EFFICIENCY OF MELATONIN ON CRYOPRESERVED SEMEN QUALITY, *HSP* 70 AND *HSP* 90 GENE EXPRESSION OF SWAMP BUFFALO IN DIFFERENT THAWING METHODS

SUBASH CHANDRA CHAUDHARY

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in (Agricultural Biotechnology)
2020

EFFICIENCY OF MELATONIN ON CRYOPRESERVED SEMEN QUALITY, *HSP* 70 AND *HSP* 90 GENE EXPRESSION OF SWAMP BUFFALO IN DIFFERENT THAWING METHODS

SUBASH CHANDRA CHAUDHARY

A Thesis Submitted to the Graduate School of Naresuan University
in Partial Fulfillment of the Requirements
for the Master of Science in (Agricultural Biotechnology)
2020

Thesis entitled "Efficiency of melatonin on cryopreserved semen quality, *hsp* 70 and *hsp* 90 gene expression of swamp buffalo in different thawing methods"

By SUBASH CHANDRA CHAUDHARY

has been approved by the Graduate School as partial fulfillment of the requirements

for the Master of Science in Agricultural Biotechnology of Naresuan University

Oral Defense Committee

..... Chair
(Assistant Professor Payungsuk Intawicha, Ph.D.)

..... Advisor
(Wilasinee Inyawilert, Ph.D.)

..... Co Advisor
(Assistant Professor Amornrat Wanangkarn, Ph.D.)

..... Co Advisor
(Assistant Professor Niran Aeksiri, Ph.D.)

..... Internal Examiner
(Associate Professor Kumrop Ratanasut, Ph.D.)

Approved

.....
(Professor Paisarn Muneesawang, Ph.D.)

for Dean of the Graduate School

Title EFFICIENCY OF MELATONIN ON CRYOPRESERVED SEMEN QUALITY, *HSP* 70 AND *HSP* 90 GENE EXPRESSION OF SWAMP BUFFALO IN DIFFERENT THAWING METHODS

Author SUBASH CHANDRA CHAUDHARY

Advisor Wilasinee Inyawilert, Ph.D.

Co-Advisor Assistant Professor Amornrat Wanangkarn, Ph.D. , Assistant Professor Niran Aeksiri, Ph.D.

Academic Paper Thesis M.S. in Agricultural Biotechnology, Naresuan University, 2020

Keywords Melatonin, Cryopreserved Semen Quality, Swamp Buffalo, hsp 70, hsp 90

ABSTRACT

The present study was undertaken with the objectives of observing the effects of melatonin on cryopreserved semen quality, *hsp* 70 and *hsp* 90 expression of swamp buffalo after thawing with different methods. Semen was collected from four swamp buffalo bulls (4years to 8 years of age) with the aid of artificial vagina. Semen was equally divided into two aliquots: one was extended and cryopreserved with tris-egg yolk-citrate-glycerol extender supplemented with 1mM of melatonin (MLT) and the other was extended with only tris-egg yolk-citrate-glycerol and cryopreserved at –196°C. Two thawing methods were applied: Method I:30°C for 60 seconds and Method II: 37°C for 30 seconds. Semen quality parameters for motility was measured by computer assisted semen analyser (CASA) and the sperm apoptosis was assessed by flow cytometry with Annexin V PE/7-AAD. The expression for *hsp* 70 and *hsp* 90 was assessed with reverse transcription polymerase chain reaction (RT-PCR). The results showed that the percentage of total motility, progressive motility, and curve line velocity of sperm in the 1 mM MLT treated groups were significantly higher than the untreated groups when thawed at 30°C for 60 seconds and 37°C for 30 seconds. However, there were no any significant differences in the other parameters such as straight line velocity (VSL), average path velocity (VAP), curve line distance (DCL),

straight line distance (DSL), average path distance (DAP), amplitude of latera head (ALH), beat cross frequency (BCF), linearity (LIN), straightness (STR), wobble (WOB). However, all the sperm parameters values, excluding the WOB, were higher when 1mM treated frozen straws were thawed at 37°C for 30 seconds. The percentage of live spermatozoa in the 1 mM MLT group was significantly higher ($p < 0.01$) and significantly lower ($p < 0.01$) early apoptotic sperms than the untreated groups in every thawing conditions. Within the treatment groups, the highest percentage of live sperm was found when thawed at 37°C for 30 seconds which is significantly higher ($p <.05$) than thawing at 30°C for 60 seconds. The lowest percentage of early apoptotic spermatozoa was found in the treatment group when thawed at 37°C for 30 seconds which was significantly lower ($p < 0.05$) than thawing at 30°C for 60 seconds. RT-PCR products of *hsp* 70 and *hsp* 90 in the MLT treated group yielded a stronger band than the control group. The band of the MLT treated group, when thawing at 37°C for 30 seconds, was the stronger than those when thawing at 30°C for 60 seconds. Hence, the overall results of the present study confirm that 1 mM MLT supplementation to the semen extender can improve the motility and viability of spermatozoa and minimize the apoptosis in the frozen-thawed semen of swamp buffalo and also promotes the expression of *hsp* 70 and *hsp* 90.

ACKNOWLEDGEMENTS

I would like to express my great appreciation to Ministry of Agriculture and Livestock development, Department of Livestock Services, Government of Nepal for providing this opportunity and granting me the study leave to pursue my master's degree. I would like honor Thailand International Cooperative Agency (TICA) for providing me the scholarship under Thailand International Postgraduate Program (TIPP). I also want to specially mention Naresuan University, Faculty of Agriculture, Natural Resources and Environment, Department of Agricultural Science, Phitsanulok, Thailand for providing me the necessary environment for accomplishing this degree smoothly.

I would like to express my deep sense of gratitude to my advisor, Dr. Wilasinee Inyawilert, for her constant guidance, valuable comments, recommendation, suggestions and her critical review to shape this dissertation. I would like to thank her for her friendly attitude, share of her knowledge, free and frank exchange of ideas which lead to the success of this study.

I am immensely grateful to Dr. Amornrat Wanaungkarn and Dr. Niran Aeksiri members of the advisory committee for their guidance, suggestions, encouragement, and editing to refine the dissertation to this quality. I wish to express my sincere thanks and gratitude to the Program Coordinator, Dr. Kumrop Ratanasut, Program of Agricultural Biotechnology and Dr. Rangsun Charoensook, Department of Agricultural Science, Naresuan University for providing me the necessary environment and equipment to conduct the research work.

Moreover, I would also like to recognize Mr. Vuttikai Paungsukpaibool (P' Wood) for his incredible and rigorous support, coordination and technical assistance for the semen sample collection and processing. Ajarn Ukrit also provided his continuous help and support in most of my activities, I would like to pay huge respect and heartfelt thanks. The HJ Thai Swamp Buffalo Farm, Uthai Thani, and its staffs are worth to mention here. They provided me the great assistance and support otherwise it would not be possible to collect the sample.

I would also like to appreciate the helping hands of all the staffs of faculty of agriculture, natural resources and environment for helping in all my academic works

and research work run smoothly especially P Kook, P Tor. and Game.

Special mention also goes to my friends Ananda, Bun, Phee, Milk, Ning, Tong, Thitimai, Tejendra and Tenzin and my dear juniors Aom and Fluk who have been always there and helped me during the study as well as entire period in the university.

I would like to express my gratitude and love to the whole family and especially my respected Father and Mother whose support, sacrifice and selfless love has always been an energetic force for my every endeavor in life. Last but not the least my heart warming appreciation and love goes to my beautiful wife Dr. Deepa Diwali and my cute daughter Samriddhi for their help in every moment, everlasting support and outstanding inspiration.

SUBASH CHANDRA CHAUDHARY

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

Symbol	Name
●O_2^-	Superoxide anion
µL	Microliter
•OH	Hydroxyl radical
7 AAD	7-Amino-actinomycin D
AFMK	N1-acetyl- N2-formyl-5-methoxykinuramine
AI	Artificial insemination
ALH	Amplitude of lateral head displacement
AMK	N1-acetyl-5-methoxykinuramine
ANOVA	Analysis of variance
ATP	Adenosine-5'-triphosphate
AV	Artificial vagina
Bax	Bcl2 associated X Protein
BCF	Beat cross frequency
Bcl2	B-cell lymphoma 2
BSA	Bovine serum albumin
bZIP	Basic leucine zipper
cAMP	Cyclic adenosine monophosphate
CASA	Computer assisted semen analyser
CAT	Catalase
cm	Centimeter
CPA	Cryoprotective agent
DAP	Average path distance
DCL	Curve line distance
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSL	Straight line distance
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPX	Glutathione peroxidase

GR	Glutathione reductase
GSH	Glutathione
GSSG	Oxidized GSH
H2O2	Hydrogen peroxide
HO-1	Heme oxygenase-1
HOST	Hypo osmotic swelling test
HSC	Constitutively expressed hsp
HSP	Heat shock protein
HSP100	Heat shock protein H and
HSP60	Heat shock protein 60 D
HSP70	Heat shock protein 70 A
HSP90	Heat shock protein 90 C
IU	International unit
kDa	Kilo dalton
KG	Kilo gram
LDH	Lactate dehydrogenase
LIN	Linearity
LPO	Lipid peroxidation
LSD	Least significant difference
mg	Milligram
min	Minute
ml	Milliliter
MLT	Melatonin
mM	Milli moles
mRNA	Messenger RNA
NADPH	Nicotinamide adenine dinucleotide phosphate
NOS	Nitrous oxide synthase
NOX5	Nicotinamide adenine dinucleotide phosphate oxidase
Nrf2	Nuclear factor erythroid 2-related factor 2
ºC	Celsius scale
OD	Optical density

OHCL	Hypochlorite radical
OS	Oxidative stress
P	Probability
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PS	Phosphatidyl serine
PT	Post-thaw
PTM	Post thaw motility
PUFAs	Polyunsaturated fatty acids
r	Correlation
RNA	Ribonucleic acid
RNS	Reacive nitrogen species
ROS	Reactive oxygen species
sec	Second
SEM	Standard error of means
SOD	Superoxide dismutase
STR	Straightness
TAE	Tris-Acetate-EDTA buffer
TAOC	Total anti-oxidant capacity
TEMED	Tetramethylethylenediamine
VAP	Average path velocity
VCL	Curve line velocity
VSL	Straight line velocity
WOB	Wobble

CHAPTER I

INTRODUCTION

1.1 Background information

The swamp buffalo (*Bubalus bubalis carabanensis*) is one of the types of Asiatic water buffalo and is indigenous in Thailand, mostly to the north-eastern part and they are well adapted with the poor condition (Triwitayakorn et al., 2006). However, the buffalo population is reducing drastically in Thailand. The number of buffaloes has decreased from 3.7 to 1.49 million from 1995 A.D. to 2004 A.D., on an average decline of 22 % per year (The Department of Livestock Development, 2004). As a consequence, conservation of the swamp buffalo and their sustainable utilization in the production system is of utmost necessity (Na-Chiangmai, 2002). In this regard, artificial insemination (AI) can play an important role to improve the genetic capability as well as for the conservation of the animal. Nevertheless, good quality of frozen-thawed semen is required to get the positively attributed AI outcomes.

AI has become one of the important reproductive and breeding biotechnology tools for enhancing the productivity of the livestock by the use of cryopreserved semen from elite males. Accordingly, semen cryopreservation plays cardinal role to upswing the sperm quality and for the application of artificial insemination. During the freezing process, the spermatozoa remains in quiescent state but retaining its biological and functional activity while the thawing process brings the sperm cell back to physiologic temperature reactivating the metabolism (Bearden, 2004). Hence, thawing procedure is also important as the freezing process in relation with the survival of spermatozoa (Nur, Dogan, Soylu, & Ak, 2003). There is about 40–50% loss in bull semen quality during the freezing-thawing process, including sperm motility, effective survival time, and antioxidant enzymes activity (Hu, Zhao, Tian, Zan, & Li, 2011; X. G. Zhang et al., 2015). Various researches have been conducted on different methods of thawing to find out the optimal thawing temperature and duration which result in higher viability and motility of spermatozoa

after thawing process. Therefore, thawing carefully at an optimal temperature for sufficient time is an integral part for minimize the loss of semen quality.

During the freezing-thawing process, there is oxidative stress and, as a result, the formation of reactive oxygen species (Bilodeau, Chatterjee, Sirard, & Gagnon, 2000). The polyunsaturated fatty acids of the sperm plasma membrane are susceptible to reactive oxygen species (ROS) damage, as its cytoplasm contains low concentrations of free radical scavenging enzymes (Saleh & Agarwal, 2002). There is also significant reduction in the level of spermatozoa antioxidants, during the cryopreservation, as a result these spermatozoa cells are susceptible to per oxidative injures (Bilodeau et al., 2000). ROS causes a chain of chemical reactions called lipid peroxidation (da Silva Maia, Bicudo, Sicherle, Rodello, & Gallego, 2010). Lipid peroxidation reduces membrane fluidity and the activity of membrane enzymes and ion channels, resulting in the inhibition of normal cellular mechanisms required for sperm motility and fertility (Pons-Rejraji et al., 2009). Therefore, adding antioxidants to the semen extender can enhance the quality of cryopreserved semen (Foote, Brockett, & Kaproth, 2002; Rather, Islam, Malik, & Lone, 2016) by reducing the amount of free radicals and oxidative stress (Breininger, Beorlegui, O'Flaherty, & Beconi, 2005) thus controlling the lipid peroxidation (da Silva Maia et al., 2010; Hu et al., 2011). Melatonin (MLT) is one of the different antioxidants which can be used as antioxidant in the semen extender.

Melatonin (MLT) is endogenously produced neuroendocrine hormone which has a natural antioxidant property. MLT protects the cells from oxidative stress by scavenging free radicals and interacting with antioxidant enzymes to promote their oxygen radical scavenging activities (Deng et al., 2017). MLT has high potency as the scavenger of hydroxyl radical (El-Sokkary, Kamel, & Reiter, 2003; Lee et al., 2002) and also detoxifies different kinds of reactive oxygen and nitrogen species (D. Tan et al., 2002) . There has been reports which substantiate that the supplementation of melatonin in the semen extender improves the quality of cryopreserved post thawed bull semen (Ashrafi, Kohram, & Ardabili, 2013), protects ram spermatozoa from the cryopreservation injuries (Succu et al., 2011), improves the quality of cryopreserved buffalo bull semen by protecting the plasma membrane, acrosomal region, mitochondria and also by maintaining the ultrastructure integrity of the spermatozoa

(El-Raey, Badr, Assi, & Rawash, 2015) and protects cryopreserved human spermatozoa from the adverse effects of cryopreservation by counteracting intracellular ROS and reverses sperm apoptosis (Espino et al., 2010; Karimfar et al., 2015). MLT can also upregulate the expression of HSP family genes (H. Shi et al., 2015) and HSP 90 (Deng et al., 2017).

Heat-shock proteins (HSPs) are the most abundant and ubiquitous soluble intracellular proteins. HSPs are a group of proteins that provide thermos-tolerance in cell and protect cells against apoptosis during injury and oxidative stress (Beere & Green, 2001). HSPs are a group of molecular chaperones whose expression is also related to sperm motility and fertility (Deng et al., 2017; Huang, Kuo, Lee, et al., 2000; X. G. Zhang et al., 2015). Among HSP family, *hsp* 70 plays a protective role in reaction to hyperthermia as well as other stress conditions (Santoro, 2000) by providing a balance between synthesis and degradation of cellular proteins (Y. Shi, Mosser, & Morimoto, 1998). It also acts as a molecular chaperone, which assists in the process of folding, transporting and assembling proteins in the cytoplasm, mitochondria and endoplasmic reticulum (Georgopoulos & Welch, 1993). Elliott et al. (2009) found that HSP 70, as sperm-binding oviductal proteins, increase longevity and viability of sperm in bull and boar. Huang, Kuo, Lee, et al. (2000) reported that the level of HSP 70 in spermatozoa may influence the semen quality in boars. Similarly, a positive correlation between hsp 70 expression and sperm motility in bull has been described by X. G. Zhang et al. (2015). Among the HSP Family, HSP90 protects cells against oxidative stress by adjusting the folding of key proteins to maintain their three-dimensional structure. It has also been shown to be involved in signal transduction related to sperm viability (Volpe et al., 2008) and is associated with improved semen quality and the freezing resistance (P. Wang et al., 2014). In one of the study Huang, Kuo, Tsou, et al. (2000) observed that HSP90 also plays an important role in regulating sperm motility through modulating the activity of protein kinases. So, this study aimed to observe the effects of MLT on the *hsp* 70 and *hsp* 90 gene expression after thawing with different thawing methods. Moreover, this type of study has not been conducted in the swamp buffaloes. Therefore, this study also aimed to observe the effects of MLT on cryopreserved semen quality of swamp buffalo in different thawing methods.

1.2 Research aim

No previous study has been reported regarding the efficiency of melatonin on cryopreserved semen quality of swamp buffalo in different thawing methods. Hence, the aims of this study were:

- To observe the effects of melatonin on cryopreserved semen quality in different thawing methods.
- To observe the effects of melatonin on the *hsp* 70 and *hsp* 90 expression after thawing with different methods.

1.3 Research hypothesis

1. $\mu 0 = 0$ The melatonin is not efficient in enhancing the cryopreserved semen quality of swamp buffalo in different thawing methods
2. $\mu 1 \neq 0$ The melatonin is efficient in enhancing the cryopreserved semen quality of swamp buffalo in different thawing methods

CHAPTER II

LITERATURE REVIEW

2.1 Semen cryopreservation

Semen cryopreservation is the complex process of preserving the semen, by cooling to subzero temperature, for a long period. It is an essential part of Artificial Insemination through which the genetic improvement and ex situ conservation of the animal can be done. Cryopreservation process involves different steps like dilution/ mixing of cryo-protective agents with semen, temperature reduction by cooling, cellular dehydration by freezing, storage and thawing.

2.1.1 Semen extender/ diluent

Semen extender is a liquid media added to semen for preserving the fertilizing ability of the spermatozoa and also for the protection of sperm cells against the various shocks during the various process of cryopreservation. The two main functions of semen extender are to prolong the fertility of sperm and to extend the volume of semen i.e. the genetic potential so that more doses of semen are produced from the limited amount of semen ejaculate (Layek, Mohanty, Kumaresan, & Parks, 2016). Semen extender should be able to provide energy to the sperm, provide protection from cold shock, control microbial contamination and protect during freezing and thawing (Raheja, Choudhary, Grewal, Sharma, & Kumar, 2018).

Semen has certain physiological properties i.e. isosmotic and near neutral pH so the semen extender should possess the appropriate pH and buffering capacity, osmolality and metabolizable substrate so that it can protect from cryo-injury (Salamon & Maxwell, 1995, 2000). The various components included in the typical semen extender are (1) non-permeating cryo-protectant (milk or milk products; egg yolk or yolk derivatives such as low density lipoproteins or phospholipids) (2) a permeating cryo-protectant (most commonly glycerol) (3) an organic buffer (typically Tris-hydroxymethylaminomethane) (4) one or more sugars as energy substrate or osmoticum (glucose, fructose, lactose, raffinose, saccharose, or trehalose) (5) solutes

to adjust pH and osmolarity (sodium citrate, citric acid); and (6) antibiotics (penicillin, streptomycin) (Layek et al., 2016).

There are different types of extender developed and used for the semen cryopreservation process. There are also different commercial extenders available in the market such as BullXcell®, BotuBov®(egg yolk based). As there is risk of disease transmission from the animal based protein sources, nowadays animal protein free extenders are also available commercially such as BIOXcell®, Biocephos plus®, OPTIXcell® and Andromed®. The most commonly used TRIS and citrate based extenders for the bovine are Tris-yolk-glycerol, Tris-fructose-yolk-glycerol and Citrate-yolk-fructose-glycerol (Raheja et al., 2018).

2.1.2 Principles of cryopreservation and cryo-injury:

Semen cryopreservation process differs from place to place, but the principles remain the same. Many authors (Gao & Critser, 2000; Jang et al., 2017; P. Mazur, 1984) have reviewed the underlying physical and biological factors which affect the survival of cells at low temperatures during the cooling and warming processes of cryopreservation. The freezing behavior of the cells can be altered in the presence of a cryo-protective agent (CPA), which affects the rates of water transport, nucleation, and ice crystal growth. According to P. Mazur (1984), the chief physical events taking place during the process of freezing is shown schematically in Figure 1. During this process, when the temperature goes down to -5°C, the cells and the surrounding medium remain unfrozen as a result of the super-cooling and the decrease in the freezing point due to the presence of the cryo-protective solutes. Ice starts to form in the external medium when the temperature is between -5°C and -15°C, but the plasma membrane does not allow the ice formation in the cytoplasm and thus cellular contents remain unfrozen. The difference in the chemical potential is created between the higher chemical potential inside the super-cooled water in the cells than that of water in the partly frozen solution outside the cell, and causing water to flow out of the cell and freeze externally. The subsequent physical events in the cells take place depending upon the rate of cooling. In a relatively slow-freezing process, ice first begins to form in the fluid surrounding the cells, and the concentration of dissolved materials in the remaining liquid increases. A concentration gradient is established

across the cell wall, and water moves out of the cell in response to the osmotic force. As freezing continues, the cell becomes relatively dehydrated (upper right of Figure 1 and left of Figure 2). But if the cooling is done very rapidly (the central and lower of Figure 1 and lower right of Figure 2) the cell is not able to lose its water fast enough to maintain equilibrium, it becomes super-cooled and finally, the cell attains equilibrium by freezing intracellularly.

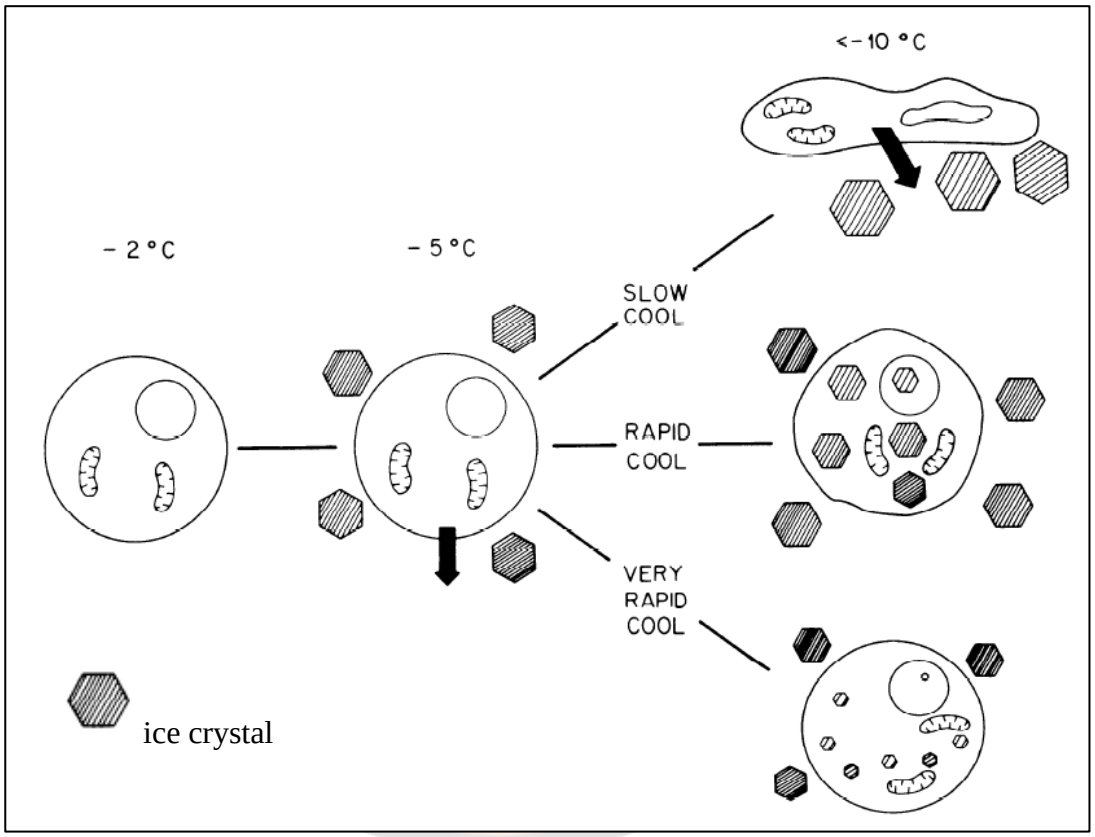


Figure 1 Schematic of physical events in cells during freezing

Source: P. Mazur, 1984

Sperms are not adapted to tolerate low temperature as mammalian sperm are very sensitive to cooling from body temperature to nearly freezing point of water. Therefore, cellular viability and normal function of the spermatozoa cells are reduced during cryopreservation. The cryopreservation process causes lethal and sub-lethal

damages to the cryopreserved sperm due to which there is reduced fertility (Watson, 2000). The process of cryopreservation exposes cells to stress-induced by low-temperature and osmotic imbalances. Exposing biomolecules to decreasing temperatures causes the cold shock which may lead to (irreversible) conformational changes. The cold shock causes damage to the cellular membranes and alteration in metabolic function, probably caused by changes in the arrangement of membrane constituents (Medeiros, Forell, Oliveira, & Rodrigues, 2002). A decrease in temperature causes a thermotropic phase transition in the membrane phospholipids from a liquid-crystalline to a gel phase, resulting in a more rigid (ordered) membrane structure (Hammerstedt, Graham, & Nolan, 1990). This leads to increased viscosity and decreased fluidity. Osmotic stress during cryopreservation is predominantly the result of extracellular ice formation. Upon extracellular ice formation, the solute concentration in the extracellular unfrozen fraction increases causing cells to dehydrate (Jang et al., 2017; Peter Mazur, 2004; Meryman, 2007). Cellular dehydration during freezing is the result of water transport out of the cell in order to retain equilibrium between the intra and extracellular solute concentration. Dehydration especially occurs when low cooling rates are used (Peter Mazur, 1963). Limited cell dehydration is crucial during cryopreservation as it limits the ice crystal formation within the cell, while excessive cell dehydration results in increased sperm abnormalities (Agca et al., 2002). When cells shrink or swell beyond their osmotic tolerance limits this can be lethal (Benson, Woods, Walters, & Critser, 2012; Woods, Benson, Agca, & Critser, 2004; Yeste, 2016). At fast cooling rates, cells do not have enough time to lose water and as a result intracellular water contents remain relatively high leading to intracellular ice formation (Peter Mazur, 1963) which causes cell injury (Jang et al., 2017) (Figure 2). Moreover, addition and removal of cryoprotective agents, also exposes cells to osmotic stress (Kashuba, Benson, & Critser, 2014; Peter Mazur, 2004; Meryman, 2007).

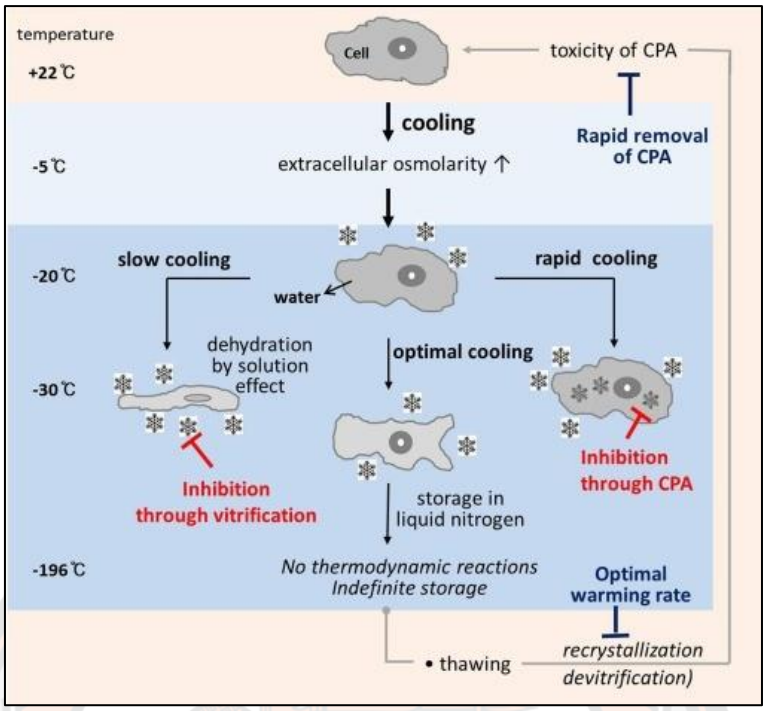


Figure 2 Physical events and cryoinjury of cells during freezing

Source: Jang et al., 2017

2.1.2 Oxidative stress during cryopreservation:

Oxidative stress is a condition associated with an increased rate of cellular damage induced by oxygen and oxygen-derived oxidants commonly known as Reactive Oxygen Species (ROS) (Sikka, Rajasekaran, & Hellstrom, 1995). Oxidative stress arises as a consequence of excessive production of ROS and impaired antioxidant defense mechanisms. Cryopreservation significantly increases production of ROS in human (Alvarez & Storey, 1992) in bovine (Bilodeau et al., 2000) and equine (Ball, 2008) sperm. In bovine semen, ROS are generated primarily by dead spermatozoa via an aromatic amino acid oxidase catalyzed reaction (Upreti, Jensen, Munday, Vishwanath, & Smith, 1994). Spermatozoa discard most of their cytoplasm during the terminal stages of differentiation, and lack the significant cytoplasmic component containing antioxidants so sperm appear to have very limited amounts of ROS scavengers, and seminal plasma is a potent source of ROS scavengers which

functions to protect ejaculated sperm from the adverse effects of ROS (Ball, 2008). The removal of seminal plasma during semen processing may increase the susceptibility of sperm to oxidative stress because of the removal of these enzyme scavengers. The ROS generated includes hydrogen peroxide(H2O2), the superoxide anion ($\bullet O_2^-$), the hydroxyl radical (OH•), and hypochlorite radical (OHCl•) (Bansal & Bilaspuri, 2011). Hydrogen peroxide appears to be the primary ROS responsible for most of the harmful effects (Aitken, Buckingham, & Harkiss, 1993; Alvarez & Storey, 1992; Baumber, Ball, Gravance, Medina, & Davies-Morel, 2000; Blondin, Coenen, & Sirard, 1997; De Lamirande & Gagnon, 1992; Griveau, Dumont, Renard, Callegari, & Le Lannou, 1995), and membrane lipid peroxidation is believed to be an important mechanism of action (Storey, 1997) by which these changes take place.

The lipid peroxidation cascade is initiated when ROS attack polyunsaturated fatty acids (PUFAs) in the sperm cell membrane (Storey, 1997). Spermatozoa are particularly susceptible to oxidative attack because they contain high concentrations of unsaturated fatty acids (Jones, Mann, & Sherins, 1979) and have limited repair mechanisms (Van Loon et al., 1991). As a consequence of lipid peroxidation, the plasma membrane loses the fluidity and integrity it requires for participation in the membrane fusion events associated with fertilization (Block, 1991; Storey, 1997).

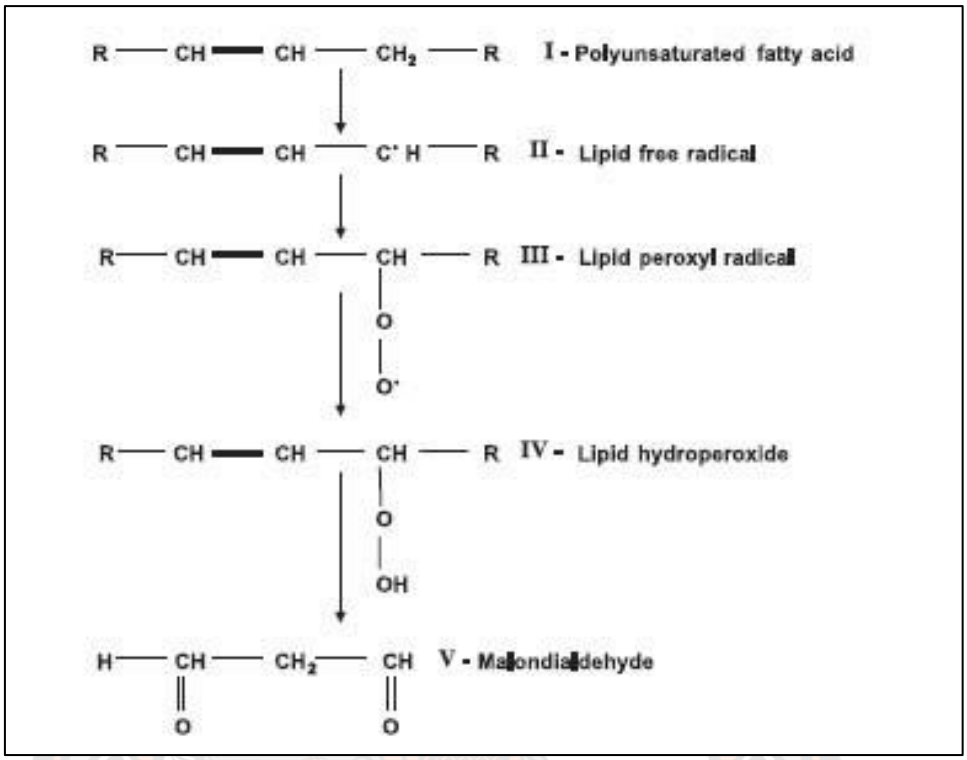


Figure 3 Schematic steps of MDA formation from polyunsaturated fatty acid

Source: Grotto et al., 2009

In addition to membrane effects, lipid peroxidation can also damage DNA. Peroxidation of DNA can lead to chromatin cross-linking, base changes, and DNA strand breaks. Several researchers have reported DNA damage in human spermatozoa associated with membrane lipid peroxidation (Z. Chen et al., 2006) and oxidative stress.

During each step, sperm structure and function are affected resulting in reduced sperm motility (Tuli, Singh, & Matharoo, 1981), acrosomal damage and alteration in sperm membrane integrity (Rasul, Ahmad, & Anzar, 2001). In general, the plasma membrane is considered to be the primary site of cryo-injury and the principal damage occurs during freezing and thawing resulting in substantial loss of viable spermatozoa.

2.2 Thawing of frozen semen

Thawing of frozen semen is the process of changing from the frozen solid-state to the liquid state by application of gradual warming. Thawing procedure is also essential as the freezing procedure with the relation to survival results of spermatozoa (Nur et al., 2003) because during both the stages spermatozoa have to pass through the critical temperature zone i.e., from -5 ℃ to -50 ℃ which causes detrimental effect on surviving spermatozoon population (Borah et al., 2015). Thawing of frozen semen brings back the frozen spermatozoa to life and to body temperature (Al-Badry, 2012; Bearden, 2004) and also in the physiologically active state. Thawing also reverses the freezing process, forcing the cell to retrace its path through the various environments encountered (J. Correa, Pace, & Zavos, 1997; Hammerstedt et al., 1990). Therefore, it is essential to carry out thawing process very carefully at an optimal temperature for sufficient time to avoid the damage to the sperm (Bearden, 2004) and to minimize the loss of semen quality during thawing procedure (Borah et al., 2015).

Many researches have been conducted to determine the optimal thawing temperature and duration to know the adequate thawing rate that may give highest percentage of viable spermatozoa after post thawing process (Borah et al., 2015; Lyashenko, 2015; Nur et al., 2003; Rastegarnia, Shahverdi, Rezaei Topraggaleh, Ebrahimi, & Shafipour, 2013; Robbins, Saacke, & Chandler, 1976). According to Lyashenko (2015) indicators of progressive motility and average survival rate of bull spermatozoa was found higher when thawed at 65°C, 67°C and 70°C temperature for 7 seconds. Robbins et al. (1976) found the maximum acrosomal retention and motility of spermatozoa in the 0.5 ml bovine straw at 8.5 % glycerol level and when thawed at 65°C for 7.5 seconds. Nur et al. (2003) showed that post thaw defected acrosome rate as well as other morphological and total morphological defects of bull spermatozoa were significantly lower when the straws were thawed at 70°C for 5 seconds compared to thawing at 37°C for 30 seconds and 50°C for 15 seconds. Similarly, Rastegarnia et al. (2013) came to the conclusion that the post thaw motility and kinematic parameters of buffalo spermatozoa were significantly improved immediately after thawing by increasing the thawing rate from 37 ℃ in 30 seconds to70 ℃ in 6 seconds. Borah et al. (2015) also found that the Post thaw motility was better when thawing at 75°C for 9 seconds. but all other parameters of good quality

semen like the percentage of live sperm, Hypo osmotic swelling test (HOST)-reacted sperm, extracellular Aspartate amino transferase (ALT) release, and incidence of separating acrosome was recorded better for the thawed semen of Yak at 35°C for 60 seconds and 37°C for 30 seconds. Similarly, significantly lesser ($p < 0.05$) incidence of swollen acrosome, total acrosomal changes, and extracellular AST release was found than the 75 °C for 9 seconds. Defrosting of sperm should be at maximum speed because increasing the speed of thawing frozen semen increases the number of sperm that restore maximum motility. According to Lyashenko (2015) the rapid thawing of semen decreases the harmful effects of recrystallization processes and hydration, preventing damage to sperm membrane and cytoplasm. In this case, when passing through the temperature danger zone (-5°~-30 °C and -30~0 °C) ice crystals do not have time to be formed and sperm switches directly from the glassy state to the liquid state.

However, Al-Badry (2012) reported that percentage of motility, livability, abnormality, intact acrosome and post thaw livability of sperm cells of Friesian Bulls were significantly ($p < 0.05$) higher for the 37°C for 30 seconds and 60°C for 8 seconds than the other thawing methods. Similarly, Dudeja (1990) found that that thawing of buffalo frozen semen in water al 35°C for 30 seconds gave satisfactory post-thaw motility than thawing at the temperature of 5°C for 4 minute and 75°C for 9±1 sec. Nicolae, Stela, Dragomir, and Hortanse (2014) showed that the structural and functional integrity of the plasma membrane of ram sperm is significantly increased when the straws were thawed at 39°C for 120 seconds and 50°C for 30 seconds as compared with other thawing temperatures of 75°C for 5 seconds & 10 seconds and 90 °C for 2 seconds. Calamera et al. (2010) reported that thawing of cryopreserved human sperm at 40 °C for 3 minutes. results in a statistically significant increase in motility recovery compared with thawing at 37°C for 3 min. Doležalová, Ptáček, Stádník, and Ducháček (2017) reported higher progressive motility and spermatozoa movement characteristics immediately after the thawing and after 2 hours of incubation in slow thawing method (30 °C, 50 s) and the lowest spermatozoa motility was recorded in rapid thawing (70°C, 3 sec.) after 2 hours of incubation in bull.

2.3 Use of antioxidants to prevent oxidative stress

Antioxidants are the compounds which react with the ROS and decompose, scavenge, suppress their formation or oppose their action (Sikka et al., 1995). Many studies have been done regarding the use of different antioxidants in the semen extender to improve the semen quality of different species and these antioxidants have shown to enhance the quality of cryopreserved semen (Foote et al., 2002; Rather et al., 2016) by reducing the amount of free radicals and oxidative stress (Breininger et al., 2005) thus controlling the lipid peroxidation (LPO) (da Silva Maia et al., 2010; Hu et al., 2011). Breininger et al. (2005) reported that addition of αTocopherol in the semen extender can be useful in preserving the boar spermatozoa against the oxidative stress generated by cryopreservation. The antioxidants like trolax-C and catalase (CAT) were able to control lipid peroxidation and hydrogen peroxide generation in ram sperm during freezing and thawing (da Silva Maia et al., 2010). Hu et al. (2011) demonstrated that vitamin E supplementation as an antioxidant in the semen extender resulted in positive effects on bovine sperm motility and movement characteristics, acrosome and membrane integrity by reducing the lipid peroxidation potential. The addition of Vitamin E and Cysteine to freezing media of ram semen also increased the percentage of sperm motility and viability and also reduced the lipid peroxidation (Andreea & Stela, 2010). Bucak, Tuncer, et al. (2010) observed the effects of antioxidants like carnitine, inositol and methionine on post thawed bovine semen and stated that supplementation of these antioxidants facilitates the enhancement of sperm cryopreservation technique and that of the sperm quality. Banday, Lone, Rasool, Rashid, and Shikari (2017) also evaluated the effects of antioxidants like taurine, quercetin and reduced glutathione on the post thaw quality of cross bred ram sperm and found out that taurine could reduce the lipid peroxidation and improved the post thaw sperm quality. Bucak, Sarıözkan, et al. (2010) determined the effects of the antioxidants like curcumin, inositol and carnitine on seminal parameters of Angora Goat and found that these antioxidants could protect the acrosome and other morphological integrity of the sperm. Martinez-Soto, de DiosHourcade, Gutierrez-Adan, Landeras, and Gadea (2010) reported that adding Genistein as an antioxidant to the freezing and thawing media of human semen decreased DNA damage caused by the process of cryopreservation.

2.4 Melatonin

N-Acetyl-5-methoxytryptamine, commonly known as melatonin, was first purified and characterized from the bovine pineal gland extract by Aron Lerner and co-workers in 1958. MLT is a ubiquitous and widely distributed molecule which can be found in all taxa (J Reiter, Tan, Rosales-Corral, & C Manchester, 2013). The reason for this universal nature could be that MLT is a highly conserved molecule which evolved in the transition from the anaerobic to the aerobic metabolism (D. X. Tan et al., 2010).

MLT is a derivative of the amino acid tryptophan and it is best known as being produced in the pineal gland. However, it was also found in different tissues and organs like retinae, extra orbital lacrimal gland, Harderian gland, bone marrow cells, platelets, lymphocytes, skin, enterochromaffin cells of gastro-intestinal tracts in vertebrates, as well as in a wide variety of organisms, ranging from invertebrates to plants (Chowdhury, Sengupta, & Maitra, 2008). Likewise, measurable MLT levels have been reported in nearly all biological fluids including cerebrospinal fluid (Young, Gauthier, Kiely, Lal, & Brown, 1984), saliva (McIntyre, Norman, Burrows, & Armstrong, 1987), bile (D. X. Tan et al., 1999), urine (Claustrat, Brun, & Chazot, 2005), synovial fluid (Maestroni, Sulli, Pizzorni, Villaggio, & Cutolo, 2002), follicular fluid (Brzezinski, Seibel, Lynch, Deng, & Wurtman, 1987), amniotic fluid (Mitchell, Sayers, Keirse, Anderson, & Turnbull, 1978), milk (Illnerova, Buresova, & Presl, 1993), and seminal plasma (Casao, Cebrian, et al., 2010; Luboshitzky, Shen-Orr, & Herer, 2002).

2.4.1 Functions of melatonin

The indoleamine was initially found to function as a mediator of circannual reproductive rhythms (R. J. Reiter, Tan, Osuna, & Gitto, 2000) as well as of circadian cycles (Kennaway & Wright, 2002). MLT was found to have many functions such as oncostatic effects (Blask, Sauer, & Dauchy, 2002), immune system stimulation (Guerrero & Reiter, 2002), anti-inflammatory functions (Cuzzocrea & Reiter, 2002). Studies in various animals, especially in mammals, have shown that MLT can regulate a wide variety of physiological functions like aging to aggression, hibernation to hypertension, sleep to stress, reproduction to tissue generation,

scavenging to synchronization of the body functions with environmental light dark cycles (Chowdhury et al., 2008) as well as a natural anti-oxidant activity so functions as an antioxidant.

2.4.2 Mechanism of anti-oxidant activity of melatonin

MLT functions to reduce the oxidative stress by different mechanisms. Deng et al. (2017) illustrated the proposed mechanism of reduction of oxidative stress by exogenous MLT supplementation in the semen extender as in the Figure 4.

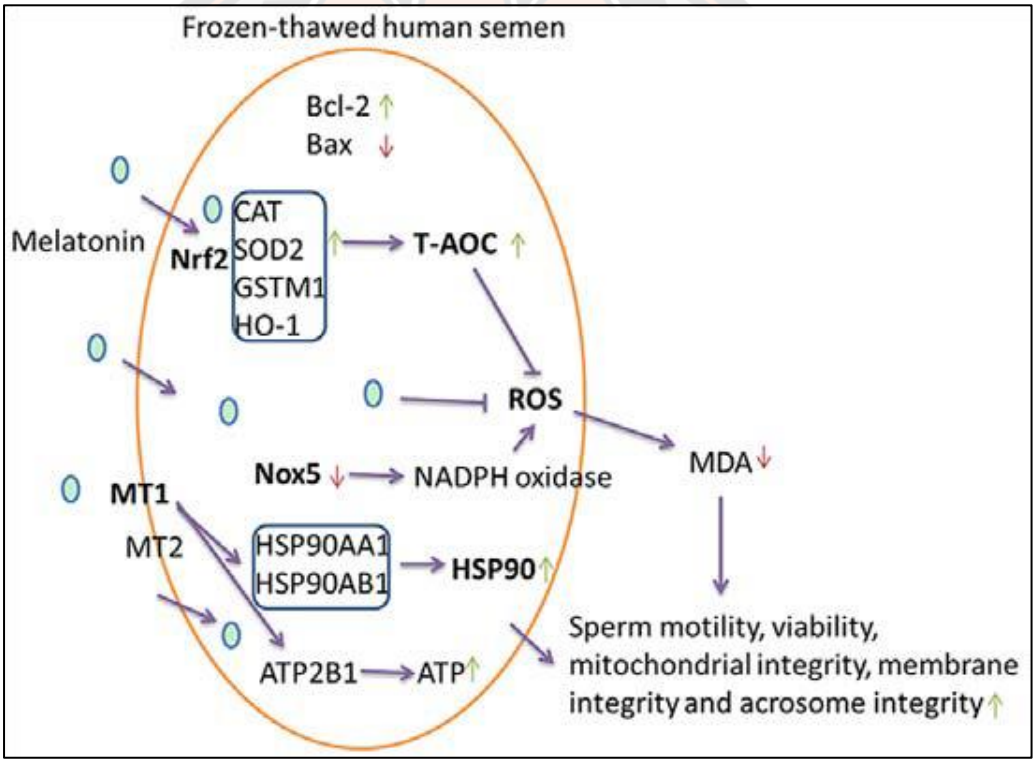


Figure 4 Schematic illustrating the proposed mechanism by which melatonin protects against oxidative damage and upregulates HSP90 expression in cryopreserved human semen.

Source: Deng et al., 2017

Deng et al. (2017) showed that MLT can suppress the ROS formation and decrease the oxidative damaging effect in frozen thawed human sperm (a) by increasing the total antioxidant capacity (TAOC) through upregulating the Bcl-2 gene and Nrf2 genes and its downstream genes (b) by downregulating nox5 gene responsible for regulating NADPH oxidase thus less formation of ROS (c) by upregulating the HSP 90 gene responsible for the motility of sperm (d) by increasing the ATPase activity, and (e) by directly acting as free radical scavenger.

A. Direct anti-oxidant mechanism of Melatonin:

It is a potent free radical scavenger (Allegra et al., 2003; Hardeland, Reiter, Poeggeler, & Tan, 1993). The Figure 5 depicts the interaction of MLT with the ROS and NRS. It interacts with reactive oxygen species and nitrogen reactive species (Allegra et al., 2003; Poeggeler et al., 2002; Russel J Reiter, Tan, Manchester, & Qi, 2001; D. X. Tan et al., 2010). MLT has the ability to neutralize the .OH hydroxyl radical (Bandyopadhyay, Bandyopadhyay, Das, & Reiter, 2002; Hardeland et al., 1995; X. J. Li, Gu, Lu, & Sun, 2002; Matuszak, Reszka, & Chignell, 1997; Poeggeler et al., 1994) and form cyclic 3-hydroxymelatonin (D. X. Tan et al., 1998), which is a footprint molecule that appears in the urine and is the indicator of in vivo .OH scavenging capacity of MLT. N1-acetyl- N2-formyl-5-methoxykinuramine (AFMK) and N1-acetyl-5-methoxykinuramine (AMK) are intermediate products of MLT and are also powerful radical scavengers and are as potent as MLT.

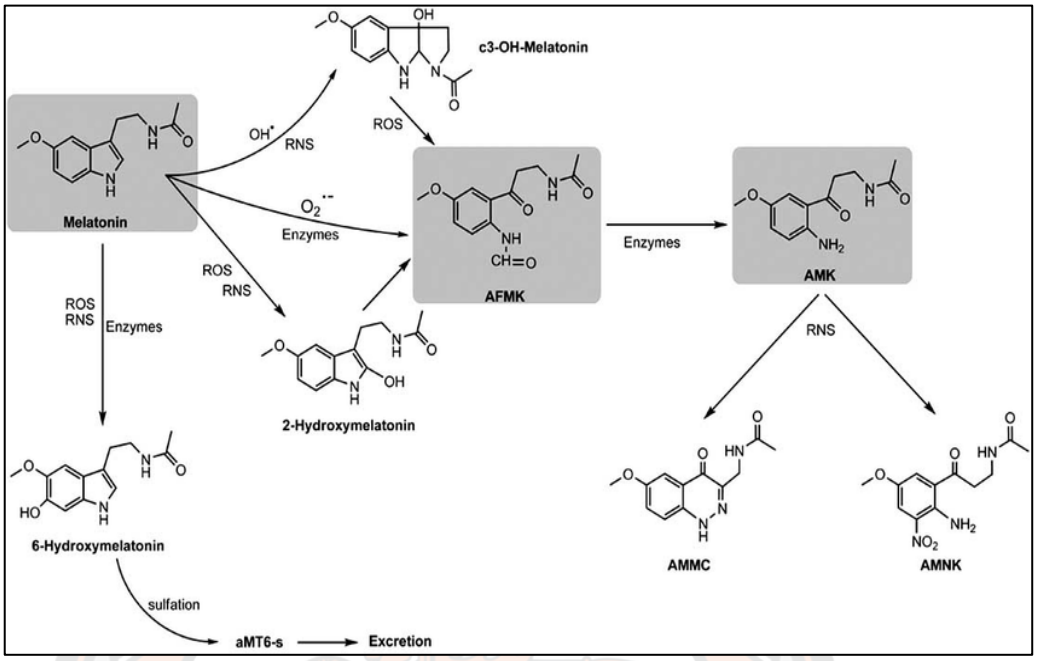


Figure 5 Chemical structure of melatonin and its best characterized reducing compounds formed by oxidation

Source: Pozo et al., 2010

The scavenging potential stems from its antioxidant reactions against ROS, including singlet oxygen, nitric oxide, hydrogen peroxide, and hydroxyl radical (Poeggeler, Reiter, Hardeland, Tan, & Barlow-Walden, 1996), and against RNS including nitric acid, peroxynitrite, and peroxynitrous acid (Zhang, Squadrito, & Pryor, 1998).

B. Indirect antioxidant mechanism of melatonin:

One of the mechanism by which MLT shows its indirect antioxidant property is by activation of antioxidant enzyme such as superoxide dismutases (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) (Deng et al., 2017; Mayo et al., 2002; R. J. Reiter et al., 2000). The cellular anti-oxidant system is shown in Figure 6. Catalase enzymes SOD and GPx are two important antioxidant enzymes, which may help sperm to resist oxidative damage and maintain the dynamic

ROS equilibrium in semen under normal physiological conditions (Salmanoglu et al., 2016; Shamsi et al., 2009). GPx reduces free radical damage because it metabolizes H2O2 (and other peroxides) to water in the process, however, glutathione (GSH) is oxidized to its disulfide, GSSG. GSSG is then quickly reduced back to GSH by GR, an enzyme which has also been shown to be stimulated by melatonin (Pablos et al., 1998).

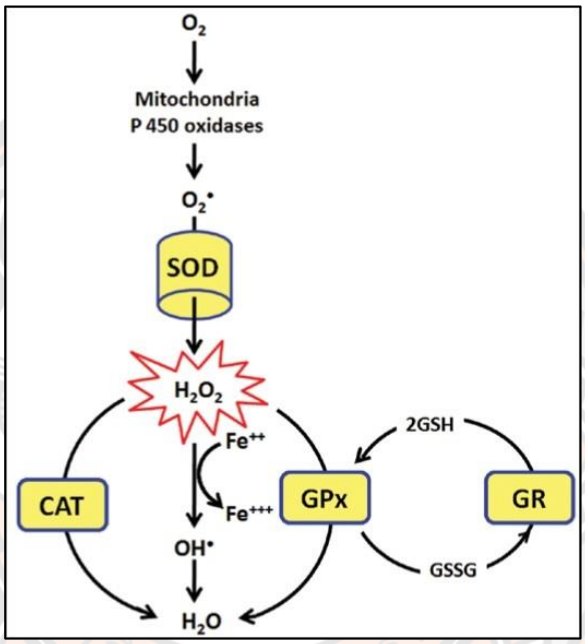


Figure 6 Cellular anti-oxidant system

Source: Pandey & Rizvi, 2010

Nrf2 is a basic leucine zipper (bZIP) protein that regulates the expression of antioxidant proteins that protect against oxidative damage triggered by injury and inflammation (Gold et al., 2012). MLT upregulates the Nrf2 genes thus enhanced resistance to the damage caused by freezing (Deng et al., 2017). Bax is the pro-apoptotic gene while Bcl2 is the anti-apoptotic gene and MLT supplementation has shown to increase the Bcl2 gene expression and decrease the Bax gene expression thus protects from damage (Deng et al., 2017). Expression of NADPH oxidase, which promotes ROS generation, is regulated by Nox5 gene and MLT downregulates the

Nox5 thus decreases the free radicals in the semen (Deng et al., 2017). HSP 90 protects cells against oxidative stress by adjusting the folding of key proteins to maintain their three-dimensional structure. MLT promotes the HSP 90 expression thereby maintaining the vitality of the sperm.

MLT also acts synergistically with other classic anti-oxidants such as Vitamin E, Vitamin C and GSH. According to Gitto et.al. (2001a), under in vitro conditions and using end products of lipid peroxidation as an indices of free radical damage, MLT augments the protective actions of vitamin E, vitamin C and GSH against free radical-mediated oxidation of polyunsaturated fatty fats.

2.4.3 Role of melatonin in spermatozoa

Several reports have substantiated that MLT has advantageous effects on the sperm. MLT supplementation in the semen extender has shown an enhancement of the semen quality parameters like Post thaw motility, viability, acrosomal integrity in different species like buffalo semen (El-Raey et al., 2015; El-Raey, Badr, Rawash, & Darwish, 2014; Husna et al., 2017), human (Deng et al., 2017; Karimfar et al., 2015), ram (Succu et al., 2011) by maintaining the ultrastructure integrity of the cryopreserved spermatozoa (El-Raey et al., 2015) increasing the total antioxidant capacity, increasing the other antioxidant enzymes like Superoxide Dismutase and glutathione reductase (El-Raey et al., 2014). It has also been demonstrated that MLT supplementation decreased the ROS level and the lipid peroxidation thereby decreasing the oxidative damage to the spermatozoa membrane (Ashrafi et al., 2013; X.-j. Chen et al., 2016; Deng et al., 2017; Karimfar et al., 2015; Succu et al., 2011). It has also been reported that addition of MLT in the semen extender improved the semen quality by increasing the Bcl2/Bax ratio thus inhibiting the apoptosis mechanism in the spermatozoa cell (X.-j. Chen et al., 2016; Deng et al., 2017; Espino et al., 2010).

Addition of MLT also has the beneficial effects in the liquid preservation of semen. It has improved the seminal parameters, enzymatic and biochemical profiles of mithun semen (Perumal, Vupru, & Khate, 2013) when stored at 5°C.

2.4.4 Concentration of melatonin used in the semen extender

Various doses of MLT have been used in the study of its efficacy in improving the semen quality. In the study of human semen, the concentration of MLT used was from 0.001 to 1 mM in which Deng et al. (2017) found that 0.1mM MLT was best for the protection of sperm during cryopreservation whereas Karimfar et al. (2015) observed that 0.01 mM MLT protected semen from the cryopreservation injuries. The concentration of MLT used as supplement in the semen extender of buffalo bull was from 0.1 to 1.5 mM and the 0.1 mM MLT showed the best result for the improvement of the semen characteristics (El-Raey et al., 2015; El-Raey et al., 2014; Husna et al., 2017) while El-Raey et al. (2015) found that 0.25 mM MLT also has the same effect and (Husna et al., 2017) observed the post thaw quality of the semen even at 0.5 mM concentration of MLT. Ashrafi et al. (2013) used 0.1, 1, 2 and 3 mM MLT in the semen extender in which 2 and 3 mM of MLT improved the quality of post thawed semen of bull. Similarly, Kapadiya, Nakhashi, Sutaria, Rathod, and Suthar (2016) used 1, 2 and 3 mM of MLT as an additive in which the 2 mM of MLT increased the seminal and acrosomal characteristics of the Kankrej Bull Semen.

Succu et al. (2011) used 0.001, 0.01, 0.1, 1 and 10 mM of MLT in the semen extender of ram to observe its role in the protection from cryopreservation injuries and concluded that 1mM of MLT addition protected spermatozoa from the cryopreservation injuries where as Khalifa (2017) found that 0.3 mM MLT efficiently maintained the post thaw motility characters. Some study has been undertaken to study the effects of melatonin on the liquid storage of the semen. Perumal et al. (2013) used 1, 2, 3 and 4 mM of MLT to assess the effects of MLT on the liquid storage (5°C) of mithun (Bos frontalis) semen and concluded that 3 mM of MLT enhanced the quality of seminal parameters, enzymatic and biochemical profiles and thus improving the keeping quality of semen preserved at 5 °C. In this study 1 mM MLT will be used as supplementation of 1 mM MLT has shown to increase the quality of cryopreserved swamp buffalo semen in the previous study done in our laboratory.

2.5 Heat shock protein (HSP)

Heat shock proteins (HSPs) are highly conserved and ubiquitous proteins found in the cells of all studied organisms. Many types of stress, including heat, induce the expression of a family of genes known as the heat shock protein (hsp) genes. The hsp genes are known to be induced by a wide variety of environmental or metabolic stresses that include the following: anoxia, ischemia, heavy metal ions, ethanol, nicotine, surgical stress, and viral agents (Whitley, Goldberg, & Jordan, 1999). HSP are a different group of proteins that are separated into families based on molecular weight. Main families of HSP comprise small chaperones and ubiquitin, HSP 60, HSP 70, HSP 90 and HSP 100. Some members of the HSP families are strictly inducible by stress, whereas others are constitutively expressed in normal temperature. The term HSP refers for inducible form of proteins while HSC for constitutively expressed HSP (Neuer et al., 2000). HSP serve two major functions: (a) They act as molecular chaperones under normal physiological conditions (intracellular housekeeping proteins) which are involved in mediating the folding and transportation of other proteins and sometimes their assembly into oligomeric structures & (b) They are induced in response to cellular stress as heat shock response and resulting in protection from damage or facilitates recovery to maintain cell survival.

2.5.1 Heat shock protein 70 (HSP 70) and its role in semen quality

HSP 70 is produced by the hsp 70 gene, and includes a family of HSP which range in size from 68 to 73 kDa. The hsp 70 gene is encoded by a single exon. HSP 70 gene family in bovines includes hsp 70-1, HSP 70-2, hsp 70-3 and hsp 70-4 gene. hsp 70-1 is an intronless gene located on chromosome 23 of bovine (BTA 23) and has 1926 nucleotides (Gade et al., 2010). Some hsp 70 are constitutively expressed, whereas others are strictly stress-inducible (Daugaard, Rohde, & Jäättelä, 2007). hsp 70 plays a protective role in reaction to hyperthermia as well as other stress conditions (Santoro, 2000) by providing a balance between synthesis and degradation of cellular proteins (Y. Shi et al., 1998). It also acts as a molecular chaperone, which assists in the process of folding, transporting and assembling proteins in the cytoplasm, mitochondria and endoplasmic reticulum (Georgopoulos & Welch, 1993). Lack of the

hsp 70 gene leads to a significant increase in apoptosis (Dix et al., 1996).

Elliott et al. (2009) found that HSP 70, as sperm-binding oviductal proteins, increase longevity and viability of sperm in bull and boar. It was reported that semen quality may be influenced by levels of HSP 70 protein in boars' spermatozoa (Huang, Kuo, Lee, et al., 2000). X. G. Zhang et al. (2015) also found the positive correlation between level of hsp 70 expression and bull sperm motility indicating that the hsp 70 plays important role in maintaining the semen quality. Govin et al. (2006) found association between HSP 70 function and spermatid DNA-packaging proteins during spermatogenesis.

2.5.2 Heat shock protein 90 (HSP 90) and its role in semen quality

HSP 90 is an abundant cytosolic protein with chaperone activity and is highly conserved throughout the eukaryotes (Csermely, Schnaider, Soti, Prohaszka, & Nardai, 1998; Pratt, 1997). HSP 90 plays an essential role in stress tolerance (Borkovich, Farrelly, Finkelstein, Taulien, & Lindquist, 1989), protein folding (Freeman & Morimoto, 1996) and signal transduction (Pratt, 1998). HSP 90 is also one of the sperm proteins which is the marker for sperm fertility. The HSP 90 protein is localized mainly in the neck, mid piece and tail regions of human sperm, the regions which drive sperm motility; therefore, HSP 90 having possible role in sperm motility and hyper-activation (K. Li et al., 2014). K. Li et al. (2014) also showed that HSP 90 is associated with the events involved in regulating human sperm function. Many proteins, including tyrosine kinases, serine-threonine kinases and other enzymes (Csermely et al., 1998; Pratt, 1997, 1998), can associate with HSP 90 and influence sperm motility.

Moreover, García-Cardeña et al. (1998) and Bender et al. (1999) reported that HSP 90 can activate nitric oxide synthase (NOS), which is beneficial to sperm motility within physiological range (Lewis et al., 1996; O'Bryan, Zini, Cheng, & Schlegel, 1998). X.-G. Zhang et al. (2015) reported HSP 90 level was decreased during the cryopreservation of bull spermatozoa and the expression level was proportional to the sperm parameters such as motility, membrane integrity and acrosome integrity, indicating the protective role of HSP 90 in semen quality during cryopreservation. Huang et al. (1999) also found the substantial decrease in HSP 90

preceding the decline in sperm motility during cooling of boar spermatozoa and also in human spermatozoa (Cao, Wang, Xiang, & Li, 2003) suggesting the cause and effect relationship. On addition of Geldanamycin, a specific inhibitor of HSP 90, to the diluent, Huang, Kuo, Tsou, et al. (2000) found that there was decrease in the porcine motility in a dose and time dependent manner suggesting that HSP 90 may play a crucial role in regulating sperm motility. P. Wang et al. (2014) observed that the high freezing resistance group showed the higher expression of HSP 90 protein indicating that level of HSP90 expression could be used to predict reliably and simply the freezing resistance of bull sperm.

CHAPTER III

RESEARCH METHODS

3.1 Site of study

The collection of semen and semen processing was done in HJ buffalo farm (Uthai Thani, Thailand) during the period of May 2019 to June 2019. The experimental study was done in the faculty of agriculture, natural resources and environment, Naresuan University from May 2019 to April 2020.

3.2 A brief outline of experimental procedure

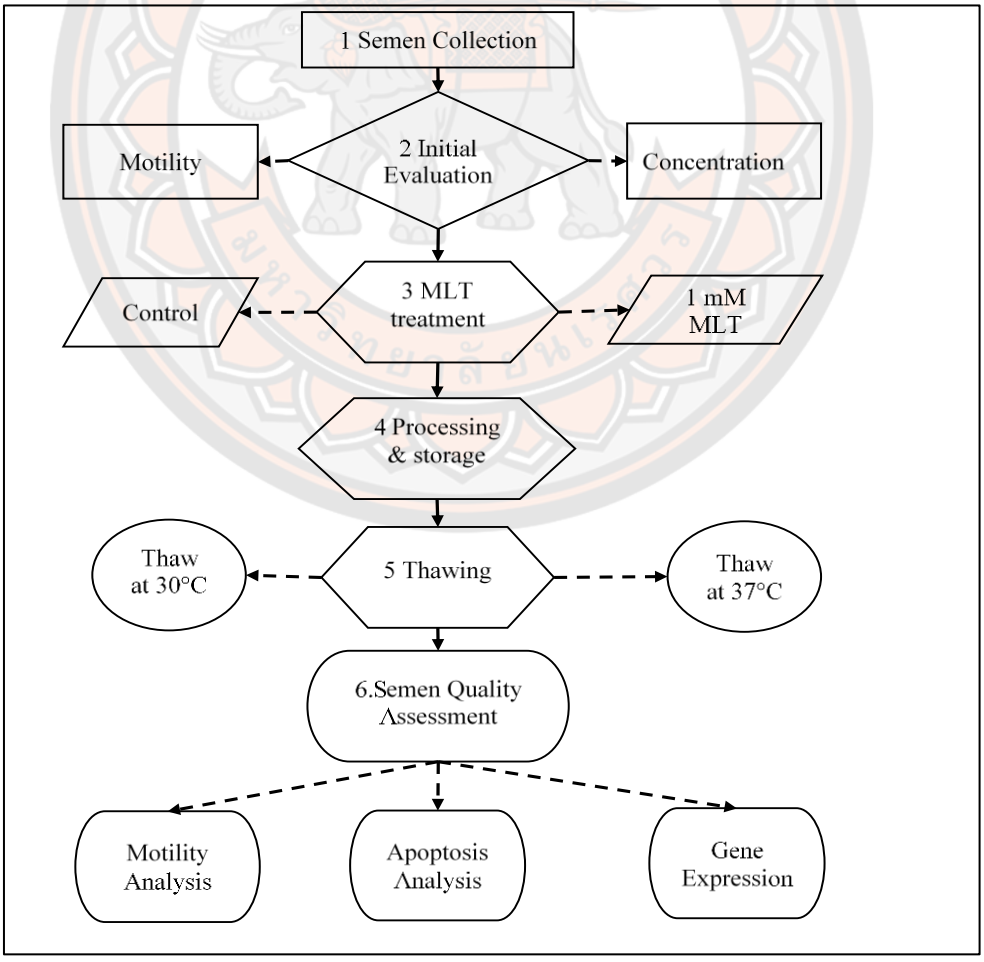


Figure 7 Flow chart of the simplified experimental protocol used in the study

3.3 Selection of animals

Four swamp buffalo bulls (4yeasrs to 8 years of age), maintained under same feeding, housing and other management system, were chosen for the collection of the semen. Semen was collected with the aid of artificial vagina according to the standard procedures.

3.4 Semen collection

Semen was collected once in a week by artificial vagina method by following the standard procedures. Maximum two ejaculates from each bull was collected at an interval of 30 minutes.

3.5 Initial semen evaluation

The semen was evaluated for the volume, color and presence of any impurities immediately after collection. Then, it was kept in the water bath at 37°C until the estimation of initial motility and concentration. The initial motility of the semen was subjectively estimated with the help of phase contrast microscope at 37°C. The concentration of the semen was evaluated with the help of haemocytometer. The ejaculates having the concentration greater than 500 x 10^6 spermatozoa/mL and initial motility greater than 70 % was included in the study and was further used in the processing and experimental process.

3.6 Semen extender preparation

The semen extender consists of 200 mM tris (hydroxymethyl) aminomethane, 70 mM citric acid, 20% egg yolk, 6.4% glycerol, 55 mM fructose in double distilled water. The antibiotics like benzylpenicillin (1000 IU/ mL) and streptomycin sulfate (1 mg/mL) was added to the extender. One mM melatonin was added to the semen extender, in the treatment group, after dissolving in 0.1% dimethyl sulfoxide (DMSO) solvent.

3.7 Semen processing and cryopreservation

The total semen extender to be added to the semen was calculated as such that the spermatozoa per straw would be 20 × 10^6 spermatozoa/ straw. Then, the

semen was divided into two groups- experimental and control group. Extender with 1mM MLT was added to the experimental group and extender without melatonin was added to the control group. The straw filled with the diluted semen was wrapped in the cotton cloth and kept at 4°C for two hours and incubated at 4°C for another two hours. Then, the rack with the semen was kept 10 cm above the liquid nitrogen (-120°C) for 15 minutes and then was plunged into the liquid nitrogen tank at -196°C and stored until the evaluation of the semen.

3.8 Thawing methods

Two thawing methods were used in this study. In thawing method, I: 30°C for 60 seconds and thawing method II: 37°C for 30 seconds were used as temperature and time combination. Thawing was done in water bath at the proper temperature for the assigned time.

3.9 Assessment of post-thaw semen quality parameters

I. Analysis of sperm motility

After thawing the frozen semen straws at 30°C for 60 seconds and 37°C for 30 seconds for each control and melatonin treated group, the aliquot of 3 µl of thawed semen was pipetted and loaded into a preheated slide (2X-CEL® Slides,Hamilton Thorne, USA) at 37°C. The semen motility and kinetic parameters were observed with the help of computer assisted semen analyser CASA (Androvision® CASA software version 1.0.0.9, Minitube, Germany). The different parameters observed were the percentage of total motility, percentage of progressive motility, Straight-Line Velocity (VSL), Curve-Line Velocity (VCL), average path velocity (VAP), distance average path (DAP), distance straight line (DSL), distance curveline (DCL), linearity (LIN), straightness (STR), beat cross frequency (BCF), wobble (WOB), amplitude of lateral head displacement (ALH). The motility pattern is illustrated in the Figure 8. At least 3 fields were observed for the evaluation of the motility. The CASA analysis was performed for 40 replications for each control and treatment groups.

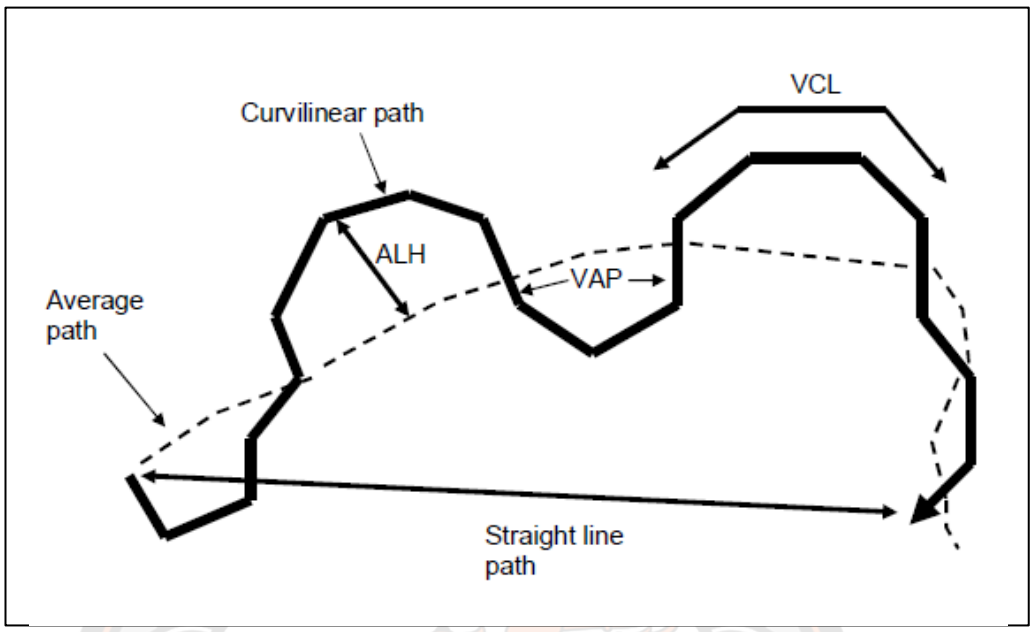


Figure 8 An illustration of different sperm motility parameters measured using CASA

Source: Lampiao & du Plessis, 2008

II. Assessment of apoptotic spermatozoa cells

Guava Nexin® reagent (Guava Technologies) containing annexin V (binds phosphatidyl serine (PS) which is translocated from the inner to the outer leaflet of the sperm plasma membrane) and 7 AAD (binds to the nuclear material within the cell after the membrane breaks down) was used to assess apoptosis according to the manufacturer's instruction with slight modification. Briefly, after thawing one semen straw for each treatment and control group, semen was emptied in 1.5 mL Eppendorf tube and centrifuged (1000 x g, 10 minutes) to remove egg yolk extender. Subsequently two washings of the semen were done with the PBS, the sperm suspensions were centrifuged at 1000 x g for 5 minutes. Then, sperms were diluted to $\leq 2 \times 10^5$ cells/mL in PBS. After guava nexin reagent was warmed to room temperature (R.T.), 100 µL of sperm suspensions was taken in an Eppendorf tube and 100 µL of reagent was added to it and incubated in dark for 20 minutes. Then, the

sample was acquired in the Guava System. The percentage of apoptotic spermatozoa cells was assessed with the help of flow cytometer (guava easyCyte TM, 5HT HPL based system). Twelve replications were done for each treatment and control.

3.10 Expression of HSP 70 and HSP 90 in Frozen-thawed Buffalo Bull Sperm (Gene Expression Assay)

1. Isolation of total RNA

Total RNA was isolated by modified hot TRIzol method according to the manufacturer's instruction with slight modification. Four semen straws were used for each sample. Semen sample after thawing by different methods, was put in RNase free Eppendorf tube and kept at 37°C in dry bath until further processing. Following the centrifugation (12000 g, 15 minutes) the supernatant was removed and the sperm pellet washed with pre-cooled 1mL phosphate-buffered saline (PBS) and centrifuged (12000 g, 5 minutes) and the procedure repeated further two times. To the sperm pellet, pre heated (65°C) 1 mL of Trizol reagent was added and homogenized with the help of 24 G needle (15-20 times), vortexed and incubated at 65°C for 30 minutes. Then, 0.2 mL chloroform per 1 mL of Trizol reagent was added, mixed vigorously, incubated for 5 minute and centrifuged at 12000 g for 15 minutes. The upper layer with RNA was transferred into fresh Eppendorf tube and 0.5 mL isopropanol was added (per 1 mL of TRIzol reagent) to precipitate the RNA. The sample was mixed by inverting the tube 20 times, incubated at room temperature for 10 minutes and centrifuged at 12000 g for 10 minutes. The pellet was washed with RNase free 1 mL of 75% ethanol and centrifuged at the rate of 12000 g for 5 minutes. The RNA pellet air dried and dissolved in 30-50 µL of Nuclease free water. Isolated RNA treated with RNase free DNase I to remove any DNA left. RNA samples was stored at –80°C.

The concentration and quality of RNA was determined by use of Nano-300 micro spectrophotometer (Allsheng, China) at OD 260 and 280 nm and stored at -80°C until further use.

2. Synthesis of first strand cDNA and PCR amplification

The first strand cDNA was obtained by reverse transcribing 1 µg total RNA in a final volume of 20 µl using Oligo (dT)18 primer and the RevertAid First Strand cDNA synthesis Kit (ThermoFisher Scientific) following manufacturer's protocol.

PCR amplification was performed with 2 µL of the previously obtained cDNA in a total volume of 20 µL with 0.5 µL (5 U/ml) of i-Taq™ DNA polymerase (iNtRON Biotechnology) per sample,10X PCR buffer and 2.5 mM of each dNTP (iNtRON Biotechnology). The final concentrations of the primers were 10 µM for HSP 70, HSP 90, and GAPDH. GAPDH amplification was used for control of the quality of the cDNAs obtained.

The primers were designed using the primer-BLAST tool (National Center for Biotechnology Information, NCBI) and checked by BLAST analysis (NCBI) to verify gene specificity. The primers used for amplifying the PCR products are given in Table 1. Using the PCR mix, 35 PCR cycles were carried out on reversetranscribed cDNA from sperm. The cycling conditions were 30 seconds at 94°C, 30 seconds at 60°C, and 30 seconds at 72°C. A 5-minute denaturation step at 94°C preceded the cycling, while at the end a final 10-minute extension at 72°C was performed. PCR products were separated on 1.5% agarose gel in 1X Tris – acetate –EDTA buffer containing 0.05 µL/mL RedSafe™ nucleic acid staining solution (iNtRON Biotechnology) and were visualized under ultraviolet light. Molecular size was estimated by using 100 bp DNA Ladder (Solis BioDyne). The band intensity of PCR amplified product in agarose gel was calculated by open source java image processing program Image J.

Table 1 Primer sequences used for amplifying PCR products

Genes	Type of Primer	Primer Sequences	Accession No.	Amplification Length
HSP 90	Forward	5’-CGATGATGAGCAGTATGCCTGGG-3’	XM_025271500.1	685
	Reverse	5’-CAGGTATTCAGGGATTAGCTCCTC-3’		
HSP 70	Forward	5'-TCGGGGACAAGTCAGAGAACG-3'	XM_006072139.2	591
	Reverse	5'- TCACCTCCTGACACTTGTCG -3'		
GAPDH	Forward	5’- GGGTCATCATCTCTGCGCCT-3’	XM_006065800.2	647
	Reverse	5’- GGAGGCCATGTGGACCATGA-3’		

3.11 Data collection

All the data from the experiment was recorded and inserted in the excel program. The data of the motility parameters like the percentage of progressive motility, straight line velocity (VSL), average path velocity (VAP) was recorded with the help of CASA. Similarly, the parameters like percentage of normal spermatozoa, the percentage of early apoptosis, percentage of late apoptotic spermatozoa data was recorded by analyzing with flow cytometer.

3.12 Data analysis

Data analysis was performed by using Statistical Product and Service Solutions (SPSS 19.0 for windows; SPSS, Chicago, IL, USA). All values were expressed as mean ± standard error of the mean. Analysis of variance with Tukey’s test was used for the comparison of the mean values at a significance level of $p < 0.05$.

CHAPTER IV

RESULTS

4.1 Effect of melatonin on sperm motility characteristics

Sperm motility characteristics are the important parameters which are assessed for the determination of the semen quality. Hence, in order to see the effects of melatonin treatment in the extender on the semen motion characteristic CASA was used and the results obtained is given in the Table 2. The percentage of total motility (Mean ± SEM) and the percentage of progressive motility (Mean ± SEM) of sperm in the 1 mM MLT treated groups at the thawing conditions of 30°C for 60 seconds (40.59 % ± 1.89 %) and 37°C for 30 seconds (43.18 % ± 1.62 %) were significantly higher ($p < 0.01$) than the untreated groups/ control groups (35.12 % ± 1.66 % & 35.88 % ± 1.54 %, respectively). The percentage of progressive motility (Mean ± SEM) of sperm in the 1 mM MLT treated groups at the thawing conditions of 30°C for 60 seconds (33.30 % ± 1.75 %) and 37°C for 30 seconds (35.87 % ± 1.54 %) were also significantly higher ($p < 0.01$) than the untreated groups/ control groups (29.18 % ± 1.72 %, and 28.05 % ± 1.58 %, respectively) Similarly, the VCL in the MLT treated groups (55.20 % ± 2.45 % and 59.69 % ± 1.60 % , respectively) were also significantly higher ($p < 0.05$) than the untreated groups (54.16 % ± 2.43 % and 51.60 % ± 2.11 %, respectively) in both the thawing conditions. However, there was no significant differences with the other velocity parameters like VSL, VAP in the MLT treated and untreated groups. Moreover, no significant differences of these parameters were observed between the two thawing conditions i.e. thawing at 30°C for 60 seconds and 37°C for 30 seconds.

The distance parameters like DCL, DSL, DAP also showed no significant differences between the MLT supplemented groups and the non-supplemented groups. In addition, ALH, BCF, LIN, STR, WOB also showed no significant difference between the MLT treated and the untreated group. However, all the sperm parameters values, excluding the WOB, were seen higher when 1mM treated frozen straws were thawed at 37°C for 30 seconds.

Table 2 Effect of melatonin on sperm characteristics at different thawing methods

Sperm Parameters	Control		Melatonin 1mM	
	Thawing at 30 °C for 60 Sec	Thawing at 37 °C for 30 Sec	Thawing at 30 °C for 60 Sec	Thawing at 37 °C for 30 Sec
T M (%)	$35.12^{b} \pm 1.66$	$35.88^{b} \pm 1.54$	$40.59^{a} \pm 1.89$	$43.18^{a} \pm 1.62$
P M (%)	$29.18^{b} \pm 1.72$	$28.05^{b} \pm 1.58$	$33.30^{a} \pm 1.75$	$35.87^{a} \pm 1.54$
VCL [µm/s]	$54.16^{b} \pm 2.43$	$51.60^{b} \pm 2.11$	$55.20^{a} \pm 2.45$	$59.69^{a} \pm 1.60$
VSL [µm/s]	24.88 ± 1.74	22.52 ± 1.30	22.86 ± 1.52	27.07 ± 1.34
VAP [µm/s]	30.03 ± 1.78	27.68 ± 1.41	28.34 ± 1.57	32.38 ± 1.27
DCL [µm]	23.54 ± 1.12	22.61 ± 0.88	24.325 ± 1.05	25.65 ± 0.90
DSL [µm]	9.79 ± 0.78	8.91 ± 0.52	9.04 ± 0.65	10.99 ± 0.59
DAP [µm]	12.22 ± 0.80	11.37 ± 0.56	11.67 ± 0.67	13.33 ± 0.58
ALH [µm]	0.65 ± 0.02	0.63 ± 0.02	0.66 ± 0.02	0.98 ± 0.29
BCF [Hz]	8.07 ± 0.61	7.06 ± 0.38	7.66 ± 0.44	9.16 ± 0.47
HAC [rad]	0.14 ± 0.00	0.15 ± 0.01	0.15 ± 0.01	0.37 ± 0.21
LIN	0.44 ± 0.01	0.43 ± 0.01	0.41 ± 0.02	0.45± 0.01
STR	0.81 ± 0.01	0.80 ± 0.01	0.79 ± 0.01	0.82 ± 0.01
WOB	0.54 ± 0.01	0.53 ± 0.01	0.51 ± 0.01	0.53± 0.01

[a,b] Values with different superscript letters in each row represent statistically significant differences ($p < 0.05$) TM: total motility, PM: progressive motility, VCL: velocity curved line, VAP: velocity average path, VSL: velocity straight line, LIN: linearity coefficient (VSL/VCL), STR: straightness coefficient (VSL/VAP), BCF: beat cross frequency, ALH: amplitude of lateral head displacement, DCL: distance curve line, DSL: distance straight line, DAP: distance average path, WOB: wobble (VAP/VCL). All data are expressed as Mean ± SEM

4.2 The effect of melatonin in apoptosis of spermatozoal cells with the different thawing methods using flow cytometry with annexin V/ 7AAD

To observe the effects of melatonin supplementation in the sperm apoptosis,

semen straws were thawed with two different temperature and time combinations and stained with annexin V/7 AAD reagent before acquiring in the flow cytometer machine. The flow cytometry dot plots of spermatozoa for the control and treatment groups (1 mM melatonin) in different thawing conditions are shown in Figure 9. As shown in Figure 9 (A) the flow cytometry dot plots have 4 quadrants showing (I) the percentage of live spermatozoa (II) the percentage of early apoptosis in spermatozoa (III) the percentage of late apoptosis in spermatozoa and (IV) the cell debris.

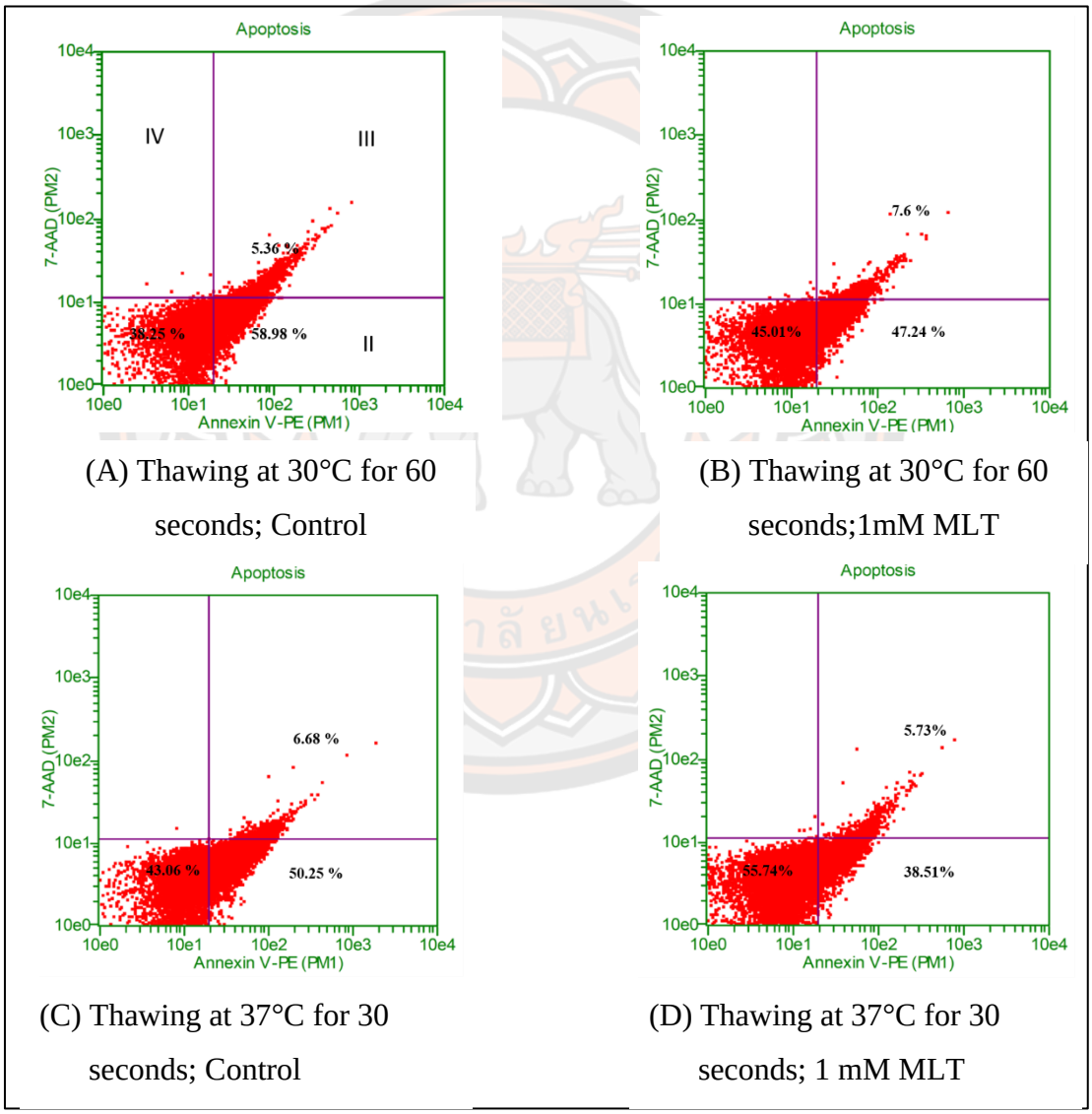


Figure 9 Flow cytometry dot plot for apoptosis analysis

Thawing at (A-B) 30°C for 60 seconds in control and treatment group (C-D) 37°C for 30 seconds in control and treatment group. Events in the lower-left quadrant represent annexin V -ve/7AAD –ve spermatozoa (viable sperm), and events in the lower-right quadrant represent early apoptotic spermatozoa (annexin V+/7AAD -). Spermatozoa in the upper-right quadrants represent late apoptotic cells (Annexin V+/7AAD +).

The bar graph depicting the percentage of live spermatozoa and early apoptosis in different thawing conditions is shown in Figure 10 and Figure 11 respectively. The percentage of live spermatozoa in the samples treated with 1 mM Melatonin was found to be significantly higher ($p < 0.01$) than the untreated samples in every thawing conditions. Within the treatment samples, the highest percentage of live sperm (55.74 ±2.41) was found when thawed at 37°C for 30 seconds which is significantly higher ($p < .05$) than thawing at 30°C for 60 seconds (45.01± 2.30).

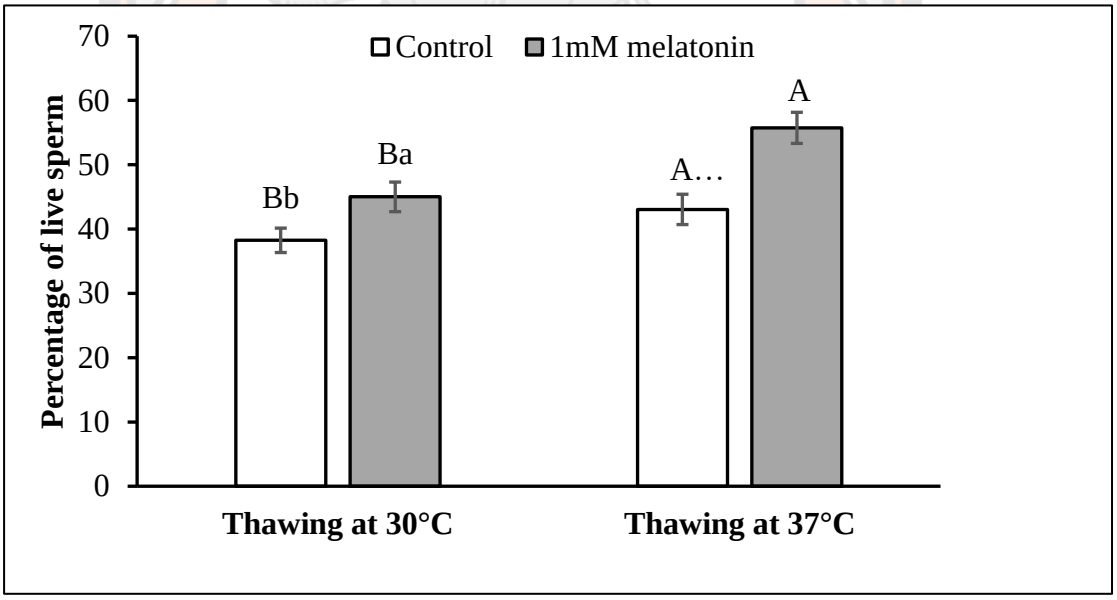


Figure 10 Effect of melatonin and thawing temperature on the percentage of sperm viability

The data expressed as mean ± SEM. Different letters in each bar represent statistically significant differences ($p < 0.01$) where (a, b) represent difference

between treatment groups and (A, B) represent difference between thawing groups. Number of replicates for each group (n) = 12

The percentage of early apoptosis was significantly lower ($p < 0.01$) in the treatment samples than the untreated samples of sperms. The lowest percentage of early apoptotic spermatozoa (38.51 ±2.55) was found in the treatment samples when thawed at 37°C for 30 seconds which was significantly lower ($p < 0.05$) than thawing at 30°C for 60 seconds (47.24 ± 2.99).

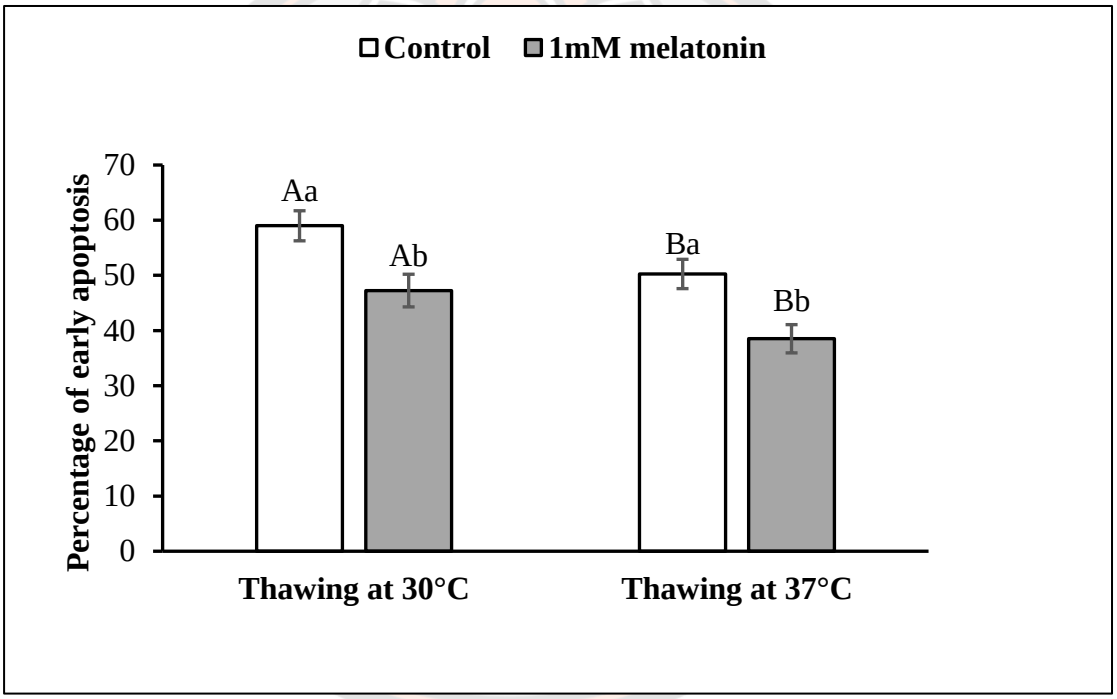


Figure 11 Effect of melatonin and thawing temperature on the percentage of early apoptotic sperm

The data expressed as mean ± SEM. Different letters in each bar represent statistically significant differences ($p < 0.01$) where (a, b) represent difference between treatment groups and (A, B) represent difference between thawing groups. Number of replicates for each group (n) = 12

The percentage of late apoptotic/dead spermatozoa in both the control and

the 1 mM MLT treated samples when thawed at 30°C for 60 minutes and 37°C for 30 seconds is shown in Figure 12. This figure illustrates that the percentage of late apoptotic sperms is independent of the treatment and the thawing method.

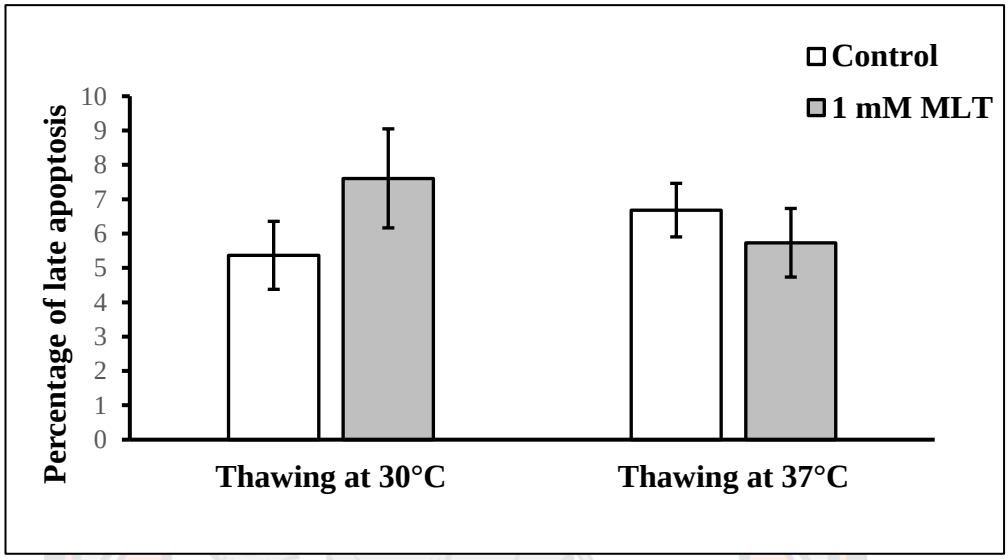


Figure 12 Effect of melatonin and thawing temperature on the percentage of late apoptotic sperm

The data expressed as mean ± SEM

4.3 Expression of *hsp* 70 and *hsp* 90 in frozen-thawed sperm:

The expression for *hsp* 70 and *hsp* 90 was analysed in the control samples and the 1 mM MLT treated samples of semen to see the effect of MLT on their expression and also to see the protective effects of these genes. The expression of *hsp* 70 and *hsp* 90 was investigated in two different thawing conditions i.e (I) 30°C for 60 seconds and (II) 37°C for 30 seconds in the MLT treated samples and the control samples. To see these expressions, the amplified PCR products corresponding to the *hsp* 70, *hsp* 90 and *GAPDH* were obtained from the sperm cDNA after 35 PCR cycles of amplification and run in 1.5% agarose gel. A single band of 591 bp was obtained (Figure 13 A) with the *hsp* 70 primer pair. The amplified PCR products in the MLT treated group yielded a stronger band than the control group (Figure 13 A and B) but

it is not significant. The band of the MLT treated sample, when thawing at 37°C for 30 seconds, was stronger than those when thawing at 30°C for 60 seconds and it is significantly different ($p \leq 0.05$) at different thawing temperature.

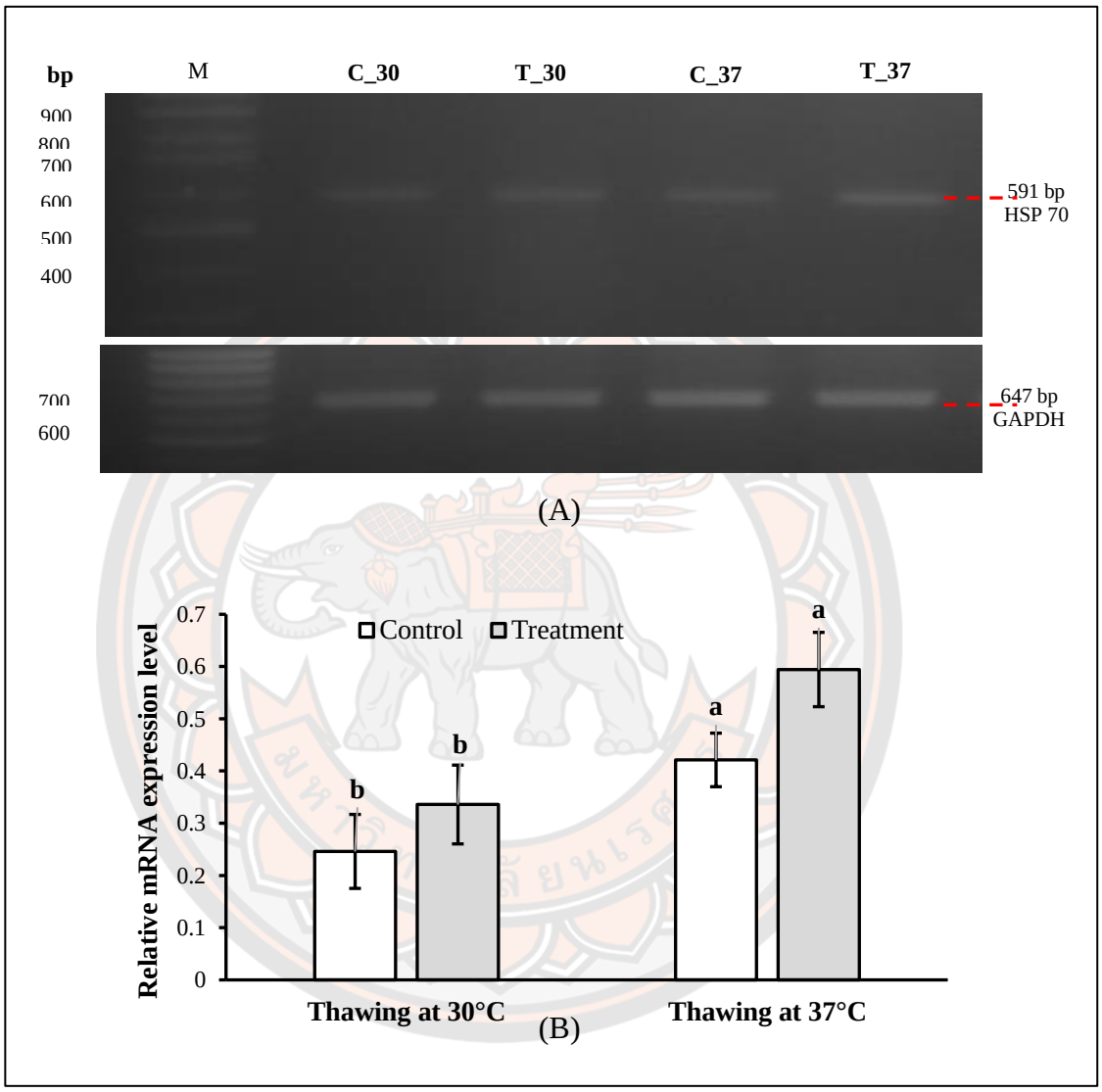


Figure 13 The effect of melatonin supplementation in cryopreserved swamp buffalo semen on hsp 70 gene expression after different thawing methods. (A) Agarose gel electrophoresis (1.5% agarose) of PCR amplified products using hsp 70 and GAPDH specific primer sets (B) Comparison of hsp 70 gene expression at different thawing temperature and treatment

Lane: C_30: Thawing of semen at 30°C for 60 seconds without MLT supplementation, T_30: with MLT supplementation, C_37: Thawing of semen at 37°C for 30 seconds without MLT supplementation, T_37: with MLT supplementation. Lane M, 100 bp DNA size marker. The data expressed as Mean ± SEM. Different superscript letters (a, b) in each bar represent statistically significant differences ($p < 0.01$)

Similarly, a single band of 685 bp was obtained (Figure 14 A) with the *hsp* 90 primer pair. The bands in the MLT treated samples demonstrated a significantly stronger band ($p \leq 0.01$) than the control sample (Figure 14 A and B). Within the MLT treated sample too, the amplified PCR product in the sample when thawed at temperature 37°C for 30 seconds yielded the stronger band than when thawing at 30°C for 60 seconds.

A single band of 647 bp with the *GAPDH* primer pair was obtained (Figure 13 A and Figure 14 A). This gene was amplified as an internal control gene. The amplified PCR products in each sample yielded the bands of the same intensity. The sequencing of the main PCR products was done by comparing them with the related databases.

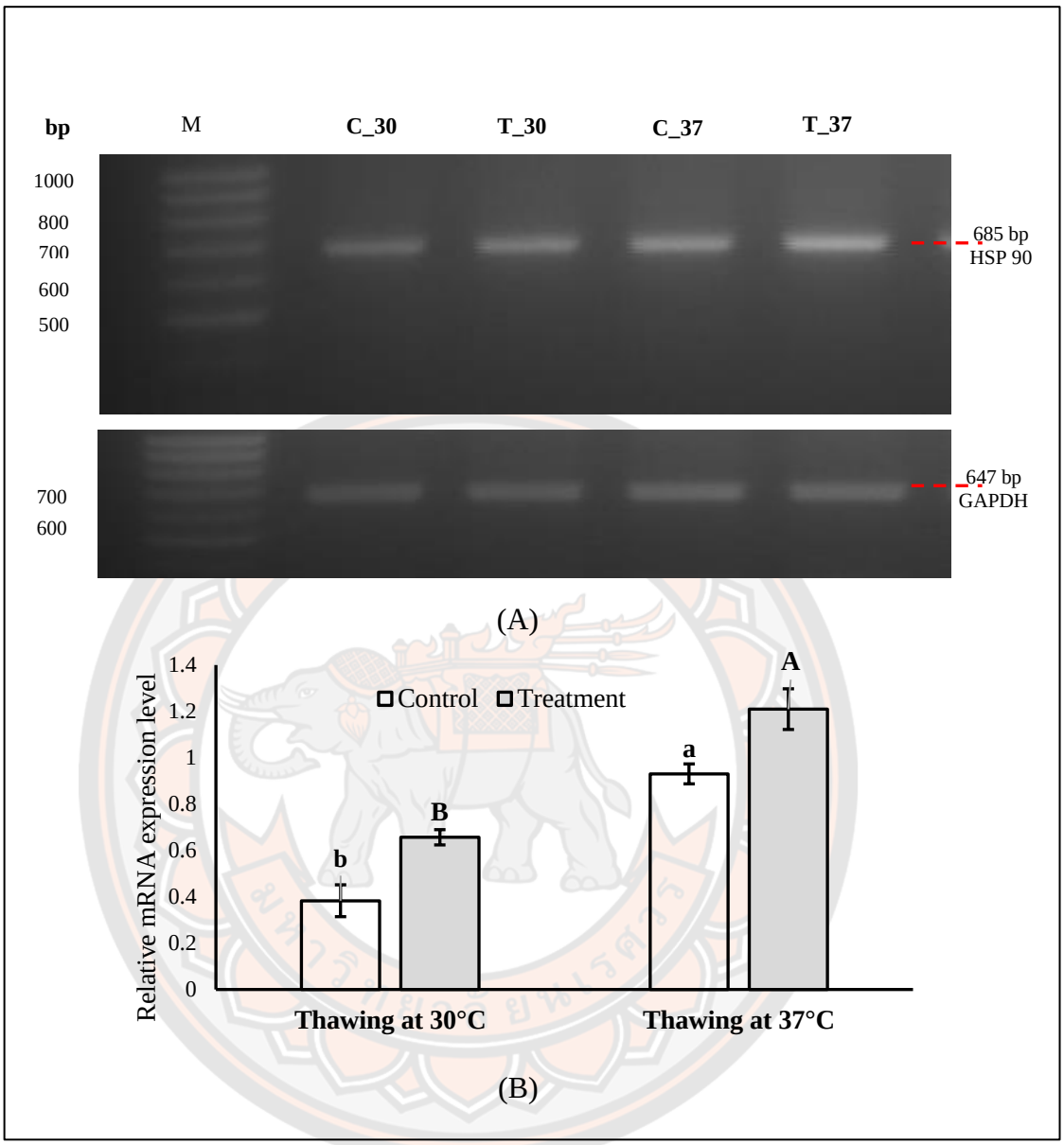


Figure 14 The effect of melatonin supplementation in cryopreserved swamp buffalo semen on hsp 90 gene expression after different thawing methods. (A) Agarose gel electrophoresis (1.5% agarose) of PCR amplified products using hsp 90 and GAPDH specific primer sets (B) Comparison of hsp 90 gene expression at different thawing temperature and treatment

Lane: C_30: Thawing of semen at 30°C for 60 seconds without MLT supplementation, T_30: with MLT supplementation, C_37: Thawing of semen at 37°C for 30 seconds without MLT supplementation, T_37: with MLT supplementation. Lane M, 100 bp DNA size marker. The data expressed as Mean ± SEM. Different superscript letters (a, b, A, and B) in each bar represent statistically significant differences ($p < 0.01$)

CHAPTER V

DISCUSSION

There is a considerable loss in semen quality during the semen cryopreservation process (X. G. Zhang et al., 2015) mostly because of excessive reactive oxygen species formation leading to oxidative stress (Bilodeau et al., 2000). Hence, it is necessary to improve the post-thaw semen quality by protecting from oxidative stress. MLT, a powerful anti-oxidant, has been found to protect the cells from oxidative stress and thus has been proved to improve the quality of post thawed semen of different species (Ashrafi et al., 2013; El-Raey et al., 2015; Karimfar et al., 2015; Succu et al., 2011). So, the present study was undertaken to see whether MLT supplemented to the semen extender of swamp buffalo semen could protect from the demerits of cryopreservation procedures in terms of the quality of the semen. This study also aimed to observe the effects of MLT on cryopreserved semen quality in different thawing methods and also to observe the effects of melatonin on the *hsp* 70 and *hsp* 90 expression after thawing with different methods. CASA and flow cytometry methods were used to assess the semen quality and RTPCR and real time PCR was used to observe the *hsp* 70 and *hsp* 90 expression. The results of our study indicates that MLT supplemented in the semen extender provides the beneficial effect to the frozen-thawed semen of swamp buffalo by improving the motility, decreasing the apoptotic sperm and also by increasing the expression of semen quality related genes i.e. *hsp* 70 and *hsp* 90.

In the present study, the motility parameters of the sperm such as the percentage of total motility and progressive motility, were seen higher when the semen extender was supplemented with 1 mM of MLT. This indicated that the swamp buffalo bull semen frozen with the 1mM MLT, yielded the superior semen quality than the untreated semen. These findings are in accordance with other studies by (Ashrafi et al., 2013; ChaithraShree et al., 2019; Kapadiya et al., 2016) where they observed the total motility of sperm increased by 5 % to 20% in bull semen; (Abdel-Khalek, El-Nagar, & Ibrahim, 2016; El-Raey et al., 2015; El-Raey et al., 2014)

observed the motility to be increased by 6 % to 15 % in buffalo bull semen; (Perumal, Chang, Sangma, Savino, & Khate, 2016; Perumal et al., 2018; Perumal et al., 2013) observed 5% to 10% increase in the sperm motility in mithun semen; (Ashrafi, Kohram, Naijian, Bahreini, & Poorhamdollah, 2011; Succu et al., 2011) observed 5 % to 10 % increase in the motility of ram semen; (Deng et al., 2017; Karimfar et al., 2015) observed 15 % to 17 % increase in the sperm motility in human semen . In all these findings when semen added to MLT improved the semen motility. These positive attributes of sperm motility in the MLT treated semen have been explained by the different authors in the different ways. According to Ashrafi et al. (2013); X.-j. Chen et al. (2016); Deng et al. (2017); Karimfar et al. (2015); Succu et al. (2011) MLT supplementation decreased the ROS level and the lipid peroxidation thereby decreasing the oxidative damage to the spermatozoa membrane and thus maintaining the sperm motility. According to El-Raey et al. (2015) MLT has the capacity to preserve and maintain the ultrastructure integrity of the plasma membrane and mitochondrial arrangement of the cryopreserved spermatozoa so that the sperms are less susceptible to the cryo-injury and thus preserving the motility. MLT also has the positive effect on the antioxidant enzymes like SOD, GSH increasing their expression and therefore increasing the total antioxidant capacity (El-Raey et al., 2014) which can resist the oxidative damage. Some studies show that the MLT has protective effect on Mitochondria by preventing from the oxidative damage, while maintaining the oxidative phosphorylation and ATP production (López et al., 2009); thus higher intracellular ATP concentrations observed (Succu et al., 2011). These all effects might be the possible causes for the increase in the motility of the sperm with the extender supplemented with MLT.

Some studies have revealed that apoptosis of spermatozoa is increased during the freezing-thawing process (Khan, Ahmad, Anzar, & Channa, 2009; G. Martin et al., 2007; G. Martin, Sabido, Durand, & Levy, 2004). The externalization of phosphatidylserine (PS) from the inner leaflet to the outer leaflet of plasma membrane occurs during the process of apoptosis (S. Martin et al., 1995; Verhoven, Schlegel, & Williamson, 1995) Annexin V is the calcium dependent phospholipid binding protein which has a high affinity for PS so it is used to study apoptosis (Koopman et al., 1994). In this study, we used Annexin V/7-AAD to detect apoptotic spermatozoa and

the late apoptotic/dead spermatozoa by the use of flow cytometry in frozen-thawed semen of swamp buffalo. 7-AAD is a fluorescent DNA intercalator that is membrane impermeant and is therefore excluded from live, healthy, and early apoptotic cells, but permeates late-stage apoptotic and dead cells. In the present study, the percentage of early apoptotic spermatozoa in the MLT treated frozen-thawed semen was found significantly lower than the untreated frozen-thawed semen in every thawing conditions. This indicates that MLT supplemented in the semen extender can reduce the apoptotic effects in the frozen-thawed semen thus enhancing the quality of the semen. Similarly, when MLT was supplemented in the semen extender, reduced percentage of apoptotic spermatozoa was observed in the various species of animals such as in mithun semen (Perumal et al., 2018), ram semen (Casao, Mendoza, et al., 2010), rooster semen (Mehaisen, Partyka, Ligocka, & Nizanski, 2020), stallion spermatozoa (Balao da Silva et al., 2011) and human spermatozoa (Deng et al., 2017; Espino et al., 2010) Oxidative stress leads to increased apoptosis of spermatozoa(X. Wang et al., 2003). Hence, the decrease in apoptotic spermatozoa in the present study might be because of the strong anti-oxidative capacity of MLT to diminish the oxidative stress by reducing the levels of intracellular ROS (Deng et al., 2017; Karimfar et al., 2015; Najafi et al., 2018) and protecting from lipid peroxidation (Ashrafi et al., 2013). MLT also increases the expression of antioxidant enzymes such as, SOD, CAT, HO-1 (Ashrafi et al., 2013), and expression of Nrf2 (Deng et al., 2017) and eventually increasing the antioxidant capacity in the frozen-thawed sperm. MLT is also able to reduce the pro-apoptotic gene bax and increase the expression of anti-apoptotic gene Bcl-2 (X.-j. Chen et al., 2016; Deng et al., 2017). Thus, the anti-apoptotic effect in the MLT treated semen might be because of all these combined mechanisms.

This study further investigated the role of MLT in the expression of semen quality related and cryo-protective related genes such as *hsp* 70 an *hsp* 90. HSPs have a protective role in the cells and HSP 70 is associated with the viability and longevity of the sperm (Elliott et al., 2009). There is a positive correlation with the motility of the sperm (X. G. Zhang et al., 2015), and the level of HSP 70 in the sperm influences the quality of the semen (Huang, Kuo, Lee, et al., 2000). In the present study, the expression of the *hsp* 70 genes is stronger in the MLT treated groups of semen in all

the thawing conditions than in those groups of semen without MLT supplementation. This indicates that MLT promotes the expression of *hsp* 70 gene. In the different studies undertaken (Cao et al., 2003; Huang et al., 1999; Huang, Kuo, Tsou, et al., 2000; X.-G. Zhang et al., 2015) it was found that the level of HSP 90 is decreased during the cryopreservation process and the expression level is proportional to the sperm parameters such as motility, membrane integrity, and acrosome integrity, indicating the protective role of HSP 90 in semen quality during cryopreservation. According to Deng et al. (2017), MLT promotes the expression of *hsp* 90 in the frozen-thawed human spermatozoa. In the present study, we also found that the *hsp* 90 gene expression was higher in the MLT treated groups in all the different thawing methods than in the MLT free group. The motility parameters and the viability parameters in the MLT treated groups was also better in the MLT treated groups. This indicates that the MLT plays protective role by promoting HSP 90 level as HSP 90 is associated with semen quality.

Apart from this, we also wanted to evaluate the two commonly used thawing temperatures and time combination. Thawing procedure is just as important as the freezing procedure in terms of its impact on the survival of spermatozoa (Nur et al., 2003) because during both the stages spermatozoa have to pass through the critical temperature zone i.e., from -5 to -50°C which causes a detrimental effect on surviving spermatozoa population (Borah et al., 2015). In this study, the thawing of frozen semen at 37 °C for 30 seconds illustrated the best result in terms of the live percentage of spermatozoa obtained from flow cytometry. At this thawing condition, the apoptotic spermatozoa were also significantly lower and the live percentage of spermatozoa higher when compared with thawing at 30 °C for 60 seconds. However, the results of the motility characteristics, as obtained by CASA evaluation, were not significant in between the two thawing methods. These results suggest that both the thawing methods are acceptable. These findings were in line with the results obtained by (Al-Badry, 2012; Chaiprasat, Benjakul, Chartchue, Joemplang, & Punyapornwithaya, 2006; J. R. Correa, Heersche, & Zavos, 1997; J. R. Correa, Rodriguez, Patterson, & Zavos, 1996; Narasimha Rao, Haranath, Soma Sekharam, & Ramamohana Rao, 1986; Nur, Ileri, & Ak, 2006; Nur et al., 2005) where thawing at 37 °C for 30 seconds was considered the best thawing procedure. Similarly, Dudeja

(1990) and Barth and Bowman (1988) found that that thawing of frozen semen in water-bath at 35 ^{0} C for 30 seconds yielded satisfactory post-thaw viability and motility. However, many of the researchers (Borah et al., 2015; Lyashenko, 2015; Nur et al., 2003; Rastegarnia et al., 2013; Robbins et al., 1976) have found out that thawing at a higher temperature (60 °C to 80 °C) for short duration yields better post-thaw motility and viability. These differences in the result might be because of the difference in the species of animal, difference in the semen processing procedures, availability of the other facilities. Therefore, it is recommended to assess the thawing methods in each and every semen stations.

CHAPTER VI

CONCLUSION

The overall results of the present study confirm that 1 mM melatonin supplementation to the semen extender can improve the motility and viability of spermatozoa and minimize the apoptosis in the frozen-thawed semen of swamp buffalo and it also promotes the expression of hsp 70 and hsp 90. Furthermore, the semen quality was better when the MLT supplemented frozen straws were thawed at 37°C for 30 seconds. However, there are other parts of experiments further needed to be studied like the parameters before and after freeze-thawing comparison, more semen quality parameters, more thawing temperatures and quantitation of the gene expression.

REFERENCES

REFERENCES

Abdel-Khalek, A., El-Nagar, H., & Ibrahim, O. M. (2016). Effect of leptin and melatonin as protective additives to tris extender on frozen semen quality of buffalo bulls. *Journal of Animal and Poultry Production, 7*(7), 225-231.

Agca, Y., Gilmore, J., Byers, M., Woods, E. J., Liu, J., & Critser, J. K. (2002). Osmotic characteristics of mouse spermatozoa in the presence of extenders and sugars. *Biol Reprod, 67*(5), 1493-1501. doi:10.1095/biolreprod.102.005579

Aitken, R. J., Buckingham, D., & Harkiss, D. (1993). Use of a xanthine oxidase free radical generating system to investigate the cytotoxic effects of reactive oxygen species on human spermatozoa. *Reproduction, 97*(2), 441-450.

Al-Badry, K. I. (2012). Effect of various thawing times and temperatures on frozen semen quality of Friesian bulls in Iraq. *Inter. J. Anim. Vet. Adv, 4*(6), 384-388.

Allegra, M., Reiter, R. J., Tan, D. X., Gentile, C., Tesoriere, L., & Livrea, M. (2003). The chemistry of melatonin's interaction with reactive species. *Journal of pineal research, 34*(1), 1-10.

Alvarez, J. G., & Storey, B. T. (1992). Evidence for increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. *Journal of Andrology, 13*(3), 232-241.

Andreea, A., & Stela, Z. (2010). Role of antioxidant additives in the protection of the cryopreserved semen against free radicals. *Romanian Biotechnological Letters, 15*(3), 34.

Ashrafi, I., Kohram, H., & Ardabili, F. F. (2013). Antioxidative effects of melatonin on kinetics, microscopic and oxidative parameters of cryopreserved bull spermatozoa. *Anim Reprod Sci, 139*(1-4), 25-30. doi:10.1016/j.anireprosci.2013.03.016

Ashrafi, I., Kohram, H., Naijian, H., Bahreini, M., & Poorhamdollah, M. (2011). Protective effect of melatonin on sperm motility parameters on liquid storage

of ram semen at 5 degrees C. *African Journal of Biotechnology, 10*(34), 6670-6674.

Balao da Silva, C. M., Macías-García, B., Miró-Morán, A., González-Fernández, L., Morillo-Rodriguez, A., Ortega-Ferrusola, C., . . . Peña, F. J. (2011). Melatonin reduces lipid peroxidation and apoptotic-like changes in stallion spermatozoa. *Journal of Pineal Research, 51*(2), 172-179.

Ball, B. A. (2008). Oxidative stress, osmotic stress and apoptosis: impacts on sperm function and preservation in the horse. *Anim Reprod Sci, 107*(3-4), 257-267.

Banday, M. N., Lone, F. A., Rasool, F., Rashid, M., & Shikari, A. (2017). Use of antioxidants reduce lipid peroxidation and improve quality of crossbred ram sperm during its cryopreservation. *Cryobiology, 74*, 25-30.

Bandyopadhyay, D., Bandyopadhyay, A., Das, P. K., & Reiter, R. J. (2002). Melatonin protects against gastric ulceration and increases the efficacy of ranitidine and omeprazole in reducing gastric damage. *Journal of pineal research, 33*(1), 1-7.

Bansal, A. K., & Bilaspuri, G. (2011). Impacts of oxidative stress and antioxidants on semen functions. *Veterinary medicine international, 2011*.

Barth, A. D., & Bowman, P. A. (1988). Determination of the best practical method of thawing bovine semen. *Can Vet J, 29*(4), 366-369.

Baumber, J., Ball, B. A., Gravance, C. G., Medina, V., & Davies-Morel, M. C. (2000). The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. *Journal of Andrology, 21*(6), 895-902.

Bearden, H. (2004). In Fuquay, JW and Willard, ST (eds). Applied Animal Reproduction, 6th Eddition: Pearson Education, Inc., Upper Saddle River, New Jersy.

Beere, H. M., & Green, D. R. (2001). Stress management–heat shock protein-70 and the regulation of apoptosis. *Trends in cell biology, 11*(1), 6-10.

Bender, A. T., Silverstein, A. M., Demady, D. R., Kanelakis, K. C., Noguchi, S., Pratt, W. B., & Osawa, Y. (1999). Neuronal nitric-oxide synthase is regulated by the Hsp90-based chaperone system in vivo. *Journal of Biological Chemistry, 274*(3), 1472-1478.

Benson, J. D., Woods, E. J., Walters, E. M., & Critser, J. K. (2012). The cryobiology of spermatozoa. *Theriogenology, 78*(8), 1682-1699.

Bilodeau, J. F., Chatterjee, S., Sirard, M. A., & Gagnon, C. (2000). Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. *Mol Reprod Dev, 55*(3), 282-288. doi:10.1002/(SICI)1098-2795(200003)55:3<282::AID-MRD6>3.0.CO;2-7

Blask, D. E., Sauer, L. A., & Dauchy, R. T. (2002). Melatonin as a chronobiotic/anticancer agent: cellular, biochemical, and molecular mechanisms of action and their implications for circadian-based cancer therapy. *Current topics in medicinal chemistry, 2*(2), 113-132.

Block, E. R. (1991). Hydrogen peroxide alters the physical state and function of the plasma membrane of pulmonary artery endothelial cells. *Journal of cellular physiology, 146*(3), 362-369.

Blondin, P., Coenen, K., & Sirard, M. A. (1997). The impact of reactive oxygen species on bovine sperm fertilizing ability and oocyte maturation. *Journal of Andrology, 18*(4), 454-460.

Borah, B. K., Deka, B. C., Biswas, R. K., Chakravarty, P., Deori, S., Sinha, S., & Ahmed, K. (2015). Effect of thawing methods on frozen semen quality of yak (Poephagus grunniens L.) bulls. *Vet World, 8*(7), 831-834. doi:10.14202/vetworld.2015.831-834

Borkovich, K., Farrelly, F., Finkelstein, D., Taulien, J., & Lindquist, S. (1989). hsp82 is an essential protein that is required in higher concentrations for growth of cells at higher temperatures. *Molecular and cellular biology, 9*(9), 3919-3930.

Breininger, E., Beorlegui, N. B., O'Flaherty, C. M., & Beconi, M. T. (2005). Alpha-tocopherol improves biochemical and dynamic parameters in cryopreserved boar semen. *Theriogenology, 63*(8), 2126-2135. doi:10.1016/j.theriogenology.2004.08.016

Brzezinski, A., Seibel, M. M., Lynch, H. J., Deng, M.-H., & Wurtman, R. J. (1987). Melatonin in human preovulatory follicular fluid. *The Journal of Clinical Endocrinology & Metabolism, 64*(4), 865-867.

Bucak, M. N., Sarıözkan, S., Tuncer, P. B., Sakin, F., Ateşşahin, A., Kulaksız, R., & Çevik, M. (2010). The effect of antioxidants on post-thawed Angora goat (Capra hircus ancryrensis) sperm parameters, lipid peroxidation and antioxidant activities. *Small Ruminant Research, 89*(1), 24-30.

Bucak, M. N., Tuncer, P. B., Sarıözkan, S., Başpınar, N., Taşpınar, M., Çoyan, K., . . . Aydos, S. (2010). Effects of antioxidants on post-thawed bovine sperm and oxidative stress parameters: antioxidants protect DNA integrity against cryodamage. *Cryobiology, 61*(3), 248-253.

Calamera, J. C., Buffone, M. G., Doncel, G. F., Brugo-Olmedo, S., de Vincentiis, S., Calamera, M. M., . . . Alvarez, J. G. (2010). Effect of thawing temperature on the motility recovery of cryopreserved human spermatozoa. *Fertility and sterility, 93*(3), 789-794.

Cao, W.-L., Wang, Y.-X., Xiang, Z.-Q., & Li, Z. (2003). Cryopreservation-induced decrease in heat-shock protein 90 in human spermatozoa and its mechanism. *Asian journal of andrology, 5*(1), 43-46.

Casao, A., Cebrian, I., Asumpcao, M. E., Perez-Pe, R., Abecia, J. A., Forcada, F., . . . Muino-Blanco, T. (2010). Seasonal variations of melatonin in ram seminal plasma are correlated to those of testosterone and antioxidant enzymes. *Reproductive biology and endocrinology, 8*(1), 59. doi:Artn 5910.1186/1477-7827-8-59

Casao, A., Mendoza, N., Perez-Pe, R., Grasa, P., Abecia, J. A., Forcada, F., . . . Muino-Blanco, T. (2010). Melatonin prevents capacitation and apoptotic-like changes of ram spermatozoa and increases fertility rate. *J Pineal Res, 48*(1), 39-46. doi:10.1111/j.1600-079X.2009.00722.x

Chaiprasat, S., Benjakul, W., Chartchue, A., Joemplang, P., & Punyapornwithaya, V. (2006). Effect of bull semen thawing methods on sperm progressive motility. *Chiang Mai Veterinary Journal, 4*(1), 25-29.

ChaithraShree, A., Ingole, S. D., Dighe, V. D., Nagvekar, A. S., Bharucha, S. V., Dagli, N. R., . . . Kharde, S. D. (2019). Effect of melatonin on bovine sperm characteristics and ultrastructure changes following cryopreservation. *Veterinary Medicine and Science*.

Chen, X.-j., Zhang, Y., Jia, G.-x., Meng, Q.-g., Bunch, T. D., Liu, G.-s., . . . Zhou, G.-b. (2016). Effect of melatonin supplementation on cryopreserved sperm quality in mouse. *Cryoletters, 37*(2), 115-122.

Chen, Z., Hauser, R., Trbovich, A. M., Shifren, J. L., Dorer, D. J., Godfrey-Bailey, L., & Singh, N. P. (2006). The relationship between human semen characteristics and sperm apoptosis: a pilot study. *J Androl, 27*(1), 112-120. doi:10.2164/jandrol.05073

Chowdhury, I., Sengupta, A., & Maitra, S. K. (2008). Melatonin: fifty years of scientific journey from the discovery in bovine pineal gland to delineation of functions in human. *Indian J Biochem Biophys, 45*(5), 289-304.

Claustrat, B., Brun, J., & Chazot, G. (2005). The basic physiology and pathophysiology of melatonin. *Sleep medicine reviews, 9*(1), 11-24.

Correa, J., Pace, M., & Zavos, P. (1997). Relationships among frozen-thawed sperm characteristics assessed via the routine semen analysis, sperm functional tests and fertility of bulls in an artificial insemination program. *Theriogenology, 48*(5), 721-731.

Correa, J. R., Heersche, G., & Zavos, P. M. (1997). Sperm membrane functional integrity and response of frozen-thawed bovine spermatozoa during the hypoosmotic swelling test incubation at varying temperatures. *Theriogenology, 47*(3), 715-721. doi:10.1016/s0093-691x(97)00029-0

Correa, J. R., Rodriguez, M. C., Patterson, D. J., & Zavos, P. M. (1996). Thawing and processing of cryopreserved bovine spermatozoa at various temperatures and their effects on sperm viability, osmotic shock and sperm membrane functional integrity. *Theriogenology, 46*(3), 413-420. doi:10.1016/0093-691x(96)00163-x

Csermely, P., Schnaider, T., Soti, C., Prohaszka, Z., & Nardai, G. (1998). The 90-kDa molecular chaperone family: structure, function, and clinical applications. A comprehensive review. *Pharmacol Ther, 79*(2), 129-168. doi:10.1016/s0163-7258(98)00013-8

Cuzzocrea, S., & Reiter, R. J. (2002). Pharmacological actions of melatonin in acute and chronic inflammation. *Curr Top Med Chem, 2*(2), 153-165. doi:10.2174/1568026023394425

da Silva Maia, M., Bicudo, S. D., Sicherle, C. C., Rodello, L., & Gallego, I. C. S. (2010). Lipid peroxidation and generation of hydrogen peroxide in frozen-thawed ram semen cryopreserved in extenders with antioxidants. *Anim Reprod Sci, 122*(1-2), 118-123.

Daugaard, M., Rohde, M., & Jäättelä, M. (2007). The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. *FEBS letters, 581*(19), 3702-3710.

De Lamirande, E., & Gagnon, C. (1992). Reactive oxygen species and human spermatozoa: I. Effects on the motility of intact spermatozoa and on sperm axonemes. *Journal of Andrology, 13*(5), 368-378.

Deng, S.-L., Sun, T.-C., Yu, K., Wang, Z.-P., Zhang, B.-L., Zhang, Y., . . . Liu, Y.-X. (2017). Melatonin reduces oxidative damage and upregulates heat shock protein 90 expression in cryopreserved human semen. *Free Radical Biology and Medicine, 113*, 347-354.

Dix, D. J., Allen, J. W., Collins, B. W., Mori, C., Nakamura, N., Poorman-Allen, P., . . . Eddy, E. (1996). Targeted gene disruption of Hsp70-2 results in failed meiosis, germ cell apoptosis, and male infertility. *Proceedings of the National Academy of Sciences, 93*(8), 3264-3268.

Doležalová, M., Ptáček, M., Stádník, L., & Ducháček, J. (2017). Effect of different thawing methods on bull's semen characteristics. *Acta Universitatis Agriculturae et Silviculturae Mendelianae Brunensis, 65*(3), 815-822.

Dudeja, S. (1990). Effect of temperature of thawing and diluent on the post--thaw physiological changes of buffalo frozen semen. *Indian journal of physiology and pharmacology, 34*(4), 267-270.

El-Raey, M., Badr, M., Assi, M., & Rawash, Z. (2015). Effect of melatonin on buffalo bull sperm freezability, ultrastructure changes and fertilizing potentials. *Assiut Vet Med J, 61*(144), 201-208.

El-Raey, M., Badr, M., Rawash, Z., & Darwish, G. (2014). Evidences for the role of melatonin as a protective additive during buffalo semen freezing. *American journal of animal and veterinary sciences, 9*(4), 252-262.

El-Sokkary, G. H., Kamel, E. S., & Reiter, R. J. (2003). Prophylactic effect of melatonin in reducing lead-induced neurotoxicity in the rat. *Cellular & molecular biology letters, 8*(2), 461-470.

Elliott, R. M., Lloyd, R. E., Fazeli, A., Sostaric, E., Georgiou, A. S., Satake, N., . . . Holt, W. V. (2009). Effects of HSPA8, an evolutionarily conserved oviductal protein, on boar and bull spermatozoa. *Reproduction, 137*(2), 191-203.

Espino, J., Bejarano, I., Ortiz, A., Lozano, G. M., Garcia, J. F., Pariente, J. A., & Rodriguez, A. B. (2010). Melatonin as a potential tool against oxidative damage and apoptosis in ejaculated human spermatozoa. *Fertil Steril, 94*(5), 1915-1917. doi:10.1016/j.fertnstert.2009.12.082

Foote, R. H., Brockett, C. C., & Kaproth, M. T. (2002). Motility and fertility of bull sperm in whole milk extender containing antioxidants. *Anim Reprod Sci, 71*(1-2), 13-23. doi:10.1016/s0378-4320(02)00018-0

Freeman, B. C., & Morimoto, R. I. (1996). The human cytosolic molecular chaperones hsp90, hsp70 (hsc70) and hdj-1 have distinct roles in recognition of a non-native protein and protein refolding. *The EMBO journal, 15*(12), 2969-2979.

Gade, N., Mahapatra, R., Sonawane, A., Singh, V., Doreswamy, R., & Saini, M. (2010). Molecular characterization of heat shock protein 70-1 gene of goat (Capra hircus). *Molecular biology international, 2010*.

Gao, D., & Critser, J. K. (2000). Mechanisms of cryoinjury in living cells. *ILAR J, 41*(4), 187-196. doi:10.1093/ilar.41.4.187

García-Cardeña, G., Fan, R., Shah, V., Sorrentino, R., Cirino, G., Papapetropoulos, A., & Sessa, W. C. (1998). Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature, 392*(6678), 821.

Georgopoulos, C., & Welch, W. (1993). Role of the major heat shock proteins as molecular chaperones. *Annual review of cell biology, 9*(1), 601-634.

Gold, R., Kappos, L., Arnold, D. L., Bar-Or, A., Giovannoni, G., Selmaj, K., . . . Sheikh, S. I. (2012). Placebo-controlled phase 3 study of oral BG-12 for relapsing multiple sclerosis. *New England Journal of Medicine, 367*(12), 1098-1107.

Govin, J., Caron, C., Escoffier, E., Ferro, M., Kuhn, L., Rousseaux, S., . . . Khochbin, S. (2006). Post-meiotic shifts in HSPA2/HSP70. 2 chaperone activity during mouse spermatogenesis. *Journal of Biological Chemistry, 281*(49), 37888-37892.

Griveau, J., Dumont, E., Renard, P., Callegari, J., & Le Lannou, D. (1995). Reactive oxygen species, lipid peroxidation and enzymatic defence systems in human spermatozoa. *Reproduction, 103*(1), 17-26.

Grotto, D., Maria, L. S., Valentini, J., Paniz, C., Schmitt, G., Garcia, S. C., . . . Farina, M. (2009). Importance of the lipid peroxidation biomarkers and methodological aspects for malondialdehyde quantification. *Quimica Nova, 32*(1), 169-174.

Guerrero, J. M., & Reiter, R. J. (2002). Melatonin-immune system relationships. *Curr Top Med Chem, 2*(2), 167-179. doi:10.2174/1568026023394335

Hammerstedt, R. H., Graham, J. K., & Nolan, J. P. (1990). Cryopreservation of mammalian sperm: what we ask them to survive. *Journal of Andrology, 11*(1), 73-88.

Hardeland, R., Balzer, I., Poeggeler, B., Fuhrberg, B., Una, H., Behrmann, G., . . . Reiter, R. J. (1995). On the primary functions of melatonin in evolution: mediation of photoperiodic signals in a unicell, photooxidation, and scavenging of free radicals. *Journal of Pineal Research, 18*(2), 104-111.

Hardeland, R., Reiter, R. J., Poeggeler, B., & Tan, D.-X. (1993). The significance of the metabolism of the neurohormone melatonin: antioxidative protection and formation of bioactive substances. *Neuroscience & Biobehavioral Reviews, 17*(3), 347-357.

Hu, J. H., Zhao, X. L., Tian, W. Q., Zan, L. S., & Li, Q. W. (2011). Effects of vitamin E supplementation in the extender on frozen-thawed bovine semen preservation. *animal, 5*(1), 107-112. doi:10.1017/S1751731110001679

Huang, S., Kuo, Y., Lee, W., Tsou, H., Lee, Y., Chang, H., . . . Yang, P. (1999). Substantial decrease of heat-shock protein 90 precedes the decline of sperm motility during cooling of boar spermatozoa. *Theriogenology, 51*(5), 1007-1016.

Huang, S., Kuo, Y., Lee, Y., Tsou, H., Lin, E., Ju, C., & Lee, W. (2000). Association of heat shock protein 70 with semen quality in boars. *Anim Reprod Sci, 63*(3-4), 231-240.

Huang, S., Kuo, Y., Tsou, H., Lee, Y., King, Y., Huang, H., . . . Lee, W. (2000). The decline of porcine sperm motility by geldanamycin, a specific inhibitor of heat-shock protein 90 (HSP90). *Theriogenology, 53*(5), 1177-1184.

Husna, A. U., Ansari, M. S., Rakha, B. A., Ejaz, R., Ullah, N., & Akhter, S. (2017). Melatonin supplementation in extender enhances the post thaw quality of buffalo bull spermatozoa. *Pakistan Journal of Zoology, 49*(1), 171-175.

Illnerova, H., Buresova, M., & Presl, J. (1993). Melatonin rhythm in human milk. *The Journal of Clinical Endocrinology & Metabolism, 77*(3), 838-841.

J Reiter, R., Tan, D.-X., Rosales-Corral, S., & C Manchester, L. (2013). The universal nature, unequal distribution and antioxidant functions of melatonin and its derivatives. *Mini reviews in medicinal chemistry, 13*(3), 373-384.

Jang, T. H., Park, S. C., Yang, J. H., Kim, J. Y., Seok, J. H., Park, U. S., . . . Han, J. (2017). Cryopreservation and its clinical applications. *Integrative medicine research, 6*(1), 12-18.

Jones, R., Mann, T., & Sherins, R. (1979). Peroxidative breakdown of phospholipids in human spermatozoa, spermicidal properties of fatty acid peroxides, and protective action of seminal plasma. *Fertility and sterility, 31*(5), 531-537.

Kapadiya, P., Nakhashi, H., Sutaria, T., Rathod, B., & Suthar, B. (2016). Melatonin as an additive to shield the hazardous effects of cryopreservation on Kankrej bull semen. *The Indian Journal of Animal Reproduction, 38*(1).

Karimfar, M. H., Niazvand, F., Haghani, K., Ghafourian, S., Shirazi, R., & Bakhtiyari, S. (2015). The protective effects of melatonin against cryopreservation-induced oxidative stress in human sperm. *Int J Immunopathol Pharmacol, 28*(1), 69-76. doi:10.1177/0394632015572080

Kashuba, C. M., Benson, J. D., & Critser, J. K. (2014). Rationally optimized cryopreservation of multiple mouse embryonic stem cell lines: I—Comparative fundamental cryobiology of multiple mouse embryonic stem cell lines and the implications for embryonic stem cell cryopreservation protocols. *Cryobiology, 68*(2), 166-175.

Kennaway, D. J., & Wright, H. (2002). Melatonin and circadian rhythms. *Curr Top Med Chem, 2*(2), 199-209. doi:10.2174/1568026023394380

Khalifa, M. A. (2017). Improving cryopreservation capacity of ram spermatozoa by supplementing the diluent with melatonin. *International Journal of Animal Research, 1*(8). doi:10.28933/ijar-2017-11-0202

Khan, D. R., Ahmad, N., Anzar, M., & Channa, A. A. (2009). Apoptosis in fresh and cryopreserved buffalo sperm. *Theriogenology, 71*(5), 872-876. doi:10.1016/j.theriogenology.2008.09.056

Koopman, G., Reutelingsperger, C. P., Kuijten, G. A., Keehnen, R. M., Pals, S. T., & van Oers, M. H. (1994). Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood, 84*(5), 1415-1420.

Lampiao, F., & du Plessis, S. S. (2008). TNF-alpha and IL-6 affect human sperm function by elevating nitric oxide production. *Reprod Biomed Online, 17*(5), 628-631. doi:10.1016/s1472-6483(10)60309-4

Layek, S., Mohanty, T., Kumaresan, A., & Parks, J. (2016). Cryopreservation of bull semen: Evolution from egg yolk based to soybean based extenders. *Animal Reproduction Science, 172*, 1-9.

Lee, Y. M., Chen, H. R., Hsiao, G., Sheu, J. R., Wang, J. J., & Yen, M. H. (2002). Protective effects of melatonin on myocardial ischemia/reperfusion injury in vivo. *Journal of pineal research, 33*(2), 72-80.

Lewis, S., Donnelly, E., Sterling, E., Kennedy, M., Thompson, W., & Chakravarthy, U. (1996). Nitric oxide synthase and nitrite production in human spermatozoa: evidence that endogenous nitric oxide is beneficial to sperm motility. *Molecular Human Reproduction, 2*, 873-878.

Li, K., Xue, Y., Chen, A., Jiang, Y., Xie, H., Shi, Q., . . . Ni, Y. (2014). Heat shock protein 90 has roles in intracellular calcium homeostasis, protein tyrosine phosphorylation regulation, and progesterone-responsive sperm function in human sperm. *PLoS One, 9*(12), e115841.

Li, X. J., Gu, J., Lu, S. D., & Sun, F. Y. (2002). Melatonin attenuates MPTP-induced dopaminergic neuronal injury associated with scavenging hydroxyl radical. *Journal of pineal research, 32*(1), 47-52.

López, A., García, J. A., Escames, G., Venegas, C., Ortiz, F., López, L. C., & Acuña-Castroviejo, D. (2009). Melatonin protects the mitochondria from oxidative damage reducing oxygen consumption, membrane potential, and superoxide anion production. *Journal of Pineal Research, 46*(2), 188-198.

Luboshitzky, R., Shen-Orr, Z., & Herer, P. (2002). Seminal plasma melatonin and gonadal steroids concentrations in normal men. *Arch Androl, 48*(3), 225-232. doi:10.1080/01485010252869324

Lyashenko, A. (2015). Effect of different thawing procedures on the quality and fertility of the bull spermatozoa. *Asian Pacific Journal of Reproduction, 4*(1), 17-21.

Maestroni, G. J. M., Sulli, A., Pizzorni, C., Villaggio, B., & Cutolo, M. (2002). Melatonin in rheumatoid arthritis - Synovial macrophages show melatonin receptors. *Neuroendocrine Immune Basis of the Rheumatic Diseases Ii, Proceedings, 966*(1), 271-275. doi:DOI 10.1111/j.1749-6632.2002.tb04226.x

Martin, G., Cagnon, N., Sabido, O., Sion, B., Grizard, G., Durand, P., & Levy, R. (2007). Kinetics of occurrence of some features of apoptosis during the cryopreservation process of bovine spermatozoa. *Hum Reprod, 22*(2), 380-388. doi:10.1093/humrep/del399

Martin, G., Sabido, O., Durand, P., & Levy, R. (2004). Cryopreservation induces an apoptosis-like mechanism in bull sperm. *Biol Reprod, 71*(1), 28-37. doi:10.1095/biolreprod.103.024281

Martin, S., Reutelingsperger, C., McGahon, A. J., Rader, J. A., Van Schie, R., LaFace, D. M., & Green, D. R. (1995). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *The Journal of experimental medicine, 182*(5), 1545-1556.

Martinez-Soto, J. C., de DiosHourcade, J., Gutierrez-Adan, A., Landeras, J. L., & Gadea, J. (2010). Effect of genistein supplementation of thawing medium on characteristics of frozen human spermatozoa. *Asian J Androl, 12*(3), 431-441. doi:10.1038/aja.2009.92

Matuszak, Z., Reszka, K., & Chignell, C. F. (1997). Reaction of melatonin and related indoles with hydroxyl radicals: EPR and spin trapping investigations. *Free Radic Biol Med, 23*(3), 367-372. doi:10.1016/s0891-5849(96)00614-4

Mayo, J. C., Sainz, R. M., Antoli, I., Herrera, F., Martin, V., & Rodriguez, C. (2002). Melatonin regulation of antioxidant enzyme gene expression. *Cell Mol Life Sci, 59*(10), 1706-1713. doi:10.1007/pl00012498

Mazur, P. (1963). Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. *The Journal of general physiology, 47*(2), 347-369.

Mazur, P. (1984). Freezing of living cells: mechanisms and implications. *Am J Physiol, 247*(3 Pt 1), C125-142. doi:10.1152/ajpcell.1984.247.3.C125

Mazur, P. (2004). Principles of cryobiology. In 'Life in the Frozen State'.(Eds BJ Fuller, NL Lane and EE Benson.) pp. 3–65: CRC Press LLC: Boca Raton, FL.

McIntyre, I. M., Norman, T. R., Burrows, G. D., & Armstrong, S. M. (1987). Melatonin rhythm in human plasma and saliva. *J Pineal Res, 4*(2), 177-183. doi:10.1111/j.1600-079x.1987.tb00854.x

Medeiros, C. M., Forell, F., Oliveira, A. T., & Rodrigues, J. L. (2002). Current status of sperm cryopreservation: why isn't it better? *Theriogenology, 57*(1), 327-344. doi:10.1016/s0093-691x(01)00674-4

Mehaisen, G. M. K., Partyka, A., Ligocka, Z., & Nizanski, W. (2020). Cryoprotective effect of melatonin supplementation on post-thawed rooster sperm quality. *Anim Reprod Sci, 212*, 106238. doi:10.1016/j.anireprosci.2019.106238

Meryman, H. T. (2007). Cryopreservation of living cells: principles and practice. *Transfusion, 47*(5), 935-945. doi:10.1111/j.1537-2995.2007.01212.x

Mitchell, M. D., Sayers, L., Keirse, M. J., Anderson, A. B., & Turnbull, A. C. (1978). Melatonin in amniotic fluid during human parturition. *Br J Obstet Gynaecol, 85*(9), 684-686. doi:10.1111/j.1471-0528.1978.tb14947.x

Na-Chiangmai, A. (2002). Current situation and development trends of beef production in Thailand. *Development strategies for genetic evaluation for beef production in developing countries*, 93-97.

Najafi, A., Adutwum, E., Yari, A., Salehi, E., Mikaeili, S., Dashtestani, F., . . . Asadi, E. (2018). Melatonin affects membrane integrity, intracellular reactive oxygen species, caspase3 activity and AKT phosphorylation in frozen thawed human sperm. *Cell Tissue Res, 372*(1), 149-159. doi:10.1007/s00441-017-2743-4

Narasimha Rao, A. V., Haranath, G. B., Soma Sekharam, G., & Ramamohana Rao, J. (1986). Effect of thaw rates on motility, survival and acrosomal integrity of buffalo spermatozoa frozen in medium French straws. *Animal Reproduction Science, 12*(2), 123-129. doi:10.1016/0378-4320(86)90052-7

Neuer, A., Spandorfer, S. D., Giraldo, P., Dieterle, S., Rosenwaks, Z., & Witkin, S. S. (2000). The role of heat shock proteins in reproduction. *Hum Reprod Update, 6*(2), 149-159. doi:10.1093/humupd/6.2.149

Nicolae, D., Stela, Z., Dragomir, C., & Hortanse, A. A. (2014). Effect of thawing time and temperature variation on the quality of frozen-thawed ram semen. *Romanian Biotechnological Letters, 19*(1), 8935-8940.

Nur, Z., Dogan, I., Soylu, M. K., & Ak, K. (2003). Effect of different thawing procedures on the quality of bull semen. *Revue De Medecine Veterinaire, 154*(7), 487-490.

Nur, Z., Ileri, I. K., & Ak, K. (2006). Effects of different temperature treatments applied to deep stored bull semen on post-thaw cold shocked spermatozoa. *Bulletin of the Veterinary Institute in Pulawy, 50*(1), 79-83.

Nur, Z., Sagirkaya, H., Dogan, I., Soylu, M. K., Ak, K., & Ileri, I. K. (2005). Effect of low temperature thawing procedure and post-thaw cold shock on frozen bull semen. *Medycyna Weterynaryjna, 61*(9), 991-993.

O'Bryan, M. K., Zini, A., Cheng, C. Y., & Schlegel, P. N. (1998). Human sperm endothelial nitric oxide synthase expression: correlation with sperm motility. *Fertility and sterility, 70*(6), 1143-1147.

Pablos, M. I., Reiter, R. J., Ortiz, G. G., Guerrero, J. M., Agapito, M. T., Chuang, J.-I., & Sewerynek, E. (1998). Rhythms of glutathione peroxidase and glutathione reductase in brain of chick and their inhibition by light. *Neurochemistry international, 32*(1), 69-75.

Pandey, K. B., & Rizvi, S. I. (2010). Markers of oxidative stress in erythrocytes and plasma during aging in humans. *Oxid Med Cell Longev, 3*(1), 2-12. doi:10.4161/oxim.3.1.10476

Perumal, P., Chang, S., Sangma, C. T. R., Savino, N., & Khate, K. (2016). Effect of melatonin on mobility and velocity parameters of mithun (Bos frontalis) semen preserved in liquid state (5° C). *Journal of Experimental Biology and Agricultural Sciences, 4*(Spl 3-ADPCIAD).

Perumal, P., Khan, M. H., Chang, S., Ezung, E., Vupru, K., & Khate, K. (2018). Anti-apoptotic effect of melatonin in sperm of mithun. *Indian Journal of Animal Sciences, 88*(4), 20-22.

Perumal, P., Vupru, K., & Khate, K. (2013). Effect of addition of melatonin on the liquid storage (5 C) of mithun (Bos frontalis) semen. *International Journal of Zoology, 2013*.

Poeggeler, B., Reiter, R. J., Hardeland, R., Tan, D.-X., & Barlow-Walden, L. (1996). Melatonin and structurally-related, endogenous indoles act as potent electron donors and radical scavengers in vitro. *Redox Report, 2*(3), 179-184.

Poeggeler, B., Saarela, S., Reiter, R. J., TAN, D. X., CHEN, L. D., Manchester, L. C., & BARLOW-WALDEN, L. R. (1994). Melatonin—A Highly Potent Endogenous Radical Scavenger and Electron Donor: New Aspects of the Oxidation Chemistry of this Indole Accessed in vitro a. *Annals of the New York Academy of Sciences, 738*(1), 419-420.

Poeggeler, B., Thuermann, S., Dose, A., Schoenke, M., Burkhardt, S., & Hardeland, R. (2002). Melatonin's unique radical scavenging properties–roles of its functional substituents as revealed by a comparison with its structural analogs. *Journal of pineal research, 33*(1), 20-30.

Pons-Rejraji, H., Sion, B., Saez, F., Brugnon, F., Janny, L., & Grizard, G. (2009). [Role of reactive oxygen species (ROS) on human spermatozoa and male infertility]. *Gynecol Obstet Fertil, 37*(6), 529-535. doi:10.1016/j.gyobfe.2009.04.015

Pozo, M. J., Gomez-Pinilla, P. J., Camello-Almaraz, C., Martin-Cano, F. E., Pascua, P., Rol, M. A., . . . Camello, P. J. (2010). Melatonin, a potential therapeutic

agent for smooth muscle-related pathological conditions and aging. *Curr Med Chem, 17*(34), 4150-4165. doi:10.2174/092986710793348536

Pratt, W. B. (1997). The role of Thehsp90-based chaperone system in signal transduction by nuclear receptors and receptors signaling via map kinase. *Annual review of pharmacology and toxicology, 37*(1), 297-326.

Pratt, W. B. (1998). The hsp90-based chaperone system: involvement in signal transduction from a variety of hormone and growth factor receptors. *Proc Soc Exp Biol Med, 217*(4), 420-434. doi:10.3181/00379727-217-44252

Raheja, N., Choudhary, S., Grewal, S., Sharma, N., & Kumar, N. (2018). A review on semen extenders and additives used in cattle and buffalo bull semen preservation. *Journal of Entomology and Zoology Studies, 6*(3), 239-245.

Rastegarnia, A., Shahverdi, A., Rezaei Topraggaleh, T., Ebrahimi, B., & Shafipour, V. (2013). Effect of Different Thawing Rates on Post-Thaw Viability, Kinematic Parameters and Chromatin Structure of Buffalo (Bubalus bubalis) Spermatozoa. *Cell J, 14*(4), 306-313.

Rasul, Z., Ahmad, N., & Anzar, M. (2001). Changes in motion characteristics, plasma membrane integrity, and acrosome morphology during cryopreservation of buffalo spermatozoa. *J Androl, 22*(2), 278-283.

Rather, H. A., Islam, R., Malik, A. A., & Lone, F. A. (2016). Addition of antioxidants improves quality of ram spermatozoa during preservation at 4°C. *Small Ruminant Research, 141,* 24-28. doi:10.1016/j.smallrumres.2016.06.007

Reiter, R. J., Tan, D.-x., Manchester, L. C., & Qi, W. (2001). Biochemical reactivity of melatonin with reactive oxygen and nitrogen species. *Cell biochemistry and biophysics, 34*(2), 237-256.

Reiter, R. J., Tan, D. X., Osuna, C., & Gitto, E. (2000). Actions of melatonin in the reduction of oxidative stress. A review. *J Biomed Sci, 7*(6), 444-458. doi:10.1007/BF02253360

Robbins, R. K., Saacke, R. G., & Chandler, P. T. (1976). Influence of freeze rate, thaw rate and glycerol level on acrosomal retention and survival of bovine spermatozoa frozen in French straws. *J Anim Sci, 42*(1), 145-154. doi:10.2527/jas1976.421145x

Salamon, S., & Maxwell, W. M. (1995). Frozen storage of ram semen I. Processing, freezing, thawing and fertility after cervical insemination. *Animal Reproduction Science, 37*(3-4), 185-249.

Salamon, S., & Maxwell, W. M. (2000). Storage of ram semen. *Anim Reprod Sci, 62*(1-3), 77-111. doi:10.1016/s0378-4320(00)00155-x

Saleh, R. A., & Agarwal, A. (2002). Oxidative stress and male infertility: from research bench to clinical practice. *J Androl, 23*(6), 737-752.

Salmanoglu, D. S., Gurpinar, T., Vural, K., Ekerbicer, N., Dariverenli, E., & Var, A. (2016). Melatonin and L-carnitin improves endothelial disfunction and oxidative stress in Type 2 diabetic rats. *Redox Biol, 8*, 199-204. doi:10.1016/j.redox.2015.11.007

Santoro, M. G. (2000). Heat shock factors and the control of the stress response. *Biochem Pharmacol, 59*(1), 55-63. doi:10.1016/s0006-2952(99)00299-3

Shamsi, M. B., Venkatesh, S., Tanwar, M., Talwar, P., Sharma, R. K., Dhawan, A., . . . Dada, R. (2009). DNA integrity and semen quality in men with low seminal antioxidant levels. *Mutat Res, 665*(1-2), 29-36. doi:10.1016/j.mrfmmm.2009.02.017

Shi, H., Tan, D. X., Reiter, R. J., Ye, T., Yang, F., & Chan, Z. (2015). Melatonin induces class A1 heat-shock factors (HSFA 1s) and their possible involvement of thermotolerance in Arabidopsis. *Journal of Pineal Research, 58*(3), 335-342.

Shi, Y., Mosser, D. D., & Morimoto, R. I. (1998). Molecularchaperones as HSF1-specific transcriptional repressors. *Genes & development, 12*(5), 654-666.

Sikka, S. C., Rajasekaran, M., & Hellstrom, W. J. (1995). Role of oxidative stress and antioxidants in male infertility. *J Androl, 16*(6), 464-468.

Storey, B. T. (1997). Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. *Molecular Human Reproduction, 3*(3), 203-213.

Succu, S., Berlinguer, F., Pasciu, V., Satta, V., Leoni, G. G., & Naitana, S. (2011). Melatonin protects ram spermatozoa from cryopreservation injuries in a dose-dependent manner. *Journal of pineal research, 50*(3), 310-318.

Tan, D., Reiter, R. J., Manchester, L. C., Yan, M., El-Sawi, M., Sainz, R. M., . . . Hardeland, R. (2002). Chemical and physical properties and potential mechanisms: melatonin as a broad spectrum antioxidant and free radical scavenger. *Current topics in medicinal chemistry, 2*(2), 181-197.

Tan, D. X., Hardeland, R., Manchester, L. C., Paredes, S. D., Korkmaz, A., Sainz, R. M., . . . Reiter, R. J. (2010). The changing biological roles of melatonin during evolution: from an antioxidant to signals of darkness, sexual selection and fitness. *Biol Rev Camb Philos Soc, 85*(3), 607-623. doi:10.1111/j.1469-185X.2009.00118.x

Tan, D. X., Manchester, L. C., Reiter, R. J., Plummer, B. F., Hardies, L. J., Weintraub, S. T., & Shepherd, A. M. (1998). A Novel Melatonin Metabolite, Cyclic 3-Hydroxymelatonin: A Biomarker ofin VivoHydroxyl Radical Generation. *Biochemical and biophysical research communications, 253*(3), 614-620.

Tan, D. X., Manchester, L. C., Reiter, R. J., Qi, W., Hanes, M. A., & Farley, N. J. (1999). High physiological levels of melatonin in the bile of mammals. *Life Sci, 65*(23), 2523-2529. doi:10.1016/s0024-3205(99)00519-6

The Department of Livestock Development. (2004). Thailand: Department of Livestock Development.

Triwitayakorn, K., Moolmuang, B., Sraphet, S., Panyim, S., Na-Chiangmai, A., & Smith, D. R. (2006). Analysis of genetic diversity of the Thai swamp buffalo (Bubalus bubalis) using cattle microsatellite DNA markers. *Asian-Australasian Journal of Animal Sciences, 19*(5), 617-621. doi:DOI 10.5713/ajas.2006.617

Tuli, R. K., Singh, M., & Matharoo, J. S. (1981). Effect of different equilibration times and extenders on deep freezing of buffalo semen. *Theriogenology, 16*(1), 99-104. doi:10.1016/0093-691x(81)90118-7

Upreti, G., Jensen, K., Munday, R., Vishwanath, R., & Smith, J. (1994). *Studies on ram spermatozoal aromatic amino acid oxidase.* Paper presented at the Proc Aust Soc Reprod Biol.

Van Loon, A., Den Boer, P., Van der Schans, G., Mackenbach, P., Grootegoed, J., Baan, R., & Lohman, P. (1991). Immunochemical detection of DNA damage

induction and repair at different cellular stages of spermatogenesis of the hamster after in vitro or in vivo exposure to ionizing radiation. *Experimental cell research, 193*(2), 303-309.

Verhoven, B., Schlegel, R. A., & Williamson, P. (1995). Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes. *J Exp Med, 182*(5), 1597-1601. doi:10.1084/jem.182.5.1597

Volpe, S., Galeati, G., Bernardini, C., Tamanini, C., Mari, G., Zambelli, D., . . . Spinaci, M. (2008). Comparative immunolocalization of heat shock proteins (Hsp)-60, -70, -90 in boar, stallion, dog and cat spermatozoa. *Reprod Domest Anim, 43*(4), 385-392. doi:10.1111/j.1439-0531.2007.00918.x

Wang, P., Wang, Y. F., Wang, H., Wang, C. W., Zan, L. S., Hu, J. H., . . . Ma, G. J. (2014). HSP90 expression correlation with the freezing resistance of bull sperm. *Zygote, 22*(2), 239-245. doi:10.1017/S096719941300004X

Wang, X., Sharma, R. K., Sikka, S. C., Thomas, A. J., Falcone, T., & Agarwal, A. (2003). Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. *Fertility and Sterility, 80*(3), 531-535. doi:10.1016/S0015-0282(03)00756-8

Watson, P. (2000). The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci, 60*, 481-492.

Whitley, D., Goldberg, S. P., & Jordan, W. D. (1999). Heat shock proteins: a review of the molecular chaperones. *J Vasc Surg, 29*(4), 748-751. doi:10.1016/s0741-5214(99)70329-0

Woods, E. J., Benson, J. D., Agca, Y., & Critser, J. K. (2004). Fundamental cryobiology of reproductive cells and tissues. *Cryobiology, 48*(2), 146-156. doi:10.1016/j.cryobiol.2004.03.002

Yeste, M. (2016). Sperm cryopreservation update: Cryodamage, markers, and factors affecting the sperm freezability in pigs. *Theriogenology, 85*(1), 47-64. doi:10.1016/j.theriogenology.2015.09.047

Young, S. N., Gauthier, S., Kiely, M. E., Lal, S., & Brown, G. M. (1984). Effect of oral melatonin administration on melatonin, 5-hydroxyindoleacetic acid, indoleacetic acid, and cyclic nucleotides in human cerebrospinal fluid. *Neuroendocrinology, 39*(1), 87-92. doi:10.1159/000123961

Zhang, H., Squadrito, G. L., & Pryor, W. A. (1998). The reaction of melatonin with peroxynitrite: formation of melatonin radical cation and absence of stable nitrated products. *Biochem Biophys Res Commun, 251*(1), 83-87. doi:10.1006/bbrc.1998.9426

Zhang, X.-G., Hu, S., Han, C., Zhu, Q.-C., Yan, G.-J., & Hu, J.-H. (2015). Association of heat shock protein 90 with motility of post-thawed sperm in bulls. *Cryobiology, 70*(2), 164-169.

Zhang, X. G., Hong, J. Y., Yan, G. J., Wang, Y. F., Li, Q. W., & Hu, J. H. (2015). Association of heat shock protein 70 with motility of frozen-thawed sperm in bulls. *Czech Journal of Animal Science, 60*(6), 256-262. doi:10.17221/8239-Cjas

APPENDIX

APPENDIX

1. Preparation of Semen Extender

Following reagents in the given quantities were used for the preparation of 100 ml of semen extender

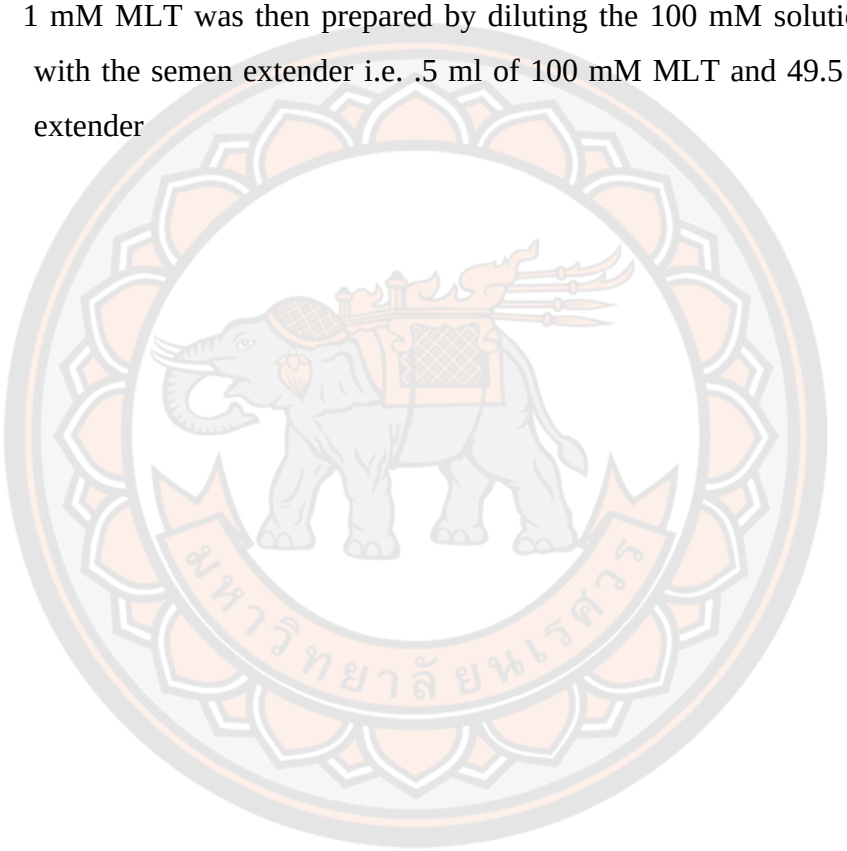
1. TRIS-HCl (VWR Life Science ®) : 2.42 gm
2. Citric acid monohydrate (HIMEDIA ®, Product code GRM 229): 1.34 gm
3. D-Fructose high purity (VWR Life Science ®) : 1.00 gm
4. Glycerin 87 % (VWR Life Science ®): 6.4 ml
5. Double distilled water: to make 80 ml
5. Egg yolk: 20 ml
6. Streptomycin sulphate (HIMEDIA ®): 0.1 gm
7. Penicillin, benzyl sodium salt (HIMEDIA ®): 100 µl

Procedures:

- TRIS-HCl, Citric acid, D-Fructose were weighted and put in a beaker.
- Glycerol was measured and added to the beaker
- DDW was added to the beaker to make the final volume of 80 ml.
- Magnet was put in the beaker and then the mixture was mixed with the help of the stirrer.
- To the beaker 20 ml of egg yok was added and stirred.
- The antibiotics were added to it and further stirred and mixed.

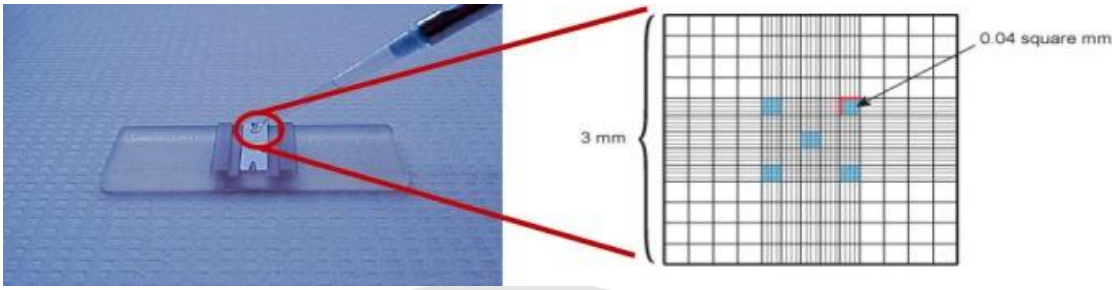
2. Preparation of 1mM melatonin solution

- 1000 mM melatonin (Sigma Aldrich ®, ≥ 98 %, product code, M5250) stock solution was prepared by adding 0.02 gm of MLT in 1.8 ml of dimethylsuphoxide (Acilabscan®, product code, AR1054)
- The mixture was vortexed and mixed and then spindled for a while
- 100 mM MLT solution was prepared by diluting 10 times with DDW by mixing 100 µl of 1000 mM MLT and 900 µl of DDW
- 1 mM MLT was then prepared by diluting the 100 mM solution 100 times with the semen extender i.e. .5 ml of 100 mM MLT and 49.5 ml of semen extender



3. Determining the concentration of sperm by haemocytometer

- Sperm concentration was calculated with the help of haemocytometer



- As shown in the figure above,
- Semen was diluted 101 times with water so that the sperms are killed and diluted properly for easy counting
- A coverslip was placed on the haemocytometer after wetting supports (rails) with water. This helps to hold down the coverslip while loading the sperm.
- 10-15 µl of diluted semen was placed under the cover slip on each side and allowed to settle in the pre wetted chamber
- The haemocytometer was placed under the microscope and observed at 40 X objective
- The sperms in the 5 squares (as shown by blue color) were counted on each side and average was taken
- The sperms falling on the left and top side of the square were also include in the counting where as those on the below and right were excluded
- The hemocytometer is 0.1 mm deep and the 25 large squares represent an area of 1 square mm. The volume above the 25 squares shown is 0.1 µl. The sperms counted in 5 squares settle out of 0.02 µl (0.1/5=0.02). Therefore, we must multiply our count in 5 squares by 50,000 in order to determine how many sperm would have been in 1.0 ml (1000/0.02=50,000). To get the concentration of the original sperm sample we must however also multiply by the dilution factor.
- **The number of sperms per ml**=

$$\textbf{dilution factor x count in 5 squares x .5 x } 10^{6}$$

4. RNA Concentration

S.N.	Sample No.	Animal No.	Concentration (ng/ul)	A260/280	A260/A230
1	N1C1	Bull 1	186.52	1.63	2.39
2	N1T1	Bull 1	302.84	1.71	2.5
3	N2C1	Bull 2	182.83	1.7	2.22
4	N2T1	Bull 2	267.81	1.71	2.35
5	N3C1	Bull 3	596.99	1.75	2.45
6	N3T1	Bull 3	1062.17	1.74	2.31
7	N4C1	Bull 4	241.78	1.62	1.19
8	N4T1	Bull 4	342.17	1.72	1.52
9	N1C2	Bull 1	648.10	1.76	2.21
10	N1T2	Bull 1	246.20	1.71	1.83
11	N2C2	Bull 2	314.86	1.7	1.88
12	N2T2	Bull 2	320.61	1.7	0.99
13	N3C2	Bull 3	1011.28	1.74	1.79
14	N3T2	Bull 3	513.52	1.74	1.13
15	N4C2	Bull 4	452.68	1.71	0.86
16	N4T2	Bull 4	195.77	1.55	0.27
17	N1C3	Bull 1	440.34	1.69	1.02
18	N1T3	Bull 1	485.94	1.74	1.98
19	N2C3	Bull 2	161.55	1.65	2.25
20	N2T3	Bull 2	333.44	1.7	2.54
21	N3C3	Bull 3	978.93	1.75	2.21
22	N3T3	Bull 3	375.58	1.72	2.41
23	N4C3	Bull 4	199.23	1.64	2.36
24	N4T3	Bull 4	198.49	1.68	1.98

5. Preparation of 1.5 % agarose gel in 1 X TAE buffer

- 1.5 gm agarose (Vivantis®, Catalogue No. PC0701) was weighed and mixed with 100 ml of 1 X TAE buffer (HIMEDIA ®, Product code, ML016) in the microwavable conical flask
- Microwave for 1-3 minutes until the agarose is dissolved completely
- Cool the agar until 50°C until we can comfortably keep our hand on the flask
- Mix 5 µl of RedSafe™ Nucleic Acid Staining Solution 20000 × (iNtRon®, Catalogue No. 21141) for 100 ml of 1.5 % agarose gel
- Now pour the agarose gel on the gel caste, fix the comb on the gel and leave to solidify at least for 20 minutes

6. Loading samples and running the agarose gel

- 6 X glycerol gel loading buffer is added to each of the cDNA samples in the ratio of 1:5
- When the agarose gel is solidified, it is kept in the electrophoresis unit
- The gel box is filled with 1 X TAE Buffer until the gel is covered
- 100 bp DNA ladder (Solis Biodyne®) is places on the first lane and on the other wells the samples are carefully loaded
- The gel is run at 100 V for 45 minutes to 1 hour until the dye line is 75-80 % way down the gel
- Power is switched off and the gel is carefully removed from the gel box and visualized under UV light source