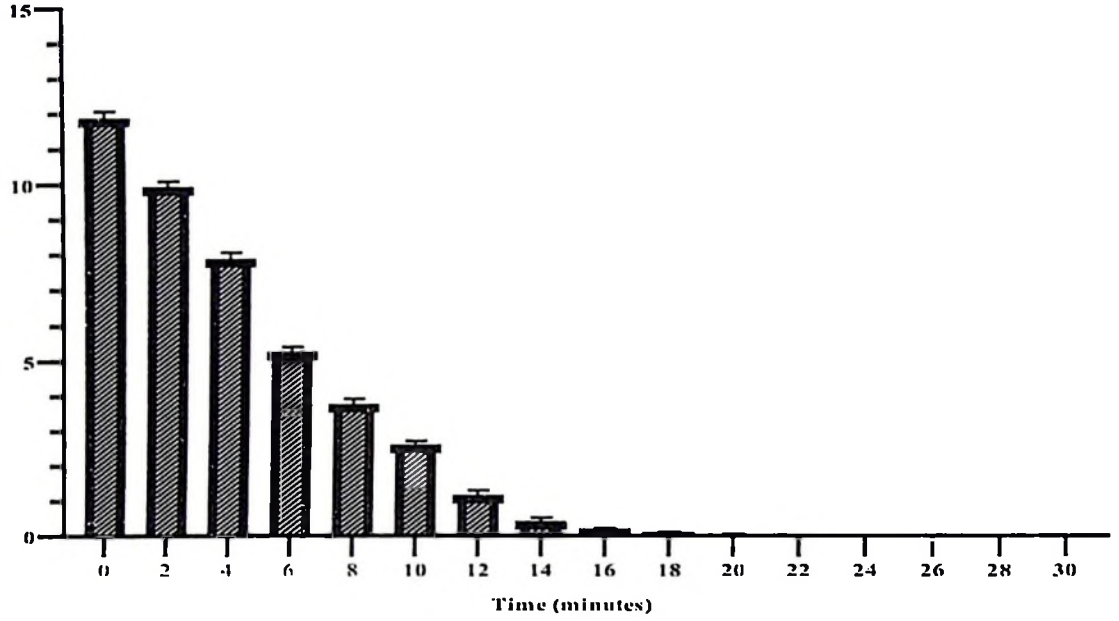


Fig. 1d: Time (min) required to obtain various levels of dissolved oxygen (ppm) in semen dilutor at 1 IU/ml of Oxyrase

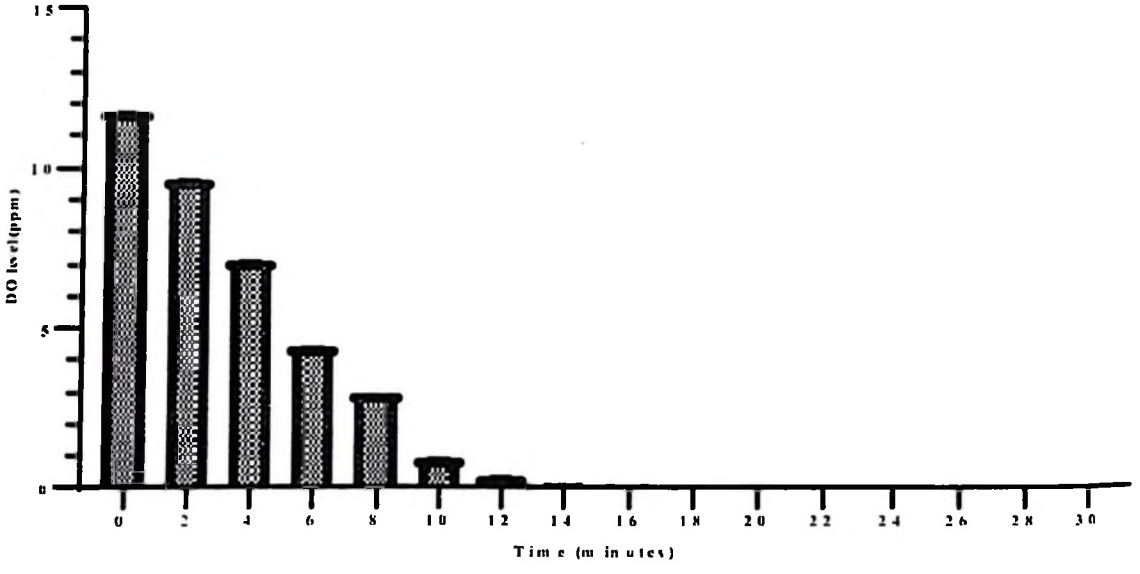


Fig. 1e: Time (min) required to obtain various levels of dissolved oxygen (ppm) in semen dilutor at 2 IU/ml of Oxyrase

4.1. Dissolved oxygen (ppm) levels in the diluted Sahiwal bull semen samples at pre and post thaw stages

Levels of DO in the semen dilutor at pre freeze stage (4 hours of incubation at 4°C) and at post thaw stage (post 24 hours of storage at -196°C) in both groups (control and treatment) are shown in Table 2 as well as Fig. 2.

Table 2: Dissolved Oxygen (ppm) levels in the diluted semen samples of Sahiwal bull at pre-freeze and post-thaw stages

	Pre Freeze		Post Thaw	
	Control	Treatment	Control	Treatment
DO (ppm)	11.52±0.19[a]	3.56±0.11[b]	8.19±0.14[a]	3.20±0.13[b]

Means across the row with different superscripts (a&b) differ significantly ($p<0.05$)

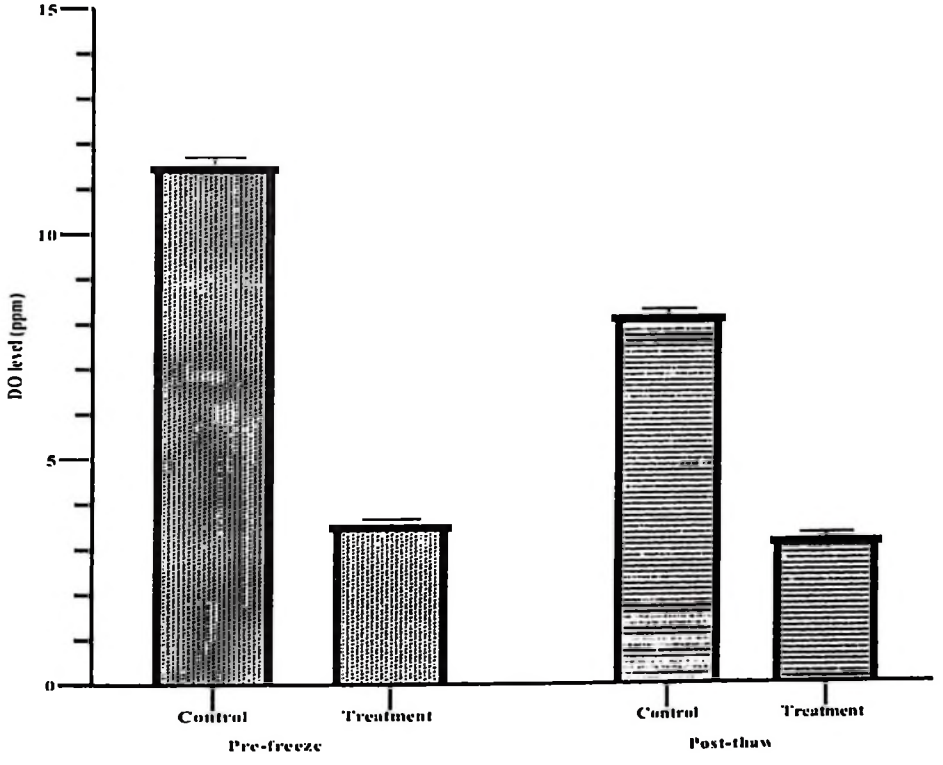


Fig. 2: Levels of dissolved oxygen in diluted semen samples of Sahiwal bull at pre-freeze and post-thaw stages

4.3. Effect of Oxyrase in dilutor on different physico-morphological characteristics of Sahiwal bull spermatozoa (Mean±S.E.M)

4.3.1. Individual progressive motility

The effect of Oxyrse addition in extender on progressive motility of spermatozoa at post dilution, pre-freeze and post- thaw stage of cryopreservation has been tabulated in Table 3 and graphically in Fig. 3.

4.3.1.1. Good ejaculates

4.3.1.1.1. Post-dilution stage

The per cent of progressive motile spermatozoa at post dilution stage in control and treatment group were 79.17±1.93 and 86.67±1.42 respectively. There was no significant difference ($p>0.05$) between the mean per cent of individual progressive motility of spermatozoa between the control and the treatment groups at post-dilution stage.

4.3.1.1.2. Pre-freeze stage

The progressive motility of spermatozoa at the pre-freeze stage was 73.33±1.42 and 80.83±1.49 per cent in control and treatment group, respectively. No statistical significance was observed at pre-freeze stage between the mean differences of the two groups, i.e control and the treatment. Furthermore, the treatment group was having slightly less (6.74%) decline in percentage of progressive motility from post dilution to pre-freeze in comparison to the control group (7.38%).

4.3.1.1.3. Post-thaw stage

The per cent of progressive motile spermatozoa at post-thaw stage in control and treatment group were 46.67±1.40 and 55.83±1.15, respectively. The treatment group was having significantly ($p<0.05$) higher progressive motile spermatozoa than the control group. The decline in per cent of progressive motility from post-dilution to post-thaw was less in the treatment group (35.58%) as compared to the control group (41.05%).

4.3.1.2. Poor ejaculates

4.3.1.2.1. Post-dilution stage

The mean value of post-dilution progressive motility of spermatozoa was 62.50±2.5 and 70.00±1.74 per cent for the control and treatment group, respectively. There was no

significant difference in the mean per cent motility of spermatozoa at post-dilution stage between the control group and the treatment group.

4.3.1.2.2. Pre-freeze stage

The spermatozoa progressive motility per cent at pre-freeze stage for the control and treatment groups were 51.67±3.86 and 65.83±3.58, respectively. The treatment group was having significantly ($p<0.05$) higher progressive motile spermatozoa in comparison to control group. Likewise, the treatment group was having less (5.96%) decline in per cent of progressively motile spermatozoa from post-dilution to pre-freeze stage in comparison to the control group (17.33%).

4.3.1.2.3. Post-thaw stage

At post-thaw stage, the per cent of progressively motile spermatozoa in the control and treatment groups were 28.33±2.07 and 37.5±2.50, respectively. The mean per cent of progressive motility of the spermatozoa between the two groups (control and treatment) did not differ significantly at this stage. However, the decline in per cent of progressive motility from post-dilution to post-thaw stage was less in the treatment group (46.43%) as compared to the control group (54.67%).

Table 3: Effect of Oxyrase in dilutor on individual progressive motility of sahiwal bull spermatozoa at post-dilution, pre-freeze and post-thaw stages of cryopreservation (Mean±S.E.M)

Stage	No. of Ejaculates (n)	Progressive motile spermatozoa (%)			
		Good		Poor	
		Control	Treatment	Control	Treatment
Post-dilution	12	79.17±1.93^{a}	86.67±1.42^{a}	62.50±2.50^{A}	70.00±1.74^{A}
Pre-freeze	12	73.33±1.42^{a}	80.83±1.49^{a}	51.67±3.86^{B}	65.83±3.58^{A}
Post-thaw	12	46.67±1.40^{b}	55.83±1.15^{a}	28.33±2.07^{A}	37.50±2.50^{A}
% Change from post-dilution to pre-freeze		7.38	6.74	17.33	5.96
% Change from post-dilution to post-thaw		41.05	35.58	54.67	46.43

Means across each row with different superscripts (a&b; A&B) differ significantly ($p<0.05$)

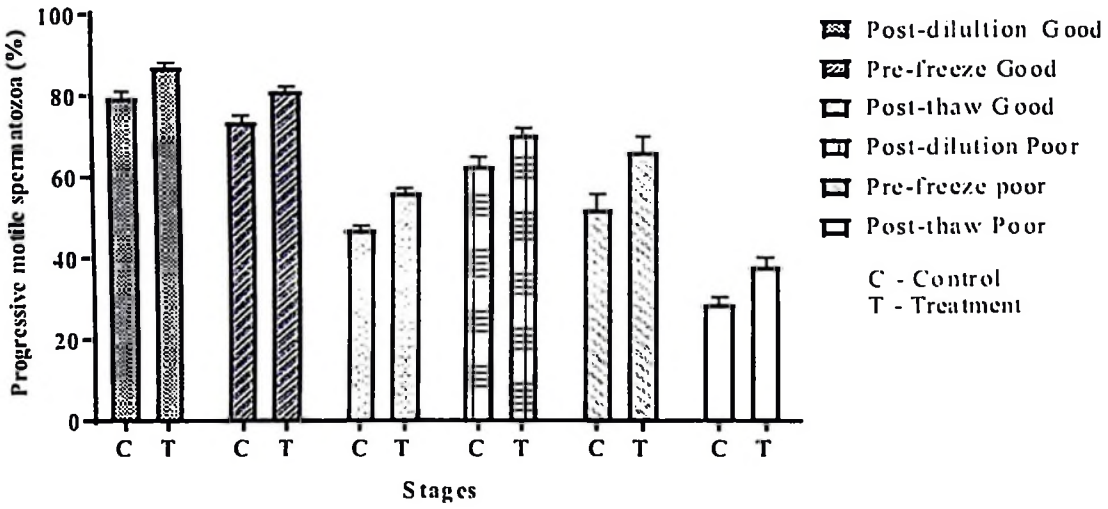


Fig. 3: The effect of Oxyrase in dilutor on individual progressive motility of Sahiwal bull spermatozoa at three different stages of cryopreservation

4.3.2. Acrosomal integrity

The effect of Oxyrase in extender on spermatozoa acrosome integrity at the three different stages of spermatozoa cryopreservation (post dilution, pre-freeze and post- thaw) is depicted in Table 4 as well as in Fig. 4.

4.3.2.1. Good ejaculates

4.3.2.1.1. Post-dilution stage

The mean per cent of acrosomal integrity of spermatozoa at post-dilution stage were 87.17±1.89 and 88.67±1.99 for the control and treatment groups, respectively. There was no significant difference between the mean of the control and the treatment groups at post-dilution stage.

4.3.2.1.2. Pre-freeze stage

The acrosome intactness of the spermatozoa at pre-freeze stage was 83.42±1.40 and 84.83±1.48, respectively. These mean values of the two groups (control and treatment) were statistically non significant. Likewise, there was no difference in change of per cent of acrosomal integrity from post-dilution to pre-freeze stage in the control (4.30%) and treatment (4.33%) groups.

4.3.2.1.3. Post-thaw stage

The mean of spermatozoa with intact acrosome at post-thaw stage for the control and treatment group were 57.25±1.78 and 65.08±1.17, respectively. The mean of the treatment group was found to be significantly ($p<0.05$) higher that of the control group. Also, the decline per cent change from the post-dilution stage to post-thaw stage for the treatment group was lesser (26.60%) in comparison to that of the control group (34.32%).

4.3.2.2. Poor ejaculates

4.3.2.2.1. Post-dilution stage

The values of the spermatozoa with intact acrosome at post dilution stage for the control and the treatment groups were 76.08±2.15 and 81.58±2.07, respectively. There was no statistical difference between the two groups at this stage.

4.3.2.2.2. Pre-freeze stage

At the pre-freeze stage, the mean per cent of spermatozoa with intact acrosome in the control and the treatment groups were 71.42±1.99 and 75.08±2.23, in that order. At this stage, the control and the treatment groups were statistically similar. However, the decline in per cent change from the post-dilution stage to the pre-freeze stage was slightly less in the control group (6.13%) as compared to that of the treatment group (7.97%).

4.3.2.2.3. Post-thaw stage

The mean values of per cent of spermatozoa with intact acrosome at post thaw stage for the control and the treatment group were 40.75±3.39 and 46.67±3.63, respectively. There was no significant difference between the mean values of the control and the treatment groups at post thaw stage. However, the decline in percentage change from the post-dilution stage to post-thaw stage was slightly less in the treatment group (42.79%) compared to that of the control group (46.44%).

Table 4: The effect of Oxyrase in dilutor on acrosomal integrity of spermatozoa at post-dilution, pre-freeze and post-thaw stages of freezing (Mean±S.E.M)

Stage	No. of Ejaculates (n)	Acrosome integrity (%)			
		Good		Poor	
		Control	Treatment	Control	Treatment
Post-dilution	12	87.17±1.89[a]	88.67±1.99[a]	76.08±2.15	81.58±2.07
Pre-freeze	12	83.42±1.40[a]	84.83±1.48[a]	71.42±1.99	75.08±2.22
Post-thaw	12	57.25±1.78[b]	65.08±1.17[a]	40.75±3.39	46.67±3.63
% Change from post-dilution to pre-freeze		4.30	4.33	6.13	7.97
% Change from post-dilution to post-thaw		34.32	26.60	46.44	42.79

Means across each row with different superscripts (a&b) differ significantly ($p<0.05$)

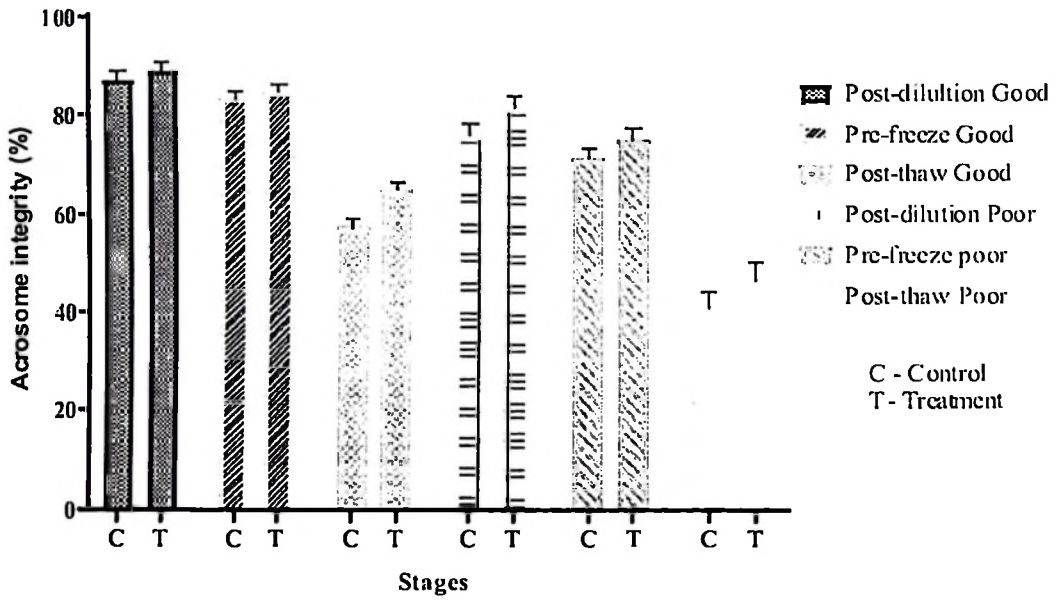
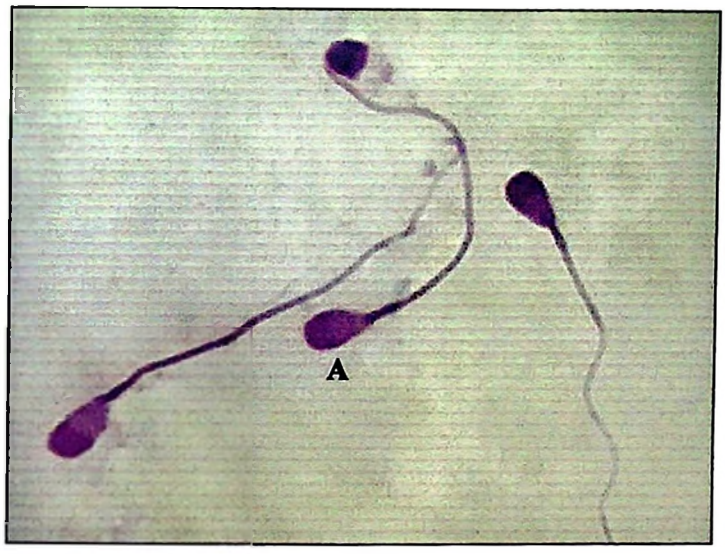


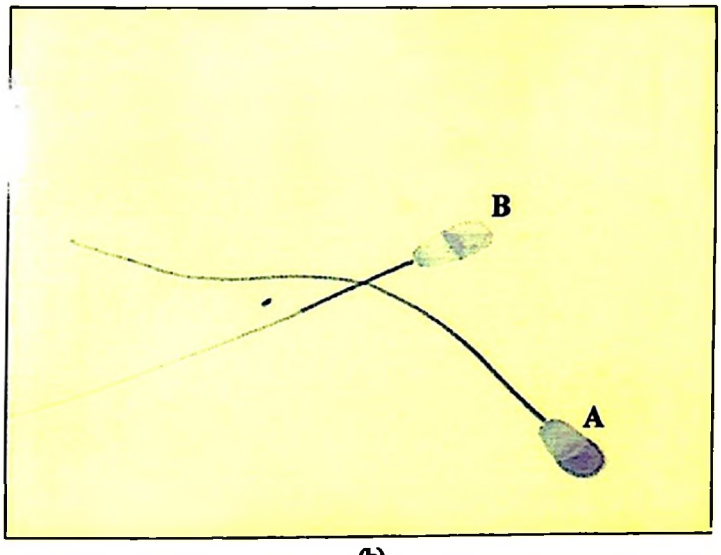
Fig. 4: The effect of Oxyrase in dilutor on acrosomal integrity of Sahiwal bull spermatozoa at three different stages of cryopreservation

4.3.3. Plasma membrane integrity

The findings for the effect of Oxyrse in dilutor on plasma membrane integrity of spermatozoa (HOST response) at three different stages (post-dilution, pre-freeze and post-thaw) of cryopreservation are tabulated in Table 5 and graphically depicted in Fig. 5.



(a)



(b)

Plate 2a,b: Photomicrographs of Sahiwal bull spermatozoa stained with Giemsa stain showing normal and abnormal acrosome (100X)

A – Intact acrosome

B – Denuded acrosome

4.3.3.1. Good ejaculates

4.3.3.1.1. Post-dilution stage

The mean values of the per cent of HOST responsive spermatozoa at post-dilution stage were 84.92±1.84 and 87.33±1.93 for the control and treatment group, respectively. There was no significant difference observed between the mean value of the control group and that of the treatment group at this stage.

4.3.3.1.2. Pre-freeze stage

The per cent of HOST responsive spermatozoa at pre-freeze stage in the control and treatment groups were 71.17±0.68 and 75.92±1.52, respectively. No statistical significant was observed when the mean value of HOST reactive per cent from control and the treatment groups were compared at pre-freeze stage. The change in per cent of HOST responsiveness from the post-dilution stage to pre-freeze stage was slightly less in the treatment group (13.07%) than that of the control (16.19%).

4.3.3.1.3. Post-thaw stage

The mean post-thaw per cent of HOST responsive spermatozoa was 53.00±1.37 and 57.25±1.78 in control and treatment group, in that order. Between the two groups, no significant difference was observed at this stage of cryopreservation. Furthermore, the per cent change from post-dilution stage to this stage was slightly less in the treatment group (34.44%) as compared to the control group (37.59%).

4.3.3.2. Poor ejaculates

4.3.3.2.1. Post-dilution stage

The HOST responsive spermatozoa per cent at post-dilution stage for the control and treatment group were 66.17±1.12 and 68.17±1.07, respectively. But the values are statistically non-significant.

4.3.3.2.2. Pre-freeze stage

At pre-freeze stage, the per cent of HOST responsive spermatozoa for the control and treatment group were 58.58±0.74 and 65.58±1.97, respectively. The value in the treatment

group was significantly ($p<0.05$) higher to that of its counter group. Also, the per cent decline of HOST responsive spermatozoa from the post-dilution stage to this stage was almost three times lesser in the treatment group (3.80%) as compared to the control group (11.47%).

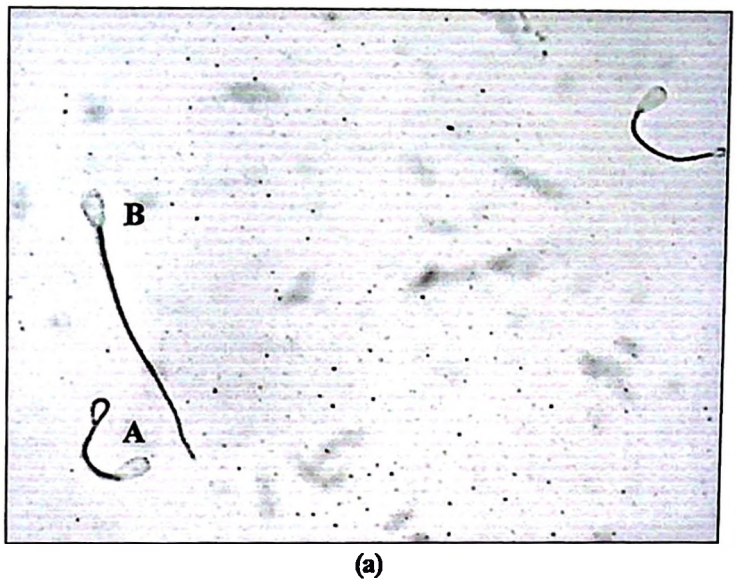
4.3.3.2.3. Post-thaw stage

The means for the per cent of HOST responsive spermatozoa at post-thaw stage was 37.17±1.97 and 41.75±2.35 for the control and treatment groups, respectively. When the mean values from the control and the treatment compared, they showed no significant difference. On the other hand the percentage decline from the post-dilution stage to post-thaw stage was slightly less in the treatment group (38.76%) than the control group (43.81%).

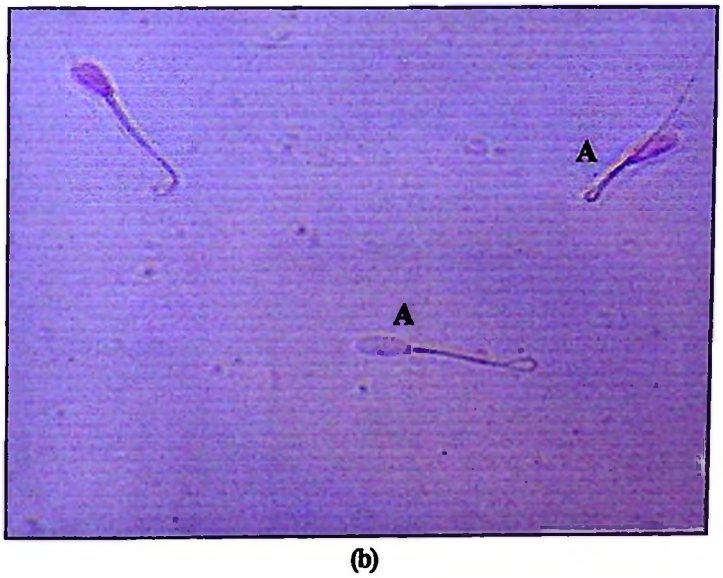
Table 5: The effect of Oxyrase in dilutor on Hypo-osmotic swelling test (HOST) of spermatozoa at three different stages of cryopreservation (Mean±S.E.M).

Stage	No. of Ejaculates (n)	HOST reactive spermatozoa (%)			
		Good		Poor	
		Control	Treatment	Control	Treatment
Post-dilution	12	84.92±1.84	87.33±1.93	66.17±1.12^{A}	68.17±1.072^{A}
Pre-freeze	12	71.17±0.68	75.92±1.52	58.58±0.74^{B}	65.58±1.97^{A}
Post-thaw	12	53.00±1.37	57.25±1.78	37.17±1.97^{A}	41.75±2.35^{A}
% Change from post-dilution to pre-freeze		16.19	13.07	11.47	3.80
% Change from post-dilution to post-thaw		37.59	34.44	43.81	38.76

Means across each row with different superscripts (A&B) differ significantly ($p<0.05$)



(a)



(b)

Plate 3a,b: Photomicrographs of Sahiwal bull spermatozoa showing response to HOST (40X)
A - HOST positive
B - HOST negative

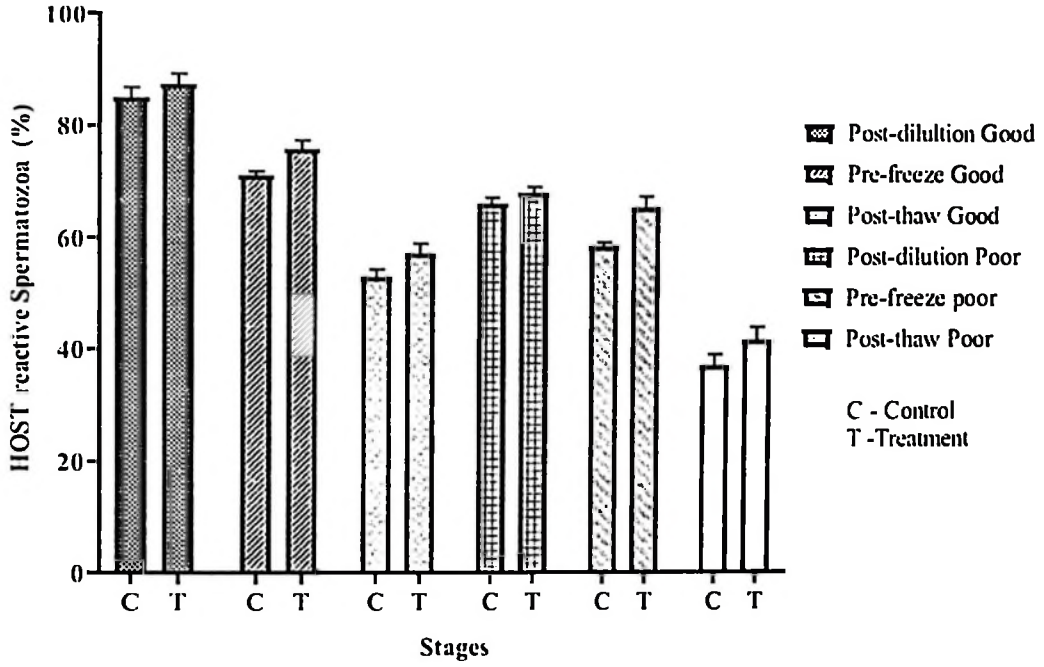


Fig. 5: The effect of Oxyrase in dilutor on Hypo-osmotic swelling test of Sahiwal bull spermatozoa at three different stages of cryopreservation

4.3.4. Seminal plasma cholesterol

The findings on the concentration of cholesterol in the seminal plasm at frest, pre-freeze and post-thaw stages of good and poor ejaculate categories are tabulated in Table 6 and graphically depicted in Fig.6.

4.3.4.1. Good ejaculates

4.3.4.1.1. Fresh stage

The average amount of cholesterol in the seminal plasma at fresh stage in this category was 36.10±0.50 mg/dL.

4.3.4.1.2. Pre-freeze stage

The concentration of cholesterol in the seminal plasma at pre-freeze stage for the Oxyrase treated and non-treated group was 82.47±0.73 mg/dL and 84.62±1.50 mg/dL, respectively. Statistically there was no significant difference between the Oxyrase treated group and its counterpart group. However, there was a significant change from fresh to pre-freeze

stage in terms of cholesterol loss from the spermatozoa to the seminal plasma in the Oxyrase treated group and non-treated group i.e. 128.45% and 134.40%, respectively. Furthermore, there was a significant ($p<0.05$) difference in concentration of seminal plasma cholesterol between the fresh stage and either of the group of pre-freeze stage.

4.3.4.1.3. Post-thaw stage

The concentration of cholesterol in the seminal plasma at this stage was 121.23±0.47 and 124.82±0.42 mg/dL for the Oxyrase treated and non-treated groups, respectively. The mean concentration of cholesterol in the seminal plasma at post-thaw stage was significantly ($p<0.05$) lower in the Oxyrase treated group in comparison to non-treated group. Also the per cent change in cholesterol concentration from fresh to post thaw stage was 245.76% and 255.82% for the control and treatment groups, respectively.

4.3.4.2. Poor ejaculates

4.3.4.2.1. Fresh stage

The mean value of cholesterol in the seminal plasma in poor ejaculate category at fresh stage was 20.33±0.74 mg/dL.

4.3.4.2.2. Pre-freeze stage

At pre-freeze stage, the mean cholesterol concentration was significantly ($p<0.05$) lower in the Oxyrase treated group (110.33±1.19 mg/dL) than in the control group (118.46±1.50 mg/dL). Also there was a significant increase in the amount of cholesterol in either of the group at this stage in comparison to the fresh stage value. Furthermore, the per cent change from fresh to pre-freeze was higher in the non-treated group than the treated one i.e. 179.85% over 153.56%, respectively.

4.3.4.2.3. Post-thaw stage

The mean concentration of cholesterol (mg/dL) was significantly ($p<0.05$) lower in the Oxyrase treated group (146.63±3.00) in comparison to not-treated group (157.98±4.71) at post-thaw stage. The per cent increase in cholesterol concentration from the fresh stage to post-thaw stage was significantly ($p<0.05$) higher in the non-treated group. Furthermore, the

concentration of cholesterol in either of the group at this stage was significantly ($p<0.05$) higher than that of the fresh stage.

Table 6: The effect of Oxyrase in dilutor on Seminal plasma cholesterol (mg/dL) at fresh, pre-freeze and post-thaw stage of cryopreservation.

Stage	No. of Ejaculates (n)	Seminal plasma cholesterol (mg/dL)			
		Good		Poor	
		Control	Treatment	Control	Treatment
Fresh	12	36.10 ± 0.50^{c}	36.10 ± 0.50^{c}	42.33 ± 0.74^{Z}	42.33 ± 0.74^{Z}
Pre-freeze	12	84.62 ± 1.50^{by}	82.47 ± 0.73^{by}	118.46 ± 1.5^{AY}	110.33 ± 1.19^{BY}
Post-thaw	12	124.82 ± 0.42^{aw}	121.23 ± 0.47^{bw}	157.98 ± 4.71^{AX}	146.63 ± 3.00^{BX}
% Change from post-dilution to pre-freeze		134.4	128.45	179.85	160.64
% Change from post-dilution to post-thaw		245.76	235.82	273.21	246.34

Means across each row with different superscripts (a&b or A&B) differ significantly ($p<0.05$)
Means across each column with different superscripts (x,y&z or X&YZ) differ significantly ($p<0.05$)

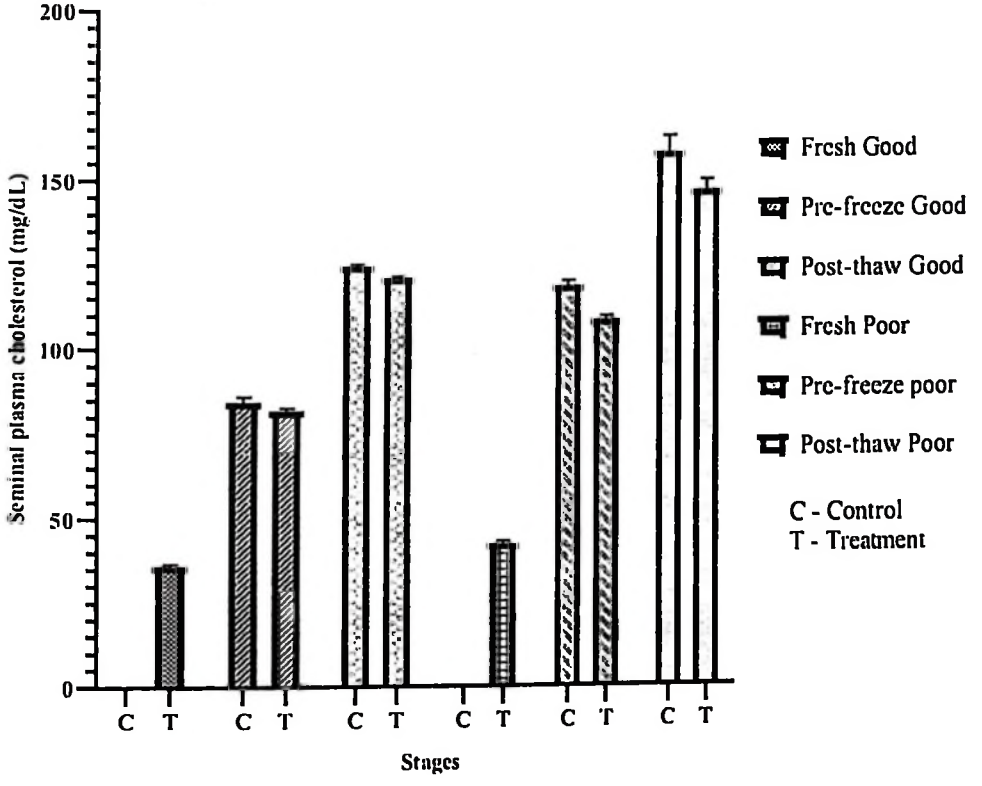


Fig. 6: Effect of Oxyrase in dilutor on seminal plasma cholesterol (mg/dL) of Sahiwal bull at three different stages of cryopreservation

4.3.5. Plasma membrane phospholipids

The findings (Mean±S.E.M) on the effect of Oxyrase on the plasma membrane phospholipids (μg/100 x 10^6) at three different stages of semen freezing (fresh, pre-freeze and post-thaw) for both good and poor ejaculates are presented in Table 7 and Fig.7.

4.3.5.1. Good ejaculates

4.3.5.1.1. Fresh stage

The mean value of plasma membrane phospholipids at fresh stage was 49.95±1.04 μg/100 x 10^6 spermatozoa.

4.3.5.1.2. Pre-freeze stage

The concentrations (μg/100 x 106 spermatozoa) of plasma membrane phospholipids for the control and treatment groups were 75.78±1.18 and 74.43±1.32, respectively. There was no significant difference in the mean concentration of phospholipids between the control and the treatment groups at pre-freeze stage. However, the per cent change from fresh stage to pre-freeze stage was slightly lower (49.01%) in the treatment group as compare to that of the control group (51.71%). Furthermore, there was a significant ($p<0.05$) increase in concentration of plasma membrane phospholipids from the fresh to pre-freeze stage.

4.3.5.1.3. Post-thaw stage

The mean concentration of phospholipids at the post-thaw stage for the control and the treatment groups were 67.44±0.99 μg/100 x 10^6 and 66.43±0.87 μg/100 x 10^6, respectively. No significant difference was observed between the mean concentrations of the two groups at this stage. The decline in phospholipids concentration from pre-freeze to post-thaw was less in the treatment group (32.99%) in comparison to the control group (35.02%). Furthermore, there was a significant ($p<0.05$) change in the concentration of phospholipids from fresh stage to either of the post-thaw groups.

4.3.5.2. Poor ejaculates

4.3.5.2.1. Fresh stage

For the poor ejaculates, the mean value of plasma membrane phospholipids at fresh stage was 45.36±1.76 μg/100 x 10^6 spermatozoa.

4.3.5.2.2. Pre-freeze stage

At pre-freeze stage, the mean values of phospholipids for the control and the treatment groups was 78.54±1.76 and 76.71±0.98 µg/100 x 10^6 spermatozoa. Between the two groups, no significant difference was observed at this stage. Also the per cent change from fresh to pre-freeze for the control and the treatment groups was 73.15% and 69.11%, respectively. Furthermore, there was a significant ($p<0.05$) difference between the mean values of the fresh and either of the group of pre-freeze stage.

4.3.5.2.3. Post-thaw stage

The mean plasma membrane phospholipids in µg/100 x 10^6 spermatozoa for the control and the treatment groups were 68.85±1.00 and 67.55±1.00, respectively. There was no significant difference observed between the treated and non-treated groups at post-thaw stage. There was a drop in the mean values of phospholipids concentration from pre-freeze to post-thaw in both groups. However, the decline from pre-freeze to post thaw was high in the control group (51.79%) than in the Oxyrase treated group (48.91%). Furthermore, there was a remarkable significant ($p<0.05$) change in phospholipids concentration between the fresh stage and the post-thaw stage.

Plasma membrane cholesterol to phospholipids (C: P) ratio could not be calculated due to some technical problems on estimation of plasma membrane cholesterol.

Table 7: The effect of Oxyrase in dilutor on plasma membrane phospholipids (µg/100 x 10^6 spermatozoa) at fresh, pre-freeze and post-thaw stage of cryopreservation.

Stage	No. of Ejaculates (n)	Plasma membrane phospholipids (µg/100 x 106)			
		Good		Poor	
		Control	Treatment	Control	Treatment
Fresh	12	49.95±1.04^{z}	49.95±1.04^{z}	45.36±1.76^{Z}	45.36±1.76^{Z}
Pre-freeze	12	75.78±1.18^{x}	74.43±1.32^{x}	78.54±1.76^{X}	76.71±0.98^{X}
Post-thaw	12	67.44±0.99^{y}	66.43±0.87^{y}	68.85±1.00^{Y}	67.55±1.00^{Y}
% Change from fresh to pre-freeze		51.71	49.01	73.15	69.11
% Change from fresh to post-thaw		35.02	32.99	51.79	48.91

Means across each column with different superscripts (x,y&z or X,Y&Z) differ significantly ($p<0.05$)

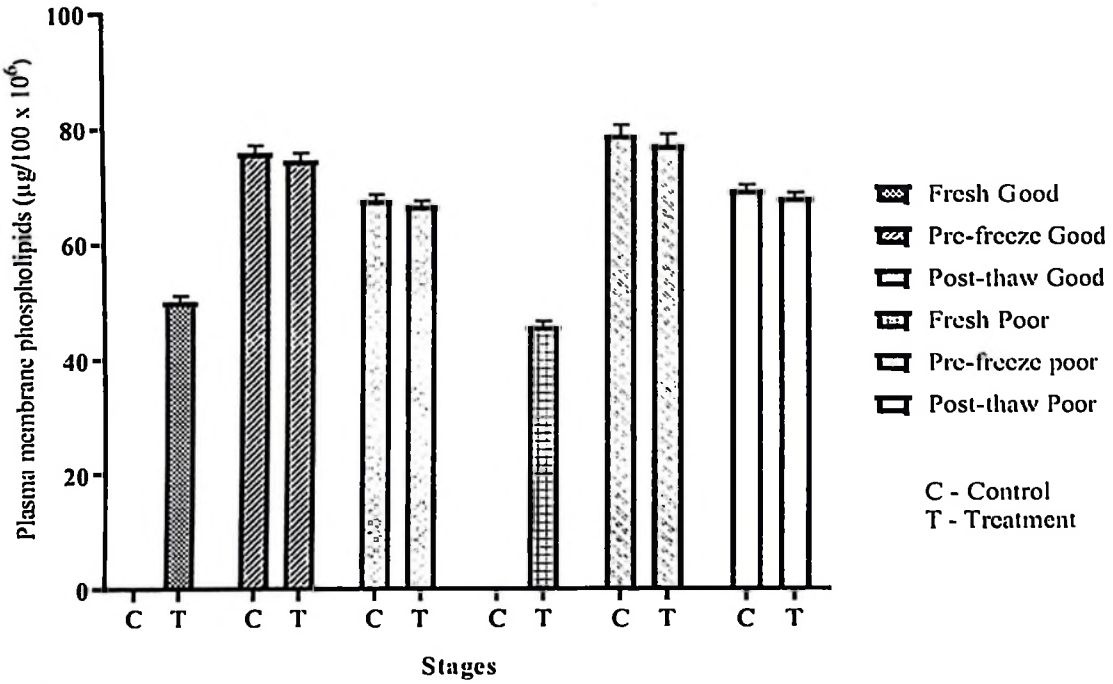


Fig. 7: Effect of Oxyrase in dilutor on plasma membrane phospholipids (µg/100 x 10^6 spermatozoa) of Sahiwal bull at three different stages of cryopreservation

4.4. Effect of Oxyrase in dilutor on oxidative stress of Sahiwal bull semen

4.4.1. Plasma membrane lipid peroxidation (LPO)

The findings on the effect of Oxyrase in dilutor on MDA prodution level (µmol/mL) of spermatozoa at pre-freeze and post-thaw stages for the good and poor ejaculates are shown in Table 8 and graphically in Fig. 8.

4.4.1.1. Good ejaculates

4.4.1.1.1. Pre-freeze stage

The mean values of MDA production (µmol/mL) at pre-freeze stage for the control and the treatment group were 0.47±0.04 and 0.35±0.038, respectively. The treatment group was found to have significantly ($p<0.05$) lower value of MDA production in comparison to that of the control group.

4.4.1.1.2. Post-thaw stage

The extent of MDA production (µmol/mL) at post-thaw stage for the control and the treatment groups were 0.72±0.04 and 0.55±0.03, respectively. It was found that the treatment

group was having significantly ($p<0.05$) lower MDA production as compared to that of the control group. Moreover, the per cent increase in MDA production from the pre-freeze stage to the post-thaw stage found to be higher in the treatment group (59.36%) than the control (52.76%).

4.4.1.2. Poor ejaculates

4.4.1.2.1. Pre-freeze stage

The level of MDA production (µmol/mL) of the spermatozoa in the control and treatment group at pre-freeze stage was 0.50±0.03 and 0.47±0.04, respectively. At this stage, no significant difference was observed when the mean values of the control and treatment groups were put into comparison.

4.4.1.2.2. Post-thaw stage

The mean MDA production (µmol/mL) in the spermatozoa at post-thaw stage was 0.75±0.03 in the control group and 0.65±0.02 in the treatment group. All the two groups showed similarity in their mean values when compared statistically. However, the per cent change in MDA production from the pre-freeze to the post-thaw stage was less in the treatment group (38.30%) in comparison to the control group (50.50%).

Table 8: The effect of Oxyrase in dilutor on spermatozoa plasma membrane lipid peroxidation (LPO) at pre-freeze and post-thaw stages of cryopreservation.

Stage	No. of Ejaculates (n)	MDA (µmol/mL)			
		Good		Poor	
		Control	Treatment	Control	Treatment
Pre-freeze	12	0.47±0.04[a]	0.35±0.04[b]	0.50±0.03	0.47±0.04
Post-thaw	12	0.72±0.04[a]	0.55±0.03[b]	0.75±0.03	0.65±0.02
% Change from Pre-freeze to post-thaw		53.19	57.14	50.50	38.30

Means across each row with different superscripts (a&b) differ significantly ($p<0.05$)

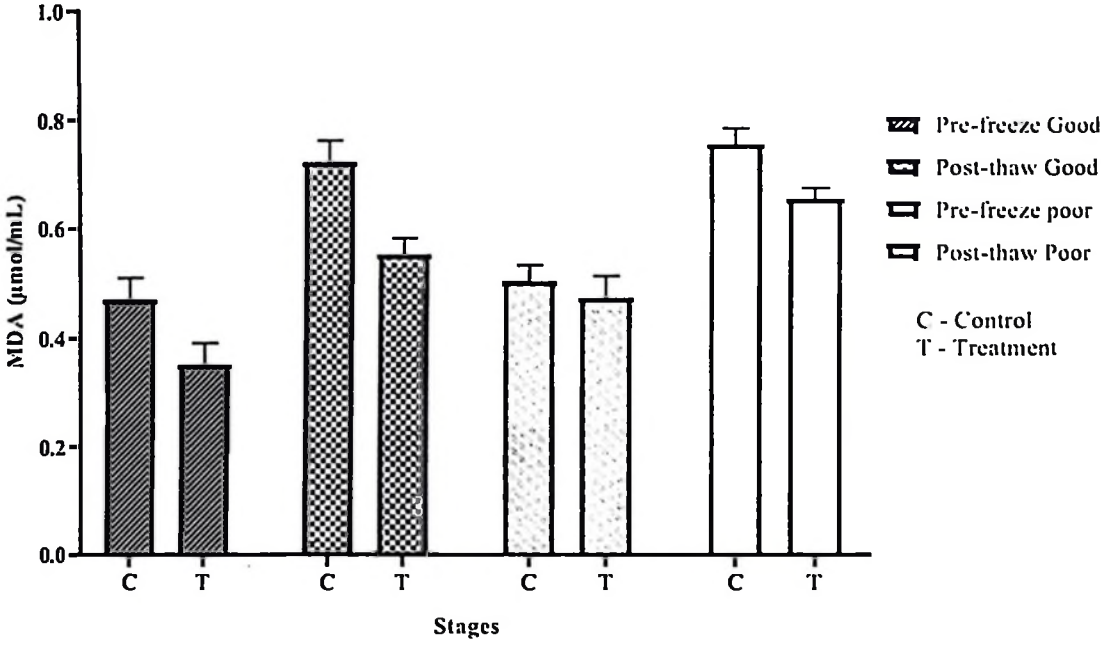


Fig. 8: Effect of Oxyrase in dilutor on spermatozoa plasma membrane lipid peroxidation at pre-freeze and post-thaw stages of cryopreservation

4.4.2. Total antioxidant capacity (TAC - nmol/mL)

The results on the effect of Oxyrase in dilutor on TAC (nmol/mL) level of seminal plasma at two different stages (pre-freeze and post-thaw) are presented in Table 9 and Fig. 9.

4.4.2.1. Good ejaculates

4.4.2.1.1. Pre-freeze stage

The mean value of TAC at pre-freeze stage was estimated to be 896.82±21.36 nmol/ mLin the control group and973.49±15.68 nmol/mL in the treatment group. The two groups were found to be statistically similar when their mean values were compared.

4.4.2.1.2. Post-thaw stage

The level of TAC (nmol/mL) in the treatment and the control groups at post-thaw stage were 553.80±29.47 and 639.50±17.13, respectively. The treatment group was found to have significantly ($p<0.05$) higher TAC mean value in comparison to that of the control group. Also, the percentage change in levels of TAC in seminal plasma from pre-freeze to post-thaw was slightly lower in the treatment group (34.31%) than that of the control (38.23%).

4.4.2.2. Poor ejaculates

4.4.2.2.1. Pre-freeze stage

The results on the TCA level in the seminal plasma of the control and the treatment group were 867.26±20.07 nmol/mL and 938.64±13.04 nmol/mL at pre-freeze stage. There was no significant difference observed between mean values of the two groups at pre-freeze stage.

4.4.2.2.2. Post-thaw stage

The seminal plasma TAC value in the control and the treatment groups at post-thaw stage were 347.61±30.85 nmol/mL and 480.90±22.65 nmol/mL, respectively. The treatment group was observed to have significantly ($p<0.05$) higher TAC values in comparison to that of the control. Moreover, the per cent change in seminal plasma TAC from pre-freeze to the post thaw stage was recorded to be lower in the treatment group (48.77%) compared to that of the control group (59.92%).

Table 9: The effect of Oxyrase in dilutor on levels of total antioxidant capacity (TAC) in Sahiwal bull seminal plasma at pre-freeze and post-thaw stages of cryopreservation

Stage	No. of Ejaculates (n)	TAC (nmol/mL)			
		Good		Poor	
		Control	Treatment	Control	Treatment
Pre-freeze	12	896.82±21.36^{a}	973.49±15.68^{a}	867.26±20.07^{A}	938.64±13.04^{A}
Post-thaw	12	553.80±29.47^{b}	639.50±17.13^{a}	347.61±30.85^{B}	480.90±22.65^{A}
% Change from Pre-freeze to post-thaw		38.23	34.31	59.92	48.77

Means across each row with different superscripts (a&b or A&B) differ significantly ($p<0.05$)

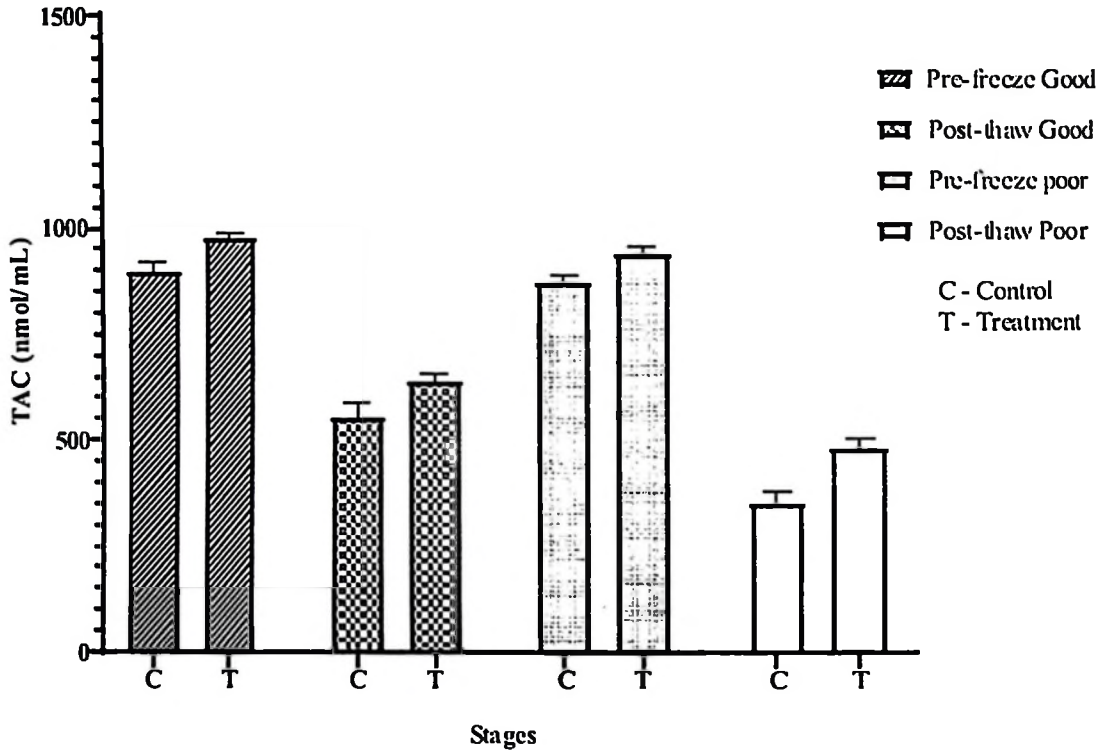


Fig. 9: Effect of Oxyrase in dilutor on TAC levels of Sahiwal bull spermatozoa at pre-freeze and post-thaw stages of cryopreservation

4.4.3. Reactive oxygen species (ROS)

The results for the ROS level (Units of H_2O_2) in seminal plasma at pre-freeze and post thaw stages are shown in Table 10 and graphically in Fig. 10.

4.4.3.1. Good ejaculates

4.4.3.1.1. Pre-freeze stage

The mean value of ROS in the seminal plasma of the control and the treatment groups were 980.7±41.57 and 895.1±46.53 Units of H_2O_2 respectively. The difference between the mean value in treated group and non-treated group was statistically non-significant at pre-freeze stage.

4.4.3.1.2. Post-thaw stage

At post-thaw stage the mean ROS was higher in the control group (1177±50.42 Units of H_2O_2) than that in the treatment group (1121±36.13 Units of H_2O_2). Though, there was

difference between the mean values of the two groups, but the difference was statistically non-significant.

4.4.3.2. Poor ejaculates

4.4.3.2.1. Pre-freeze stage

During pre-freeze the mean value of ROS (Units of H_2O_2) in the seminal plasma was 1037±41.61 in the control group and 962.4±41.51 in the treatment group. The difference between the treated and non-treated groups at pre-freeze was statistically non-significant.

4.4.3.3. Post-thaw stage

The mean values of ROS (Units of H_2O_2) in the control and treatment groups at post-thaw stage were 1207±29.10 and 1164±28.46, respectively. However, mean values of ROS in both groups were statistically non-significant.

Table 10: The effect of Oxyrase in dilutor on reactive oxygen species (ROS) levels in Sahiwal bull seminal plasma at pre-freeze and post-thaw stages of cryopreservation (Mean±S.E.M)

Stage	No. of Ejaculates (n)	ROS (Units of H_2O_2)			
		Good		Poor	
		Control	Treatment	Control	Treatment
Pre-freeze	12	980.7±41.57	895.1±46.53	1037±41.61	962.4±41.51
Post-thaw	12	1177±50.42	1121±36.13	1207±29.10	1164±28.46
% Change from Pre-freeze to post-thaw		20.02	22.93	16.39	20.95

No significant difference between the values across each row in all stages of both categories

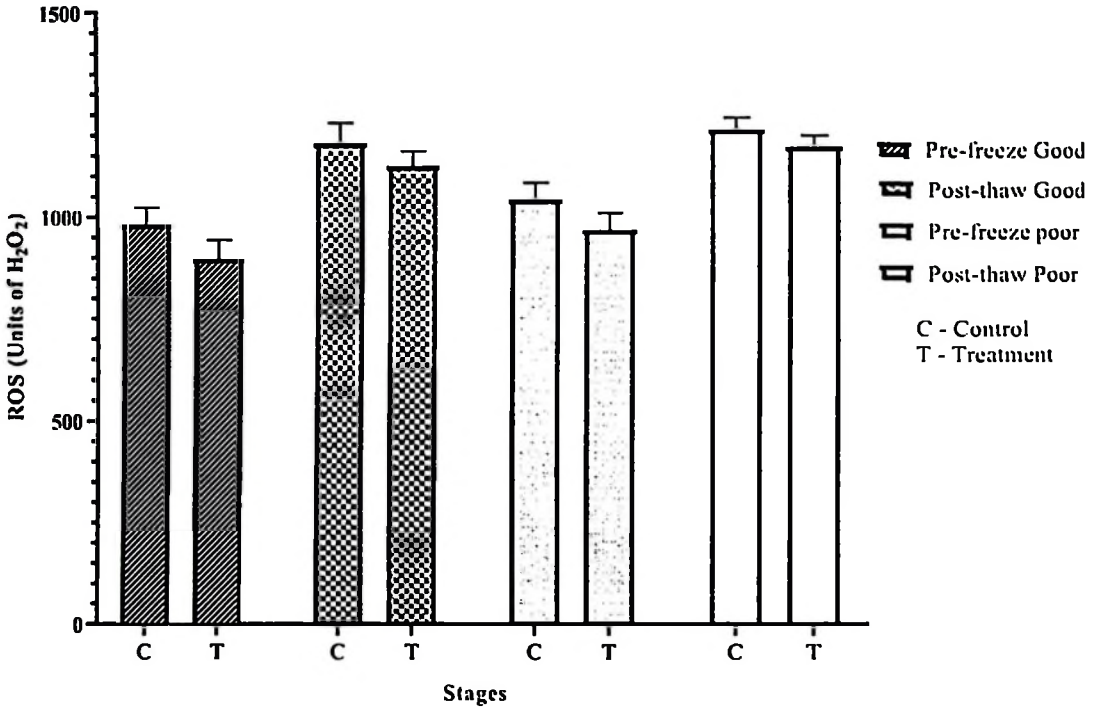


Fig. 10: Effect of Oxyrase in dilutors on ROS levels of Sahiwal bull seminal plasma at pre-freeze and post-thaw stages of cryopreservation

4.5. Bacterial load

The extent of bacterial contamination (CFU/mL) at fresh stage as well as post-thaw stage for the good and poor ejaculates, are shown in Table 11 and diagrammatically in Fig. 11.

4.5.1. Good ejaculates

4.5.1.1. Fresh stage

The mean value of bacterial load (CFU/mL) at fresh stage was 108.3±31.28.

4.5.1.2. Post-thaw stage

The mean bacterial contamination (CFU/mL) in the control and treatment groups at post-thaw stage was 300.0±62.76 and 83.33±32.18, respectively. At this stage, the control group had significantly ($p<0.05$) higher bacterial load in comparison to not only its counter group but also to the fresh stage. The per cent change from the fresh to post-thaw stage was lower in the treatment group (23.06%). However, the per cent change of bacterial load from fresh stage to the control group of the post-thaw stage was very high (177.01%).

4.5.2. Poor ejaculates

4.5.2.1. Fresh stage

The mean level of bacterial contamination at fresh stage in the poor ejaculates was 216.7±81.49 CFU/mL of semen.

4.5.2.2. Post-thaw stage

During the post-thaw stage, the average bacterial load for the control group was 333.3±76.21 CFU/mL and the treatment group was 225.0±46.26 CFU/mL. There was no significant difference between the control and the treatment groups at this stage. Also, there was no significant change between the mean value of the fresh stage in comparison to either group of the post-thaw stage. However, the per cent change from the fresh stage to post-thaw was lower in the treatment group (3.83%) and the control group (53.81%).

The bacterial load in the treatment group of good ejaculates category has been decreased from fresh (108.3±31.28 CFU/mL) to post-thaw stage (83.33±32.18 CFU/mL) to a great extent (23.06%). However, in control group it has been increased from fresh (108.3±31.28 CFU/mL) to post-thaw stage (300.0±62.76 CFU/mL) by (177.01%).

Table 11: The effect of Oxyrase in dilutor on semen bacterial load (CFU/mL) at fresh and post-thaw stages of cryopreservation (Mean±S.E.M)

Stage	No. of Ejaculates (n)	Bacterial load (CFU/mL)			
		Good		Poor	
		Control	Treatment	Control	Treatment
Fresh	12	108.3 ± 31.28^{by}	108.3 ± 31.28^{y}	216.7 ± 81.49^{Y}	216.7 ± 81.49^{Y}
Post-thaw	12	300.0 ± 62.76^{ax}	83.33 ± 32.18^{by}	333.3 ± 76.21^{X}	225.0 ± 46.26^{Y}
% Change from Fresh to post-thaw		177.01	23.06	53.81	3.83

Means across each row with different superscripts (a&b) differ significantly ($p<0.05$)
Means across each column with different superscripts (x&y or X&Y) differ significantly ($p<0.05$)

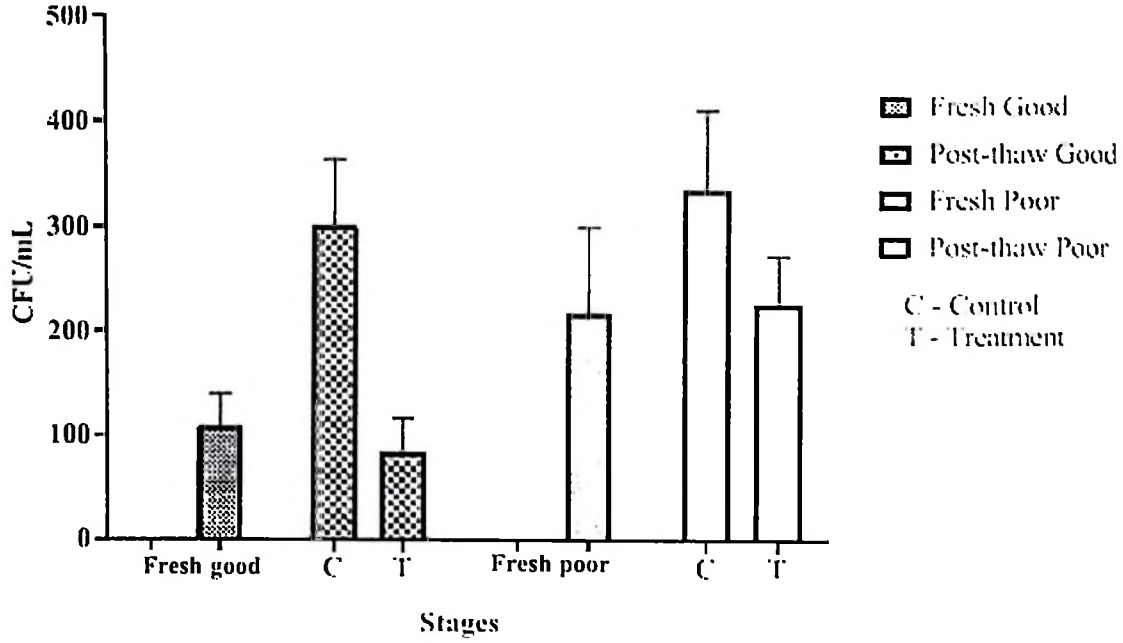


Fig. 11: Effect of Oxyrase in dilutor on bacterial load (CFU/mL) at pre-freeze and post-thaw stages of cryopreservation

Discussion

5 DISCUSSION

The current study was carried out to evaluate the effect of Oxyrse in semen extender as a partial deoxygenating agent on freezability of Sahiwal bull semen.

5.1. Standardization of Oxyrase in dilutor

The present study intended to standardize the concentration of Oxyrase in semen dilutor that would give DO level of around 4ppm. To achieve so, five different concentrations were used *i.e.* 0.125, 0.25, 0.5, 1 and 2 IU/mL. Among those, 0.125 IU/mL was found to suit the purpose as it reduces DO in dilutor to around 4pm in about 16-18 min at 35°C.

The concentration of Oxyrase was standardized in the semen dilutor, in order to get the DO level of around 4ppm at pre-freeze and post-thaw stages. This is because 4ppm was found to improve semen freezability via decreasing ROS levels and increased the fertilizing ability of crossbred bull spermatozoa (Mustapha, 2017). In that study, the technique was based on the principle of physical displacement of oxygen molecules using partially inert gas like nitrogen which does not have any adverse effect on the osmotic status and pH of the medium (Parkinson *et al.*, 2009).

Oxyrase effectively reduces the level of dissolved oxygen (DO) in the solution to low levels was known earlier by Alder, (1990). In the later years, studies on the use of Oxyrase in semen dilutor and its effect on the freezability of frozen-thawed spermatozoa started (Koshimoto *et al.*, 2000; Mazur *et al.*, 2000; Dong *et al.*, 2010; Darr *et al.*, 2016 and London *et al.*, 2017).

5.2. The levels of DO at pre freeze and post thaw stages

In the current study the level of DO was slightly low at pre-freeze and post-thaw stages for the treatment group in comparison to when nitrogen gassing was used in previous studies (Mustapha, 2017; Amin *et al.*, 2018; Bhutia, 2018 and Kumar *et al.*, 2018). However, in previous studies when Oxyrase was incorporated in the semen dilutor, the level of DO at pre-freeze and post-thaw stages was not mentioned (Koshimoto *et al.*, 2000; Mazur *et al.*, 2000; Dong *et al.*, 2010; Darr *et al.*, 2016 and London *et al.*, 2017).

The lower levels of DO at pre-freeze could be explained by the fact that, it takes some time (60 to 90 minutes) for the temperature of the diluted semen in the straws to equilibrate. During this time the Oxyrase will be acting though at a reduced rate, due to temperature decline. That is why, when we measured the DO level at pre-freeze it was slightly less than 4ppm. On the other hand when the temperature of the medium reaches 4°C, the Oxyrase activities are stopped and therefore no further reduction of DO level.

However, the DO level in the medium was further reduced after thawing due to resumption of Oxyrase activities. In addition to that, oxygen consumption is high in frozen thawed semen because spermatozoa metabolic activities are resumed after thawing (Garrett *et al.*, 2008; Pelaez *et al.*, 2006 and Thomas *et al.*, 2006). In our study, the DO level at post-thaw stage was low (3.2 ppm) in the first two minutes after thawing.

5.3. The effect of Oxyrase in diluted semen on physico-morphological characteristics of Sahiwal bull spermatozoa at different stages of cryopreservation

5.3.1. Individual progressive motility at post-dilution, pre-freeze and post-thaw stages

The individual progressive motility of the Sahiwa bull spermatozoa was significantly higher in the treatment group at post-thaw stage in good ejaculate category. Also, was significantly higher in the treatment group at pre-freeze in poor ejaculate category. However, at post-dilution stage for both categories, no significance difference between the control and treatment group was noted. The same was observed at pre-freeze and post-thaw stages of the good and poor ejaculates, respectively.

5.3.1.1. Post-dilution stage

The mean values of individual progressive motility of the Sahiwal bull spermatozoa for the control and treatment groups were 79.17±1.93% and 86.67±1.42% from good/freezable ejaculates. However, from the poor category/non-freezable ejaculates were 62.50±2.50% and 70.00±1.74 for the control and treatment, respectively.

These values for the freezable ejaculates match with findings from the previous studies in Sahiwal bull semen parameters which reported progressive motility at post-dilution stage was ranging from 65-85% (Ahmad *et al.*, 2003; Rehman *et al.*, 2016). The same was reported in crossbred bulls when nitrogen gassing method was used (Mustapha, 2017; Amin *et al.*, 2018; Bhutia, 2018 and Kumar *et al.*, 2018).

In this study, the influence of Oxyrase on progressive motility of the spermatozoa observed at post-dilution stage was statistical non-significant. This means that Oxyrase has no beneficial effect on progressive motility of spermatozoa at post-dilution stage. Similar findings were reported by previous studies in our Lab on effect of reduced DO (4ppm) on progressive motility at post dilution stage (Mustapha, 2017; Amin *et al.*, 2018; Bhutia, 2018 and Kumar *et al.*, 2018).

The reports on the effect of Oxyrase on progressive motility of good ejaculates in different species are contradicting. Oxyrase is having positive benefit to spermatozoa motility in mice (Koshimoto *et al.*, 2000 and Mazur *et al.*, 2000). However, other study in stallion ejaculates with good tolerance to cryodamage, Oxyrase had no significant effect on spermatozoa motility of freshly diluted semen (London *et al.*, 2017).

For the poor ejaculates category, the current study reported no beneficial effect of Oxyrase on spermatozoa progressive motility at post-dilution stage in comparison to the control. This is contrary to previous report where Oxyrase had beneficial effect on non-freezable ejaculates of stallion and rhesus monkey (Darr *et al.*, 2016 and Dong *et al.*, 2010) at post-dilution stage. This could be contributed by species difference as well as the concentration of oxyrase used. The concentration of Oxyrase used in the current study was low compared to those in the previous studies. Although, oxyrase demonstrated non-significant improvement in spermatozoa mortality but the treated group has relatively high motality than the control.

5.3.1.2. Pre-freeze

After filling and sealing, semen straws from good and poor ejaculate categories were incubated for four hours at 4°C. This practice is a standard protocol for proper glycerolisation before freezing. The metabolic activity of the spermatozoa is gradually lowered as the temperature decreases (from 35°C to 4°C) during equilibration period. This goes hand to hand with the gradual decrease of Oxyrase action in the treated group as the temperature is lowered during the period of equilibration. During the early phases of equilibration, spermatozoa are metabolically active and produce more ROS as a result of oxidative phosphorylation particularly under elevated levels of DO within the dilutor (Gibb and Aitken, 2016; Mustapha, 2017). In addition to this, the spermatozoa are subjected to phase transition of membrane lipids rendering them vulnerable to oxidative stress (Jones and Mann, 1977).

In this study, no significant difference was observed on spermatozoa motility between the Oxyrase treated group and non-treated group in good ejaculates category at pre-freeze stage. While on the other hand, Oxyrase treated group in poor category semen demonstrated significantly high spermatozoa motility in comparison to the non-treated group. Previous studies reported the improvement of spermatozoa progressive motility in mice, stallion and rhesus monkey in the Oxyrase treated groups at pre-freeze stage (Koshimoto *et al.*, 2000; Mazur *et al.*, 2000; Dong *et al.*, 2010 and Darr *et al.*, 2016). In comparison to these reports, the current study result from the poor ejaculates category is in agreement with the previous studies.

On the other hand, in good ejaculates category the present result deviate from previous studies in mice, stallion and rhesus monkey. However, there is one report by London *et al.*, (2017) where our findings match with their study in stallion with history of good tolerance to cryodamage at pre-freeze stage.

Also, per cent change in progressive motility from post-dilution to pre-freeze stage was similar in the good ejaculate category between control and treatment groups. However, it was significantly different in the poor ejaculate category i.e. the decline in progressive motility was less in the Oxyrase treated group. This means Oxyrase has beneficial effect on the poor ejaculate semen as it significantly maintains the spermatozoa progressive motility from post-dilution to pre-freeze in comparison to the control group.

5.3.1.3. Post-thaw

Single freeze-thaw cycle is adequate to drop the spermatozoal motility by 50 % (Pena *et al.*, 2009). In the present study, a reduction of 41.05% and 54.67% in progressive motility in the control group of good and poor category, respectively have been observed from the post-dilution to post-thaw stage. However, the treatment group of good ejaculate category was having significantly less decline in progressive motility. No significant difference was revealed in the poor ejaculates category between treated and no-treated groups at post-thaw.

At post-thaw stage, in good ejaculate category significant difference was noted between the treatment and the control group. This point out that Oxyrase in the treated group had significant benefit on the progressive motility of spermatozoa in comparison to the non-treated group. On the other side, there was no significant difference in the mean progressive motility of spermatozoa observed between the treated and non treated group in poor ejaculate category,

With regard to good ejaculate category, the present study concurs with the report in mice which pointed out the positive beneficial effect of Oxyrase on progressive motility of spermatozoa at post thaw stage (Koshimoto *et al.*, 2000 and Mazur *et al.*, 2000). On the other side, our results differ from the previous study in stallion with history of producing semen with good tolerance to cryodamage (London *et al.*, 2017). The difference could be due to species difference as the current study was done in bovine (Sahiwal breed) and the previous study was conducted in Equine.

The other finding we have experienced in our study during the time of evaluation of individual progressive motility of spermatozoa at post-thaw stage was vigorous motility of spermatozoa just after semen thawing, in the treated group. However, there is drastic drop in spermatozoa motility in the treated group in comparison to the non-treated group. This could be due to resumption of spermatozoa metabolic activities after thawing of frozen semen. Furthermore, Oxyrase resumes its activity of reducing oxygen due to the increase in temperature (37°C) and ultimately the DO level is reduced. This could be the cause of exhaustion of the spermatozoa *i.e.* reduced oxygen in the medium makes its availability to be less for the energy apparatus to generate more ATP to sustain the motility.

5.3.2. Acrosomal integrity by Giemsa staining at post-dilution, pre-freeze and post-thaw

The process of freezing-thawing raises the maturation of sperm membranes resulted from the damage rooted by unwarranted ROS production and capacitated acrosome (Medeiros *et al.*, 2002). The damage caused by ROS to cell phospholipids is of little doubt, being one of the constituent of the acrosomal membrane is liable to this adverse effect. Amusingly, among most important physiological roles of ROS are induction of capacitation and the acrosomal reaction (Satorre *et al.*, 2007; Aitken, 2017). Though, the aforementioned processes are critical for fertilization of ovum by the spermatozoa, their early induction during spermatozoa processing can prove unfavorable to the longevity of spermatozoa in the female reproductive tract (Medeiros *et al.*, 2002).

In the present study, significantly higher per cent of intact acrosome was observed in the treated group of the good ejaculate category at post thaw stage. However, at post-dilution and pre-freeze stages in both categories and the post-thaw stage in poor category, the Oxyrase treated groups showed numerically higher mean values but no significant difference was observed between the treated and non-treated groups.

These findings at post-thaw stage in the good ejaculates category provide the same opinion with the previous studies which also suggested the benefit of reduced DO to the spermatozoa acrosomal intactness at post-thaw stage in bovine semen (Pande *et al.*, 2015; Mustapha, 2017; Amin *et al.*, 2018, Bhutia, 2018 and Kumar *et al.*, 2018).

5.3.3. Plasma membrane integrity by HOS test at post-dilution, pre-freeze and post-thaw

Intactness of the spermatozoa plasma membrane is obligatory for its fertilizing ability (Amirat *et al.*, 2004). The functional plasma membrane is essential for the spermatozoa capacitation, acrosome reaction and binding to the ovum surface (Satorre *et al.*, 2007 and Aitken, 2017). Hypo-osmotic swelling test evaluates the functional integrity of the spermatozoa plasma membrane and also serves as a useful indicator of its fertility potential.

In the current study, significantly higher percentage of HOST reactive spermatozoa observed in the treatment group of poor ejaculates category at pre-freeze stage. While in other stages, no statistical significant difference was observed between the treated and non-treated groups from both categories. Furthermore, previous study in stallion semen by London *et al.* (2017), reported increase in membrane damage in the Oxyrase treated groups in comparison to non-treated groups at post-thaw stage.

The values obtained in the current study did not match with previous studies evaluating the benefit of reduced DO by nitrogen gassing method on plasma membrane integrity in the good ejaculates category at post-thaw stage (Mustapha, 2017; Amin *et al.*, 2018, Bhutia, 2018 and Kumar *et al.*, 2018).

Although, our mean values of plasma membrane intactness were statistically not significant at all stages in good ejaculates category but numerically the treated groups demonstrated high values of plasma membrane intactness in comparison to the control groups. With this regard, there is a beneficial effect provided by Oxyrase to the protection of plasma membrane integrity but it was not enough to impart the statistical difference in comparison to the control group in this study.

5.3.4. Seminal plasma cholesterol at fresh, pre-freeze and post-thaw

Cholesterol plays a special role in the sperm membrane because its release from the sperm membrane initiates the key step in the process of capacitation and acrosome reaction that is crucial for fertilization (Witte and Schäfer-Somi, 2007). It is well established that during cryopreservation cholesterol efflux occurs from the spermatozoa plasma membrane. This upshot calcium influx which triggers intracellular signaling cascade associated with capacitation (Leahy and Gadella, 2015).

In this study, we have estimated seminal plasma cholesterol and found that cholesterol efflux increases tremendously during cryopreservation process i.e. highest cholesterol was recorded at post-thaw followed by pre freeze and the lowest was at fresh stage.

Significantly low cholesterol efflux was observed in the Oxyrase treated group than non-treated group at post-thaw stage in the good ejaculates category. While during pre-freeze

the extent of cholesterol efflux was similar in Oxyrase treated and non-treated groups. This finding, agrees with previous studies stated the increase in cholesterol efflux during semen cryopreservation (Rajoriya *et al.*, 2013; Leahy and Gadella, 2015 and Amin *et al.*, 2018, Ramamoorthy, 2018).

On the other hand, significantly lower concentrations of cholesterol were noted in Oxyrase treated groups in comparison to control groups at pre-freeze and post-thaw stages in the poor ejaculates category. This indicates the decrease in cholesterol efflux in the Oxyrase treated groups hence less spermatozoa plasma membrane damage. This finding matches with other studies in rhesus monkey and stallion with low tolerance to cryodamage where oxyrase was reported to protect plasma membrane of the spermatozoa (Dong *et al.*, 2010 and Dar *et al.*, 2016).

5.3.5. Plasma membrane phospholipids at fresh, pre-freeze and post-thaw

The mean spermatozoa plasma membrane phospholipids content at fresh stage from present study was 49.95±1.04 µg/100x10^6 spermatozoa in the good ejaculates category. However, in the poor category slightly low mean value 45.36±1.76 µg/100 million spermatozoa was noted.

In this study, the mean value of plasma membrane phospholipids obtained in the good category is similar to those reported in buffalo and crossbred bulls at fresh stage (Rajoriya *et al.*, 2016 and Amin *et al.*, 2018). However, the value observed in the poor ejaculates category at fresh stage is slightly lower. No specific report on the level of plasma membrane phospholipids in Sahiwal bull spermatozoa at fresh stage could be traced.

In the present study, phospholipids concentration at pre-freeze and post-thaw stages in treated and non-treated groups in both categories were similar but higher than fresh stage. The increased values at pre-freeze and post-thaw stages in both categories could be due to incorporation of egg yolk lipids present in semen dilutor into the spermatozoa plasma membrane (Vishwanathan *et al.*, 1992 and Amin *et al.*, 2018). However, there was a distinct decline in the mean value of phospholipids from pre-freeze to post-thaw stage. The decline from pre-freeze stage to post-thaw stage is due to freezing and thawing damages.

The same trend of increased plasma membrane lipids at pre-freeze and post-thaw stages was observed in other study when egg yolk based extender was used in boar (Cerolini *et al.*, 2001). Yolk lipoproteins can strongly bind to plasma membrane of the spermatozoa (Vishwanathan *et al.*, 1992). Lipids from semen dilutor can reversibly bind to plasma membrane hence providing physical barrier to cryodamages without affecting the lipid content of spermatozoa plasma membrane (De Leeuw *et al.*, 1993 and Zeron *et al.*, 2002). Stabilization of the spermatozoa against cold shock is the outcome of replacing the lost lipids with the exogenous lipids occurring between plasma membrane and semen dilutor (Graham and Foote, 1987; Buhr *et al.*, 1994).

There is scanty of literature on the effect of reduced DO on spermatozoa plasma membrane phospholipids in bovine semen, except that by Amin *et al.* (2018) who reported the similar findings in cross-bred bull spermatozoa.

5.4. Oxidative stress status

5.4.1. Lipid peroxidation by MDA assay at pre-freeze and post-thaw

Extent of oxidative stress manifested by peroxidative injure due to ROS can be computed indirectly in spermatozoa by MDA assay. Numerous studies have detailed MDA assay to have outstanding association with impairment of spermatozoa function in terms of motility and capacity to fertilize (Sidhu *et al.*, 1998; Chatterjee and Gagnon, 2001).

In this study, the mean values of MDA (μmol/mL) was significantly low in the Oxyrase treated groups in comparison to no-treated groups at pre-freeze and post-thaw stages of good ejaculates category. The results in the current study, matches with previous workers when the effects of DO in dilutor were evaluated in bovine semen (Mustapha, 2017; Bhutia, 2018; Kumar *et al.*, 2018 and Amin *et al.*, 2018). However, the findings in our study were slightly lower. This could designate less production of free radicals or protection of the membrane against the action of the free radicals in the Oxyrase treated groups at pre-freeze and post-thaw in good ejaculates category.

In the poor ejaculates category, no significant difference was observed between the treated and non-treated groups at pre-freeze and post-thaw stages of croyopreservation.

These results agree with previous studies in stallion whereby, Oxyrase had no significant protection to spermatozoa against peroxidation by the free radicals (Dar *et al.*, 2016 and London *et al.*, 2017).

5.4.2. Total antioxidant capacity at pre-freeze and post-thaw

Assessment of total antioxidants in Sahiwal bull seminal plasma is prophetic tool for the estimation of oxidative stress in frozen thawed spermatozoa (Gürler *et al.*, 2015). In this study, significantly high total antioxidant capacity was estimated in Oxyrase treated group at post-thaw stage in comparison to non-Oxyrase group in both categories of ejaculate. However, at pre-freeze stage in both categories the FRAP values showed no statistical significance between the Oxyrase treated and non-treated groups regardless of their numerical differences.

Our findings in the good ejaculate category match with the previous studies done in our lab except the values obtained in this study are slightly higher. This could be due to differences in breeds under each study (Amin *et al.*, 2018; Bhutia, 2018 and Kumar *et al.*, 2018).

Higher TAC mean values in the Oxyrase treated groups indicate suppression of free-radicals formation due to decreased oxidative phosphorylation because of low DO in the media. On the other side of the non-treated groups, FRAP values could result from utilization of available antioxidants in scavenging the generated free radicals. Furthermore, less oxidative stress was noted in the Oxyrase treated groups during the process of cryopreservation and thawing. This observation could be supported by the results on MDA at post-thaw stage.

5.4.3. Reactive oxygen species at pre-freeze and post-thaw

Normal spermatozoa functions such as capacitation, acrosome reaction and preservation of fertilizing ability do require optimal amount of ROS. Nevertheless, increase in ROS production causes oxidative stress which further damages the spermatozoa (Sofikitis *et al.*, 1995). Oxidative stress reduces sperm motility, viability, ionophore-induced acrosome reaction and sperm oocyte fusion. Furthermore, hydrogen peroxide appears to be the primary ROS responsible for those changes via lipid peroxidation (Alvarez and Storey, 1983; Barbas and Mascarenhas, 2009; Tatone *et al.*, 2010; Aitken and Curry, 2011).

In the current study, no significant difference was observed between the control and Oxyrase treated groups at pre-freeze and post-thaw stages in both categories. These findings are contrary with previous studies in our lab, bur the trend of decrease in ROS levels in groups with low DO matches. Those studies revealed the reduction of DO (4ppm) in semen dilutor significantly reduces ROS at both pre-freeze and post-thaw stage (Amin *et al.*, 2018 and Kumar *et al.*, 2018). However, findings in this study are almost ten times the result obtained by previous workers in our lab. This could be contributed by breed difference as this study was conducted in Sahiwal and they evaluated ROS in crossbred bulls. On the other hand, results of this study are within range with reference to results obtained from human seminal plasma (Moein *et al.*, 2007). In different species the level of ROS productions vary widely (1.5 to 9700 units) among species, among individuals and among ejaculates. (Griveau and Le Lannou, 1997; Padron *et al.*, 1997; Moein, *et al.*, 2007; Amin *et al.*, 2018 and Kumar *et al.*, 2018).

The report by Dar *et al.*, (2016) in stallion semen observed dose dependent function of Oxyrase. The benefits of Oxyrase were less in lower doses i.e. 0.6 IU/mL and 0.12 IU/mL while higher dose (2.4 IU/mL) was found to be the best and the saturation point was 5 IU/mL. Considering the dose we used in this study (0.125 IU/mL), it is more likely that concentration of Oxyrase was not enough to significantly trim down ROS production.

Previous studies done in mice, monkeys and stallion with low tolerance to cryodamage reported four types of protections that Oxyrase do provide to spermatozoa during cryopreservation process. These benefits include elimination of damages from centrifugation and reduction of osmotic damage from the cryoprotectants. Third, Oxyrase allows the use of higher concentration of cryoprotectant *i.e.* glycerol up to three times and lastly it imposes anaerobic effect which substantially increases the motility of spermatozoa at post-thaw (Mazur *et al.*, 2000, Dong *et al.*, 2010, Dar *et al.*, 2016 and London *et al.*, 2017).

5.4.4. Bacterial load at fresh and post-thaw stage

Bacterial contamination in the semen could not only have impact on the female side after deposition, but also, it has a devastating effect to the spermatozoa as it is the good source

of ROS. As per Minimum Standard Protocol (MSP) of cryopreserved bovine semen, maximum permissible bacterial load should be 5000 cfu/mL. The level of bacterial contamination in the fresh bovine semen ranges from 5.0 to 444 x 10^4 cfu/mL (Almquist *et al.*, 1949; Ronald and Prabhakar, 2001). While the bacterial load of frozen-thaw semen was reported to be 1.77±0.13 cfu/mL in Sahiwal and 0.85±0.09 cfu/mL in Crossbred bulls (Ankesh, 2017).

The results obtained in the current study were significantly ($p<0.05$) low in the Oxyrase treated group at post-thaw stage in good ejaculates category. Furthermore, our results match up with the MSP recommendation. The maximum bacterial load in our study was 333.30±76.21 cfu/mL in the control group of poor ejaculates category it is still very low i.e. 6.67% of the recommended maximum value by MSP.

The numerical values of bacterial load in Oxyrase treated groups at post thaw in both categories are low in comparison to non-treated groups. Moreover, the per cent change from fresh to post-thaw was very low in Oxyrase treated groups of both categories. This point out the chances of contamination during semen processing in the Oxyrase treated group are less and the opposite happens in the non-treated groups.

Due to the fact that, most of the semen extenders i.e. TYG and INRA96 contain antibiotics like sodium penicillin, streptomycin, gentamicin, amphotericin B and these antibiotics cover a broad spectrum of action to minimize or eliminate the bacterial contaminants in the semen used for artificial insemination (Dar *et al.*, 2016).

Summary and Conclusions

6 SUMMARY AND CONCLUSIONS

The current study was carried out in order to evaluate the effect of incorporating Oxyrase in the dilutor on freezability of Sahiwal bull spermatozoa. Intention was to reduce troubles originating from oxygen metabolism during the time of semen processing. This was achieved by first standardizing the concentration of Oxyrase to be added in the semen dilutor. Secondly, evaluation of semen quality parameters such as physic-morphological functional and oxidative stress at different stages of semen cryopreservation after addition of Oxyrase in the extender.

On the basis of previous reports, the level of dissolved oxygen (DO) of 4ppm in the semen dilutor found to improve cryopreserved semen quality parameters. Moreover, Oxyrase was reported to have the capacity of scavenging oxygen in the media and finally reduces the DO to low level. This triggered this study to test different concentration of Oxyrase on the rate of reduction of DO in semen extender. Finally, one concentration of Oxyrase was chosen and used for the study.

A total of 24 ejaculates were collected from four Sahiwal bulls maintained at Germ-Plasm Centre, IVRI. After collection the ejaculates were evaluated for initial progressive motility, this enabled us to group the ejaculates into two broader categories. First was the poor ejaculates category having initial progressive motility of 55 to 65% and second was good ejaculates category with $\geq$70% initial progressive motility.

Whenever there was a need of separation of spermatozoa pellet and seminal plasma, representative semen samples from each experimental group were centrifuged at 4000 g for

20 minutes at 5°C in a refrigerated centrifuge (Sigma). Then, the supernatant and pellet were stored at -20°C till their use. The semen pellets were utilized for plasma membrane phospholipids and plasma membrane lipid peroxidation (LPO) while the supernatant (seminal plasma) was used for the estimation of cholesterol, total antioxidant capacity (TAC) and reactive oxygen species (ROS).

Each ejaculate was further evaluated for spermatozoa concentration (by Photometer), bacterial load (CFU/mL), seminal plasma cholesterol (kit method) and plasma membrane phospholipids at fresh stage. Thereafter, the remaining fresh semen was split into two portions and final dilution was done with different extender. One group was extended with dilutor without Oxyrase while the other group was extended with dilutor containing 0.125 IU/mL of Oxyrase.

Different semen quality parameters were evaluated at different stages of semen cryopreservation. Parameters like individual progressive motility, acrosomal integrity (Giemsa staining) and plasma membrane integrity (HOST responsiveness) were assessed at post-dilution stage. After the final dilution the extended semen was filled in 0.25 mL French min straws, sealed and kept at 4°C for 4 h in cold-handling cabinet for equilibration and glycerolisation.

During pre-freeze stage, in addition to the post-dilution parameters, evaluated for oxidative stress status *i.e.* spermatozoa plasma membrane lipid peroxidation (MDA assay), seminal plasma total antioxidant capacity (FRAP assay) and reactive oxygen species in the seminal plasma. Other parameters like seminal plasma cholesterol, plasma membrane phospholipids and DO level of extended semen in each group were evaluated at this stage.

From cold-handling cabinet straws were taken for freezing in the Biological Freezer where temperature was reduced from 4°C to -140°C in 7 minutes. Then the straws were plunged directly into liquid nitrogen (-196°C) for cryopreservation and storage.

During the post-thaw stage, all the parameters evaluated at pre-freeze were repeated and bacterial load was determined. This was performed after thawing of representative number of straws from each group in water bath at 37°C for 30s.

There was a significant ($p<0.05$) reduction in the DO level in Oxyrase treated groups at pre-freeze (3.56±0.11) and post-thaw (3.20±0.13) stages. While, in the control group significant ($p<0.05$) decrease in the DO was observed at post thaw stage.

Individual progressive motility of the spermatozoa was significantly ($p<0.05$) higher in the Oxyrase treated group (65.83±3.58%) than non-treated group at pre-freeze in poor ejaculates category. Also the treated group (55.83±1.15%) in the good ejaculates category at post-thaw stage showed significantly higher motility of the spermatozoa than the control group (46.67±1.40%). Also, the per cent change in progressive motility in the Oxyrase treated groups from post-dilution to pre-freeze was significantly ($p<0.05$) low (5.96%) in the poor ejaculates category and from post-dilution to post-thaw (35.58%) stage in the good ejaculates category.

Significantly ($p<0.05$) higher per cent of spermatozoa with intact acrosome was observed in Oxyrase treated group (65.08±1.17%) in comparison to control (57.25±1.78%) at post-thaw stage in the good ejaculate category. There were no significant difference between the control and the treatment groups at post-dilution and pre-freeze stages in all ejaculate categories and at the post-thaw stage of the poor ejaculates category. Also, the per cent decline was significantly ($p<0.05$) lower from post-dilution to post-thaw in the good ejaculates category.

The response of the spermatozoa to HOST was significantly ($p<0.05$) higher in the treatment group at pre-freeze stage of the poor ejaculates category in comparison to its control. Even though, there was a slightly higher values in the Oxyrase treated groups in good ejaculates category but the difference was statistically non-significant.

The extent of cholesterol efflux from the spermatozoa was significantly ($p<0.05$) lower in the Oxyrase treated groups in good ejaculates (121.23±0.47 mg/dL) and in poor ejaculates (146.63±3.00 mg/dL) category compared to control groups *i.e.*, 124.82±0.42 in good and 157.98±4.71 in poor ejaculates at post-thaw stage. Furthermore, cholesterol effluxes were observed to increase from fresh stage to pre-freeze and to post thaw stage.

There was a significant ($p<0.05$) increase in the spermatozoa plasma membrane phospholipids from fresh stage to pre-freeze and post-thaw in the treated and non-treated groups in all ejaculates categories.

A significantly ($p<0.05$) lower MDA values were recorded in the Oxyrase treated groups at pre-freeze stage (0.35±0.04µmol/mL) and post-thaw stage (0.55±0.04µmol/mL) of good ejaculates category in comparison to their control groups. No statistical difference was observed in the poor ejaculates categories.

The mean TAC values in the Oxyrase treated groups were significantly ($p<0.05$) higher at post-thaw stage in good ejaculates category (639.50±17.13 nmol/mL) and in poor ejaculates category (480.90±22.65 nmol/mL) than control groups. At pre freeze stage, the TAC values between groups showed no statistical significance in both categories.

No significant difference in ROS production was observed between the Oxyrase treated groups and non-treated groups at all stages and both ejaculate categories.

A significant ($p<0.05$) decreased of bacterial load was observed in the Oxyrase treated group compared to the control group at post-thaw in the good ejaculates category. There was no statistical difference in the poor ejaculates category observed.

Conclusion

- 0.125 IU/mL of Oxyrase concentration can significantly reduce DO in the extender to 4 ppm in 16-18 minutes at 35°C.
- Addition of Oxyrase in dilutor significantly increases freezability of Sahiwal bull spermatozoa by improving post-thaw motility, acrosome integrity, TAC and reducing cholesterol efflux, LPO and bacterial load in good ejaculates.
- Addition of Oxyrase in poor ejaculates reduces cholesterol efflux and increase TAC but do not improve the freezability to a significant level.

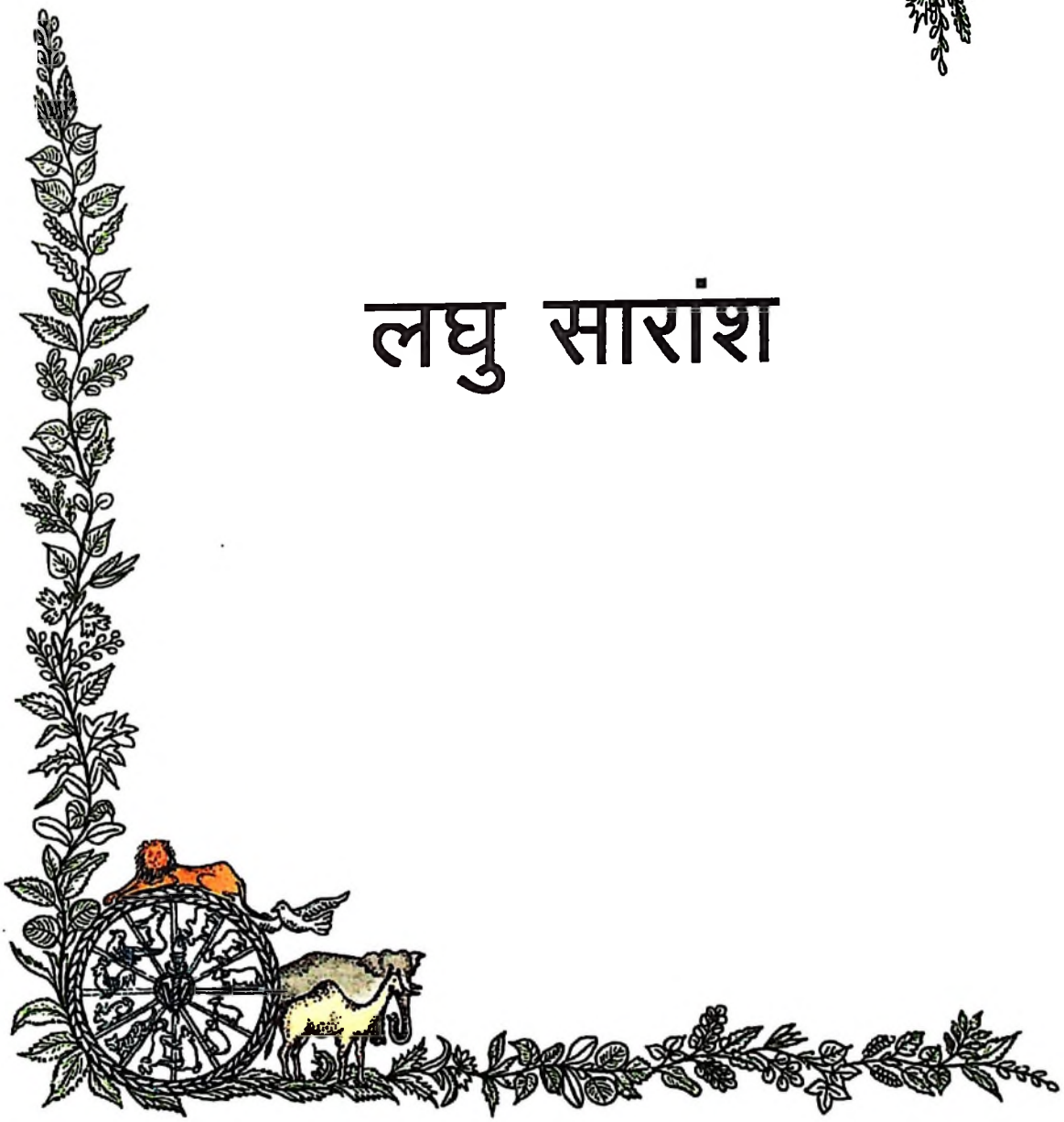
Future perspectives

- A detailed study on the effect of higher concentrations of Oyrase in bull semen
- *in vitro* and *in vivo* fertility trials
- Assessment of the technique on semen of other species like buffalo and porcine

Mini Abstract

7 MINI ABSTRACT

Poor survival of the spermatozoa after cryopreservation is vastly contributed by damages resulted from temperature stress, osmotic stress and oxidative stress. The current study intended to optimize the concentration of Oxyrase (partial deoxygenating agent) in the semen dilutor and to evaluate its effect on freezability of Sahiwal bull spermatozoa. The selected ejaculates (n=24) were categorized into poor ejaculates (n=12) and good ejaculates (n=12) based on their initial progressive motility. Besides that, each selected ejaculate was divided into two groups and diluted with tris-egg yolk extender containing no Oxyrase or 0.125 IU/mL of Oxyrase for the control and treatment groups, respectively. Parameters evaluated were bacterial load, seminal plasma cholesterol (SPC) and plasma membrane phospholipids (PMP) at fresh stage while individual progressive motility (IPM), acrosomal integrity and plasma membrane integrity (PMI) at post-dilution stage. At pre-freeze stage dissolved oxygen (DO), IPM, acrosomal integrity, PMI, SPC, PMP and oxidative stress (LPO, TAC and ROS) were evaluated. Bacterial load and all the pre-freeze parameters were again evaluated at post-thaw stage. Oxyrase at 0.125 IU/mL concentration significantly reduced DO in the dilutor to 4 ppm in 16-18 minutes at 35°C. Significantly higher progressive motility was observed in the Oxyrase treated groups at post-thaw stage of good ejaculates category in comparison to control. The lowest decline in progressive motility from post-dilution to post-thaw was observed in the Oxyrase treated group of good ejaculates category and the highest decline was in the control group of poor ejaculates category. The per cent intact acrosome differed significantly between treatment and control groups of good ejaculates category at post-thaw stage. Significant ($p<0.05$) difference in plasma membrane integrity was observed at pre-freeze stage of poor ejaculates category between the treatment group and control group. The lowest decline in plasma membrane intactness from post-dilution to post-thaw was observed in treatment group of good ejaculates category while the highest decline was in control group of poor ejaculates category. The trend of increase in cholesterol efflux was significantly observed from fresh to pre-freeze stage and post-thaw stage. However, the treatment groups at post-thaw stage in both categories had significantly less cholesterol efflux in comparison to their control groups. No significant difference in plasma membrane phospholipids concentration was observed between the Oxyrase treated and non-treated groups at all stages of both ejaculate categories. The treatment groups of good ejaculate category had significantly ($p<0.05$) lower levels of lipid peroxidation in comparison to their control group at pre-freeze and post-thaw stages. The level of antioxidant capacity was significantly ($p<0.05$) higher in the treatment groups compared to control groups at post-thaw stage of both categories. Moreover, the lowest decline in antioxidant capacity from pre-freeze to post-thaw was estimated in the treatment group of good category and the highest decline found in the control group of poor ejaculate category. No statistical difference in ROS production was observed between the control and the treatment groups at all stages of both ejaculate categories. The level of bacterial contamination was significantly lower in treatment group than in control at post-thaw in good ejaculates category. Consequently, addition of Oxyrase in dilutor significantly increases freezability of Sahiwal bull spermatozoa by improving post-thaw motility, acrosome integrity, TAC and reducing cholesterol efflux, LPO and bacterial load in good ejaculates. Furthermore, addition of Oxyrase in poor ejaculates reduces cholesterol efflux and increase TAC but do not improve the freezability to a significant level. Therefore, reduced dissolved oxygen in dilutor before cryopreservation improves post-thaw semen quality parameters and may have the potential to overcome freeze-thaw damages due to excess reactive oxygen species.



लघु सारांश

लघु सारांश

8 लघु सारांश

हिमांक संरक्षण के बाद शुक्राणुओं के अस्तित्व में कमी का मूल कारण तापमान के तनाव, प्रसारण तनाव और ऑक्सीकृत तनाव होता है। वर्तमान अध्ययन का उद्देश्य वीर्य विस्तारक में आक्साईड (आंशिक डीऑक्सीजनेटिंग एजेंट) को अनुकूलित करना और साहीवाल सांड के शुक्राणुओं की हिमांक क्षमता पर इसके प्रभाव का मूल्यांकन करना है। चयनित वीर्य को (n=24) को उनके प्रारंभिक प्रगतिशील गतिशीलता के आधार पर खराब वीर्य (n=12) और अच्छे वीर्य (n=12) में वर्गीकृत किया गया था। इसके अलावा, प्रत्येक चयनित वीर्य को दो समूहों में विभाजित किया गया था और क्रमशः नियंत्रण और उपचार समूहों के लिए आक्साईड के 0.0 IU/mL या 0.125 IU/mL युक्त ट्रिस अण्डे की जर्दी वाले विस्तारक से पतला किया गया था। ताजा अवस्था में बैक्टीरिया–लोड, सेमिनल प्लाज्मा कोलेस्ट्रॉल (एसपीसी) और प्लाज्मा झिल्ली फॉस्फोलिपिड्स (पीएमपी) तथा पतला करने के पश्चात व्यक्तिगत प्रगतिशील गतिशीलता (आईपीएम), एक्रोसोमल अखंण्डता, पीएमआई, एसपीसी, पीएमपी और ऑक्सीडेटिव तनाव (एलपीओ, टीएसी और आरओएस) का मूल्यांकन किया गया था। बैक्टीरियल लोड और सभी पूर्व–हिमांक मापदण्डों का फिर से विघलन के बाद भी मूल्यांकन किया गया था। नतीजतन, ऑक्सीरेज सांद्रण का 0.125 IU/mL स्तर वीर्य विस्तारक में 16–18 मिनट में 35°C पर 4 पीपीएम तक डीओ को कम कर सकता है। नियंत्रण समूह की तुलना में आक्सिडेज उपचारित अच्छे वीर्य समूह में विघलन पश्चात प्रगतिशील गतिशीलता महत्वपूर्ण रूप से अधिक देखी गई थी। वीर्य को पतला करने से लेकर विघलन पश्चात तक की शुक्राणु प्रगतिशील गतिशीलता में सबसे कम गिरावट अच्छी श्रेणी के ऑक्सीरिज उपचारित समूह (35.58%) में देखी गई और सबसे अधिक गिरावट खराब श्रेणी के नियंत्रण समूह में हुई। विघलन पश्चात अच्छे श्रेणी के वीर्य की अक्रोसोमल अखण्डता में नियंत्रण एवं उपचार श्रेणी के शुक्राणुओं की प्रतिशतता में काफी अंतर पाया गया। खराब वीर्य श्रेणी के पूर्व–हिमांक चरण में प्लाज्मा झिल्ली अखण्डता में उपचार समूह और नियंत्रण समूह के बीच महत्वपूर्ण अंतर (पी<0.05) देखा गया था। पतला करने से विघलन पश्चात तक प्लाज्मा झिल्ली की अक्षुण्णता में सबसे कम गिरावट अच्छी वीर्य श्रेणी के उपचार समूह जबकि सबसे अधिक गिरावट खराब वीर्य श्रेणी के नियंत्रण समूह में देखी गयी थी। कोलेस्ट्रॉल के प्रवाह में पतला करने से लेकर पूर्व–हिमांक और विघलन बाद के चरण में महत्वपूर्ण रूप से वृद्धि देखी गयी। हालांकि, दोनों श्रेणियों में विघलन बाद कोलेस्ट्रॉल का प्रवाह उपचार समूहों में उनके नियंत्रण समूहों की तुलना में कम था। प्लाज्मा झिल्ली फॉस्फोलिपिड सांद्रता में दोनो वीर्य श्रेणियों के सभी चरणों में तथा दोनों समूहों के बीच में काई महत्वपूर्ण अंतर नही देखा गया था। पूर्व हिमांक और विघलन पश्चात चरणों में अच्छी वीर्य वाली श्रेणी के उपचार समूहों में लिपिड पेरॉक्सिडेशन का स्तर उनके नियंत्रण समूहों की तुलना में कागी का था (P<0.05)। दोनों समूहों के विघलन पश्चात चरण में नियंत्रण समूहों की तुलना में उपचार समूहों में एंटीऑक्सिडेंट क्षमता का स्तर काफी अधिक था। इसके अलावा, पूर्व–हिमांक से लेकर विघलन पश्चात चरण में नियंत्रण समूहों की तुलना में उपचार समूहों में टीऑक्सिडेंट क्षमता का स्तर काफी अधिक था। इसके अलावा, पूर्व–हिमांक से लेकर विघलन पश्चात तक एंटीऑक्सिडेंट क्षमता में सबसे कम गिरावट अच्छी श्रेणी के उपचार समूह तथा सबसे अधिक गिरावट खराब श्रेणी के नियंत्रण समूह में दर्ज की गई। आरओएस उत्पादन में कोई सांख्यिकीय अंतर दोनों वीर्य श्रेणियों के सभी चरणों में बीच नहीं देखा गया था। विघलन बैक्टीरियल संदूषण का स्तर तथा दोनों समूहों उपचार समूह में नियंत्रण से कम था। इसके अलावा, वीर्य विस्तारक में ऑक्सिरेस विघलन पश्चात गतिशीलता, एक्रोसोम अखंण्डता, टीएसी में सुधार और अच्छी श्रेणी के वीर्य में कोलेस्ट्रॉ के बहाव, एलपीओ और बैक्टीरिया के भार को कम करने से साहीवाल सांड के शुक्राणुओं की हिमांक क्षमता को काफी बढ़ा देता है। इसके अलावा, खराब श्रेणी के वीर्य में ऑक्सीरिज कोलेस्ट्रॉल के प्रवाह को कम करता है और टीएसी को बढ़ाता है लेकिन एक महत्वपूर्ण स्तर तक हिमांक क्षमता में सुधार नहीं करता है। इस प्रकार निष्कर्ष निकाला जाता है कि हिमांक संरक्षण से पहले धुली हुई ऑक्सीजन को कम करके विघलन पश्चात के चरण में वीर्य की गुणवत्ता के मापदण्डों में सुधार होता है और अधिक प्रतिक्रियाशील ऑक्सीजन प्रजातियों के कारण हिमीकरण–विघलन के दौरान होने वाले नुकसान को दूर करने की क्षमता बढ़ती है।

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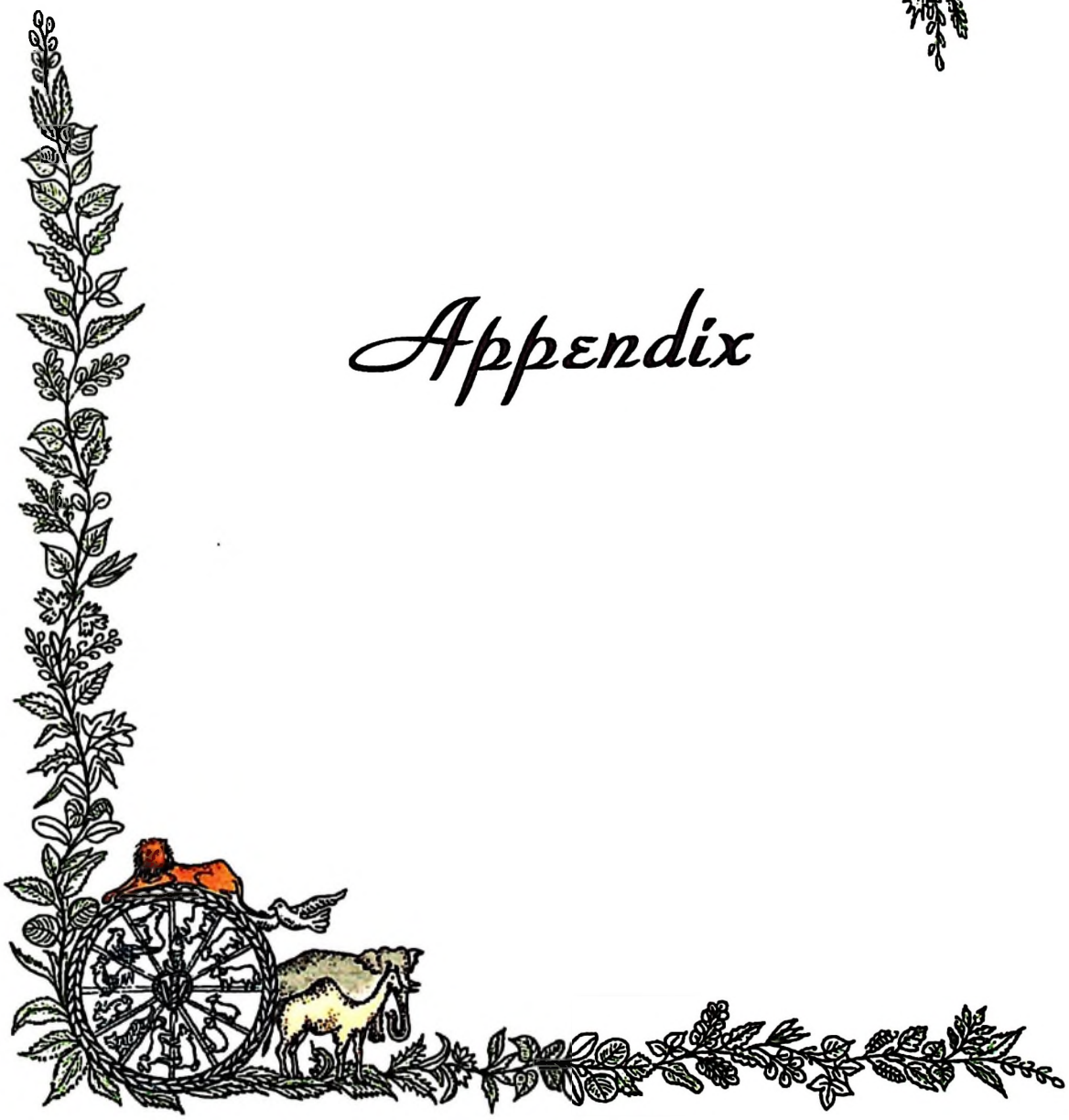
Appendix



APPENDIX

1. Distilled water (DW)

Double distilled water

2. Normal saline

Sodium chloride	9.0 g
Double distilled water	1000 ml

3. Phosphate buffer saline (PBS)

Sodium chloride	8 g
Potassium chloride	0.2 g
Disodium hydrogen phosphate	1.44 g
Potassium dihydrogen phosphate	0.24 g
Double distilled water up to	1000 mL

pH adjusted to 7.2 0 - 7.4

4. Tris-egg yolk glycerol extender

Tris(hydroxymethyl)amino-methane	3.028 g
Citric acid monohydrate	1.675 g
D-fructose	1.250 g
Penicillin G Sodium	28 mg
Glycerol	7 mL
Egg yolk	10 mL
Distilled water upto	100 mL

All the components of the extender except egg yolk were mixed a day before collection. Egg yolk was added on the day of semen collection. All the components were mixed thoroughly for 20 to 30 min with the help of a magnetic stirrer before use.

5. Hypo – osmotic solution for HOST (100 mOsm)

Sodium citrate dehydrate	4.9 g
Fructose	9.9 g
Double glass distilled water up to	1000 ml

6. FRAP reagent

a) Acetate buffer 300 mM (pH 3.6)

0.31 g Sodium Acetate Trihydrate

1.6 mL Acetic Acid

Distilled water up to 100 mL.

b) TPTZ (2, 4, 6-tripyridyl-*s*- triazine) (cat. No. T1253, Sigma-Aldrich, USA) (M.W. 312.34) 10 mM in 40mM HCl (M.W. 36.46)

- 40 mM HCL - 343.3 µL of HCL (M.W. 36.46, N= 11.65; Sp gr= 1.18) in 100 mL distilled water.
- 10 mM in 40 mM HCL – 312 mg in 100 mL 40 mM HCL

c) 20 mM $FeCl_3$. $6H_2O$ (M.W. 270.30)

- 541 mg $FeCL_3$ $6H_2O$ in 100 mL distilled water.

The working FRAP reagent was prepared by mixing a, b and c in the ratio of 10:1:1 at the time of use.

Standard: 1M Ascorbic Acid (M.W. 176.13)

- 17.61 g in 100 mL distilled water

7. TBA- TCA reagent

a. Trichloroacetic acid (TCA) 99.9% w/w (Cat. No. 27242, Sigma-Aldrich, USA) diluted to make 40% solution in distilled water.

b. Thiobarbituric acid (TBA) (Cat. No. T5500 Sigma-Aldrich, USA).

c. 1 N Hydrochloric Acid (100 mL) - 8.6 mL of HCL (N= 11.65; Sp gr= 1.18) with 91.4 mL distilled water.

• TCA	37.5 mL
• TBA	0.375 g
• 1 N HCL	25 mL
• Distilled Water upto	100 mL

8. Solutions for Giemsa staining

a. Giemsa stock solution:

• Giemsa stain	1 g
• Methanol	98 mL
• Glycerol	32 mL

Giemsa stain powder was grounded along with methanol in a glass pestle mortar. Glycerol was added to the mixture and stored at 37°C for a week. During this period the stain mixture was mixed well for few min every day.

b. **Sorenson's 0.1 M phosphate buffer (pH 7.0)**

- **Solution A**: 0.1 M Potassium dihydrogen phosphate solution

Potassium dihydrogen phosphate (Anhydrous)	13.609 g
Double glass distilled water	1000 mL

- **Solution B:** 0.1 M Disodium hydrogen orthophosphate solution

Disodium hydrogen phosphate	14.198 g
Double glass distilled water	1000 mL

Sorenson's 0.1 M phosphate buffer (pH 7.0) was prepared by mixing 17 mL of solution 'A' and 33 mL of solution 'B' and the pH was adjusted to 7.0

c. **Hancock's fixative:**

Sodium chloride	10 g
Sodium bicarbonate	0.5 g
Formalin	125 mL
Distilled water up to	1000 mL

d. **Giemsa working solution:**

Giemsa Stock solution	3.0 mL
Sorenson's 0.1M phosphate buffer	2.0 mL
Double glass distilled water	35.0 mL

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