NITROSATIVE STRESS MEDIATED STRUCTURAL AND FUNCTIONAL CHANGES IN CROSSBRED BULLS CRYOPRESERVED SPERMS

THESIS SUBMITTED TO THE

ICAR- NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL

(DEEMED UNIVERSITY)

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE AWARD OF THE DEGREE

OF

MASTER OF VETERINARY SCIENCE

IN

ANIMAL PHYSIOLOGY

BY

Dr. KULKARNI NITISH ARUN

B.V.Sc & A.H.

ANIMAL PHYSIOLOGY DIVISION

ICAR- NATIONAL DAIRY RESEARCH INSTITUTE

(DEEMED UNIVERSITY)

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Approved by

(EXTERNAL EXAMINER)

Dr. A.K.ROY

MAJOR ADVISOR & CHAIRMAN GUIDE

MEMBERS OF ADVISORY COMMITTEE

1. **Dr. SUJATA PANDITA**
   Principal Scientist, AP Division
2. **Dr. A.K.DANG**
   Principal Scientist, AP Division
3. **Dr. SUDARSHAN KUMAR**
   Scientist, ABTC Division
4. **Dr. T.K.MOHANTY**
   Principal Scientist, ARGO Division, ABRC
   (Joint Director's Nominee)

DIVISION OF ANIMAL PHYSIOLOGY
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
KARNAL-132001 (HARYANA), INDIA

Dr. A.K.ROY
(Senior Scientist)

CERTIFICATE

This is to certify that the thesis entitled, **"NITROSATIVE STRESS MEDIATED STRUCTURAL AND FUNCTIONAL CHAN GES IN CROSSBRED BULLS CRYOPRESERVED SPERMS"** submitted by **Dr. KULKARNI NITISH ARUN** towards the partial fulfillment of the award of the degree of **MASTER OF VETERINARY SCIENCE IN ANIMAL PHYSIOLOGY** of the **ICAR-National Dairy Research Institute (Deemed University)**, Karnal (Haryana), India, is a bonafide research work carried out by him under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

Dated: /08 / 2020

(Dr. A. K. ROY)
MAJOR ADVISOR & GUIDE

DEDICATED TO

ALMIGHTY

FOR MAKING ME WHAT I AM

MY PARENTS

FOR THE FAITH AND SUPPORT EXTENDED

&

MY GUIDE

FOR VALUABLE GUIDANCE

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Place: Karnal, Haryana

(Kulkarni Nitish Arun)

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LIST OF ABBREVIATIONS

+ve	Positive
-ve	Negative
%	Percentage
°C	Degree Centigrade
µg	Microgram
µl	Microliter
µM	Micromole
mM	Mill molar
mOsm	Milli Osmol
nM	Nano molar
ad lib	Ad libitum
ABRC	Artificial Breeding Research Centre
AM	Ante meridiem
AI	Artificial Insemination
ANOVA	Analysis of Variance
ART	Assisted Reproductive Technology
AV	Artificial vagina
Conc.	Concentration
DABCO	1, 4-diazabicyclo[2.2.2]octane

DAI	Dead Acrosome Intact
DAR	Dead Acrosome Reacted
DPBS	Dulbecco's phosphate buffer saline
eNOS	Endothelial Nitric Oxide Synthase
et.al.	Co-workers
EYC	Egg Yolk Citrate
EYTG	Egg Yolk Tris Glycerol
Ft.	Feet
Fig	Figure
FITC-PNA	Fluorescein Isothiocyanate-Peanut Agglutinin
h	Hour
HF	Holstein Friesian
HOST	Hypo Osmotic Swelling Test
i.e.	id est./that is
ICAR	Indian Council of Agricultural Research
ILFC	Instructional Livestock Farm Complex
iNOS	Inducible Nitric Oxide Synthase
IU	International unit
Kg	Kilogram
KH2HPO4	Potassium dihydrogen phosphate
L	Litre
LAI	Live Acrosome Intact
LAR	Live Acrosome Reacted
LN2	Liquid nitrogen

LPO	Lipid Peroxidation
MA	Mass Activity
MDA	Malondialdehyde
mg	Milligram
mg/m	Milligram Per Milliliter
min	Minutes
MMP	Mitochondrial Membrane Potential
mtNOS	Mitochondrial Nitric Oxide Synthase
N2	Nitrogen
Na2HPO4	Disodium hydrogen phosphate
NDRI	National Dairy Research Institute
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
$O^{2\bullet-}$	Superoxide
ODQ	1H-(1,2,4) Oxadiazolo (4,3-a) quinoxalin-1-one
OH•	Hydroxyl
$ONOO^-$	Peroxynitrite
PBS	Phosphate Buffer Saline
PM	Progressive motility
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
rpm	Revolution per minute
S.E.	Standard error

SEM	Standard error mean
SIN-1	3-Morpholinosydnonimine
SNP	Sodium Nitroprusside
SPSS	Statistical package for the social sciences
TALP	Tyrode's Albumen Lactate Pyruvate
TBARS	Thiobarbituric Acid Reactive Substances
TDN	Total Digestible Nutrient
Tris	Hydroxy methyl amino methane
v/v	Volume/ volume
Vit.	Vitamin
viz.	Videlicet
vs.	Versus

Nitrosative stress mediated structural and functional changes in crossbred bulls cryopreserved sperms

ABSTRACT

The present research was aimed to investigate the effect of nitric oxide (NO) supplementation in extender on post thaw seminal attributes so that the quality semen from superior breeding bulls can be kept longer without much decline in its fertilizing capacity. The study was designed to cover the maximum facets of cryopreservation with two objectives. Under first objective, the semen from 6 Karan-Fries bulls was taken and assessed for the percentage of progressive motility, viability, membrane integrity (HOST +ve), acrosome integrity and total abnormalities, and other semen biomarkers before and after cryopreservation in different time intervals. A sharp reduction ($p<0.01$) in the semen quality was observed at 24 h after cryopreservation in comparison to fresh semen. However, there was not much variation in the sperm functions at 15 days or 1 month after freezing. The lipid peroxidated sperm percentage was greater ($p<0.01$) in the cryopreserved semen as compared to fresh ejaculate. The second objective emphasizes on elucidating the efficacy of adding SNP and SIN-1 in extender to improve the post-thaw semen quality. The standardization and optimization of SNP and SIN-1 concentration indicated that the different concentrations of SNP and SIN-1 showed positive effect on the progressive motility and viability in a time and dose dependent manner. The percentage of progressive motility and viability improved significantly ($p<0.05$) with the addition of 100 nmol/ml SNP and 80 µM SIN-1, which was used further for evaluating other seminal attributes and *in vitro* sperm function tests.

There was no significant difference between control and modified extender in fresh semen. A significant increase ($p<0.05$) was observed in progressive motility, viability and membrane integrity in SNP and SIN-1 treated extender at 24 h and 1 month while a significant ($p<0.05$) increase in acrosome integrity only after 24 hours for SNP and SIN-1 treated extender in comparison to control.

There was no significant difference in sperm abnormality in extended and control group. The seminal plasma of SNP treated extender had less ($p<0.05$) lipid peroxidation as compared to SIN-1 treated and control groups. There was a significant ($p<0.05$) increase in the percentage of Mitochondrial membrane potential (MMP) +ve spermatozoa in both the treatment groups (SNP and SIN-1) in comparison to control. It may be concluded that the addition of SNP and SIN-1 in cryopreservation media at 100 nmol/ml and 80 µM respectively improved the seminal attributes significantly.

नाइट्रोसेटिव तनाव से संकर सांड के हिमीकृत वीर्य में संरचनात्मक एवं कार्यात्मक परिवर्तन

सारांश

वर्तमान शोध का उद्देश्य हिमीकृत वीर्य संबंधी विशेषताओं पर एक्सटेंडर में नाइट्रिक ऑक्साइड मिलाकर इसके प्रभाव की जांच करना था ताकि बेहतर प्रजनन के सांड से उपयुक्त गुणवत्ता वाले वीर्य को निषेचन क्षमता में अधिक गिरावट के बिना लंबे समय तक संरक्षित रखा जा सके। यह अध्ययन दो उद्देश्यों हेतु हिमीकरण के अधिकतम पहलुओं को सम्मिलित करने के लिए किया गया था। पहले उद्देश्य के अनुसार, अलग-अलग समय में हिमीकरण से पहले और बाद में 6 कर्ण-फ्रिस सांडों से वीर्य लिया गया और शुक्राणु प्रगतिशील गतिशीलता, व्यवहार्यता, झिल्ली अखंडता (होस्ट पॉजिटिव), एक्रोसोम अखंडता, कुल असामान्यताएं तथा अन्य वीर्य सम्बंधित गुणों के प्रतिशत हेतु मूल्यांकन किया गया। ताजा वीर्य की तुलना में हिमीकृत वीर्य की गुणवत्ता में तीव्र कमी (पी <0.01) 24 घंटे के परिरक्षण के बाद देखी गई। हालांकि, ठंडा करने के बाद 15 दिनों या 1 महीने बाद शुक्राणुओं के गुणों में बहुत अधिक भिन्नता नहीं थी। ताजा वीर्य की तुलना में हिमीकृत वीर्य में लिपिड पेरोक्सीडेटेड शुक्राणु प्रतिशत (पी <0.01) अधिक था। दूसरा उद्देश्य एसएनपी और एसआईएन-1 को एक्सटेंडर में मिलाने के बाद वीर्य की गुणवत्ता में सुधार होने की प्रभावशीलता का अनुमान लगाने हेतु किया गया था। एसएनपी और एसआईएन-1 एकाग्रता के मानकीकरण और अनुकूलन के अनुसार एसएनपी और एसआईएन-1 की विभिन्न सांद्रता ने एक समय और खुराक आधारित विधि से प्रगतिशील गति और व्यवहार्यता पर सकारात्मक प्रभाव दिखाया। प्रगतिशील चालन और व्यवहार्यता का प्रतिशत 100 नैनोमोल / मिली.ली. एसएनपी और 80 माइक्रोरो-मोल एसआईएन-1 के साथ सार्थक रूप से (पी <0.05) सुधर गया, जिसका उपयोग अन्य वीर्य विशेषताओं और इन-विट्रो शुक्राणु कार्यविधि परीक्षणों के मूल्यांकन हेतु किया गया। ताजा वीर्य के नियन्त्रित और संशोधित एक्सटेंडर समूह में कोई सार्थक अंतर नहीं था। एसएनपी और एसआईएन-1 में 24 घंटे और 1 महीने में उपचार समूह की प्रगति, व्यवहार्यता और झिल्ली अखंडता में एक सार्थक वृद्धि (पी <0.05) देखी गई, जबकि 24 घंटे बाद ही एसएनपी व एसआईएन-1 उपचारित वीर्य की एक्रोसोम अखंडता में एक सार्थक (पी <0.05) वृद्धि हुई है। एक्सटेंडर और नियन्त्रित समूह में शुक्राणु असामान्यता में कोई सार्थक अंतर नहीं था। एसआईएन-1 उपचारित और नियन्त्रित समूहों की तुलना में एसएनपी उपचारित वीर्य के प्लाज्मा में कम (पी <0.05) लिपिड पेरॉक्सिडेशन हुआ था। नियन्त्रित समूह की तुलना में दोनों उपचार समूहों (एसएनपी और एसआईएन-1) में माइटोकॉन्ड्रियल झिल्ली क्षमता या एमएमपी पॉजिटिव के प्रतिशत में सार्थक (पी <0.05) वृद्धि हुई थी। निष्कर्षतः हिमीकृत मीडिया में एसएनपी और एसआईएन-1 के क्रमशः 100 नैनो-मोल/ मिली.ली. और 80 माइक्रो-मोल/ मिली-ली. मात्रा ने सेमीनल विशेषताओं में अधिक सुधार किया।

CHAPTER-1

Introduction

INTRODUCTION

Cryopreservation is one of the major technique with lot of advantages, mainly in the field of assisted reproductive technology (ART) in livestock. Artificial Insemination (AI) has achieved greater heights due to this technique. It has led to the genetic improvement of livestock and selection of high quality animals. Time and location no longer remains barrier for storage of semen which has led to widespread expansion of this technique to all parts of the country. However, there is a considerable damage to the sperm morphology during cryopreservation that compromises its functionality. Many factors such as cold shock, cryoinjuries, ice-crystal formation, oxidative stress, osmotic changes etc. cause this detrimental activity during freezing and thawing. These factors cause decrease in sperm viability and motility (Punyatanasakchai et al., 2008) due to several impacts that produce cellular damage, like excessive dehydration, deleterious changes in sperm structure, morphological alterations, damage to plasma membrane and acrosome cap, mitochondrial injury, apoptosis, and sperm DNA fragmentation (Merino et al., 2015; Sharma et al., 2015).

Cross-bred bulls have got 40% inferior quality of semen ejaculates which are not suitable for cryopreservation. Hence, it leads to sub-fertility that takes a toll on the economic benefits of the farmers. Cryopreserved semen of crossbreds may further lead to structural and functional damages to the sperm which results in enormous wastage of exotic crossbred bulls due to their poor quality semen, poor freezability (consistently giving below 35% post thaw motility; 10.87% bulls) resulting in huge economic losses as well as reduction in genetic gain. The main reason behind male infertility is the defective sperm functions that are hard to treat (Sikka et al., 2001).

Oxidative stress is emerging as a promising field in sperm physiology that plays a major role in causing infertility problems of livestock. When the reactive

Oxygen species (ROS) and nitrogen species (RNS) largely outnumber the natural Antioxidants in the semen, it leads to oxidative stress. ROS includes superoxide(O^{2-}), hydrogen peroxide(H_2O_2), peroxyl (ROO^-) and hydroxyl radicals (OH). ROS cause damage to the proteins, lipids and DNA in spermatozoa resulting in an impaired sperm function which ultimately causes infertility problems (Gharagozloo and Aitken, 2011). ROS and RNS are generated in aerobic conditions during the process of spermatogenesis, epididymal sperm maturation and exposure to toxic chemicals and environmental pollutants. The sperm cells have been reported to be highly susceptible to reactive oxygen species (ROS) (Saraswat *et al.,* 2013) due to the presence of high lipid content in their plasma membrane. The mammalian spermatozoa have high polyunsaturated fatty acids, plasmalogens and sphingomyelins, which are responsible for sperm flexibility and functional ability. Therefore, any attack by ROS will have an adverse impact on the fertility potential of spermatozoa.

RNS are often considered as sub-class to ROS, which are included in the family of antimicrobial molecules. RNS are derived from nitric oxide and superoxide produced by the enzymatic activity of nitric oxide synthase 2 (NOS-2) and NADPH oxidase respectively. RNS includes nitric oxide (NO^-), nitrogen dioxide (NO_2) and peroxynitrite anion ($ONOO^-$; Sikka*,* 2001). Reactive nitrogen radicals may have beneficial and detrimental effects on sperm functions based upon their nature and concentration. NO plays an important role in intercellular and intracellular messenger activities controlling many physiological processes. Several in-vitro studies reveal that NO as a signalling molecule is involved in the physiological functions of spermatozoa. NO affects sperm motility (O'Bryan et al., 1998), acts as chemoattractant (Miraglia et al., 2007), regulates the tyrosine phosphorylation of different sperm proteins (Thundathil et al., 2003) which results in sperm capacitation, enhances the sperm-zona pellucida binding ability, hyperactivation of sperm and modulates the acrosomal reaction (Revelli et al., 2001). Low concentration of NO results in increase in motility, viability and reduces lipid peroxidation of sperm cells while higher concentration leads to alterations in the sperm function, particularly reduced motility, inhibition of cellular

respiration, mitochondrial damage and DNA fragmentation; RNA damage and axonemal proteins' damage (Agarwal & Prabakaran, 2005). The most of the detrimental effects attributed to NO are the results of its reaction with superoxide($O2^-$), producing peroxynitrite (Pacher et al., 2007) which is responsible for inducing nitrosative stress. Peroxynitrite ($ONOO^-$) covalently interacts with most biomolecules and its cytotoxicity is mediated through protein oxidation and nitration, lipid peroxidation, activation of matrix metalloproteinases and inactivation of several mitochondrial enzymes; and also DNA oxidation and fragmentation. Excess of ONOO- concentration results in reduced viability and cell function leading to cell death by apoptosis and necrosis, as reported in several diseased conditions (Szabo et al., 2007). There is also a negative correlation between ONOO- levels and Na+/K+ ATPase, Ca^{+2} ATPase activity, and total thiol content (Vignini et al., 2009). There is NO donor- Sodium Nitroprusside (SNP) and Peroxynitrite (ONOO-) generator- 3-Morpholinosydnonimine (SIN-1), both have got similar biphasic functions as that of Nitric Oxide (Domple et al., 2016 and Khodaei et al., 2016). These compounds have beneficial effects at normal physiological levels.

There are few reports regarding the impact of SNP and SIN-1 on fresh as well as frozen semen in crossbred KF bulls. Reports regarding structural changes in spermatozoa caused by these two compounds during pre-freezing and post-thawing are very limited in number. Therefore, the present study was planned with the following objectives.

1. To assess the impact of ROS generated in fresh and cryopreserved semen on structural changes and sperm quality

2. To assess the impact of SIN-1 and/or SNP supplemented semen extender on post-thaw structural changes and sperm quality

CHAPTER-2

Review of Literature

REVIEW OF LITERATURE

Genetic improvement of the livestock off-springs is of utmost importance which brings about an increase in productive and reproductive performance. Cryopreservation plays a major role in this regard. It is an excellent technique for the perpetuation of livestock and human spermatozoa for decades to conserve male fertility and reproductive capacity. The ultimate goal of cryopreservation of semen is to maintain sperm viability, structural integrity and functionality at sub-zero temperatures, which improves the success rate of fertility in cows (Pegg, 2015). Artificial Insemination (A.I.) is preferably the most effective method to use cryopreserved semen to enhance animal production. By A.I., from one ejaculate of superior quality bull, we can impregnate multiple females which in turn help in the transmission of dominant and favorable genes. Thus, effective cryopreservation with minimal damage to sperm helps in successful A.I. in comparison to natural breeding. A lot of progress has been made in the storage of sperm for a long time, but a complete success has not been in this aspect. About 50% loss of viability and fertility has been reported after freezing and thawing of bull semen (Lessard *et al.,* 2000). The success of this technique depends on many factors like synergy between type of extender, cooling rate, thawing rate, cryoprotectant, and packaging, as well as the variation between individual animal (Cooter *et al.*, 2005; Andrabi, 2007; Clulow *et al.*, 2008).

2.1 Sperm damage during Cryopreservation process

Generally, 40%–50% of the sperm population does not survive during the cryopreservation process, even when “optimized” cooling/thawing protocols are used (Holt, 2000; Watson, 2000). In comparison to fresh semen, based on a similar number of motile and viable cells, the results obtained are lower, which indicates

the viable subpopulation is compromised after cryopreservation (Watson, 2000). Cryopreservation results in damage to sperm's intra- and extracellular environment related to two main factors: on one hand, formation of ice-crystals and on the other hand, addition and removal of cryoprotective agents (Morris *et al.,* 2006; Ozkavukcu *et al.,* 2008). The most common cryoinjuries reported deal with the evident reduction of sperm motility and viability (Hammadeh *et al.,* 1999; O'Connell *et al.,* 2002). Major damages during freezing and thawing includes damage to plasma membrane fluidity and integrity, oxidative stress results in variations in acrosomal content and state, lipid peroxidation, mitochondrial functionality, chromatin decondensation and DNA fragmentation resulting in inferior semen quality which consequently reduces fertility in cows (Holt *et al.,* 1994; Chatterjee *et al.,* 2001; Chohan *et al.,* 2004; Boitrelle *et al.,* 2012).

Table 2.1 Stress factors causing sperm damage

DURING COOLING	DURING FREEZING
Metabolic decoupling	Ice crystal formation
Deprivation of energy	Concentration of solutes
Ionic imbalances	Hyperosmolarity
Cellular acidosis	Alterations of cell volume
Activation of proteases	Protein denaturation

(Adopted from Baust *et al.,* 2009)

2.2 Plasma membrane damage

Different cooling speeds, lipid transition phase passing and ionic imbalances result in changes in composition and response capacity of sperm membranes and determine its damage. This phenomenon is commonly known as "cold shock" which

causes changes in the distribution of phospholipids through the double membrane, resulting in an alteration of its function and capacities (De Leeuw *et al.,* 1990; Parks *et al.,* 1992).

Cryopreservation primarily induces damage to the plasma membrane of spermatozoa (Parks *et al.,* 1992; Watson, 1995). Cryomicroscopic structure of ram spermatozoa with a marker for membrane integrity was observed which revealed partial effect to the plasma membranes of principal –piece, mid-piece, and head, with the head membrane, inevitably damaged (Holt and North, 1994). Certain indications related to Morphometry measurements depicted that the cryopreserved bull sperm has reduced head size compared with unfrozen sperm that probably reflects the permanent modification of the plasma membrane's architecture (Gravance *et al.,* 1998). Lipid composition and organization of sperm plasma membranes are affected by cryopreservation in rams (Hinkovska- Galcheva *et al.,* 1989) and boars (Buhr *et al.,* 1994). Cryopreservation destabilizes membranes due to ultrastructural damages which makes sperm susceptible to gross morphological defects, which includes missing and abnormal acrosomes.

Phase transitions and other ultrastructural modifications of the plasma membranes during freezing and thawing may play a role in the poor fertility of cryopreserved sperm. Sperm do not survive in sudden cold i.e. cold shock. Early evidence of cold shock causing irreversible depression of sperm motility and metabolic activity (White, 1993) included plasma membrane disruption due to cooling or freezing favoring the loss of enzymes and cations from sperm (Harrison and White, 1972). It also destroys the selective permeability of sperm membranes to calcium, leading to exorbitant intracellular levels decreasing motility and lead to necrosis (Simpson and White, 1986; Robertson *et al.,* 1990).

2.3 Cryocapacitation

Significant numbers of cells undergo death or severe damages during the cooling or freezing process. Above all this, a sub-population of sperm is induced partially or completely to undergo capacitation like changes. Hence, this

phenomenon is called “Cryocapacitation” (Bailey *et al.,* 2000). Non capacitated subpopulation maintains its fertilizing ability. “Cryo-capacitation” additionally produces a subpopulation of spermatozoa with a shorter lifespan, thus reduces the complete fertilization efficiency of the frozen spermatozoa population sample (Bailey *et al.,* 2000). Cryopreserved bovine sperm undergo capacitation easily than fresh semen. This was confirmed by in-vitro fertilization studies with setting of fertilization media in which cryopreserved semen were allowed to react with oocytes and they were able to fertilize them even in absence of heparin (Cormier *et al.,* 1997), an “active” agent to induce in vitro capacitation of bovine sperm (Parrish, 2014). Changes in membrane fluidity are responsible for the above changes. There is an increase in intracellular calcium concentrations which is responsible for premature sperm capacitation independent of heparin concentration in fertilization media (Cormier & Bailey, 2003; Green & Watson, 2001).

Efflux of cholesterol concentrations from the membranes was mainly responsible for capacitation changes. Hence, comparisons were made between cholesterol levels of fresh and frozen semen, which indicated an increased cholesterol loss in frozen semen with different viability assessments (Cerolini, Maldjian, Pizzi, & Gliozzi, 2001), which increases membrane fluidity and is responsible for premature capacitation like changes (Cormier *et al.,* 1997). This leads to increased absorption and elevation of intracellular calcium concentrations during the cooling process, a major predisposing factor for capacitation like changes and development of fusion events between the plasma membrane and the external acrosomal membrane. As a result of this, there is an expected decrease in the semen fertilizing capacity (Bailey *et al.,* 2000; Watson, 2000). A greater evidence of “cryo-capacitation” is provided by phosphorylation pattern of tyrosine protein that was confirmed by several experiments, revealing that the majority of cryopreserved sperm exhibited a characteristic distribution of phosphotyrosine-containing proteins identical to that observed in fresh semen after induced capacitation through heparin (Cormier & Bailey, 2003). A study by Naresh

and Atreja, (2015) reported an increase in tyrosine protein phosphorylation and actin polymerization during the cooling and cryopreservation processes. The above processes can be explained by the fact that freezing/thawing results in cold shock or osmotic stress on the sperm plasma membrane, concluding in its damage and increased permeability leading to the influx of calcium which increases the intracellular concentration of cAMP and 1,2-Diacylglycerol (DAG), finally leading to phosphorylation of protein tyrosine(Naresh and Atreja, 2015).

2.4 Oxidative stress and Reactive Oxygen Species (ROS)

Major changes take place in the structural and functional integrity of the sperm after cryopreservation due to the formation of reactive oxygen species (ROS). Oxidative stress is by the consequence of excessive production of pro-oxidants or less amount of antioxidants (Favier, 2003) with free oxygen radicals subduing the radical scavengers antioxidants (du Plessis *et al.,* 2008), affecting sperm functionality parameters, like motility deprivation, plasma membrane damage and inactivation of enzymes (Sikka *et al.,* 2004). Oxidative stress is responsible for compromising the integrity of the sperm structural, functional and physiological components which may affect sperm viability and impair its fertilizing capacity (Aitken *et al.,* 2012). Sperm cell becomes susceptible to damage due to its structure, its plasma membrane, a higher number of mitochondria and a low amount of cytoplasmic antioxidants (Bollwein *et al.,* 2008). ROS contains broad range of molecules including free radicals' collection with an unpaired electron (du Plessis *et al.,* 2008) such as superoxide ($O2^{\bullet-}$), Hydroxyl ($OH^{\bullet}$), Peroxyl ($RO2^{\bullet}$), Alkoxyl ($RO^{\bullet\cdot}$), Hydroperoxyl ($HO2^{\bullet\cdot}$) and non-radicals without an unpaired electron but have strong oxidizing potential viz Hydrogen peroxide (H_2O_2), Hypochlorous acid ($HOCl^{-}$), Ozone (O_3), Singlet oxygen ($^1O_2$), endogenous entities such as peroxisomes, lipoxygenases, mitochondria (Raha *et al.,* 2001), NADPH oxidase and cytochrome P450 as well as exogenous factors such as ultraviolet light, ionizing radiation, chemotherapeutics inflammatory cytokines, and environmental toxins are considered to be the major factors that generate ROS in living cells

(Finkel *et al.,* 2000).

The mechanisms of the ROS vary depending upon the nature of free radicals and their molecular targets (Therond *et al.,* 2006). Oxidative damage of sperm cells can be estimated by using formed oxidative products as biomarkers of oxidative stress caused. For example, 8-oxo-guanine (8-oxodeoxyguanosine) serves as a sensitive marker to estimate the level of DNA damage caused by hydroxyl radicals (Shigenaga *et al.,* 1989; Cundy *et al.,* 1988)**.** Apoptotic pathways are induced by lower antioxidant enzyme and ROS production that can lead to decrease in sperm viability (Di Santo *et al.,* 2012). ROS has got a dual effect and they act as a double-edged sword. ROS at normal physiological levels have got major functions to play viz. spermatozoa maturation, sperm capacitation, acrosome reaction, binding to zona pellucida and signaling process (Bucak *et al.,* 2010; Zhang *et al.,* 2012), stabilization of the mitochondrial capsule in the midpiece (Goncalves *et al.,* 2010) to ensure fertilization. Paradoxically the higher amount of ROS has got negative effects resulting in loss of viability, motility, ATP depletion, inhibit mitochondrial membrane potential, insufficient axonemal phosphorylation and lipid peroxidation (Bansal *et al.,* 2011) which leads to low fertilization rates (Jang *et al.,* 2010; Ghaleno *et al.,* 2014; Sapanidou *et al.,* 2015). Normal metabolism is seen in cells without any stress-producing physiological levels of ROS. Male gametes themselves generate supra-physiological levels of ROS and get exposed to it during in-vitro manipulations in assisted reproductive techniques (du Plessis *et al.,* 2008).

2.5 Reactive Nitrogen Species (RNS)

Reactive Nitrogen Species (RNS) are included as sub-class of ROS belongs to the family of antimicrobial molecules which are derived from nitric oxide and superoxide, produced by the enzymatic activity of nitric oxide synthase-2 (NOS-2) and NADPH oxidase respectively. RNS are considered as sub-group of ROS and are free nitrogen radicals (Sikka *et al.,* 2001). RNS include nitric oxide

($NO^{\bullet}$), Nitrogen dioxide ($NO_2^{\bullet}$). Non-radicals include Peroxynitrite ($ONOO^-$), Alkyl peroxynitrites (ROONO), Dinitrogentrioxide (N_2O_3), Dinitrogen tetroxide (N_2O_4), Nitrous acid (HNO_2), Nitronium anion ($NO2^+$), Nitroxyl anion (NO^-), Nitrosylation (NO^+), Nitryl chloride (NO_2Cl; Forman *et al.,* 2008). Similar to that of ROS, RNS also plays a major role in male reproduction and fertilization (Maneesh and Jayalekshmi, 2006). RNS in excess concentration are responsible for Nitrosative stress as that of Oxidative stress. This nitrosative stress ultimately leads to lipid peroxidation, loss of motility and viability and further damage to several biomolecules (Nash *et al.,*2012) and it also contributes to impaired sperm functions (Khosravi *et al.,* 2014; Salvolini *et al.,* 2012). RNS is reported to be highly toxic and hence cause protein nitration that leads to protein dysfunction at high levels (Bergendi *et al.,* 1999).

RNS are of two types i.e. radicals and non-radicals.

Radicals

$NO^{\bullet}$**-** Nitric Oxide

$NO^{\bullet}$ -Nitrogen dioxide

Non-Radicals

$ONOO^-$ **-**Peroxynitrite

N_2O_3- Dinitrogen trioxide

ROONO-Alkyl peroxynitrite

NO^- -Nitroxyl anion

$NO2^+$ -Nitronium anion

N_2O_4 -Dinitrogentetraoxide

NO^+ -Nitrosylation

2.5.1 Nitric oxide– A powerful oxidant

Nitric oxide (NO) is a diatomic free radical which is hydrophobic and biologically active in almost all cells of the organism and can easily diffuse through biological membranes due to its low molecular weight (Pacher *et al.,* 2007). NO is

referred to as an endothelium-derived relaxing factor (EDRF) because it causes vasodilation via a cascade of events that leads to vascular smooth muscle relaxation. In vivo synthesis occurs during the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS), with the help of co-factors such as the reduced form of NADP, FMN, FAD, and tetrahydrobiopterin (Lewis *et al.,* 1996). After making a band with sulfur-iron complexes NO changes the activity of these enzymes. NO is an important transmitter molecule in mammalian cells including humans and plays a main role in physiological and pathological processes (Dixit *et al.,* 2001; Hou *et al.,* 2008). NO has a very short half-life (3 to 10 seconds) in tissues because it reacts with oxygen (O2) and converts into nitrite and nitrate. It also reacts with superoxide ion ($O2^{-\cdot}$**)** and thiol sites to yield peroxynitrite and nitrosothiols respectively.

Many of the research findings suggest the presence of nitric oxide in the male reproductive tract (Ehren *et al.,* 1994; Zini *et al.,* 1996; Uckert *et al.,* 2003). It was reported to be present in accessory glands, epididymis, testis, penis, seminal ejaculate and other ducts (Archer *et al.,* 1993). Its presence in human and animal spermatozoa (Herrero *et al.,* 1996; Lewis *et al.,* 1996) and seminal plasma (Burnett *et al.,* 1993; Zini *et al.,* 2001) has also been reported, hence it proves that it supports several sperm functions at physiological levels (Revelli *et al.,* 1999; Belen Herrero *et al.,* 2000; Miraglia *et al.,* 2011) indicating NO to be a novel mediator of sperm function.

2.5.2 Nitric oxide synthase (NOS)

NOS is a major enzyme that catalyzes the conversion of L-arginine (L-Arg) into L-citrulline, through oxidative reactions (Moncada and Higgs, 1993) to form NO. There are four isoforms of NOS, out of which three i.e .neuronal (nNOS), endothelial (eNOS), mitochondrial (mtNOS) are calcium-dependent. The following three are responsible for the continuous basal release of NO and both require calcium/calmodulin for activation (Griffith and Stuehr, 1995; Snyder, 1995). The fourth isoform (iNOS or NOS2) is an inducible calcium-independent form

(Moncada *et al.,* 1991; Rebeca López-Úbeda and Carmen Matás, 2015). The co-factors like NADPH, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD) and tetrahydrobiopterin (BH4) and substrates are required for NOS activity. The rates of cellular NO synthesis depends on the availability of these factors.

Numerous research studies have determined the presence and localization of various isoforms of NOS in sperm from several species. For example, Herrero *et al.*, (1996) located nNOS in the head of freshly ejaculated human spermatozoa, with a more concentrated fluorescent staining toward the equatorial region. O'Bryan *et al.*, (1998) described the pattern of eNOS expression in human spermatozoa, finding that morphologically normal spermatozoa exhibited post-acrosomal and equatorial eNOS immunostaining. In other mammalian spermatozoa such as mice (Herrero *et al.,* 1997), bull (Meiser and Schulz, 2003), and boar spermatozoa (Hou *et al.,* 2008) activating the biosynthesis of NO. NOS participation in bull sperm capacitation was depicted by Go and Wolf, (1985), but the mechanism by which NOS was activated during this process could not be established by these authors. NOS activity with regards to sperm maturity and sperm apoptosis signaling in mature and immature human spermatozoa was evaluated (Roessner *et al.,* 2010). The maturity of human spermatozoa was positively associated with NOS activity, while activation of apoptosis signaling was significantly negatively correlated with NO production, hence, indicating an anti-apoptotic effect of NO. Thus, NO was associated with physiological sperm signal transduction during capacitation.

2.5.3 **Role of NO on sperm functionality**

Several in-vitro studies were conducted to determine the role of nitric oxide on sperm physiology and they found a significant role of nitric oxide in this regard. It has been shown that under basal physiological levels, nitric oxide affects sperm motility, viability (Lewis *et al.,* 1996; Donnelly *et al.,* 1997), acts as chemoattractant (Miraglia *et al.,* 2007; Sliwa *et al.,* 2000), regulates the tyrosine phosphorylation of different sperm proteins (Herrero *et al.,* 1999; Thundathil *et*

al., 2003), enhances the sperm-zona pellucida binding ability (Sengoku *et al.,* 1998), and modulates the acrosomal reaction (Revelli *et al.,* 2001), hyperactivation of spermatozoa in female reproductive tract and spermatozoa-egg fusion (Sapanidou *et al.,* 2015; Cruz *et al.,* 2015). Basal release of NO by spermatozoa from normozoospermic samples tended to be greater than that from asthenozoospermic samples, suggesting a physiological and beneficial role for endogenous NO in the preservation of sperm motility (Donnelly *et al.,* 1997). These observations agree with a previous report that normozoospermic spermatozoa express more NOS and generate more nitrite than asthenozoospermic spermatozoa (Lewis *et al.,* 1996). When millimolar concentrations of exogenous NO donors were added to sperm samples they showed detrimental effects on motility (Rosselli *et al.,* 1995; Weinberg *et al.,* 1995).

Nitric Oxide may exert a chemoattractant effect on spermatozoa. In a study, the percentage of mouse sperm migrating toward the medium containing an NO donor increased significantly (Sliwa *et al.,* 2000). Similar results were obtained when human spermatozoa were exposed to an NO donor (Miraglia *et al.,* 2007). It was proposed that NO exerts its chemoattractant effect through the activation of the NO/sGC/cGMP pathway since the use of an NO scavenger and/or ansGC and cGMP-dependent protein kinase inhibitor reverted the NO donor-induced migration of sperm.

The involvement of NO in capacitation was further confirmed by a study that involved the determination of tyrosine phosphorylation in different sperm proteins associated with capacitation process (Emiliozzi *et al.,* 1997). NO-releasing compound increased tyrosine phosphorylation which in turn accelerated human sperm capacitation (Herrero *et al.,* 1999). On the opposite hand, when sperm capacitation was inhibited by L-NAME, there was an attenuation within the tyrosine phosphorylation of sperm proteins. Also, a study by Thundathil et al., (2003) reported that L-NAME prevented, and a NO donor promoted, the increase in

threonine, glutamine, and tyrosine phosphorylation in human spermatozoa. Furthermore, the addition of L-arginine reversed the inhibitory effect of L-NAME on the capacitation and the associated increase in phosphorylation.

Sengoku et al., (1998) investigated the correlation between NO and sperm-zona pellucida binding ability, who reported that when treated with low concentrations of a NO donor, the number of spermatozoa which binds to the hemizona, is above in sperm treated with a better concentration. Additionally, a NO quencher lowered the enhancement of sperm binding by the NO donor.

NO also modulates the acrosome reaction. A study was conducted regarding the determination of the percentage of acrosomal loss induced by human follicular fluid or by calcium ionophore, which was studied when human spermatozoa were capacitated in the presence/absence of NO-releasing compounds or NOS inhibitors (Herrero et al., 1999). NOS inhibitors decreased the percentage of acrosome reaction whereas NO donors induced sperm cells to respond faster to the human follicular fluid. Revelli et al., (1999), obtained similar results who showed that different NO-releasing compounds were able to increase the percentage of reacted spermatozoa in the presence of protein-enriched extracts of human follicular fluid. Along with it, hemoglobin, a NO scavenger, inhibited the follicular fluid-induced acrosomal reaction. In-depth analysis of the signaling pathway of the nitric oxide-induced acrosome reaction in human spermatozoa (Revelli et al., 2001) was carried out, which concluded that the effect of exogenous NO on capacitated human spermatozoa is accomplished via the NO/sGC/cGMP pathway, which results in the activation of cGMP-dependent protein kinase (PKG). On incubation with SNP, there was a significant increase in both the intracellular cGMP levels and the percentage of reacted spermatozoa. Furthermore, in the presence of sGC inhibitors, the SNP-induced acrosome reaction was significantly reduced, a reduction that was reverted by the addition of a cell-permeating cGMP analog to the incubation medium. Finally, PKG inhibition reduced the SNP-induced acrosome reaction.

Hyperactivation can be considered as a subcategory of capacitation. Hyperactivation of spermatozoa exhibits high amplitude and asymmetric flagellar movement, non-linear motility and penetrate the oocyte with strong propulsive force (Miraglia et al., 2007; Kothari et al., 2010). The effect of NO on sperm motility and hyperactivation was found to be quite similar where little concentrations of NO increased spermatozoa hyperactivation, whereas excessive concentrations decreased the hyperactivated spermatozoa motility (Otasevic et al., 2013; Miraglia et al., 2011).

2.6 Peroxynitrite, ($ONOO^-$)- A potent anti-oxidant

Peroxynitrite is a potent oxidant as well as a nitrating agent with very short half-life (~10ms) (Beckman et al., 1990; Radi et al., 2001). It is formed by the diffusion-controlled reaction of superoxide radicals and nitric oxide (Vinten-Johansen, 2000) due to its short half-life and relatively stable nature. Neither superoxide radicals nor NO are toxic in vivo because of efficient body systems that minimize their accumulation. SOD isoforms remove superoxide radicals and NO is removed by its rapid diffusion through tissues (Beckman, 1996 and Butler et al., 1998). Under pro-inflammatory conditions, the production of NO and superoxide radicals lead to the generation of significant amounts of ONOO-, which causes injury to different cellular structures. Furthermore, it affects mitochondrial function and triggers cell death via nitration and oxidation reactions (Radi, 2013). The biological chemistry of peroxynitrite is modulated by Endogenous antioxidants and synthetic compounds modulate the biological chemistry of ONOO- and its scavenging activity to neutralize its damaging effects (Uribe et al., 2014). The rate of peroxynitrite production in vivo has been estimated to be as high as 50–100 lM per min.

2.6.1 Sperm attributes- concerning peroxynitrite

Similar to NO, $ONOO^-$has been associated with both detrimental and beneficial effects (Moro *et al.,* 1995; Vinten-Johansen, 2000), based on its concentration and redox environment (Mallozzi *et al.,* 1997). For instance,

Herrero *et al.,* (2001) have demonstrated that ONOO−at lesser concentrations (5–50 µmol/l) stimulates capacitation in human spermatozoa, whereas at greater concentrations ONOO− produce an excess of spontaneous tyrosine nitration,
Affecting sperm cell function (Vignini *et al.,* 2006). Peroxynitrite derived radicals cause oxidation and-or nitration of various biological macromolecules including proteins, DNA and unsaturated fatty-acid-containing phospholipids. Peroxynitrite can also cause deoxyribose oxidation and break DNA strands (Kennedy *et al.,* 1997). Peroxynitrite induced oxidative and nitrosative changes in lipids result in their peroxidation (Radi *et al.,* 1991) and the formation of nitrito-, nitro-, nitrosoperoxo- and/or nitrated lipid oxidation adducts (malondialdehyde, conjugated diene and lipid hydroperoxide formation) (Villa *et al.,* 1994; Rubbo *et al.,* 1994; Violi *et al.,* 1999). Further, it causes the oxidation of arachidonic acid and leads to the formation of F2-isoprostanes through the oxidation of low-density lipoprotein (Moore *et al.,* 1995). The nitration of fatty acids may lead to the secondary inhibition of protein function via thiol-based modifications (Wright *et al.,* 2006).

2.6.2 Mass motility

Mass motility of fresh semen sample is accessed by graded estimation of the vigor of swirls, eddies, and waves formed in the undiluted semen sample. A small drop (10µl) of undiluted semen is viewed without cover-slip and graded under the low magnification of phase-contrast microscope (10x) with the heated stage at 37oC considering the mass activity of spermatozoa from 0 to 5 point scale. For Karan Fries (KF) bulls, the average mass activity was reported to be 2.6±0.09 (Bhakat *et al.,* 2014), 1.81±0.78 (Soren *et al.,* 2016), 2.20±0.09 (Mallick *et al.,* 2016), 1.85±0.14 (Jalmeria *et al.,* 2018). For HF bulls it has been reported to be 2.80±0.26, 2.40±0.11 and 1.94 by Tomar *et al.,* (1985), Panwar and Nagpaul, (1994) and Keshava, (1996). Mathur *et al.,* (2002) reported the average mass activity of fresh semen as 2.94±0.02 for Frieswal bulls.

2.6.3 Individual motility

Individual motility of sperm is one of the major functional biomarkers to

detect sperm function, mainly in AI laboratories. Evaluation by a light microscope is widely used as the simplest method and does not require any expensive equipment. Highly subjective evaluation method and it's not a reliable assay to predict the fertility of the animal (Martinez, 2004). Various in-vitro studies have shown the relationship between high peroxynitrite levels and impaired sperm motility. Uribe *et al.,* (2014) reported that peroxynitrite mediated nitrosative stress is time and concentration-dependent reduction in progressive and individual motility of spermatozoa. The decrease in progressive motility was significant compared with the untreated control from 3h of incubation with 1.0 mmol/l of SIN-1 in human spermatozoa (Uribe *et al.,* 2014). Cryopreserved spermatozoa are highly susceptible to structural and physiological alterations that are induced by free radicals (Medeiros *et al.,* 2002). A decrease in the motility of cryopreserved bull spermatozoa was reported (Rodriguez and Beconi, 2009) after incubation with SIN-1 (160 mmol/l).

Increased level of lipid peroxidation is negatively associated with sperm motility (Oztezcan *et al.,* 1999). Peroxynitrite alters sperm motility by resulting in lipid peroxidation, interfere with the ATP production by spermatozoa and also damages the structural and contractile proteins that are responsible for the movement of spermatozoa (Uribe *et al.,* 2017).

2.6.4 Sperm viability

Eosin-Nigrosin staining is used for the estimation of live and dead spermatozoa. Live and dead ones' can be differentiated by their ability to retain stain. Dead spermatozoa retain the dye while live sperm are colorless. A study by Uribe *et al.,* (2014) reported that the viability of sperm cells remains unchanged after treatment with concentrations up to 1.0 mmol/l of SIN-1 during an incubation time up to 4 h. A previous study conducted on cryopreserved bovine spermatozoa, where incubation for 45 min at 370C with up to 1.0 mmol/l of SIN-1 did not significantly reduce sperm viability (Rodriguez and Beconi, 2009).

2.6.5 Sperm morphology

Classification of sperm abnormalities is based on their location of defects (head, mid-piece, tail) and site of origin ((primary- occur during spermatogenesis in the testis; secondary- during maturation in the epididymis; tertiary abnormality- accessory glands/post ejaculation). sperm abnormalities were found to be correlated with fertility (Sekoni and Gustafsson,1987). Bhakat *et al.,* (2014) reported total sperm abnormalities, head, mid-piece and tail abnormalities in KF bulls as 3.16 ±0.02; 2.23 ±0.007; 7.53 ±0.001; 12.97±0.001 respectively. Jain, (2004) reported head, mid-piece, and tail abnormalities in KF bulls as 4.73%, 4.58%, and 4.95% respectively. In human patients, a negative correlation has been reported between peroxynitrite concentration and sperm morphology and motility. More the production of reactive oxygen species, more were the abnormal sperms such as immature or cytoplasmic droplets (Khosravi *et al.,* 2014).

2.6.6 Acrosomal Integrity

Determination of the acrosome status in cryopreserved semen is of utmost importance, as cryopreservation directly damages the sperm membrane, which could lead to the loss of acrosome's matrix contents. This leads to an inability of acrosome reaction to occur in the female reproductive tract which further results in reduced fertility. Hence, the acrosome should be intact for the acrosome reaction to occur to facilitate fertilization (Esteves *et al.,* 2007). Most workers examined the acrosome abnormality by using the Giemsa stain method (Bhosrekar *et al.,* 1994 and Ramakrishnan and Ariff, 1994). Lower concentration peroxynitrite is required to accelerate acrosome reaction in cryopreserved bull semen (Rodriguez and Beconi, 2009).

2.6.7 Mitochondrial Membrane Potential (MMP)

Changes in mitochondrial membrane potential (Δψm) were reported as a brilliant indicator of sperm motility. Peroxynitrite can react with key components of mitochondria, such as proteins of the electron transport chain, cytochrome-c, the

TCA cycle enzyme aconitase, mitochondrial creatine kinase, as well as nicotinamide nucleotide transhydrogenase (Radi *et al.,* 2013). Thus, oxidative damage has the potential to affect virtually every critical function of these organelles (Pacher *et al.,* 2007). When crucial targets are reached by peroxynitrite, mitochondrial permeability transition pores (MPT) are induced, resulting in the dissipation of mitochondrial membrane potential (ΔΨm), mitochondrial swelling and rupture of the outer membrane, with the subsequent efflux of proapoptotic molecules (Szabo *et al.,* 2007) and subsequent cell apoptosis.

The dye, JC-1 was used to study the effect of cryopreservation on bovine sperm organelle function and viability (Thomas *et al.,* 1998). Uribe and co-workers conducted in-vitro studies using SIN-1 as a peroxynitrite precursor which resulted in a decrease in sperm mitochondrial membrane potential after incubation with SIN-1. Thus peroxynitrite has a negative effect on the mitochondrial function of human spermatozoa (Uribe *et al.,* 2014).

2.6.8 Plasma membrane integrity

Sperm plasma membrane integrity is an essential parameter that determines the structural and functional integrity of spermatozoa, whose abnormality leads to a negative impact on fertility and sperm survival in the female reproductive tract (Oura and Toshimori, 1990). Sperm tail is particularly more susceptible to the hypo-osmotic medium (Jeyendran *et al.,* 1984). Viable spermatozoa in a hypo-tonic medium have been reported to show bent or coiled tails (Pursley and Herman, 1950), whereas the dead sperm had straight tails. Therefore, it was hypothesized that the ability of sperm cells to swell up in a hypo-tonic solution indicates its membrane integrity and normal function activity (Takahasi *et al.,* 1990). Spermatozoa with compromised membranes were unable to swell as they lacked the water influx regulation system. Zhou *et al.,* (2010) reported that the semen quality can be detected by evaluating the functional integrity of the sperm plasma membrane.

2.6.9 Lipid Peroxidation

Lipid peroxidation results in a subsequent loss in membrane integrity, induction of sperm apoptosis, impaired sperm motility and impaired cell functions (Bucak *et al.,* 2010). Once LPO in spermatozoa has been initiated, it propagates like a chain reaction, leading to the accumulation of lipid peroxides in the sperm plasma membrane (Sharma *et al.,* 1996) which are then released by the action of enzyme phospholipase A2. Lipid peroxides are capable of inducing DNA damage (Twigg *et al.,* 1998). Although the peroxides reduce sperm functions and viability, it significantly enhanced the binding of spermatozoa with heterologous and homologous zona pellucida (Aitken *et al.,* 1989). The end product of LPO is malondialdehyde (MDA) which can be measured through thiobarbituric acid (TBA) assay (Sanocka *et al.,* 2004).

Peroxynitrite induced oxidation of plasma results in the production of lipid hydroperoxides (Radi, 2013). The continuous accumulation of plasma lipid hydro-peroxides is mediated by peroxynitrite and other oxidants such as hydroxyl radical, nitrogen dioxide (Morita *et al.,* 2016). In a study done to correlate MDA concentration to sperm parameters, it was concluded that MDA concentration has a positive correlation with abnormalities (r = 0.478) and negative correlation with the hypo-osmotic swelling test (r = –0.359) and sperm concentration (r = –0.257) (Soren *et al.,* 2016). A negative relationship was reported between semen quality parameters and lipid peroxidation in ram spermatozoa (Kasimanickam *et al.,* 2006).

2.7 SIN-1 or 3-Morpholinosydnonimine

SIN-1, also known as 3-Morpholinosydnonimine is an *in-vitro* generator of peroxynitrite radical. SIN-1 in solution and the presence of oxygen decomposes spontaneously releasing NO and superoxide free radicals, which react together and thus increase the intracellular formation of peroxynitrite spontaneously (Blanco Garcia *et al.,* 2009). Peroxynitrite generated through SIN-1 was demonstrated by

oxidation of DHR, which was evidenced by the increase in absorbance at 500 nm. Absorbance of DHR increased exponentially in direct proportion to the concentration of SIN-1 (Uribe *et al.,* 2014).

2.8 Sodium Nitroprusside

Sodium Nitroprusside (SNP) is one of the most effective nitric oxide donor used for *in vitro* studies, and its capability of producing NO seems to depend on its interaction with sulfhydryl-containing molecules present *in vivo* leading to the formation of the corresponding disulfides and S-nitrosothiols; NO, and cyanide ions (Grossi and Angelo, 2004). SNP is involved in the regulation of a variety of cell signals in spermatozoa including the motility, hyperactivation, capacitation, and acrosome reaction (AR) of spermatozoa. SNP has got congenial effects as that of nitric oxide concentration in sperm cells. A lot of studies have been carried out on spermatozoa of different species regarding a variety of parameters which helps to explore the structural and functional integrity of sperm cells.

2.8.1 Sperm motility and viability

NO has got dual action which depends on its concentration, while the lower concentrations increase motility and higher concentration reduces the motility (Weinberg *et al.,* 1995; Herrero and Gagnon, 2001; Miraglia *et al.,* 2011; Doshi *et al.,* 2012). In a study by Rahman *et al.,* (2014), sodium nitroprusside dose-dependently decreased the motility and hyperactivation of spermatozoa, which were significantly decreased at the highest concentration of sodium nitroprusside tested (100 µM). Khodaei *et al.,* (2016) reported a significant increase in sperm motility after thawing when exogenous SNP concentration was about 100nmol/ml and also SNP affected sperm viability and improved this parameter 1 hour after thawing. Rodriguez *et al.,* (2005) demonstrated that a higher concentration of SNP reduced progressive motility in a dose-dependent manner and lower concentrations of SNP failed to affect either motility or sperm viability in the case of bulls. Contrastingly a study by Domple *et al.,* (2016) on buffalo spermatozoa revealed a

significant decrease in sperm motility and viability even at 100nmol/ml of SNP and there was an obvious negative effect on these parameters at high concentrations of SNP (100µM). Naskar (2018) and Panth (2017), reported that the motility percentage remained static up to 1µM SNP concentration followed by a significant decline at 10 µM indicating that higher concentrations of NO were detrimental to sperm motility while the percent viability increased significantly up to 1µm SNP concentration followed by a decline.

2.8.2 Sperm morphology

A study by Revelli *et al.,* (2001) reported that NO had an impact on normal sperm morphology and it aids in accurate prediction of fertility status and pregnancy outcomes during assisted reproductive techniques. High concentrations of NO-induced abnormal sperm morphology in human spermatozoa as reported by Wu *et al.,* (2004). Domple *et al.,* (2016) reported a negative correlation between sperm morphology and SNP concentration. Naskar, (2018) and Panth, (2017) reported that only the highest concentration of SNP (10 µM), significantly enhanced the percent abnormal spermatozoa from 15 minutes post-treatment, whereas lower concentrations (0.1 and 1µM), increased the percent abnormalities only after 60 minutes of exposure.

2.8.3 Acrosome Integrity and acrosome reaction

Weinberg (1995), reported that high concentrations of % NO lead to a reduction in motility, induce toxicity and affect acrosomal membrane integrity in human spermatozoa. Vanasco *et al.,* (2010) reported an increased percentage of spermatozoa undergoing the acrosome reaction in the presence of NO-releasing compound (SNP). Naskar, (2018) and Panth, (2017), demonstrated that the membrane integrity and acrosome integrity of cryopreserved semen was reduced ($P<0.01$) at 60 minutes of exposure to different concentrations of either SNP (0.1, 1 and 10 µM) in buffalo and crossbred KF bulls respectively. Khodaei *et al.,* (2016) reported that SNP (100nmol/ml) increased the acrosome integrity of Holstein bulls spermatozoa pre and post thawing.

2.8.4 Lipid Peroxidation

Membrane lipid peroxidation (LPO) is considered as the best indications of a healthy sperm membrane. Mammalian spermatozoa contain a significant amount of polyunsaturated fatty acids (PUFAs) which makes them highly susceptible to damage mediated by LPO. Lipid peroxidation results in a subsequent loss in membrane integrity, impaired sperm motility, impaired cell functions and induction of sperm apoptosis (Bucak *et al.,* 2010). Malondialdehyde (MDA) is the end product of LPO which can be measured through thiobarbituric acid (TBA) assay (Sanocka *et al.,* 2004). It was reported by Wang *et al.,* (1997) that there was not any significant difference between MDA levels in fresh and frozen semen of normal men. Chatterjee *et al.,* (2001) demonstrated that TBARS level was increased in frozen-thawed bull sperm but not in cooled sperm. Khodaei *et al.,* (2016) reported that different treatments of SNP (10nmol/ml and 50nmol/ml) reduced this LPO significantly and damaged sperm membrane at 1 and 2 hours after thawing, but in fresh semen, only 50 nmol caused a significant reduction.

2.8.5 Capacitation and hyperactivation

Sperm capacitation and acrosome reaction are important reactions for successful fertilization to take place, both in-vivo and in-vitro. Particularly some of the studies have shown that NO donors are responsible for significant acceleration of these reactions while NO inhibitors have an opposite effect (Zini *et al.,* 1995; Herrero *et al.,* 1999; O'Flaherty *et al.,* 2006). Sodium nitroprusside generating low NO was shown to act as a capacitation inducer in cryopreserved bovine spermatozoa, reaching the concentrations similar to those of heparin treated samples (Rodriguez *et al.,* 2004). There was an increase in the percentage of capacitated sperm by incubating spermatozoa with a low concentration of SNP (Choudhary *et al.,* 2010). These authors also depicted the involvement of NO in regulating lipoxygenase and cyclooxygenase activities during capacitation thus

implicating nitric oxide in sperm functions. The effect of NO donor on hyperactivation was found to be similar to that on sperm motility, as little concentrations of NO increased spermatozoa hyperactivation, whereas excessive concentrations decreased the hyperactivated spermatozoa motility (Otasevic *et al.,* 2013; Miraglia *et al.,* 2011).

2.8.6 Mitochondrial membrane potential (MMP)

During the process of oxidative phosphorylation, protons are being pumped from inner mitochondria to the outer side, which creates an electrochemical gradient called the inner Δψm (Evenson *et al.,* 1982). The ability to distinguish the mitochondria exhibiting higher mitochondrial membrane potential from those having low Δψm provides an estimate of the metabolic functions of the cells. Measuring Δψm in spermatozoa provides useful information about fertility potential. There is a positive correlation between sperm motility and viability with Δψm (Auger *et al.,* 1989; Troiano *et al.,* 1998). Δψm is an important indicator of the functional integrity of spermatozoa.

Wang *et al.,* (2003) reported that the semen samples having abnormal semen parameters had a significantly lower Δψm [1337.7(1066.38, 1879.2)] as compared to normal semen [Δψm: 2482.9 (2162.5, 3520.6)]. There is a positive correlation between sperm concentration (r=0.62) and Δψm while Δψm is negatively correlated with ROS (reactive oxygen species) produced (r=-0.45). Hence, an increased ROS production by spermatozoa was associated with a decreased Δψm.

CHAPTER–3

Materials and Methods

MATERIALS AND METHODS

3.1 Location of the study

The present study was carried out on the Karan Fries (Tharparkar x HF) cattle bulls maintained at Artificial Breeding Research Centre, National Dairy Research institute, Karnal, Haryana. This institute is located at an altitude of 250 meters above the mean sea level on 29.704°N latitude and 76.982°E longitude. The maximum ambient temperature rises up to 45°C during the summer and minimum about 4°C during winter. The annual rainfall is about 650 to 780 mm, most of which occurs during July and August. Relative humidity ranges from 41 to 85 per cent.

3.2 Experimental period

The experiment was conducted from November 2019 to May 2020 for a period of 10 months. The animal experiments performed were acceptable to the ethical standards of the National Dairy Research Institute, Karnal.

3.3 Selection, housing and management of the bulls

For the current study, 6 adult (3–4.5 years) Karan Fries bulls maintained at ABRC, ICAR-NDRI, Karnal were selected. The body weights of these bulls ranged between 350 and 450 kg. They were kept in concrete floored individual pens (30×10m) under loose housing system with corrugated asbestos roofed shed, having east-west directional orientation through its long axis. The pens were separated by solid partitions to restrict physical and visual contact of the bulls in adjacent pens but allow free movements within the shed. Cleaning of the shed was done early in the morning every day. Bulls were exercised once a week, the day before the semen collection in the rotary exerciser to maintain the sexual vigor of bulls and ensure quality semen production. All bulls were tied under bull washing shed and thoroughly washed,

groomed and cleaned an hour before the actual time of semen collection. The semen was collected by artificial vagina (A.V.) technique between 7.30 to 9.00 A.M. Vaccination, deworming and other herd health programs were followed as per farm schedule, to protect the animals from diseases.

3.3.1 Feeding of the bulls

Around 2.0 to 2.5 kg concentrate mixture with 21% CP and 70% TDN per bull per day was offered at 9.30 am throughout the duration of experiment. Institute grown green fodder and water *ad lib* was provided throughout the day.

3.3.2 Cleaning and sterilization of articles

The following procedure was followed in washing of all the glasswares used for semen collection, processing and specific experiments.

- Washed thoroughly with soap water.
- Dipped in 10% HCL for 12 h
- Washed vigorously with tap water.
- Rinsed twice in triple distilled water.
- Dried and then sterilized in hot-air oven at 160°C for 1 h.
- Buffer solutions, rubber articles, artificial vagina were autoclaved at 15 lb pressure at 120 °C for 20 minutes. The stains, polyvinyl alcohol and other polyethylene particles were exposed to ultraviolet rays for an hour before use.

3.4 Sample collection and processing

As per the routine, semen ejaculates were collected from the bulls using artificial vagina (Temperature 42- 45 °C; from IMV, L'Aigle, France) at the weekly intervals with two ejaculates on the same day at 20 minutes interval and were immediately, placed in a water bath (37 °C) to carry out semen evaluation parameters. Standard semen characteristics were evaluated using microscopy, and only those ejaculates which qualified the standard quality control measures (≤+3 mass motility and ≤ 70% individual

motility) were selected for further processing and cryopreservation.

3.5 Preparation of extender for cryopreservation

The extender was always prepared fresh, generally an hour before collection of semen so that the medium gets stabilized. Fresh eggs were procured from the market for the preparation of semen extender.

Table 3.1: Composition of semen extender

Buffer solution	**For 100 mL**	**For 250 mL**
Tris	2.422g	6.055g
Citric Acid	1.36g	3.4g
Fructose	1.25g	2.5g
Millipore water	60mL	150Ml
Streptomycin	0.1g	0.25g
Benzyl Penicillin	100,000 IU	250000 IU
Make the volume up to	73.6mL	184mL

The pH of the dilutor was adjusted to 6.8 with 10% NaOH

Final extender		
Buffer solution	73.6 mL	184 mL
Egg yolk	20 mL	50 mL
Glycerol	6.4 mL	16 mL
Total	100 mL	250 mL

3.6 Cryopreservation of semen

The processing method for the freezing of semen was as per the procedure followed in the Artificial Breeding Research Centre, ICAR-National Dairy Research Institute.

The method followed is described as follows.

- **Dilution rate:** It was decided on the basis of sperm concentration and initial motility (%), the extender was divided into two equal parts, i.e., Part-A and Part-B. Both parts were kept in water bath at 30^{o}C.

  The glycerol (12.8 per cent, v/v) was added in part-B of the extender. So that the final concentration was maintained at 6.4 per cent (v/v).

  Part-B was added to Part-A. The contents were mixed by gentle rotation and kept it in the plastic tray filled with water maintained at 30^{o}C for filling and sealing.
- **Printing the straws:** Straws were printed with details like- bull number, breed, date of freezing, treatment given, and the name of the station by computerized auto printing through Domino Ink Jet Printer A 200 Pinpoint (Domino UK Ltd, Bar Hill, Cambridge, CB3 8TU, England).
- **Filling and Sealing of Straws**: French Top Bull mini straws (0.25 mL; 135 mm length and 2 mm diameter, IMV) of different colours were used. Automatic straw filling and sealing machine (IMV, France) was used for filling of semen into the straws and sealing it. Filling and sealing were done at room temperature in Semen Processing Lab.
- **Equilibration time**: The filled and sealed straws were placed in a cold room at 4 to 5^{o}C in a goblet. The temperature of the samples decreased to 5^{o}C during 4 hours with an average cooling rate of 0.25^{o}C per min.

  After completion of equilibration time, the straws were placed horizontally in a freezing rack. The rack along with straws was kept in the liquid nitrogen vapour, 6 cm above the level of liquid nitrogen for 10 min. The temperature of semen reached -130^{o}C to -150^{o}C by about 10 min.
- **Storage:** The straws were transferred into goblets with the help of pre-cooled forceps and the goblets were stored in separate canisters in the liquid nitrogen (LN_2) in cryovessel. Cryovessel was always kept three-fourth full by replenishing liquid nitrogen from time to time.

- **Thawing and examination of frozen semen**: The straws were thawed in a 37^{o}C water bath (tested in Thaw Monitor, World-Wide Sires, Inc. California, USA) for 30 seconds after removing from the LN_2 container. Immediately after thawing, motility of semen was checked. Semen was taken in the micro-centrifuge tube and kept at 37^{o}C until further examination. The thawed semen samples were subjected to different tests for evaluation of motility, non-eosinophilic count, physical abnormalities, acrosome integrity, HOST reacted spermatozoa, etc, as described under different headings in section.

3.7 Objective 1: To assess the impact of ROS generated in fresh and cryopreserved semen on structural changes and sperm quality

The ejaculates were brought to the laboratory immediately after collection, and kept in water bath at 32°C. Soon the semen samples were classified according to the mass activity and colour. The semen sample were subsequently examined at 0 hour (before cryopreservation) for volume, colour, mass-activity, individual motility, sperm concentration, non-eosinophilic (live), hypo-osmotic swelling test, acrosome reaction and subsequent experimentation. From remaining semen samples, straws were prepared for further assessing the above mentioned seminal attributes at 24 hour, 1 month and 2 month intervals.

3.7.1 Evaluation of conventional seminal attributes

Semen quality parameters that determine the quality of semen include the color, pH, volume, progressive motility, viability, sperm concentration, morphology and membrane integrity (Arifiantini *et al.,* 2006; Zaenuri *et al.,* 2014).

3.7.2 Volume

The semen was collected in 15 ml graduated sterilized glass conical centrifuge tube with 0.1 ml accuracy.

3.7.3 Colour

Colour of the neat semen was determined by visual inspection and recorded immediately after collection. It was scored from creamy to watery.

3.7.4 Mass activity

Mass activity was determined immediately after the semen collection. It was graded on the basis of wave movement under a warmed stage (37°C) of a phase contrast microscope (200 X, Nikon eclipse E600, Tokyo, Japan). Two drops of undiluted semen were placed at two spots on a slide placed in stage warmer (37^{o}C) and scored on a scale of 0-5 using 10X objective lens. Mass motility was expressed qualitatively in 0-5 scale as per the description given by Tomar *et al*., (1966). The average of the two score was considered as final value.

Table 3.2: Grading of mass activity based on wave movement

Grade	Observation
0	Immotile spermatozoa and absence of waves
+	Sperm cells motile but waves absent
++	Swirls are absent. Individual movement of spermatozoa are more evident from the field
+++	Presence of waves in moderate motion
++++	Less rapid swirls and eddies Dark intense waves in rapid motion waking eddies
+++++	Rapid waves and swirls

3.7.5 Sperm Concentration

The concentration of spermatozoa (millions per mL) in the fresh semen was determined by photometer (IMV, L'Aigle, France). The concentration was assessed with the aim of ascertaining the final dilution rate of semen.

3.7.6 Individual Motility estimate (%)

The progressive motility and percentage of motile spermatozoa were determined manually by placing 100 µl of undiluted semen into pre-warmed tubes in digital control heat blocks containing 900 µl of Tris buffer and mixed thoroughly. 10 µl of diluted semen was placed on a warmed glass slide (37^{0}C) and was allowed to spread uniformly

under the coverslip (18x18 mm). Initial progressive motility rating was scored using 200X magnifications with phase contrast microscope (Nikon Eclipse E600, Tokyo, Japan) equipped with a heated stage.

Percent progressive motility was measured at five representative areas of the slide. The average of the five scores for each category was recorded.

3.7.7 Non-eosinophilic sperm count

A method described by Blom (1950) and Hancock (1951) was followed to assess non-eosinophilic or live sperm count. Dead spermatozoa could be differentiated by their ability to get stained by eosin dye. The live spermatozoa, which are alive at the time of staining, remain colourless. Live spermatozoa are impermeable to the eosin stain. Nigrosine provided a blue-black background.

3.7.7.1 Preparation of eosin- nigrosin stain

The staining solution contained one part of 5% eosin-Y and four parts of 10% nigrosin. Thus, 5 g of eosin-Y yellow was dissolved in 100ml of 2.94% sodium citrate buffer by heating it up to boiling point. Nigrosin (10 g) was dissolved in 100ml of 2.94% sodium citrate buffer by boiling for 15 min. The solution was allowed to cool to room temperature after w**h**ich it was filtered through Whatsman filter paper no. 40 and stored in a dark glass bottle and sealed. Before use, the staining solution was brought to room temperature.

3.7.7.2 Procedure

- 2µl of neat semen and 20µl eosin-nigrosin staining solution were mixed properly on a clean grease free pre-warmed (37ºC) glass slide.
- 5 µL of the above mixture was drawn on another pre-warmed (37ºC) glass slide and smear was made. Duplicate smears were made from each sample.
- The smear slide was allowed to air dry at room temperature.
- About 200 sperms were assessed under bright field at 100X under oil immersion.

- The unstained sperms were classified as non-eosinophilic and those showing any pink or red colouration were classified as dead (eosinophilic), with the sole exception for sperms with a slight pink or red appearance restricted to the neck region ('leaky necks') were assessed as non-eosinophilic.
- ***Number of live sperms*:**

- ***Percentage of live spermatozoa = Number of live sperms/ Total number of sperms counted × 100***

3.7.8 Morphological abnormality

The slide prepared as discussed above was used for screening morphological abnormalities. A drop of oil was applied on the slide and different types of abnormalities (Head/Midpiece/tail abnormalities) were examined under bright field 100X objectives. About 200 spermatozoa were counted in different fields and percentage of abnormal spermatozoa was calculated as follows.

$$\textbf{Abnormality (Head/Mid-piece/Tail) (\%)} = \frac{\textbf{Number of abnormal sperm (Head/ mid-piece/Tail)}}{\textbf{Total spermatozoa counted}} \times 100$$

$$\textbf{Total Abnormality \%} = \frac{\textbf{Total number of abnormal spermatozoa}}{\textbf{Total spermatozoa counted}} \times 100$$

3.7.9 Hypo-osmotic swelling test (HOST)

HOST is an indirect method of assessing membrane integrity of the sperm. This test was performed according to the method described by Correa and Zavos (1994). Hypo-osmotic solution of 150 mOsmol/l was prepared as follows:

Table 3.3 Composition of hypo-osmotic swelling test solution

	HOST solution	Control solution
Sodium citrate (g)	0.735	2.94
Fructose (g)	1.351	5.40
Millipore water upto (ml)	100	100
Osmolarity (mOsm/ kg)	150	300

3.7.9.1 Procedure

- 10 µl of semen was mixed with 0.9 ml of hypo-osmotic solution, having an osmotic strength of 150 mOsm/l and incubated at 37ºC for 1 hour in a CO_2 incubator.
- Following incubation, a drop of well mixed solution was taken on a clean dry sterilized glass slide and a cover-slip was put over it.
- Sperm tail curling was recorded as an effect of swelling due to influx of water.
- A total of about 200 spermatozoa were counted in different fields at 40X magnification under phase contrast microscope.
- These spermatozoa were classified in four different groups according to the presence of following swelling pattern.

Table 3.4 Swelling pattern of spermatozoa in HOS Test

Sl. No.	Observations/patterns	Interpretation
1.	No swelling, no membrane reaction	-ve
2.	Swelling of tip of tail	+ve
3.	Different type of hair pin like swelling pattern or swelling of mid-piece	+ve
4.	Complete tail swelling	+ve

The percent swollen spermatozoa were calculated by dividing the number of reacted cells by the total spermatozoa counted in the same area and multiplying the figure by 100.

3.7.10 Acrosomal integrity

Acrosome integrity was judged by Giemsa staining technique as per the methodology described by Hancock (1952), Saacke *et al*. (1968) and Watson (1975).

Table 3.5: Composition of stain used for acrosomal integrity test

Giemsa stain	3.8 g
Absolute Alcohol (GR grade)	375 mL
Glycerol (AR grade)	125 mL

3.7.10.1 Preparation method

Giemsa stain was grounded with absolute Methanol in a pestle and mortar.

Glycerol was added to it. The stain mixture was stored at 37°C for one week. During this storage period, it was shaken for few minutes each day. After 7 days the stain was filtered in Whatman filter paper No. 40 for further use.

3.7.10.2 Preparation of Hancock's fixative, working Giemsa solution and Sorenson's phosphate buffer- These were prepared as follows.

Table 3.6 : Composition of Hancock's fixative used for acrosomal integrity test

10 % Formaline solution was used for fixation of spermatozoa	
NaCl	8.5 g
NaH2PO4.H2O	4.0 g
NaH2PO4	6.5 g
40 % Formaldehyde	100 mL
Distilled water	900 mL

Table 3.7: Composition of Giemsa working solution for acrosomal integrity test

Giemsa stain	3.0 ml
Sorenson's M/15 phosphate buffer (pH=7.0)	2.0 ml
Distilled water	35 ml

Table 3.8: Composition of Sorenson's phosphate buffer

3.7.10.3 Buffer Composition

Solution A

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

Solution B

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

100 ml of 0.1 M Sorenson phosphate buffer was prepared by adding 33 mL of solution A and 17mL of solution B with the desired pH (7.0)

3.7.10.4 Staining procedure

- A thin smear of diluted semen drop was made on a clean, grease-free slide.
- The smear was air dried and kept in Hancock's fixative solution for 30 min. at 37°C.
- The slides were washed with double distilled water and air-dried.
- In a staining jar, 3 mL of Giemsa stock solution was added drop by drop in 2 mL
- Sorenson phosphate buffer solution and 35 mL of double distilled water was added and the resulting mixture was mixed thoroughly.
- The slides were kept in the staining jar solution for 90 minutes at 37°C in the incubator.
- The stained slides were washed with distilled water and air-dried.

- A total of 200 spermatozoa per slide were examined under high power of phase contrast-microscope. This method stains the acrosomes dark purple.

Acrosome integrity (%) = Number of acrosome stained spermatozoa/ Total number of spermatozoa counted X 100

3.7.11 Advanced *in vitro* sperm function tests

3.7.11.1 Spermatic lipid peroxidation estimation

The spermatic lipid peroxidation was measured based on the malondialdehyde (MDA) concentration by following the procedures of Buege and Aust (1978) and modified by Suleiman *et al*. (1996).

3.7.11.1.1 Procedure

- Take 1mL of semen suspension in an eppendorf tube and centrifuge at 4000 rpm for 10 minutes.
- The pellet and supernatant are separated, further, the sperm pellet is dissolved in 900µL of PBS.
- Centrifuge both seminal plasma and sperm pellet tubes for 10 minutes at 4000 rpm.
- Take one milliliter of sperm suspension (100 million sperm) was added 2 mL of TCA–TBA reagent. (Tri-chloro acetic Acid 15% (w/v), TBA 0.375% (w/v) in 0.25N HCl.
- The mixture was boiled for 15 minutes and allowed to cool, then centrifuged (5000 rpm;15 min).
- The supernatant was separated and the absorbance was measured at 535 nm using UV-VIS spectrophotometer (DBS; Model-UV 3092, LABINDIA).
- The concentration of the MDA was determined by the specific absorbance coefficient (1.56×10^5/ mol/ cm^3).
- LPO (n M MDA/10^8 sperm or mL seminal plasma) was calculated by the following formula;

OD X 10 X Total volume (3mL)/ 1.56 X Test volume (1mL) =

OD X 30/1.56

3.7.11.2 Test for Mitochondrial membrane potential (JC-1)

5,5´,6,6´-tetrachloro-1,1´,3,3´tetraethylbenzimidazolyl-carbocyanin iodide (JC-1) was used to estimate the population of spermatozoal mitochondrial potential as per the method described by De Tauw et al., (2002) and Saraf et al., (2017) with minor modifications. Thawed semen sample was washed and diluted as previously described in 3.1.3.2. To 10 million spermatozoa, 10 mL of 5,5´,6,6´-tetrachloro-1,1´,3,3´tetraethylbenzimidazolyl carbocyanine iodide (0.153 m*M*) was added in dark conditions and incubated for 15 minutes at 37 ˚C and 1 µL PI (0.3 mg/mL) was added, mixed properly and further incubated for 2 minutes. After incubation, 100 µL sperm-TALP was added and centrifuged at 800 x g for 3 minutes. A thin smear was made out of the pellet, DABCO was added, and spermatozoa were observed under a fluorescence microscope using FITC and TRITC filters and images of the two filters were merged to obtain the final image.

3.8 Objective 2- To assess the impact of SIN-1 and/or SNP supplemented semen extender on post-thaw structural changes and semen quality

3.8.1 Preparation of chemicals

3.8.1.1 Preparation of SNP

The chemical compound used is sodium nitroprusside dihydrate (SIGMA-ALDRICH 71778-25G). It has molecular weight of 297.95 g/mol and it is water soluble. From these three different concentrations of SNP is prepared viz. 10 nmol/mL, 50 nmol/mL and 100 nmol/mL.

3.8.1.2 Preperation of SIN-1 (3-Morpholinosydnonimine hydrochloride)

The chemical compound used is SIN-1 (3-Morpholinosydnonimine hydrochloride) (SIGMA-ALDRICH M5793-25MG). It is a crystalline solid with molecular

weight of 206.63 g/mol. From this three different concentrations are prepared viz., 80 µmol/L, 160 µmol/L and 200 µmol/L.

3.8.2 Standardization of SNP and SIN-1 concentration to be used for modifying semen extender

Semen will be collected once every week from 6 Karan Fries bulls for one month and assessed for ejaculate volume, sperm concentration and progressive motility. The semen samples will be divided into 8 aliquots containing various concentrations of SNP (0, 10, 50, 100 nmol/ml) and SIN-1 (0, 80, 160, 200 µM). These aliquots will be cooled at 4 degree Celsius and evaluated at 0, 24, 48 & 72 hrs. The concentration giving highest motility is selected for further investigation.

3.8.3 Effect of modified semen extender on seminal attributes of spermatozoa during cryopreservation of KF semen

Again from the same 6 KF bull semen sample is collected at weekly interval for 2 months. The semen is cryopreserved with optimum level of SNP (10 nmol/mL) and SIN-1 (80 µmol/L) from the above results by adding it on extender and assessed for quality before and at 24hr, 15 days and 1 months after cryopreservation. Semen, seminal plasma was separated after 2 months of cryopreservation and aliquoted in different vials and preserved at -20 degree Celsius till analysis of biochemical indicators of semen quality i.e. lipid peroxidation status (TBARS).

3.8.3.1 Following parameters were evaluated for semen quality:

- **Sperm motility** (Procedure described in 3.7.6)
- **Sperm viability** (Procedure described in 3.7.7)
- **Sperm membrane integrity** (Procedure described in 3.7.9)
- **Acrosomal integrity** (Procedure described in 3.7.10)
- **Mitochondrial membrane potential**
- **Lipid peroxidation status** (Procedure described in 3.7.11.1)

- **Sperm Abnormalities** (Procedure described in 3.7.8)
- **Acrosomal reaction status (FITC-PNA)**

3.8.3.2 Advanced *in vitro* sperm function tests

3.8.3.2.1 Mitochondrial membrane potential (MMP)

Mitochondrial Apoptosis staining Kit with MitoStain dye was used to estimate the population of spermatozoal mitochondrial membrane potential as per the method described by De Tauw et al., (2002) and Saraf et al., (2017) with minor modifications. Thawed semen sample was washed and diluted as previously described in 3.6. To 10 million spermatozoa, 1X MitoStain Reagent working solution was added in dark conditions and incubated for 15 minutes at 37 ˚C. After incubation, 100 µL sperm-PBS was added and centrifuged at 800 x g for 5 minutes. A thin smear was made out of the pellet, DABCO was added, and spermatozoa were observed under a fluorescence microscope using FITC and TRITC filters and images of the two filters were merged to obtain the final image. Mid-piece with red/orange florescence is positive for MMP while green mid-piece depicted negative for MMP.

3.8.3.2.2 Assessment of sperm acrosome reaction

The sperm acrosome reaction assessment was done by fluorescein isothiocyanate peanut agglutinin (FITC-PNA) staining.

Procedure:

1. The frozen straws were thawed at 37 °C for 30 seconds.
2. The straws were cut and semen was poured into 1.5 mL Eppendorf tubes and washed twice with 1 mL sperm-Phosphate buffered saline (PBS) by centrifugation (800 rpm for 5 min).
3. From the sperm pellet, 10 million sperm were taken in 1.5 mL amber colour Eppendorf tube, 5 µL of FITC-PNA (0.04 mg / mL) was added under dark conditions and incubated at 37 °C for 15 min, followed by addition of 2 µL of PI (0.3 mg / mL).

4. After incubation for 5 min, 100 µL of sperm-PBS was added, and, centrifuged at 800 rpm for 3 min.
5. The supernatant was discarded and a thin smear was made from 10 µL of the pellet.
6. The smear was dried and antifading agent 1, 4-diazabicyclo [2.2.2] octane (DABCO) was added and coverslip (22 × 40 mm) applied.
7. Sperm was observed under fluorescent microscope (Nikon ECLIPSE, Japan and OLYMPUS SC-180) using FITC filter (Emission: 515–555 nm and Excitation: 465–495 nm) and tetramethylrhodamine (TRITC) filter (Emission: 554–576 nm; Excitation: 540 nm).
8. Images of the two filters were merged to obtain the final image.
9. Sperm was observed under fluorescent microscope. A total of 200 sperm per slide were observed and classified into four different staining patterns and expressed in percentage.

3.9 STATISTICAL ANALYSIS

The data was analysed for mean ± SE. The variations in semen parameters were quantified using one-way ANOVA. Pearson's correlation was used to correlate the seminal attributes with biochemical indicators (TBARS). Unpaired t-test was used to assess levels of TBARS in fresh and extended semen after cryopreservation. For the second objective the data was analysed by one-way and two-way ANOVA (Tukey's multiple comparison test). GraphPad prism (version 7) and SPSS software was used for statistical analysis.

CHAPTER–4

Results and Discussion

RESULTS AND DISCUSSION

The present investigation was carried out at Animal Physiology Division and Artificial Breeding Research Centre, ICAR-National Dairy Research Institute, Karnal (Haryana), on clinically normal and sexually mature Karan Fries bulls .It is the need of the hour to bridge down the gap between the production of frozen semen and its quality in an economical manner. The simplest technique is to modify the extender in order to obtain high quality semen ejaculates from superior bulls, which could be preserved for longer along with optimum fertility. In order to satisfy this need, the present investigation was carried out in two parts. Part 1 explains the impact of duration of cryopreservation on different seminal attributes and assesses the structural and functional changes, which are the indicators of semen quality. The data has been put forth in tables 4.1 to 4.4 and graphically depicted in figures from 4.1 to 4.4. The second part dealt with *in vivo* effects of adding Sodium Nitroprusside (SNP) and 3-Morpholinosydnonimine HCl (SIN-1) in extender to investigate the impact of nitrosative stress/bimodal action of nitric oxide on fresh and cryopreserved sperm functions and their structure. The data has been presented in tables 4.5 to 4.15 and graphically. The results of the said investigation are presented under following headings:

4.1 Objective 1: To assess the impact of ROS generated in fresh and cryopreserved semen on structural changes and sperm quality

4.1.1 Mass activity

The mean of mass activity based on 0-5 scale was found to be 2.54±0.066 from 6 different KF bull over the whole period of research work.

4.1.2 Concentration

Total (Mean±S.E.) sperm concentration was found to be 1119.32±34.298 x 10^6/ ml.

4.1.3 Progressive Motility

Percent progressive motility (Mean±S.E.) before freezing and 24 h, 15 days and 1 month after freezing was found to be 61.6±1.17, 40.2±1.46, 38.00±1.29 and 36.56±1.25 respectively. The only major significant ($p<0.05$) change was observed between fresh and cryopreserved semen samples at 24 hours after freezing interval, however, when talking about the cryopreserved samples after 15 days and 1 month interval there was only slight non-significant reduction observed in the % progressive motile spermatozoa.

4.1.4 Viability

The viability of spermatozoa, assessed as non-eosinophilia sperm count (% Mean±S.E.) was 70.99±0.86, 46.85±1.15, 46.40±1.40 and 42.12±1.40 in fresh semen and at 24 h, 15 days and 1 month after freezing respectively. The significant ($p<0.05$) change was observed both between fresh and cryopreserved semen samples and cryopreserved semen samples of 24 h and 1 month interval.

4.1.5 Membrane integrity

Hypo-osmotic swelling test positive spermatozoa (% Mean±S.E.) expressed as percentage was observed 63.5±1.142, 42.30±1.17, 40.08±1.11, and 38.16±1.21 before freezing and 24 h, 15 days and 1 month after freezing respectively. The only major significant ($p<0.05$) change was observed between fresh and cryopreserved semen samples at 24 hours after freezing however when talking about the cryopreserved samples there was only non-significant reduction observed in HOST positive spermatozoa at different intervals.

4.1.6 Acrosome integrity

Acrosomal integrity, expressed as a percentage of spermatozoa with intact acrosome showed significant ($P<0.01$) change between fresh and cryopreserved semen and further there was a significant ($p>0.05$) change observed between cryopreserved

samples at all the time intervals (24hr, 15 days, 1 month). The Mean±S.E. of sperm with intact acrosome was found to be 76.6±1.13, 66.16±1.22, 61.44±2.03 and 58.92±2.01 before freezing and 24 h, 15 days and 1 month after freezing respectively.

Table: 4.1 Effect of cryopreservation (Mean ±SEM) on seminal attributes

Attributes	Fresh semen (0h)	Cryopreserved semen		
		24 hours	15 days	1 month
Progressive Motility	61.60^{x}±1.17	40.20^{y}±1.46	38.00^{y} ±1.29	36.56^{y} ±1.25
Viability	70.99^{x} ±0.86	46.85^{y} ±1.15	46.40^{y} ±1.12	42.12^{z}±1.12
HOST +ve	63.50^{x}±1.15	42.30^{y}±1.17	40.08^{y}±1.12	38.16^{y} ±1.22
Acrosome Integrity	76.60^{x}±1.14	66.16^{y}±1.22	61.44^{y}±2.03	58.92^{z}±2.01

Values with different superscripts i.e, x, y and z within the rows differ significantly (P<0.05).

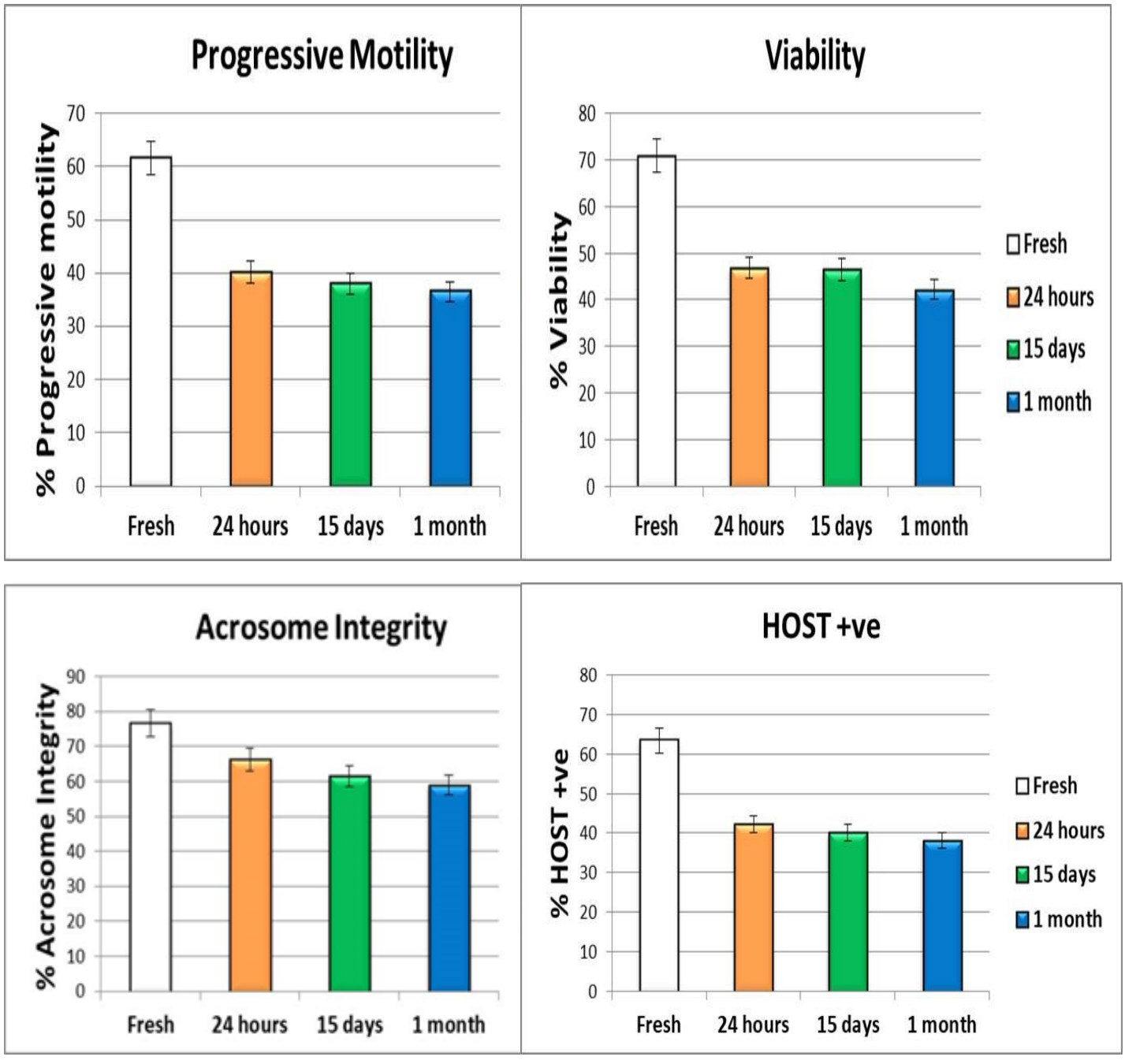
4.1.7 Total abnormality

Abnormal morphology of Semen was expressed as total abnormal spermatozoa (% Mean±S.E.) which includes head abnormality, mid-piece abnormality and tail abnormality. Total abnormality (%) was found to be 9.60±1.00, 16.52±1.13, 18.32±1.65 and 20.40±1.71 before freezing and 24 h, 15 days and 1 month respectively after freezing. Percentage of total abnormal spermatozoa showed significant (P<0.05) change between fresh and cryopreserved semen 24 hours after freezing and further there was non-significant (p<0.05) change was noticed between cryopreserved samples of different time interval.

4.1.7.1 Head abnormality

There was a Significantly (P<0.01) lower percentage of abnormal sperm heads found in fresh semen (3.92 ± 0.51) compared to cryopreserved semen which were found to be 5.78 ±0.64, 5.40 ±0.49 and 6.14±0.51 at 24 h, 15 days and 1 month respectively.

Fig. 4.1: Effect of cryopreservation on seminal attributes at different time interval



4.1.7.2 Mid-piece abnormality

Significantly (P<0.01) lower percentage of abnormal Mid-piece were found in fresh semen (1.96±0.13) compared to cryopreserved semen which were found to be 3.58±0.23, 3.56±0.29 and 2.83±0.21 at 24 h, 15 days and 1 month respectively.

4.1.7.3 Tail abnormality

Similar result was also found in case of tail abnormality with Significantly (P<0.01) lower percentage of abnormal tail in fresh semen (4.72±0.80) and cryopreserved semen which was found to be 7.16±0.60, 9.36±1.05 and 10.36±1.08 at 24 h, 15 days and 1 month respectively.

Table 4.2: Effect of cryopreservation on percent head, mid-piece, tail and total abnormality (Mean ±SEM)

Attributes	Fresh semen (0h)	Cryopreserved semen		
		24 hours	15 days	1 month
Head	3.92^{x}±0.51	5.78^{xy}±0.64	5.40^{xy}±0.49	6.14^{y}±0.51
Mid Piece	1.96^{x}±0.13	3.58^{y}±0.23	3.56^{y}±0.29	3.92^{y}±0.29
Tail	4.72^{x}±0.80	7.16^{xy}±0.60	9.36^{yz}±1.05	10.36^{z}±1.08
Total	9.60^{x}±1.00	16.52^{y}±1.13	18.32^{y}±1.65	20.40^{y}±1.71

Values with different superscripts i.e, x, y and z within the rows differ significantly (P<0.05).

Fig. 4.2: Effect of cryopreservation on percent head, mid-piece, tail and total abnormality at different time intervals

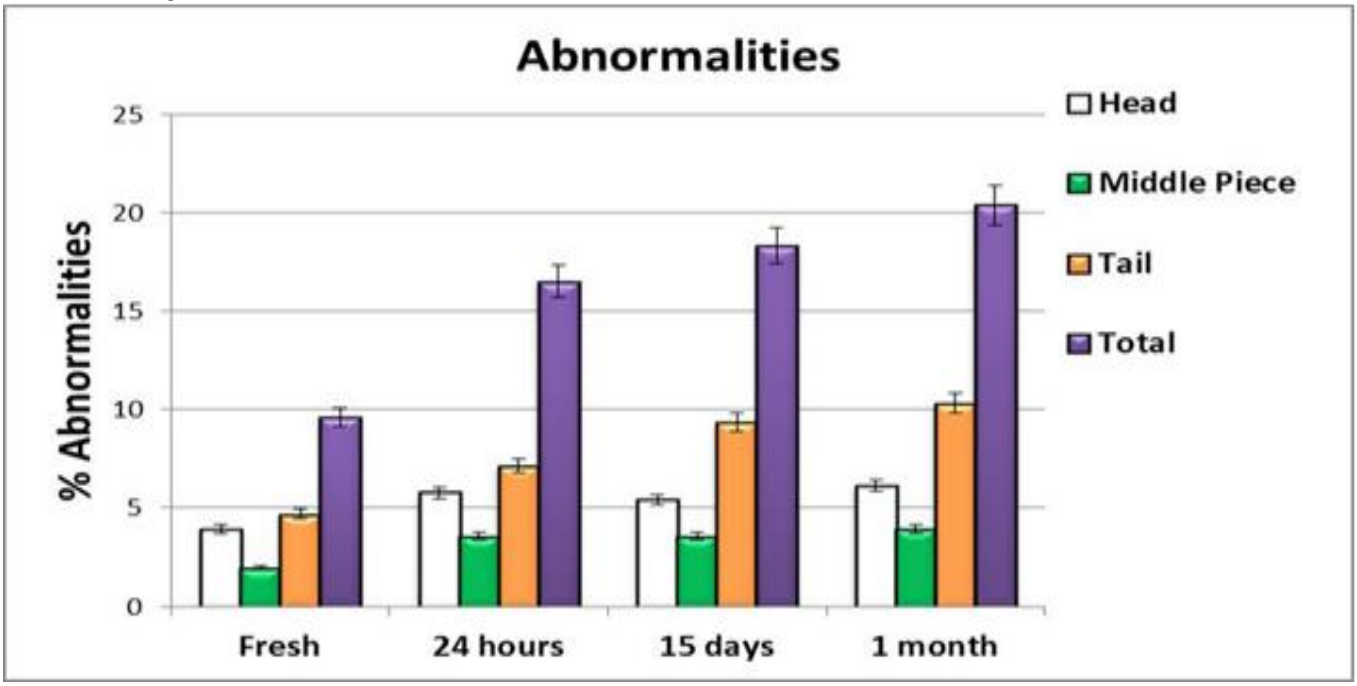
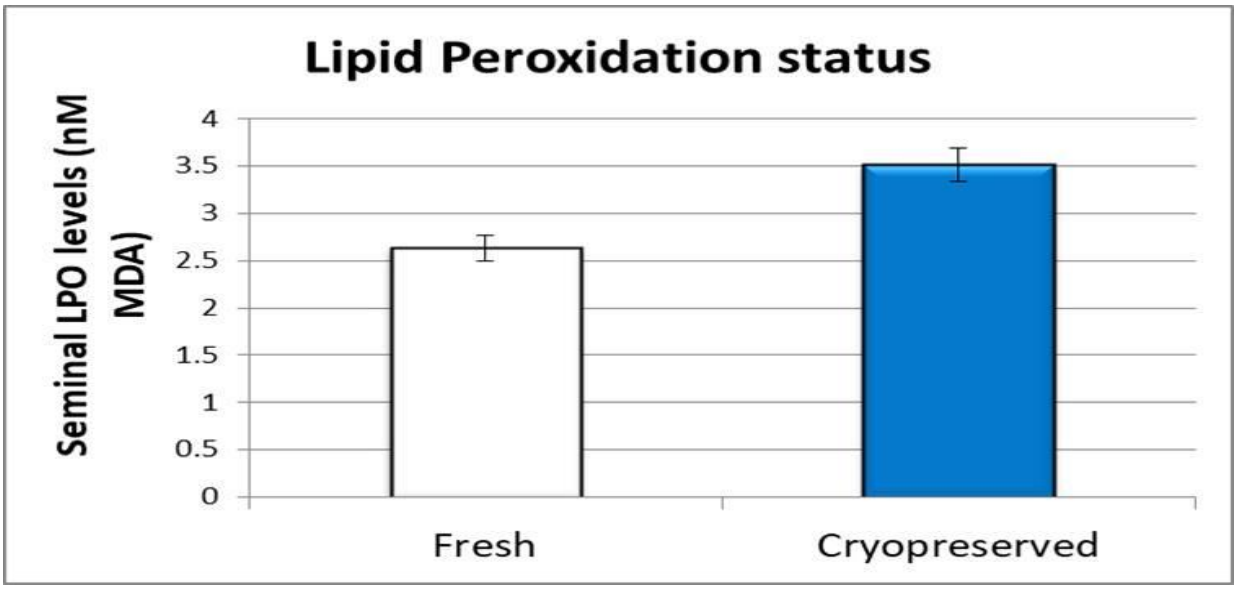


Fig 4.3: TAC and TBARS levels in deep freeze seminal plasma and cryopreserved extended seminal plasma



4.1.8 Lipid peroxidation status

The lipid peroxidation status (MDA) in seminal plasma of neat semen (2.63±0.22) was noticed to be lower in comparison to extended seminal plasma (3.51±0.34) received after cryopreservation.

Table 4.3: TBARS levels in Fresh seminal plasma and Cryopreserved (Mean ±SEM) extended seminal plasma

Seminal Plasma	TBARS levels (nM MDA)
Fresh	2.63±0.22
Cryopreserved	3.51±0.34

4.2- Objective 2- To assess the impact of SIN-1 and/or SNP supplemented semen extender on post-thaw structural changes and sperm quality

4.2.1 Standardization of SNP and SIN-1 concentration to be used to modifying semen extender

The semen samples were divided into 8 aliquots comprising of various concentrations of SNP (0, 10, 50, 100 nmol/ml) and SIN-1 (0, 80, 160, 200 µM). These aliquots will be cooled at 4 degree Celsius and evaluated at 0, 24, 48 & 72 hrs. During optimization and standardization of SNP and SIN-1 concentration for modifying semen extender it was noticed that progressive motility and viability was affected by SNP and SIN-1 in both time and dose dependent manner. Progressive motility (%) and Viability improved significantly ($P<0.05$) with the addition of 100 nmol/ml SNP and 80 µM SIN-1 as compared to control, 10 and 50 nmol/ml of SNP and control, 160 and 200 µM of SIN-1 concentration respectively at different time intervals.

Table 4.4: Time and dose dependent responses of SNP on % progressive motility (Mean ±SEM) of spermatozoa

Treatment (SNP)	Control	10 nmol/ml	50 nmol/ml	100 nmol/ml
0 Hour	73.61^{xa}±1.22	74.167^{xa}±1.14	74.722^{xa}±1.04	75.833^{xa}±0.91
24 Hours	52.50^{xab}±1.26	51.11^{xab}±2.22	51.389^{xab}±1.70	64.167^{yb}±0.99
48 Hours	48.05^{xab}±1.36	42.22^{xab}±2.46	43.889^{xab}±1.53	50.00^{xc}±1.61
72 Hours	41.38^{xb}±1.57	32.50^{xyb}±2.02	33.889^{xyb}±2.52	43.05^{xd}±2.14

Values with different superscripts i.e., x , y and z within same rows and a, b, c and d within same columns differ significantly (P<0.05)

Table 4.5: Time and dose dependent responses of SIN-1 on % progressive motility (Mean ±SEM) of spermatozoa

Treatment (SIN-1)	Control	80 µM	160 µM	200 µM
0 Hour	73.611^{xa}±1.22	76.20^{xa}±0.84	74.44^{xa}±1.10	74.30^{xa}±1.22
24 Hours	55.833^{xab}±1.49	57.22^{xab}±1.14	51.11^{xab}±1.96	56.944^{xab}±1.34
48 Hours	48.05^{xab}±1.36	50.00^{xab}±1.21	46.11^{xab}±1.86	50.277^{xab}±1.45
72 Hours	36.388^{xb}±1.18	45.55^{yb}±1.04	41.94^{xb}±1.61	45.277^{yb}±1.59

Values with different superscripts i.e., x and y within same rows and a and b within same columns differ significantly (P<0.05)

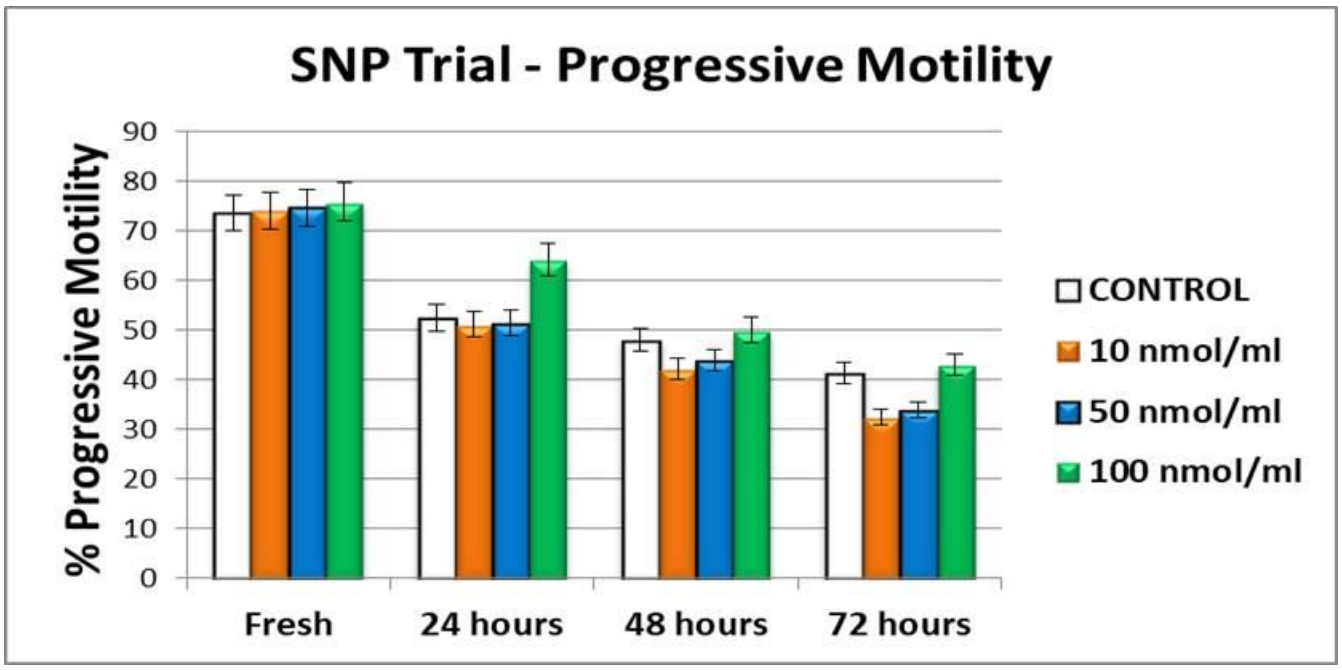


Fig. 4.4: Time and dose dependent responses of SNP on progressive motility of spermatozoa

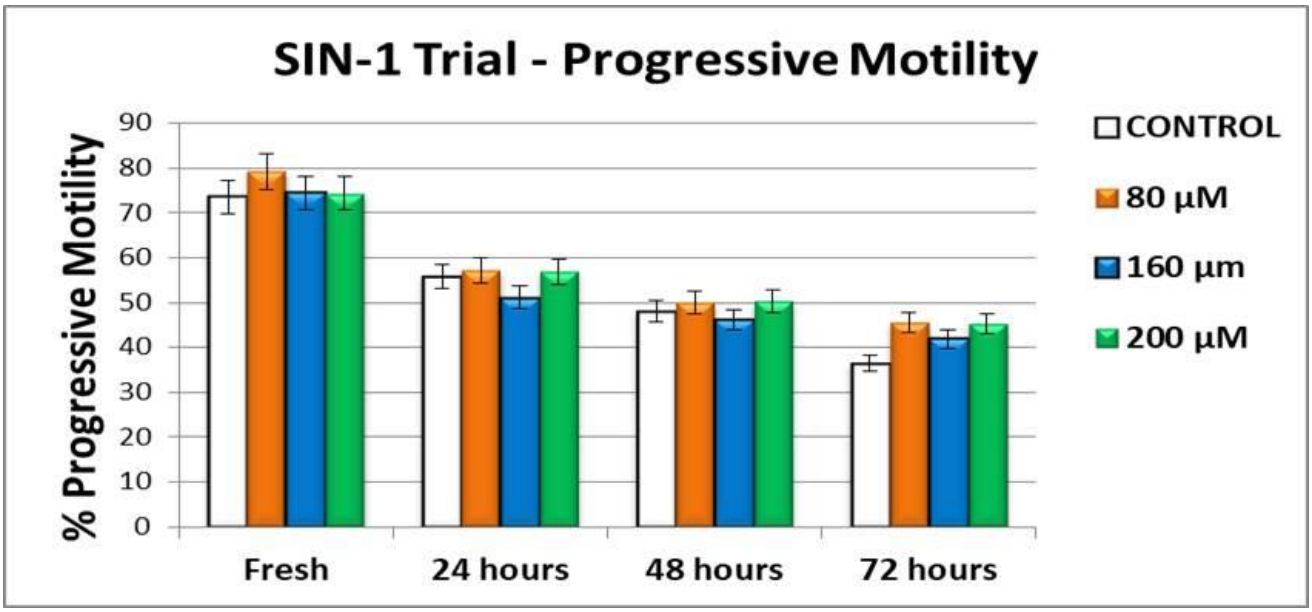


Fig. 4.5: Time and dose dependent responses of SIN-1 on progressive motility of spermatozoa

Table 4.6: Time and dose dependent responses of SNP on % Viability (Mean ±SEM) of spermatozoa

Treatment (SNP)	Control	10 nmol/ml	50 nmol/ml	100 nmol/ml
0 Hour	80.678^{xa}±0.89	80.84^{xa}±0.89	80.317^{xa}±1.00	82.92^{xa}±0.57
24 Hours	64.92^{xab}±0.96	69.367^{xb}±1.59	67.97^{xb}±1.36	72.22^{ybc}±0.86
48 Hours	57.76^{xab}±1.67	64.57^{xb}±1.59	64.85^{xb}±2.11	67.08^{ybc}±1.23
72 Hours	52.344^{xb}±2.43	61.57^{yb}±2.19	63.82^{yb}±2.31	60.18^{yc}±2.56

Values with different superscripts i.e., x and y within same rows and a, b and c within same columns differ significantly (P<0.05)

Table 4.7: Time and dose dependent responses of SIN-1 on % Viability (Mean ±SEM) of spermatozoa

Treatment (SIN-1)	Control	80 µM	160 µM	200 µM
0 Hour	80.67^{xa}±0.89	82.155^{xa}±0.70	80.95^{xa}±0.86	81.113^{xa}±0.81
24 Hours	70.31^{xab}±1.30	70.658^{xb}±1.03	69.87^{xb}±1.34	68.343^{xb}±1.60
48 Hours	65.333^{xab}±2.21	70.221^{yb}±1.05	67.511^{xb}±1.06	67.981^{xb}±1.28
72 Hours	55.85^{xb}±2.61	66.876^{yb}±1.20	67.36^{yb}±1.34	66.67^{yb}±1.54

Values with different superscripts i.e., x and y within same rows and a and b within same columns differ significantly (P<0.05)

Fig. 4.6: Time and dose dependent responses of SNP on viability of spermatozoa

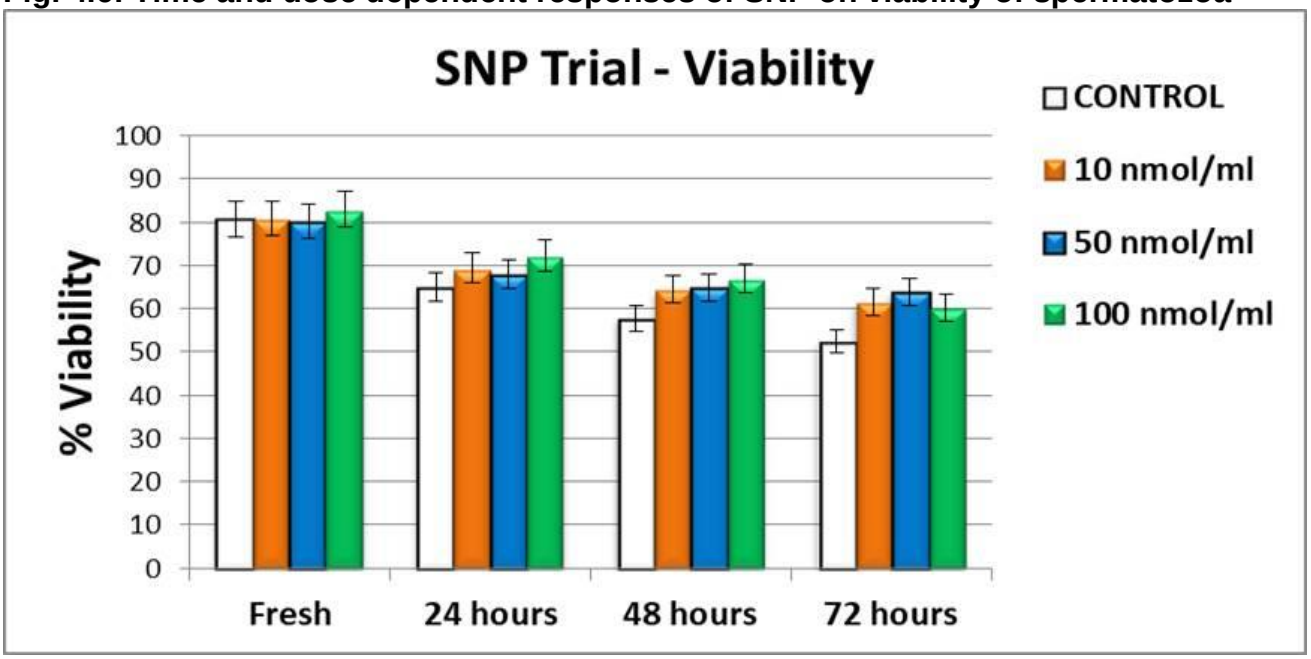
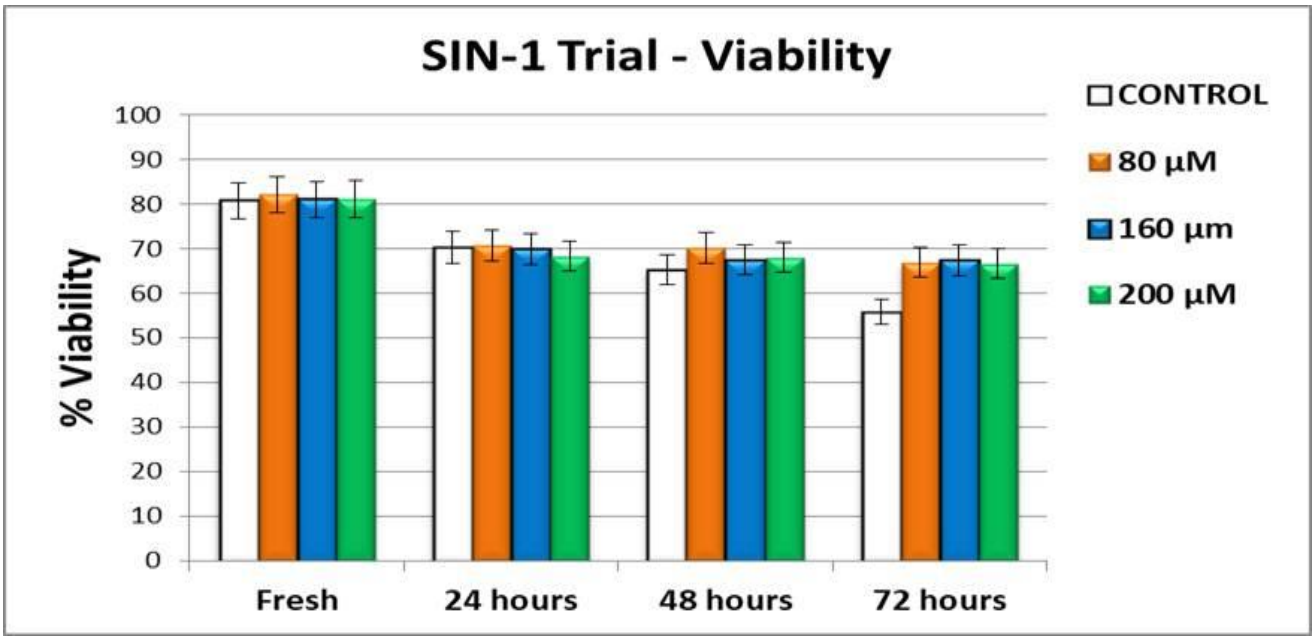


Fig. 4.7: Time and dose dependent responses of SIN-1 on viability of spermatozoa



4.2.2 Progressive Motility

Percent progressive motility (Mean±S.E.) in the three groups viz. Control (61.96±1.17), SNP (62.92±1.09) and SIN-1 (64.33±1.049) treatment had insignificant effect in the fresh semen. After cryopreservation there is significant ($p<0.01$) decrease in progressive motility as compared to fresh semen in control, SNP and SIN-1 treated groups at 24 h (40.58±1.54, 50.75±1.36 and 49.46±1.423), 15 days (38.33±1.293, 47.21±1.27 and 46.71±1.23) and 1 month (36.92±1.25, 44.52±1.21 and 44.43±1.12) respectively. The ANOVA disclosed that there was significant ($p<0.05$) increase in percent progressive motility between control (40.58±1.54, 38.33±1.293, 36.92±1.25) and SNP (50.75±1.36, 47.21±1.27 and 44.52±1.21) and SIN-1(49.46±1.423, 46.71±1.23, 44.43±1.12) treated extender at 24 h, 15 days and 1 month time intervals respectively.

Table 4.8: Effect of modified semen extender on progressive motility (Mean ±SEM)

TREATMENT GROUPS	FRESH SEMEN (0hr)	CRYOPRESERVED SEMEN		
		24 hours	15 days	1 month
CONTROL	61.96^{ax}±1.17	40.58^{ay}±1.54	38.33^{ay}±1.293	36.92^{ay}±1.25
SNP	62.92^{bx}±1.09	50.75^{by}±1.36	47.21^{by}±1.27	44.52^{by}±1.21
SIN-1	64.33^{bx}±1.049	49.46^{by}±1.423	46.71^{by}±1.23	44.43^{by}±1.12

Values with different superscripts i.e., x and y within same rows and a and b within same columns differ significantly ($P<0.05$)

4.2.3 Viability

Percent viability (Mean±S.E.) in the three groups viz. control (70.99±0.86), SNP (73.04±0.72) and SIN-1 (70±0.56) treated fresh semen were insignificant. Post cryopreservation there is significant ($p<0.05$) reduction in viability in comparison to fresh semen in control, SNP and SIN-1 treated groups at 24 h (48.20±1.16, 55.08±1.20, 54.04±1.22), 15 days (46.00±1.08, 51.80±0.81 and 50.48±0.92); and 1 month

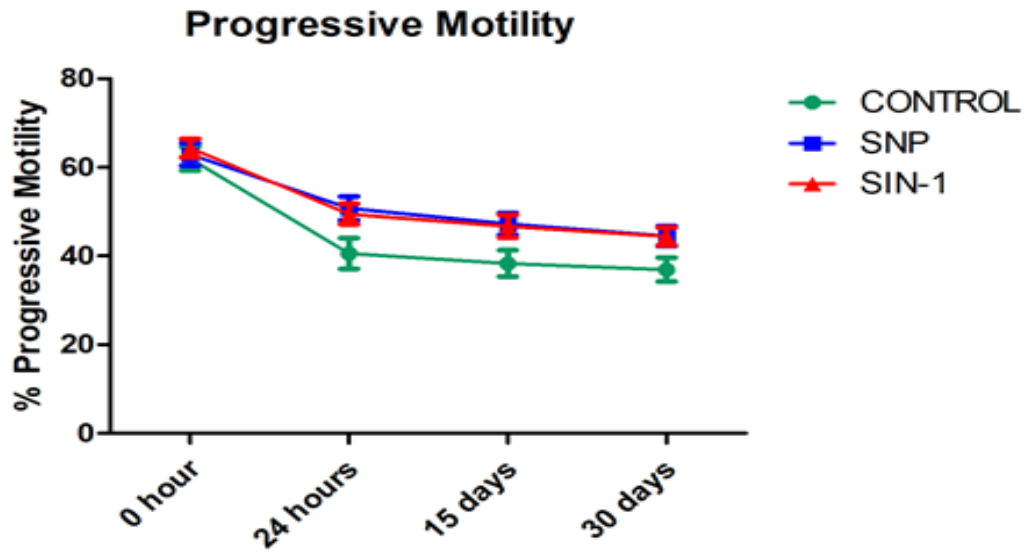
(42.64±1.09, 48.76±1.046 and 47.64±1.21) respectively. On the other side, it was observed that there was significant ($p<0.05$) increment in percent viability between control (48.20±1.16, 46.00±1.08 and 42.64±1.09) and SNP (55.08±1.20, 51.80±0.81 and 48.76±1.046) and SIN-1(54.04±1.22, 50.48±0.92 and 47.64±1.21) treated extender at 24 h, 15 days and 1 month time intervals respectively. There was significant ($p<0.05$) decrease in percent viability between 24 h, 15 days and 1 month intervals in control, SNP and SIN-1 modified extender.

Table 4.9: Effect of modified semen extender on viability of spermatozoa (Mean ±SEM)

TREATMENT GROUPS	FRESH SEMEN (0hr)	CRYOPRESERVED SEMEN		
		24 hours	**15 days**	**1 month**
CONTROL	70.99^{aw}±0.86	48.20^{ax}±1.16	46.00^{ay}±1.08	42.64^{az}±1.09
SNP	73.04^{aw}±0.72	55.08^{bx}±1.20	51.80^{by}±0.81	48.76^{bz}±1.046
SIN-1	70.56^{aw}±0.89	54.04^{bx}±1.22	50.48^{by}±0.92	47.64^{bz}±1.21

Values with different superscripts i.e., w, x, y and z within same rows and a, b, c and d within same columns differ significantly ($P<0.05$)

Fig. 4.8: Effect of control and treated semen extenders on progressive motility of spermatozoa



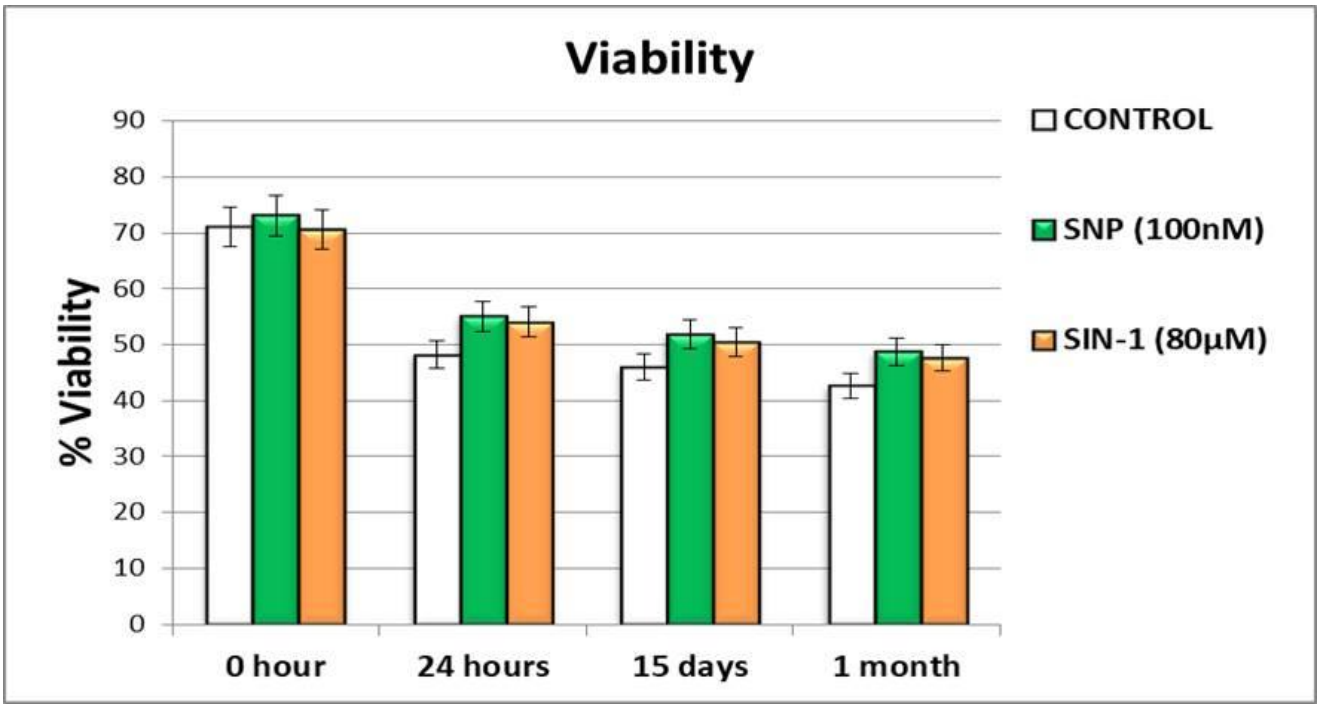


Fig. 4.9: Effect of control and treated semen extender on viability of spermatozoa

4.2.4 Membrane integrity

The average percentage of HOST positive spermatozoa in three groups *viz*. control (63.50±1.15), SNP (65.02±0.93) and SIN-1(64.72±1.07) treated semen from the fresh semen did not vary significantly. After cryopreservation, there was significant ($p<0.01$) decline in the percent of HOST +ve spermatozoa as compared to fresh semen in control, SNP and SIN-1 treated groups at 24 h (41.32±1.17, 48.08±1.42 and 44.92±1.37), 15 days (40.08±1.12, 47.28±0.58 and 42.84±1.17) and 1 month (38.16±1.22, 45.20±0.56 and 40.36±1.24) respectively. Meanwhile it was observed that, there was significant ($p<0.05$) increase in percent HOST +ve between control (41.32±1.17, 40.08±1.12 and 38.16±1.22) and SNP(48.08±1.42, 47.28±0.58 and 45.20±0.56) treated extender at 24 hours, 15 days and 1 month time interval respectively. There was no significant change after cryopreservation between control and SIN-1 treated extender at 24 h, 15 days and 1-month intervals respectively.

Table 4.10: Effect of modified semen extender on HOST +ve (Mean ±SEM) % of spermatozoa

TREATMENT GROUPS	FRESH SEMEN (0hr)	CRYOPRESERVED SEMEN		
		24 hours	**15 days**	**1 month**
CONTROL	63.50^{xa}±1.15	41.32^{ya}±1.17	40.08^{ya}±1.12	38.16^{ya}±1.22
SNP	65.02^{xa}±0.93	48.08^{yb}±1.42	47.28^{yb}±0.58	45.20^{yb}±0.56
SIN-1	64.72^{xa}±1.07	44.92^{ya}±1.37	42.84^{ya}±1.17	40.36^{ya}±1.24

Values with different superscripts i.e., x and y within same rows and a and b within same columns differ significantly ($P<0.05$)

4.2.5 Acrosome Integrity

The ANOVA revealed that the acrosome integrity (% Mean±S.E.) in three groups *viz*. control (78.90±1.14), SNP (78.94±0.79) and SIN-1(78.13±0.85) treated semen from the fresh semen did not vary significantly. After cryopreservation there was significant ($p<0.01$) reduction in acrosome integrity as compared to fresh semen in control, SNP and SIN-1 treated groups at 24 h (61.04±2.03, 67.94±1.23 and 67.53±0.83), 15 days (60.19±1.50, 65.54±1.59 and 65.08±0.92) and 1 month (58.56±2.00, 62.30±1.34 and 61.72±1.08) respectively.. There was significant ($p<0.05$) increase in acrosome integrity between control (61.04±2.03), SNP (67.94±1.23) and SIN-1 (67.53±0.83) treated extenders at 24 hours interval.

Table 4.11: Effect of modified semen extender on acrosome integrity (Mean ±SEM) of spermatozoa

TREATMENT GROUPS	FRESH SEMEN (0hr)	CRYOPRESERVED SEMEN		
		24 hours	15 days	1 month
CONTROL	76.90^{xa}±1.14	61.04^{ya}±2.03	60.19^{ya}±1.50	58.56^{ya}±2.00
SNP	78.94^{xa}±0.79	67.94^{yb}±1.23	65.54^{ya}±1.59	62.30^{ya}±1.34
SIN-1	78.13^{xa}±0.85	67.53^{yb}±0.83	65.08^{ya}±0.92	61.72^{ya}±1.08

Values with different superscripts i.e., x and y within same rows and a and b within same columns differ significantly ($P<0.05$)

Fig. 4.10: Effect of control and treated semen extender on membrane integrity of spermatozoa

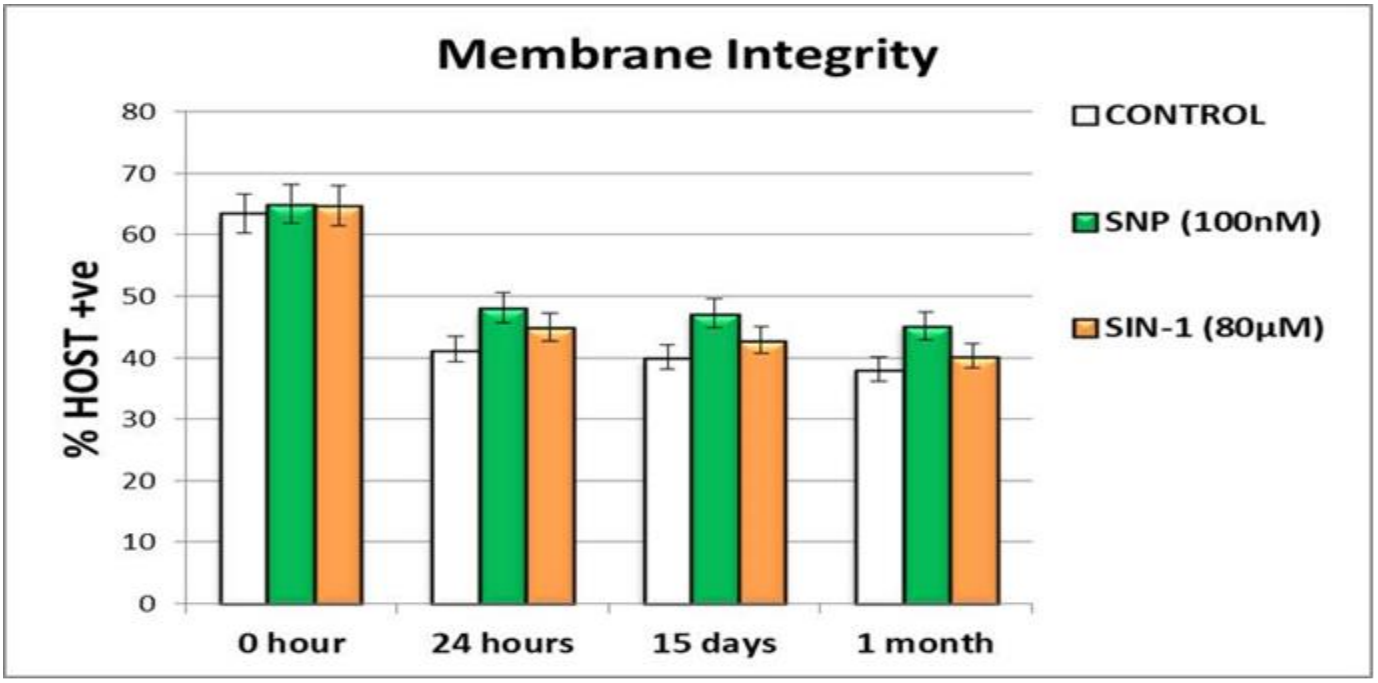
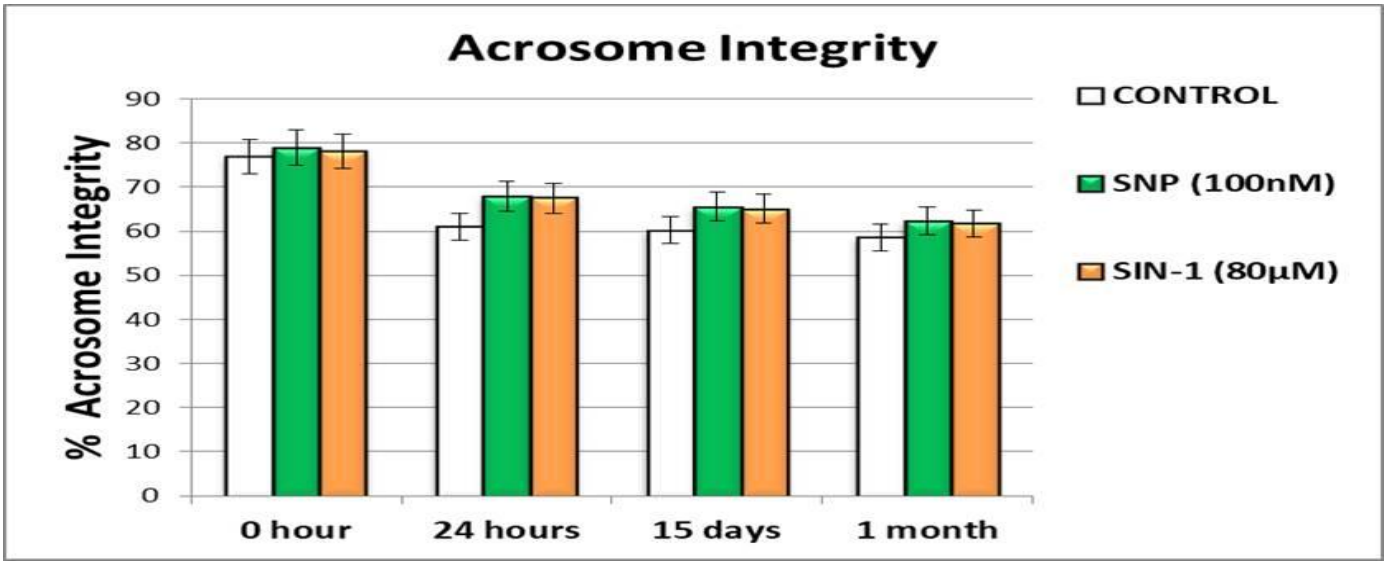


Fig 4.11: Effect of control and treated semen extender on acrosome integrity of spermatozoa



4.2.6 Abnormality

In fresh semen percent, total abnormalities (Mean±S.E.) vary non-significantly between the control (9.71±1.00), SNP (9.40±0.94) and SIN-1 (9.91±0.82) treated extenders. After cryopreservation there is significant ($p<0.01$) increase in total abnormalities in comparison to fresh semen in control, SNP and SIN-1 treated groups at 24 h (16.76±1.13, 15.25±0.98 and 16.16±1.35), 15 days (18.45, 16.78±1.78 and 18.52±2.08) and 1 month (20.61±1.71, 18.02±1.66 and 20.40±1.98) respectively. There was no significant change after cryopreservation between 24 h, 15 days and 1 month intervals respectively.

Table 4.12: Effect of modified semen extender on total abnormality (Mean ±SEM) of spermatozoa

TREATMENT GROUPS	FRESH SEMEN (0hr)	CRYOPRESERVED SEMEN		
		24 hours	15 days	1 month
CONTROL	9.71^{xa}±1.00	16.76^{ya}±1.13	18.45^{ya}±1.65	20.61^{ya}±1.71
SNP	9.40^{xa}±0.94	15.25^{ya}±0.98	16.78^{ya}±1.79	18.02^{ya}±1.66
SIN-1	9.91^{xa}±0.82	16.16^{ya}±1.35	18.52^{ya}±2.08	20.40^{ya}±1.98

Values with different superscripts i.e., x and y within same rows and a and b within same columns differ significantly ($P<0.05$)

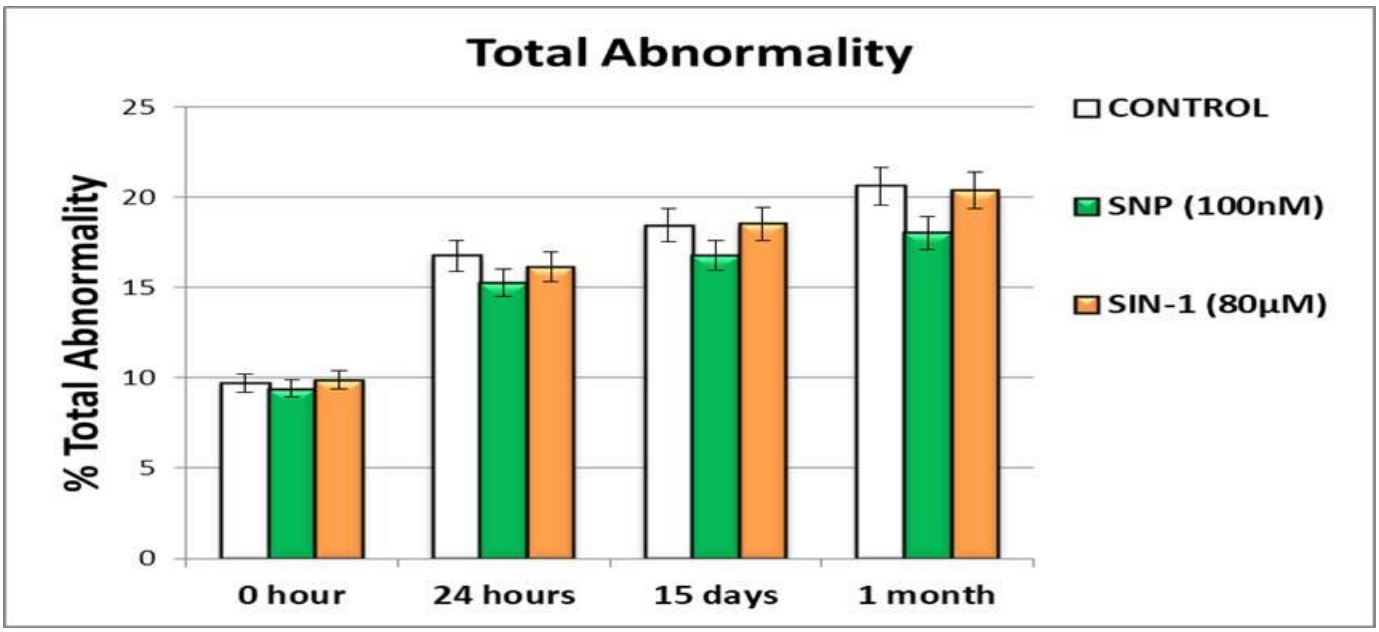
4.2.7 Lipid peroxidation

In SNP treated extender (0.53±0.02) there was significantly ($p<0.05$) less lipid peroxidation in comparison to SIN-1 treated (3.30±0.47) and control (3.51±0.34) groups.

Table 4.13: Effect of modified semen extender on Lipid Peroxidation (Mean ±SEM) status of spermatozoa

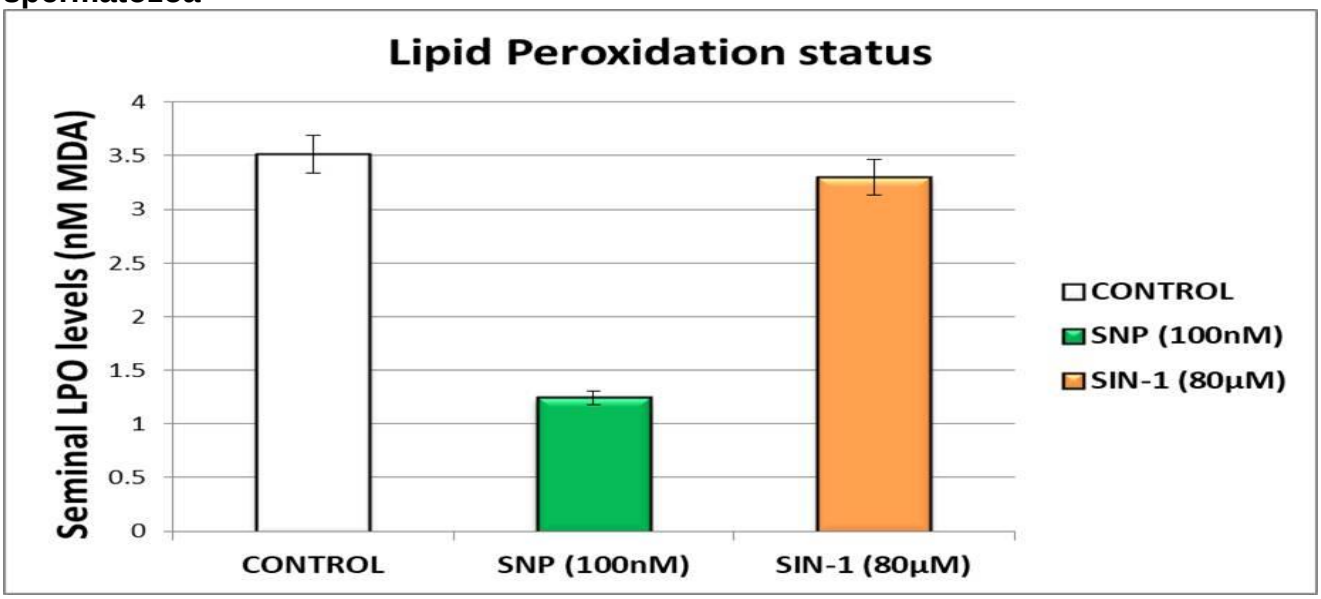
ATTRIBUTE	CONTROL	SNP	SIN-1
POST-THAW	3.51^{a}±0.34	1.24^{b}±0.09	3.30^{a}±0.47

Fig. 4.12: Effect of control and treated semen extender on total abnormality of spermatozoa



Values with different superscripts i.e., a and b within same columns differ significantly (P<0.05)

Fig 4.13: Effect of modified semen extender on Lipid Peroxidation status of spermatozoa



4.2.8 Mitochondrial membrane potential (MMP)

Mitochondrial membrane potential (MMP) in spermatozoa was evaluated by using Mitochondrial Apoptosis Staining kit and results obtained have been depicted in Table 4.14 and Fig. 4.14. Post-thaw evaluation of spermatozoa revealed a significant increase in % of sperm cells having positive mitochondrial membrane potential (MMP) in SNP (57.24±0.27) and SIN-1 (54.8±0.95) as compared to control (51.4±0.27). The process of cryopreservation and thawing induce irreversible modifications in spermatozoa leading to loss of spermatozoa structural and functional dynamics.

Table 4.14: Effect of modified semen extender on mitochondrial membrane potential (Mean ±SEM) of spermatozoa

MMP STATUS	CONTROL	SNP	SIN-1
MMP +ve	51.4^{a}±0.28	57.24^{b}±0.27	54.8^{c}±0.95
MMP –ve	48.6^{a}±0.28	42.76^{b}±0.27	45.2^{c}±0.95

Values with different superscripts i.e., a, b and c within same columns differ significantly (P<0.05)

4.2.9 Acrosome Reaction

The sperm acrosomal reaction status was observed using the acrosome-specific fluorochrome fluorescein isothiocyanate-labeled peanut (*Arachis hypogaea*) agglutinin (FITC-PNA) in concurrence with DNA-specific fluorochrome propidium iodide (PI) as a viability test. Sperm cells were classified into 4 categories on the basis of the FITC-PNA staining as follows: i) Live acrosome intact (LAI) – Absence of green fluorescence on acrosome cap and no red fluorescence in post-acrosomal region, ii) Live acrosome reacted (LAR) - uniform green fluorescence of acrosome cap without red fluorescence in post-acrosomal region III) Dead acrosome intact (DAI) - no green fluorescence of acrosome cap but red fluorescence in post-acrosomal region and iv) Dead acrosome reacted (DAR) - uniform green fluorescence of acrosome cap and red

fluorescence in post-acrosomal region (Fig 4.15).

The analysis of variance has revealed that there was significant ($p<0.05$) increase in mean percentage of Live acrosome intact (LAI) spermatozoa in SNP (45.77±0.82) and SIN-1 (41.8±0.37) treated extender as compared to control (38.2±0.29) after freezing. There is a significant difference ($p<0.05$) of the mean percentage of Live Acrosome reacted (LAR) spermatozoa, between SNP (7.96±0.10) and SIN-1 (8.9±0.14) treated extenders as compared to control (9.83±0.23) after thawing. On the other hand, the proportion of DAI spermatozoa was significantly lower ($p<0.05$) in SNP (28.32±0.25) and SIN-1 (29.04±0.23) as compared to the control (33.92±0.24). There was a significant increase ($p<0.05$) in the % of DAR spermatozoa in SIN-1 (20.24±0.20) as compared to the control (18.04±0.27). While, there was no significant difference between SNP (17.92±0.396) treated extender and control (18.04±0.27) in the % of DAR spermatozoa.

Table 4.15: Effect of modified semen extender on Acrosome Reaction status(Mean ±SEM) of spermatozoa

TREATMENT GROUPS	**ACROSOMAL REACTION STATUS**			
	Live Acrosome Intact	**Live Acrosome Reacted**	**Dead Acrosome Intact**	**Dead Acrosome Reacted**
CONTROL	38.2^{a}±0.29	9.83^{a}±0.23	33.92^{a}±0.24	18.04^{a}±0.27
SNP	45.77^{b}±0.82	7.96^{b}±0.10	28.32^{b}±0.25	17.92^{a}±0.396
SIN-1	41.8^{c}±0.37	8.9^{c}±0.14	29.04^{b}±0.23	20.24^{b}±0.20

Values with different superscripts i.e., a, b and c within same columns differ significantly ($P<0.05$)

Fig. 4.14: Effect of modified semen extender on mitochondrial membrane potential (Mean ±SEM) of spermatozoa

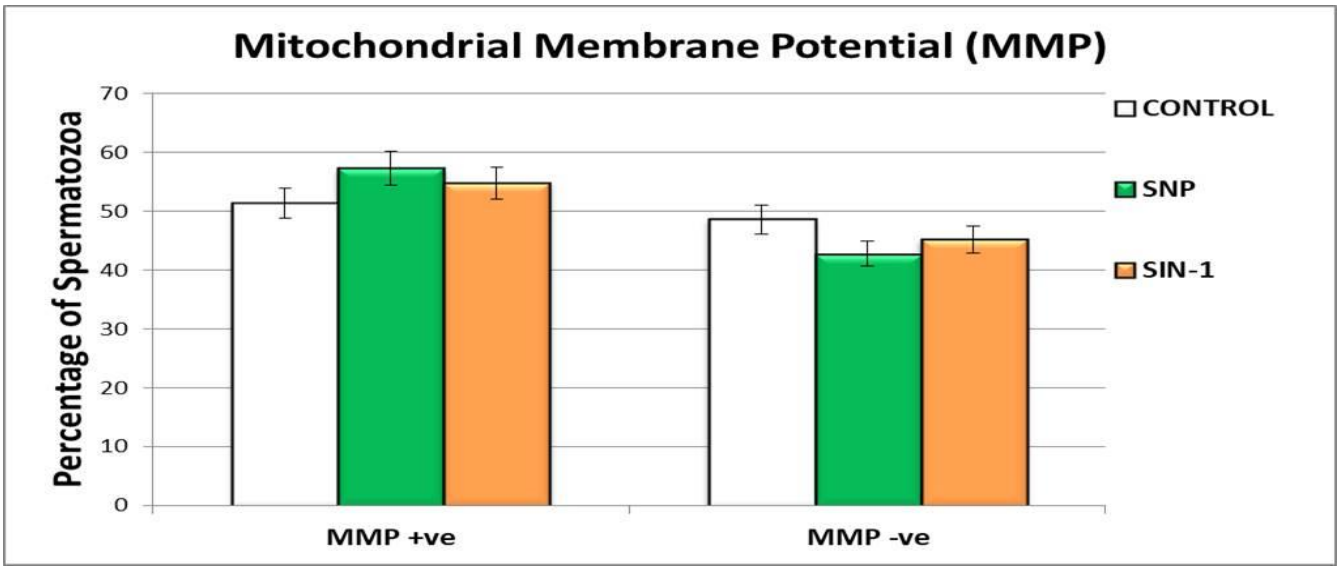
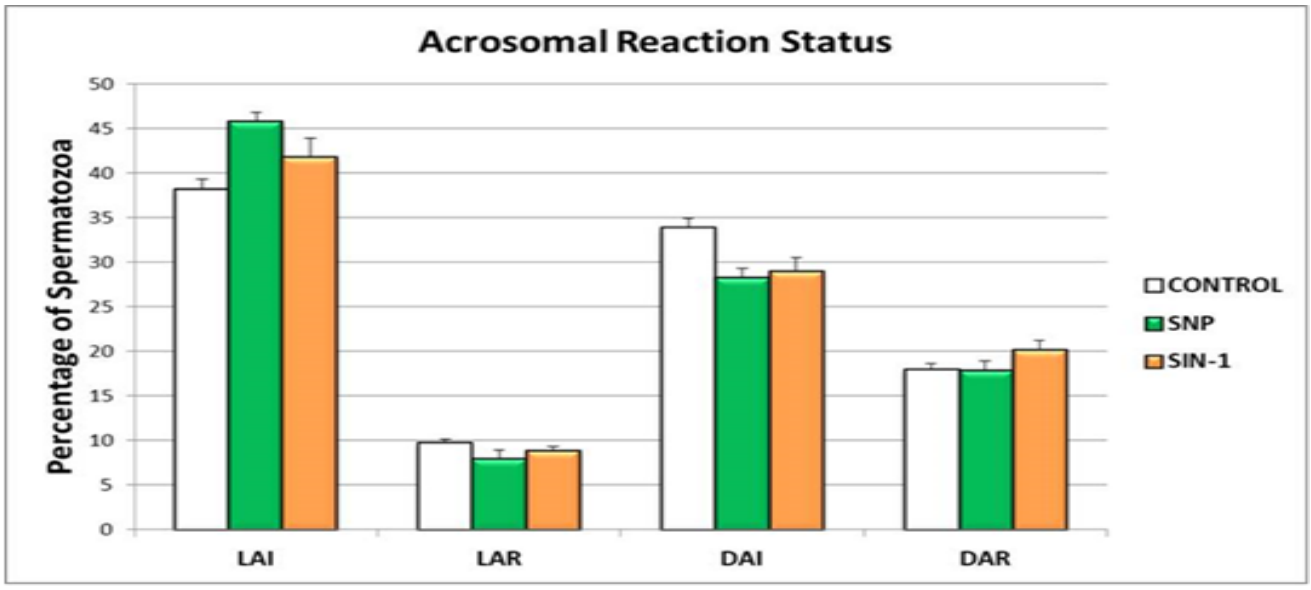


Fig. 4.15: Effect of modified semen extender on Acrosome Reaction status (Mean ±SEM) of spermatozoa



(LAI/LAR- Live Acrosome Intact/Reacted; DAI/DAR- Dead Acrosome Intact/ Reacted)

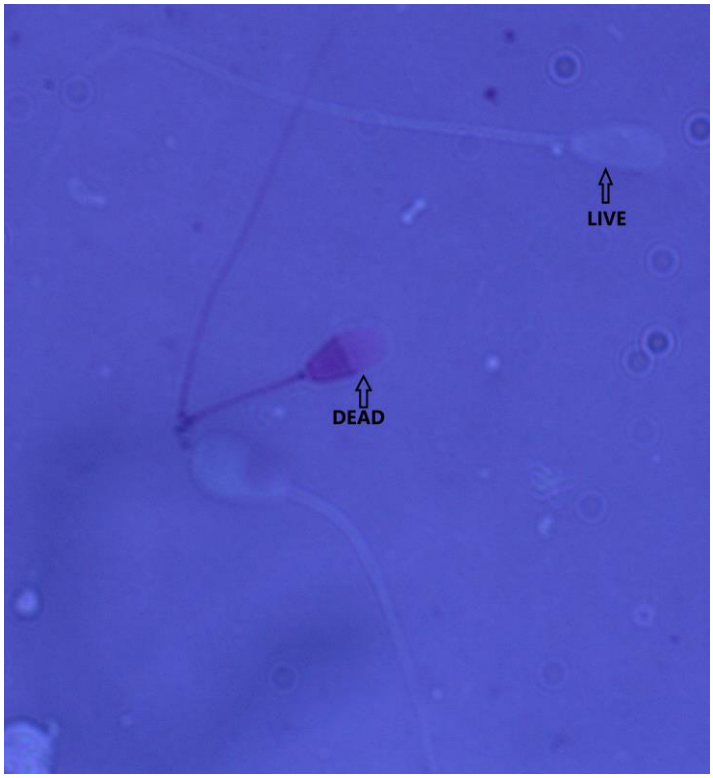


Plate-4.1: Eosin-Nigrosin Staining

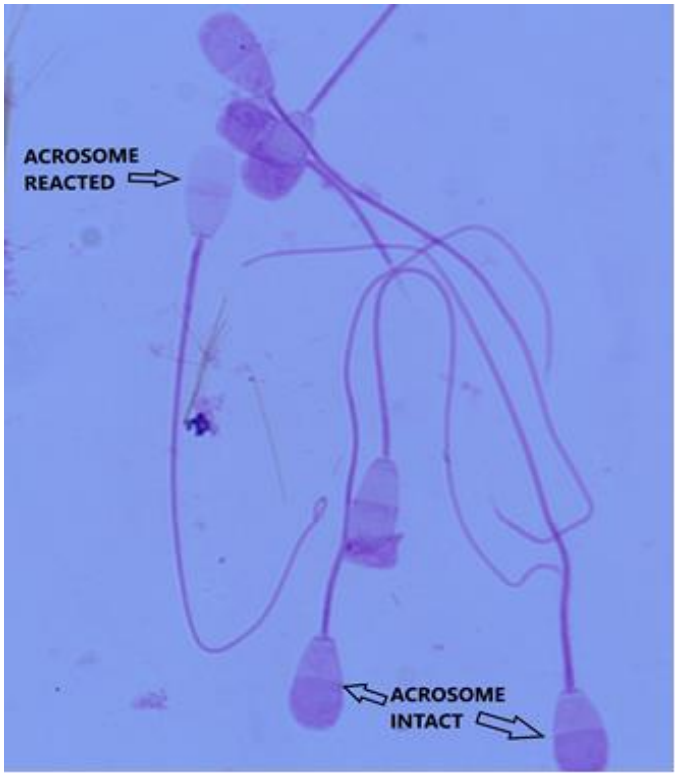


Plate-4.2: Giemsa staining- Acrosome Integrity

Plate-4.3: HOST Responses

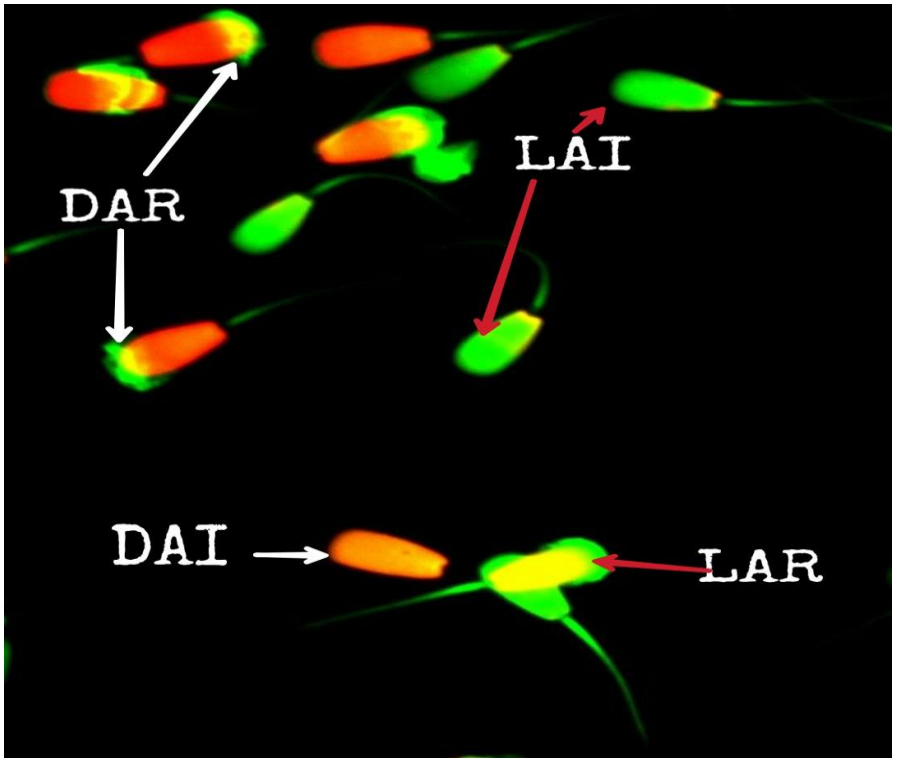


Plate-4.4

ACROSOME REACTION

LAI/ LAR - Live Acrosome Intact/Reacted;

DAI/ DAR - Dead Acrosome Intact/ Reacted

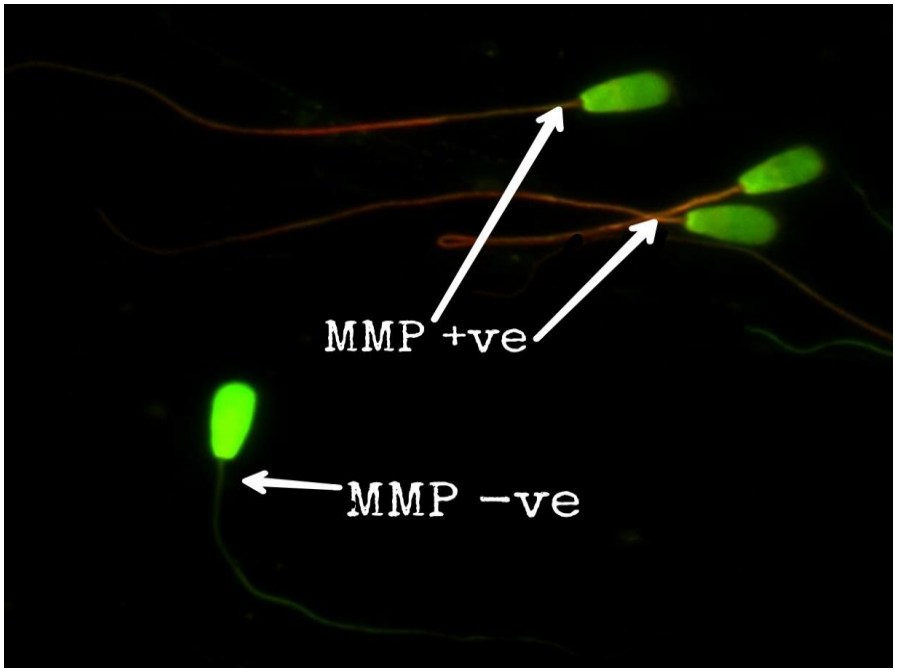


Plate-4.5

MITOCHONDRIAL MEMBRANE POTENTIAL (MMP)

Table 4.16: Pearson's Correlation between seminal attributes in unaltered control extender

Attributes	Progressive Motility	Viability	HOST +ve	Acrosome Integrity	Abnormality	TBARS	MMP
Progressive Motility	1	0.667** 0.000	0.812** 0.000	0.475** 0.000	-0.275** 0.000	-0.036 0.613	-0.342** 0.000
Viability	0.667** 0.000	1	0.720** 0.000	0.572** 0.000	-0.320** 0.000	-0.016 0.819	0.071 0.001
HOST +ve	0.812** 0.000	0.720** 0.000	1	0.485** 0.000	-0.226** 0.001	-0.023 0.748	-0.224** 0.001
Acrosome Integrity	0.475** 0.000	0.572** 0.000	0.485** 0.000	1	-0.467** 0.000	0.053 0.459	0.066 0.356
Abnormality	0.275** 0.000	-0.320** 0.000	-0.226** 0.001	-0.467** 0.000	1	-0.026	-0.117
TBARS	-0.036 0.613	-0.016 0.819	-0.023 0.748	0.053 0.459	-0.026	1	-0.030
MMP	-0.342** 0.000	0.071 0.001	-0.224** 0.001	0.066 0.356	-0.117 0.100	-0.030 0.678	1

**. Correlation is significant at the 0.01 level (2-tailed)

Table 4.17: Pearson Correlation between seminal attributes in SNP treated extender

Attributes	Progressive Motility	Viability	HOST +ve	Acrosome Integrity	Abnormality	TBARS	MMP
Progressive Motility	1	0.618** 0.000	0.590** 0.000	0.485** 0.000	-0.253** 0.000	-0.018 0.803	0.107 0.133
Viability	0.618** 0.000	1	0.807** 0.000	0.601** 0.000	-0.167* 0.018	0.076 0.285	0.180* 0.011
HOST +ve	0.590** 0.000	0.807** 0.000	1	0.490** 0.000	-0.126 0.074	0.090 0.205	0.139* 0.050
Acrosome Integrity	0.485** 0.000	0.601** 0.000	0.490** 0.000	1	-0.158* 0.025	0.149* 0.035	0.067 0.349
Abnormality	-0.253** 0.000	-0.167* 0.018	-0.126 0.074	-0.158* 0.025	1	0.037 0.604	0.258** 0.000
TBARS	-0.018 0.803	0.076 0.285	0.090 0.205	0.149* 0.035	0.037 0.604	1	-0.054 0.451
MMP	0.107 0.133	0.180* 0.011	0.139* 0.050	-0.067 0.349	0.258** 0.000	-0.054 0.451	1

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 4.18: Pearson Correlation between seminal attributes in SIN-1 treated extender

Attributes	Progressive Motility	Viability	HOST +ve	Acrosome Integrity	Abnormality	TBARS	MMP
Progressive Motility	1	0.636** 0.000	0.654** 0.000	0.538** 0.000	-0.372** 0.000	-0.023 0.750	-0.114 0.109
Viability	0.636** 0.000	1	0.727** 0.000	0.630** 0.000	-0.234** 0.001	-0.073 0.304	-0.049 0.493
HOST +ve	0.654** 0.000	0.727** 0.000	1	0.587** 0.000	-0.195** 0.006	-0.071 0.319	-0.103 0.050
Acrosome Integrity	0.538** 0.000	0.630** 0.000	0.587** 0.000	1	-0.233** 0.001	0.033 0.643	0.018 0.799
Abnormality	-0.372** 0.000	-0.234** 0.001	-0.195** 0.074	-0.233** 0.001	1	0.065 0.363	-0.030 0.677
TBARS	-0.023 0.750	-0.073 0.304	-0.071 0.319	0.033 0.643	0.065 0.363	1	0.063 0.377
MMP	-0.114 0.109	-0.049 0.493	-0.103 0.147	0.018 0.799	-0.030 0.677	0.063 0.377	1

**. Correlation is significant at the 0.01 level (2-tailed)

DISCUSSION

There was a highly significant decline ($p<0.01$) in Progressive motility, viability, membrane integrity (HOST +ve), acrosome integrity and total abnormalities at 24 hours of cryopreservation as compared to the fresh semen. These results are in compliance to the observations of many researchers (Watson, 2000, Batellier *et al.*, 2001; Medeiros *et al.*, 2002). Watson, 2000, has accounted for 50% of the reduction in the quality of spermatozoa due to irreversible damage at post-thaw stage, which are in conformity with the current results. The cryopreservation accelerated biochemical and biophysical alterations in the quality of semen, that results in ultra-structural damages to the membranes and destabilizes them; predisposing sperms to complete morphological defects, such as abnormal and missing acrosomes. The freezing has induced biophysical changes due to extracellular ice crystal formation, super cooling, concentration of solutes, like sugars, proteins and salts (Lemma, 2011) and finally dehydration (Woelders, 1997; Watson, 2000; Andrabi, 2007). The excessive super cooling results in a rapid ice formation and physical defects resulting into significant deteriorations in seminal parameters as compared to fresh semen.

The higher levels of ROS can damage normal spermatozoa by inducing lipid peroxidation and DNA damage (Ollero *et al*., 2001 and Saleh *et al*., 2002) during freezing which deteriorates its quality by compromising chromatin, membrane integrity and blocking oxidative metabolism (Lone *et al*., 2016). The sperm competency is challenged as compared to their detoxification (Aitken, 2006) due to the increased ROS production. The attack of ROS decreases intracellular ATP concentration which leads to reduced sperm viability and motility; increased sperm morphological abnormalities and axonemal damage (Lector, 1996; Bansal and Bilaspuri, 2007; Bansal and Bilaspuri, 2011). These changes are due to the polyunsaturated fatty acids (PUFAs) rich sperm plasma membrane (Sarlos *et al.,* 2002), and its binding to the oxygen (Gadea *et al.*, 2013). The spermatozoa become more sensitive to the free radicals (Jones & Mann, 1977) due to reduced seminal antioxidant levels and the inefficiency of sperm to synthesize important constituents of the membrane.

Production of higher levels of reactive nitrogen species (RNS) leads to a nitrosative stress, causing modifications to several biomolecules (Nash *et al.,* 2012). The attack of RNS makes PUFA very susceptible due to the abstraction of hydrogen by NO that leads to the production of free radicles further triggering lipid peroxidation (Semenova *et al.*, 2005; Makker *et al*., 2009).

The lipid peroxidation status in seminal plasma of fresh semen (2.63±0.22) was observed to be lower in comparison to extended seminal plasma (3.51±0.34) of the cryopreserved semen. The LPO levels increased by 30% in frozen-thawed sperm compared to fresh semen, which is in agreement with earlier reports of Slaweta *et al.,* (1998), Chatterjee, and Gagnon (2001). It could be attributed to the fact that the frozen-thawed semen of bulls are more easily peroxidized as compared to fresh semen (Trinchero *et al.,* 1990) due to the decrease in the antioxidant enzyme level occuring during freeze-thaw process (Ball *et al.,* 2000; Bilodeau *et al.,* 2000). Previous studies have indicated that the aromatic amino oxidase enzyme activity in morbid sperm and the higher number of dead sperm may be the attributing factors for higher LPO levels (Upreti *et al.,* 1998).

There is a significant ($P<0.05$) reduction in the sperm viability upto 1 month of cryopreservation. The toxin production should be avoided to maintain the viability of spermatozoa for longer duration, the metabolic rate must be slowed down and the cryo-injury should be kept at the minimum level. However, finest combinations of cryoprotective agents are required with adequate freeze-thaw protocols to minimize cryo-injuries.

While standardizing and optimizing SNP and SIN-1 concentration for semen extender during the second part of the study, the progressive motility and viability was affected by SNP and SIN-1 in both dose and time dependent manner. Percent motility and viability improved significantly ($p<0.05$) with the addition of 100 nmol/ml SNP and

80 µM SIN-1 as compared to 10, 50 nmol/ml SNP and 160, 200 µM SIN-1 concentration. There was a significant ($p<0.05$) reduction in motility and viability with increase in the refrigeration time (viz. 24 hours, 48 hours and 72 hours). At 100 nmol/ml SNP, concentration sperm motility increased as compared to control, but at lower concentrations, there was no change in motility, as reported earlier by Panth, (2017), Naskar, (2018) and Upadhyay, (2019). Balercia *et al.,* (2004) found that Nitric oxide (NO) at lower concentrations (<1 µM) improved the sperm motility while higher concentrations (>1 µM) reduced the sperm motility in a dose dependent manner. Khodaei *et al.,* (2016), reported similar result. Rahman *et al.,* (2014) found that there was a significant decrease in sperm motility particularly at the highest concentration of 100 µm. However, higher doses of SNP decreased the sperm motility and caused hyperactivation of mice spermatozoa. Meanwhile, similar investigations were found in human semen that SNP decreased sperm motility both in a dose and time dependent manner.

The motility and viability improved significantly ($p<0.05$) with the addition of 80 µm SIN-1 as compared to 160 and 200 µM concentration, which was in harmony with the results of Jalmeria, (2017) and Kshetrimayum, (2019). However, Vignini *et al.,* 2006 and Khosravi *et al.,* 2014 have reported a negative correlation between peroxynitrite concentration and sperm morphology and motility. Similar, results were observed in the study undertaken by Uribe *et al.,* 2014 indicating significantly lower sperm motility on incubation with SIN-1 for 4 hour. The results of the present studies were similar to those reported by Rodriguez and Beconi (2009).

They conducted in-vitro studies on bovine semen using SIN-1 and reported a significant decrease in the sperm motility in the cryopreserved bull semen after exposure to 160 µM or high SIN-1 concentration. However, a significant ($p<0.05$) increase in sperm motility on addition of 80 µM SIN-1 has been observed in the present study, which differs with Rodriguez and Beconi (2009). The damage at higher SIN-1

concentration due to the presence of peroxynitrite could be attributed to the damage of contractile proteins of spermatozoa responsible for its motility or lipid peroxidation or due to varied ATP levels (Souza and Radi, 1998; Buchczyk *et al*., 2003).

The present research has elucidated that fresh semen had insignificant ($p<0.05$) changes in % progressive motility, HOST +ve, viability, acrosome integrity and total abnormalities between control and treated semen samples. Thereafter, a significant ($p<0.05$) increase in progressive motility, viability, membrane integrity for SNP as well as SIN-1 treated extender was observed after 24 hours, 15 days and 1 month interval while there was significant ($p<0.05$) increase in acrosome integrity after 24 hours for SNP and SIN-1 treated extender in comparison to control. These observations are in agreement with the investigations made by Naskar, (2018) and Upadhyay, (2019) in buffaloes, who reported that the percent viability increased significantly ($p<0.05$) at 1 µM concentration which is in consonance with the present findings. Rosselli *et al.,* (1995) found an improvement in post-thaw sperm viability in human subjects, at lower concentration of NO. Khodaei *et al.,* (2016), reported a significant ($p<0.05$) increase in viability of spermatozoa after 1, 2 and 3 hours post-thawing at the SNP concentrations of 10, 50 and 100 nmol/ml, while there was no significant variation before freezing and immediately after thawing. Balercia *et al.,* (2004), has reported similar results that SNP maintain sperm viability in cryopreserved semen. Few researchers have cited that SNP reduces sperm viability at 0.1 µM concentration (Tomlinson *et al.,* 1992) and at 0.25-2.5 µm concentration (Bolanos *et al.,* 2008). There was non-significant difference in sperm viability in SNP treated (1 µM -100 µM) sperms in comparison to control (Tomlinson *et al.,* 1992). Doshi et al., (2012) also found that there was no effect of SNP on sperm viability at lower concentration.

The findings of the present study disagree with the results of Rodriguez and Beconi (2009), as they reported that SIN-1 did not significantly reduce viability of sperm upto 1mmol/l on in-vitro incubation at 37^{o}C for 45 minutes. This variation could be

attributed to the addition of SIN-1 in-vitro at higher concentrations that causes higher susceptibility of cryopreserved sperm cells to both structural and physiological alterations caused by the free radicals. This showed that longer exposure of spermatozoa to increased peroxynitrite concentrations was highly dangerous for their survival and normal sperm morphology. Uribe *et al.,* 2014 also reported that there is no decrease in human sperm viability on addition of SIN-1.

Roy and Atreja (2008) and Rahman *et al.,* (2014) observed that increased concentrations of SNP modify membrane integrity of the sperm, hence leading to premature acrosome reaction and modify overall sperm function. Makker *et al.,* (2009) reported that the increased levels of NO (>1 µM) results in the loss of sperm membrane integrity due to the induction of lipid peroxidation of sperm plasma membrane. Present study has indicated that the lipid peroxidation levels were significantly ($p<0.05$) lower in SNP and SIN-1 treated extenders in comparison to the control. Khodaei *et al.,* (2016) reported that the varying concentrations of SNP (10, 50 and 100 nmol/ml) decreased the lipid peroxidation of sperm plasma membrane and improved integrity of acrosomal membranes in time and dose dependent manner. It depicts that SNP increased sperm membrane integrity, particularly in 50 and 100 nmol/ml concentrations. Hellstrom *et al.,* (1994) has also reported similar results. On the other hand, Roselli *et al.,* (1995), observed a decrease in sperm membrane integrity on addition of NO inhibitors in human semen. The results of the current study on sperm morphology differs with the findings of Vignini *et al.,* (2006), Khosravi *et al.,* (2014) , as they have reported negative correlation between the human sperm morphology and peroxynitrite concentration. This may be due to the addition of lower concentration of SIN-1 as compared to their experiment. Jalmeria, (2017) and Kshetrimayum, (2019), observed similar findings regarding membrane integrity and sperm morphology in bull spermatozoa.

It has been observed that, there is no significant difference in the sperm abnormalities between the groups treated with SNP and SIN-1 as compared to control.

A positive correlation was reported by Wu *et al.,* (2004) between NO of seminal plasma and defects in sperm morphology ($r=0.4$; $p<0.05$). Ramya *et al.,* (2011) reported that increased abnormalities of the spermatozoa are related to higher levels of NO. Ambrosini *et al*., (2006) and Vidya *et al*., (2011) reported similar findings that higher NO concentrations and sperm morphology have a negative correlation. Mitochondrial membrane potential (MMP) improved significantly ($p<0.05$) on addition of SNP and SIN-1 treated extenders in comparison to the control. Uribe *et al.,* (2014) reported that peroxynitrite (SIN-1) mediated nitrosative stress decreases MMP along with reduction in motility as compared to control. However, there is no report on a comparative study on MMP on addition of SNP in different concentrations of semen doses.

CHAPTER–5

Summary and Conclusions

SUMMARY AND CONCLUSIONS

Current study was designed to evaluate the effects of cryopreservation and nitric oxide compounds on the quality of semen in Karan-Fries bulls, for which 6 bulls of similar age were selected from the herd that was maintained by Artificial Breeding Research Centre (ABRC), NDRI Karnal. The semen samples were collected from these bulls at weekly intervals. The semen ejaculates were examined for conventional functional attributes like concentration, progressive motility, mass motility, sperm viability, acrosome integrity, sperm abnormality and membrane integrity. The levels of TBARS were also determined in the seminal plasma. The foremost standardization of SNP and SIN-1 concentration for modifying semen extender was followed to evaluate the effect of reactive nitrogen species (RNS) on the sperm functional parameters. The results of these compounds were examined on seminal attributes before and after 24 hours, 15 days and 1 month of freezing intervals. The major findings are summarized below in objective-wise manner.

Objective-1. To assess the impact of ROS generated in fresh and cryopreserved semen on structural changes and sperm quality

1. Progressive motility, viability, membrane integrity (HOST +ve), acrosome integrity and total abnormalities declined significantly ($P<0.01$) after 24 h of cryopreservation as compared to the freshly ejaculated semen.
2. After 24 hours of freezing, there was not much variation in the seminal attributes at 15 days or 1-month interval. However, sperm viability and acrosome integrity reduced significantly up to 1 month of cryopreservation.
3. The lipid peroxidation status was 2.63±0.22 in nM MDA in fresh seminal plasma which is lower in comparison to that of extended seminal plasma (3.51±0.34) after cryopreservation.

Objective-2 To assess the impact of SIN-1 and/or SNP supplemented semen extender on post-thaw structural changes and sperm quality

1. During standardization of SNP and SIN-1 concentration for modifying semen extender it was found that progressive motility and viability was affected by SNP and SIN-1 in both dose and time dependent manner.
2. The percent progressive motility and Viability improved significantly with the addition of 100 nmol/ml and 80 µM/ml as compared to 10, 50 nmol/ml and 160, 200 µM concentrations respectively at different time intervals.
3. In fresh semen, there was no significant difference in % progressive motility, viability, HOST +ve and total abnormalities between control and treated semen samples.
4. There was significant ($p<0.05$) increase in % progressive motility, viability and membrane integrity for SNP treated extender after 24 hours, 15 days and 1 month interval while there was significant ($p<0.05$) increase in acrosome integrity after 24 hours for SNP treated extender in comparison to control.
5. There was significant ($p<0.05$) increase in % progressive motility and viability for SIN-1 treated extender after 24 hours, 15 days and 1 month interval while there was significant ($p<0.05$) increase in acrosome integrity after 24 hours for SIN-1 treated extender in comparison to control.
6. There was no significant ($p<0.05$) difference in % total abnormality between control and SNP and SIN-1 treated extenders.
7. In SNP treated extender (1.244±0.09) there was significantly ($p<0.05$) less lipid peroxidation levels (nM MDA) in comparison to SIN-1 treated (3.30±0.47) and control (3.51±0.34) groups.
8. There was a significant ($p<0.05$) increase in the percentage of Mitochondrial membrane potential (MMP) +ve spermatozoa in both the treatment groups (SNP and SIN-1) in comparison to control.

9. The mean percentage of Live acrosome intact sperm differed significantly ($p<0.05$) between both the treatment groups (SNP and SIN-1) in comparison to the control.
10. There was a significant ($p<0.05$) increase in the percentage of Live acrosome reacted spermatozoa in both the treatment groups (SNP and SIN-1) in comparison to control.
11. The mean percentage of dead acrosome intact sperm differed significantly ($p<0.05$) between both the treatment groups (SNP and SIN-1) in comparison to the control.
12. There was a significant ($p<0.05$) increase in the percentage of dead acrosome reacted spermatozoa in SIN-1 treated extender in comparison to control while there was no significant ($p<0.05$) difference between SNP treated extender and control.

CONCLUSIONS

1. A drastic reduction ($p<0.01$) in semen quality was observed at 24 hours after cryopreservation in comparison to fresh semen. Meanwhile, no significant variation was observed in sperm quality after 15 days or 1-month time interval of freezing.
2. Addition of SNP and SIN-1 in cryopreservation media at 100 nmol/ml and 80 µM/ml concentrations respectively improved seminal attributes significantly at 24h and 1 month of freezing in both the cases.
3. Improvement in the seminal attributes on addition of SNP to cryopreservation media was better than that of SIN-1 treated samples. Thus, SNP could be a better choice if the semen is required to be cryopreserved for longer durations.

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