# IMPACT OF OXIDATIVE STRESS ON BULL SPERM PROTEOME AND FUNCTIONAL ATTRIBUTES

**THESIS SUBMITTED TO THE**
**ICAR-NATIONAL DAIRY RESEARCH INSTITUTE**
**(DEEMED UNIVERSITY)**
**KARNAL (HARYANA)**
**IN PARTIAL FULFILLMENT OF THE REQUIREMENTS**
**FOR THE AWARD OF THE DEGREE OF**

**MASTER OF VETERINARY SCIENCE**
**IN**
**VETERINARY GYNAECOLOGY AND OBSTETRICS**

**BY**
**Dr. APOORVA VERMA**
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**VETERINARY GYNAECOLOGY AND OBSTETRICS**
**SOUTHERN REGIONAL STATION**
**ICAR-NATIONAL DAIRY RESEARCH INSTITUTE**
**ADUGODI, BENGALURU - 560030, INDIA**
**2022**

**Regd. No: 20-M-GO-05**

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02/09/2022

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## CERTIFICATE

This is to certify that the thesis entitled **"IMPACT OF OXIDATIVE STRESS ON BULL SPERM PROTEOME AND FUNCTIONAL ATTRIBUTES"** submitted by **Dr. Apoorva Verma** in partial fulfilment of the requirement for award of the degree of **MASTER OF VETERINARY SCIENCE** in **VETERINARY GYNAECOLOGY AND OBSTETRICS** of the **NATIONAL DAIRY RESEARCH INSTITUTE (Deemed University), Karnal (Haryana)** is a bonafide research work carried out by her under my supervision and guidance and no part of thesis has been submitted for any other degree or diploma.

**(Dr. A. KUMARESAN)**
**MAJOR ADVISOR & CHAIRMAN**

**Place: Bengaluru**

**Date:** 13/07/2022

*Dedicated to*

*My Beloved Parents,*

*Respected Guide*

*and*

*Theriogenology Family*

## ACKNOWLEDGEMENT

*First and foremost, I would like to express my deepest gratitude to the almighty God and beloved parents for always bestowing me with his blessings without which I could not have achieved anything that I have today and showing me the right path at life's cross roads.*

*It was a great pleasure to carry out my research work under the guidance of* ***Dr. A. Kumaresan****, Principal Scientist (VGO), SRS-NDRI. In his unique way, he provided me with conscientious, valuable and inspiring guidance, persistent support and suggestions throughout the process of this research. I admire his expertise in planning the projects and immense ability of reasoning and appreciate his perseverance in tackling research problems. He understood me thoroughly and has been very kind to me throughout the period. I worked with him and I have greatly benefited from his knowledge and experience, His vision, sincerity and motivation have deeply inspired me. Sir you will remain my mentor forever.*

*I express my heartfelt gratitude to the members of advisory committee* ***Dr. S. Jeyakumar,*** *Principal scientist (VGO),* ***Dr. A. Manimaran*****,** *scientist (LPM),* ***Dr. G. V. Vedamurthy*****,** *Scientist (ABC) and* ***Dr. M. A. Kataktalware*****,** *Senior Scientist (LPM) for their valuable suggestions, encouragement, support throughout the research work. I extend my sincere thanks to* ***Dr. K. P. Ramesha****, Head, SRS of ICAR-NDRI, Bangaluru for providing necessary facilities. I gratefully acknowledge the NDRI fellowship awarded to me during the course of study.*

*I would like to extend my regards to* ***Dr. T. K. Mohanty, Dr. Nishant Kumar*** *and* ***Dr. Rubina Kumari Baithalu*** *for their teaching and encouragement during my course work period.*

*I am fortunate to have been a part of the therio family, thank you for the lessons, skills, I have learned during my post-graduation life. I feel blessed to have seniors like* ***Nilendu Sir****,* ***Elango Sir****,* ***Kathan Sir*** *and* ***Shivanagouda Sir****, You guys were always there for me whenever I needed and being a constant inspiration to me. I will never forget your helpfulness to carry out my research in a smooth way.*

*I acknowledge with gratitude the valuable suggestions and moral support rendered by* ***Dr. Thirumala Rao Talluri Sir****,* ***Pradeep Nag Sir, Ankur Sharma Sir*** *and* ***Mohua Ma'am****.*

*I could not have embarked on my voyage without the assistance of* ***Nilendu Sir****, who provided me with the initial glimpse of research and always support me. I wish to add cordial thanks to* ***Shivanagouda Sir*** *for his assistance and dedicated involvement in every step throughout the process, and have been unwavering in personal and professional support during the research. I sincerely record my gratitude to* ***Manish sir****, who act like the crucial component of my research work in data analysis and tolerate my crazy talks, offering me advice, and supporting me*

*through this entire research. I'd like to thank **Ebenezer Sir** for improving my reasoning ability and upraising my thinking.*

*I would like to thank **Elango Sir** and **Kathan Sir** for all their support and cooperation, discussion, reviewing my progress constantly, and polishing my writing skills.*

*I wish to thank **Murthy Uncle**, **Mujeeb bhaiya**, **Kiran uncle**, **Santhosh ji**, **Ramalingam ji** for their help during sample collection, all the security personnel & cleaning staff who are working tirelessly.*

*It is pleasure for me to offer thanks to my friends **Akanksha**, **Deeksha** and **Nripendra** for their immense support, love and affection which always inspired me to face the challenges. Special appreciation to **Pritee ma'am** and **Spoorthy ma'am** for your constant encouragement and always reminding me to have my meals and fun whenever I was stressed. Finally, my thanks go to all the people who have supported me to complete the research work directly or indirectly.*

*Words do not suffice to express my devotion and gratitude to my parents who dreamt big for me and supported me through thick and thin of my life. I would like to thank my father **Mr. Sushil kumar Verma**, mother **Mrs**. **Rekha Verma** and my sweet brother **Dr. Anand Verma**, for encouraging me in all of my pursuits and inspiring me to follow my dreams. I always knew that you believed in me and wanted the best for me.*

***Not everyone is mentioned but none is forgotten***

*Place: Bengaluru*
*Date: 2022* *APOORVA VERMA*

## Abstract

Reactive oxygen species (ROS) are involved in several sperm functions, but when their levels exceed beyond physiological concentrations, leads to oxidative stress and associated alterations in sperm fertilizing potential. Recent studies suggested that ROS is the major cause of infertility in case of human being, however the consequence of oxidative stress on bovine sperm are unclear, and the alterations in the spermatozoa at the protein level due to oxidative stress is not known. In this regard, the current study aimed to explore sperm functional attributes and proteome of the oxidative stress induced spermatozoa. Freshly ejaculated spermatozoa from Deoni (*Bos indicus*) bulls were subjected to oxidative stress (50µM $H_2O_2$) either in the presence or absence of seminal plasma. Flow cytometric analysis revealed that oxidative stress resulted in higher ($p<0.05$) proportion of ROS positive spermatozoa, dead spermatozoa, altered intra-cellular calcium concentrations and lipid peroxidation status but did not have any significant effect on sperm acrosome reaction status and mitochondrial membrane potential. Global proteomic analysis detected a total of 1441 proteins in Deoni bull spermatozoa, which are involved in 70 pathways, 118 molecular functions and 141 biological processes. The major pathway in which the identified proteins were involved was metabolic process (26%) and oxidative phosphorylation (7%). In the absence of seminal plasma, a total of 1434 proteins were identified in oxidative stress induced bull spermatozoa; among dysregulated proteins, 260 were upregulated while 191 were downregulated. A total of 78 proteins were identified to be specific to oxidative stressed spermatozoa. Among the differentially expressed proteins, 27% were involved in metabolic process, 8% were involved in reactive oxygen species and 7% were involved in oxidative phosphorylation. Significant number of proteins were involved in biological process like response to oxidative stress, spermatogenesis, protein stabilization and folding, and sperm motility. In the presence of seminal plasma, a total of 1325 proteins were identified in oxidative stress induced bull spermatozoa; among dysregulated proteins, 32 were upregulated while 21 were downregulated. It is concluded that oxidative stress significantly altered the proteomic profile of bull spermatozoa and presence of seminal plasma did not have any beneficial effect on sperm oxidative stress, functional attributes and protein profile in bulls. The findings provide valuable information regarding functional and subcellular changes in sperm due to oxidative stress.

## सारांश

प्रतिक्रियाशील ऑक्सीजन प्रजातियां (आरओएस) कई शुक्राणु कार्यों में शामिल होती हैं, लेकिन जब उनका स्तर शारीरिक सांद्रता से अधिक हो जाता है, तो ऑक्सीडेटिव तनाव और शुक्राणु निषेचन क्षमता में संबंधित परिवर्तन होते हैं। हाल के अध्ययनों ने सुझाव दिया कि आरओएस मानव के मामले में बांझपन का प्रमुख कारण है, हालांकि गोजातीय के मामले में आरओएस से संबंधित अध्ययन अस्पष्ट हैं, और ऑक्सीडेटिव तनाव के कारण प्रोटीन स्तर पर शुक्राणु में परिवर्तन ज्ञात नहीं है। इस संबंध में, वर्तमान अध्ययन का उद्देश्य शुक्राणु कार्यात्मक विशेषताओं और ऑक्सीडेटिव तनाव प्रेरित शुक्राणुजोज़ा के प्रोटिओम का पता लगाना है। देवनी (बॉस इंडिकस) सांडों से ताजा स्खलित शुक्राणुओं को ऑक्सीडेटिव तनाव ($50\mu M$ $H_2O_2$) या तो सेमिनल प्लाज्मा की उपस्थिति या अनुपस्थिति के अधीन किया गया था। फ्लो साइटोमेट्रिक विश्लेषण से पता चला कि ऑक्सीडेटिव तनाव के परिणामस्वरूप आरओएस पॉजिटिव स्पर्मेटोजोआ, डेड स्पर्मेटोजोआ, परिवर्तित इंट्रा-सेलुलर कैल्शियम सांद्रता और लिपिड पेरोक्सीडेशन स्थिति का उच्च (पी $<0.05$) अनुपात हुआ, लेकिन शुक्राणु एक्रोसोम प्रतिक्रिया स्थिति और माइटोकॉन्ड्रियल झिल्ली क्षमता पर कोई महत्वपूर्ण प्रभाव नहीं पड़ा। . वैश्विक प्रोटिओमिक विश्लेषण ने देवनी बुल स्पर्मेटोजोआ में कुल 1441 प्रोटीन का पता लगाया, जो 70 मार्गों, 118 आणविक कार्यों और 141 जैविक प्रक्रियाओं में शामिल हैं। मुख्य मार्ग जिसमें पहचाने गए प्रोटीन शामिल थे, चयापचय प्रक्रिया (26%) और ऑक्सीडेटिव फास्फारिलीकरण (7%) थे। सेमिनल प्लाज्मा की अनुपस्थिति में, ऑक्सीडेटिव तनाव प्रेरित बुल स्पर्मेटोजोआ में कुल 1434 प्रोटीनों की पहचान की गई; विकृत प्रोटीनों में से 260 को अपग्रेड किया गया जबकि 191 को डाउनग्रेड किया गया। ऑक्सीडेटिव तनावग्रस्त शुक्राणु के लिए विशिष्ट होने के लिए कुल 78 प्रोटीन की पहचान की गई थी। विभेदित रूप से व्यक्त प्रोटीनों में, 27% चयापचय प्रक्रिया में शामिल थे, 8% प्रतिक्रियाशील ऑक्सीजन प्रजातियों में शामिल थे और 7% ऑक्सीडेटिव फास्फारिलीकरण में शामिल थे। ऑक्सीडेटिव तनाव की प्रतिक्रिया, शुक्राणुजनन, प्रोटीन स्थिरीकरण और तह, और शुक्राणु की गतिशीलता जैसी जैविक प्रक्रिया में महत्वपूर्ण संख्या में प्रोटीन शामिल थे। सेमिनल प्लाज्मा की उपस्थिति में, ऑक्सीडेटिव तनाव प्रेरित बुल स्पर्मेटोजोआ में कुल 1325 प्रोटीन की पहचान की गई; विकृत प्रोटीनों में, 32 को अपग्रेड किया गया जबकि 21 को डाउनग्रेड किया गया। यह निष्कर्ष निकाला गया है कि ऑक्सीडेटिव तनाव ने बैल शुक्राणुजोज़ा के प्रोटिओमिक प्रोफाइल को महत्वपूर्ण रूप से बदल दिया और वीर्य प्लाज्मा की उपस्थिति का शुक्राणु ऑक्सीडेटिव तनाव, कार्यात्मक विशेषताओं और बैल में प्रोटीन प्रोफाइल पर कोई लाभकारी प्रभाव नहीं पड़ा। निष्कर्ष ऑक्सीडेटिव तनाव के तहत शुक्राणु के कार्यात्मक और उप-कोशिकीय परिवर्तनों के बारे में बहुमूल्य जानकारी प्रदान करते हैं।

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| | | |
|---|---|---|
| % | : | Percentage |
| °C | : | Degree Centigrade |
| µL | : | Micro liter |
| µM | : | Micromole |
| AI | : | Artificial Insemination |
| AMP | : | Adenosine mono phosphate |
| ANOVA | : | Analysis of Variance |
| ATP | : | Adenosine tri-phosphate |
| Ca | : | Calcium |
| CR | : | Conception Rate |
| DAVID | : | Database for Annotation, Visualization and Integrated Discovery |
| DEPs | : | Differentially Expressed Proteins |
| DFI | : | DNA Fragmentation Index |
| DNA | : | Deoxyribonucleic Acid |
| et al. | : | Co-workers |
| FITC | : | Fluorescein Isothiocyanate |
| G | : | Gram |
| GO | : | Gene ontology |
| $H_2O_2$ | : | Hydrogen peroxide |
| hr | : | Hour |
| ICSI | : | Intracytoplasmic Sperm Injection |
| IVF | : | In Vitro Fertilization |
| LC-MS/MS | : | Liquid Chromatography/ Mass Spectrometry |

| | | |
|---|---|---|
| LPO | : | Lipid peroxidation |
| MDA | : | Malondialdehyde |
| mg | : | Milligram |
| min | : | Minutes |
| mL | : | Milliliter |
| mM | : | Millimole |
| MMP | : | Mitochondrial Membrane Potential |
| MW | : | Molecular weight |
| pH | : | Potential of Hydrogen ion |
| PI | : | Propidium Iodide |
| Pi | : | Isoelectric point |
| PNA | : | Pea Nut Agglutinin |
| PPI | : | Protein-protein interaction |
| PSA | : | *Pisum sativum* Agglutinin |
| PSMs | : | Peptide spectrum matches |
| PUFA | : | Poly Unsaturated Fatty Acids |
| ROS | : | Reactive Oxygen Species |
| RPM | : | Revolution Per Minute |
| S | : | Seconds |
| SE | : | Standard Error |
| SP- | : | $H_2O_2$ treated only spermatozoa |
| SP+ | : | $H_2O_2$ treated spermatozoa in presence of seminal plasma |
| SP-TALP | : | Modified Tyrode's Albumin, Lactate and Pyruvate |

# Chapter- 1

# Introduction

*Chapter 1*

# INTRODUCTION

Reduction in dairy cattle reproductive efficiency has become a global challenge (Barkema *et al*., 2015). Although both dam and sire contribute to reproductive success, much of the dairy cattle research has been focused on cow fertility. Bull fertility, on the other hand, has received far less attention and the bull's fertility have largely been ignored (Butler *et al*., 2020). According to recent study, infertility or subfertility in bulls accounts for a major portion of reproductive failure in dairy cattle. Despite the fact that bulls produce plenty of sperm with normal sperm function characteristics; certain bulls may indeed be infertile. Although there are various tools for estimating bull fertility (microscopic and macroscopic), these methods are unable to explain and reliably predict individual bull`s reduced fertility. Poor fertility is the single most important problem restricting animal reproductive performance, influencing the dairy economy.

Bull fertility is essential because a single bull produces several thousands of sperm doses, which are then utilized to inseminate many thousands of cows. Currently, cryopreserved sperm is primarily used in artificial insemination, and it has been proved that spermatozoa can suffer significant damage during cryopreservation (Mostek *et al*., 2017). Numerous cellular and subcellular modifications deteriorate overall post-thaw sperm quality. Even with optimised protocols, 50% of the bull sperm does not survive after freezing and thawing (Grötter *et al*., 2019) resulting in lower conception rates with cryopreserved semen as compared to fresh semen.

Increasing evidence indicate that cryopreservation significantly lowers sperm fertility by lipid peroxidation (LPO), osmotic stress and excessive reactive oxygen species (ROS) formation (Aitken and Drevet, 2020), which adversely affect sperm functional attributes like membrane integrity (Petrunkina and Harrison, 2011), acrosome integrity (Birck *et al*., 2010; Kumaresan *et al*., 2017), capacitation (Layek *et al*., 2016) and oviduct/zona binding ability (Saraf *et al*., 2019) ultimately leading to reduced fertility. Among these factors, oxidative stress is one that significantly affect sperm fertility in human beings (Agarwal *et al*., 2008; Thomson *et al*., 2009; Aitken and Baker, 2020), cattle bull (Bucak *et al*., 2010; Bollwein and Bittner, 2018), ram (Bucak *et al*., 2008), stallion (Ball, 2008), and goat (Bucak *et al*., 2010). The

presence of polyunsaturated fatty acids (PUFAs) in spermatozoa's plasma membrane makes them vulnerable to oxidative stress (Kelso *et al*., 1997; Koppers *et al*., 2010). Although substantial progress has been made in identifying the numerous sources of ROS in these cells, a complete explanation of the genetic, epigenetic and environmental variables that cause oxidative stress in subfertile males is still awaited. Sperm functions are disrupted by oxidative stress, which damages sperm proteins, lipids and DNA (Alahmar, 2019) leading to lipid peroxidation (Aitken *et al*., 2022), protein degradation (Sharma *et al*., 2013), DNA fragmentation (Kasimanickam *et al*., 2007) and apoptosis (Aitken and Baker, 2013), lowering sperm survival and fertility (Tvrda *et al*., 2017). Oxidative stress in the male germ line compromises integrity of sperm DNA, potentially affecting embryo development and offspring health (Tremellen, 2008; Aitken, 2020). Due to oxidative stress, phosphorylation of proteins occurs and lipid metabolites cause damage to spermatozoa, as they will bind to flagellar axonemal proteins and create adducts (Moazamian *et al*., 2015). ROS alters proteins by oxidizing the amino acid side chain, leading to covalent protein-protein cross-linkages (Zhang *et al*., 2013). Mitochondrial electron transport is disrupted when mitochondrial proteins are altered, resulting in an electron outflow (Bulkeley *et al*., 2021). Although the impairment of sperm fertility by ROS is reported consistently in several species, the molecular alterations induced by the oxidative stress have not been understood thoroughly.

Proteins play an important role in reproductive processes and systematic identification of sperm proteins is necessary for gaining new insights into spermatogenesis, male fertility and infertility. Several studies are available with regard to combating the oxidative damage; fortifying semen with enzymatic antioxidants (e.g., glutathione peroxidase, glutathione reductase, catalase, superoxide dismutase and mitoquinone), non-enzymatic antioxidants (e.g., reduced glutathione, Vit-E, Vit-C and Vit-B12), miscellaneous agents (e.g., carotenoids, melatonin, coenzyme Q, resveratrol etc.) and antioxidant preservatives (e.g., BHT and BHA) resulted in varied level of success in decreasing oxidative burden of sperm and improving post thaw sperm motility (Michael *et al*., 2007; Yue *et al*., 2010; Ghallab *et al*., 2017; Partyka and Niżański, 2021; Arjun *et al*., 2022; Liman *et al*., 2022). However, the molecular functions and biological process, pathways related to the oxidative stress induced damages in sperm need to be explored for better

understanding of oxidative damage before developing strategies to combat sperm oxidative stress.

In the current situation, an update on current research addressing ROS production, diagnostics and the relationship between oxidative stress and male infertility, as well as pregnancy and assisted reproductive techniques result, is critical. Although the global protein profile of bull spermatozoa is available, the alterations in the proteomic profile of spermatozoa due to oxidative stress are not known. Using the "OMICS" approach, limited numbers of studies have evaluated the molecular alterations in the sperm of human being due to oxidative stress, but this altered mechanism is unclear in animals. Therefore, identifying the cellular, functional and proteomic alterations associated with oxidative stress in bulls will broaden our understanding of poor fertility.

Hence, we hypothesized that inducing oxidative stress in sperm results in altered functional attributes and protein modifications. With this backdrop, the present study was envisaged with the following objectives.

1. Assessment of cellular and functional phenomes of bull spermatozoa before and after induction of oxidative stress
2. Comparative proteomic profiling of normal and oxidative stress induced bull spermatozoa
3. Identification of potential proteins that could serve as biomarkers for oxidative stress in bull spermatozoa

# Chapter - 2

# Review of Literature

*Chapter 2*

# REVIEW OF LITERATURE

Artificial insemination (AI) has played a pivotal role in genetic improvement of indigenous/non-descript cows. Currently, more than 90% of dairy cows in developed countries are inseminated artificially (Ombelet and Van Robays, 2015). The adoption of AI is higher than any other assisted reproductive technique. AI encompasses semen collection, semen processing, and deposition of semen in the female reproductive tract using AI, a bull can be effectively used to breed more than 20,000 cows per year. Currently, cryopreserved sperm is primarily used in AI, and it has shown that spermatozoa can suffer significant damage during cryopreservation (Yousef *et al*., 2003; Mostek *et al*., 2017).

## 2.1: Fertility outcome of cryopreserved semen

Spermatozoa undergo several detrimental changes during cryopreservation and lose their fertility (Bilodeau *et al*., 2000; Bilodeau *et al*., 2002; Ezzati *et al*., 2020). Fertile lifespan of sperm is significantly lower as compared to fresh semen (Holt *et al.,* 2007). Numerous cellular and subcellular modifications during cryopreservation deteriorate overall post-thaw sperm quality. Even with optimised protocols, 50% of the bull sperm does not survive after freezing and thawing (Grötter *et al*., 2019). Cryopreserve semen reduces Pregnancy rates in cattle as compared to fresh semen (Saha *et al*., 2014). Shannon and Vishwanath (1995) found 8-10 times more frozen-thawed bull sperm hence required than fresh sperm to attain equal fertility rates *in vivo*, suggesting that cryopreservation significantly lowers sperm fertility because of the changes including LPO, osmotic stress, and excessive ROS formation (Aitken and Drivet, 2020). Spermatozoa cryopreservation is related to physical and oxidative stress (Chatterjee and Gagnon, 2001), with the latter being the major cause of poor quality frozen-thawed sperm due to increased formation of ROS (Lone *et al*., 2018), which is the predominant cause of male infertility, accounting to about 30-80% in human infertility cases (Iwasaki and Gagnon, 1992; Zini *et al*., 1993; Ochsendorf, 1994; Shekarriz *et al*., 1995; de Lamirande and Gagnon, 1995; Agarwal *et al*., 2017).

## 2.2: Cryopreservation induced alterations in sperm functions

Since the sperm from one bull is used to inseminate a larger female population, the quality of the male factor has been given substantial weight in dairy cattle production. During the cryopreservation process, spermatozoa are subjected to drastic changes such as temperature, ice crystal formation, and a variety of stresses (physical, chemical, osmotic and oxidative) (Ezzati *et al*., 2020; Lavanya *et al*., 2021) that cause membrane phase transition, metabolic decoupling, ionic imbalance, protease activation, energy deprivation, cytoskeleton destabilization and production of ROS (Grötter *et al*., 2019). It is well known that during the last stages of spermatogenesis, spermatozoa lose much of their cytoplasm and lack the major cytoplasmic component carrying antioxidants that fight the detrimental effects of ROS and lipid peroxidation (Bucak *et al*., 2007*)*.

The plasma membrane of bovine sperm is composed of PUFA, in which docosahexaenoic acid (DHA) accounts for up to 55–60% of total PUFAs (Kelso *et al*., 1997). Cryopreservation alters the composition of PUFAs in the sperm membrane lowering motility, viability, and acrosome integrity (Schiller *et al*., 2000; O'Connell *et al*., 2002). In comparison to buffalo bull spermatozoa, are more prone to oxidative damage than cattle bull spermatozoa due to high PUFA (Kumaresan *et al*., 2006). The process of cryopreservation is reported to alter the functions such as membrane integrity (Petrunkina and Harrison, 2011), acrosomal integrity (Birck *et al*., 2010; Kumaresan *et al*., 2017), capacitation (Layek *et al*., 2016) and oviduct/zona binding ability (Saraf *et al*., 2019) which ultimately lead to reduced fertility. The interesting plethora of sperm cryodamage is that $Ca^{2+}$ influx would produce extended openings of mitochondrial permeability transition pore (MPTP), results in ROS and $Ca^{2+}$ release, loss of mitochondrial membrane potential, reduced ATP content, Cytochrome c (Cytc) release (Gualtieri *et al*., 2021), rendering sperm non-functional. Significant losses in farm animals occur as a result of oxidative stress caused by cryopreservation, although it is unclear how this influences subcellular alterations. The ROS thus produced further lads to disruption of sperm mitochondria, increased lipid peroxidation, protein modifications and DNA damage, thus affecting initial fertilization and post-fertilization early embryo survival. Although the global protein profile of bull spermatozoa is available, the alterations in the proteomic profile of spermatozoa due to oxidative stress are unclear. Identifying the molecular alterations

in the spermatozoa due to oxidative stress would improve our understanding of poor fertility.

### 2.3: Reactive oxygen species and its chemistry

In order to understand oxidative stress as a cause of male infertility, it is necessary to grasp the leading players, such as free radicals and ROS. Free radicals are highly reactive molecules or atoms with an unpaired electron and may readily join with any molecule to promote protein, lipid, or nucleic acid oxidation. Indeed, ROS are a harmless consequence of metabolism that, to a certain extent, are essential for proper cell signaling. ROS are a diverse group of molecules that include: oxygen-free radicals such as superoxide anion ($O_2^-$), hydroxyl radical ($OH^-$), and Non-radical species include hydrogen peroxide ($H_2O_2$) (Agarwal *et al*., 2014). Reactive nitrogen species and free nitrogen radicals include nitroxyl ion, nitrous oxide, and peroxynitrite (Agarwal *et al*., 2014). The most abundant ROS are $O^{-2}$ and $H_2O_2$ (Dong *et al*., 2020). Superoxide dismutase in the mitochondria quickly converts excess $O^{-2}$ to $H_2O_2$. In the presence of protons, $O^{-2}$ interacts with itself, culminating one molecule in oxidation while the other in reduction to yield $H_2O_2$. The Fenton's reactions occur when $H_2O_2$ comes in contact with unbound ferrous and copper ions in the proper environment, producing the highly reactive hydroxyl radical (Valko *et al*., 2005). Fenton's chemistry may be responsible for the onset of lipo-peroxidative damages inside the sperm plasma membrane (Aitken, 2017). The potential of ROS to initiate lipid peroxidation cascades that result in the formation of small-molecular-mass aldehydes such acrolein, malondialdehyde, and 4-hydroxynonenal are the metabolic foundation of this cyclic activity (Moazamian *et al*., 2015). These aldehydes are strong electrophiles that covalently attach to susceptible histidine, lysine, and cysteine residues on target proteins, causing protein malfunction, followed by functional and genetic damage (Aitken *et al*., 2016).

### 2.4: Why are sperm prone to oxidative damage?

Spermatozoa are most vulnerable to oxidative stress because their plasma membranes are rich in PUFA and they have low levels of cytoplasmic antioxidant enzymes. Sperm also creates ROS, especially during transit through the epididymis, and these isolated cells in both the male and female genital tracts cause oxidative

stress through their metabolism. Sperm cells, however, lack DNA repair mechanisms (Sabeti *et al*., 2016).

### 2.5: Etiology of reactive oxygen species

In sperm cells, the mitochondria and the sperm plasma membrane are two key contributors of ROS, it causes mitochondrial membrane damage, and the affected mitochondrial membrane causes an increase in ROS production (Sanocka and Kurpisz, 2004; Bedard and Krause., 2007). ROS is created throughout the reproductive system, including during spermatogenesis, although not at the same rate because at that time antioxidant defence and DNA repair pathways, fortunately, exist. During spermatogenesis, Superoxide ($O_2^-$) is produced naturally as a byproduct of cellular respiration. Once spermatozoa are discharged from the germinal epithelium, they are vulnerable to oxidative damage because they are no longer shielded by the Sertoli cell defense system (Aitken, 2009). The produced one-electron reduction product of oxygen combines with itself via dismutation process, which is considerably accelerated by Superoxide dismutase (SOD), to produce $H_2O_2$. The major sources of ROS in cattle are the aromatic amino acid oxidase, produced by dead spermatozoa, the immature and residual cytoplasm in sperm. Factors associated with oxidative stress shown in **Table 2.1**.

**Table 2.1: Potential factors associated with oxidative stress in the male reproductive system**

| **Intrinsic Factors** | **Extrinsic Factors** |
|---|---|
| Sperm metabolism - Mitochondrial enzymes, NADPH synthase, Aromatic amino acid oxidase, Nitric oxide synthase | Environmental, Climate change, Seasonality |
| Sperm morphology and viability - Immature, abnormal, or dead spermatozoa, - Presence of cytoplasmic droplets | Iatrogenic, Sperm handling, Sperm storage, Media composition |
| Leukocyte activation following inflammation or infection | Pathological, Bacteriospermia |
| Genetics and phenotypic traits, Age | |

### 2.5.1: Thermotropic changes

Thermotropic changes during cryopreservation causes membrane phase transitions, which in turn causes lipid molecule rearrangement and result in cholesterol and PUFA loss (Maldjian *et al*., 2005). These changes cause calcium ion influx, which causes protein phosphorylation and causes capacitation. Overexposure to these changes during cryopreservation, causes oxidative stress, which reduces the fertilizing potential of these gametes. Santiani *et al*. (2014) reported that ROS generation gradually increases from the start of the cooling process until it reaches maximum values when samples are cooled to 5 °C hence high quantities of ROS are produced during spermatozoa incubation at 5 °C.

### 2.5.2: Aromatic L- amino acid (Egg yolk and milk)

The generation of $H_2O_2$ by spermatozoa was originally documented in 1940s. The addition of egg yolk as cryodiluent to total semen (spermatozoa and seminal plasma) boosted oxygen intake initially, but respiration gradually reduced as the reaction progressed (Aitken *et al*., 2015). The sperm enzyme L-amino acid oxidase oxidatively deaminated the aromatic amino acids in egg yolk to produce $H_2O_2$ L-amino acid oxidase (LAAO) (Tosic & Walton *et al*., 1950; Aitken *et al*., 2016). When LAAO is liberated from dead spermatozoa, aromatic amino acids are deaminated, and $H_2O_2$ is produced as a consequence. ROS induced death is caused when produced $H_2O_2$ reacts with regenerating viable spermatozoa and this process has an adverse effect on sperm viability.

$$\text{L amino acid} + H_2O + O_2 \rightarrow\rightarrow \overset{AAAO}{\rightarrow\rightarrow} NH_3 + H_2O_2 + \text{2 oxo acid}$$

### 2.5.3: Leukocytes as a source of ROS

Activated leukocytes release ROS, protease, and cytokines, which can harm sperm via lipid peroxidation and DNA fragmentation (Agarwal *et al*., 2014). It should be emphasized that ejaculate is devoid of leucocytes for use in AI, and the bulk of these cells are removed using a simple density gradient centrifugation procedure.

### 2.5.4: Effect of dilution on spermatozoa

At higher dilution rates, important seminal plasma components are lacking, adding to the negative effects of semen dilution (Garner *et al*., 2001). In nutshell, it is

obvious that the effective antioxidant present in seminal plasma decreases during cryopreservation while the ROS increases.

### 2.5.5: Freezing/Thawing

The findings of Bilodeau *et al.* (2000) clearly support the presence of an oxidative stress during a freeze/thaw cycle, which is in accordance with the idea that ROS produced during such a cycle are detrimental to sperm function. In bovine spermatozoa, the levels of antioxidants diminished during freeze/thaw cycles as sperm Glutathione (GSH) levels were lowered by 78% and SOD activity was reduced by 50% after cryopreservation (Bilodeau *et al*., 2000). The formation of superoxide radicals increases when spermatozoa are frozen.

## 2.6: Generation of reactive oxygen species

Spermatozoa gain the ability to migrate when they go through the epididymis, but they lack the ability to fertilize, until a physiological shift known as 'capacitation' occurs in the female tract. Mammalian sperm capacitation is a redox-controlled process that necessitates the formation of several forms of ROS in order to enhance the fertilization of spermatozoa into mature oocytes. To create energy, spermatozoa use glycolysis, the citric acid cycle (Krebs cycle), and the oxidative phosphorylation. Horses, rely heavily on aerobic routes like oxidative phosphorylation, while bovine, ovine, and porcine sperm mostly on anaerobic pathways like glycolysis (Nesci *et al*., 2020). Two ROS-producing mechanisms may be involved: nicotinamide adenine dinucleotide phosphate (NADPH) oxidase at the sperm membrane (O'flaherty *et al*., 2003) and sperm diaphorase (mitochondrial nicotinamide adenine dinucleotide–hydrogen, NADH-dependent oxidoreductase) in the mitochondria (Koppers *et al*., 2008).

### 2.6.1: Mitochondria's role in reactive oxygen species generation

The accumulation of potentially harmful quantities of ROS is a by-product of normal mitochondrial metabolism and homeostasis. The formation of ROS from complex I or III in spermatozoa was caused by disruption of mitochondrial electron transport flow via processes that were independent of mitochondrial membrane potential (Koppers *et al*., 2008). The opening of mPTP, which includes the creation of a "hole" in the inner mitochondrial membrane (IMM), is known to result in the

dissipation of as well as an increase in ROS levels hence, Openings of the mPTP are crucial for maintaining mitochondrial homeostasis (Zorov *et al*.,2014). Longer mPTP openings at greater ROS levels may cause a ROS burst, resulting in mitochondrial damage and, it may spread from mitochondrion to mitochondrion (Zorov *et al*., 2014).

The mitochondrial response to oxidative stress, forms mPTP, it enhances ROS signal which has been demonstrated to promote $H_2O_2$ production via conformational changes to complex I of electron transport chain (ETC) (Batandier *et al*.,2004*)*. The fast release of $H_2O_2$ into the extracellular space was caused by the activation of ROS production at complex III (Adam, 2003).

## 2.7: Physiological role of reactive oxygen species in sperm cells and capacitation

Capacitation is a complex biochemical process that gives mammalian sperm the ability to fertilise an egg. In the recent past, it has been discovered that sperm capacitation is a reduction-oxidation (redox) regulated process that is aided by the de novo creation of modest quantities of ROS. The physiological amount of ROS is required for proper germ cell growth and capacitation (Du Plessis *et al*., 2015), however excessive ROS generation causes oxidative stress and apoptosis in spermatozoa (He *et al*., 2017). Cyclic adenosine monophosphate (CAMP) drives this signaling pathway, which is controlled by the cells' redox state (de Lamirande, & Gagnon, 1998). The production of ROS is expected to have a favourable effect on tyrosine phosphorylation, as seen in the spermatozoa of human (Villegas *et al*.,2003), bull (O'Flaherty *et al*., 2003), horses (Baumber *et al*., 2002), and boars (Boerke *et al*., 2013). ROS stimulate sperm capacitation by a variety of processes, including the activation of adenylyl cyclase activity (Rivlin *et al*., 2004) followed by protein kinase A activation (O'Flaherty *et al*., 2006), oxidation of cholesterol and as a result, cholesterol is removed from the plasma membrane (Boerke *et al*., 2013).

ROS is required for capacitation, hyperactivation, motility, acrosome reaction, and fertilization. ROS elevates intracellular cAMP, which subsequently activates protein kinase (**Fig. 2.1**). These modifications promote tyrosine phosphorylation (Yamagishi *et al*., 2001). Capacitation is also linked to increased thiol content on

some proteins on the sperm surface (O'Flaherty, 2015). Thiol oxidation, which forms disulfide bridges, and an increase in thiol content are required to regulate certain sperm proteins linked with capacitation (O'Flaherty, 2015). Despite this, there are still numerous gaps in our knowledge of the biochemical processes linking ROS, cAMP production, Protein Kinase A activation, protein phosphorylation, and capacitation. The ideal $H_2O_2$ concentration for initiating protein phosphorylation is 50–200μM, but inhibition was detected at higher doses (0.5 and 5 mM) (Rivlin *et al*., 2004).

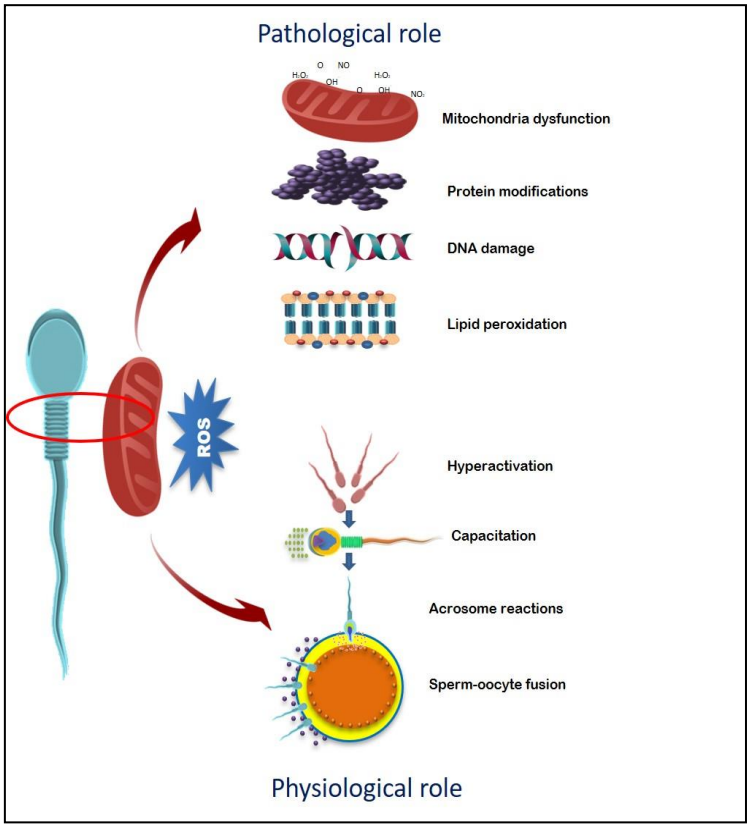



**Figure 2.1:** Physiological and pathological role of ROS

## 2.8: Impact of oxidative stress on spermatozoa

### 2.8.1: Lipid peroxidation

When free radicals destroy cell membranes, the protective mechanism is lost, exposing the entire cell to risk. In this context, increased ROS generation causes lipid peroxidation in spermatozoa. The plasma membrane of spermatozoa is composed of polyunsaturated fatty acid, which contribute to membrane elasticity

and fluidity that is essential for fertilization (Aurich *et al*., 2018) that is readily oxidized by ROS (Kasimanickam *et al*., 2007; Aurich *et al*., 2018) and form further reactive by-products like acrolein, malondialdehyde and 4- hydroxynonenal (Jones *et al*., 1979; Ayala *et al*., 2014; Sabeti *et al*., 2016) which consequently affects membrane fluidity, integrity and ability to fuse with oocyte**.**

### 2.8.2: Protein dysfunction

The lipid metabolites cause damage to spermatozoa as they will bind to flagellar axonemal proteins and create adducts (Moazamian *et al*., 2015). ROS alters proteins by oxidizing the amino acid side chain, leading to covalent protein-protein cross-linkages. Oxidative cascade results in perturbing post translational modification and primarily causing change in protein structure, folding and protein carbonylation. Mitochondrial electron transport is disrupted when mitochondrial proteins are altered, resulting in an electron outflow**.**

### 2.8.3: DNA damage

It is critical to safeguard the integrity and correctness of DNA in the sperm nucleus in order to transmit genetic material completely from one generation to the next. Free radicals damage DNA in numerous ways as hydroxyl radicals bind to the double-bonds of DNA bases and extract hydrogen from the deoxyribose sugar (Breen and Murphy, 1995). ROS damages DNA at the base level, causing DNA modification and fragmentation (Kasimanickam *et al*., 2007). Simoes *et al*. (2013) found that increasing Oxidative stress susceptibility affected sperm DNA integrity, and the sperm samples with reduced sensitivity to oxidative stress were also less sensitive to DNA damage. Furthermore, embryo *in vitro* production demonstrated that the embryo cleavage rate reduced as the oxidative stress elevated. When the sperm from bull with oxidative DNA damage was used to fertilise oocyte, it reduced cleavage rate significantly (Simoes *et al*., 2013).

When spermatozoa were triggered by oxidative stress, infertility may be exacerbated by DNA damage (Hosen *et al*., 2015). Cryopreservation has deleterious effects on sperm DNA by inducing DNA fragmentation and oxidation (Thomson *et al*., 2009). Sperm DNA damage has been associated with high levels of ROS in fresh and cryopreserved/thawed semen. Cryopreservation enhanced the DNA damage in

cells considerably, as measured by the comet test or DNA fragmentation. ROS can also cause DNA fragmentation, further damaging the paternal genome and resulting in male infertility (Das *et al*., 2013).

### 2.8.4: Apoptosis

Apoptosis is a type of planned cell death in which genetically defective cells are removed. The mitochondria are at the centre of the apoptotic cascade, providing key components such as those that activate caspases and fragmentation of DNA (Adams, 2003). When mitochondrial DNA is exposed to ROS, it promotes apoptosis and thus DNA fragmentation. 4-HNE and acrolein, which are byproducts of lipid peroxidation, causes apoptosis and DNA fragmentation (Aitken *et al*., 2012). Excessive mitochondrial ROS leads to induction of intrinsic apoptosis as ROS metabolites can bind and either activate or deactivate protein for a pro-apoptotic effect (Galimov *et al.,* 2012).

## 2.9: Effect of reactive oxygen species on sperm functions

### 2.9.1: Sperm kinematics

One of the first and most profound effects of oxidative stress to be observed was a loss of motility (de Lamirande *et al*., 1997; Maia *et al*., 2014). When compared to cryopreserved sample, sperm subjected to moderate sublethal stress had greater motility and viability characteristics. The effects of nitric oxide (NO) and $H_2O_2$ on sperm motility and viability were studied earlier, in which, all freezing groups had considerably lower motility indices and viability than the fresh sample (Khosrozadeh *et al.*, 2022). Sperm viability and kinetics were drastically lowered at $H_2O_2$ concentrations of 200 µM and 250 µM (Pujianto *et al*., 2021). At all doses tested, $H_2O_2$ decreased sperm capacity to penetrate cervical mucus and also affect cell membrane integrity (Pujianto *et al*., 2021). According to the findings, modest quantities of $H_2O_2$ (10 µM and 100 µM) stimulate the defense system and motility of human sperm (Evdokimov *et al*., 2015). Incubating sperm samples with 10 µM and 100 µM $H_2O_2$ enhanced the proportion of spermatozoa with progressive motility by 20% and 18%, respectively (Evdokimov *et al*., 2015). Low amounts of $H_2O_2$ are thought to activate the pentose phosphate pathway, culminating in NADPH production and glutathione reductase reduction of the oxidised glutathione, giving

GSH (Glutathione). The GSH decreases the oxidised cysteine residues of the GAPDS active site, enhancing the enzyme's activity and, as a result, increasing the content of sperm cells with progressive motility (Evdokimov *et al*., 2015). Sharafi *et al* (2015) reported that inducing sub-lethal oxidative stress with 1 µM nitric oxide (NO) might be advantageous for the cryopreservation of bull sperm, as when sperm was exposed to nitric oxide before freezing, it improved overall motility, progressive motility and average path velocity much more than other extenders. The administration of 50 µM $H_2O_2$ causes capacitation and related protein tyrosine phosphorylation in human spermatozoa without reducing motility (O'Flaherty *et al*., 2006).

### 2.9.2: Viability

Mahfouz *et al*. (2010) found that $H_2O_2$ exposure dramatically decreased viability and increased the proportion of dead sperm (Mahfouz *et al*., 2010). Furthermore, Pujianto *et al*. (2021) discovered that higher concentrations of $H_2O_2$ (150–250 µM) significantly reduced sperm viability and motility parameters. At 50 µM, $H_2O_2$ sperm viability dropped to 61% ± 1.87%, at 100 µM was 58.20% ± 1.64%, at 150 µM was 55.80% ± 2.16%, at 200 µM was 54.0% ± 3.39%, and at 250 µM was 48.40% ± 6.98%. Significant alterations were observed at the concentration of 200 µM and 250 µM (Khosrozadeh *et al*., 2022). Khosrozadeh *et al*. (2022) reported that sperm exposed to 1 µM NO and 10µM $H_2O_2$ showed significantly increased percentages of viability ($P < 0.05$). The most viable sperm (85.6 ± 2.3 percent) was generated by sublethal oxidative stress with1 µM Nitric oxide treatment.

### 2.9.3: Mitochondrial membrane potential (MMP)

Increased ROS levels boost UCP2 (Uncoupling protein) activity in mitochondria, either directly or indirectly (Mailloux and Harper, 2011). The mitochondria are the predominant generator of ROS in the spermatozoa. Increased ROS production by spermatozoa is associated with a decreased MMP (Wang *et al*., 2003). Khosrozadeh *et al*. (2022) reported that sperm exposed to 1 µM NO and 10µM $H_2O_2$ showed significantly increased percentages of mitochondria activity ($P < 0.05$) as the activation of mitochondrial inner membrane proteins such as UCP2 can raise mitochondrial membrane potential by blocking proton gradient disintegration.

UCP2 governs not only mitochondrial ATP synthesis but also ROS creation (Toda and Diano, 2014).

### 2.9.4: Plasma membrane integrity

As cryopreservation changes plasma membrane fluidity, 40% of frozen-thawed sperm exhibited a swollen plasma membrane, in cattle (Khalil *et al*., 2019). Khosrozadeh *et al.* (2022) reported that membrane integrity was considerably lower in the frozen control group (54.4 ± 1.95) than in the fresh group (85.2 ± 1.95) and when compared to the frozen control group, the NO-0.1µM and $H_2O_2$-10µM induced groups had considerably greater percentages of membrane integrity (61.7 ± 1.9 and 62.3 ± 1.95, respectively).

### 2.9.5: Early embryonic development

It has been reported that excessive ROS could seriously harm developing embryos (Covarrubias *et al*., 2008). However, it has been revealed that sublethal exposure of gametes to specific stresses might improve gamete performance in processes such as cryopreservation, making it a helpful tool in assisted reproductive technology. The insemination of oocytes with spermatozoa treated to 200 µM $H_2O_2$ enhanced the rates of fertilization, cleavage, and blastocyst formation (Rahman *et al*., 2012). Wyck *et al*. (2018) found that sperm with oxidative stress can impede epigenetic reprograming in early cow embryos owing to impaired active DNA demethylation on the male pronucleus, which may lead to improper embryo development and low embryo quality. Wyck *et al*. (2018) demonstrate that fertilization using oxidatively stressed sperm resulted in a significant developmental halt at the period of embryonic genome activation. De Castro *et al*. (2016) treated cryopreserved bull sperm with increasing $H_2O_2$ dosages (0, 12.5, 25, and 50 µM) and found that the control group (0 µM) had a blastocyst formation rate of (40.3 ± 4.4 %) and it was severely decreased (9.0 ± 2.7 %) in sperm treated with $H_2O_2$. These results indicate that $H_2O_2$ treatment of sperm results in severe abnormalities after the first cleavage. Base excision repair is the major cellular repair pathway involved in fixing oxidative DNA damage (Markkanen *et al*., 2017). The BER machinery is recruited to the paternal genome of zygotes produced from sperm with oxidative DNA damages. Active DNA demethylation in the paternal pronucleus is impaired by

oxidative stress in sperm (De Castro *et al*., 2016). At the moment of fertilization, spermatozoa with high amounts of 8-hydroxy-2' –deoxyguanosine (8-OHdG) might experience inadequate DNA repair in the zygote, which can impede preimplantation embryo development (Khokhlova *et al*., 2020) and foetal growth (Lane *et al*., 2014).

### 2.10: Methods to detect oxidative stress

Oxidative stress can be measured in a variety of biological samples, including plasma, serum, urine, and follicular/peritoneal/seminal fluid. Oxidative stress can be measure using either direct or indirect assays. Various method to measure oxidative stress are mentioned in **Table 2.2**. It can be measured in semen using several methods such as chemiluminescence, nitro blue tetrazolium (NBT) test, cytochrome c reduction test, and electron spin resonance (Dutta *et al*., 2019). Several studies, however, highlighted the importance of using flow cytometry to measure ROS generation by using fluorescent probes such as $H_2$DCFDA (Silva *et al*., 2016), CellROX Green (De Castro *et al*., 2016; Lançoni *et al*., 2018) and MitoSOX Red (Riley *et al*., 2021). H2DCFDA is a cell-permeable, stable, nonfluorescent probe that de-esterified in the presence of intracellular $H_2O_2$ to generate a fluorescent 2,7-dichlorofluorescein (DCF) (Gosalvez *et al*., 2017) while Dihydroethidium (DHE) were effective in detecting intracellular ROS produced by sperm mitochondria (Gallo *et al*., 2018). LPO is regarded as a specific biomarker of oxidative stress in sperm cells, and it has mostly been evaluated by detecting end products of lipid peroxidation such as malondialdehyde; C11-BODIPY581/591 has been frequently utilised to measure LPO in sperm cells (Aitken *et al*., 2007; Ferrusola *et al*., 2009; Gallo *et al*., 2021). Thiobarbituric acid reactive substances (TBARS) assay uses thiobarbituric acid (TBA) as a reagent to detect byproducts of lipid peroxidation that react with MDA. It is an indirect method of detecting ROS (Sanocka and Kurpisz, 2004). 8-oxo-7,8-dihydro 2′ deoxyguanosine (8-OHdG) levels, a direct marker of sperm oxidative stress appears to have diagnostic value (Loft *et al*., 2003). It is widely recognised that oxidative stress is a major cause of sperm DNA damage (Gharagozloo and Aitken, 2011). The measurement of the oxidised deoxynucleoside, 8-OHdG, provides an excellent direct assessment of sperm DNA oxidative damage (Jeng *et al*., 2015). As ROS can cause direct DNA damage by forming oxidised DNA adducts such as 8-OHdG (Gualtieri *et al*., 2021). 4-hydroxynonenal (4-HNE) protein adducts are

increased in oxidatively stressed conditions (Shoeb *et al*., 2014). Therefore, measuring 4-HNE protein adducts might suggest systemic oxidative stress. Cytotoxic aldehydes (4-HNE) can be measured by HPLC and ELISA.

The chemiluminescence method is the most extensively used approach for measuring the formation of ROS by spermatozoa (Homa *et al*., 2015). The luminol (5-amino-2,3, -dihydro-1,4-phthalazinedione) and lucigen are the two probes for Chemiluminescence method in which, luminol-dependent chemiluminescence approach employs a sensitive luminous probe that interacts with several free radicals in entire sperm, including $H_2O_2$, $O^-$, and $OH^-$, allowing both intracellular and extracellular ROS to be quantified on a luminometer where as, lucigenin is more specific for superoxide anions released extracellularly (Aitken *et al*., 1992). The results can be expressed as relative units (RLU), counted photons per minute (CPM), or millivolts/second (Kashou *et al*., 2013). The NBT measures the amount of ROS generated by spermatozoa and leucocytes within their cells (Robert *et al*., 2021). When the NBT reagent is exposed to superoxide, it is reduced and transformed into blue formazan crystals (Aitken *et al.,* 2018). One important disadvantage of the NBT OxiSperm test is that seminal plasma contains large quantities of reductase, which can convert NBT to formazan, resulting in false positive findings in clean semen assays (Aitken, 2018). Electron transfer from oxidants to antioxidants is measured as static oxidation reduction potential (sORP) by MiOXSYS analyzer (Agarwal *et al*., 2016). Using the MiOXSYS System, Agarwal *et al.* (2017) developed a standardized technique for assessing oxidative stress (as oxidative reduction potential; ORP) in sperm. Total antioxidant capacity (TAC) levels can be determined using chemiluminescence or colorimetric methods (Martins and Agarwal, 2019).

**Table 2.2: Various assay to measure oxidative stress**

| Direct assays | Indirect assays |
|---|---|
| Chemiluminescence assays | Myeloperoxidase test |
| Cytochrome c reduction | Lipid peroxidation level |
| Nitroblue tetrazolium test | TAC/Antioxidants, micronutrients, vitamins (vitamin E, vitamin C) |
| Electron spin resonance | Chemokines |
| Flow cytometry | Oxidation reduction potential |
| Fluorescein isothiocyanate (FITC)-labelled lectins | Myeloperoxidase or Endtz test |
| 8-OHdG levels | DNA damage |
| C11-BODIPY assay | TBARS assay |

### 2.11: Effect of cryopreservation on sperm bio-molecules

It is evident that cryopreservation alters the functional and phenotypic changes in the spermatozoa, several studies have shown that cryopreservation affects expression level of sperm biomolecules like gene and protein. Various "OMICS" technologies in sperm cryo-biology are now being used, particularly proteomics (Soggiu *et al*., 2013; Scott *et al*., 2018) and transcriptomics (Saraf *et al*., 2021) which has made significant contributions to the study of molecular changes produced by cryopreservation-induced damage, as well as the identification of several stress indicators and particular proteins that might lead to spermatozoa deterioration. The generation of huge data related to functional attributes, including genomes, proteomics, metabolomics, transcriptomics, and epigenomics, assists researchers in generating substantial information to better grasp the unraveling physiological factors behind oxidative stress.

#### 2.11.1: Sperm proteomics

Currently, researchers are looking into the molecular components associated with spermatozoa that could interfere with fertilization, with a particular focus on the proteome of ejaculated spermatozoa. Recent interest in bovine sperm proteomics

has grown because it provides detailed information on the functional state of spermatozoa. Proteomic study of spermatozoa is required to explore functionally significant proteins and their role in the regulation of different fertilization processes. Mark Wilkins in 1994 coined the term proteome to define the complete set of proteins expressed by a genome, cell, tissue, or organism. In proteomics, denaturing polyacrylamide gel electrophoresis (PAGE) or sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional gel electrophoresis, and high-performance liquid chromatography are the preferred methods for separation of complicated protein or peptide samples (HPLC). MALDI-TOF and liquid chromatography tandem mass spectrometry (LC-MS/MS) are two advanced proteomic techniques for analysing sperm proteins, these tools can detect the greatest number of proteins, even in low-concentration samples (Agarwal *et al*., 2020).

#### 2.11.1.1: Stress associated sperm proteins dysfunctions

ROS are thought to directly alter seminal plasma and sperm proteins by oxidizing amino acid residue side chains, cleaving peptide bonds and forming covalent protein-protein cross linkages (Bollwein and Bittner, 2018). Reduced antioxidant levels alter the expression of sperm and seminal plasma proteins (O'flaherty *et al*., 2017). They are highly redox-dependent which affect sperm signaling pathways and lead to failure of vital sperm functions. Protein conformation and functionality can be affected by oxidation of sulfhydryl groups and thiol groups culminate with structural as well as functional collapse of Tyrosine phosphatase enzyme and cysteine containing proteins respectively. The enzyme tyrosine phosphatase, which has a thiol group in sperm and is thus sensitive to oxidation by ROS, plays an important role in sperm capacitation, therefore inhibition of its activity initiates a cascade and finally inducing capacitation followed by acrosome reaction. Prolonged oxidative stress causes over-oxidation of protamine thiol groups, resulting in hyper-condensation of DNA, which has a negative impact on sperm function (De Lamirande *et al*., 1997). As fertilization necessitates the fusing of gamete regarding their relevance, some proteins are known to be required for sperm-egg membrane fusion (Lamas-Toranzo *et al*., 2020).

Agarwal *et al*. (2014) reported that changes in protein concentrations in seminal plasma samples exposed to oxidative stress were linked to stress responses

and regulatory pathways, whereas changes in sperm proteins were largely linked to metabolic (carbohydrate metabolism) and stress responses. Peroxiredoxins, are the primary antioxidants in spermatozoa and are affected protein modifications (O`Flaherty and Fournier, 2017). Reversible redox signaling and irreversible nonenzymatic processes are part of the cellular response to ROS, depending on the form and concentration of the ROS. Changes in thiol/disulfide pairs affect protein conformation, enzymatic activity, ligand binding and protein-protein interactions. Significant redox-dependent protein alterations such as thiol oxidation (Liu and O'Flaherty, 2017), tyrosine nitration (Morielli and O'Flaherty, 2015), and S-glutathionylation (Morielli and O'Flaherty, 2015) have been linked to sperm dysfunction and changes in the paternal genome thus resulting in infertility. Bilodeau *et al.* (2001) found that freezing bull spermatozoa in egg yolk Tris extender (EYTG) lowered the amount of thiols in the cells substantially. The cystein, a sulfur-containing amino acid, possesses reactivity owing to thiol group oxidation. As definite proportion of disulphide bridge and thiol group is required for optimal operation. As oxidative stress oxidizes free thiol and it prevents the formation of disulphide bonds. Sperm motility is reduced in a time and dosage dependent way as a result of oxidative stress. Furthermore, it was discovered that thiol oxidation by hydrogen peroxide can inactivate glyceraldehyde 3-phosphate dehydrogenase, a critical enzyme in the glycolytic system (Tossounian *et al*., 2020). Oxidative stress-induced infertility cases would help in identifying alterations in the protein expression and/or translational modifications that may occur during sperm maturation and the functions of proteins involved.

### 2.11.2: Protein alterations in spermatozoa as a result of reactive oxygen species

Significant redox-dependent protein alterations such as thiol oxidation (Liu and O'Flaherty, 2017), tyrosine nitration (Morielli and O'Flaherty, 2015), and S-glutathionylation (Morielli and O'Flaherty, 2015) have been linked to sperm dysfunction and changes in the paternal genome thus resulting in infertility (**Fig. 2.2**).

#### 2.11.2.1: S-Glutathionylation

S-glutathionylation modifies protein function, interactions, and localization in a variety of physiological processes, and its pathological function. Protein S-

glutathionylation is a post-translational modification that happens both under normal and oxidative stress circumstances (Morielli and O'Flaherty, 2015). Glutathione-containing spermatozoa provide a protective mechanism against oxidative damage caused by $H_2O_2$. Protein S- glutathionylation only occur when reduced glutathione (GSH) react with SH group and resulting in enzyme inactivation. Sulfenic, sulfinic, and sulfonic acids are formed when cysteine residues in proteins are oxidised. Protein sulfenic and sulfinic acids can be reduced or conjugated to GSH to generate S-glutathionylated proteins, either enzymatically or nonenzymatically, using glutathione S-transferases, Glutaredoxins (Grx). Higher quantities of glutathionylated proteins were found in spermatozoa treated with $H_2O_2$ than in untreated controls (Morielli and O'Flaherty, 2015). Glutathione peroxidases carry out GSH's antioxidant activities, reducing hydrogen peroxide and lipid hydroperoxides by oxidizing GSH to oxidized glutathione (GSSG) (Toborek and Hennig, 1994). ROS and modulators such as glutaredoxins and glutathione transferases can target cysteine thiols (-SH) of proteins in particular. Protein glutathionylation is thought to be a protective process against oxidative stress. Nonenzymatic and enzymatic reactions can both lead to glutathionylation. The availability of GSH/GSSG is required for nonenzymatic glutathionylation. Glutathionylation in target proteins can be easily reversed by releasing GSH from cysteine residues (Cha *et al*., 2017).

### 2.11.2.2: S- Nitrosylation and tyrosine nitration

The majority of nitrosative protein is formed by nitration and nitrosylation of the side chains of tyrosine, tryptophan, methionine, and cysteine. Excess or unregulated nitric oxide reacts with ROS, resulting in an increase in reactive nitrogen species (Yeo *et al*., 2008). 3-nitrotyrosine in proteins is a byproduct of the reactions of nitric oxide-derived oxidants, which are commonly linked with oxidative stress (Batthyány *et al*., 2017). Nitrogen oxygen species cause tyrosine nitration, a protein alteration. High concentrations of various ROS cause an increase in tyrosine nitration in spermatozoa, researchers used peroxides ($H_2O_2$ or tert-BHP) and DA-NONOate (NO donor) to produce a dose-dependent increase in S-glutathionylation and tyrosine nitration (O'Flaherty and Fournier, 2017).

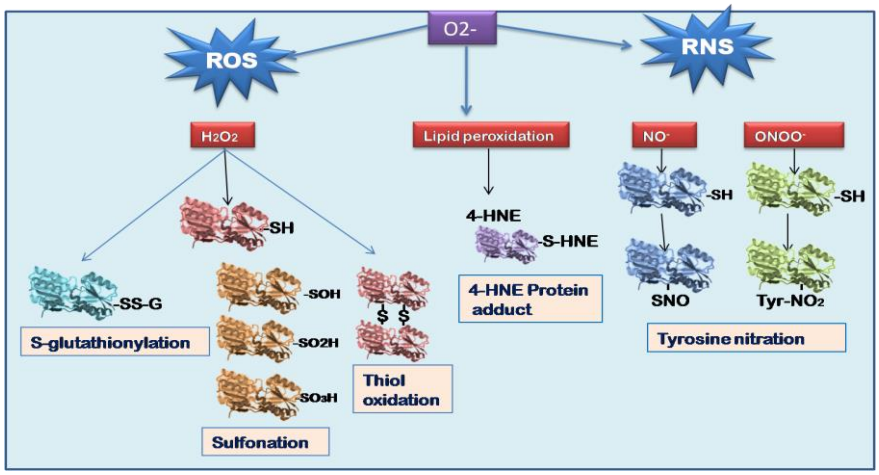



**Figure 2.2**: Redox-dependent protein modifications

### 2.11.3: Proteomic alterations due to oxidative stress in relation to fertility

Despite the well-established relevance of oxidative stress in the aetiology of male infertility, there have been few studies that have used proteome analysis to study the relationship between ROS-induced oxidative stress and a distinct protein expression profile in bovine ejaculate. Previous studies revealing that heat shock protein family A (Hsp70) member 2/arylsulfatase A/sperm adhesion molecule 1 protein complex (HSPA2/ARSA/SPAM1), which is required for sperm-egg identification, are the prime illustration of an oxidatively induced loss of sperm function (Gibb *et al*., 2020). Sharma *et al*. (2013) identified 14 proteins: seven that were found in both ROS+ and ROS samples, three that were only found in ROS samples (fibronectin I isoform 3 preprotein (FN1), macrophage migration inhibitory factor-1 peptide (MIF), and galectin 3 binding protein (G3BP), and four that were only found in ROS+ samples (cystatin S precursor, albumin preprotein, lactotransferrin precursor 1 peptide and prostate-specific antigen isoform 4 preprotein) in seminal fluid from men exhibiting oxidative stress. DJ-1, a protein specifically reported to be involved in the modulation of oxidative stress in Asthenozoospermic men seminal plasma (Wang *et al*., 2009) as DJ-1 self-oxidizes three cysteine residues to combat ROS (Yasuda *et al*., 2013) hence it aids in the management of oxidative stress and protects the sperm cell from the harmful effects of high ROS levels. PSA isoform 1 preprotein was found to be down-regulated in ROS+ patients, whilst PSA isoform 4 preprotein was shown to be exclusive to ROS+ samples (Sharma *et al*., 2013). Aslam *et al*. (2018) indicated that ENO1 is overexpressed in high fertile bull, it help to

protect sperm against oxidative stress. Wang *et al*. (2009) illustrate that Clusterin expression was shown to be greater in asthenozoospermic patients with higher ROS levels. Sharma *et al*. (2013) found down-regulation of clusterin preprotein in seminal ejaculates with increased ROS levels. Semen samples from 11 infertile idiopathic oligoasthenoteratozoospermia patients were used in a study by Herwig *et al*. (2013), which revealed that highly expressed 24 proteins in ROS+ patients were involved in biological processes focussed on metabolism, inflammation, immunity, and stress response. Only the glycerolipid metabolism pathway was shown to be enriched in pathway analysis of the proteins found exclusively.

Summary of different sperm protein expression in relation to oxidative stress mentioned in **(Table 2.3).** Hamada *et al*. (2013) studied that major proteins that are highly expressed in spermatozoa in semen samples with OS include A-kinase anchor protein 4 (AKAP4), Heat shock protein HSP 90-beta (HSP 90), and endoplasmin. DJ-1, TBCB (tubulin-folding cofactor B), AACT (anti-a1-antichymotrypsin), ALDR (alcohol dehydrogenase), DGK (diacylglycerol kinase), and PIP are overexpressed while DJ-1, underexpressed in seminal plasma with high oxidative stress seminal plasma (Agarwal *et al*., 2014).Yoon *et al*. (2016) reported that there were nine proteins that were differentially expressed before and after cryopreservation of bull sperm out of these nine, two proteins [Outer dense fiber protein 2(ODF2) and (LOC616410)] decreased and seven proteins Superoxide dismutase (SOD2), F-actin-capping protein subunit beta (CAPZB), NADH dehydrogenase flavoprotein 2(NDUFV2), Nucleoside diphosphate kinase (NDPK), (TPI), F1-ATPase, and AKAP increased after cryopreservation and they found that ROS may have caused an increase in NDPK.

Various protein markers that may be suggestive of oxidative stress-induced male infertility are given in **Table 2.4**

**Table 2.3: Proteins expression in semen samples with oxidative stress compared to semen samples without oxidative stress (Human being)**

| Protein | Expression | Reference |
|---|---|---|
| AKAP4 | Overexpressed | Hamada *et al*., 2013 |
| HSP90AB1 or HSP90B | Overexpressed | |
| HSP90B1 | Overexpressed | Hamada *et al*., 2013; Sharma *et al*., 2013 |
| GAPDH, GAPDHS, ALDOA, MDH2, TPI1, HIST1H2BA, HSPA5, GLUL, OLFM4, SPACA4, APMAP, C20orf3, TGM4, GPX4, LDHC | Overexpressed | Sharma *et al*., 2013 |
| PRDX1 | Under expressed | Hamada *et al*., 2013 |
| SEMG2, PRDX6, CLTC, EEF2, ENO1, ACE, ODF1, PGK2, HSPB1 | Under expressed | Sharma *et al*., 2013 |

**Table 2.4: Candidate protein biomarkers that may be indicative of oxidative stress-induced male infertility (Human being)**

| Reported protein biomarker | Reference |
|---|---|
| Seminal plasma DJ-1 | Wang *et al*., 2009 |
| Tubulin-folding cofactor B (TBCB)<br>Alpha-1- antichymotrypsin (AACT)<br>Aldose reductase (ALDR)<br>Diacylglycerol kinase (DGK) | Herwig *et al*., 2013 |
| Spermatozoa Lactotransferrin-2<br>Peroxiredoxin-1 (PRDX1) | Hamada *et al*., 2013 |
| Prolactin-inducible protein (PIP)<br>Histone cluster 1, H2ba (HIST1H2BA)<br>Malate dehydrogenase 2, NAD (mitochondrial) (MDH2) precursor<br>Transglutaminase 4 (TGM4)<br>Glutathione peroxidase 4 (GPX4) isoform A precursor<br>Glutamate-ammonia ligase (GLUL)<br>Heat shock protein 90 kDa beta (HSP90B1)<br>Heat shock protein 70 kDa protein 5 (HSPA5) | Sharma *et al*., 2013 |

# Chapter - 3

# Materials & Methods

*Chapter 3*

# MATERIALS AND METHODS

The present study was conducted at the Theriogenology laboratory, Southern Regional Station of Indian Council of Agriculture Research- National Dairy Research Institute, Adugodi, Bengaluru. The study was duly approved by the Institute Animal Ethics Committee (Approval number CPCSEA/IAEC/LA/SRS-ICAR-NDRI-2019/ NO. 04). The study consisted of three objectives,

1. Assessment of cellular and functional phenomes of bull spermatozoa before and after induction of oxidative stress
2. Comparative proteomic profiling of normal and oxidative stress induced bull spermatozoa
3. Identification of potential proteins that could serve as biomarkers for oxidative stress in bull spermatozoa

## 3.1: Experimental animals

The present investigation was conducted on Zebu (*Bos indicus)* bulls of Deoni breed maintained at the Livestock Research Centre, SRS of ICAR-NDRI, Adugodi, Bengaluru, India. Experimental bulls had qualified the breeding soundness evaluation and were routinely used for artificial breeding. The age of bulls ranged between 5 and 8 years. The experimental bulls were housed in individual pens under a loose housing system. Vaccination, deworming, diagnostic tests for communicable diseases and other herd health programs were done as per the standard practices of the farm.

## 3.2: Semen collection and sample preparation

The present study utilized semen ejaculates from three bulls (n=6 ejaculates/bull). The bulls were properly washed and dried before brought to the collection site. Ejaculates were collected using Danish model standard artificial vagina (IMV Technologies, France) with a smooth neoprene liner (IMV Technologies, France) twice a week by allowing the bull to mount over a male teaser bull. The temperature of AV was maintained between 42-45 °C with sufficient pressure and non-spermicidal KY jelly was used as lubricant. Two ejaculates were obtained on a

day with a minimum gap of 15 to 30 min between ejaculates; each collection was preceded by a period of sexual preparation that included at least two false mounts separated by a one-minute restrain. Immediately after collection, each ejaculate was placed in a water bath at a temperature of 37 ˚C and assessed for quality parameters.

### 3.3: Routine semen analysis

Mass activity was assessed by placing a drop (10 µL) of neat semen on prewarmed slide (37 °C) without applying coverslip and observed at 10x on DIC (differential interference contrast) phase contrast microscope (Nikon eclipse E600). Mass activity was scored in 0-5 scale as per the method described by Tomar and Singh (1996). Concentration was estimated by using haemocytometer. Initial progressive motility was evaluated by placing a thin drop of neat semen diluted in Tyrode's Albumin Lactate Pyruvate medium (SP-TALP; 3.1mM KCl, 100 mM NaCl, 0.29 mM $NaH_2PO_4$, 25 mM $NaHCO_3$, 2.0 mM $CaCl_2$, 21.6 mM $C_3H_5NaO_3$, and 1.5 mM $MgCl_2$) on a prewarmed clean glass slide. The drop was allowed to spread uniformly under the cover slip (18 x 18mm). Motility was assessed under 20x magnification in phase contrast microscope with thermostatically controlled warm stage. Average of five representative area of slide was evaluated and percentage of progressive motile sperm was recorded. All the ejaculates used in the study had mass activity >+3, a minimum 500 million/ml concentration and ≥70 % progressive motility.

### 3.4: Standardization of $H_2O_2$ dose for inducing oxidative stress in spermatozoa

The sperm and seminal plasma of fresh semen were separated by centrifugation (800 x g for 8 min) and sperm pellets washed with SP-TALP. Hydrogen peroxide ($H_2O_2$) (Thermo Scientific$^{TM}$) was used to induce oxidative stress. Thirty percent stock solution of $H_2O_2$ was diluted to 10 µM, 25 µM, 50 µM and 100 µM (final concentration) with SP-TALP. We took 100 million sperm in one ml of each working solution and incubated at 37°C for 0 hr, 0.5 hr and 1 hr. At least three aliquots of samples were used for each concentration of $H_2O_2$ (n=3 ejaculates/bull). Sperm suspension without the addition of $H_2O_2$ served as a control. After incubation of sperm with $H_2O_2$, samples were centrifuged at 800 x g for 8 min; supernatant was

removed. The pellet obtained in both the groups, homogenized on ice to prepare cell lysate. The Thiobarbituric Acid Reactive Substances (TBARS) Assay Kit (Cayman, Item No. 10009055) was used to measure oxidative stress (lipid peroxidation). This method indirectly measures the amount of sperm oxidative stress by analyzing the concentration of MDA.

### 3.4.1: Calorimetric standard preparation

A stock solution of 125 µM was prepared by diluting 250 µl of the MDA Standard (Item No. 10009202) with 750 µl of Milli-Q® water. Clean glass test tubes (eight) were taken and labelled as A-H. Stock solution (125 µM MDA) and water is added to each tube as described in **Table 3.1**.

**Table 3.1:** MDA colorimetric standards

| Tube | MDA (µl) | Water (µl) | MDA Concentration (µM) |
|---|---|---|---|
| A | 0 | 1,000 | 0 |
| B | 5 | 995 | 0.625 |
| C | 10 | 990 | 1.25 |
| D | 20 | 980 | 2.5 |
| E | 40 | 960 | 5 |
| F | 80 | 920 | 10 |
| G | 200 | 800 | 25 |
| H | 400 | 600 | 50 |

### 3.4.2: TBARS Assay Procedure

Cell lysate of 100 µl or standard was added to appropriately labeled 5 ml tubes. Then, 100 µl of Sodium Dodecyl Sulphate (SDS) solution was added to tubes and swirled to mix them properly. Color reagent (4 ml) was carefully poured down along the edge of each tube. Then the tubes were placed in a holder to keep them upright during boiling for one hour. After one hour, tubes were immediately removed and placed in ice bath to stop the reaction. Incubation of tubes in ice was done for 10 min. After 10 min, tubes were centrifuged for 10 min at 1,600 x g at 4 °C. Then 150 µl (in duplicate) was added from each tube to the clear plate (colorimetric version), followed by measurement of absorbance at 530-540 nm (Thermo Scientific Multiskan GO Microplate Spectrophotometer; model no. 51119200).

The results were compared to a standard curve prepared with a standard solution of malondialdehyde (MDA). Among the different levels of $H_2O_2$, the 50µM

$H_2O_2$ incorporated group had significantly higher malondialdehyde (MDA) level than the other groups. Based on these results, we selected the dose and time of incubation for $H_2O_2$ i.e. 50 µM for 1 h for further experiments.

### 3.5: Evaluation of sperm functional attributes

A total of 18 ejaculates (six ejaculates per bull) were subjected to flow cytometric analysis. Each ejaculate was divided into three aliquots for further processing. The first group served as a control, which contained fresh spermatozoa without $H_2O_2$. In the second group, seminal plasma was removed by centrifugation at 800 x g for 8 min; the sperm pellet was washed using SP-TALP. Approximately 100 million washed sperm were suspended in 500 µL of SP-TALP, to which 50 µM of $H_2O_2$ was added and incubated for one hour. In the third group, 50 µM $H_2O_2$ was added to spermatozoa with seminal plasma and incubated for one hour.

Sample from all the three groups were subjected to flow cytometric assessment for sperm functional attributes including sperm acrosome integrity, plasma membrane integrity, mitochondrial superoxide, mitochondrial membrane potential, lipid peroxidation and sperm intracellular calcium status. Flow cytometric analyses were performed using a CytoFLEX S flow cytometer (Beckman Coulter Life Sciences, Indianapolis, IN). A total of 10,000 spermatozoa were assessed for each sample. Acquisition and analyses were made using CytExpert software (Beckman Coulter).

#### 3.5.1: Sperm Viability

Sperm viability was assessed using fluorochromes SYBR-14 (Invitrogen™, Thermo Fisher Scientific, USA) and Propidium Iodide (Invitrogen™, Thermo Fisher Scientific, USA) as described by Kumaresan *et al*. (2017). Briefly, 1 µL of SYBR-14 working solution (1:50 in DMSO) was added to 2 million spermatozoa in 300 µL of SP-TALP and incubated at 37$^{o}$C for 10 min in the dark. Further, 1 µL of propidium iodide (2.4 mM) was added and incubated for 2 min before assessing in flow cytometer, and excitation was done by blue laser (488 nm). Fluorescence emitted was observed in fluorescence channel (FL) 1 (BP 525/40 nm), and PI fluorescence was measured in FL2 (BP 585/42 nm) and the resultant population were divided into live, moribund and dead spermatozoa.

### 3.5.2: Sperm Acrosome Integrity

Sperm acrosome integrity was assessed using Peanut Agglutinin (PNA) tagged with fluorochrome fluorescein isothiocyanate (FITC) (Invitrogen™, Thermo Fisher Scientific, USA) as described by Kumaresan *et al*. (2017) with minor modifications. Briefly, 1 µL of FITC-PNA (1 mg/ml) was added to 2 million spermatozoa in 300 µL of SP-TALP and incubated in the dark for 10 min at 37ºC. To the sperm suspension, 1 µL of propidium iodide (2.4 mM) was added and further incubated for 2 min prior to sample acquisition in flow cytometer. The fluorochrome was excited by blue laser (488 nm), and the fluorescence emitted was detected in FL 1 (BP 525/40 nm) and PI fluorescence was measured in FL2 (BP 585/42 nm). The percentage of live and dead sperm population with intact and reacted acrosome were assessed.

### 3.5.3: Mitochondrial Membrane Potential

Mitochondrial membrane status of spermatozoa was assessed using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbo-cyanine iodide (JC-1) fluorochrome (Invitrogen™, Thermo Fischer Scientific, USA) as described by Mocé and Graham (2008) with minor modifications. Briefly, 1 µL of 0.2 mM JC-1 solution was added to 2 million spermatozoa in 300 µL of SP-TALP and incubated for 30 min at 37ºC in the dark. The fluorochrome was excited by blue laser (488 nm). The fluorescence emitted from JC1 monomer (green) was detected in FL1 (BP 525/40 nm) and fluorescence emitted from JC1 aggregate (orange red) was detected in FL2 (BP 585/42 nm) and the resultant population were divided into two types viz. sperm with high MMP and sperm with low MMP.

### 3.5.4: Intracellular Calcium Level

Intracellular calcium concentration in spermatozoa was assessed using Fluo 3 AM (Invitrogen™, Thermo Fischer Scientific, USA) as described by Nag *et al*. (2021). Briefly, 3 µL of Fluo 3 AM (1 mM) was added to 2 million spermatozoa in 300 µL of SP-TALP was added and incubated in the dark at 37ºC for 30 min. After incubation, 1 µL of PI (2.4 mM) was added to the samples and incubated in the dark at 37ºC for 2 min and analyzed in the flow cytometer. The fluorochrome was excited by blue

laser (488 nm). The fluorescence emitted from Fluo 3 AM and PI was detected in FL 1 (BP 525/40 nm) and FL 2 (BP 585/40 nm) respectively. The resultant population was divided into live sperm with low calcium, live sperm with high calcium, dead sperm with low calcium and dead sperm with high calcium.

### 3.5.5: Mitochondrial Superoxide Level

Mitochondrial superoxide level of sperm was measured by using MitoSOX™ Red (Thermo Fisher Scientific, USA, M36008) as previously done by Kumaresan *et al*. (2017) with little modifications. Briefly, 1 µL of 500 µM MitoSOX™ Red solution was added to 2 million sperm in 300 µL SP-TALP. The samples were incubated in the dark at 37°C for 20 minutes and analyzed in flow cytometer. After excitation with blue laser (480 nm), the fluorescence was measured in the FL 2 (BP 585/42 nm) channel. The resultant population was divided into sperm with high ROS and low ROS.

### 3.5.6: Lipid Peroxidation

Sperm membrane lipid peroxidation was measured with the fluorescent probe BODIPY™ 581/591 C11 (Thermo Fisher Scientific, USA, D3861) in bovine sperm as described by De Andrade *et al*. (2012) with little modifications. Briefly, 0.5 µL of 2mM (1 mg/mL) fluorochrome was added to 2 million spermatozoa in 300 µL SP-TALP. The samples were incubated in the dark at 37°C for 30 minutes and analyzed in flow cytometer. The fluorochrome was excited using a blue laser (488 nm) and the emitted fluorescence was detected in FL 3 (690/50 nm), and the population were categorized into LPO positive and LPO negative.

## 3.6: Oxidative stress induced proteomic alterations in bull spermatozoa

Six ejaculates from three *Deoni* breeding bulls were utilized for proteomic studies. Each ejaculate was divided into three equal aliquots. Seminal plasma was removed from all the ejaculates by centrifugation at 800 x g for 8 min; supernatant were collected and sperm pellet was washed using SP-TALP. Purification of spermatozoa was done as per the procedure describe by Peddinti et al. (2008), i.e. sperm pellets were purified using 90-45% discontinuous percoll gradient centrifugation by adding 0.9 ml of 45% of percoll fraction in SP-TALP over 0.2 ml of

90% fraction in 1.5 ml micro centrifuge tube. The sperm were carefully layered over the top of prepared percoll gradient fraction. Spermatozoa than pelleted by centrifugation at 950 x g for 15 min at room temperature to obtain sperm pellet. The first group served as a control, which contained sperm pellet suspended in 500 µL of SP-TALP without $H_2O_2$. In second group sperm were suspended in 500 µL of SP-TALP, to which 50 µM of $H_2O_2$ was added and incubated for one hour. In the third group, spermatozoa with seminal plasma and 50 µM of $H_2O_2$ was incubated for one hour, followed by a wash to remove remaining $H_2O_2$ and stored at -20˚C with 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich, USA) until further use for proteomic analysis as shown in the **Fig. 3.1** below.

### 3.6.1: Sample preparation

Each sample's 25 µg protein was reduced with 5 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), alkylated with 50 mM iodoacetamide, and then digested with Trypsin (1:50 Trypsin/lysate ratio) for 16 hours at 37°C. To eliminate the salt, digests were cleaned with a C18 silica cartridge and dried with a speed vac. The dried pellet was resuspended in buffer A (5 % acetonitrile, 0.1 % formic acid).

### 3.6.2: Mass Spectrometric Analysis of Peptide Mixtures

Experiments were carried out using an Ultimate 3000 RSLCnano system in conjunction with a Thermo QE Plus. 1ug of C18 column 50 cm, 3.0µm Easy-spray column was loaded (Thermo Fisher Scientific). Peptides were eluted at a flow rate of 300 nl/min using a 0-40% gradient of buffer B (80 % acetonitrile, 0.1 % formic acid) and injected for MS analysis. For 100 minutes, LC gradients were run. MS1 spectra were collected in the Orbitrap at a resolution of 70k. For 10 seconds, dynamic exclusion was used to exclude all charge states for a given precursor. MS2 spectra were collected at a resolution of 17500.

### 3.6.3: Data Processing

All samples were processed, and the resulting RAW files were analyzed using Proteome Discoverer (v2.4) against the Uniprot reference proteome database. The precursor and fragment mass limitations for the Sequest and Amanda searches were established at 10 ppm and 0.5 Da, respectively. The protease utilized to create the

peptides, i.e. the enzyme specificity, was set to trypsin/P (cleavage at the C terminus of "K/R: unless followed by "P") with a maximum missed cleavages value of two. Carbamidomethyl on cysteine was regarded a permanent modification, while methionine oxidation and N-terminal acetylation were considered variable modifications for database search. The peptide spectrum match and the protein FDR were set to 0.01 FDR.

### 3.6.4: Differential analysis

To identify the differentially expressed proteins (DEP's) statistical analysis were performed using the abundance values of each sample. Protein Abundance measurements were filtered based on their valid values. Log2 transformation of abundance ratios were calculated followed by Z-score standardization. To compare the means of two groups the student T-Test was utilized and statistical significance was considered with a P values less than or equal to 0.05. The Benjamini Hochberg FDR (cut-off = 0.05) is used to calculate significance. Based on the observed abundance values the significant ($p<0.05$) proteins were then employed for bioinformatics data visualization like volcano plot, PCA, Scree plot and heatmap were generated using in-house R programming scripts.

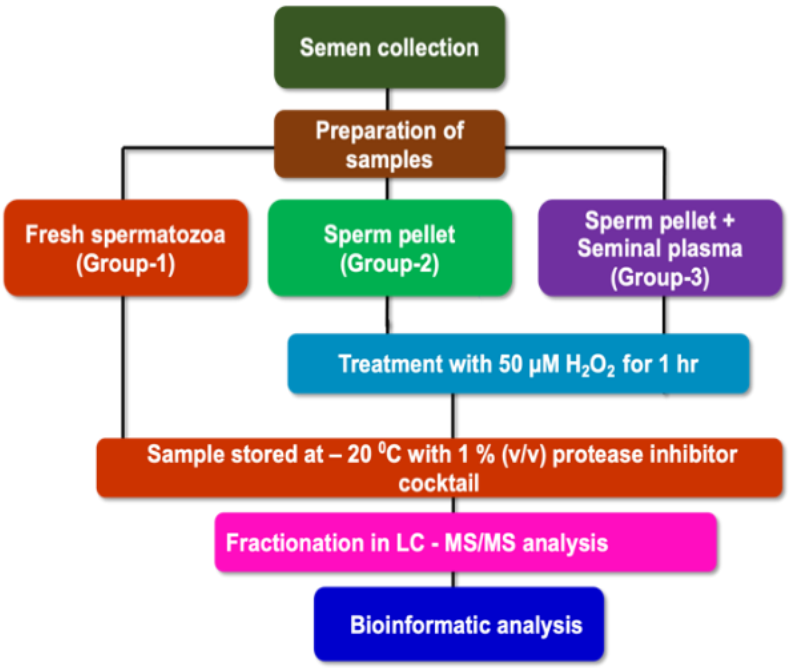



**Figure 3.1:** Workflow of sample collection, processing and proteomic analysis

### 3.6.5: Gene ontology and pathway analysis of proteins

The functional annotation of all the differentially expressed proteins was performed using advanced bioinformatics tools available online. Web sources like Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.8 (http://david.abcc.ncifcrf.gov) was utilized for functional annotation and pathways analysis. Gene names were uploaded in the above-mentioned software for gene ontology (GO) analysis. Further, Cytoscape 3.7.1, an open source software platform with ClueGO plugin was used for the visualization of protein-protein interaction networks and biological pathways at the molecular level.

### 3.7: Statistical analysis

All the statistical analysis was performed using GraphPad Prism V8.4.3. Mixed effect model ANOVA was used to assess the differences in sperm functional attributes between control and treated samples. All the differences were considered significant when $p \leq 0.05$.

# Chapter - 4

# Results & Discussion

*Chapter 4*

# RESULTS AND DISCUSSION

The current study aimed to detect changes in sperm proteome and functional attributes due to oxidative stress in order to better understand the underlying processes of poor fertility in males with oxidative stress. For that we compared the global protein profile of fresh spermatozoa with that of protein profile of oxidative stress induced spermatozoa. Also, we compared the cellular and functional phenomes of bull spermatozoa before and after oxidative stress induction, to the best of our knowledge, this is the first study to identify the proteomic changes in *Bos indicus* bull spermatozoa due to oxidative stress.

## 4.1: Objective-1: Assessment of cellular and functional phenomes of bull spermatozoa before and after induction of oxidative stress

### 4.1.1: Standardization of dose of Hydrogen peroxide for induction of oxidative stress

Since it has been shown that $H_2O_2$ induce oxidative stress in spermatozoa, we expose *Bos indicus* spermatozoa with different concentrations of $H_2O_2$ to find out optimum concentration to induce oxidative stress to spermatozoa. The level of oxidative stress was measured by MDA levels using TBARS assay. The results of the experiment revealed that MDA levels in fresh spermatozoa (Control) was 0.361±0.02µM/$10^9$ spermatozoa, while after 30 min in 10µM, 25µM, 50µM and 100µM of $H_2O_2$, it was 0.376±0.03, 0.382±0.08, 0.397±0.07 and 0.388±0.04 µM/$10^9$ spermatozoa respectively. After 60 min of incubation, lipid peroxidation in 10µM, 25µM, 50µM and 100µM of $H_2O_2$ incubated groups was, 0.462±0.04, 0.449±0.02, 0.842±0.07 and 0.572±0.06µM/$10^9$ spermatozoa respectively **(Fig. 4.1)**. Our results confirmed that among the different doses of $H_2O_2$ concentration (10µM, 25µM, 50µM and 100µM) the 50µM $H_2O_2$ group had significantly ($p<0.05$) higher malondialdehyde (MDA) level at 60 minutes when compared with the other groups. Based on our finding we fixed 50µM concentration of $H_2O_2$ at 60 min duration of incubation with spermatozoa for further studies.

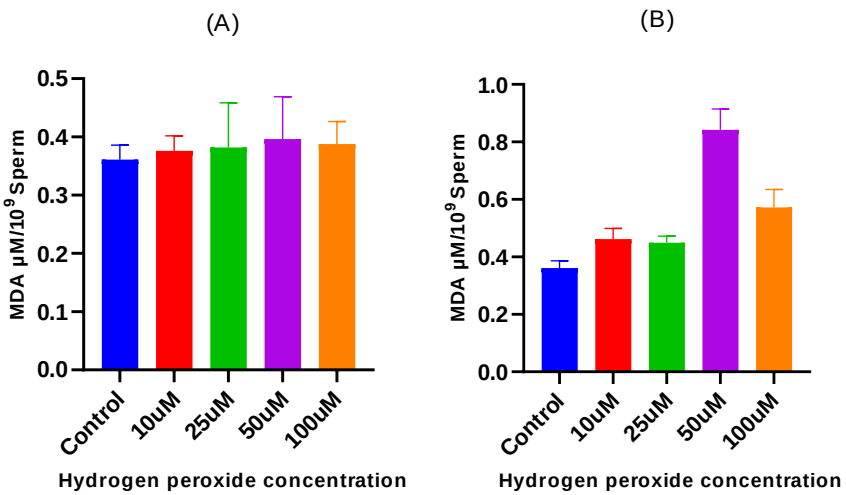



**Figure 4.1:** Lipid peroxidation levels (MDA) in Zebu spermatozoa after exposing to H2O2 at 30 minutes (A) and at 60 minutes (B)

Similar to this, Garg *et al*. (2009) reported that fresh buffalo sperm incubated at 50µM $H_2O_2$ for 60 minutes at 37°C produce highest lipid peroxidation. In canines, Lucio *et al*. (2016) measured MDA concentration using TBARS assay and found that spermatozoa that were refrigerated, glycerolized and thawed had MDA concentrations of 0.411, 0.448 and 0.609 µM/$10^9$, further, concluded that the formation of free radicals promotes oxidative stress.

### 4.1.2: Effect of oxidative stress on sperm functional attributes

Sperm functional attributes play an important role in deciding the fertilizing potential of a sperm. We incubated spermatozoa with $H_2O_2$ and evaluated the vital sperm attributes such as viability, acrosome integrity, intracellular calcium, mitochondrial membrane potential, lipid peroxidation and mitochondrial superoxide production using flow cytometry. The results and their possible explanation are discussed below **(Fig. 4.2)**.

#### 4.1.2.1: Sperm Viability

Sperm viability was evaluated in control and the treatment groups. The treatment groups include the sperm without seminal plasma (seminal plasma negative/SP-ve) and the sperm with seminal plasma (seminal plasma positive/SP+ve) group. The percentage of live and moribund sperm did not differ significantly between control and treatment groups.

However, dead sperm count was significantly ($p<0.05$) higher in the treatment group containing seminal plasma (34.70 ± 3.9%) compared to control group (21.41 ± 1.9%). The significance of dead spermatozoa is that they potentially act as source of ROS, through an aromatic amino acid oxidase-catalyzed process and further damage other live sperm. According to Bansal and Bilaspuri (2011), excessive oxidative stress directly alters the plasma membrane integrity of spermatozoa by promoting lipid peroxidation. Mahfouz *et al*. (2010) also reported that acute exposure of spermatozoa to $H_2O_2$ results in increased percentage of dead spermatozoa. Our findings are in agreement with the previous study of De la Rosa *et al*. (2006) who also reported $H_2O_2$ induced cell death.

#### 4.1.2.2: Acrosome integrity

In the present study, we did not observe any significant effect of $H_2O_2$ exposure on sperm acrosome reaction status. It has been reported that high level of ROS can promote the acrosome reaction with the mechanism of ROS-modulated tyrosine phosphorylation (Wagner *et al*., 2018). We observed that Live acrosome intact population in control group was (53.68±2.44%) higher than the SP+ve (44.27 ± 2.89%) and SP-ve (49.65± 3.08%) groups. Baumber *et al*. (2000) also reported that increased reactive oxygen species lead to decrease in acrosomal integrity with increased lipid peroxidation. The oxidative stress is positively correlated with acrosome abnormalities and have a deleterious influence on fertility (de Lamirande and O'Flaherty, 2008). The species difference of the dose of $H_2O_2$ used might be a reason for difference in findings among different studies.

#### 4.1.2.3: Mitochondrial membrane potential

Our study found no significant difference between control and treatment groups in terms of sperm MMP. It is indicating that oxidative stress might not have a significant effect on mitochondrial membrane potential. Our results are in contrast to prior findings that reported a decline in sperm mitochondrial activity associated with rise in free radical generation (Kadirvel *et al*., 2009; Lucio *et al*., 2016).

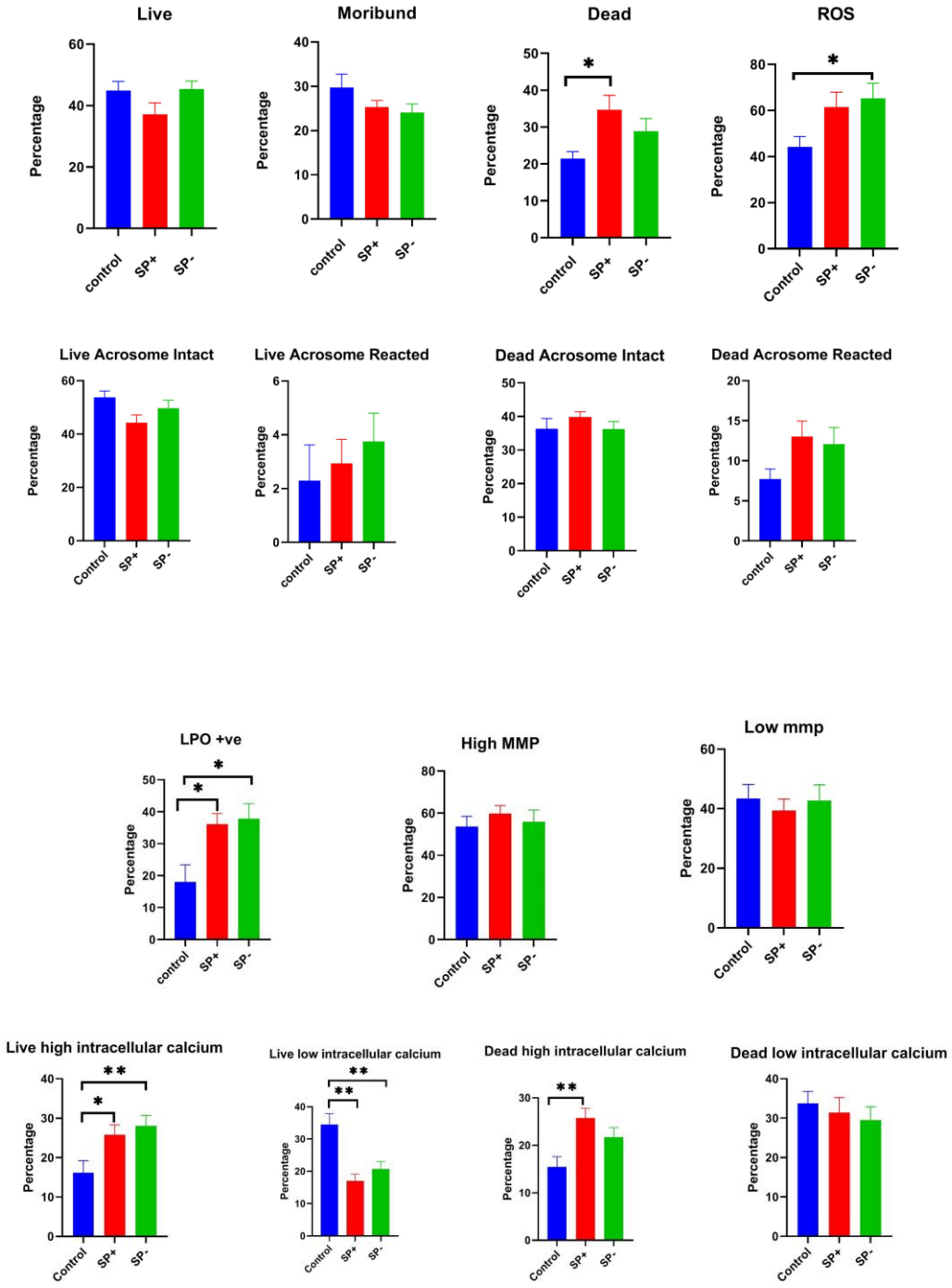



**Figure 4.2:** Sperm functional attributes after induction of oxidative stress in Zebu bull spermatozoa (Control) = no $H_2O_2$, (SP+) = Sperm incubated with $H_2O_2$ in the presence of seminal plasma and (SP-) =Sperm incubated with $H_2O_2$ in the absence of seminal plasma

### 4.1.2.4: Intracellular calcium

The percentage of live low intracellular calcium sperm was significantly ($p<0.01$) low in both the treatment groups (SP+ve: 16.99 ± 2.1%; SP-ve: 20.77 ± 2.2%), when compared to control group (34.54 ± 3.3%). The percentage of live high intracellular calcium sperm increased significantly in $H_2O_2$ treated groups ($p<0.01$) (SP+ve: 25.81 ± 2.5%; SP-ve: 28.05 ± 2.6%) when compared to control group (16.18 ± 3%). Dead high intracellular calcium population was also significantly ($p<0.05$) greater in SP+ve (25.73 ± 2%) when compared with the control group (15.49 ± 2.1%). Dead low intracellular calcium sperm population did not vary significantly. It is known that spermatozoa cannot fertilize oocyte without undergoing capacitation (Jin and Yang, 2017). The capacitation should happen in female reproductive tract immediately before fertilization. However, capacitation is deleterious if it is found in freshly ejaculated and frozen thawed sperm as capacitation-induced changes can compromise the life of sperm (Bucher *et al*., 2019). Many studies revealed that exogenous ROS addition (Xanthine oxidase, $H_2O_2$ and Nitric oxide) induces capacitation (Thundathil *et al*., 2003; O'Flaherty *et al*., 2006). Increased intracellular $Ca^{+2}$ level in sperm is the important indicator of capacitation (Jagannathan *et al*., 2002). The cryopreservation induced cryo-capacitation like changes in sperm happens through ROS production (Leahy and Gadella, 2011). The semen of low-fertile bulls contain high proportion of sperm with high intracellular calcium than the high-fertile bull semen (Collin *et al*., 2000). While some reports suggested that apoptotic cell death in acrosome-reacted sperm cells mediated by high intracellular calcium levels (Engel *et al*., 2018). Overall, our results indicate that oxidative stress induced premature capacitation like changes in Zebu bull spermatozoa.

### 4.1.2.5: Lipid peroxidation

The percentage of sperm cells positive for lipid peroxidation was significantly higher in $H_2O_2$ treated groups (SP +ve: 36.16 ± 3.3%; SP -ve: 37.8 ± 4.7%) as compared to control group (18.04 ± 5.4%). Reports from many studies highlighted the positive correlation between the sperm cells with high ROS levels and LPO (Kadirvel *et al*., 2009; Ahmed *et al*., 2018; Bassiri *et al*., 2020). Aitken *et al*. (2006) demonstrated the presence of PUFA induced ROS production and lipid peroxidation in spermatozoa. LPO may also cause an 'apoptotic-like' event, resulting in premature

ageing and a shorter life for processed spermatozoa (Ferrusola *et al*., 2009). Our results confirmed ROS induction increase lipid peroxidation in spermatozoa, which might have deleterious effect on sperm functions.

**4.1.2.6: Mitochondrial superoxide production / Mitochondrial ROS**

The proportion of sperm with high mitochondrial superoxide was significantly higher ($p<0.05$) in $H_2O_2$ treated group (SP-ve: 65.30 ± 6.5%) compared to control group (44.26 ± 4.4%). Tvrda *et al*. (2019) also reported that the exposure of bovine sperm to ferrous ascorbate (FeAA) lead to increased generation of ROS ($p<0.001$). However, there was no significant difference between SP+ve and control group. It could be possible that, various antioxidants present in seminal plasma might have reduced ROS generation in sperm. Potts *et al*. (2000) reported that presence of seminal plasma reduces oxidative damage to the sperm caused by external ROS. Our finding aligns with these previous studies as sperm ROS was not increased significantly when the sperm was with seminal plasma.

Collectively, flow cytometric analysis of sperm functional attributes revealed the significant increase in intracellular ROS and LPO levels leading to premature capacitation like changes (high intracellular $Ca^{+2}$) and increased dead spermatozoa population. All of these compromised sperm functions could be responsible for the oxidative stress induced low fertilizing ability in spermatozoa.

**4.2: Global proteomic profile of Deoni (Zebu) bull spermatozoa**

The present study utilized freshly ejaculated spermatozoa from three *Bos indicus* bulls. Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) technique was used to investigate proteins in fresh spermatozoa, A total of 1366, 1382 and 1379 proteins were found in fresh 1, fresh 2 and fresh 3 spermatozoa respectively as shown in **Fig. 4.3**. Among them 1302 proteins were found to be common in all the three biological replicates, However 21, 12 and 24 were found to be specific in fresh1, fresh 2 and fresh 3 respectively, while 29, 39 and 14 were uniquely common between fresh1, fresh 2 and fresh 3. Hence a total of 1441 proteins were obtained in fresh sample. Collective of 1,99,365 peptide spectrum matches (PSMs) and 11,409 peptides corresponding to 1,441 proteins in Zebu bull spermatozoa.

The molecular weight (MW) of different proteins detected in Zebu bull spermatozoa ranged from 4.1 to 770.8 kDa; a majority of proteins were belongs 100 kDa MW as represented in **Fig. 4.4 (A)**, while isoelectric point (pI) values ranged from 4.02 to 12.66 as shown in **Fig. 4.4 (B)**. Among all listed proteins (1,441), 955 were characterized according to the *UniProt* database, and 486 were defined as uncharacterized.

Out of the total proteins (1441) in fresh spermatozoa, to identify the significant proteins; three criteria viz. PSM >2, coverage ≥10 and unique peptide >2, were used and a total of 777 proteins were filtered out.

### 4.2.1: Functional analysis of Zebu bull sperm proteins using Gene Ontology (GO)

GO annotation was used to gain insight into the biological processes, cellular components and molecular functions related to proteins found in spermatozoa. The proteins identified were found to be involved in 141 biological processes, 97 cellular components and 118 molecular functions. The top 10 GO terms are depicted in **Fig. 4.5**.

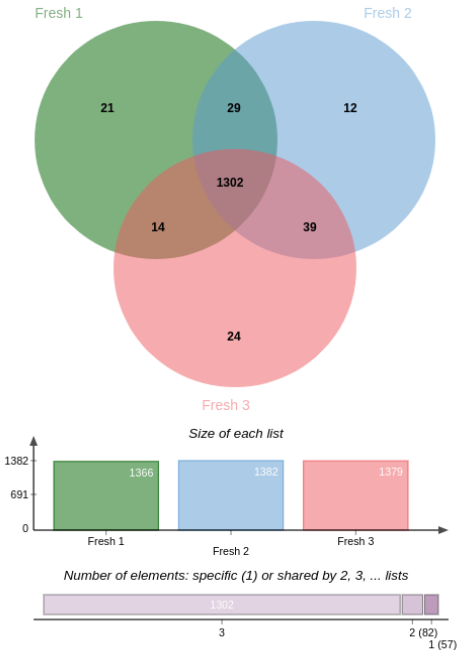
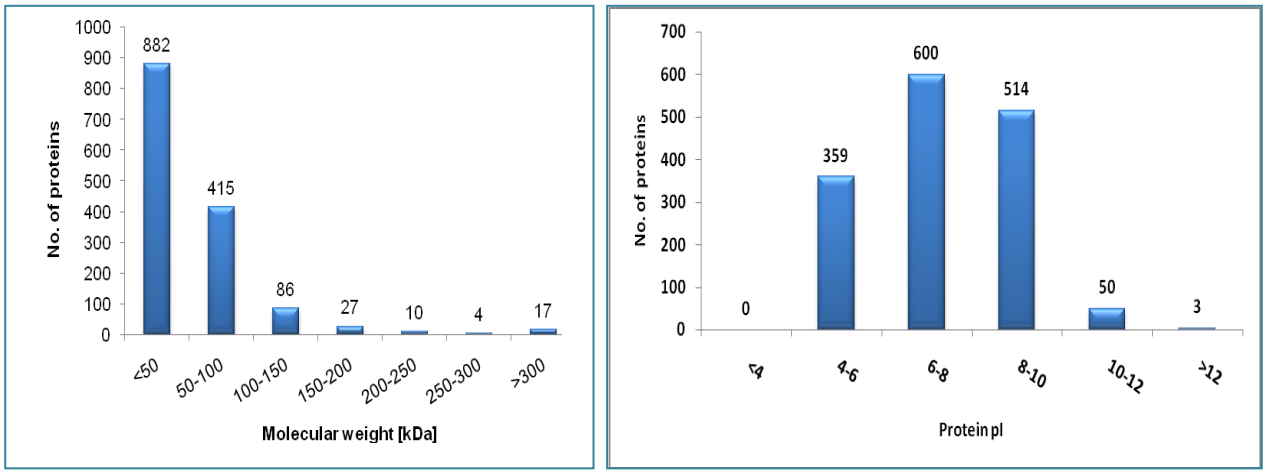



**Figure 4.3:** Venn diagram depicted the proteins indentified in freshly ejaculated spermatozoa of three Zebu bulls




**(A)** **(B)**

**Figure 4.4:** Distribution of proteins based on molecular weight (A) and isoelectric points (B) in freshly ejaculated spermatozoa of Zebu bulls

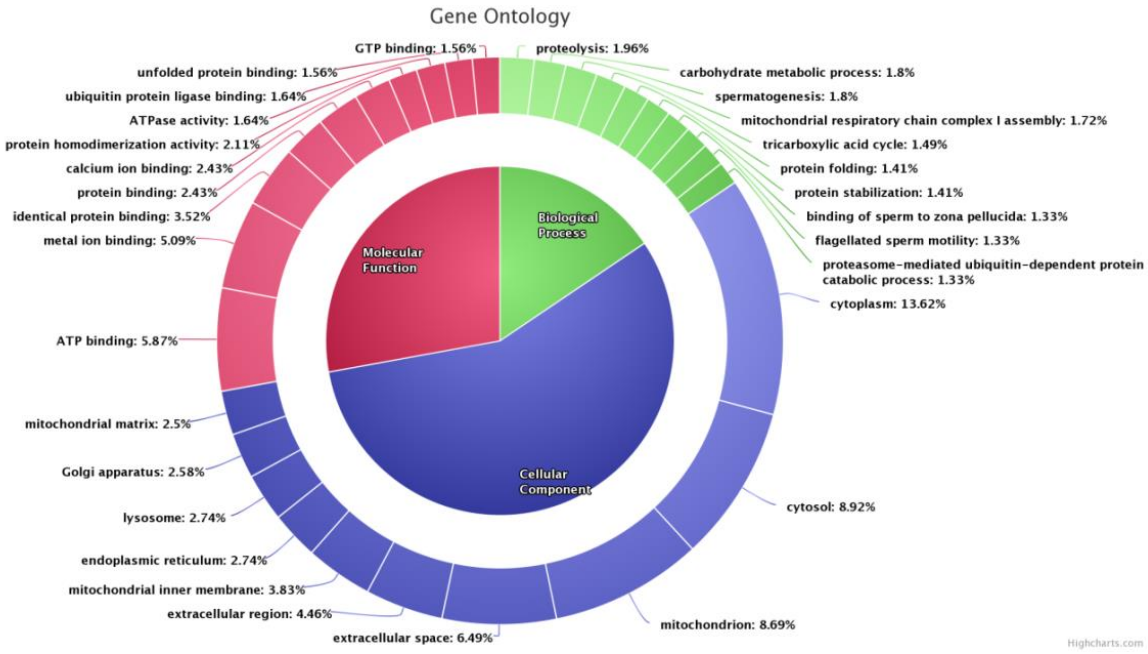



**Figure 4.5:** Top 10 gene ontology functional classification of proteins identified in freshly ejaculated spermatozoa of Zebu bulls

In the current study, the detected proteins were enriched in biological process like proteolysis (3.52%), carbohydrate metabolic process (3.23%), spermatogenesis (3.23%), mitochondrial respiratory chain complex I assembly (3.09%), tricarboxylic acid cycle (2.67%), protein folding (2.53%), protein stabilization (2.53%), binding of sperm to zona pellucida (2.39%), flagellated sperm motility (2.39%) and proteasome-mediated ubiquitin-dependent protein catabolic process (2.39%). The list of proteins involved in different biological processes is given in the **Table 4.1**.

The analysis also categorized the proteins with respect to cellular components such as cytoplasm (24.47%), cytosol (16.03%), mitochondrion (15.61%), extracellular space (11.67%), extracellular region (8.02%), mitochondrial inner membrane (6.89%), endoplasmic reticulum (4.92%), lysosome (4.92%), Golgi apparatus (4.64%) and mitochondrial matrix(4.50%).

The major molecular functions were related to ATP binding (10.55%), metal ion binding (9.14%), identical protein binding (6.33%), protein binding (4.36%), calcium ion binding (4.36%), protein homodimerization activity (3.80%), ATPase activity (2.95%), ubiquitin-protein ligase binding (2.95%), unfolded protein binding (2.81%) and GTP binding (2.81%) shown in **Table 4.2**.

**Table 4.1:** Top 10 Molecular functions enriched by sperm proteins

| S. No. | Molecular Function | Count | P Value | Percentage |
|---|---|---|---|---|
| 1 | ATP binding | 75 | 4.38E-06 | 10.55 |
| 2 | Metal ion binding | 65 | 0.020412 | 9.14 |
| 3 | Identical protein binding | 45 | 0.013199 | 6.33 |
| 4 | Protein binding | 31 | 1.02E-12 | 4.36 |
| 5 | Calcium ion binding | 31 | 0.016980 | 4.36 |
| 6 | Protein homodimerization activity | 27 | 0.031097 | 3.80 |
| 7 | ATPase activity | 21 | 7.09E-13 | 2.95 |
| 8 | Ubiquitin protein ligase binding | 21 | 6.71E-05 | 2.95 |
| 9 | Unfolded protein binding | 20 | 4.93E-11 | 2.81 |
| 10 | GTP binding | 20 | 0.040546 | 2.81 |

**Table 4.2:** Top 10 biological processes enriched by the sperm proteins

| S. No. | Biological Process | Percent | P Value | Genes |
|---|---|---|---|---|
| 1 | Proteolysis | 3.51 | 8.00E-08 | PRSS1, ENPEP, CPQ, PRCP, PARK7, CNDP2, DPP4, DNPEP, DPP7, CAPNS1, CTSD, CTSA, GGH, ASRGL1, PRSS54, ADAM2, AFG3L2, QPCT, TPP1, LAP3, UQCRC2, DLD, CFB, LGMN, LTF |
| 2 | Carbohydrate metabolic process | 3.23 | 5.00E-10 | MANBA, MDH1, B3GAT3, IDUA, MDH2, HEXB, LANCL1, HEXA, SLC3A2, SMRP1, LDHC, CS, GANAB, LDHA, GLB1L, GLB1, LANCL2, GUSB, SIAE, FBP1, B4GALT4, SPAM1, LDHAL6B |
| 3 | Spermatogenesis | 3.23 | 2.01E-05 | ACE, ODF1, GPX4, ODF2, ODF3, ATP1A4, SEPTIN12, HSPA2, SMRP1, SPATA6, SUN5, SEPTIN7, OAZ3, SOD1, TSGA10, TPPP2, PRDX4, CCIN, SLC25A31, CYLC2, TXNDC8, CYLC1, SPATA19 |
| 4 | Mitochondrial respiratory chain complex assembly | 3.09 | 1.40E-15 | NDUFA9, NDUFB9, NDUFA8, NDUFA13, NDUFA5, NDUFB10, NDUFB6, NDUFB11, NDUFA12, NDUFA10, NDUFA2, ACAD9, NDUFC2, TIMM21, BCS1L, NDUFS8, NDUFS7, NDUFS5, NDUFS4, NDUFS3, NDUFS2, NDUFS1 |
| 5 | Tricarboxylic acid cycle | 2.67 | 1.84E-18 | FH, MDH1, MRPS36, IDH3G, MDH2, IDH2, DLST, PDHB, SDHA, SDHB, CS, SUCLA2, OGDH, IDH3B, SUCLG2, SUCLG1, ACO2, DLAT, IDH3A |
| 6 | Protein folding | 2.53 | 4.07E-07 | PDIA3, CCT3, CCT2, HSP90AA1, HSPE1, CCT6B, HSPD1, HSP90B1, TCP1, QSOX1, CALR, CCT8, P4HB, PPIB, CCT7, PPIA, CCT5, CCT4 |
| 7 | Protein stabilization | 2.53 | 3.04E-05 | CCT3, CCT2, PHB1, HSP90AA1, HIP1, APOA1, ATP1B3, PARK7, PHB2, CLU, EFNA1, TCP1, CALR, PPIB, CCT7, GAPDH, CCT5, CCT4 |
| 8 | Binding of sperm to zona pellucida | 2.39 | 3.00E-13 | CCT3, CCT2, ARSA, HSPA1L, TEX101, ADAM32, PRSS55, ZPBP, ADAM2, TCP1, VDAC2, SPA17, GLIPR1L1, CCT7, ALDOA, CCT5, CCT4 |
| 9 | Flagellated sperm motility | 2.39 | 2.71E-10 | CFAP52, ATP1A4, SORD, PRSS55, AKAP4, ENKUR, LDHC, TEKT2, TEKT3, TEKT4, ROPN1L, TEKT5, TSSK4, DNAI1, CFAP45, ROPN1, GAS8 |
| 10 | Proteasome-mediated Ub-dependant catabolic process | 2.39 | 2.51E-04 | VCP, GSK3A, PSMA8, SOD1, PSMB6, PSMB7, PSMC5, PSMB4, PSMA3, PSMA4, PSMB5, PSMA1, UBXN11, PSMB2, PSMA2, PSMB3, PSMB1 |

KEGG pathway analysis was used to execute pathway enrichment to identify the important biochemical and signal transduction pathways. We observed that a total of, 70 pathways were enriched in spermatozoa, the top 10 pathways are depicted in **Fig. 4.6**. The sperm proteins were mainly involved the metabolic pathway (189 proteins), Oxidative phosphorylation (52 proteins), Glycolysis / Gluconeogenesis (25 proteins), Citrate cycle (TCA cycle) (20 proteins) and Estrogen signalling pathway (12 protein). Metabolic pathways are crucial in sperm physiology, such as motility, energy metabolism, and metabolic activity regulation (Miki *et al*., 2004). Two metabolic pathways, glycolysis (Magdanz *et al*., 2019) and oxidative phosphorylation (Du Plessis *et al*., 2015), are involved in production of ATP, which provides energy to maintain the spermatozoa's important functions. The process of oxidative phosphorylation appears to be favoured in bovine sperm (Garrett *et al*., 2008). This finding supports that sperm motility is the primary attribute that allows them to reach the fertilization site requires energy. In our study, we found that ATP6V1A, ATP5MG, ATP5PD, ATP5PB, ATP5F1C, ATP5F1D, and ATP5F1E are involved in energy production through the oxidative phosphorylation pathway **(Fig. 4.7)**. The processes that have been mentioned are crucial for regulating the homeostasis of spermatozoa.

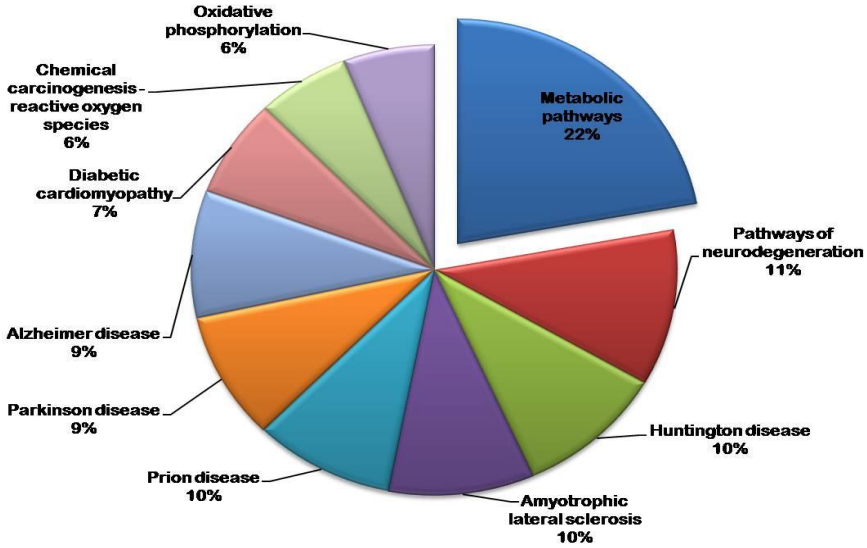



**Figure 4.6:** The top 10 pathways where in freshly ejaculated sperm proteins of Zebu bulls are enriched

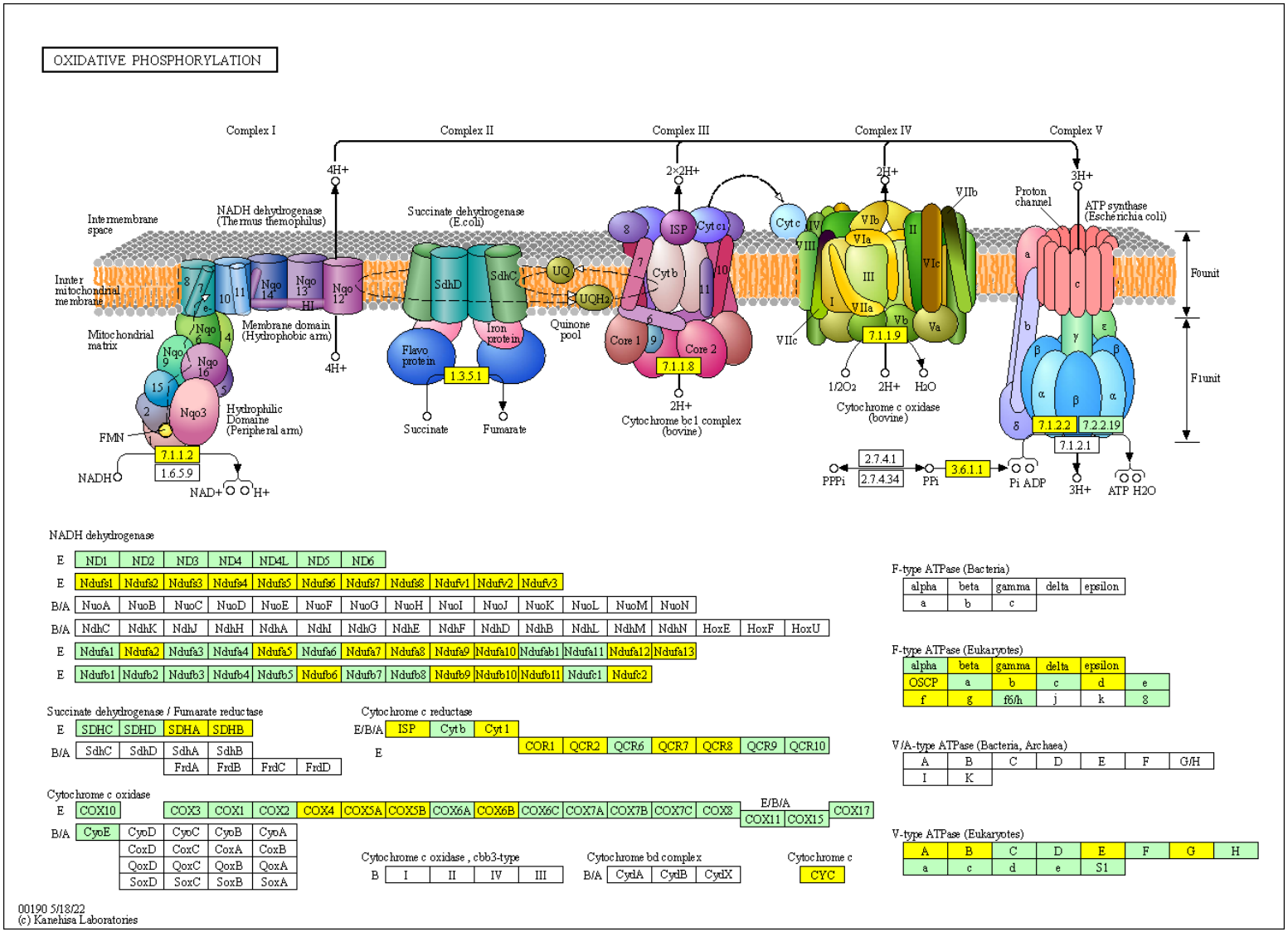



**Figure 4.7**: Involvement of identified Zebu bull sperm proteins in Oxidative phosphorylation pathway

### 4.2.2: Top 10 most abundant proteins in spermatozoa of Zebu bull

We observed that Seminal plasma protein A3, A-kinase anchoring protein 4 (AKAP4), C-type natriuretic peptide (NPPC/CNP), Cationic trypsin (PRSS1), Outer dense fiber protein 2 (ODF2), Seminal ribonuclease (SRN), Seminal plasma protein PDC-109, Vasoactive intestinal peptide (VIP), Caltrin (PYY2) and Metalloproteinase inhibitor 2 (TIMP2) were abundantly expressed in spermatozoa of Zebu bulls **(Table 4.3)**. Earlier reports suggested that AKAP4, CNP, SRN and ODF2 are essential for sperm motility in mice (Tarnasky *et al*., 2010; Xia *et al*., 2016; Codognoto *et al*., 2018; Blommaert *et al*., 2019). Ito *et al*. (2019) reported that decapitated and decaudated spermatozoa result from ODF2 haploinsufficiency. Huang *et al*. (2011) reported that CNP could considerably enhance the mRNA expression of the androgen-binding protein and transferrin in the rat Sertoli cells.

SRN (seminal ribonuclease) is a protein correlated to the trait of high freezability (Gomes *et al*., 2020). SRN plays a role in sperm capacitation and has a catalytic activity. PDC-109 has previously been recognized to promote sperm-to-oviductal epithelial interaction (Gwathmey *et al*., 2003) and enhances bull sperm capacitation by promoting lipid efflux from the plasma membrane (Manjunath and Thérien, 2002). Yu *et al*. (2003) reported that PDC-109 inhibits protein kinase C activity (*in vitro*), and it is considered to prevent premature acrosome reaction. In our study, we found that these previously reported functions are interrelated because they are all involved in keeping sperm in a healthy state in the female system until oocyte fertilization.

TIMPs are endogenous inhibitors of Matrix metalloproteinases (Brew *et al*., 2000) that cause a decrease in proteolytic activity (Shimokawa *et al*., 2003). When compared to the low sperm DNA fragmentation group, the high sperm DNA fragmentation group had lower seminal plasma levels of TIMP-2 (Belardin *et al*., 2019). According to our findings, most of these proteins confer protection and a favorable environment for spermatozoa.

**Table 4.3:** List of top 10 most abundant proteins in freshly ejaculated spermatozoa of Zebu bulls

| S. No. | Uniprot ID | Protein name | Gene symbol |
|---|---|---|---|
| 1 | P04557 | Seminal plasma protein A3 | - |
| 2 | F1MYH5 | A-kinase anchoring protein 4 | AKAP4 |
| 3 | P55206 | C-type natriuretic peptide | NPPC |
| 4 | P00760 | Cationic trypsin | PRSS1 |
| 5 | Q2T9U2 | Outer dense fiber protein 2 | ODF2 |
| 6 | P00669 | Seminal ribonuclease | SRN |
| 7 | P02784 | Seminal plasma protein PDC-109 | - |
| 8 | P81401 | Vasoactive intestinal peptides | VIP |
| 9 | P06833 | Caltrin | PYY2 |
| 10 | F1N430 | Metalloproteinase inhibitor 2 | TIMP2 |

### 4.2.3: Proteins associated with spermatogenesis

In the current study, we identified 23 proteins related to spermatogenesis; ACE, ODF1, GPX4, ODF2, ODF3, ATP1A4, SEPTIN7, SEPTIN12, HSPA2, SMRP1, SUN5, OAZ3, SOD1, TSGA10, TPPP2, PRDX4, CCIN, SLC25A31, CYLC2, TXNDC8, CYLC1, SPATA6 and SPATA19. Spermatogenesis is a controlled process of germ cell multiplication and differentiation that results in the production of spermatozoa (Staub and Johnson, 2018). Bovine testis angiotensin-converting enzyme (tACE) was found in post-pubertal bull germ cells near spermiation, suggesting that this enzyme is involved in spermatogenesis. It was reported that men with fertilization failure had lower ACE content than men with a normal fertilization rate (Li *et al*., 2014) and it could be a potential field fertility indicator (Ojaghi *et al*., 2018). ODF2 is a key component of the outer dense fibres (ODF) of spermatozoa's flagellum, and its tyrosine phosphorylation is crucial for sperm motility (Tarnasky *et al*., 2010), ODF1/HSPB10 (outer dense fibre of sperm tails 1) is required for the tight connection of sperm head to tail (Yang *et al*., 2012). Thus, it appears to be functionally implicated in spermatid differentiation and sperm tail

formation. ATP1A4 was found in the sperm plasma membrane and activated certain signalling molecules (Caveolin-1, EGFR, Src and ERK1/2) during sperm capacitation (Thundathil *et al*., 2018). Lin *et al*. (2009) revealed that SEPT12 is required for the formation of acrosome, mitochondria, tail and perhaps nuclei during post meiotic germ cell terminal differentiation. In humans, sperm with aberrant SEPT 7 and SEPT12 expression patterns are more prevalent in infertile males (Lin *et al*., 2011). HSPA2 is testis-specific and is most abundant in pachytene spermatocytes; it is essential for male fertility (Scieglinska and Krawczyk, 2015). Matsuoka *et al*. (2008) found that SMRP1 may be relevant as a functional protein interacting with manchette proteins. Yuan *et al*. (2015) found that spermatogenesis-associated protein 6 (SPATA6) is required for normal assembly of the sperm connecting piece and tight head-tail conjunction and also essential for regulation of proliferation, apoptosis, and testosterone production (Li *et al*., 2021). It has been indicated that SPATA19 proteins aids mitochondrial function in spermatid differentiation, which is critical in sperm motility (Nourashrafeddin *et al*., 2014). SPATA19-cKO males were infertile, as their sperm had disordered mitochondrial structure (Mi *et al*., 2015).

Recent studies found that SUN domain-containing protein 5 (SUN5) is involved in the attachment of the sperm head and tail (Shang *et al*., 2017). SLC25A31 protein is considered involved in spermatogenesis, where it is expected to interact with a component of the flagellar cytoskeleton and with glycolytic enzymes. Christensen *et al*. (2006) studied extensively on spermatogenesis and concluded OAZ3 is a testis-specific antizyme paralog, the primary antizyme expressed throughout the mid to late stages of spermatogenesis and play a significant role in spermatogenesis by regulating ornithine decarboxylase (ODC) and polyamine levels.

### 4.2.4: Proteins associated with sperm motility

In freshly ejaculated sperm proteome, we identified 17 potential proteins associated with sperm motility, which are CFAP52, ATP1A4, SORD, PRSS55, AKAP4, ENKUR, LDHC, TEKT2, TEKT3, TEKT4, ROPN1L, TEKT5, TSSK4, DNAI1, CFAP45, ROPN1 and GAS8. Earlier studies, suggested that A-kinase anchoring protein 4 (AKAP4) is a component of the sperm fibrous sheath essential for the flagellar structure, chemotaxis, capacitation, and sperm motility (Blommaert *et al*.,

2019). During sperm capacitation, AKAP4 is actively phosphorylated in tyrosine (Pereira *et al*., 2017) and complete lack of AKAP4 has been reported in case of necrozoospermia (Moretti *et al*., 2006).

Furthermore tektins are important components of sperm flagella and shown to be highly expressed in high fertility group which might increase motility due to higher expression of AKAP4 (Peddinti *et al*., 2008). Likewise Bhilawadikar *et al*. (2013) found that Tektin 2 levels are lower in oligoasthenozoospermic men's spermatozoa than in normozoospermic men, suggesting that it plays an important role in motility. However, TEKT5 is associated with the development of flagella during spermiogenesis and is also related to sperm motility (Cao *et al*., 2011). Tektin 4, PRSS55 deficiency in male mice, causes asthenozoospermia (Roy *et al*., 2007; Holmlund *et al*., 2022). Rhophilin-associated tail protein 1, found in the fibrous sheath of mammalian sperm flagella, has been shown to play a vital role in sperm motility, it binds to AKAP3 in a phospho-regulated manner and participates in sperm motility (Fiedler *et al*., 2013).

### 4.2.5: Proteins associated with sperm-egg recognition

In Zebu bull spermatozoa we detected a total of five proteins, which are related to sperm egg recognition, includes IZUMO1, SPESP1, SPACA3, CD9 and FOLR1. Several studies have reported that, before sperm-oolemma union, IZUMO1 is necessary for binding (Inoue *et al*., 2005; Bianchi and Wright, 2015). SPESP1 is involved in sperm-egg binding and fusion (Wolkowicz *et al*., 2008; Fujihara *et al*., 2010).

### 4.2.6: Proteins associated with sperm capacitation

We identified few proteins, CABYR, ROPN1L, ELSPBP1, PRKACA, DLD, and ROPN1, which are involved in capacitation. Capacitation refers to the functional changes that impart fertilizing potential to sperm, such as the ability to bind the zona pellucida and then to undergo the acrosome reaction, hyperactivate motility, and the ability to fuse with the oocyte. Calcium-binding tyrosine phosphorylation-regulated protein (CABYR) is a sperm protein of sperm flagella that acquires calcium-binding capacity when phosphorylated during capacitation (Naaby-Hansen *et al*., 2002). Fiedler *et al*. (2013) documented that, Ropporin-1-like protein (ROPN1L) mutations

can lead to anomalies in fibrous sheath integrity, sperm motility, and PKA-dependent signalling mechanisms, resulting in male infertility.

### 4.2.7: Protein-protein interaction network

To find additional important proteins linked in critical pathways, we formed a protein-protein interaction network utilizing Search Tool for the Retrieval of Interacting Proteins (STRING v11.0) (http://string.embl.de/) and Cytoscape 3.9.2. The PPI network of fresh spermatozoa consists 717 nodes, and 8444 edges were found to be associated with sperm PPI networks. According to the results from the string analysis the expected of edges were 2526 which is less than the actual interaction this means that proteins have more interaction among themselves. The average node degree, avg. local clustering coefficient and PPI enrichment p-value were 23.6, 0.389 and <1.0e-16 respectively. Network analysis revealed that the proteins were commonly involved in various pathways Citrate cycle (TCA cycle), Glutathione metabolism, PPAR signaling pathway, Pentose phosphate pathway, Glycolysis/Gluconeogenesis and oxidation-reduction process.

## 4.3: Objective-2: Comparative proteomic profiling of normal and oxidative stress induced bull spermatozoa

LC-MS/MS technique was used to investigate the proteome profile of fresh, oxidative stress induced spermatozoa with and without seminal plasma of bull semen. A total of 1526 proteins were detected in Deoni bull spermatozoa, among which 1441, 1434 and 1458 proteins were found in fresh, oxidative stress induced spermatozoa with and without seminal plasma, respectively. Among these, 1341 proteins (87.9%) were common to all three groups, while 42 (2.8%), 11 (0.7%), and 7 (0.5%) proteins were unique to fresh, oxidative stress induced spermatozoa with and without seminal plasma respectively as shown in **Fig. 4.8**.

The number of proteins observed in our study is in consonance with previous reports that reported, 1974 proteins identified in indigenous Malnad Gidda breed of cattle (Ramesha *et al*., 2020) and 2,147 protein in Buffalo (Fu *et al*., 2019), and 1,343 proteins in ROS negative and 1,265 proteins in ROS positive human being (Hamada *et al*., 2013).

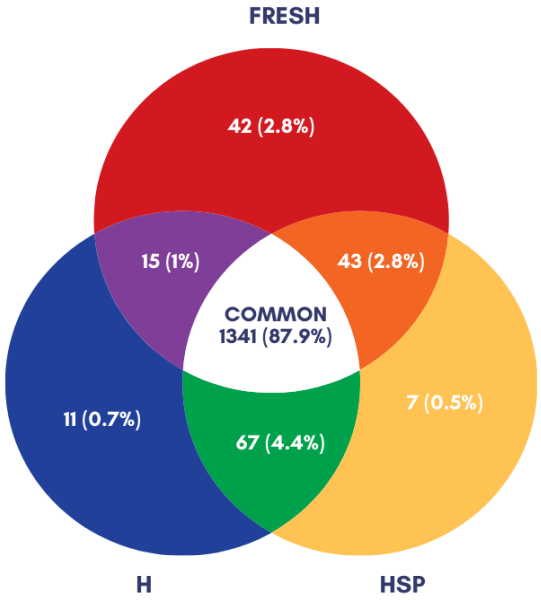



**Figure 4.8:** Venn diagram showing number of common and unique proteins present in fresh, oxidative stress induced spermatozoa with and without seminal plasma

### 4.3.1: Differential expression of proteins between freshly ejaculated and oxidative stress induced spermatozoa of Zebu bulls

Based on the p-value ($p<0.05$) and fold change, upregulated (>1 fold change) and down-regulated (<-1 fold change) proteins were determined. A total of 451 proteins were dysregulated in oxidative stress induced spermatozoa compared to fresh spermatozoa based on fold change (>1 or <-1); among these, 260 were upregulated (>1 fold change), while 191 were downregulated (<-1 fold change). Differentially expressed proteins (DEPs) were plotted using Heat map in freshly ejaculated Vs oxidative stress induced spermatozoa shown in **Fig. 4.9**. Based on log2 (fold change) and p value, the DEPs were plotted using Volcano plot **(Fig. 4.10)** in freshly ejaculated Vs oxidative stress induced spermatozoa**.**

### 4.3.2: Top 10 upregulated proteins in oxidative stress-induced spermatozoa compared to freshly ejaculated spermatozoa of Zebu bulls

Among the dysregulated proteins topmost upregulated proteins are MYCBP, NDUFS7, LETM2, SEPTIN4, FAM71F2, ECH1, SEPTIN10, CCDC81, LYPLA1 and CYB5R2. Top 10 upregulated proteins in oxidative stressed spermatozoa represented in Table. 4.4. C-Myc Binding Protein (MYCBP) is usually present in the

cytoplasm, but during the S phase of the cell cycle, it translocates to the nucleus. Selvaraju *et al*. (2018) reported that MYCBP is required for spermatogenesis. McReynolds *et al*. (2014) found that the proteins MYCBP had lower levels of expression in sperm from infertile men, which led to poor blastocyst formation. This protein is also important in the development and activities of male gametes are underexpressed in spermatozoa from infertile normozoospermic men. MYCBP was a highly upregulated protein, which could elucidate our observations regarding spermatogenesis and fertility.

NADH:ubiquinone oxidoreductase core subunit S7 (NDUFS7) found in complex I mitochondrial respiratory chain. Earlier studies reported that Complex I (NADH:ubiquinone oxidoreductase) is a major contributor to mitochondrial superoxide generation (Hirst *et al*., 2008). Furthermore, NDUFS7 dysfunction has been linked to mitochondrial complex I of the electron transport chain impairment (Triepels *et al*., 2001). A study conducted by Scott *et al*. (2018) reported that, overexpression of NDUFS7 protein in sexed sorted spermatozoa with X-bearing chromosomes may lead to increased oxidative stress and decreased motility. Alvarez-Rodriguez *et al*. (2021) reported that NDUFS7 protein (upregulated) in high-fertile boars and claimed that decreased levels of complex I (and NDUFS7) promote mitochondrial ROS generation. Our findings are in agreement with Scott *et al*. (2018) and Alvarez-Rodriguez *et al*. (2021) who reported that over expression of NDUFS7 enhance oxidative stress through mitochondria dysfunction contributes to mitochondrial superoxide generation.

It is well documented that, SEPTIN 4 proteins are required for mitochondrial architecture and annulus formation (Kissel, 2005). Septin is flagellar protein that produces energy in annular region and aids sperm movement in the female reproductive tract (Touré *et al*., 2021). Several studies found SEPTIN 4 expression was downregulated in asthenozoospermic men (Dar *et al*., 2018; Netherton *et al*., 2018). Our study found upregulation of SEPTIN 4 suggesting regulation of sperm motility. LETM1 and LETM2 have been identified as important proteins in mitochondrial protein translation and expressed from spermatocyte to spermatozoon (Tamai *et al*., 2008). Recent study conducted by Zhang *et al*. (2012) found LETM1 is a highly stress-inducible gene, Our results showed that, LETM2 dysregulation have direct control over the mitochondrial function.

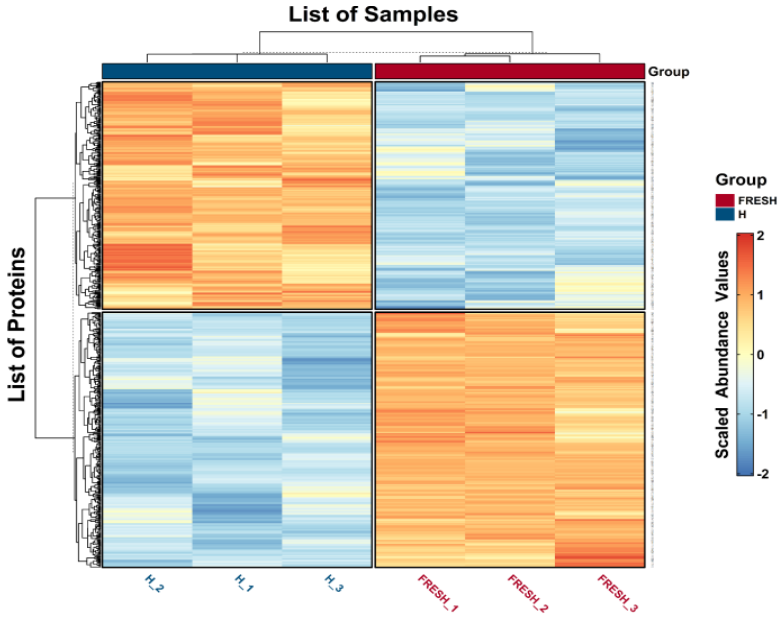



**Figure 4.9:** Heat map representing the DEPs in freshly ejaculated Vs oxidative stress induced spermatozoa of Zebu bulls

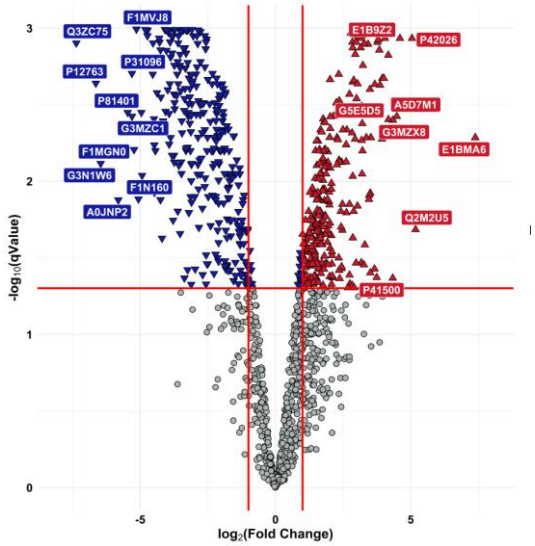



**Figure 4.10**: Volcano plot representing the DEPs in freshly ejaculated Vs oxidative stress induced spermatozoa of Zebu bulls

Updated insights found on Golgi-associated RAB2 interactor protein 1A (FAM71F2) concluded that it is essential for male fertility as the acrosome was abnormally enlarged at the round spermatid stage in FAM71F1-mutant mice (Morohoshi *et al*., 2021). Previously, RAB2A was reported to be correlated with the membrane of developing proacrosomic and acrosomic secretory vesicles during acrosomal biogenesis in bull spermatozoa (Mountjoy *et al*., 2008). Considering these findings, it is possible that Golgi-associated RAB2 is involved in anchoring the acrosome to the nuclear envelope during sperm head assembly mediated by interaction with other RAB proteins.

ECH1 (Enoyl-CoA hydratase 1) is located in sperm mitochondria, ECH is required for the beta oxidation of fatty acids to produce acetyl CoA and energy in the form of ATP. Over expression of these proteins in asthenozoospermic males could act as a possible biomarker (Martínez-Heredia *et al*., 2008). We observed overexpression of ECH1 possibly related to oxidation as well as impaired motility. Septins are GTP-binding proteins that polymerize to form non-polar filaments can be organized into bundles, rings, and mesh-like structures (Valadares *et al*., 2017). Septin 10 isoform 1 is involved in the formation and maturation of spermatozoa and their motility (Gao *et al*., 2012). Coiled-coil domain containing 81 (CCDC81) proteins are centrosome or cilia-associated proteins related to centrosome organization (Priyanka and Yenugu, 2021) and plays a role in protein-protein interactions during centrosome assembly and, when combined with Dynein-VII, could act as a cargo-binding protein (Burroughs *et al*., 2017).

In human spermatozoa, cytochrome-b5 reductase (CYB5R2) is a crucial enzyme that mediates NADH-induced redox activity (Baker *et al*., 2005). Overexpression of CYB5R2 resulted in increased mitochondrial functions such as production of ATP, oxygen consumption, and complex I and II activities (Hyun and Lee, 2015). According to Aitken and Baker (2004), cytochrome b5 reductase is a possible candidate for ROS production. Our findings are in agreement with Hyun and Lee (2015) who reported that overexpression of CYB5R2 promotes oxidative stress.

Collectively, majority of upregulated proteins were involve in energy metabolism, oxidation, motility, increase oxygen consumption and mediates mitochondrial dysfunction.

### 4.3.2.1: Important upregulated proteins in oxidative stress induced spermatozoa

Enolase 1 (ENO1) is one of the proteins expressed variably before and after stress exposure; ENO1 functions as a stress protein and induces the production of hypoxia-inducible factor-1 (HIF-1). Aaronson *et al*. (1995), implying that ENO1 may enhance cell protection by increasing anaerobic metabolism. Our study found that upregulation of ENO1 indicates enhanced energy metabolism due to stress response. Glutathione peroxidase 4 isoform A precursor (GPX4) was an overexpressed protein in the ROS+ samples compared to the ROS- samples. It is involved in reducing oxidative stress. In our study, we found overexpression of GPX4, a well-known antioxidant enzyme, its enhanced expression in ROS+ sperm reflects its activation on genes that synthesize this enzyme. Another study suggested that, Cytochrome b-c1 complex subunit 2 (UQCRC2) is highly expressed in bull spermatozoa with low fertility (Park *et al*., 2012), our study found upregulation of UQCRC2, which suggest negative correlation with fertility.

### 4.3.3: Top 10 downregulated proteins in oxidative stress induced spermatozoa compared to freshly ejaculated spermatozoa of Zebu bulls

Topmost downregulated proteins are AHSG, VIP, SPP1, SEMA3F, OLFM4, BPIFB1, LGMN, PSAP, LGALS3BP and AZGP1 shown in **Table 4.5**. The protein alpha-2-HS-glycoprotein (AHSG) is synthesized in the liver and released into the bloodstream (Mori *et al*., 2012). Following testosterone treatment, AHSG mRNA expression increases in Human liver hepatocellular carcinoma (Voelkl *et al*., 2014). Vasoactive intestinal peptide (VIP) was found to regulate testis activity that could be employed as a biomarker in spermatogenesis and apoptosis (Sweett *et al*., 2020). Previous reports revealed that a lack of VIP reduces testosterone levels (Lacombe *et al*., 2007). These studies implicit that VIP has an important role in controlling testosterone synthesis. VIP inhibits NADPH oxidase, which decreases oxidative stress in pancreatic acinar cells (Fujimori *et al*., 2011), it appears to have a positive effect by downregulating a wide range of inflammatory cytokines, chemokines and oxidative stress mediators (Delgado and Ganea, 2013). VIP prevents inflammatory cells from producing superoxide radicals (Delgado *et al*., 2008). Previous reports suggested its role as an antioxidant in scavenging free radicles and could alter

testosterone production. However, we observe that downregulation of VIP deregulate scavenging mechanism.

**Table 4.4:** List of top 10 upregulated proteins in oxidative stress-induced spermatozoa compared to freshly ejaculated spermatozoa of Zebu bulls

| S. No. | Gene symbol | Protein name | Log 2 fold change |
|---|---|---|---|
| 1 | MYCBP | C-Myc-binding protein | 5.52 |
| 2 | NDUFS7 | NADH dehydrogenase [ubiquinone] iron-sulfur protein 7 | 5.04 |
| 3 | LETM2 | Leucine zipper and EF-hand containing transmembrane protein 2 | 4.60 |
| 4 | SEPTIN4 | Septin 4 | 4.04 |
| 5 | FAM71F2 | Golgi associated RAB2 interactor protein 1A | 4.02 |
| 6 | ECH1 | Enoyl-CoA hydratase 1 | 3.95 |
| 7 | SEPTIN10 | Septin-10 | 3.94 |
| 8 | CCDC81 | Coiled-coil domain containing 81 | 3.93 |
| 9 | LYPLA1 | Lysophospholipase 1 | 3.79 |
| 10 | CYB5R2 | Cytochrome-b5 reductase | 3.74 |

We found downregulation of Osteopontin (OPN) which may inferred as to deregulate fertility based on the following findings which are in agreement with our study. OPN was previously identified as high fertility marker (Erikson *et al*., 2007), further, it prevents polyspermy by limiting the quantity of spermatozoa attached to the Zona pellucida (Hao *et al*., 2006). The presence of OPN and its receptors in spermatozoa and seminal fluid suggests that these molecules have a role in the male reproductive system, such as sperm motility and capacitation (Moura, 2018).

The genes Olfactomedin (OLFM 4) is known to control cellular proliferation, differentiation and pathological processes (Kodithuwakku *et al*., 2011). It is well known that, the lack of OLFM 4 gene expression has been linked to the progression of human prostate cancer. However, the relevance of this gene and the molecular mechanisms involved in this process are yet unknown. On the other hand, very few studies have reported that OLFM 4 promote $H_2O_2$ induced NADPH oxidase activation and apoptosis in mouse neutrophils (Liu *et al*., 2018). Ochiai *et al*. (2008) found that Prosaposin (PSAP) can reduce hydrogen peroxide-induced apoptotic cell

death by regulating neurotoxic pathways. Interestingly, in the present study, prosaposin is downregulated and it could be suggestive of stress induced injury.

**Table 4.5:** List of top 10 downregulated proteins in oxidative stress induced spermatozoa compared to freshly ejaculated spermatozoa of Zebu bulls

| S. No. | Gene symbol | Protein name | Log 2 fold change |
|---|---|---|---|
| 1 | AHSG | Alpha-2-HS-glycoprotein | -6.64 |
| 2 | VIP | Vasoactive intestinal peptide | -5.46 |
| 3 | SPP1/OPN | Secreted Phosphoprotein 1/ Osteopontin | -5.30 |
| 4 | SEMA3F | Semaphorin 3F | -5.23 |
| 5 | OLFM4 | Olfactomedin 4 | -5.14 |
| 6 | BPIFB1 | BPI fold-containing family B member 1 | -4.95 |
| 7 | LGMN | Legumain | -4.93 |
| 8 | PSAP | Prosaposin | -4.88 |
| 9 | LGALS3BP | Lectin galactoside-binding soluble 3-binding protein | -4.81 |
| 10 | AZGP1 | Zinc-alpha-2-glycoprotein | -4.73 |

### 4.3.3.1: Important downregulated proteins in oxidative stress induced spermatozoa

A natural antioxidant defense mechanism exists in sperm, which is made up of protein players called peroxiredoxins (PRDX) (Ritchie and Ko, 2021). It has been indicated that PRDX 1 act as scavengers of ROS when oxidative stress levels are high (Lee *et al*., 2017). According to Kumsta and Jakob (2009), when a human spermatozoon is exposed to high ROS concentrations, peroxiredoxin is transformed into high molecular mass complexes to escape protein modification or becoming molecular chaperones. Our findings are consistent with the studies of Hamada *et al*. (2013) and Ritchie and Ko, (2021) arrived at a conclusion; PRDX1 and PRDX4 get downregulated, implicating reduced oxidant mechanism. Hamada *et al*. (2013) reported that Peroxiredoxin 1 have reduced expression in samples with oxidative stress compared with those without oxidative stress.

Among dysregulated proteins we found upregulation of Glutathione peroxidase 4 isoform A precursor (GPX4), while downregulation of HSP90B1 protein. Earlier Sharma *et al*. (2013) revealed that candidate proteins serve

biomarkers of oxidative stress, HSP90B1 is overexpressed which is contradictory with our findings, however, GPX4 upregulation is similar with his finding could serve as a biomarker.

### 4.3.4: Functional classification of DEPs between freshly ejaculated Vs oxidative stress-induced spermatozoa of Zebu bulls

Gene ontology analysis of differentially expressed proteins revealed their involvement in 69 molecular functions **(Table 4.6)**, 59 cellular components and 84 biological processes **(Table 4.7)**.

Topmost molecular function associated with ATP binding (9.51%), calcium ion binding (4.63%), protein homodimerization activity (4.15%), protein binding (3.90%), ATPase activity (2.44%), unfolded protein binding (2.20%), magnesium ion binding (2.20%), serine-type endopeptidase inhibitor activity (1.95%), serine-type endopeptidase activity (1.95%) and receptor binding (1.95%). In the current study, the detected proteins were enriched in biological process like carbohydrate metabolic process (3.90%), mitochondrial respiratory chain complex I assembly (3.41%), negative regulation of endopeptidase activity (2.93%), proteolysis (2.93%), mitochondrial electron transport, NADH to ubiquinone (2.68%), flagellated sperm motility (2.68%), protein folding (2.44%), protein stabilization (2.44%), spermatogenesis (2.44%) and response to oxidative stress (2.20%).

Likewise looking into the biological process, increased proteolysis could be responsible for the alteration in protein, and most oxidative protein modifications are irreversible. Mitochondrial respiratory chain complex I assembly dysfunction leads to stress response in cells (Leman *et al*., 2015). Therefore, in our finding, most of the altered genes involved in mitochondrial respiratory chain complex I assembly are upregulated, indicating enhanced activity of mitochondria. Furthermore, PRDX1, PRDX5, and GPX5 are down-regulated in response to oxidative stress. Taken together all these GPX, PRDX5, and PRDX1 serve as a sensor for hydrogen peroxide-mediated damage and act as a direct eliminator and neutralizer of reactive oxygen species (Imai and Nakagawa, 2003; Yuan *et al*., 2004). In our finding, GPX5, PRDX5, and PRDX1 are downregulated in cells exposed to stress.

With regards to cellular location, these proteins belongs mainly to extracellular space (15.61%), mitochondrion (15.37%), extracellular region (10.49%), mitochondrial inner membrane (8.78%), lysosome (5.85%), endoplasmic reticulum (5.85%), membrane (5.61%), golgi apparatus (5.37%), mitochondrial matrix (5.12%) and perinuclear region of cytoplasm (4.39%).

**Table 4.6:** Top 10 Molecular functions in which the DEPs between freshly ejaculated Vs oxidative stress induced spermatozoa of zebu bulls were involved.

| S. No. | Molecular function | Count | % | P Value |
|---|---|---|---|---|
| 1 | ATP binding | 39 | 9.51 | 0.007540 |
| 2 | Calcium ion binding | 19 | 4.63 | 0.043689 |
| 3 | Protein homodimerization activity | 17 | 4.15 | 0.052237 |
| 4 | Protein binding | 16 | 3.90 | 4.06E-06 |
| 5 | ATPase activity | 10 | 2.44 | 1.89E-05 |
| 6 | Unfolded protein binding | 9 | 2.19 | 2.63E-04 |
| 7 | Magnesium ion binding | 9 | 2.19 | 0.033548 |
| 8 | Serine-type endopeptidase inhibitor activity | 8 | 1.95 | 0.009874 |
| 9 | Serine-type endopeptidase activity | 8 | 1.95 | 0.062146 |
| 10 | Receptor binding | 8 | 1.95 | 0.084826 |

**Table 4.7:** Top 10 biological processes in which the DEPs between freshly ejaculated Vs oxidative stress induced spermatozoa of Zebu bulls were involved

| S. No. | Biological process | Count | P Value | Genes |
|---|---|---|---|---|
| 1 | Carbohydrate metabolic process | 16 | 3.18E-08 | MANBA, B3GAT3, IDUA, LANCL1, HEXA, SLC3A2, SMRP1, LDHC, CS, GANAB, GLB1L, GLB1, GUSB, SIAE, B4GALT4, LDHAL6B |
| 2 | Mitochondrial respiratory chain complex I assembly | 14 | 2.58E-10 | NDUFB9, NDUFA8, NDUFA13, NDUFB6, NDUFA12, NDUFA10, NDUFA2, NDUFC2, TIMM21, BCS1L, NDUFS8, NDUFS7, NDUFS5, NDUFS2 |
| 3 | Negative regulation of endopeptidase activity | 12 | 2.79E-06 | C3, CST3, LOC112442231, SERPINA1, SERPINE2, AHSG, TFPI2, TIMP2, NGF, A2M, SERPINA5 |
| 4 | Proteolysis | 12 | 0.0016 | DPP4, CTSA, AFG3L2, ENPEP, QPCT, TPP1, UQCRC2, CTSD, DLD, CFB, LGMN, PRSS54 |
| 5 | Mitochondrial electron transport, NADH to ubiquinone | 11 | 8.88E-11 | NDUFB9, NDUFA8, NDUFA7, NDUFS8, NDUFB6, NDUFS7, NDUFS6, NDUFA10, NDUFS2, NDUFC2, DLD |
| 6 | Flagellated sperm motility | 11 | 3.64E-07 | LDHC, CFAP52, TEKT2, TEKT4, TEKT5, TSSK4, PRSS55, AKAP4, GAS8, CFAP45, ENKUR |
| 7 | Protein folding | 10 | 4.17E-04 | PDIA3, HSP90AA1, QSOX1, CALR, P4HB, CCT7, PPIB, CCT5, HSP90B1, HSPD1 |
| 8 | Protein stabilization | 10 | 0.003 | EFNA1, PHB1, HSP90AA1, APOA1, CALR, CCT7, PPIB, PHB2, CLU, CCT5 |
| 9 | Spermatogenesis | 10 | 0.039 | TSGA10, PRDX4, ACE, GPX4, SEPTIN12, SLC25A31, SMRP1, CYLC1, SUN5, SEPTIN7 |
| 10 | Response to oxidative stress | 9 | 6.87E-05 | NDUFS8, PRDX4, GPX4, NDUFA12, PRDX1, GPX5, ETFDH, LOC112441537, NDUFS2 |

### 4.3.5: KEGG pathway analysis of oxidative stress induced Vs freshly ejaculated sperm proteins of Zebu bull

Pathway analysis of differentially expressed proteins revealed involvement of 54 different pathways. Among these top 10 pathways are shown in **Fig. 4.11**. Among the 54 pathways, apart from metabolic pathways (27.12%), Pathways of neurodegeneration (11.55%), Huntington's disease (10.61%), Amyotrophic lateral sclerosis (10.14%), Diabetic cardiomyopathy (8.96%), reactive oxygen species (7.78%), Thermogenesis (7.07%), Oxidative phosphorylation (6.83%), Lysosome (5.18%) and Carbon metabolism (4.71%) are identified. Metabolic pathways (bta01100) were the major pathway enriched with dysregulated protein involving 118 proteins. While reactive oxygen species pathway involves 33 proteins among them, all these proteins are upregulated. We found 23 proteins involved in oxidative phosphorylation and all of them are upregulated except ATP6V1A, which was downregulated.

Majority of the sperm ATP is produced by oxidative phosphorylation (OXPHOS) mechanism, located in the inner mitochondrial membrane and modulated by respiratory enzyme complexes I–V (Faccenda and Campanella, 2012). Mitochondria are the most important energy source for sperm movement, while mitochondrial dysfunction is the primary source of ROS. Wong *et al*. (2019) reported that Mitochondria produce 45% of extracellular H2O2; NADPH oxidases (NOXs) produce 40% ROS. Out of which, mitochondria contribute 30% from site complex III and 15% from site complex I. Complex I, also called NADH-ubiquinone oxidoreductase, is the subunit complex in the ETC (Urra *et al*., 2017). In our study we found, in complex I NDUFS5, NDUFS6, NDUFS7, NDUFS8, NDUFV3, NDUFA2, NDUFA7, NDUFA10, NDUFA12, NDUFA13, NDUFB6, NDUFB9 and NDUFC2 are upregulated. Overexpression of these proteins in complex I have the potential to increase the activity of mitochondrial complex I, which is one of the primary sites of superoxide production.

Our study found upregulation of SDHB in complex II consistent with the reported finding. Huang and Lemire (2009) suggested that SDH ubiquinone-binding site can become a superoxide source, leading to pathological consequences. Guzy *et al*. (2008) reported that increases in ROS generation at complex II caused by

SDHB dysregulation could thus activate the hypoxia-inducible factor by simulating the hypoxia signalling pathway. Complex III, also known as cytochrome C reductase, UQCRFS1, and CYC1 predicted to enable oxidoreductase activity involved in mitochondrial respiratory chain complex III assembly of electron transport chain (Sánchez *et al*., 2013). Aguilera-Aguirre *et al*. (2009) reported that ubiquinol-cytochrome c reductase core II protein (UQCRC2) was involved in the formation of mitochondrial ROS by respiratory complex III. In complex 4 we found upregulation of COX4i1 and COX5a are involve in mitochondrial respiration. The terminal enzyme of the electron transport chain, cytochrome c oxidase (COX), catalyses the transfer of electrons from cytochrome c to oxygen (Sinkler *et al*., 2017).

Our findings highlighted the downregulation of ATP6V1A in the oxidative phosphorylation pathway **(Fig. 4.12)**. Similar results were reported by Zhou *et al*. (2021) regarding low ATP6V1A in oxidative phosphorylation. Likewise, our findings supported the likelihood that low expression of ATP6V1A participated in oxidative phosphorylation. These finding clearly shows that any dysfunction in oxidative phosphorylation leads to impaired sperm functional activities and even impaired mitochondrial organization leads to reduced oxygen consumption, could be the predominant factor for bull infertility.

### 4.3.6: Protein–protein interaction network

The PPI network comprised 413 nodes and 2440 edges in oxidative stress-induced spermatozoa. According to the results from the string analysis the expected of edges were 686 which is less than the actual interaction this means that proteins have more interaction among themselves. The average node degree, avg. local clustering coefficient and PPI enrichment p-value were 11.8, 0.414 and <1.0e-16 respectively. However, the network analysis involves 8 biological process, 8 cellular components, 4 molecular functions and 26 Kegg pathway**.** Furthermore, molecular functions related to antioxidant activity (GPX4, GPX5, PRDX1, PRDX4, S100A9, TXNRD1) and oxidoreductase activity (55 proteins), implies activation of proteins against stress. GPX and PRDX possesing antioxidant activity involve in reducing antioxidant activity.

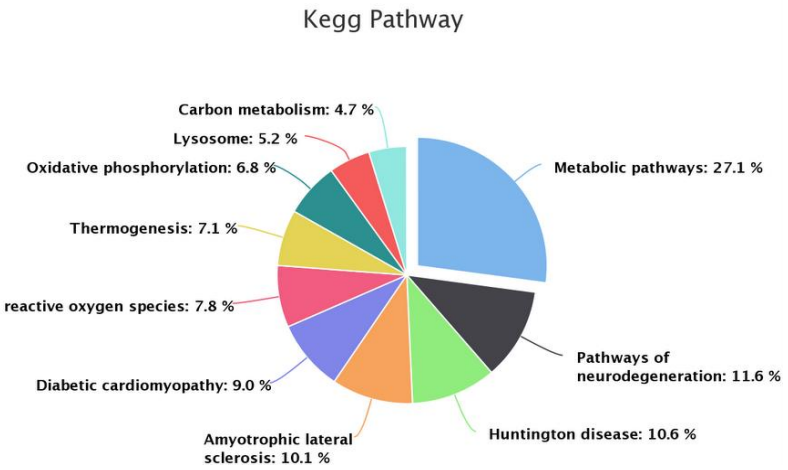



**Figure 4.11:** The Top 10 pathways associated with DEPs between freshly ejaculated Vs oxidative stress induced spermatozoa of Zebu bulls

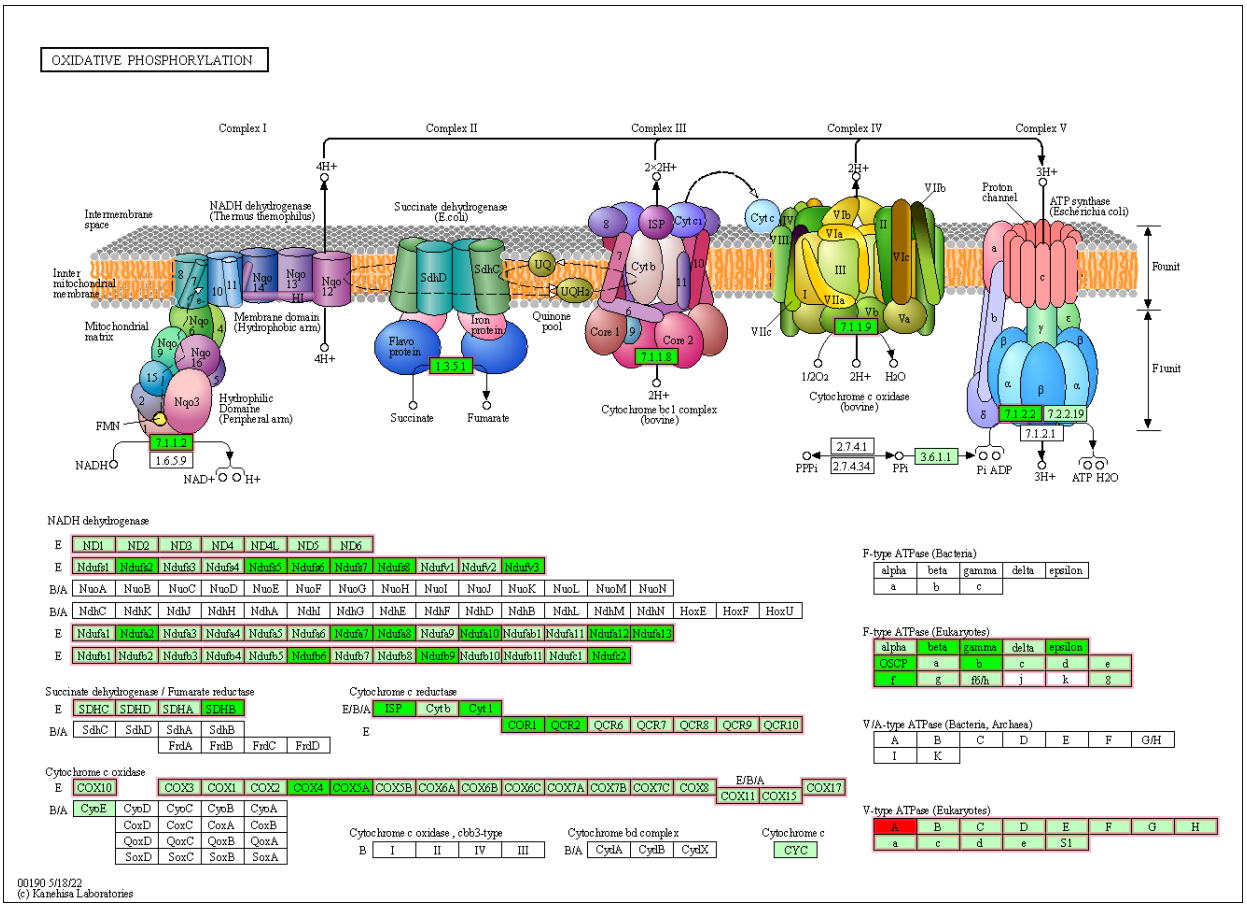



**Figure 4.12:** Oxidative phosphorylation pathway of DEPs in freshly ejaculated Vs oxidative stress induced spermatozoa of Zebu bull

## 4.4: Effect of seminal plasma on oxidative stress mediated proteomic alterations in Zebu bull spermatozoa

Cumulative of 1325 proteins were identified in both the groups i.e. only spermatozoa (SP-) and spermatozoa in presence of seminal plasma (SP+) after induction of oxidative stress. Out of the total proteins (1325), to identify the significant proteins three criteria viz. PSM >2, coverage ≥10 and unique peptide >2, were considered. A total of 769 significant proteins were identified, among them 716 proteins were neutrally expressed, 32 were upregulated while 21 are downregulated in oxidative stress induced groups. DEPs were plotted using Heatmap in SP+ Vs SP- group as shown in **Fig. 4.13**. Based on log2 (fold change) and p value, the DEPs were plotted using Volcano plot **(Fig. 4.14)** in SP+ Vs SP- group**.**

### 4.4.1: Gene ontology classification of dysregulated proteins in SP+ Vs SP- oxidative stress induced groups

Gene ontology classification of differentially expressed proteins revealed their involvement in 29 biological processes **(Table 4.8)**, 15 cellular components and 11 molecular functions **(Table 4.9)**.

In the current study, the detected proteins were enriched in 29 biological processes among them topmost are involve in innate immune response (12%), antibacterial humoral response (8%), positive regulation of bone resorption (6%), iron ion transport (6%), antimicrobial humoral immune response mediated by antimicrobial peptide (6%), regulation of iron ion transport (4%), blood coagulation (4%), cellular response to iron ion (4%), cellular response to interleukin-7 (4%) and plasminogen activation (4%).

While considering the cellular components, the proteins were located in 15 different components mainly in extracellular space (46%), extracellular region (16%), endoplasmic reticulum (12%), Golgi apparatus (12%), external side of plasma membrane (8%), cell surface (8%), recycling endosome (6%), early endosome (6%), HFE-transferrin receptor complex (4%) and extrinsic component of external side of plasma membrane (4%).

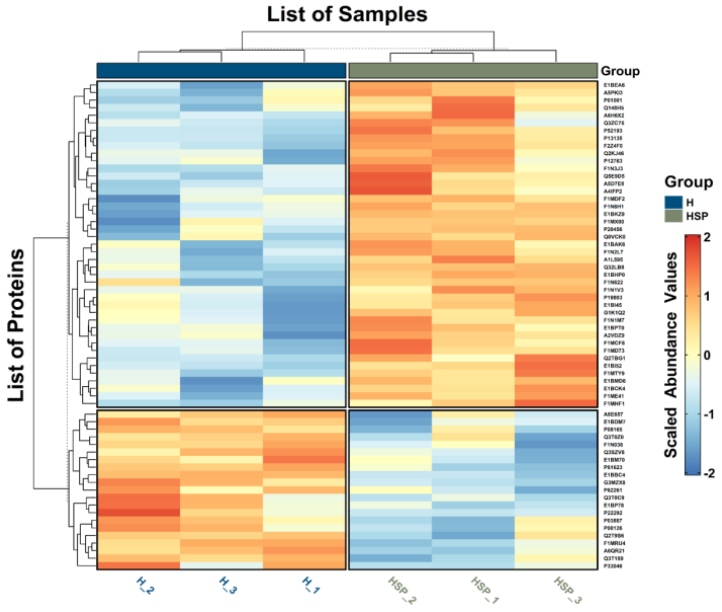



**Figure 4.13:** Heat map representing the DEPs in SP+ Vs SP- oxidative stress induced spermatozoa of Zebu bulls

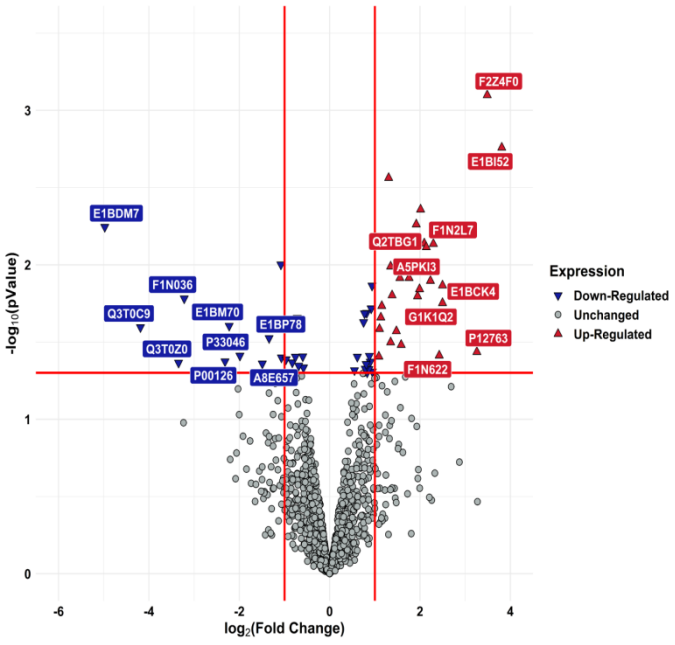



**Figure 4.14:** Volcano plot representing the DEPs in SP+ Vs SP- oxidative stress induced spermatozoa of Zebu bulls

**Table 4.8:** Top 10 biological process in which DEPs between SP+ Vs SP- oxidative stressed spermatozoa of Zebu bulls were involved

| S. No. | Biological Processes | % | P Value | Gene list |
|---|---|---|---|---|
| 1 | Innate immune response | 12 | 0.001274 | FGB, BPIFA1, GARIN5A, LOC512548, S100A12, S100A9 |
| 2 | Antibacterial humoral response | 8 | 1.20E-04 | BPIFA1, TF, LOC512548, F3 |
| 3 | Positive regulation of bone resorption | 6 | 8.44E-04 | TF, SPP1, F3 |
| 4 | Iron ion transport | 6 | 0.002202 | TF, MELTF, F3 |
| 5 | Antimicrobial humoral immune response mediated by antimicrobial peptide | 6 | 0.007473 | BPIFA1, S100A12, S100A9 |
| 6 | Regulation of iron ion transport | 4 | 0.007206 | TF, F3 |
| 7 | Blood coagulation, fibrin clot formation | 4 | 0.011982 | FGB, FGG |
| 8 | Cellular response to iron ion | 4 | 0.014361 | TF, F3 |
| 9 | Cellular response to interleukin-7 | 4 | 0.021466 | FGB, FGG |
| 10 | Plasminogen activation | 4 | 0.021466 | FGB, FGG |

**Table 4.9:** Top 10 Molecular function in which DEPs between SP+ Vs SP- oxidative stressed spermatozoa of Zebu bulls were involved

| S. No. | Molecular function | % | P value | Gene list |
|---|---|---|---|---|
| 1 | Structural molecule activity | 8 | 0.002242 | FGB, KRT17, PSMD13, FGG |
| 2 | Protein binding | 8 | 0.009263 | DNAJC3, FGB, AHSG, S100A12 |
| 3 | Chaperone binding | 6 | 0.011797 | DNAJC3, FGB, BAG5 |
| 4 | Lipid binding | 6 | 0.029304 | BPIFA1, BPIFB1, NGF |
| 5 | Receptor binding | 6 | 0.072322 | FGB, FGG, NGF |
| 6 | Iron chaperone activity | 4 | 0.008532 | TF, F3 |
| 7 | Transferrin receptor binding | 4 | 0.025382 | TF, F3 |
| 8 | Ferric iron binding | 4 | 0.046054 | TF, F3 |
| 9 | Cysteine-type endopeptidase inhibitor activity | 4 | 0.058251 | CST3, AHSG |
| 10 | Cell adhesion molecule binding | 4 | 0.078243 | FGB, FGG |

### 4.4.2: KEGG pathway analysis of SP+ and SP- oxidative stress induced spermatozoa

The principle KEGG pathways of DEPs protein were found to be enriched in 7 different pathways **(Fig. 4.15)** are Huntington disease (10%), Amyotrophic lateral sclerosis (10%), Pathways of neurodegeneration - multiple diseases (10%), Prion disease (8%), Parkinson disease (8%), Complement and coagulation cascades (6%) and Protein processing in endoplasmic reticulum (6%).

Among the pathway enriched by dysregulated proteins, the most represented top 5 pathways involving ACTR1A, PSMB2, NDUFA5, PSMD13, TUBB genes in which ACTR1A is upregulated while others are downregulated. Earlier reports suggested that typical hallmark of neurodegenerative disorders is neuro inflammation and mitochondrial dysfunction can enhance oxidative stress by excessive release of harmful reactive oxygen (Fischer and Maier, 2015). Pathways of neurodegeneration is represented in **Fig. 4.16**. Mitochondrial structure and functions like motility are governed by actin cytoskeletons, in our study Actin Related Protein 1A (ACTR1A) expression was upregulated, which could enhance motility.

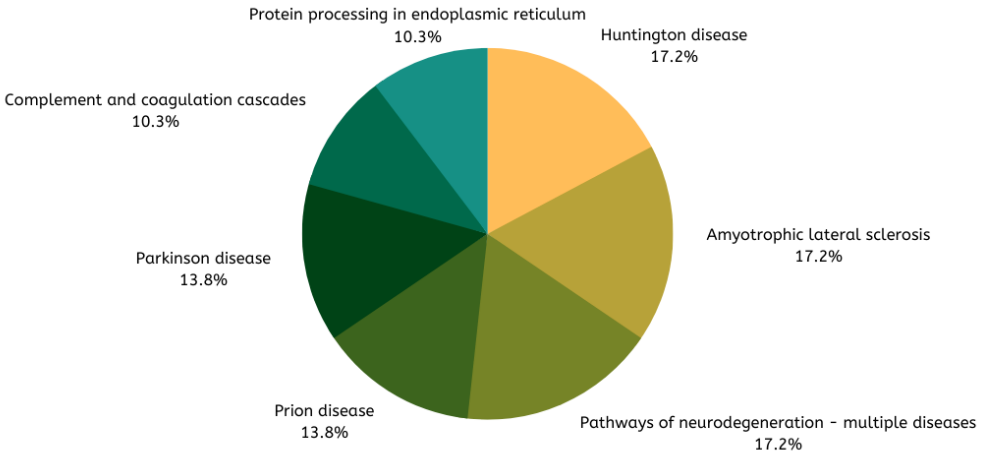



**Figure 4.15:** Topmost pathways associated with DEPs between SP+ Vs SP- oxidative stress induced spermatozoa of Zebu bulls

Numerous proteins and organelles are degraded during spermatogenesis, and the ubiquitin-proteasome pathway is vital for the development of condensed spermatozoa. However, in spermatozoa with reduced motility, various proteasome subunits displayed altered expression (Siva *et al*., 2010). In our study Proteasome subunit beta type-2 (PSMB2) and proteasome 26S subunit (PSMD13) altered expression seems to be associated with deregulated sperm motility. Several studies have shown that the proteasome is essential for the targeted destruction of oxidised proteins. Moreover, it has been demonstrated that overexpression of the proteasome assembly protein indicate enhances cell survival after exposure to a variety of oxidants (Chen *et al*., 2006). In our study downregulation of PSMB2 and PSMD13 might indicate inhibition of degradation of oxidized proteins.

NDUFA5 subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I). We observed that drop in NDUFA5 proteins coincided with considerable decrease in the activity of complexes I and dysfunction of mitochondrial oxidative phosphorylation.

Tubulin beta chain (TUBB) is a structural component of the cytoskeleton, specifically microtubules (Fu *et al*., 2019). The expression of TUBB family member (TUBA3C, TUBA4A, and TUBB4B) is downregulated in teratozoospermic patients (Amargant *et al*., 2019). Taking these findings together, we conclude that oxidative stress-induced microtubule derangement and alterations of cytoskeletal proteins may affect the cell shape, adhesion and motility of spermatozoa.

All these pathways enriched by dysregulated gene were reported to have a significant role in oxidative stress indicating that seminal plasma can not mitigate $H_2O_2$ induced oxidative stress.

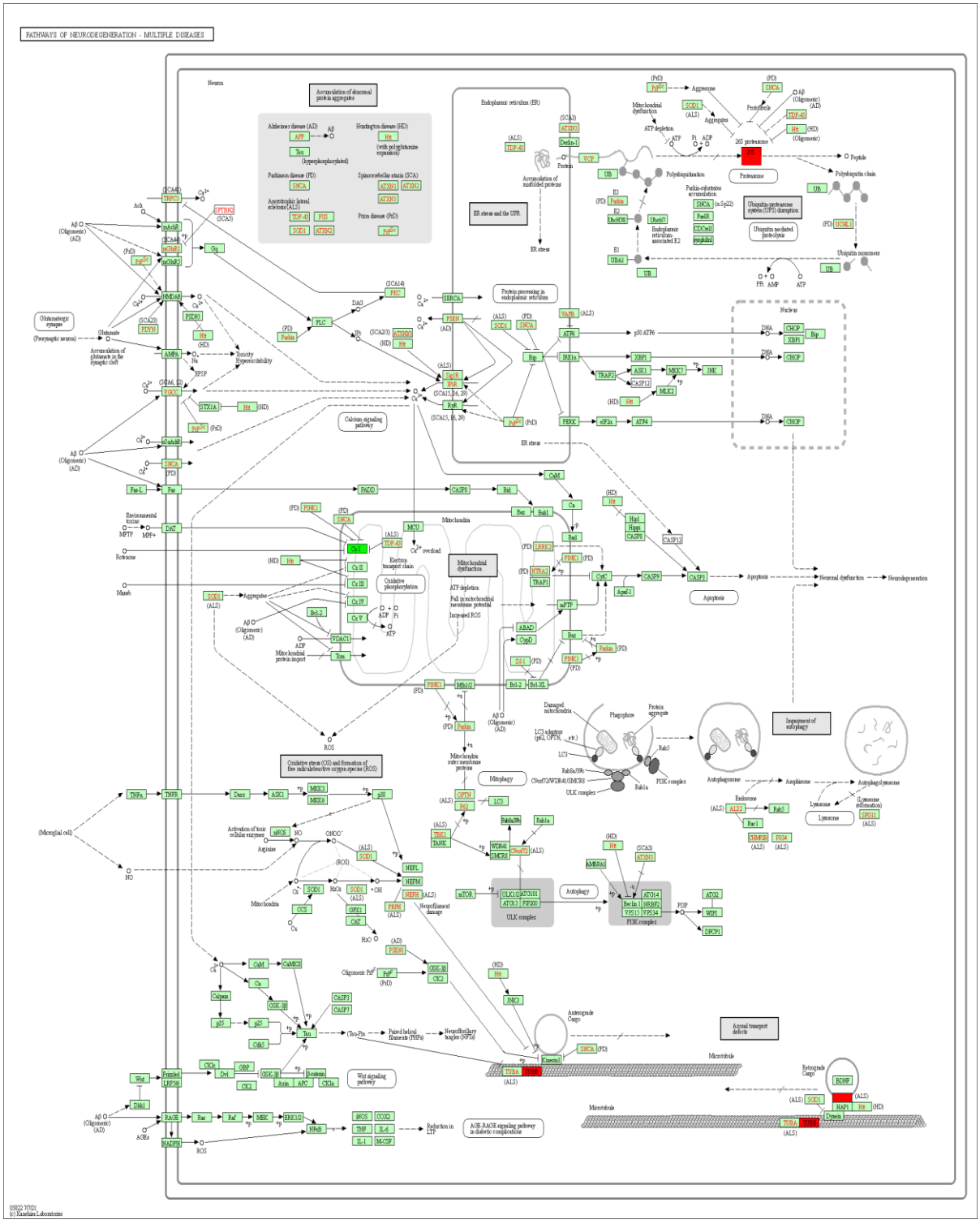



**Figure 4.16**: Neurodegeneration - multiple diseases pathway of DEPs in SP+ Vs SP- oxidative stress induced spermatozoa of Zebu bulls

### 4.4.3: Top 10 upregulated proteins in SP+ group as compared to SP- oxidative stress induced spermatozoa

The top 10 upregulated proteins were represented in the **Table 4.10**. Actin Related Protein 1A (ACTR1A) binds to both microtubules and cytoplasmic dynein and are involved in spindle formation, chromosome movement, nuclear positioning and furthermore play a role during sperm differentiation (Merdes *et al*., 2000). Previous reports suggested that member of Actin related protein showing differences in protein carbonylation in response to cryopreservation induced oxidative stress (Mostek *et al*., 2017). This is striking similarity with our study as dysregulation of ACTR1A is suppose to impair spermatogenesis, motility and could be a reason to induce oxidative stress due to protein oxidative modifications.

AHSG is a member of the protease inhibitors cystatin superfamily. Prior reports suggest that AHSG is more abundant in sperm from high-fertility bulls (Park *et al*., 2012). Rudloff *et al*. (2021) suggested that when cells are under hypoxic stress, fetuin-A (AHSG) can be locally increased. Testosterone helps to control production of fetuin-A (AHSG), this effect is partially mediated by androgen receptor activation (Voelkl *et al*., 2014). Our study correlate dysregulation of ASHG mediate impaired testosterone regulation as well as hypoxia associated cell changes induces oxidative damage.

Keratin, type I cytoskeletal 17 (KRT17) was upregulated in our study. A plethora of studies have shown that keratin type I family members proteins (KRT1) were upregulated in asthenozoospermia (Hashemitabar *et al*., 2015). Keratin protein phosphorylation modulated by stress activated kinase leads to dramatic organization of keratin filament network (Jin and Wang, 2014; Snider, 2016). Collectively, relating with above mentioned studies, oxidative stress hamper motility may be associated with phosphorylation of proteins.

Thioredoxin reductase 1, cytoplasmic (TXNRD1) was found to be upregulated in our study and it is an intracellular antioxidant that protects against oxidative stress when overexpressed or upregulated (Liu *et al*., 2022). Our findings are in agreement with the former studies and reveals compensatory redox mediated action in response to protect sperm from ROS mediated damage.

S100-A9 and S100-A12 were overexpressed after induction of oxidative stress. They are $Ca^{2+}$ binding proteins members of S100 family and play an important role in controlling the inflammatory response through cytokine secretion. A study performed by Camargo *et al*. (2013) reported that S100-A9 have overexpressed functions such as cellular response to reactive oxygen species in adult men before and after varicocelectomy. Ghavami *et al*. (2010) documented that S100 A9 exerts apoptosis activity through cross-talk of mitochondria and lysosomes via ROS. Studies also found that incubation of sperm *in vitro* with S100A9 leads to significantly decreased sperm motility and viability. Cell stress or inflammation cause S100 proteins to be released into the acellular compartment, where they bind cell surface receptors such as RAGE, TLR4, CD147 and GPCR, initiating a variety of cellular processes such as cell differentiation, migration, apoptosis, proliferation and inflammation (Xia *et al*., 2018). Our results are in line with these finding as ROS leads to decreased motility, viability and increase apoptosis. Our findings on Beta-microseminoprotein (MSMB) are in line with the findings of Wu *et al*. (2013); considerably higher MSMB levels in oligoasthenoteratozoospermic patients compared to fertile controls. Collectively these findings highlighted alterations in the expression of numerous upregulated proteins implicated enhance oxidative stress.

**Table 4.10:** Top 10 upregulated proteins in SP+ compared to SP- oxidative stressed spermatozoa of Zebu bulls

| S. No. | Uniprot ID | Gene Symbol | Protein name | Log fold change |
|---|---|---|---|---|
| 1 | F2Z4F0 | ACTR1A | Actin Related Protein 1A | 3.49 |
| 2 | P12763 | AHSG | Alpha-2-HS-glycoprotein | 3.26 |
| 3 | G3N2P6 | KRT17 | Keratin, type I cytoskeletal 17 | 2.69 |
| 4 | G1K1Q2 | TXNRD1 | Thioredoxin reductase 1, cytoplasmic | 2.50 |
| 5 | F1MHS5 | S100A9 | Protein S100-A9 | 2.26 |
| 6 | A5PKI3 | FAM3C | Protein FAM3C | 2.23 |
| 7 | F1MDF2 | MSMB | Beta-microseminoprotein | 2.10 |
| 8 | P79105 | S100A12 | Protein S100-A12 | 1.99 |
| 9 | Q0VCQ9 | RCN2 | Reticulocalbin 2, EF-hand calcium binding domain | 1.96 |
| 10 | G3MXB5 | LOC524810 | Uncharacterized protein | 1.93 |

### 4.4.4: Top 10 downregulated proteins in SP+ compared to SP- oxidative stress induced spermatozoa

The top 10 downregulated proteins represented in **Table 4.11**. DJ homolog subfamily C member 3 (DJC3) is downregulated in our finding. DJC3 is HSP40/ DNAJ family member, which is involved in biological processes like protein metabolism and stress response. DJ-1 is an important redox-reactive signaling intermediate controlling oxidative stress (Zhang *et al*., 2020). Our study revealed that DJC3 dysregulation redox mediated action involving directly in stress response.

Other proteins like Golgi associated RAB2 interactor protein 2 are located in sperm midpiece, seems to play a role in sperm motility. A novel Golgi-resident RAB2 B-specific binding protein called Golgi-associated RAB2 B interactor-like 4 whose suppression also caused the fragmentation of Golgi (Aizawa and Fukuda, 2015). Translocase of Outer Mitochondrial Membrane 34 (TOMM34) is found downregulated in our study, and it was originally identified as a component of the mitochondrial import machinery and has been found to form a complex with both Hsp70 and Hsp90 as a scaffolding co-chaperone (Trcka *et al*., 2014). Zhang *et al*. (2019) found mitochondrial origin of the increased levels of ROS observed in cells exposed to inhibitor of proteasome leads to decrease expression of TOMM34. Our study is in agreement with previous finding, we suppose that TOMM34 could have been linked to dysfunction of mitochondria that impaired transport machinery. FAM3B is an another protein downregulated in our findings, and its overexpression results in resistance to cell death corresponding to a recent study by Maciel-Silva *et al*. (2018).

Pro-adrenomedullin (ADM) is downregulated in SP- group on comparing SP+ group. As reported earlier which is purified from adrenal medulla, increased the flagellar/ciliary beating of human spermatozoa through stimulating the release of Nitric oxide (Qiu *et al*., 2021). Furthermore, studies revealed that it is a crucial protein for the implantation of human embryos (Karli *et al*., 2020). Zhao *et al*. (2021) reported that, Cytochrome b5 domain-containing protein 1 (CYB5D1) mutation causes uncoordinated ciliary beating by altering the redox status of flagella. CYB5D1 is differentially expressed between smoking and control group spermatozoa protein suggesting smoking induces oxidative stress (Chen *et al*., 2015). Whereas, COP9

signalosome complex subunit 4 (COPS4) was downregulated in our finding. Nahlik *et al*. (2010) reported that COP9 is required for protection against oxidative stress and defects in COP9 result in embryonic lethality impacts; a variety of cellular activities, including the ubiquitin-proteasome system. COP9 is emerging as a crucial contributor in the response to DNA damage (Wei and Deng, 2003).

Cystatin C (CST3) is another downregulated protein. It is a cysteine protease inhibitor in seminal plasma, which prevents the acrosome response, premature capacitation, maintaining the capacity of sperm to fertilize (Lee *et al*., 2018). This is striking similarity with our study as all the downregulated proteins create unfavourable environment for maintaining fertility of the spermatozoa.

**Table 4.11:** Top 10 downregulated proteins in SP+ compared to SP- oxidative stressed spermatozoa of Zebu bulls

| S. No. | Uniprot ID | Gene Symbol | Protein name | Log fold change |
|---|---|---|---|---|
| 1 | F1N036 | DJC3 | DJ homolog subfamily C member 3 | -3.22 |
| 2 | Q32L49 | FAM71E1 | Golgi associated RAB2 interactor protein 5A | -2.20 |
| 3 | E1BGD1 | TOMM34 | Translocase of outer mitochondrial membrane 34 | -1.65 |
| 4 | O77588 | PLOD1 | Procollagen-lysine,2-oxoglutarate 5-dioxygese 1 | -1.49 |
| 5 | E1BQ21 | FAM3B | FAM3 metabolism regulating signaling molecule B | -1.44 |
| 6 | O62827 | ADM | Pro-adrenomedullin | -1.34 |
| 7 | E1BP78 | CYB5D1 | Cytochrome b5 domain-containing protein 1 | -1.34 |
| 8 | Q3SZA0 | COPS4 | COP9 signalosome complex subunit 4 | -1.34 |
| 9 | P01035 | CST3 | Cystatin-C | -1.30 |
| 10 | G3X6N3 | TF | Serotransferrin | -1.29 |

### 4.4.5: Protein–protein interaction network

The PPI network comprised 42 nodes and 19 edges in oxidative stress-induced spermatozoa shown in **Fig 4.17**. According to the results from the string analysis the expected of edges were 6 which is less than the actual interaction this means that proteins have more interaction among themselves. The average node degree, avg. local clustering coefficient and PPI enrichment p-value were 0.905, 0.337 and 2.85e-05 respectively. However, the network analysis revealed

involvement of 2 biological processes (protein activation cascade and negative regulation of hemostasis), 1 pathway (Proteasome) and 4 cellular components as shown in **Fig. 4.18**. Proteasome pathway involve PSMB2 and PSMD13 gene. Additionally, it has been found that sperm energy metabolism and motility are related to proteasome pathways (Dietrich *et al.,* 2016). Asthenozoospermic samples of human semen exhibited lower proteasome alpha complex concentrations than normozoospermic patients (Siva *et al*., 2010).

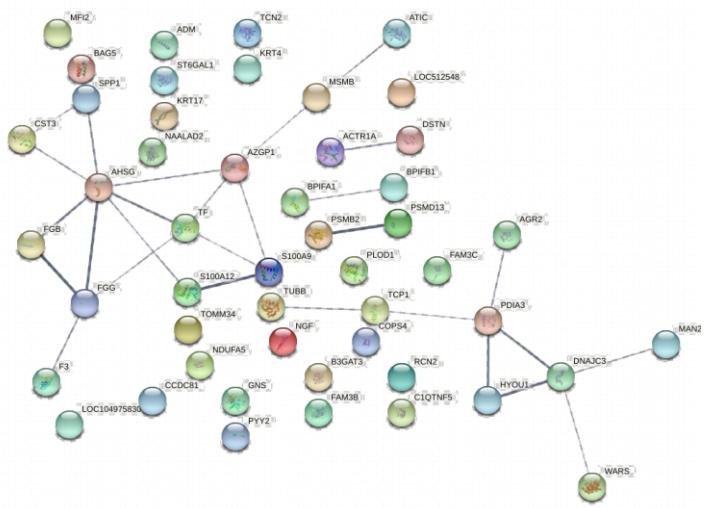



**Figure 4.17:** PPI network of DEPs in SP+ Vs SP- oxidative stress induced spermatozoa of Zebu bulls developed using search tool for the retrieval of interacting genes

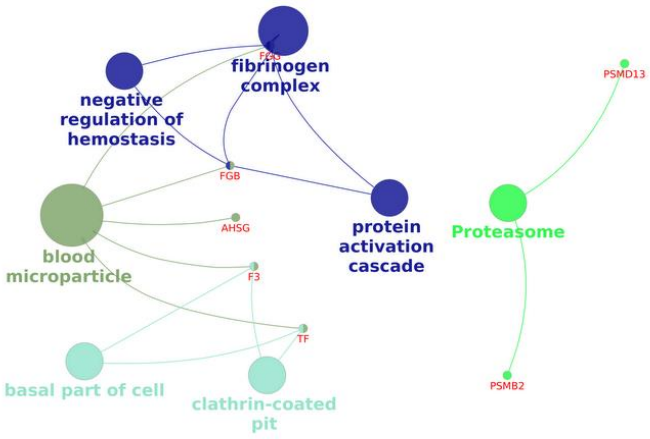



**Figure 4.18:** Interaction network of dysregulated proteins in SP+ Vs SP- oxidative stress induced spermatozoa of Zebu bulls

## 4.5: Objective-3: Identification of potential proteins that could serve as biomarkers for oxidative stress in bull spermatozoa

One of the main emphasis of this study was to uncover possible biomarkers that might be used as indicators of oxidative stress. In the current study, among all the proteins, we found 42, 11 and 7 proteins unique to fresh, oxidative stress induced spermatozoa with and without seminal plasma, respectively.

### 4.5.1: Unique proteins in freshly ejaculated spermatozoa

Topmost unique proteins in freshly ejaculated spermatozoa are Olfactomedin-like protein 2A (OLFML2A), Iduronate 2-sulfatase (IDS), SHBG protein (SHBG), Heparan-sulfate 6-O-sulfotransferase (HS6ST1), Heterogeneous nuclear ribonucleoproteins A2/B1 (HNRNPA2B1), FAT atypical cadherin 2 (FAT2), Lysosomal thioesterase PPT2 (PPT2), Endoplasmic reticulum aminopeptidase 2 (ERAP2), Cytohesin-2 (CYTH2) and Myeloid-derived growth factor (MYDGF) **Table 4.12**. In our study, OLFM-2 was found unique to fresh spermatozoa; it has been linked to cellular growth, differentiation, and pathological processes (Kodithuwakku *et al*., 2011). Viana *et al*. (2018) found that in high fertile bulls, olfactomedin-like 2A-like protein was overexpressed. IDS is necessary for the breakdown of glycosaminoglycans (GAGs), heparan sulfate (HS) and dermatan sulfate (DS) (Kuzenkova *et al*., 2020). Sex hormone binding globulin (SHBG) is a testicular androgen-binding protein (ABP) produced by the sertoli cells in the lumen of the seminiferous tubules, where it is assumed to primarily serve as a transporter of testosterone throughout the male reproductive system (Joseph, 1994). SHBG also perform important functions in spermatogenesis, and sperm maturation and its expression in the epididymis were only positively associated with plasma membrane integrity (Dalmazzo *et al*., 2019). SHBG alleles were also attributed to higher sperm concentration, emphasizing the presence of these genes in spermatogenesis and sperm quality (Lazaros *et al*., 2008). In our study, SHBG is thought to be directly correlated with testicular androgen-binding and transport and is associated with higher sperm concentrations. As previously reported, vitamin D-binding showed the highest negative correlations with fertility ranking (Viana *et al*., 2018). Earlier finding suggested that IGFBP-6 interacts with pro inflammatory cytokine genes to regulate the immune system (Alzaid *et al*., 2016) and causes inhibition of proliferation,

metastasis and induction of apoptosis of sarcoma (Qiu *et al*., 2018). Altogether all proteins unique to freshly ejaculated spermatozoa are elucidating protective functions to spermatozoa.

**Table 4.12:** Top 10 unique proteins in freshly ejaculated spermatozoa of Zebu bulls

| S. No. | Uniprot ID | Protein name | Gene symbol |
|---|---|---|---|
| 1 | E1B818 | Olfactomedin-like protein 2A | OLFML2A |
| 2 | F1N2D5 | Iduronate 2-sulfatase | IDS |
| 3 | A5PKC2 | SHBG protein | SHBG |
| 4 | E1BNW3 | Heparan-sulfate 6-O-sulfotransferase | HS6ST1 |
| 5 | Q2HJ60 | Heterogeneous nuclear ribonucleoproteins A2/B1 | HNRNPA2B1 |
| 6 | F1MPF3 | FAT atypical cadherin 2 | FAT2 |
| 7 | Q1JQA0 | Lysosomal thioesterase PPT2 | PPT2 |
| 8 | A6QPT7 | Endoplasmic reticulum aminopeptidase 2 | ERAP2 |
| 9 | Q2KI41 | Cytohesin-2 | CYTH2 |
| 10 | P62248 | Myeloid-derived growth factor | MYDGF |

### 4.5.2: Unique proteins in oxidative stress induced spermatozoa of Zebu bull

Dynein axonemal heavy chain 3, Dynein axonemal intermediate chain 3, Oxysterol-binding protein, Actin-related protein 2/3 complex subunit 4, Tryptophan-tRNA ligase, mitochondrial, Mitochondrial pyruvate carrier-like protein, DnaJ homolog subfamily B member 1, Phosphomannomutase 2, DnaJ homolog subfamily B member 1 and 26S protease regulatory subunit 6B are uniquely expressed in oxidative stress induced spermatozoa **Table 4.13**. Previous reports suggest that defects in the axonemal genes dynein axonemal heavy chain (DNAH) and dynein axonemal intermediate chain 1 (DNAI1) cause reduced sperm motility. 4-HNE prevents sperm capacitation by targeting the tyrosine phosphorylation pathway and causing motility loss, which could be through a direct adduct on the dynein heavy chain (Baker *et al*., 2015). Interestingly, Dynein axonemal heavy chain 3 and Dynein axonemal intermediate chain 3 uniquely express relates spermatozoa with dysregulation of motility. OSBP is involved in choslesterol metabolism, its expression increase in response to stress, and induce cellular oxidative stress and cytotoxicity,

mainly via apoptosis (Arnal-Levron *et al*., 2019). Dias *et al*. (2020) reported that mitochondrial pyruvate carrier 1, 2 differentially expressed proteins associated with decreased semen quality in patients with testicular seminoma. It is involved in selective transport of pyruvate across the inner mitochondrial membrane by the mitochondrial pyruvate carrier (MPC). DNAJB5, a member of the heat shock protein 40 chaperone family (Hageman *et al*., 2011), is a regulator of cellular protein homeostasis through its interaction with histone deacetylases and is activated in response to oxidative stress (Hageman *et al*., 2010). Moreover, previous reports suggested that the expression of DNAJB5 is known to increase in response to other cellular stressors. We found DNAJB1 uniquely expressed in oxidative stressed spermatozoa.

### 4.5.3: Unique proteins in oxidative stress induced spermatozoa in presence of seminal plasma in Zebu bulls

SLC25A13, CLGN, TNP2, DRC1, HDHD5 and BBS1 are uniquely expressed in oxidative stressed spermatozoa in presence of seminal plasma as shown in the **Table 4.14**. SLC25A13 is a mitochondrial family member involved in exchanging aspartate for glutamate across the inner mitochondrial membrane. We suggest that any dysregulation associated with SLC25A13 could hamper mitochondrial respiratory activity. Previous reports suggested that male mice deficient in calmegin (CLGN) exhibit poor sperm migration into the oviduct and lose their capacity to bind sperm to the zona pellucida (Yamaguchi *et al*., 2006). Transition nuclear proteins (TNPs) are involved in packaging sperm nuclear DNA during spermiogenesis. TNP2 is a DNA-compacting protein translation necessary for the correct differentiation of round spermatids into mature spermatozoa and for male fertility (Tseden *et al*., 2007). TNP2 were overexpressed in spermatozoa from high-fertile boars (Alvarez-Rodriguez *et al*., 2021). Hammadeh *et al*. (2010) reported that oxidative stress induced by cigarette smoking causes a poor protamine replacement process. In the present study, we found dysregulation of TNP2 is related to defective protamine packaging due to oxidative stress.

Among all the uniquely expressed proteins in the present study, we report Solute carrier family 25 member 13 (SLC25A13), Nuclear transition protein 2 (TNP2), Mitochondrial pyruvate carrier-like protein (Q2M2T3), DNAJB1 (DnaJ

homolog subfamily B member 1), Calmegin (CLGN) could serve as indicators of oxidative stress in spermatozoa due to involvement of these proteins in stress response biological process**.**

**Table 4.13:** Unique proteins in oxidative stress induced spermatozoa of Zebu bulls

| S. No. | Uniprot ID | Protein name | Gene symbol |
|---|---|---|---|
| 1 | F1MV72 | Dynein axonemal heavy chain 3 | DNAH3 |
| 2 | F1MJ69 | Dynein axonemal intermediate chain 3 | DNAI3 |
| 3 | E1BPW1 | Oxysterol-binding protein | OSBP |
| 4 | Q148J6 | Actin-related protein 2/3 complex subunit 4 | ARPC4 |
| 5 | Q3T099 | Tryptophan--tRNA ligase, mitochondrial | WARS2 |
| 6 | Q2M2T3 | Mitochondrial pyruvate carrier-like protein | - |
| 7 | F6QYE2 | DnaJ homolog subfamily B member 1 | CCDC37/ CFAP100 |
| 8 | Q3SZJ9 | Phosphomannomutase 2 | PMM2 |
| 9 | Q3MI00 | DnaJ homolog subfamily B member 1 | DNAJB1 |
| 10 | F1MG70 | 26S protease regulatory subunit 6B | PSMC4 |

**Table 4.14:** Unique proteins in oxidative stress induced spermatozoa in presence of seminal plasma in Zebu bulls

| S. No. | Uniprot ID | Protein name | Gene symbol |
|---|---|---|---|
| 1 | F1MX88 | Solute carrier family 25 member 13 | SLC25A13 |
| 2 | Q3SYT6 | Calmegin | CLGN |
| 3 | P26377 | Nuclear transition protein 2 | TNP2 |
| 4 | Q32KY1 | Dynein regulatory complex protein 1 | DRC1 |
| 5 | F1N6K5 | Haloacid dehalogenase like hydrolase domain containing 5 | HDHD5 |
| 6 | E1BN34 | BBS1 domain-containing protein | BBS1 |

# Chapter - 5

# Summary & Conclusion

*Chapter 5*

# SUMMARY AND CONCLUSION

Oxidative stress occurs due to excessive production of prooxidant relative to antioxidant. Numerous researches indicated that production of ROS increases significantly during the cryopreservation in bull spermatozoa, which affects sperm membranes, proteins, and DNA thereby having a potential negative effect on fertility. Therefore, in order to sustain male fertility, it is essential to keep the equilibrium between ROS and antioxidants. Sperm fertility can be restored in spermatozoa affected with oxidative stress if mechanism behind ROS generation is understood thoroughly. Identification of the most crucial cellular targets, biological process, molecular functions underlying pathways and impaired sperm functions in response to ROS would help in elucidating idiopathic male infertility. Since sperm proteins are essentially involved in sperm-zona binding and fertilization, and it was shown that ROS could possibly affect cell protein composition, identification of sperm proteomic alterations induced by oxidative stress could help in understanding the oxidative stress mediated infertility and also to evolve measures to prevent the negative effects of oxidative stress on sperm fertility.

Therefore, in the present study, we assessed cellular and functional phenomes of bull spermatozoa before and after induction of oxidative stress, and compared proteomic profiling of normal and oxidative stress induced bull spermatozoa with the aim to identify potential proteins that could serve as biomarkers for oxidative stress in bull spermatozoa. The salient findings are as under:

- High throughput global proteomic analysis detected a total of 1441 proteins in Deoni bull spermatozoa; a majority of the significantly identified proteins were involved in a total of 70 pathways, 118 molecular functions and in 141 biological processes.
- The enriched biological processes included spermatogenesis, proteolysis, protein folding, protein stabilization, metabolic process and sperm binding to zona pellucida; around 30% of identified proteins were involved in ATP binding, metal ion binding and protein binding.

- The major pathway in which the identified proteins were involved was metabolic process (26%) and oxidative phosphorylation (7%).
- Incubation of bull spermatozoa with $H_2O_2$ at the concentration of 50µM for a period of 60 minutes induced a greater ($P<0.05$) oxidative stress.
- Oxidative stress increased ($P<0.05$) the proportion of ROS positive spermatozoa, dead spermatozoa and lipid peroxidation status and altered the intra-cellular calcium concentrations in bull spermatozoa but did not affect the acrosome reaction status and mitochondrial membrane potential.
- A total of 1434 and 1458 proteins were detected in oxidative stress induced spermatozoa with and without seminal plasma, respectively.
- When compared to freshly ejaculated spermatozoa, oxidative stressed spermatozoa showed dysregulation of 451 proteins, of which 260 were upregulated while 191 were downregulated. A total of 78 proteins were identified to be specific to oxidative stressed spermatozoa.
- Among the differentially expressed proteins (DEPs), 27% were involved in metabolic process, 8% were involved in reactive oxygen species and 7% were involved in oxidative phosphorylation. Significant number of proteins were involved in biological process like response to oxidative stress, spermatogenesis, protein stabilization and folding, and sperm motility.
- The top 10 upregulated proteins in oxidative stressed spermatozoa included MYCBP, NDUFS7, LETM2, SEPTIN4, FAM71F2, ECH1, SEPTIN10, CCDC81, LYPLA1 and CYB5R2, which are involved in spermatogenesis, ROS production, sperm motility and oxidation of fatty acids.
- The top 10 downregulated proteins in oxidative stressed spermatozoa included AHSG, VIP, SPP1, SEMA3F, OLFM4, BPIFB1, LGMN, PSAP, LGALS3BP and AZGP1, which are involved in ROS production, sperm motility and sperm-zona interaction.
- A total of 53 proteins were differentially expressed between SP+ and SP- oxidative stressed spermatozoa; 32 were upregulated while 21 were downregulated.
- The DEPs were involved in a total of 7 pathways, 11 molecular functions, 15 cellular components and in 29 biological processes.

- Among the DEPs, 10% were involved in Huntington disease, 10% were involved in neurodegenerative disease and 8% were involved in Parkinson disease. Significant number of proteins were involved in biological process like innate immune response, iron ion transport and cellular response to inteukin-7.
- The top 10 upregulated proteins in oxidative stressed spermatozoa with seminal plasma included ACTR1A, AHSG, KRT17, TXNRD1, S100A9, FAM3C, MSMB, S100A12, RCN2 and LOC524810.
- The top 10 downregulated proteins in oxidative stressed spermatozoa with seminal plasma included DJC3, FAM71E1, TOMM34, PLOD1, FAM3B, ADM, CYB5D1, COPS4, CST3 and TF.
- We found 42, 11 and 7 proteins unique to fresh, oxidative stress induced spermatozoa with and without seminal plasma, respectively.

# Conclusion

- ✓ Oxidative stress negatively influenced sperm viability, lipid peroxidation and intracellular calcium and altered the expression of sperm proteins involved in sperm motility, ROS production, oxidation of fatty acids and sperm-zona interaction.
- ✓ Presence of seminal plasma did not have beneficial effect on sperm functions and proteome during *in vitro* induction of oxidative stress.
- ✓ Solute carrier family 25 member 13 (SLC25A13), Nuclear transition protein 2 (TNP2), Mitochondrial pyruvate carrier-like protein (Q2M2T3), DNAJB1 (DnaJ homolog subfamily B member 1), Calmegin (CLGN) could serve as indicators of oxidative stress in bull spermatozoa.

# Chapter - 6

# Bibliography

*Chapter 6*

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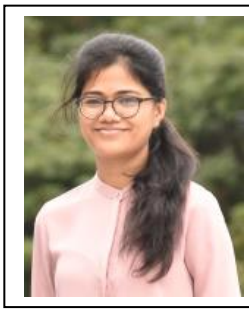
# CURRICULUM VITAE




## Dr. Apoorva Verma

**M.V.Sc in Veterinary Gynaecology and Obstetrics**

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## *Career Objective*

Looking forward to employ my skills to contribute to the profession of veterinary and animal science.

### *Technical skill sets on research*

Routine semen evaluation and cryopreservation of semen.

Artificial insemination and advanced sperm function tests (Microscopic examination to Flow cytometric analysis).

Handling of fresh and cryopreserved sperm and $LN_2$ containers

### *Proteomics*

Proteomic analysis

### *Bioinformatics*

DAVID and Cytoscape (ClueGO)

## *Academic Qualifications*

| Degree | University/Board | Year | Percentage/ Grade |
|---|---|---|---|
| M.V.Sc. | National Dairy Research Institute, Karnal | 2022 | 8.7/10.0 |
| B.V.Sc. & AH | NDVSU, Jabalpur, M. P. | 2020 | 8.7/10.0 |
| XII class | CBSE Board | 2013 | 82.4 % |
| X class | M. P Board | 2011 | 83.5 % |

## *Research Project*

***Masters' Programme:* Impact of oxidative stress on bull sperm proteome and functional attributes**

***Awards and Fellowships:***

1. Secured Gold medal in B.V.Sc & A.H (Batch-2015) NDVSU Jabalpur M. P.
2. Received Institutional Scholarship funded by ICAR (Indian Council for Agriculture & Research) during Post graduation
3. Academic excellence award: Merit Certificate in Veterinary Gynaecology & Obstetrics during M.V.Sc. degree programme (2020-22), ICAR-National Dairy Research Institute

**DATE: Sept 2022** **(Apoorva Verma)**