DISSERTATION TITLE

ASSESSMENT OF FACTORS AFFECTING THE MOTILITY AND VIABILITY OF FROZEN-THAWED BULL SPERMATOZOA

September, 2023

NGUYEN THANH HAI

Graduate School of Environmental and Life Science

(Doctor's Course)

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GENERAL ABSTRACT

Artificial insemination using frozen-thawed bull semen has been the most widely used and has had the greatest impact on the genetic improvement of both dairy and beef cattle, as compared to other domestic animal species. However, the conception rate after artificial insemination in developed countries including Japan has been on a steady decline. Rapid genetic improvement of cattle could be thought to have caused this decline, it is considered necessary to develop advanced feeding management techniques and highly accurate estrus detection technologies suited to high-performance cattle. Furthermore, since no major improvements have been attempted in the process of freezing and thawing semen for a long time, reconsidering techniques during the freezing and thawing process and examination of factors considered necessary for selecting bull sperm suitable for freezing and thawing would greatly contribute to improving the conception rate of frozen-thawed bovine spermatozoa. Good quality parameters, especially motility, of frozen bull semen after thawing are much important components to improve the success rate following artificial insemination, since the motility parameters are positively correlated with bull fertility. Although there are a variety of potential factors affecting bull semen frozen in straws during the freezing-thawing process, it may be valuable to reconsider if there is any space to be reanalyzed the effect of the thawing process on the quality of frozen-thawed bull semen to overcome inevitably existing disadvantages under conventional thawing methods and further improve the motility and other vital parameters of post-thawed spermatozoa. Furthermore, mitochondria of spermatozoa, especially mitochondrial content and mtDNA copy number, are universally known as the principal multitasking organelles for energy production and other numerous key physiological functions, thus significantly associating with quality characteristics of frozen-thawed bull spermatozoa. Therefore, the general main research purpose was to evaluate the motility and other various important parameters of frozen-thawed bull spermatozoa under various conditions and their correlations to find the possible potential approaches to further improve the motility and other important quality parameters of frozen bull semen after thawing, including specific objectives in two following separated studies in this research.

Firstly, up to now, the definitive conclusion of the positive effects of rapid transient thawing at higher temperatures for shorter durations has not been obtained yet and is still under discussion due to some contradictory findings and limited assessment of post-

thawed parameters. The aim of the study was to evaluate the effectiveness of rapid thawing in water at 70°C by using various post-thawed parameters of frozen bull spermatozoa. Results showed that the time it took for the temperature inside the straw to warm up to 15°C was nearly twice as faster when the straw was thawed in 70°C water compared with 37 or 39°C. Viability, motility, and mitochondrial membrane potential (MMP) of spermatozoa thawed at 70°C for 8 seconds and stabilized at 39°C for 52 seconds (RT70) were significantly higher than controls thawed and stabilized at 39°C for 60 seconds (CT39) or thawed at 37°C for 46 seconds and stabilized at 39°C for 14 seconds (CT37). Just after thawing, however, there were no differences in acrosome integrity and phospholipase C zeta 1 distribution, whereas mitochondrial reactive oxygen species production in all cells was significantly lower in spermatozoa thawed at RT70. Although there were differences among bulls, viability, motility, and MMP of spermatozoa thawed at RT70 were also significantly higher than those of CT39 at 0 and 3 h after thawing. From these results, it is concluded that rapid thawing at 70°C for 8 seconds and then stabilization at 39°C for 52 seconds significantly improves viability, motility, and mitochondrial health of bull spermatozoa rather than conventional thawing at 37 or 39°C, whereas no significant differences between two conventional thawing procedures. Thus, this rapid thawing method is recommended to make significant differences in the viability, motility, and MMP of frozen bull spermatozoa. The beneficial effect of rapid transient thawing could be due to shorter exposure to temperatures outside the physiological range, and consequently less damage of the biological membrane associated with mitochondrial health.

Secondly, mtDNA copy number and mitochondrial content are well known to be significantly related to semen quality, but their features are likely to be altered by many potential factors due to the different findings from various recent studies and species. The aim of the study was to investigate the relationship between mitochondrial content of commercial frozen-thawed bull spermatozoa and conventional quality parameters. Firstly, mitochondrial DNA copy number per spermatozoon (MDCN), mitochondrial content (MC), the percentage of spermatozoa with high mitochondrial membrane potential (HMMP), intracellular reactive oxygen species (ROS) and motility parameters of frozen-thawed spermatozoa from five bulls were determined by using qPCR, flow cytometry and CASA, respectively, and analyzed the relationships. Results showed that all parameters examined, including MDCN, MC, HMMP, ROS, and motility indicators,

significantly differed among frozen spermatozoa from different bulls. Both MDCN and MC were negatively correlated with HMMP and motility indicators, but positively with ROS, of course, whereas there was a highly positive relationship between MDCN and MC. Secondly, when MDCN and MC were examined in frozen spermatozoa prepared at different points in the lives of four bulls, those did not correlate overall throughout their lives (1.3-14.3 years old), but did correlate significantly in two sires. From these results, it is concluded that MDCN and MC of commercial frozen-thawed spermatozoa differ among sire bulls, and have significantly negative correlations with HMMP and a majority of sperm motility parameters, probably due to significantly positive correlations with mitochondrial oxidative stress resulted in the presence of ROS, demonstrating that these appear to be useful markers to assess sires' spermatozoa. It should be noted that the MDCN and MC of commercially available frozen-thawed bull spermatozoa do not vary overall with the age of the sire from which the semen was collected, whereas they change with age in some individuals and may also affect sperm motility. Furthermore, the current simple method of measuring sperm MC by fluorescence intensity under flow cytometry is a sufficiently reliable sperm evaluation method, since there is a significant correlation with MDCN.

Overall, the results of the first series of the current studies demonstrate that there are rooms to further improve the viability and motility of frozen bull spermatozoa by controlling temperature during the thawing process, especially by allowing it to pass as quickly as possible through areas outside the physiological temperature range. In particular, this study showed that thawing temperature also affected sperm mitochondrial membrane potential and the motility. However, further research will be needed to further clarify the details of the effects of thawing rate and exposure to non-physiological temperatures on sperm mitochondria. In addition, since the results of the second series of studies showed that a positive correlation existed between the amount of sperm mitochondrial DNA/mitochondrial content and the amount of ROS, which in turn negatively affects the motility, the simple measurement protocols used in this study. The current study proposes that mitochondrial content will be a useful information in evaluating the reproductive activity of sire bulls. On the other hand, since this study used commercially available frozen bull semen prepared from ejaculated spermatozoa for artificial insemination, it was not possible to use semen collected at a defined time during the life span of the sire bulls, and more systematic research on how much sperm

mitochondrial DNA content changes during the life span of the sire bulls. More systematic research may be required to determine how much sperm mitochondrial DNA levels change during the life of a bull. Anyhow, the results of studies in this dissertation indicate that it is still possible to provide more motile frozen-thawed spermatozoa through further improvement of thawing conditions of frozen sperm and selection of bulls using mitochondrial content as an indicator, and that fertility after artificial insemination can still be improved through modifying sperm thawing process and the bull selection conditions.

DECLARATION

This dissertation contains no materials which have been accepted for the award of any other degree or diploma in any other tertiary institution and to the best of my knowledge and belief, contains no materials previously published or written by another person, except for the references that have been included in this text.

Date

06th June, 2023

Signature

Nguyen Thanh Hai

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PUBLICATIONS ARISING FROM THIS DISSERTATION

1. **Rapid thawing of frozen bull spermatozoa by transient exposure to 70 °C improves the viability, motility and mitochondrial health**.

   NGUYEN Thanh Hai, DO Quang Son, ATHURUPANA Rukmali, WAKAI Takuya and FUNAHASHI Hiroaki.

   Animal Reproduction (Accepted on 13th July, 2023).

2. **Negative correlations of mitochondrial DNA copy number in commercial frozen bull spermatozoa with the motility parameters after thawing**.

   NGUYEN Thanh Hai, DO Quang Son, KOBAYASHI Hiroshi, WAKAI Takuya and FUNAHASHI Hiroaki.

   Theriogenology (Accepted on 22nd July, 2023).

CONFERENCE PROCEEDINGS

1. **Rapid thawing by transient exposure to 70°C water improves the viability and motility, but not acrosome integrity and PLC zeta-1 distribution of frozen bull spermatozoa**.

   NGUYEN Thanh Hai, DO Quang Son, ATHURUPANA Rukmali, WAKAI Takuya and FUNAHASHI Hiroaki.

   International Symposium on Animal Bioscience 2021 (ISAB), Oral presentation, 3rd November 2021, Okayama, Japan. Proceedings of the International Symposium on Animal Bioscience 2021 (ISAB), ISSN: 2186-7755, Volume111 Supplement[53]: PB-13 (page 39). https://ousar.lib.okayama-u.ac.jp/en/63175

2. **Migration of PLCZ1 distribution in frozen-thawed bull spermatozoa occurs during acrosomal exocytosis, but its translocation is not influenced by thawing protocols**.

   NGUYEN Thanh Hai, DO Quang Son, Fonseka LAKSHITHA, WAKAI Takuya and FUNAHASHI Hiroaki.

   Kansai Society of Animal Science Meeting 2022. Oral presentation, 16th October, 2022, Okayama University, Japan. Page 21 in book of abstracts. Proceeding of Kansai Society of Animal Science Meeting 2022, pp. 21, oral presentation on 16th October, 2022 at Okayama University, Japan.

3. **Redistribution of PLCZ1 in frozen bull spermatozoa appears through acrosomal exocytosis, but its dynamic is not affected by thawing methods**.

   NGUYEN Thanh Hai, DO Quang Son, Fonseka LAKSHITHA, WAKAI Takuya and FUNAHASHI Hiroaki.

   The International Conference on Sustainable Agriculture and Environment 2022 (SAE), oral presentation, from 17th to 19th November 2022 at Nong Lam University, Ho Chi Minh City, Vietnam. Book of abstracts of the 4th International Conference on Sustainable Agriculture and Environment, session 7, O-702, page 69.

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LIST OF ABBREVIATIONS

AI	artificial insemination
ALH	amplitude of lateral head displacement
ANOVA	analysis of variance
ATP	adenosine triphosphate
BCF	beat cross frequency
BSA	bovine serum albumin
CASA	a computer-assisted sperm analysis system
COX1	mtDNA-encoded OXPHOS protein
CT37	thawed at 37°C for 46 seconds and stabilized at 39°C for 14 seconds
CT39	thawed and stabilized at 39°C for 60 seconds
DAG	diacylglycerol
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
ER	endoplasmic reticulum
FITC-PNA	fluorescein isothiocyanate-conjugated peanut agglutinin
FITC-PSA	fluorescein-conjugated pisum sativum agglutinin
FC	flow cytometry
h	hour(s)
H_2O_2	hydrogen peroxide
ICC	indirect immunocytochemistry
IP_3	inositol 1,4,5-trisphosphate
JC-1	5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl-carbocyanine iodide
MC	mitochondrial content
MDCN	mtDNA copy number
MFI	mean fluorescence intensity
min	minute(s)
MMP	mitochondrial membrane potential

mtDNA	mitochondrial deoxyribonucleic acid
MTG	MitoTracker Green FM
OXPHOS	oxidative phosphorylation
PBS	phosphate-buffered saline solution
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator-1 alpha
PI	propidium iodide
PIP_2	phosphatidylinositol 4,5-bisphosphate
PLCZ1	phospholipase C zeta1
PM	progressive motility
qPCR	a quantitative real-time PCR (polymerase chain reaction)
r	correlation coefficient
ROS	reactive oxygen species
RPM	rapid progressive motility
RT70	thawed at 70°C for 8 seconds and stabilized at 39°C for 52 seconds
sec	second(s)
SEM	standard error of the mean
TFAM	transcription of mitochondrial transcript factor A
TL-HEPES-PVA	Tyrode lactate-HEPES-polyvinyl alcohol buffered medium
TM	total motility
VAP	velocity of average path
VCL	velocity of curved line
VSL	velocity of straight line
WHO	Organization World Health
°C	degree(s) Celsius
$[Ca^{2+}]i$	intracellular cytosolic free calcium

CHAPTER 1

GENERAL INTRODUCTION

1.1 Preface

Artificial insemination (AI) using frozen-thawed bull semen has been the most widely used, and has had many advantages (Lima et al., 2010; Mohammed, 2018; Mengistu, 2019) and the greatest impact on the genetic improvement for more productive and profitable (Vishwanath, 2003; Baruselli et al., 2018; Mohammed, 2018), as compared to other domestic animal species. However, conception and pregnancy rates following AI with frozen-thawed bull semen in developed countries including Japan have been on a steady decline (López-Gatius, 2003; Royal et al., 2008; Dochi et al., 2010; Göncü and Koluman, 2019) and are still the main concerns in cattle industry (Vartia et al., 2017). Rapid genetic improvement of cattle could be thought to have caused this decline, it is considered necessary to develop advanced feeding management techniques (Bisinotto et al., 2012; Hasan et al., 2021) and highly accurate estrus detection technologies (Bombardelli et al., 2016; Wang et al., 2020b; Ali et al., 2022; Furukawa et al., 2022; Santos et al., 2022) suited to high-yielding cattle. Furthermore, since no major improvements have been attempted in the process of freezing and thawing semen for a long time, reconsidering techniques during the freezing and thawing process and examination of factors considered necessary for selecting bull sperm suitable for freezing and thawing would greatly contribute to improving the conception rate of frozen-thawed bovine sperm.

Although the concerns of the low conception and pregnancy rates can originate from many factors and aspects (Kebede, 2018), it is necessary to have higher quality parameters, especially motility, of frozen bull semen after thawing to ensure the success rate following AI. It has been universally recognized that better motility of post-thawed bull spermatozoa is the most popular and important feature for higher semen quality, subsequent higher conception and pregnancy rates after AI, since sperm motility parameters are found to have positive correlations with bull fertility (Kathiravan et al., 2008; Nagy et al., 2015; Sellem et al., 2015; Kumaresan et al., 2017; Mapel et al., 2022). There may be many potential factors affecting bull semen frozen in straws during the freezing-thawing process, but it may be valuable to reconsider if there is any space to be reanalyzed the effect of the thawing procedure on the quality of frozen-thawed bull semen to overcome existing disadvantages

under conventional thawing methods and further improve the motility and other vital quality characteristics of post-thawed spermatozoa. Thawing process of frozen semen brings spermatozoa from the inactively frozen status to the physiological temperature reactivating metabolism (Athurupana et al., 2015a), inevitably resulting in adverse effects on the sperm motility and viability (Correa et al., 1996; Lyashenko, 2015; Gürler et al., 2016). Currently, it has been demonstrated that very slow thawing ways of spermatozoa seem to render recrystallization and consequently induce injury to the organelles (Hammerstedt et al., 1990) and thawing at higher temperatures can improve the quality (motility and viability) of post-thawed spermatozoa of sheep (Paulenz et al., 2004; Nicolae et al., 2014), horse (Snoeck et al., 2012), pig (Córdova-Izquierdo et al., 2006; Tomás et al., 2014; Athurupana et al., 2015a), buffalo (Dhami et al., 1996; Rastegarnia et al., 2013) and cattle (Nur et al., 2003; Lyashenko, 2015); but there have still been some contradictory results (Yilmaz et al., 2019). Therefore, further comprehensive investigations to reanalyze the effect of the thawing process on more crucial parameters associated with the quality of frozen-thawed bull semen are needed for the final conclusion.

Furthermore, mitochondria of mammalian spermatozoa, recognized as necessary multitasking organelles for energy production besides other principal physiological functions (Mukai and Travis, 2012), play a central role in semen quality and potential sperm fertilizability by energy synthesis in adenosine triphosphate (ATP) form for sperm motility and other key pathways (Boguenet et al., 2021; Bulkeley et al., 2021; Madeja et al., 2021). It is located in sperm midpiece with its genome in the form of mitochondrial DNA (mtDNA) from some specific encoded genes (Hirata et al., 2002) and each mitochondrion carries, on average, from 0 to 11 copies of mtDNA (May-Panloup et al., 2003; Song and Lewis, 2008; Benkhalifa et al., 2014; Boguenet et al., 2022). Although there have been a large number of various studies in human (Díez-Sánchez et al., 2003; May-Panloup et al., 2003; Kao et al., 2004; Amaral et al., 2007; Song and Lewis, 2008; Gabriel et al., 2012; Tian et al., 2014; Zhang et al., 2016; Wu et al., 2019a; Faja et al., 2019; Wu et al., 2019b; Boguenet et al., 2022), mouse (Luo et al., 2013), boar (Guo et al., 2017), stallion (Orsztynowicz et al., 2016; Darr et al., 2017) and bull spermatozoa (Madeja et al., 2021) focused on the relationships of mitochondrial content and mtDNA copy number with semen quality and fertilizing capability, due to several contrary results among studies and species, the eventual conclusion has not been attained yet and is still under discussion. In fact, the majority of previous research results in mammalian spermatozoa mentioned above have reported the inverse

correlations of mtDNA copy number and mitochondrial content with the conventional quality of semen, but contrasted with one in human (Kao et al., 2004). Interestingly, less semen quality from decreased mitochondrial membrane potential, reduced mtDNA integrity and increased mtDNA copy number is significantly associated with elevated ROS production of human spermatozoa (Bonanno et al., 2016).

Therefore, the general research goal was to assess the motility and other numerous vital parameters of frozen bull spermatozoa and their relationships under different conditions to find out the possible potential approaches to enhance the current low motility and other crucial quality parameters of frozen bull semen after thawing.

1.2 Literature review

1.2.1 Cryopreservation

Cryopreservation is the process to use very low temperatures to preserve structurally intact living cells, tissues and organelles (Jang et al., 2017). In spermatozoa, it is a sequential process of reduction in temperature, dehydration of the cell, freezing in subzero temperatures and storage as frozen straws for long-term preservation in liquid nitrogen by which stop the biological activity and metabolism (Ugur et al., 2019; Upadhyay et al., 2021). Unlike other cells, spermatozoa own a low water content and high membrane fluidity, so they should be less susceptible to cryodamage or oxidative stress (Ugur et al., 2019). In spite of this, the freeze-thawing process still triggers injury to subcellular organelles associated with the integrity of spermatozoa because of the negative alterations to the membrane structure functions and metabolic pathways (Hammerstedt et al., 1990). It has also been reported that the freezing-thawing procedures could induce the substantial remodeling of proteomes involved in key structural and functional roles for fertilization capability of mammalian spermatozoa (Westfalewicz et al., 2015; Pini et al., 2018; Perez-Patiño et al., 2019; Peris-Frau et al., 2019), due to multifactorial causes (Chen et al., 2014; Wang et al., 2014; Bogle et al., 2016; Perez-Patiño et al., 2019) at every stage of cryopreservation process (Bogle et al., 2016) for triggering the biological changes (Peris-Frau et al., 2019). Indeed, changes during the freezing-thawing process can deleteriously alter plasma membrane, acrosomal integrity, mitochondria, intracellular reactive oxygen species (ROS) and DNA status, consequently cause lower motility, kinematics and viability of post-thawed spermatozoa (Chatterjee et al., 2001; Dziekońska et al., 2009; Kim et al., 2011; Miranda et al., 2017). By

cooling spermatozoa, it can successfully lower the risk of adverse effects related to cryopreservation in order to effectively lower sperm metabolic rates and extend its cryosurvival (Upadhyay et al., 2021). Sperm are susceptible to detrimental effects during freezing, including the formation of ice crystals, hyper-osmolarity, volume alterations and protein denaturations (Jang et al., 2017). In order for spermatozoa to retain their fertility for many years, semen is widely frozen at -196°C in liquid nitrogen (Kumar et al., 2019).

Cryoprotectants are used to reducing the rate of dehydration and alleviate the formation of ice crystals during freezing (Oldenhof et al., 2010). Until now, there are a wide variety of cryoprotectants and extenders used in frozen semen straws such as glycerol, ethylene glycol, dimethyl sulfoxide, egg yolk, trehalose, citrate, skim milk, coconut milk, etc (Ugur et al., 2019). The cryopreservation of bull semen for AI has been demonstrated with many advantages (Lima et al., 2010; Mohammed, 2018; Mengistu, 2019), and has significantly contributed to the genetic improvement and efficient reproduction for decades in the dairy and beef production industries (Vishwanath, 2003; Baruselli et al., 2018; Mohammed, 2018), but poor conception and pregnancy rates following AI with frozen-thawed bull semen (López-Gatius, 2003; Royal et al., 2008) has still been reported as big concerns (Upadhyay et al., 2021).

1.2.2 Thawing process

Contrary to the freezing process, thawing of frozen semen has been extensively used to bring spermatozoa from the inactively frozen status to the physiological temperature reactivating metabolism (Hammerstedt et al., 1990; Athurupana et al., 2015a), inevitably resulting in adverse effects on sperm cells (Hammerstedt et al., 1990; Correa et al., 1996; Meyers, 2005; Lyashenko, 2015; Gürler et al., 2016). In fact, it is remarkably concerned that these changes during the thawing process could harmfully change the integrity of the biological membrane of spermatozoa, and consequently may reduce the motility and viability (Senger, 1980; Hammerstedt et al., 1990; Chatterjee et al., 2001). In general, lower quality of frozen bull semen after thawing probably comes from decreased important parameters, such as motility (Stradaioli et al., 2007; Harayama et al., 2010; Gangwar et al., 2018), viability (Anzar et al., 2002; Stradaioli et al., 2007; Gangwar et al., 2018), acrosome integrity (Harayama et al., 2010; Shah et al., 2017; Gangwar et al., 2018) or DNA integrity (Anzar et al., 2002; Shah et al., 2017), as compared to the fresh semen.

For decades, the thawing temperatures between 37 and 40°C for from 15 to 60 seconds have been the popular applications for frozen semen straws (Pace et al., 1981; Rasul et al., 2000; Sariözkan et al., 2009; Contri et al., 2010; Lymberopoulos and Khalifa, 2010; Faezah et al., 2012; Athurupana et al., 2015a). In recent years, a thawing method with different temperatures and osmotic pressures has been interested to overcome subfertility in cattle due to low post-thawed spermatic quality parameters. In reality, there were further improvements in frozen-thawed sperm motility and viability under rapid thawing methods at higher temperatures (60 - 80°C) for cat (Chatdarong et al., 2010), dog (Peña and Linde-Forsberg, 2000; Kim et al., 2011), sheep (Paulenz et al., 2004; Nicolae et al., 2014), horse (Snoeck et al., 2012), pig (Eriksson and Rodriguez-Martinez, 2000; Córdova-Izquierdo et al., 2006; Tomás et al., 2014) and cattle (Senger, 1980; Lyashenko, 2015) due to the detrimental effect reduction of recrystallization and rehydration at the high thawing speeds (Fiser and Fairfull, 1990; Fiser et al., 1993; Athurupana et al., 2015a). However, some other authors have still reported no positive effects with post-thawed parameters of spermatozoa thawed at rapid thawing methods as compared with thawing at around physiological temperatures (Yilmaz et al., 2019). Therefore, the final definitive conclusion with rapid thawings has not been obtained yet and is still needed for more detailed investigations and discussions.

1.2.3 Fertility of cattle

1.2.3.1 Fertility ability

The AI technique for transferring semen collected from a male animal into the female genital tract in the 1950s (Vishwanath, 2003) to finally get the fertilized egg cell is widely used in modern animal reproduction (Pagl et al., 2006). It has been demonstrated with many advantages in comparison with the direct mating service by males (Lima et al., 2010; Lamb and Mercadante, 2016), resulting in higher genetic progress with more productive and profitable (Rodgers et al., 2012; Baruselli et al., 2017). Reproductive efficiency has a substantial economic effect on cattle husbandry (Anchordoquy et al., 2017) with approximately five times more important in production practice than milk yield or growth performance (Trenkle and Willham, 1977).

However, AI after the cryopreservation of bull semen has significantly contributed to genetic improvement and efficient reproduction for decades in cattle production industries, but a poor fertilizability of frozen-thawed spermatozoa has still been reported and seems to

be the big problem (Upadhyay et al., 2021) in dairy and beef husbandry. At present, the low conception and pregnancy rates in cattle following AI with frozen-thawed bull semen (López-Gatius, 2003; Royal et al., 2008) originated from many problems and factors (Kebede, 2018) have still been occurred over the world (Anchordoquy et al., 2017; Vartia et al., 2017), currently low-quality parameters, especially motility, of frozen bull semen after thawing are one of the most important components needed to be improved. Indeed, the post-thawed quality parameters, such as motility or viability, are very important for bull fertility, since they are positively correlated with fertilization capacity (Hamano et al., 2001; Kathiravan et al., 2008; Sellem et al., 2015; Kumaresan et al., 2017). In addition to the motility and viability, acrosome integrity (Oliveira et al., 2014; Bernecic et al., 2021), high mitochondrial membrane potential (Marchetti et al., 2002, 2012; Espinoza et al., 2009; Agnihotri et al., 2016) and intracellular ROS level (Bahmyari et al., 2020) are the crucial characteristics, and also significantly correlated with not only the motility and viability but also fertilizability of spermatozoa. Furthermore, some studies reported that the expression of crucial proteins of spermatozoa could be changed in post-thawed spermatozoa, compared to fresh semen (Rezaie et al., 2021), since membrane lipids may be damaged, resulting in the membrane instability and reduction of many proteins, which are necessary for sperm quality and bull fertility (Hezavehei et al., 2021). Besides, several new parameters, such as mtDNA copy number and mitochondrial content are being investigated as potential indicators to assess spermatozoa in different mammals (Darr et al., 2017; Guo et al., 2017; Madeja et al., 2021; Boguenet et al., 2022; Shi et al., 2022).

1.2.3.2 Some conventional post-thawed parameters for semen quality

It has been well demonstrated that a significant positive correlation was found between post-thawed sperm characteristics and male fertility (Correa et al., 1996; Kim et al., 2011; Dogan et al., 2015; Sellem et al., 2015; Kumaresan et al., 2017). To date, there have been a wide variety of post-thaw sperm parameters used for assessing the frozen-thawed quality of mammalian spermatozoa in general and of bull semen in particular, such as motility, kinematics, viability, morphology, acrosomal status, mitochondrial activity, oxidative stress, DNA status and others (Kjrestad et al., 1993; Januškauskas and Žilinskas, 2002; Ahmed et al., 2016; Caamaño et al., 2021).

Motility: Sperm motility and dynamic parameters are one of the most popular crucial indicators that are measured when analyzing sperm quality parameters for the fertilizing

ability of spermatozoa (Nagy et al., 2015; Chakraborty and Saha, 2022). It refers to sperm movement ability to swim and is an indispensable activity for successful sperm transport in the female reproductive tract. Indeed, numerous previous studies have indicated that sperm motility parameters are found to have significant positive associations with other important semen quality characteristics (Stradaioli et al., 2007; Harayama et al., 2010; Gangwar et al., 2018) and bull fertility (Kathiravan et al., 2008; Nagy et al., 2015; Sellem et al., 2015; Kumaresan et al., 2017; Mapel et al., 2022). Therefore, sperm motility and dynamic parameters are undoubtedly essential for semen quality and bull fertilization ability. According to the WHO criteria (Organization World Health, 2010) of standard parameters for semen, sperm motility is classified into 4 main types: type A (rapid progressive motility), type B (slow progressive motility), type C (non-progressive motility), and type D (immotility). Percentage of motile spermatozoa is the total of types A, B and C (often called the total motility), and the proportion of progressive motile spermatozoa is the total of types A and B (often termed the progressive motility) (Qiu et al., 2016). Besides, it is often set up in a computer-assisted sperm analysis system (CASA) to measure the dynamic parameters related as follows: curvilinear velocity (μm/s) with 10 < slow < 40 < medium < 75 < rapid; average path velocity (μm/s) with 10 < slow < 40 < medium < 75 < rapid (Qiu et al., 2016). The common kinematics measured by CASA include velocity straight line (VSL, μm/s), velocity curved line (VCL, μm/s), average path velocity (VAP, μm/s), linearity (%, ratio between VSL and VCL), straightness of trajectory (%, ratio between VSL and VAP), wobble coefficient (%, ratio between VAP and VCL), amplitude of lateral head displacement (ALH, μm) and beat cross frequency (BCF, Hz) (Tomás et al., 2014; Kourouma et al., 2015).

Viability (survival rate): The ratio of live subpopulation in total spermatozoa. Over the last several decades, it has widely been recognized that a loss of transport function and physical integrity of the plasma membrane is one of the main characteristics of dead cells (Januškauskas and Žilinskas, 2002). Indeed, when spermatozoa have an intact plasma membrane, they are considered as viable (Amanda, 2014) because the permeability of the plasma membrane is the main index to evaluate the viability of spermatozoa. Previous investigations have indicated that the viability of post-thawed bull semen is significantly correlated with sperm motility and bull fertility (Hamano et al., 2001; Kathiravan et al., 2008; Kumaresan et al., 2017). At present, there are various available commercial kits for determination of the mammalian viability such as SYBR and propidium iodide (PI) (Athurupana et al., 2015a; Gonzalez-Castro et al., 2019), Hoechst and PI (Cai et al.,

2005), M540 and YOPRO1 (Bergstein-Galan et al., 2018), eosin-nigrosin (Samplaski et al., 2015) and others by fluorescence microscopy (Athurupana et al., 2015a; b) or flow cytometry (Gonzalez-Castro et al., 2019; Šterbenc et al., 2019; Llavanera et al., 2020). It has been demonstrated that the viability of spermatozoa is strongly correlated with DNA fragmentation and male fertility (Samplaski et al., 2015; Kumaresan et al., 2017). Interestingly, the post-thawed viability of frozen spermatozoa could be improved by rapid thawing methods in mammalian species (Ahmad, 1984; Snoeck et al., 2012; Tomás et al., 2014; Lyashenko, 2015).

Morphology: It is one important element related to the shape, appearance and size of spermatozoa that regularly examines as part of a semen analysis to evaluate male fertility. In cattle, the assessment of sperm morphology is based on the relation between the incidence of abnormal spermatozoa and types of particular morphological defects with fertility (Söderquist et al., 1991). There was a strong correlation between the percentage of morphologically normal bull sperm with conception rate and fertility in both beef (Holroyd et al., 2002; Perry, 2021) and dairy herds (Al-Makhzoomi et al., 2008; Nagy et al., 2013; Attia et al., 2016).

Acrosome integrity (intactness of the acrosome membrane): It is one of the principal determinant drivers of mammalian sperm quality for normal fertilization success. Acrosome of spermatozoa must be intact before capacitation and acrosome reaction. It often needs to undergo capacitation and acrosome reaction to release acrosomal content before binding and penetrating the zona pellucida of oocytes (Morales et al., 1989; Reckova et al., 2015). It has been demonstrated that sperm acrosome is really sensitive to cryopreservation and positive strategies need to be applied to minimize cryodamage during the freeze-thawing process (Esteves and Verza, 2011). Several previous studies reported that acrosomal status is positively associated with the motility, viability, and subsequent fertilizability of spermatozoa (Oliveira et al., 2014; Kumaresan et al., 2017; Bernecic et al., 2021). Acrosome status of spermatozoa is often evaluated by specific lectins such as fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) (Funahashi, 2002; Nagy et al., 2003; Fàbrega et al., 2012; Águila et al., 2015; Athurupana and Funahashi, 2016; Gürler et al., 2016; Ogata et al., 2022), fluorescein-conjugated pisum sativum agglutinin (FITC-PSA) (Cross and Watson, 1994; De Andrade et al., 2007; Wolkowicz et al., 2008; Celeghini et al., 2010; Lee et al., 2014) and others (Robles-Gómez et al., 2021) by flow cytometry (Nagy et

al., 2003; Águila et al., 2015; Gürler et al., 2016; Byrne et al., 2017) or fluorescence microscopy (Funahashi, 2002; Siciliano et al., 2008; Hossain et al., 2011; Athurupana and Funahashi, 2016; Ogata et al., 2022).

Mitochondrial activity (mitochondrial membrane potential, MMP): It is also one of the most crucial features widely recognized for the comprehensive assessment of sperm quality along with the spermiogram parameters (Alamo et al., 2020). Mitochondria are the main place for energy production in ATP form, obtained through the electron transport chain and oxidative phosphorylation, used for sperm motility, kinematics, survival, capacitation, acrosome reaction and other events (Hirata et al., 2002; Oliveira et al., 2014; Davila et al., 2016; Nesci et al., 2020). In fact, MMP is defined from the voltages in the internal mitochondrial membrane (Wang et al., 2003), so spermatozoa with high MMP possess the potential of better sperm quality (Wang et al., 2003) rather than those with low MMP showed worse sperm motility, which reflects the capacity of energy production in ATP form (Marchetti et al., 2002). Thus, it has been demonstrated that MMP positively correlated with sperm motility, viability and male fertilization rates (Marchetti et al., 2002, 2012; Espinoza et al., 2009; Agnihotri et al., 2016). Until now, JC-1 (5',6,6'-tetrachloro-1,1',3,3'-tetraethyl benzimidazolyl-carbocyanine iodide) is one of the most popular dyes for measuring the MMP of sperm besides the rhodamine 123 (R123), MitoTracker Green FM (MTG) and others (Gillan et al., 2005; Hossain et al., 2011). Particularly, it can form aggregates making an orange fluorescence at 590 nm (called high MMP) and monomers giving a green wavelength at 527 nm (termed as low MMP) (Alamo et al., 2020) by using either fluorescence microscopy (Athurupana et al., 2015a; Kato and Nagao, 2015; Umezu et al., 2020; Saraf et al., 2021) or flow cytometry (Gravance et al., 2000; Macías-García et al., 2012; Rodriguez et al., 2012; Gürler et al., 2016; Madeja et al., 2021).

Intracellular reactive oxygen species (ROS): In spermatozoa, the primary sources of intracellular ROS synthesis come from the mitochondrial metabolism, plasma membrane nicotinamide adenine dinucleotide phosphate oxidases and L-amino acid oxidases (Aitken, 2017; Gibb et al., 2020). It has been well documented that although ROS are by-products generated by cells during metabolic pathways in mitochondria, they have a central role to transduce signals, maintain homeostasis and other biological functions (Zhang et al., 2021). Even though adequate intracellular ROS generation is necessary for the fertilization process, such as hyper-activation, capacitation and acrosome reaction (Baumber et al., 2003); an

excessive or imbalanced ROS level could impair motility, plasma membrane, DNA and mitochondria homeostasis, and subsequent infertility (Ball et al., 2001; Gil-Guzman et al., 2001; Aitken and Baker, 2004; Bahmyari et al., 2020) because its overproduction can overwhelm the protective mechanism for sperm itself and trigger alterations in the plasma membranes layers of lipid and/or protein (Sanocka and Kurpisz, 2004; Gibb et al., 2020). Indeed, an excessive generation of ROS leads to oxidative stress (Gürler et al., 2016; Murphy, 2016; Mislei et al., 2020), detrimental impacts on sperm quality (DNA integrity, membrane stability, MMP) and functions (energy generation, motility, dynamics, capacitation) as well as subsequently lower fertilization rates (Barroso et al., 2000; Agca et al., 2005; Nesci et al., 2020; Zhang et al., 2021) due to peroxidation of polyunsaturated fatty acid of sperm membrane (Amaral et al., 2016; Nowicka-Bauer and Nixon, 2020). The higher percentage of motility and dynamics (Gibb et al., 2014), survival and high MMP (Gonzalez-Castro et al., 2019) could significantly contribute to higher ROS production due to higher metabolic activity (Valeanu et al., 2015) and energy requirements (Nesci et al., 2020) from these spermatozoa. It has been reported that the intracellular ROS was produced during and/or after the freezing and thawing processes because of changes in temperature, induction of osmotic shock, and formation of ice crystals (Kadirvel et al., 2009; Macías-García et al., 2012; Gürler et al., 2016); as well as, this level is significantly escalated with an expansion in the thawing time (Zhang et al., 2021). In fact, it is highlighted that intracellular ROS are generated in response to osmotic stress conditions and to alters in osmolarity in live sperm (Macías-García et al., 2012; Kim et al., 2013), so the positive correlation link between osmotic shock and ROS production has been found in spermatozoa from previous studies (Burnaugh et al., 2010; McCarthy et al., 2010; Macías-García et al., 2012). Sperm ROS generation is extensively detected by an oxidation-sensitive fluorescent probe of various dyes originating from 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, a non-fluorescent probe) (Kim et al., 2013; Valeanu et al., 2015; Gürler et al., 2016; Gonzalez-Castro et al., 2019) which permeates cell membrane and is intracellularly de-esterified to release DCFH form by hydrogen peroxide (H_2O_2) which emits green fluorescence (Gürler et al., 2016; Gonzalez-Castro et al., 2019).

Chromatin structure (chromatin integrity): Up to date, a variety of potential factors have been found to trigger the abnormal shape of spermatozoa (Januškauskas and Žilinskas, 2002). Abnormal chromatin structure has a positive correlation with abnormal morphometry of bovine spermatozoa and a negative relationship with bull male fertility (Sailer et al.,

1996). Indeed, bull subfertility significantly associates with DNA fragmentation, chromatin structure instability and morphological abnormalities following AI (Nagy et al., 2013). Spermatozoa from low-fertility bulls have significantly higher fragmentation index of DNA and lower chromatin maturity than those from high-fertility bulls (Dogan et al., 2015; Kumaresan et al., 2017). In other mammalian species, it has also been reported that DNA fragmentation rates remarkedly correlate with motility, viability of spermatozoa and subsequent fertility (Kadirvel et al., 2009; Samplaski et al., 2015). The *in vitro* manipulation techniques during and/or after the cryopreservation for bovine semen can notably impact the incidence of sperm chromatin instability and subsequent male infertility, so the appropriate techniques with a careful manipulation of frozen bull semen to minimize the possible damage of chromatin integrity of spermatozoa (Lymberopoulos and Khalifa, 2010). It has also been revealed that changes in DNA integrity in frozen-thawed spermatozoa could be remarkably related to ROS synthesis (H_2O_2) (Gürler et al., 2016) and oxidative stress (Barroso et al., 2000). The percentage of spermatozoa with DNA fragmentation index is often determined by sperm chromatin structure assay (Larson-Cook et al., 2003; Sellem et al., 2015; Gürler et al., 2016; Kumaresan et al., 2017; Šterbenc et al., 2019) or by the terminal deoxy-nucleotidyl transferase-mediated dUTP nick-end labeling assay (Sergerie et al., 2005; Takeda et al., 2015; Sharma et al., 2021; Ogata et al., 2022).

1.2.4 Mitochondrial content and mtDNA copy number

Mitochondria, are the multitasking organelles, necessary for many physiological events in spermatozoa. Morphologically, it is characterized by two specific membranes including the outer and inner mitochondrial membranes, and the mitochondrial matrix is the intermembrane space of two membranes (Boguenet et al., 2021). Mitochondria of mammalian spermatozoa, known as the principal powerhouse for energy production besides other numerous important physiological functions, have a central role in fertility by energy supply in ATP form for sperm motility and other key pathways (Boguenet et al., 2021; Bulkeley et al., 2021; Madeja et al., 2021). In cattle, especially, mitochondrial respiration known as the oxidative phosphorylation (OXPHOS) pathway in the flagellar midpiece, are the main ATP source for bull sperm motility, functions and fertilizability, even though the anaerobic glycolysis pathway is also utilized effectively in the principle piece of sperm flagellum (Garrett et al., 2008; Losano et al., 2017).

Mitochondria situated along the midpiece of mature spermatozoa flagellum with its genome in the form of mitochondrial DNA (mtDNA) from some specific encoded genes (Hirata et al., 2002), and each mitochondrion contains, on average, from 0 to 11 copies of mtDNA (May-Panloup et al., 2003; Song and Lewis, 2008; Benkhalifa et al., 2014; Boguenet et al., 2022). It has been reported that a certain and optimal content of mitochondrial and mtDNA in spermatozoa is needed to secure mammalian male fertility (Wai et al., 2010). For several last decades, it has been well documented that mitochondrial activity is positively related to the motility, viability and fertilizing capacity of mammalian spermatozoa (Marchetti et al., 2002, 2012; Espinoza et al., 2009; Agnihotri et al., 2016). In horses, intriguingly, mtDNA copy number in spermatozoa is inversely correlated with high mitochondrial activity index (Orsztynowicz et al., 2016). Mitochondrial and mtDNA contents can be altered by several factors besides the spermatogenesis, such as the habitat altitudes (Luo et al., 2011), ROS levels (Bonanno et al., 2016; Guo et al., 2017; Shi et al., 2022), microsurgical varicocelectomy after clinically palpable varicocele (Gabriel et al., 2012), etc. The previous study with mammalian spermatozoa from 10 species (bull, goat, pig, human, mouse, rat, golden hamster, rabbit, dog and guinea pig) indicated that swimming velocities and ATP production are positively correlated with mitochondrial volumes (Gu et al., 2019). However, although there have been a large number of various studies in human (Díez-Sánchez et al., 2003; May-Panloup et al., 2003; Kao et al., 2004; Amaral et al., 2007; Song and Lewis, 2008; Gabriel et al., 2012; Tian et al., 2014; Zhang et al., 2016; Bonanno et al., 2016; Wu et al., 2019a; b; Faja et al., 2019; Boguenet et al., 2022; Sun et al., 2022), mouse (Luo et al., 2013), dog (Hesser et al., 2017); boar (Guo et al., 2017), stallion (Orsztynowicz et al., 2016; Darr et al., 2017) and bull spermatozoa (Madeja et al., 2021) focused on the correlations of mitochondrial content and mtDNA copy number with semen quality and fertilizing capability, due to some contradictory results among studies and species, the definitive conclusion has not been obtained yet and is still in discussion. Indeed, the majority of investigations in humans mentioned above showed the significant inverse relationships of mitochondrial and mtDNA contents with the seminal quality and fertilizability, except for one investigation (Kao et al., 2004). Similarly, the opposite relationships are mostly observed in domestic mammalian species (Orsztynowicz et al., 2016; Darr et al., 2017; Guo et al., 2017), while the positive correlation was found in froze-thawed spermatozoa of two bull groups separated by the artificial insemination performance and the sperm parameters determined by using CASA (Madeja et al., 2021).

MitoTracker Green FM (MTG) is one the most common fluorescent compounds for mitochondrial content measurement regardless of MMP (Poot et al., 1996; Métivier et al., 1998; Poot and Pierce, 1999; Pendergrass et al., 2004) in spermatozoa by flow cytometry (Zhou et al., 2011; Doherty and Perl, 2017; Clutton et al., 2018; Monteiro et al., 2020). Recently, mitochondrial content in mammalian spermatozoa has been determined by MTG with its specific fluorescence by flow cytometry (Kao et al., 2004; Guo et al., 2017; Madeja et al., 2021). Indeed, MTG is classified as cell-permeant mitochondrion selective dyes (Doherty and Perl, 2017), containing a thiol-reactive chloromethyl moiety, leading to stable peptide and protein conjugates after this accumulation in mitochondria (Zhao et al., 2012).

The quantification of mtDNA copy number per spermatozoon is commonly determined by using a quantitative real-time PCR (qPCR). It has also been demonstrated that less mitochondrial and mtDNA contents do not affect the mitochondrial activity and conventional motion characteristics (Darr et al., 2017; Guo et al., 2017; Boguenet et al., 2022) in good-quality semen due to higher MMP and mitochondrial biosynthesis, COX1 protein (mtDNA-encoded OXPHOS protein), PGC-1α (peroxisome proliferator-activated receptor gamma coactivator-1 alpha) and TFAM (transcription of mitochondrial transcript factor A) protein levels in better motile spermatozoa (Amaral et al., 2007; Guo et al., 2017). It has been reported that expression of COX1 protein in human spermatozoa is negatively associated with mtDNA copy number and positively correlated with TFAM or PGC-1α (Amaral et al., 2007); while better motile spermatozoa in boar semen have greater levels of PGC-1α, TFAM, COX1, mitochondrial biosynthesis and MMP (Guo et al., 2017). It has also been mentioned that TFAM gene expression negatively correlates with total motility, but positively associates with abnormal forms, sperm DNA fragmentation and mtDNA copy number (Faja et al., 2019).

1.2.5 Phospholipase C Zeta1 protein in spermatozoa

Phospholipase C zeta1 (PLCZ1) is a kind of soluble cytosolic protein (Swann, 1990; Nomikos et al., 2013) and is first identified in mice (Saunders et al., 2002); subsequently in monkeys, humans, rats (Cox et al., 2002; Ito et al., 2008), chickens (Coward et al., 2005), pigs (Kurokawa et al., 2005; Yoneda et al., 2006), fishes (Ito et al., 2008), bulls (Cooney et al., 2010; Kasimanickam et al., 2012), horses (Bedford-Guaus et al., 2011) and buffaloes (Atabay et al., 2019). It is one of the PLC family members (Cooney et al., 2010; Torra-Massana et al., 2019), universally recognized as a strongly potential candidate responsible

for the resumption of the meiosis egg activation and early embryonic development, through initiating intracellular free calcium ($[Ca^{2+}]_i$) oscillations at fertilization (Cooney et al., 2010; Bedford-Guaus et al., 2011; Hachem et al., 2017; Gonzalez-Castro et al., 2019). PLCZ1 is often termed as a sperm-derived oocyte-activating factor (SOAF) (Cooney et al., 2010) due to mechanisms for oocyte activation during fertilization in mammalian eggs (Ito and Kashiwazaki, 2012) (Figure 1.2). Briefly, it is widely documented that sperm PLCZ1 is liberated into the ooplasm after the sperm-egg membrane fusion process, where it catalyzes the hydrolysis of organelle membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate 2 products of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Nomikos et al., 2005; Kashir et al., 2012; Amdani et al., 2013). DAG is known as an activator of protein kinase C (Swann and Lai, 2013; Antal and Newton, 2014; Yeste et al., 2016) whereas IP_3 triggers cytoplasmic Ca^{2+} oscillations through binding to its receptors (IP_3R) on the endoplasmic reticulum (ER) by which allowing oocyte activation, meiotic resumption, and embryogenesis development (Nomikos et al., 2005; Heytens et al., 2009; Cooney et al., 2010; Amdani et al., 2015; Hachem et al., 2017).

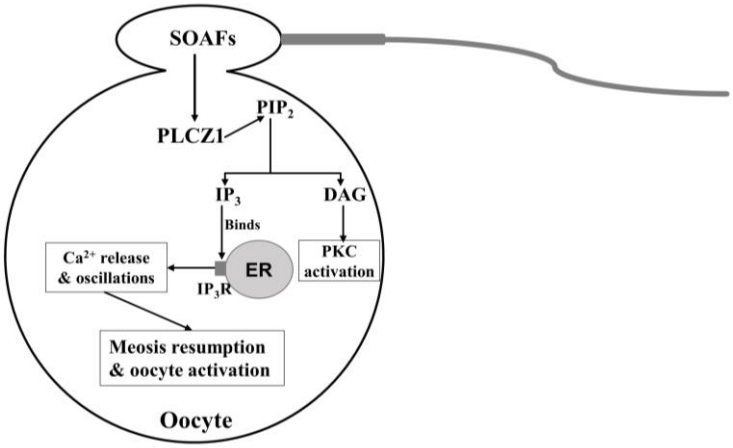

Figure 1.1 The molecular-underlying mechanism of sperm PLCZ1 for oocyte activation during fertilization in mammalian eggs.

In bulls, PLCZ1 was often detected in the acrosome region in the majority of frozen-thawed bull spermatozoa at the initial post-thawed time (Kasimanickam et al., 2012) because its synthesis was demonstrated as an integrated part of the acrosome during the Golgi phase of spermiogenesis (Aarabi et al., 2012). In rodents and humans, PLCZ1 has been detected in the acrosome region of intact spermatozoa (Grasa et al., 2008; Young et al., 2009), although other reports have been observed in both regions of acrosome and equatorial

segment of sperm head (Bedford-Guaus et al., 2011; Durban et al., 2015). It was also found that PLCZ1 in human spermatozoa could be likely associated along with the inner acrosomal membrane (Escoffier et al., 2015). Distribution of PLCZ1 is known to be affected by not only the progression of capacitation and acrosome reaction (Grasa et al., 2008; Young et al., 2009; Mejía-Flores et al., 2017) but also the freezing-thawing process (Heytens et al., 2009; Kashir et al., 2011; Moreau et al., 2019) due to a damaged membrane and/or a loss of integrity (Lasso et al., 1994; Hammadeh et al., 2001), thus leading to subfertility or infertility in humans (Kashir et al., 2011; Dai et al., 2019; Moreau et al., 2019; Wang et al., 2020a). A strongly closed correlation between the intact acrosome and immunoreactive exhibition of sperm PLCZ1 (Grasa et al., 2008; Young et al., 2009) was found in the acrosomal region. To present, there have been valuable evidences indicating that oocyte activation at fertilization is driven by sperm-specific PLCZ1 through inducing intracellular free calcium ($[Ca^{2+}]_i$) oscillations during the sperm-oocyte fusion (Saunders et al., 2002; Heytens et al., 2009; Cooney et al., 2010; Bedford-Guaus et al., 2011; Gonzalez-Castro et al., 2019), but several pieces of evidence still point out that this protein property is not incorporated as SOAF (Aarabi et al., 2012) or not sufficient for oocyte activation event (Ferrer-Vaquer et al., 2016). Besides, some previous studies have reported that acrosome reaction process just changes PLCZ1 distribution in spermatozoa (Grasa et al., 2008; Young et al., 2009; Mejía-Flores et al., 2017), although another one mentioned that PLCZ1 in pig spermatozoa is completely disappeared during acrosomal exocytosis (Aarabi et al., 2012).

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Chemicals and media

All chemicals used in the research were purchased from Sigma–Aldrich Japan G.K. (Tokyo, Japan), unless specified otherwise. The basic medium used for washing, manipulating, and incubating spermatozoa was modified TL-HEPES-PVA solution, composed of 114 mmol/L NaCl, 3.2 mmol/L KCl, 2 mmol/L $NaHCO_3$, 0.34 mmol/L KH_2PO_4, 10 mmol/L Na-lactate, 0.5 mmol/L $MgCl_2 \cdot 6H_2O$, 2 mmol/L $CaCl_2 \cdot 2H_2O$, 10 mmol/L HEPES, 0.2 mmol/L Na-pyruvate, 12 mmol/L sorbitol, 0.1% (w/v) polyvinyl alcohol, 25 μg/mL gentamicin, 65 μg/mL potassium penicillin G and 0.005 mg/mL phenol red (pH = 7.4) (Akaki et al., 2009).

Phosphate-buffered saline (PBS, Sigma) solution, consisted of 2.7 mM KCl, 137 mM NaCl and 10 mM phosphate buffer from 8.54 mM Na_2HPO_4 and 1.46 mM KH_2PO_4 (one tablet of PBS was dissolved in 200 mL Milli-Q water, pH = 7.4), was also used for sub-experiments with the specific purposes.

2.2 Animals and frozen bull semen

Animals used for semen collection were Japanese Black bulls in the Okayama Prefectural Center for Animal Husbandry and Research. These bulls are raised for both research and commercial purposes with commercially proven fertility for AI.

Frozen semen collected from these bulls with excellent fertility scores and prepared for commercial purpose in 0.5 mL straws with a conventional semen-cryopreserved extender (Watson and Martin, 1975) was obtained from the Okayama Prefectural Center for Animal Husbandry and Research (a local public AI center). The procedure of semen collection at the center has been according to the standard protocols authorized from point of view on animal welfare and ethics. No ethics approvals were required because commercial frozen semen was used in the current research. The collected semen was processed and frozen by using a protocol (Hamano, 2016). Briefly, semen was mixed with extender solution (112.7 mM tris(hydroxymethyl)aminomethanol, 39.7 mM citric acid, 20.8 mM fructose, 43.8 mM lactose and 53.5 mM raffinose containing 20% (v/v) egg yolk) and cooled to 4°C in

refrigerator. After the concentration of glycerol in the extender solution was gradually increased to 6.5% (v/v) at 4°C, the diluted semen was filled in 0.5 mL straws. The straws were frozen by cooling to -7°C in 1 - 2 minutes and then to -80°C in 3 minutes before being thrown into liquid nitrogen (-196°C).

2.3 Evaluation of post-thawed spermatozoa quality

2.3.1 Flow cytometry

Sperm suspension was analyzed for viability, MMP, acrosome status, mitochondrial content and ROS by using a Gallios flow cytometer (Beckman Coulter Inc., Brea, CA, USA) after repeatedly washing before and after staining with specific dyes to remove non-sperm particles which can affect the results. The flow cytometer was equipped with three argon lasers and a total of ten fluorescence channels (five 488 nm, three 638 nm, and two 405 nm). Green fluorescence emissions were detected in 525 nm band pass filter (FL1), as well as orange fluorescence wavelength in 575 nm band pass detector (FL2), and red fluorescence in 620 nm filter (FL3). For each sperm suspension sample, one test tube containing 0.5 mL of the diluted sperm suspension at $5x10^6$ cells/mL was acquired the result of 10,000 events per replicate at the forward and side scatter channels. The other non-sperm events such as debris (Rodriguez et al., 2012; Gürler et al., 2016) or alien particles (Petrunkina et al., 2010) were also excluded from the cell population of interest by gating out on the basis of the forward and side scatter dot plots. The analysis of flow cytometry data was performed by using Kaluza analysis software (Beckman Coulter Inc., Brea, CA, USA).

2.3.2 Viability of spermatozoa

The viability of frozen-thawed spermatozoa was determined according to the commercial protocol of a LIVE/DEAD sperm viability kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). In brief, 25 µL of sperm samples were mixed with 471.2 µL TL-HEPES-PVA with a final concentration of 100 nmol/L SYBR safe DNA and 10 µmol/L PI and incubated in the dark for 1.5 minutes at 39°C (Athurupana et al., 2015a; Gonzalez-Castro et al., 2019). The viability of 10,000 spermatozoa was determined by the proportion of SYBR+/PI- gates in flow cytometry (Gonzalez-Castro et al., 2019).

2.3.3 Mitochondrial activity of spermatozoa

Sperm mitochondrial activity determined as mitochondrial membrane potential (MMP) was assessed by using JC-1 (Molecular Probes/Thermo Fisher Scientific, Waltham, MA, USA) by which forms aggregates making an orange fluorescence at 590 nm (high MMP) and monomers giving a green wavelength at 527 nm (low MMP) (Garner and Thomas, 1999). The samples of sperm suspension were incubated with JC-1 dye at a final concentration of 0.76 µmol/L in TL-HEPES-PVA at 39°C for 8 minutes in the dark (Macías-García et al., 2012; Athurupana et al., 2015a). The percentage of spermatozoa with high MMP (HMMP) was determined by the ratio of cells in the orange fluorescence gate in flow cytometry (Macías-García et al., 2012) of 10,000 events by using Kaluza software installed with the flow cytometer.

2.3.4 Acrosome integrity of spermatozoa

Acrosome integrity (intactness of the acrosome membrane) of spermatozoa was evaluated by using PI and FITC-PNA in TL-HEPES-PVA. Sperm samples were incubated with a final concentration of 10 µmol/L PI and 1 µg/mL FITC-PNA at 39°C for 8 minutes in the dark (Athurupana et al., 2015a; Gürler et al., 2016). The percentage of spermatozoa with intact acrosome membrane was calculated by the ratio of cells in the PNA- gates (Gürler et al., 2016) in flow cytometry of 10,000 events.

2.3.5 Reactive oxygen species in spermatozoa

Reactive oxygen species (ROS) in spermatozoa were extensively detected by an oxidation-sensitive fluorescent probe of various dyes originating from 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Kim et al., 2013; Valeanu et al., 2015; Gürler et al., 2016; Gonzalez-Castro et al., 2019) which permeates cell membrane and is intracellularly de-esterified to release DCFH. Particularly, DCFH-DA is a non-fluorescent probe, but it could be transformed by hydrogen peroxide (H_2O_2) into a DCFD form which emits green fluorescence (Gürler et al., 2016; Gonzalez-Castro et al., 2019). For the detection of ROS, sperm suspension samples were incubated with a final concentration of 10 µmol/L PI (Gonzalez-Castro et al., 2019) and highly sensitive DCFH-DA reagent (Dojindo Laboratories, Kumamoto, Japan) diluted at 1:1,000 with the loading buffer for 30 minutes at 39°C in the dark. The ROS levels in a total (live and dead) of 10,000 spermatozoa and/or the only live subpopulation were determined by using a flow cytometer.

2.3.6 Mitochondrial content

Mitochondrial content (MC) of spermatozoa was determined by using MitoTracker Green FM (MTG), according to the manufacturer's protocols (Molecular Probes, Eugene, OR, USA) and a previous report (Madeja et al., 2021) with small modifications. In brief, sperm samples were fixed in 2% paraformaldehyde in PBS for 15 minutes, washed once by centrifugation at 700 ×g for 3 minutes with fresh TL-HEPES-PVA and then stained with a final concentration of 20 nM MTG in TL-HEPES-PVA for 60 minutes at 39°C. After washing once by centrifugation (700 ×g, 3 minutes), MC of frozen-thawed bull spermatozoa was analyzed by using flow cytometer and presented as mean fluorescence intensity (MFI) from 10,000 events by using Kaluza software installed with the flow cytometer (Figure 2.1A).

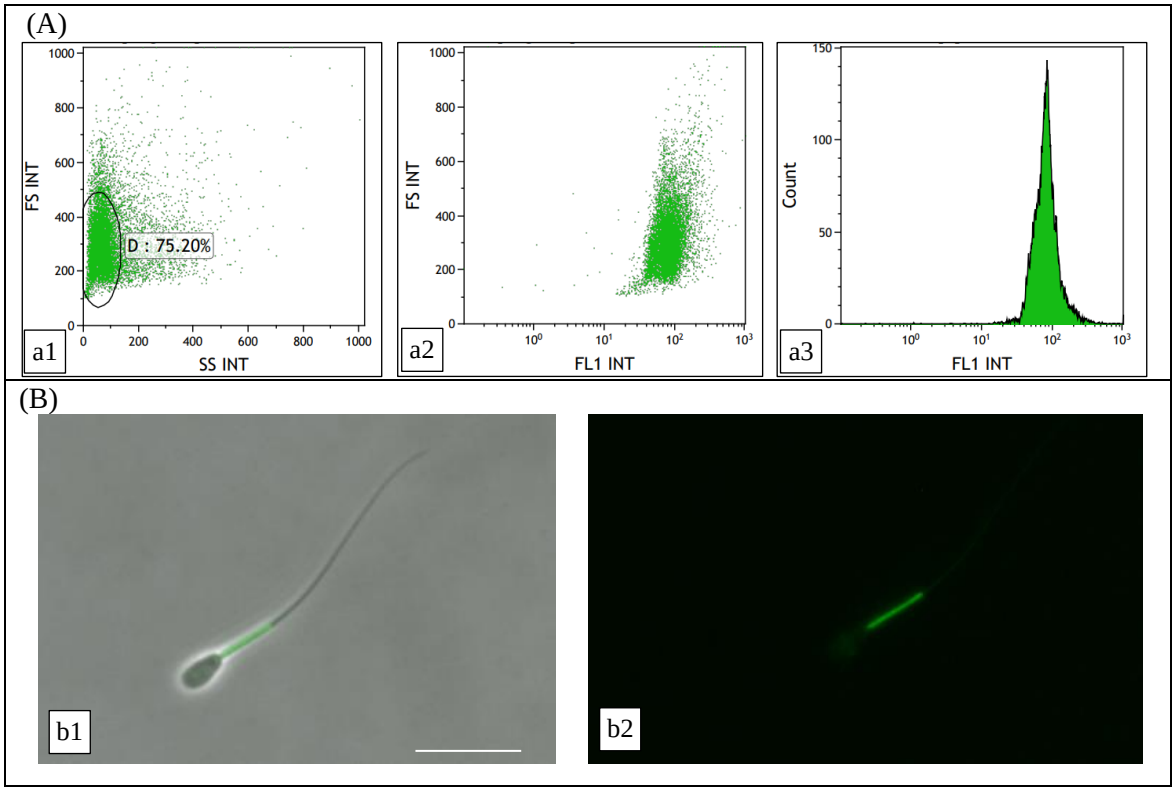

Figure 2.1 Representative fluorescence images of mitochondrial content evaluation in frozen-thawed bull spermatozoa were obtained by using flow cytometry (A) and fluorescence microscopy (B). a1, dot plot at the forward and side scatter channels for gating out the non-sperm events; a2 and a3, dot plot and histogram plot at 525 nm long pass detector in green fluorescence emission; b1 and b2, specificity of the fluorescence signal of MTG dye for mitochondrial content detection at spermatozoa midpiece in bright field and dark field, respectively, at ×400 magnification. Scale bar = 25 µm.

Then, aliquots of 10 μL of the sperm suspension were smeared onto the glass slide and covered by a glass coverslip to confirm the specificity of the fluorescence signal of MTG dye for mitochondrial content detection at spermatozoa midpiece. Fluorescence images were obtained by using a fluorescence microscope (BZ-X710, KEYENCE Inc., Osaka, Japan) at ×400 magnification with a green fluorescence channel at excitation wavelengths of 488 nm (Figure 2.1B).

2.3.7 Motility of spermatozoa

A computer-assisted sperm analysis (CASA) system with 60 digital images per second (Sperm Motility Analysis System, Digital Image Technology, Tokyo, Japan) was used according to the manufacturer's protocols basically based on the WHO criteria (Organization World Health, 1999, 2010) and previously described in more specifications for bull semen (Gürler et al., 2016; Bulkeley et al., 2021) (Spermvision standard bulls, average orientation change <2.5, straightness <0.5, linearity <0.35, distance straight line <4.5, distance average path/radius ≥3 and linearity <0.5) to identify the conventional motion characteristics such as the total motility (TM), progressive motility (PM) and rapid progressive motility (RPM) of spermatozoa (%) (Qiu et al., 2016), and other kinetic parameters (Ogata et al., 2022; Sapanidou et al., 2022). The kinematics assessed in the present research were velocity of straight line (VSL, μm/s), velocity of curved line (VCL, μm/s), velocity of average path (VAP, μm/s), linearity (LIN, %), straightness of trajectory (STR, %), wobble coefficient (WOB, %), amplitude of lateral head displacement (ALH, μm), and beat cross frequency (BCF, Hz) (Tomás et al., 2014; Kourouma et al., 2015). Frozen-thawed sperm suspension was centrifuged once (700 ×g, 3 minutes, 39°C) and diluted with TL-HEPES-PVA for a final suspension concentration of 1 x 10^7 cells/mL. The suspension was analyzed in a Makler counting chamber (FUJIFILM Irvine Scientific, Santa Ana, CA, USA) at 39°C, according to the sperm analysis system's procedures. A minimum of 300 spermatozoa from three different microscopic fields were analyzed by the system.

2.3.8 Relative quantification of mtDNA copy number

Extraction of DNA: Sperm samples were extensively washed again in PBS pre-warmed at 39°C to completely clean spermatozoa and then pelleted by centrifugation (1,900 ×*g*, 5 minutes, 39°C) for DNA isolation. Any somatic cells contained in the washed sperm sample were removed by a cell strainer (PluriSelect, San Diego, CA, USA) according to a

protocol described previously (Darr et al., 2017). Total genomic DNA of spermatozoa was extracted with the High Pure PCR Template Preparation Kit (11796828001, Roche, Nippon Genetic Co., Ltd, Tokyo, Japan) according to the manufacturer's protocols with modifications. In fact, a principal difference in the given protocol was the addition of dithiothreitol (2 mM) in the lysis step to break the nuclear disulphide bonds of mitochondrial capsule and to dissociate mitochondria from the mitochondrial sheath in bull and human spermatozoa (Sutovsky et al., 1997; Carter et al., 2000; May-Panloup et al., 2003; Boguenet et al., 2022). In brief, sperm samples were lysed with 200 µL tissue lysis buffer, 5 µL of 100 mM dithiothreitol and 40 µL of proteinase K at 55°C for 1 h. Then, after incubation at 70°C for 10 minutes following adding 200 µL of binding buffer, the lysed samples were cultured on ice for 10 minutes with 100 µL of isopropanol (100%) to adjust DNA binding conditions. Subsequently, sperm mixture was loaded in the filter column tube and centrifuged at 8,000 ×g for 1 minute. After the flow-through liquid was discarded, inhibitor removal buffer (500 µL) was added into the column and centrifuged at 8,000 ×g for 1 minute, followed by washing twice with silica membrane (500 µL wash buffer and centrifugation at 8,000 ×g for 1 minute each time). The column was again centrifuged for 30 seconds at 13,000 ×g to dry the silica membrane before DNA elution step. The pre-warmed elution buffer (50 µL) was added into the column, incubated at 37°C for 2 minutes and centrifuged at 8,000 ×g for 1 minute to collect all DNA into a 1.5-mL microcentrifuge tube. DNA concentration was quantified by using a NanoDrop OneC spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and the total DNA was stored at -20°C until PCR analysis.

The relative quantification of mitochondrial DNA copy number per spermatozoon (MDCN) was determined by using a quantitative real-time PCR (qPCR). Primers (Fasmac Co., Ltd. Kanagawa, Japan) specific for mtDNA (F: 5'-ATATGCACGTAGGACGAGGC-3', R: 5'-TGCCGATGTATGGGATTGCT-3') and a reference (F: 5'-TTATGGTCGACAACGGGCTC-3', R: 5'-CCGTGCTCAATGGGGTACTT-3') were synthesized from the reference genomic DNA sequence of mitochondrial cytochrome b gene and nuclear β-actin gene, respectively, using the online tool Primer-BLAST (NIH, National Center for Biotechnology Information, USA). The specificity of each primer pair was confirmed by using UCSC In-Silico PCR online tool (UCSC Genome Browser). DNA samples were diluted to a final concentration of 10 ng/µL for qPCR. The qPCR reactions contained 1 µL of DNA solution, 0.5 µM of each primer, and FastStart Essential DNA Green Master (Roche, Nippon Genetic Co., Ltd, Tokyo, Japan) in 10 µL total volume (final DNA

concentration of 1 ng/µL for qPCR reactions). Reactions were performed in LightCycler 96 (Roche) with the heat profile as followed and every reaction was repeated twice, including the pre-incubation (95°C for 5 minutes), 2-step amplification of 40 cycles (95°C for 10 seconds, 58°C for 30 seconds), melting analysis at ramping temperatures from 65 to 95°C with continuous acquisition and cooling (37°C for 5 seconds). The amplification efficiency of each primer pair was checked by performing a standard dilution series of six points of 10-fold dilutions from 10 ng/µL of a sample DNA. MDCN was calculated with the $2^{-\Delta\Delta Ct}$ method (Guo et al., 2017).

2.3.9 Detecting distribution of PLCZ1 in spermatozoa

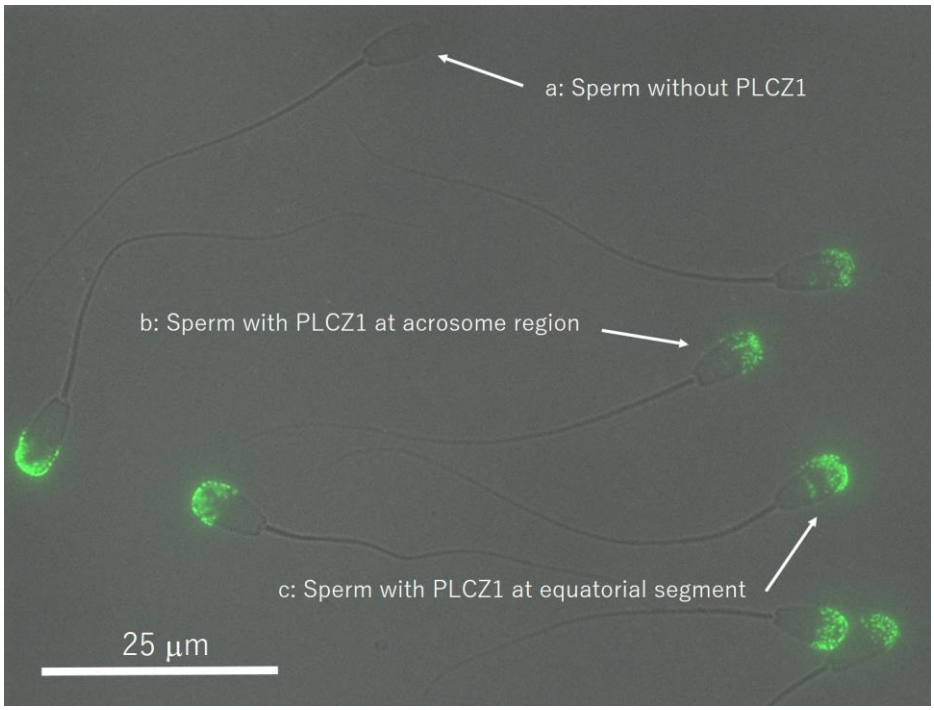

Figure 2.2 Fluorescence image including three fluorescence patterns (a, b and c) of PLCZ1 distribution in frozen-thawed bull spermatozoa was taken at ×1,000. Bar indicates 25 µm.

Indirect immunocytochemistry (ICC) was performed on frozen-thawed spermatozoa with a protocol as described previously by our laboratory (Ioki et al., 2016). In brief, aliquots of 10 µL of sperm samples were smeared onto the glass slide and maintained for 30 minutes at room temperature for air-drying. Then, specimen slides were dipped in methanol (100%) for 15 minutes at room temperature (about 28°C) for fixation and permeabilization. The samples were incubated in blocking solution, TL-HEPES-PVA containing 5% (w/v) bovine serum albumin (BSA), for 1 hour at 37°C to block the nonspecific antibody binding sites, followed by incubation in the presence or absence of primary antibody of Anti-PLCZ1

Rabbit Polyclonal Antibody (Sigma) at a 1:100 dilution in the blocking solution for 2 hours at 37°C. Subsequently, the specimens were incubated with secondary antibody of Alexa Fluor 488 Goat Anti-Rabbit IgG (Molecular Probes/Thermo Fisher Scientific, Waltham, MA, USA) diluted at 1:200 with the blocking solution for 1 hour at 37°C. Thereafter, sperm samples were extensively washed three times with TL-HEPES-PVA and mounted with VECTASHIELD mounting medium (H-1000, Vectorlabs, Burlingame, CA, USA), and a 24 x 50 mm glass coverslip. Images were observed by using a fluorescence microscope (BZ-X710, KEYENCE Inc., Osaka, Japan) at ×400 and ×1,000 magnifications with a specific green fluorescence channel at excitation wavelength of 488 nm. Totally 300 spermatozoa were observed per sample by ICC and the percentages of sperm with three specific categories were calculated, including (1) sperm without PLCZ1 fluorescence exhibition, (2) sperm with PLCZ1 distributing at the acrosome region only, and (3) sperm with PLCZ1 detected at both the acrosomal and equatorial segment regions (Figure 2.2).

CHAPTER 3

RAPID THAWING OF FROZEN BULL SPERMATOZOA BY TRANSIENT EXPOSURE TO 70°C IMPROVES THE VIABILITY, MOTILITY AND MITOCHONDRIAL HEALTH

Abstract

Up to now, the definitive conclusion of the positive effects of rapid transient thawing at higher temperatures for shorter durations has not been obtained yet and is still under discussion due to some contradictory findings and limited assessment of post-thawed parameters. The aim of the study was to evaluate the effectiveness of rapid thawing in water at 70°C by using various post-thawed parameters of frozen bull spermatozoa. Experiment 3.1, monitoring the temperature change inside frozen bull straw thawed in water at different temperatures. Experiment 3.2, evaluation of various post-thawed characteristics of frozen bull spermatozoa thawed in water at different temperatures by using CASA, flow cytometry and immunocytochemistry. The time it took for the temperature inside the straw to warm up to 15°C was nearly twice as faster when the straw was thawed in 70°C water compared with 37 or 39°C. Viability, motility, and MMP of spermatozoa thawed at 70°C for 8 seconds and immediately stabilized at 39°C for 52 seconds (RT70) were significantly higher than controls thawed and stabilized at 39°C for 60 seconds (CT39) or thawed at 37°C for 46 seconds and then stabilized at 39°C for 14 seconds (CT37). Just after thawing, however, there were no differences in acrosome integrity and PLCZ1 distribution, whereas mitochondrial ROS production in all cells was significantly lower in spermatozoa thawed at RT70. Although there were differences among bulls, viability, motility, and MMP of spermatozoa thawed at RT70 were also significantly higher than those of CT39 at 0 and 3 hours after thawing. From these results, we conclude that rapid thawing at 70°C and then stabilization at 39°C significantly improves viability, motility and mitochondrial health of bull spermatozoa rather than conventional thawing at 37 or 39°C, whereas there are similar results between CT37 and CT39. The beneficial effect of rapid transient thawing could be due to shorter exposure to temperatures outside the physiological range, consequently maintaining mitochondrial health.

3.1 Introduction

Cryopreservation of bull semen has contributed significantly to the genetic improvement and efficient reproduction for decades in the dairy and beef production industries, but a low fertilizability of frozen-thawed spermatozoa seems to be still the main concerns (Upadhyay et al., 2021). It is recommended that frozen bull semen straws are thawed under the physiological temperature (Correa et al., 1996), even though slow thawing of frozen spermatozoa seems to promote recrystallization and consequently damages the organelles (Hammerstedt et al., 1990; Sharafi et al., 2022). Although thawing at higher temperatures for shorter durations has been tried with better results for some important parameters to overcome a low quality of frozen-thawed buffalo (Rastegarnia et al., 2013) and cattle spermatozoa (Lyashenko, 2015), due to some inconsistent results (Yilmaz et al., 2019), the definitive conclusion has not been obtained yet and is still in discussion. Intriguingly, subsequent stabilization at 39°C for a while following rapid thawing could be an ideal condition to recover spermatozoa integrity and functions after the quick status change of frozen spermatozoa (Athurupana et al., 2015a).

In the previous reports mentioned above (Rastegarnia et al., 2013; Lyashenko, 2015), motility and viability of spermatozoa have already been assessed after thawing at 70°C for 6 - 7 seconds, but it is not yet clear if there are any differences among bulls in the effect of thawing by rapid thawing on frozen sperm quality. Additionally, since other crucial parameters associated with mitochondrial function, such as MMP and mitochondrial ROS, have not also been made clear yet, the effectiveness of rapid thawing at a high temperature has never been discussed with evidences of mitochondrial activity, oxidative stress and others. Mitochondrial membrane potential is well known to be positively correlated with the viability, motility, and fertilizability of spermatozoa (Marchetti et al., 2012). An excessive ROS level during the freeze-thawing process could impair plasma membrane, mitochondria homeostasis, motility, and consequent penetrability (Bahmyari et al., 2020). Furthermore, cryopreservation is known to significantly affect PLCZ1, one of the PLC family members universally recognized as a potential candidate responsible for the resumption of oocyte meiosis at fertilization (Amdani et al., 2015; Thanassoulas et al., 2022), and consequently fertility (Moreau et al., 2019).

Therefore, the aim of the present study was to investigate the effects of thawing of frozen bull spermatozoa at different temperatures (37, 39, or 70°C) and subsequent

stabilization at 39°C on not only the motility and viability but also MMP, acrosome integrity, ROS, and PLCZ1 distribution.

3.2 Materials and Methods

3.2.1 Chemicals and frozen bull semen

All chemicals used in the current study were purchased from Sigma-Aldrich Japan G.K. (Tokyo, Japan), unless specified otherwise. The basic medium used for washing, manipulating, and incubating sperm was modified TL-HEPES-PVA (Akaki et al., 2009).

Frozen semen collected from four Japanese Black Bulls (3-8 years old) with excellent fertility scores and prepared for commercial purpose in 0.5 mL straws with a conventional semen-cryopreserved extender (Watson and Martin, 1975) was donated from a local public AI center, the Okayama Prefectural Center for Animal Husbandry and Research.

3.2.2 Experimental design

3.2.2.1 Experiment 3.1: Monitoring the change of temperature inside frozen straw thawed in water at different temperatures

The change of temperature inside straws of frozen bull semen was observed every two-second interval (a minimum recording interval of the thermometer) during the thawing period by using a two-channel digital record thermometer (TNA-140; TASCO Inc., Tokyo, Japan). The first sensor wire of the thermometer was inserted through the cut end of that straw and corked to prevent the semen inside from leaking, and then the straw was immersed back into liquid nitrogen to be frozen again. The second sensor wire was used to monitor the temperature of water for thawing (37, 39, or 70°C) in a temperature control bath. The recording of the change of the temperature inside straws was started just before the straw of bull semen in liquid nitrogen was plunged into the water at 37, 39, or 70°C and continued until the temperature inside the straw reached at least 37 or 39°C. The recording was replicated eight times with different straws of frozen semen in each experimental group.

3.2.2.2 Experiment 3.2: Evaluation of post-thawed characteristics of spermatozoa from frozen bull semen thawed in water at different temperatures

The straws containing bull spermatozoa were thawed in water at 37°C (CT37) and 70°C (RT70) for 46 and 8 seconds, respectively, and promptly followed by the preserving process in water at 39°C for 14 and 52 seconds, respectively. As controls, other straws were

thawed and preserved in water at 39°C for 60 seconds (CT39); these protocols were adopted from the results in experiment 3.1. Thawed sperm suspension was immediately diluted and washed following a protocol previously described by our laboratory (Athurupana et al., 2015a). Briefly, the suspension was diluted with 5 mL of TL-HEPES-PVA pre-warmed at 39°C and washed once by centrifugation with 700 ×*g* for 3 minutes at 39°C. The precipitate re-suspended in the pre-warmed TL-HEPES-PVA at a final concentration of 1 x 10^8 cells/mL (called a sperm sample) was evaluated after incubating at 39°C for 5 minutes (termed as 0 hour after thawing, or just after thawing). The sperm samples were analyzed on viability, high MMP, acrosome integrity, intracellular ROS, motility, and distribution of PLCZ1. Experiments were replicated 6 times (6 different frozen straws) for each thawing temperature.

To determine if the similar effect could be obtained with semen from several bulls, basically, a 2 x 4 factorial design (two thawing methods with CT39 and RT70; frozen semen from 4 different bulls) was applied to examine the effects of thawing methods, bulls, or interaction between thawing methods and bulls on some parameters at just after thawing (0 hour) and after culture for 3 hours at 39°C in TL-HEPES-PVA supplemented with 6 mg/mL BSA (Sigma). The thawed sperm samples (0 and 3 hours) were analyzed on the viability, high MMP, motility, and some dynamics. Experiments were replicated 6 times (6 different frozen straws from each bull semen) in all experimental groups.

3.2.3 Statistical analysis

Statistical analyses were carried out by one-way or two-way ANOVA followed by Turkey's multiple range tests using GraphPad Prism 8.3 statistical software (GraphPad Software Inc., San Diego, CA, USA). Before the statistical analysis, to fit a normal distribution, all the percentage data for the experiment were subjected to arc-sine transformation if there were values > 90% or < 10%, then they were posteriorly transformed back into percentages for the tables and figures. The continuous-valued data such as VSL, VCL, VAP, ALH and BCF were transformed by logarithmic transformations if there were non-normal distributions. All results were expressed as mean ± SEM. Differences were considered significant at $P < 0.05$.

3.3 Results

3.3.1 Monitoring the change of temperature inside frozen straw thawed in water at different temperatures (experiment 3.1)

As shown in Figure 3.1, there were different phases of the change of temperature inside frozen semen straws during thawing before and after the melting point. Before the melting point, temperature rate was very fast, the solid phase, and relatively slower after the point, the liquid phase.

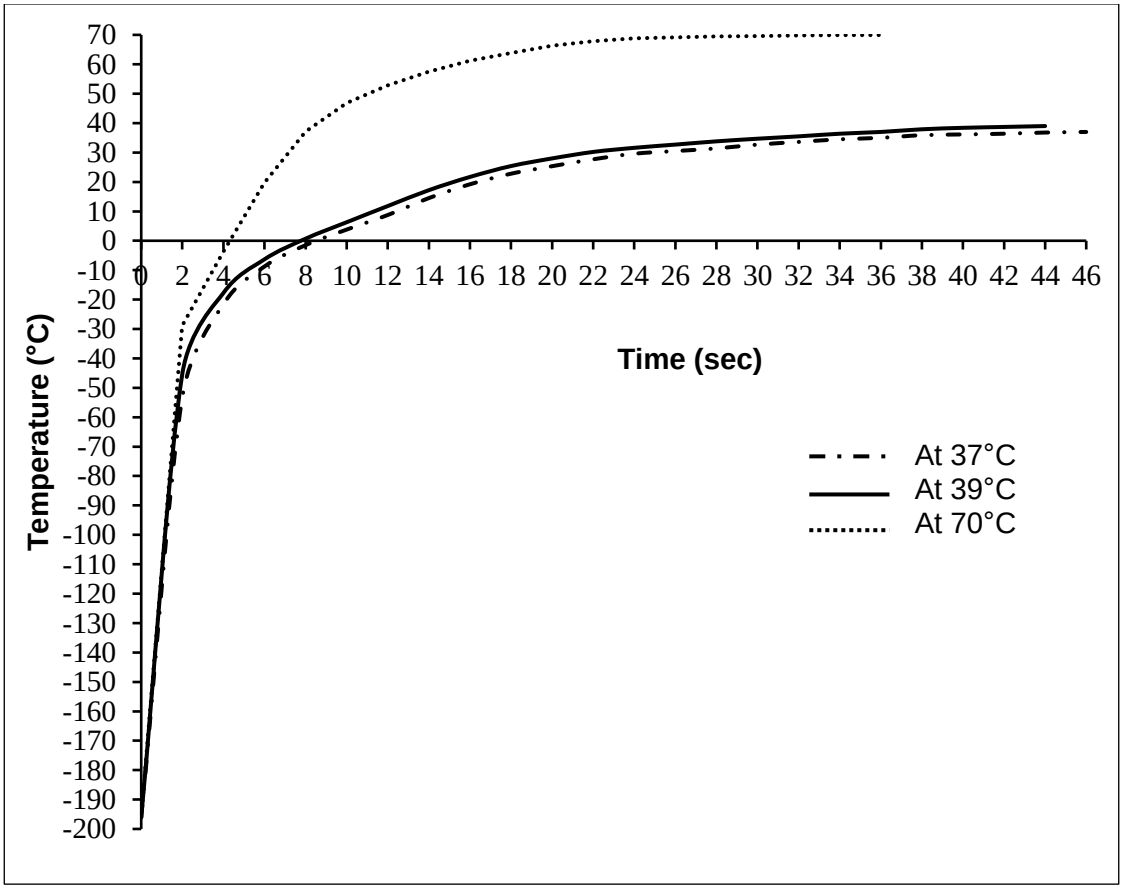

Figure 3.1 Change of temperature inside the frozen bull straws during thawing in water at 37, 39, or 70°C. The temperature inside the frozen straw was recorded every 2 seconds. The points represent the mean value from 8 times replicated data, and the expected curves were manually drawn. The temperature rate was very fast at the beginning and then relatively slower at the liquid phase in spite of the thawing temperature.

When frozen straws were thrown into the water at 37, 39, or 70°C, the change of temperature inside the straw monitored for every 2 seconds differed significantly ($P < 0.01$) in 70°C water compared with 37 or 39°C, whereas no significant differences were found

between 37 and 39°C (Table 3.1). Although there were no significant differences in warming rates inside the semen straw within 10 seconds when the frozen straw was thawed in water at 37 and 39°C, the rates were significantly faster ($P < 0.01$) when frozen semen was thawed in water at 70°C, without those at 4 and 6 seconds after the start of thawing. The estimated average time for the temperature inside the straw to reach from -196 to 0 or to 15°C was significantly faster ($P < 0.01$) only when the frozen semen was thawed in water at 70°C (5.1 ± 0.2 or 6.5 ± 0.2 seconds, respectively), as compared with 37°C (10.0 ± 0.6 or 14.6 ± 0.8 seconds, respectively) or 39°C (9.6 ± 0.3 or 13.0 ± 0.4 seconds, respectively). Furthermore, the time from -196 to 37°C differed significantly ($P < 0.01$) among three thawing temperatures (45.8 ± 1.3 seconds at 37°C, 39.8 ± 0.3 seconds at 39°C, and 7.6 ± 0.2 seconds at 70°C). The estimated average time for the temperature inside the frozen straw to reach from -196 to 39°C was also significantly faster ($P < 0.01$) when frozen semen was thawed at 70°C (8.2 ± 0.3 seconds) than at 39°C (43.8 ± 0.8 seconds). Since the temperature inside the frozen semen straw thawed in 70°C water reached 39°C in 8.2 seconds, the method of stabilizing with 39°C water after thawing at 70°C for 8 seconds was adopted in the following experiments.

3.3.2 Evaluation of post-thawed characteristics of spermatozoa from frozen bull semen thawed in water at different temperatures (experiment 3.2)

As shown in Table 3.2, when frozen semen straws from one bull were thawed at RT70, the percentages of viable and high MMP spermatozoa at 0 hour after thawing were significantly higher ($P < 0.05$) than those of spermatozoa thawed at CT37 or CT39. The percentage of spermatozoa with high ROS level was significantly lower ($P < 0.05$) when frozen semen was thawed at RT70 than at CT37 or CT39, whereas there were no differences in the percentage of "live" spermatozoa with high ROS level among three thawing temperatures. Furthermore, there were no significant differences in the percentage of spermatozoa with intact acrosome among thawing conditions examined.

As shown in Table 3.3, there were significant differences in percentages of viable and high MMP spermatozoa not only among bulls ($P < 0.01$) but also between CT39 and RT70 ($P < 0.05$) at 0 and 3 hours after thawing. Importantly, no significant interactions were also found between the thawing methods and multiple bulls (0 and 3 hours) under these post-thawed parameters.

When frozen semen straws from one bull were rapidly thawed at RT70, post-thawed motility parameters at 0 hour after thawing, such as the total motility, progressive motility, VSL, and VAP, were significantly higher ($P < 0.05$) than thawing at CT37 or CT39 (Table 3.4). The rapid progressive motility of spermatozoa thawed at RT70 was not different from that thawed at CT39, but significantly higher ($P < 0.05$) than that thawed at CT37. However, there were no differences in VCL, ALH, and BCF among the thawing temperatures.

When frozen semen straws from four bulls were examined to make sure the effects of rapid thawing, although all motility parameters examined in this study were significantly different ($P < 0.05$) among bulls (only an exception of VAP at 0 hour), some parameters, such as the total motility, progressive motility, and VAP, were significantly different ($P < 0.05$) between controls (CT39) and RT70 at 0 (Table 3.5) and 3 hours after thawing (Table 3.6). However, the rapid progressive motility, VSL, VCL, ALH, and BCF of spermatozoa were not different between RT70 and CT39. Moreover, there were no significant interactions between two thawing conditions and bull semen at 2 points of time after thawing in all motility parameters.

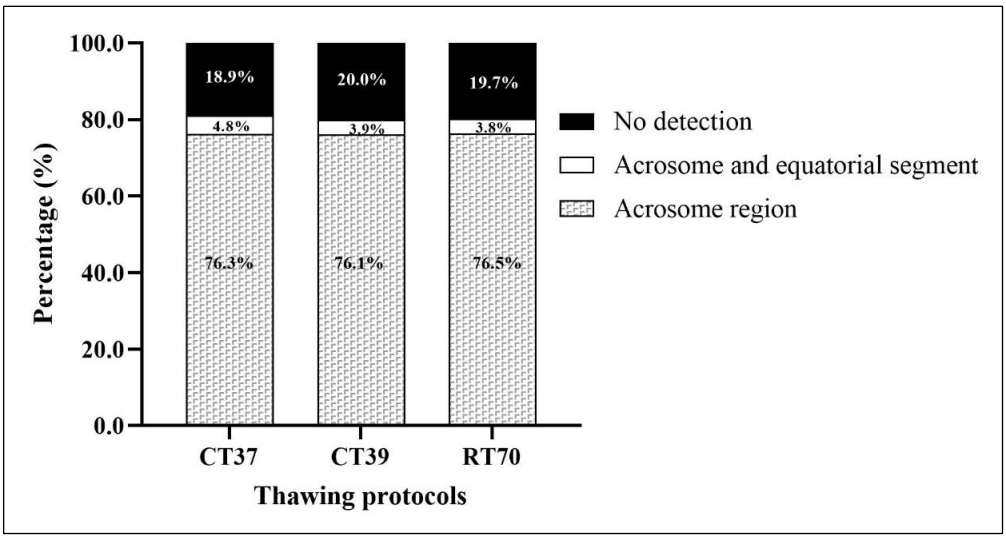

Figure 3.2 Distribution of PLCZ1 in frozen spermatozoa from one bull semen thawed at different temperatures ($n = 6$, $P > 0.05$). CT37: Spermatozoa were conventionally thawed at 37°C for 46 seconds and stabilized at 39°C for 14 seconds; CT39: Spermatozoa were conventionally thawed for totally 60 seconds; RT70: Spermatozoa were rapidly thawed at 70°C for 8 seconds and then stabilized at 39°C for 52 seconds.

When the distribution of PLCZ1 in frozen bull spermatozoa thawed at CT37, CT39, or RT70 was observed by immunocytochemistry, a majority of spermatozoa in all

experimental groups showed the presence at the acrosome region (76.1 - 76.5%; Figure 3.2). A few parts of spermatozoa were detected with PLCZ1 at both the acrosome and equatorial segment regions (3.8 - 4.8%), whereas the others (18.9 - 20.0%) were not detected the presence. However, these percentages were not different among three thawing temperatures examined.

Table 3.1 Average temperature and warming rates (mean ± SEM) inside the frozen bull straw during thawing in water at different temperatures

Time (sec) after plunged into thawing water	Temperature of water in which the straws of frozen bull semen were thawed at					
	37°C		39°C		70°C	
	Temperature (°C) in the straw	Warming rate (°C/sec)	Temperature (°C) in the straw	Warming rate (°C/sec)	Temperature (°C) in the straw	Warming rate (°C/sec)
0	-196.0	–	-196.0	–	-196.0	–
2	-53.2 ± 3.0^{B}	71.4 ± 1.5^{b}	-45.2 ± 5.6^{B}	75.4 ± 2.8^{b}	-28.9 ± 3.9^{A}	83.6 ± 1.9^{a}
4	-14.6 ± 6.8	19.3 ± 3.2	-10.8 ± 1.0	17.2 ± 2.3	-5.3 ± 0.9	11.8 ± 1.6
6	-4.0 ± 5.6^{B}	5.3 ± 3.2	-6.3 ± 0.7^{B}	2.2 ± 0.6	11.9 ± 2.3^{A}	8.6 ± 1.3
8	-2.1 ± 0.4^{B}	1.0 ± 0.5^{b}	-4.7 ± 0.4^{B}	0.8 ± 0.4^{b}	37.2 ± 3.2^{A}	12.6 ± 1.7^{a}
10	1.1 ± 1.5^{B}	1.6 ± 0.6^{b}	2.2 ± 1.5^{B}	3.4 ± 0.7^{b}	47.7 ± 2.3^{A}	5.3 ± 0.9^{a}
12	5.8 ± 2.1	2.4 ± 0.9	10.0 ± 2.0	3.9 ± 1.0	–	–
14	13.1 ± 3.1	3.7 ± 0.8	20.2 ± 1.8	5.1 ± 1.0	–	–
16	20.0 ± 2.5	3.4 ± 0.7	26.3 ± 1.0	3.1 ± 1.2	–	–
The change sphere of temperature	**Estimated average time (sec) to take the change of temperature inside the straw thawed at**					
	37°C		**39°C**		**70°C**	
-196 to 0°C	10.0 ± 0.6^{A}		9.6 ± 0.3^{A}		5.1 ± 0.2^{B}	
-196 to 15°C	14.6 ± 0.8^{A}		13.0 ± 0.4^{A}		6.5 ± 0.2^{B}	
-196 to 37°C	45.8 ± 1.3^{A}		39.8 ± 0.3^{B}		7.6 ± 0.2^{C}	
-196 to 39°C	–		43.8 ± 0.8^{A}		8.2 ± 0.3^{B}	

Note: Different small and capital superscripts indicate a significant difference in temperature and the warming rate within the same row, respectively ($P < 0.05$, mean $\pm$ SEM, $n = 8$).

Table 3.2 Post-thawed quality parameters at 0 h after thawing frozen spermatozoa from one bull semen thawed at different temperatures

Thawing temperature and period	Percentage (mean ± SEM) of spermatozoa at 0 h after thawing with				
	Viability	High MMP	Intact acrosome	High ROS level (in total sperm)	High ROS level (in live sperm only)
37°C for 46 sec and then 39°C for 14 sec	61.8 ± 0.5^{b}	44.5 ± 1.2^{b}	71.9 ± 1.0	44.3 ± 2.7^{b}	28.0 ± 2.9
39°C for 60 sec	62.6 ± 1.0^{b}	46.7 ± 0.9^{b}	71.4 ± 2.0	44.0 ± 2.8^{b}	27.7 ± 3.4
70°C for 8 sec and then 39°C for 52 sec	67.0 ± 1.7^{a}	51.9 ± 1.8^{a}	75.0 ± 1.3	35.0 ± 2.7^{a}	25.1 ± 2.0

Note: Different superscripts indicate a significant difference in the parameter within the same column ($P < 0.05$, $n = 6$).

Abbreviations: MMP, mitochondrial membrane potential; ROS, reactive oxygen species; SEM, standard error of the mean.

Table 3.3 Post-thawed quality parameters at 0 and 3 h after thawing frozen spermatozoa thawed at different protocols

Thawing protocols	Bull	Percentage (mean ± SEM) of spermatozoa with			
		Viability at 0 h (%)	Viability at 3 h (%)	High MMP at 0 h (%)	High MMP at 3 h (%)
39°C for 60 sec	A	57.7 ± 4.2^{c}	40.7 ± 0.4^{c}	46.9 ± 1.5^{d}	41.5 ± 0.4^{bc}
	B	73.4 ± 0.4^{ab}	69.7 ± 0.7^{a}	82.5 ± 1.1^{a}	65.1 ± 1.7^{a}
	C	65.2 ± 1.9^{bc}	54.3 ± 0.6^{b}	55.1 ± 2.2^{bc}	38.1 ± 0.5^{c}
	D	36.5 ± 0.9^{d}	27.9 ± 1.3^{d}	49.8 ± 2.2^{cd}	25.2 ± 1.1^{d}
	Total	58.2 ± 3.1	48.1 ± 3.3	58.6 ± 3.1	42.4 ± 3.1
70°C for 8 sec and then 39°C for 52 sec	A	65.1 ± 4.6^{bc}	43.2 ± 1.2^{c}	52.6 ± 2.2^{cd}	45.2 ± 0.5^{b}
	B	77.2 ± 0.5^{a}	73.9 ± 0.5^{a}	83.3 ± 1.2^{a}	69.4 ± 1.4^{a}
	C	68.8 ± 1.2^{ab}	58.8 ± 1.1^{b}	61.3 ± 1.6^{b}	41.5 ± 1.7^{bc}
	D	37.4 ± 0.9^{d}	27.5 ± 1.5^{d}	47.9 ± 1.9^{cd}	26.1 ± 0.6^{d}
	Total	62.1 ± 3.3	50.8 ± 3.7	61.3 ± 3.0	45.5 ± 3.3
P among bulls		< 0.01	< 0.01	< 0.01	< 0.01
P between thawing protocols		< 0.05	< 0.01	< 0.05	< 0.01
P of thawing protocols x bulls		0.62	0.06	0.09	0.44

Note: Different small superscripts indicate a significant difference among bulls, respectively ($P < 0.05$, $n = 6$). P values from two-way ANOVA analysis ($n = 6$) are shown the bottom of the table.

Abbreviations: MMP, mitochondrial membrane potential; SEM, standard error of the mean.

Table 3.4 Post-thaw motility parameters at 0 h after thawing frozen spermatozoa from one bull semen thawed at different temperatures

Thawing temperature and period	**Post-thaw motility parameters (mean ± SEM) at 0 h after thawing**							
	Total motility (%)	**Progressive motility (%)**	**Rapid progressive motility (%)**	**VSL (µm/s)**	**VCL (µm/s)**	**VAP (µm/s)**	**ALH (µm)**	**BCF (Hz)**
37°C for 46 sec and then 39°C for 14 sec	45.1 ± 1.6^{b}	41.2 ± 1.3^{b}	35.4 ± 1.6^{b}	51.7 ± 1.5^{b}	199.1 ± 7.0	73.4 ± 1.5^{b}	4.2 ± 0.1	7.3 ± 0.2
39°C for 60 sec	45.5 ± 1.6^{b}	41.7 ± 1.8^{b}	36.8 ± 2.1^{ab}	51.9 ± 3.0^{b}	202.5 ± 6.7	74.5 ± 2.0^{b}	4.1 ± 0.3	7.4 ± 0.3
70°C for 8 sec and then 39°C for 52 sec	52.5 ± 1.5^{a}	48.8 ± 2.0^{a}	42.4 ± 1.7^{a}	60.1 ± 2.6^{a}	211.6 ± 4.9	80.8 ± 1.3^{a}	4.4 ± 0.1	7.7 ± 0.5

Note: Different superscripts indicate a significant difference in the parameter within the same column ($P < 0.05$, $n = 6$).

Abbreviations: VSL, velocity straight line; VCL, velocity curved line; VAP, velocity average path; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; SEM, standard error of the mean.

Table 3.5 Post-thawed motility parameters at 0 h after thawing frozen spermatozoa from four different bulls thawed at different protocols

Thawing protocols (TPS)	Bull	Post-thaw parameters (mean + SEM) at 0 h after thawing							
		Total motility (%)	Progressive motility (%)	Rapid progressive motility (%)	VSL (µm/s)	VCL (µm/s)	VAP (µm/s)	ALH (µm)	BCF (Hz)
39°C for 60 sec	A	43.9 ± 2.0^{bc}	38.8 ± 2.3^{cd}	32.2 ± 2.7^{cd}	58.2 ± 4.9^{ab}	195.9 ± 6.5^{ab}	73.8 ± 1.6	4.0 ± 0.2^{abc}	8.8 ± 1.0^{b}
	B	69.2 ± 2.2^{a}	57.1 ± 3.8^{ab}	49.0 ± 3.9^{ab}	48.8 ± 5.3^{b}	207.0 ± 10.7^{ab}	73.2 ± 5.8	4.3 ± 0.2^{ab}	6.6 ± 0.4^{b}
	C	44.2 ± 1.6^{bc}	36.4 ± 1.2^{cd}	30.0 ± 1.7^{cd}	65.2 ± 5.0^{ab}	192.2 ± 9.4^{ab}	78.8 ± 4.8	3.3 ± 0.2^{c}	11.5 ± 0.2^{a}
	D	34.5 ± 1.7^{d}	27.8 ± 1.9^{d}	22.8 ± 1.7^{d}	62.6 ± 2.8^{ab}	198.1 ± 3.4^{ab}	77.8 ± 2.5	3.9 ± 0.1^{abc}	8.4 ± 0.3^{b}
	Total	47.9 ± 2.8	40.0 ± 2.5	33.5 ± 2.4	58.7 ± 2.5	198.3 ± 3.9	75.9 ± 2.0	3.9 ± 0.1	8.8 ± 0.4
70°C for 8 sec and then 39°C for 52 sec	A	50.9 ± 2.8^{b}	45.1 ± 2.9^{bc}	38.1 ± 2.4^{bc}	64.8 ± 3.4^{ab}	203.1 ± 7.5^{ab}	81.0 ± 2.7	4.0 ± 0.2^{abc}	9.0 ± 0.9^{ab}
	B	75.8 ± 1.7^{a}	67.1 ± 4.9^{a}	55.0 ± 4.5^{a}	52.0 ± 2.3^{b}	218.8 ± 4.6^{a}	82.9 ± 2.7	4.4 ± 0.2^{a}	7.1 ± 0.3^{b}
	C	48.6 ± 2.5^{b}	42.2 ± 3.0^{c}	34.5 ± 2.4^{cd}	69.9 ± 3.3^{a}	191.6 ± 8.5^{ab}	83.6 ± 3.3	3.5 ± 0.1^{bc}	11.6 ± 0.7^{a}
	D	35.7 ± 1.6^{cd}	27.5 ± 1.8^{d}	21.9 ± 2.2^{d}	58.4 ± 3.2^{ab}	184.1 ± 6.7^{b}	78.2 ± 4.6	3.5 ± 0.2^{bc}	8.4 ± 0.3^{b}
	Total	52.8 ± 3.2	45.5 ± 3.3	37.4 ± 2.8	61.3 ± 2.0	199.4 ± 4.2	81.4 ± 1.7	3.8 ± 0.1	9.0 ± 0.4
P among bulls		< 0.01	< 0.01	< 0.01	< 0.01	< 0.05	0.74	< 0.01	< 0.01
P between TPS		< 0.01	0.01	0.06	0.36	0.84	< 0.05	0.73	0.65
P of TPS x bulls		0.48	0.31	0.59	0.55	0.34	0.65	0.50	0.97

Note: Different small superscripts indicate a significant difference among bulls, respectively (P < 0.05, n = 6). P values from two-way ANOVA analysis (n = 6) are shown the bottom of the table.

Abbreviations: VSL, velocity straight line; VCL, velocity curved line; VAP, velocity average path; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; SEM, standard error of the mean.

Table 3.6 Post-thawed motility parameters after 3 h incubation of frozen spermatozoa from four different bulls thawed at different protocols

Thawing protocols (TPS)	Bull	Post-thaw parameters (mean + SEM) after 3 h incubation of spermatozoa at 39°C							
		Total motility (%)	Progressive motility (%)	Rapid progressive motility (%)	VSL (µm/s)	VCL (µm/s)	VAP (µm/s)	ALH (µm)	BCF (Hz)
39°C for 60 sec	A	29.9 ± 0.8^{c}	24.2 ± 1.3^{b}	20.9 ± 1.5^{bc}	50.4 ± 3.0^{ab}	161.1 ± 8.6^{ab}	73.2 ± 4.1^{ab}	2.9 ± 0.1^{ab}	12.5 ± 0.7^{ab}
	B	51.4 ± 1.5^{b}	42.7 ± 3.0^{a}	35.6 ± 3.1^{a}	48.6 ± 3.1^{ab}	159.8 ± 15.2^{ab}	67.3 ± 4.1^{ab}	3.3 ± 0.3^{a}	12.7 ± 0.7^{ab}
	C	28.4 ± 0.9^{c}	22.5 ± 0.9^{bc}	18.9 ± 1.6^{bcd}	56.4 ± 4.8^{a}	164.4 ± 11.6^{ab}	76.2 ± 4.7^{a}	2.5 ± 0.3^{b}	15.1 ± 0.6^{a}
	D	20.6 ± 1.4^{d}	14.2 ± 1.2^{d}	10.6 ± 1.6^{d}	40.9 ± 3.7^{b}	134.5 ± 8.3^{b}	61.1 ± 7.3^{b}	2.7 ± 0.3^{b}	10.8 ± 0.4^{b}
	Total	32.6 ± 2.5	25.9 ± 2.3	21.5 ± 2.1	49.1 ± 2.8	154.9 ± 5.8	69.4 ± 2.7	2.9 ± 0.2	12.8 ± 0.4
70°C for 8 sec and then 39°C for 52 sec	A	34.0 ± 1.2^{c}	29.2 ± 1.0^{b}	24.8 ± 1.8^{b}	57.2 ± 7.1^{ab}	171.2 ± 11.8^{ab}	77.9 ± 5.6^{ab}	3.2 ± 0.2^{ab}	13.6 ± 1.0^{ab}
	B	57.3 ± 2.0^{a}	48.9 ± 2.6^{a}	38.3 ± 3.2^{a}	51.0 ± 7.3^{ab}	180.1 ± 6.9^{a}	81.6 ± 2.6^{ab}	3.5 ± 0.3^{a}	13.0 ± 0.3^{ab}
	C	30.7 ± 1.1^{c}	26.3 ± 1.3^{b}	21.6 ± 1.1^{bc}	62.6 ± 3.2^{a}	161.3 ± 7.0^{ab}	80.0 ± 3.7^{a}	2.6 ± 0.2^{b}	14.2 ± 0.5^{a}
	D	21.8 ± 1.2^{d}	15.4 ± 1.9^{cd}	12.3 ± 2.1^{cd}	46.6 ± 6.5^{b}	151.7 ± 5.4^{ab}	67.0 ± 6.0^{b}	2.6 ± 0.2^{b}	11.0 ± 0.6^{b}
	Total	36.0 ± 2.8	29.9 ± 2.7	24.3 ± 2.2	54.3 ± 3.2	166.1 ± 4.4	76.6 ± 2.5	3.0 ± 0.1	12.9 ± 0.4
P among bulls		< 0.01	< 0.01	< 0.01	< 0.05	< 0.05	< 0.05	< 0.01	< 0.01
P between TPS		< 0.01	< 0.01	0.07	0.15	0.12	< 0.05	0.71	0.72
P of TPS x bulls		0.30	0.55	0.96	0.97	0.64	0.70	0.96	0.54

Note: Different small superscripts indicate a significant difference among bulls, respectively ($P < 0.05$, $n = 6$). P values from two-way ANOVA analysis ($n = 6$) are shown the bottom of the table.

Abbreviations: VSL, velocity straight line; VCL, velocity curved line; VAP, velocity average path; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; SEM, standard error of the mean.

3.4 Discussion

For long decades, frozen semen of domestic animals has been mostly thawed around body temperature, from 37 to 39°C (Correa et al., 1996; Mehmood et al., 2017; Ogata et al., 2022; Ömür, 2022). Currently, frozen bull semen has been thawed by immersing it in water at 37°C for 30 seconds or at 40°C for 7 seconds (Faezah et al., 2012). Since the harmful temperature zone in frozen spermatozoa is often occurred critically, due to the deleterious recrystallization of intracellular ice, between -60 and 0°C (around the melting point, switching from glassy to liquid phase) during thawing, it needs to pass through the dangerous temperature range as soon as possible. However, our observation demonstrated when frozen bull semen was thawed in water at 70°C, the temperature inside the straw rose from -196 to 0°C significantly faster than the other groups (37 or 39°C) whereas no significant differences when the straws were thawed in water at 37 and 39°C. Besides, mammalian spermatozoa are also known to be susceptible to detrimental shock temperature ranges (< 15°C) (Pursel et al., 1973). It has also been demonstrated that the positive effects of rapid thawing methods could come from passing faster through not only the deleterious temperature zone from -60°C to the melting point but also the warm shock temperature range to below 15°C (Athurupana et al., 2015a). In our results, the estimated average time for the temperature change inside the straw to reach from -196 to 15°C was much faster when the frozen semen was thawed in water at 70°C than that of thawing at 37 or 39°C. Furthermore, the time required for warming from -196 to 37°C was significantly faster when thawed in water at 70°C than at 37 and 39°C. Therefore, when frozen semen was thawed at 37 and 39°C, as compared with thawing at 70°C, spermatozoa inside the straw seem to expose longer to the dangerous temperature range and also to the low temperatures outside the physiological range. Furthermore, it has been well known that changes in temperature, osmotic stress, toxicity of glycerol and formation of ice crystals during not only freezing but also thawing process make damage spermatozoa (Hammerstedt et al., 1990; Samper et al., 1991; Lessard et al., 2000; Cerolini et al., 2001), resulting in less sperm viability (Royere et al., 1996). In the present study, since the frozen semen thawed at 37, 39 and 70°C was not diluted with the fresh medium until totally 60 seconds after thawing, the condition exposed to osmotic stress and glycerol was similar in all groups. Therefore, three thawing rates in the present study may make differences in ice crystal reformation within the semen and the effects of exposure to non-physiological temperatures on thawing spermatozoa.

Rapid thawing did not seem to have a firm evaluation, with a report that viability and acrosome integrity of bull spermatozoa did not differ between thawing at 60°C for 8 seconds and 37°C for 30 seconds (Al-Badry, 2012) while another report demonstrated that rapid thawing at 65 - 70°C for 6 - 7 seconds maintained higher viability and motility of frozen bull spermatozoa compared with thawing at 35°C for 20 seconds (Lyashenko, 2015). Interestingly, the percentages of sperm viability at 2 and 4 hours incubation at 37°C after thawing at 60°C for 8 seconds were significantly higher than those of thawing at 37°C 30 seconds although no significant differences were found at immediately after thawing and at 0 hour (Al-Badry, 2012). Similar better results have been reported in different species, including pigs (Tomás et al., 2014; Athurupana et al., 2015a), horses (Snoeck et al., 2012), and buffaloes (Ahmad, 1984; Dhami et al., 1996; Rastegarnia et al., 2013). In the present study, not only the viability but also the percentage of spermatozoa with high MMP were significantly higher when frozen straws from one bull semen were thawed in water at RT70, as compared with thawing at CT37 or CT39. It was also confirmed similar results of significant differences at 0 and 3 hours after thawing when frozen semen straws from totally 4 bulls were thawed at RT70 and CT39, which is in line with other recent observations (Tomás et al., 2014; Lyashenko, 2015). In addition, the interactions between the thawing methods and four bulls on the viability and MMP (at 0 and 3 hours) did not show any significant statistical effects, so the means displayed separately correspond to each of the effects (Tomás et al., 2014). Since we also observed that the percentage of spermatozoa with intact acrosome did not differ among different thawing temperatures (CT37, CT39 and RT70), there does not appear to be a difference in effect between rapid and conventional thawing methods examined that would damage the acrosome membrane. Furthermore, since observation by using both JC-1 and PI was very hard to detect the percentage of “live” spermatozoa with high MMP due to the interference of fluorescence wavelengths, we assessed the percentage of “live” spermatozoa with relatively high ROS contents by using DCFH-DA and PI in flow cytometry. The results showed that although the percentages in “live” cells with high ROS level were not different after thawing at different temperatures, those of all spermatozoa were significantly lower when frozen semen was thawed at RT70 as compared with at CT37 or CT39. These results suggest that rapid thawing method (RT70) maintains the viability of frozen bull spermatozoa rather than conventional thawing at 37 or 39°C, and conventional thawing may induce more oxidative stress related to ROS production in spermatozoa. Although intracellular ROS production has been reported to be

induced even during and/or after the thawing process of frozen spermatozoa (Gürler et al., 2016), therefore, it is also possible that our results indicate that intracellular ROS level increased during sperm mortality, rather than that ROS elevation induced sperm mortality (Correa et al., 1996).

Our present results on CASA evaluation of frozen semen derived from four bulls revealed that the percentages of post-thawed motility (total and progressive motility) and VAP at 0 hour after thawing were higher in rapid thawing by transient exposure to 70°C water (RT70), which was consistent with the previous results (Nur et al., 2003; Lyashenko, 2015), but was contrasted with an other one (Yilmaz et al., 2019). In our study, we did not find any significant differences in some kinetic parameters at 0 hour after thawing, such as VSL, VCL, ALH, and BCF. Especially, no differences in ALH and BCF may suggest that kinetics itself of motile spermatozoa is not affected by the thawing temperatures examined (37, 39, and 70°C), whereas higher viability and motility are obtained after thawing at RT70 rather than at CT37 or CT39. It was also confirmed the maintenance of these parameters with similar results up to 3 hours after thawing which is coincident with other previous investigations (Tomás et al., 2014; Lyashenko, 2015). Additionally, we found that the main effects could come from the main experimental factors themselves (thawing conditions or bulls) on motility parameters due to no significant statistical differences in the interactions (Tomás et al., 2014) (at 0 and 3 hours). As described above, furthermore, since the percentage of spermatozoa with high MMP was also significantly different between CT39 and RT70 at 0 and 3 hours after thawing, our results on sperm motility could be speculated to reflect positively the state of mitochondria in spermatozoa thawed under the RT70 condition.

In this study, the percentage of spermatozoa with intact acrosome did not differ when frozen semen was thawed in water at 37, 39, and 70°C, in contrast to a previous report that rapid thawing improves the acrosome integrity of frozen spermatozoa (Dhami et al., 1992). Furthermore, when we observed the distribution of PLCZ1 in frozen-thawed spermatozoa, the result was also not different among spermatozoa thawed at CT37, CT39, and RT70. Previous studies reported that the freezing-thawing process could change expression of important proteins of spermatozoa (Rezaie et al., 2021). PLCZ1, known as a sperm-derived oocyte-activating factor (Cooney et al., 2010), is a kind of soluble cytosolic protein (Nomikos et al., 2013), and the distribution is known to be affected by not only the

progression of capacitation and acrosome reaction (Mejía-Flores et al., 2017) but also cryopreservation (Kashir et al., 2011; Moreau et al., 2019) due to a damaged membrane and/or a loss of integrity (Lasso et al., 1994; Hammadeh et al., 2001). In fact, during cryopreservation, membrane lipids are damaged, leading to instability of the membrane and reduction of many proteins, which are important for sperm fertility (Hezavehei et al., 2021). In the present study, PLCZ1 was detected at the acrosome region in the majority of frozen-thawed bull spermatozoa, which was in agreement with a previous study (Kasimanickam et al., 2012) because its synthesis was demonstrated as an integrated part of the acrosome during the Golgi phase of spermiogenesis (Aarabi et al., 2012). In rodents and humans, PLCZ1 has been detected in the acrosome region of intact spermatozoa (Grasa et al., 2008; Young et al., 2009; Ferrer-Vaquer et al., 2016), although other reports have been observed in both regions of acrosome and equatorial segment of sperm head (Bedford-Guaus et al., 2011; Durban et al., 2015). Combining the evidence that the percentage of spermatozoa with intact acrosome did not differ among different thawing protocols (CT37, CT39, and RT70), therefore, our results suggest that the distribution of PLCZ1 seems to be similarly maintained in spermatozoa under three thawing conditions we examined.

In conclusion, rapid thawing (RT70) rather than conventional thawing conditions (CT37 or CT39) is recommended to make significant differences in the viability, motility, and MMP of frozen bull spermatozoa, whereas no differences are found between CT37 and CT39. The beneficial effects of rapid thawing could be due to shorter exposure to temperatures from -196°C to the physiological temperature range, and consequently less damage of the biological membrane associated with mitochondrial health.

CHAPTER 4

NEGATIVE CORRELATIONS OF MITOCHONDRIAL DNA COPY NUMBER IN COMMERCIAL FROZEN BULL SPERMATOZOA WITH THE MOTILITY PARAMETERS AFTER THAWING

Abstract

The purpose of the current study was to investigate the relationship between mitochondrial content of commercial frozen-thawed bull spermatozoa and motility. Firstly, mitochondrial DNA copy number per spermatozoon (MDCN), mitochondrial content (MC), the percentage of spermatozoa with high mitochondrial membrane potential (HMMP), intracellular reactive oxygen species (ROS) and motility parameters of frozen-thawed spermatozoa derived from five bulls were determined by using qPCR, flow cytometry and CASA, respectively, and analyzed the relationships. Results showed that all parameters examined, including MDCN, MC, HMMP, ROS and motility indicators, significantly differed among frozen spermatozoa from different bulls. Both MDCN and MC were negatively correlated with HMMP and motility indicators, but positively with ROS, of course, whereas there was a highly positive relationship between MDCN and MC. Secondly, when MDCN and MC were examined in frozen spermatozoa prepared at different points in the lives of four bulls, those did not correlate overall throughout their lives (1.3-14.3 years old), but did correlate significantly in two sires. From these results, we conclude that MDCN and MC of frozen spermatozoa differ among sires, and are negatively correlated with HMMP and sperm motility parameters, probably due to mitochondrial oxidative stress resulted in the presence of ROS, demonstrating that these appear to be useful markers to assess sires' spermatozoa. It should be noted that the MDCN and MC of bull spermatozoa may not vary overall with the age of the sire, whereas those changes with age in some individuals and may affect sperm motility.

4.1 Introduction

Mitochondria are an important organelle to have multiple functions, including synthesis of adenosine triphosphate (ATP), production of intracellular reactive oxygen species (ROS), calcium signaling, thermogenesis and apoptosis (Durairajanayagam et al., 2021). Although energy production in bull spermatozoa appears to be carried out not only

through the oxidative phosphorylation in the midpiece (mitochondria) but also through the anaerobic glycolysis in the principle piece (Losano et al., 2017), sperm mitochondria still play an important role in energy production for progressive motility, and other functions, such as capacitation, hyper-activation and acrosome reaction (Mukai and Travis, 2012). In fact, mitochondrial membrane potential has been reported to be a potent indicator of sperm motility (Agnihotri et al., 2016). Mitochondrial membrane potential and DNA integrity in buffalo (Kadirvel et al., 2009) and human spermatozoa (Jing et al., 2023) seem to be significantly associated with the presence of intracellular ROS, since oxidative stress induced by an overproduction of ROS in mitochondria (Gürler et al., 2016; Mislei et al., 2020; Nowicka-Bauer and Nixon, 2020) could impair plasma membrane, mitochondrial homeostasis, and consequent penetrability of spermatozoa (Bahmyari et al., 2020).

On the other hand, the mitochondrial DNA copy number (MDCN) and mitochondrial content (MC) per spermatozoon are known to drastically reduce through mammalian spermatogenesis, especially between the late spermatocyte and mature spermatozoa stages (Otani et al., 1988; Gu et al., 2019; Boguenet et al., 2021). Recently, a study on the MDCN from two groups of bulls separated by the artificial insemination performance and the sperm parameters determined by using CASA has reported a higher copy number in the superior scoring bull group (Madeja et al., 2021). However, MDCN has been known to be negatively correlated with motility parameters in stallion (Orsztynowicz et al., 2016; Darr et al., 2017) and boar spermatozoa (Guo et al., 2017), as well as clinical studies in human (May-Panloup et al., 2003; Song and Lewis, 2008; Tian et al., 2014; Wu et al., 2019a; Boguenet et al., 2022). If MC is, as reported in equine, swine and human, negatively correlated with sperm motility, then an incomplete reduction in MC during spermatogenesis would have a negative effect on the sperm motility. However, only bull spermatozoa may require a contrary interpretation, although the previously reported study with bovine sperm was conducted in two groups of cows (Madeja et al., 2021) and did not reveal correlations for each individual. Furthermore, although it has been demonstrated that motility and fertility of bull spermatozoa could decrease with the age of sires (Hallap et al., 2006), it is also not clear whether the ability to regulate MDCN and MC to a constant level varies across the bulls' lives.

Therefore, the objective of the present study was to determine (1) what correlation exists between MDCN or MC and the percentage of spermatozoa with high mitochondrial

membrane potential (HMMP), ROS or sperm motility indicators in commercial frozen-thawed spermatozoa from multiple sires, and (2) if MDCN, MC, HMMP and motility parameters vary among spermatozoa collected at various times during the lives of sire bulls.

4.2 Materials and Methods

4.2.1 Chemicals, media and frozen semen samples

Unless otherwise indicated, all chemicals used in this study were obtained from Sigma-Aldrich Japan K.K. (Tokyo, Japan). The basic media used for washing and manipulating the frozen-thawed bull spermatozoa was modified TL-HEPES-PVA or PBS solution (PBS only used for washing and pelleting sperm samples again in DNA extraction protocol for mtDNA copy number analysis).

Commercial frozen semen derived from totally nine Japanese Black bulls (prepared at 1.3-14.3 years old, bulls A-I) with proven excellent fertility quality and prepared in 0.5 mL straws through the national standard protocols (Hamano, 2016) was obtained from a local public AI center, the Okayama Prefectural Center for Animal Husbandry and Research.

Straws of frozen semen were thawed individually in water bath at 70°C for 8 seconds and then stabilized at 39°C for 52 seconds. Immediately, the semen was washed three times with modified TL-HEPES-PVA by centrifugation at 700 ×g for 3 min at 39°C. Thawed spermatozoa were re-suspended in pre-warmed TL-HEPES-PVA at a concentration of 1 x 10^8 cells/mL and used in each experiment.

4.2.2 Experimental design

4.2.2.1 Experiment 4.1: Relationships between MDCN, MC, HMMP, ROS and motility parameters of frozen-thawed spermatozoa from five bulls

The frozen semen straws, which had been prepared using the same ejaculate from each of five sire bulls (A-E), were used in this experiment. After thawing and washing, spermatozoa were evaluated for MDCN, MC, HMMP, ROS and conventional motility parameters. Correlations among those were also examined. Experiments were replicated 6 times with different frozen straws from each bull semen in all experimental groups.

4.2.2.2 Experiment 4.2: MDCN, MC, HMMP and motility parameters of frozen-thawed semen prepared at various points in the lives of four bulls

Frozen semen straws prepared at three different times during the lifetime of each of four bulls (F-I) were used in this experiment. Thawed and washed spermatozoa were evaluated for the parameters described above to determine if MDCN and MC in spermatozoon varied across the lifetime of the bulls and affected the motility. After thawing and washing, spermatozoa were evaluated for MDCN, MC, HMMP and conventional motility parameters. Experiments were replicated 5 times using different frozen straws at each time point during the lives of the bulls.

4.2.3 Statistical analysis

Data from five or six replicated trials were evaluated using one-way analysis of variance (ANOVA) in GraphPad Prism 8.3 statistical software (GraphPad Software Inc., San Diego, CA, USA). Pearson correlation coefficients were applied to characterize relationships among MDCN, MC, HMMP, ROS and conventional motility parameters in sperm samples. Since all percentage data in the current study consequently distributed within 10-90%, those data were not subjected to the arc-sin transformation for fitting to a normal distribution before analyses. All data were expressed as the mean ± SEM. Findings were considered significantly different at $P < 0.05$ and, when there was a significant effect, values were compared with a Turkey's multiple range post hoc test.

4.3 Results

4.3.1 Relationships between MDCN, MC, HMMP, ROS and motility parameters of frozen-thawed spermatozoa from five bulls (experiment 4.1)

As shown in Tables 4.1 and 4.2, not only MDCN, MC, HMMP and ROS, but also a majority of motility parameters, such as TM, PM, RPM, VCL, WOB, ALH and BCF, significantly differed ($P < 0.01$) among commercial frozen-thawed spermatozoa from five bulls, whereas no significant differences were found in VSL, VAP, LIN and STR (Table 4.2). When correlation matrix analyses were performed using these data (Table 4.3), MDCN had a significant positive correlation with MC ($r = 0.45$, $P < 0.05$), but HMMP had a negative correlation with MDCN ($r = -0.59$, $P < 0.01$) and MC ($r = -0.66$, $P < 0.01$). Both MDCN and MC were found to have a significant negative relationship ($P < 0.05$) with a majority of

motility parameters, except for VSL (r = -0.35, P = 0.06), VAP (r = -0.28, P = 0.13), LIN (r = -0.01, P = 0.95), STR (r = -0.26, P = 0.16) and WOB (r = 0.33, P = 0.08) in MC, whereas MDCN and MC had positive correlations with BCF (r = 0.37, P < 0.05 and r = 0.50, P < 0.01, respectively). Contrary to the characteristics of MDCN and MC, HMMP was found to have significantly positive correlations (P < 0.01) with a majority of motility parameters, except for VSL (r = 0.31, P = 0.10) and VAP (r = 0.30, P = 0.10), whereas it was a negative correlation with BCF (r = -0.73, P < 0.01). Whereas ROS had positive correlations with MDCN (r = 0.41, P < 0.05) and MC (r = 0.58, P < 0.01), it was significantly negative correlations with HMMP (r = -0.84, P < 0.01) and a majority of motility parameters, except for VSL (r = -0.30, P = 0.11), VAP (r = -0.31, P = 0.10), LIN (r = 0.07, P = 0.70), STR (r = -0.30, P = 0.11) and WOB (r = 0.34, P = 0.06). Within motility parameters, only VSL was not found any correlations with many parameters, except for VCL (r = 0.55, P < 0.01), VAP (r = 0.43, P < 0.05), LIN (r = 0.75, P < 0.01) and STR (r = 0.62, P < 0.01).

4.3.2 MDCN, MC, HMMP and motility parameters of frozen-thawed semen prepared at various points in the lives of four bulls (experiment 4.2)

Interestingly, in only two of the four bulls examined, significant differences were observed (P < 0.05) in MDCN, MC, HMMP (one bull increased MDCN and MC with age and decreased HMMP, while another did the opposite; Table 4.4), and even a majority of motility parameters (P < 0.05), except for VSL, VAP, STR and WOB (from these two bulls), or LIN and ALH (from only one bull), among frozen-thawed sperm samples prepared at three different points in bull's lives (Table 4.5). When correlation matrix analyses were performed using these data (Table 4.6), BAC in the present study (from 1.3 to 14.3 years old) did not affect any with MDCN, MC, HMMP and all motility parameters examined. Results of correlations among MDCN, MC, HMMP, and motility parameters in this experiment (Table 4.6) were similar with those in Table 4.3. The scattering plots for MDCN and MC in frozen-thawed spermatozoa prepared at three different points during four bulls' lives again showed no clear trend between the age of the bulls and MDCN or MC at the time of semen collection (Figure 4.1). Furthermore, even when the age of bulls at semen collection was classified into three groups (< 5 years old, 5-10 years old and ≥ 10 years old), there were no significant differences in both MDCN (< 5 years old, 1.10 ± 0.05; 5-10 years old, 1.22 ± 0.05; ≥ 10 years old, 1.12 ± 0.04; P = 0.15) and MC (< 5 years old, 90.4 ± 1.7; 5-10 years old, 93.6 ± 1.4; ≥ 10 years old, 89.3 ± 1.6; P = 0.11).

Table 4.1 mtDNA copy number (MDCN), mitochondrial content (MC), high mitochondrial membrane potential rate (HMMP) and ROS detection rate (ROS) of frozen-thawed spermatozoa derived from five bulls

Bulls	MDCN*	MC (MFI)	HMMP** (%)	ROS** (%)
A	1.00 ± 0.04^{c}	84.0 ± 0.4^{c}	83.0 ± 0.9^{a}	24.7 ± 1.8^{c}
B	1.03 ± 0.04^{c}	86.6 ± 1.1^{bc}	79.5 ± 2.5^{a}	29.3 ± 3.1^{c}
C	1.36 ± 0.05^{a}	98.6 ± 3.2^{a}	54.4 ± 2.3^{b}	39.5 ± 2.8^{b}
D	1.28 ± 0.08^{ab}	94.6 ± 4.1^{abc}	50.2 ± 2.4^{b}	48.5 ± 1.9^{ab}
E	1.12 ± 0.07^{bc}	96.7 ± 2.9^{ab}	53.3 ± 1.2^{b}	50.9 ± 2.1^{a}

*MDCN was expressed as a relative amount to the mtDNA copy number in spermatozoa collected from sire bull A.

**HMMP and ROS were shown as percentages of spermatozoa with high mitochondrial membrane potential and with detected intracellular ROS, respectively.

Different superscripts indicate significant differences within the same column ($P < 0.01$, $n = 6$). Abbreviation: MFI, mean fluorescence intensity.

Table 4.2 Motility parameters of frozen-thawed spermatozoa derived from five bulls

Bull	TM (%)	PM (%)	RPM (%)	VSL (μm/s)	VCL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)	ALH (μm)	BCF (Hz)
A	78.5 ± 1.5^{a}	73.2 ± 1.9^{a}	63.9 ± 1.8^{a}	68.9 ± 2.7	204.2 ± 5.5^{ab}	81.4 ± 2.0	34.0 ± 2.0	84.5 ± 2.9	39.9 ± 0.7^{b}	4.3 ± 0.1^{a}	8.0 ± 0.3^{b}
B	71.0 ± 2.3^{a}	64.9 ± 3.4^{a}	55.6 ± 2.5^{a}	70.3 ± 3.8	205.4 ± 7.3^{a}	83.2 ± 3.7	34.5 ± 2.2	84.0 ± 3.3	40.6 ± 1.5^{ab}	4.3 ± 0.2^{a}	7.5 ± 0.4^{b}
C	37.6 ± 1.6^{b}	31.6 ± 1.7^{b}	27.2 ± 1.9^{b}	60.7 ± 3.8	178.8 ± 5.9^{b}	75.6 ± 1.7	33.9 ± 1.6	80.1 ± 4.4	42.5 ± 1.7^{ab}	3.1 ± 0.1^{c}	11.7 ± 0.4^{a}
D	41.9 ± 2.5^{b}	37.2 ± 2.8^{b}	31.5 ± 2.4^{b}	60.8 ± 2.0	183.8 ± 4.3^{ab}	76.0 ± 2.0	33.1 ± 0.6	79.9 ± 1.3	41.4 ± 0.4^{ab}	3.7 ± 0.1^{b}	9.2 ± 0.3^{b}
E	39.2 ± 1.5^{b}	33.4 ± 1.4^{b}	27.7 ± 1.9^{b}	66.3 ± 5.1	179.4 ± 7.7^{b}	80.7 ± 4.4	36.7 ± 1.3	81.7 ± 2.0	44.9 ± 0.9^{a}	3.2 ± 0.1^{c}	11.6 ± 0.7^{a}

Different superscripts indicate significant differences within the same column ($P < 0.01$, $n = 6$).

Abbreviations: TM, total motility; PM, progressive motility; RPM, rapid progressive motility; VSL, velocity straight line; VCL, velocity curved line; VAP, velocity average path; LIN, the percentage of linearity; STR, the percentage of straightness; WOB, wobble index; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

Table 4.3 Correlation coefficients from linear relationship matrix analysis of mitochondrial characteristics and motility parameters of frozen-thawed spermatozoa derived from five bulls

	MDCN	MC	HMMP	ROS	TM	PM	RPM	VSL	VCL	VAP	LIN	STR	WOB	ALH	BCF
MDCN	1.00														
MC	0.45*	1.00													
HMMP	-0.59**	-0.66**	1.00												
ROS	0.41*	0.58**	-0.84**	1.00											
TM	-0.61**	-0.67**	0.93**	-0.80**	1.00										
PM	-0.60**	-0.67**	0.92**	-0.78**	0.99**	1.00									
RPM	-0.59**	-0.65**	0.91**	-0.78**	0.99**	0.99**	1.00								
VSL	-0.38*	-0.35	0.31	-0.30	0.34	0.33	0.33	1.00							
VCL	-0.47*	-0.53**	0.58**	-0.57**	0.68**	0.68**	0.71**	0.55**	1.00						
VAP	-0.39*	-0.28	0.30	-0.31	0.37*	0.37*	0.41*	0.43*	0.69**	1.00					
LIN	-0.11	-0.01	-0.07	0.07	-0.11	-0.13	-0.16	0.75**	-0.14	-0.03	1.00				
STR	-0.27	-0.26	0.27	-0.30	0.24	0.23	0.21	0.62**	0.31	-0.09	0.51**	1.00			
WOB	0.12	0.33	-0.36	0.34	-0.42*	-0.42*	-0.41*	-0.21	-0.46*	0.33	0.12	-0.54**	1.00		
ALH	-0.55**	-0.64**	0.76**	-0.68**	0.83**	0.81**	0.83**	0.24	0.69**	0.40*	-0.24	0.19	-0.40*	1.00	
BCF	0.37*	0.50**	-0.73**	0.59**	-0.74**	-0.72**	-0.70**	-0.14	-0.38*	0.03	0.12	-0.21	0.51**	-0.75**	1.00

Abbreviations: MDCN, relative mtDNA copy number; MC, mitochondrial content; HMMP, percentage of spermatozoa with high mitochondrial membrane potential; ROS, percentage of spermatozoa with detected intracellular ROS; TM, total motility; PM, progressive motility, RPM, rapid progressive motility; VSL, velocity straight line; VCL, velocity curved line; VAP, velocity average path; LIN, the percentage of linearity; STR, the percentage of straightness; WOB, wobble index; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

Significant correlations were represented by * ($P < 0.05$) and ** ($P < 0.01$).

Table 4.4 mtDNA copy number (MDCN), mitochondrial content (MC) and high mitochondrial membrane potential rate (HMMP) of frozen-thawed spermatozoa prepared at three different time points in the lives of four bulls

Bulls	Bull's age at semen collection (years old)	MDCN*	MC (MFI)	HMMP (%)
F	2.3	1.00 ± 0.05	87.3 ± 2.2	73.0 ± 2.4
	8.7	1.05 ± 0.07	87.7 ± 3.7	69.1 ± 1.3
	14.3	1.12 ± 0.10	89.3 ± 2.3	67.1 ± 2.3
G	7.7	1.10 ± 0.10	88.3 ± 1.8	67.5 ± 4.5
	10.0	1.18 ± 0.09	90.1 ± 3.3	61.2 ± 4.4
	12.1	1.17 ± 0.10	89.7 ± 5.3	61.8 ± 2.1
H	2.7	1.29 ± 0.04^{a}	96.5 ± 2.5^{a}	49.2 ± 4.2^{b}
	7.4	1.34 ± 0.13^{a}	96.9 ± 1.5^{a}	48.8 ± 2.6^{b}
	11.2	0.99 ± 0.06^{b}	88.2 ± 1.8^{b}	75.0 ± 2.1^{a}
I	1.3	1.01 ± 0.09^{b}	87.5 ± 2.5^{b}	69.2 ± 3.1^{a}
	6.2	1.25 ± 0.07^{ab}	96.8 ± 1.1^{a}	49.4 ± 2.1^{b}
	9.6	1.38 ± 0.11^{a}	98.2 ± 3.3^{a}	43.6 ± 2.9^{b}

*MDCN was expressed as a relative amount to the mtDNA copy number in spermatozoa collected when sire bull F was 2.3 years old.

Different superscripts indicate significant differences within each bull in the same column ($P < 0.05$, $n = 5$).

Abbreviations: MFI, mean fluorescence intensity.

Table 4.5 Motility parameters of frozen-thawed spermatozoa prepared at three different time points in the lives of four bulls

Bulls	Bull's age at semen collection (years old)	TM (%)	PM (%)	RPM (%)	VSL (μm/s)	VCL (μm/s)	VAP (μm/s)	LIN (%)	STR (%)	WOB (%)	ALH (μm)	BCF (Hz)
F	2.3	62.9 ± 1.9	55.6 ± 1.8	47.0 ± 1.9	58.8 ± 1.1	210.7 ± 4.1	83.4 ± 1.2	27.9 ± 0.2	70.6 ± 1.1	39.6 ± 0.6	4.2 ± 0.1	8.1 ± 0.1
	8.7	58.9 ± 3.3	51.0 ± 3.9	43.3 ± 3.5	60.6 ± 2.5	196.5 ± 7.6	83.6 ± 3.1	30.8 ± 0.4	72.4 ± 0.6	42.6 ± 0.5	3.9 ± 0.1	8.4 ± 0.2
	14.3	58.1 ± 2.4	50.0 ± 2.3	43.3 ± 2.7	57.3 ± 3.6	193.5 ± 2.3	81.4 ± 2.0	29.7 ± 2.0	70.2 ± 2.9	42.1 ± 1.3	3.9 ± 0.1	8.1 ± 0.4
G	7.7	58.2 ± 3.1	52.3 ± 2.8	47.0 ± 2.1	70.5 ± 2.1	215.3 ± 7.0	91.8 ± 4.8	33.0 ± 2.0	77.4 ± 3.5	42.8 ± 2.3	4.2 ± 0.2	9.6 ± 0.3
	10.0	57.3 ± 6.5	51.1 ± 6.8	43.7 ± 7.6	64.5 ± 4.1	206.3 ± 7.9	88.6 ± 5.8	31.5 ± 1.5	73.0 ± 2.2	43.3 ± 2.2	4.0 ± 0.2	9.8 ± 0.2
	12.1	57.7 ± 2.8	50.6 ± 3.0	42.1 ± 2.6	63.2 ± 0.6	191.7 ± 2.3	85.0 ± 1.3	33.0 ± 0.5	74.4 ± 0.8	44.4 ± 0.9	3.8 ± 0.1	10.2 ± 0.4
H	2.7	43.6 ± 3.6^{b}	36.5 ± 4.3^{b}	30.2 ± 5.0^{ab}	61.8 ± 3.7	180.5 ± 4.1^{ab}	82.6 ± 4.4	34.3 ± 2.0^{a}	74.8 ± 1.6	45.8 ± 2.2	3.3 ± 0.2	11.1 ± 0.8^{a}
	7.4	38.7 ± 2.0^{b}	30.0 ± 2.4^{b}	23.9 ± 2.8^{b}	55.2 ± 3.0	170.5 ± 5.2^{b}	71.9 ± 1.6	32.4 ± 1.7^{ab}	76.4 ± 3.2	42.2 ± 1.1	3.3 ± 0.2	10.2 ± 1.0^{ab}
	11.2	63.3 ± 1.7^{a}	54.1 ± 1.7^{a}	41.6 ± 2.4^{a}	50.7 ± 3.2	187.4 ± 3.0^{a}	75.5 ± 2.3	27.1 ± 1.6^{b}	67.6 ± 5.8	40.3 ± 1.4	3.9 ± 0.1	7.8 ± 0.5^{b}
I	1.3	63.8 ± 2.8^{a}	55.4 ± 3.6^{a}	46.5 ± 2.5^{a}	58.3 ± 3.3	199.5 ± 5.0^{a}	76.5 ± 4.0	29.2 ± 1.5	76.2 ± 5.2	38.6 ± 2.8	3.9 ± 0.1^{a}	9.1 ± 0.3^{b}
	6.2	47.4 ± 3.1^{b}	38.5 ± 4.2^{b}	32.4 ± 4.1^{b}	61.2 ± 5.3	172.2 ± 11.1^{ab}	78.0 ± 5.6	35.5 ± 2.0	78.2 ± 1.9	45.3 ± 1.5	3.2 ± 0.2^{b}	10.7 ± 0.8^{ab}
	9.6	40.7 ± 4.3^{b}	32.4 ± 4.5^{b}	25.6 ± 3.9^{b}	56.5 ± 5.3	169.2 ± 6.6^{b}	74.7 ± 6.3	33.1 ± 2.1	75.4 ± 1.2	43.8 ± 2.4	2.8 ± 0.1^{b}	12.0 ± 0.8^{a}

Different superscripts indicate significant differences within each bull in the same column ($P < 0.05$, $n = 5$).

Abbreviations: TM, total motility; PM, progressive motility; RPM, rapid progressive motility; VSL, velocity straight line; VCL, velocity curved line; VAP, velocity average path; LIN, the percentage of linearity; STR, the percentage of straightness; WOB, wobble index; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

Table 4.6 Correlation coefficients from linear relationship matrix analysis of bull's age at semen collection, mitochondrial characteristics and motility parameters of frozen-thawed spermatozoa prepared at different time points in the lives of four bulls

	BAC	MDCN	MC	HMMP	TM	PM	RPM	VSL	VCL	VAP	LIN	STR	WOB	ALH	BCF
BAC	1.00														
MDCN	0.05	1.00													
MC	-0.05	0.36**	1.00												
HMMP	0.06	-0.46**	-0.55**	1.00											
TM	0.04	-0.45**	-0.49**	0.80**	1.00										
PM	0.03	-0.42**	-0.49**	0.79**	0.98**	1.00									
RPM	0.02	-0.39**	-0.42**	0.75**	0.93**	0.97**	1.00								
VSL	-0.06	0.05	0.05	0.14	0.24	0.33**	0.43**	1.00							
VCL	-0.07	-0.29*	-0.39**	0.63**	0.60**	0.67**	0.70**	0.51**	1.00						
VAP	0.05	0.08	-0.07	0.35**	0.45**	0.53**	0.62**	0.75**	0.62**	1.00					
LIN	-0.03	0.02	0.01	-0.24	-0.21	-0.15	-0.07	0.72**	-0.22	0.15	1.00				
STR	-0.17	0.01	0.16	-0.25	-0.22	-0.19	-0.16	0.54**	-0.04	-0.13	0.65**	1.00			
WOB	0.12	0.11	0.23	-0.21	-0.05	-0.01	0.07	0.24	-0.24	0.60**	0.19	-0.07	1.00		
ALH	0.01	-0.41**	-0.46**	0.68**	0.72**	0.75**	0.75**	0.30*	0.78**	0.47**	-0.29*	-0.14	-0.21	1.00	
BCF	-0.10	0.50**	0.42**	-0.56**	-0.39**	-0.30*	-0.23	0.35**	-0.28*	0.14	0.13	0.18	0.50**	-0.40**	1.00

Abbreviations: BAC, bull's age at semen collection; MDCN, relative mtDNA copy number; MC, mitochondrial content; HMMP, percentage of spermatozoa with high mitochondrial membrane potential; TM, total motility; PM, progressive motility, RPM, rapid progressive motility; VSL, velocity straight line; VCL, velocity curved line; VAP, velocity average path; LIN, the percentage of linearity; STR, the percentage of straightness; WOB, wobble index; ALH, amplitude of lateral head displacement; BCF, beat cross frequency.

Significant correlations were represented by * ($P < 0.05$) and ** ($P < 0.01$).

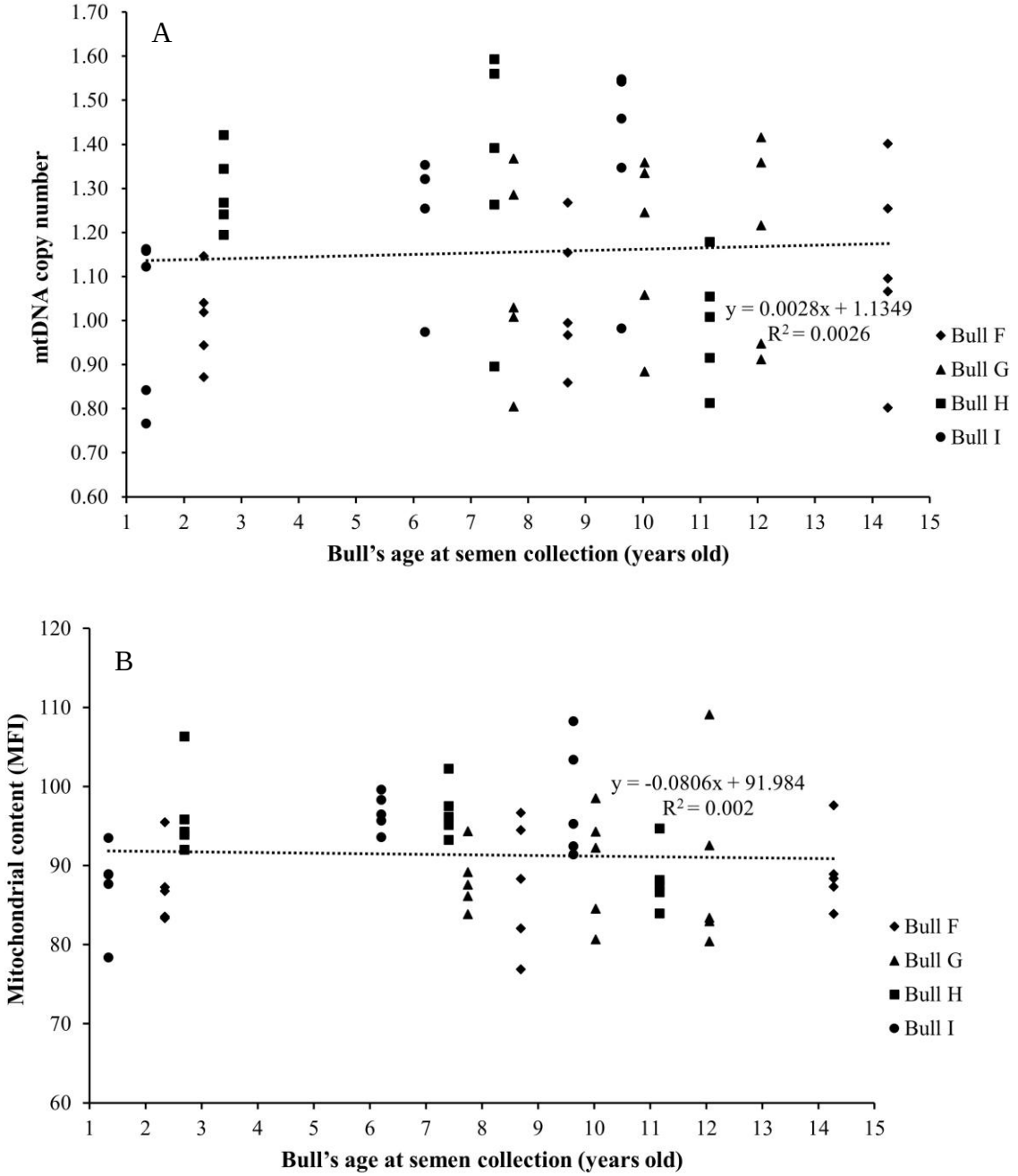

Figure 4.1 The scattering plots for the relation between (A) the mtDNA copy number or (B) mitochondrial content in frozen-thawed spermatozoa from four bulls (F-I) and the age of bulls when their semen were collected and cryopreserved. The dot points represent the one value in the replicated data.

4.4 Discussion

In the present study, we assessed MC by staining sperm mitochondria with MTG and then measuring their fluorescence by flow cytometry, according to previous reports (Kao et al., 2004; Guo et al., 2017; Madeja et al., 2021). However, there have been few reports on how accurately this method can assess MC, including comparative assessments with MDCN and other methods. Our current results demonstrated that MC had a significant positive correlation with MDCN determined by qPCR in frozen-thawed bull spermatozoa, indicating that this simple method of measuring sperm MC by fluorescence intensity under flow cytometry made clear to be a sufficiently reliable sperm evaluation method. On the other hand, HMMP had significant negative correlations with both MDCN and MC. This result was consistent with a previous report demonstrating a negative relation between MDCN and HMMP in stallion spermatozoa (Orsztynowicz et al., 2016). In our current study, the percentage of spermatozoa detected intracellular ROS not only had significant positive correlations with both MDCN and MC but also had negative correlations with HMMP. Since the intracellular ROS seems to be strongly associated with HMMP and DNA integrity in buffalo (Kadirvel et al., 2009) and human spermatozoa (Jing et al., 2023), therefore, in spermatozoa with higher MDCN and MC, it is quite possible that oxidative stress due to excessive mitochondrial ROS production can significantly reduce sperm HMMP and motility. This interpretation is consistent with a previous report in human spermatozoa of patients with severe asthenozoospermia showing the relation between increased MDCN and elevated ROS level (Bonanno et al., 2016).

Furthermore, the current results in both experiments 1 and 2 demonstrated that both MDCN and MC had significant negative correlations with a majority of motility parameters in commercial frozen-thawed spermatozoa from a total of nine sires. Recently, an analysis of frozen bull semen, divided into two groups based on artificial insemination performance, reported that larger MDCN were observed in spermatozoa with higher motility (Madeja et al., 2021). Our current results were in contrast to their report (Madeja et al., 2021), but were consistent with previous reports in stallion (Orsztynowicz et al., 2016; Darr et al., 2017) and boar spermatozoa (Guo et al., 2017) and clinical studies in human (May-Panloup et al., 2003; Song and Lewis, 2008; Tian et al., 2014; Wu et al., 2019a; Boguenet et al., 2022). Therefore, it seems reasonable to conclude that both MDCN and MC are negatively correlated with sperm motility, similar to what has been reported in other species, rather than that only

bovine sperm require a reverse special interpretation. Since MDCN and MC drastically decrease through the mammalian spermatogenesis, especially between the late spermatocyte and mature spermatozoa stages (Otani et al., 1988; Gu et al., 2019; Boguenet et al., 2021), spermatozoa with relatively larger copy numbers and contents may be immature during the process of spermatogenesis. It has also been demonstrated that less mitochondrial and mtDNA contents did not affect the mitochondrial activity and conventional sperm motility (Darr et al., 2017; Guo et al., 2017; Boguenet et al., 2022). Again, in the present study, we found that the incidence of spermatozoa with detected ROS significantly correlated with both MDCN and MC positively and with HMMP negatively. Therefore, MDCN- and MC-rich spermatozoa may have further reduced mitochondrial membrane potential due to severe oxidative stress from relatively more ROS production, consequently resulting in lower sperm motility.

In the second experiment, when we observed whether MDCN, MC or HMMP in frozen-thawed bull spermatozoa varied among the time points when the semen was collected during bulls' lives (1.3-14.3 years old), there were significant variations in two of the four bulls examined (one bull increased MDCN and MC with age and decreased HMMP, while another did the opposite), but no significant variations overall. There were no correlation between bull's age at time of semen collection and MDCN, MC or HMMP, whereas both MDCN and MC differ with age at semen collection in a few individuals and may also affect sperm motility. To our knowledge, no study has examined whether MDCN, MC and HMMP in frozen bull spermatozoa from the same sires vary with the age of the sire at the time when the semen is collected. Our results are consistent with the results on sperm MDCN when collected from a large number of males of various ages in human (Song and Lewis, 2008) and stallion (Orsztynowicz et al., 2016; Darr et al., 2017). Although it has been demonstrated that motility and fertility of bull spermatozoa could decrease with the age of sires (Hallap et al., 2006; Salimiyekta et al., 2023), our current results showed that bull's age at semen collection did not correlate with all sperm motility parameters examined. Therefore, within the range of semen collection ages of the four Japanese Black bulls examined in the present study, the sperm MDCN and MC, as well as the HMMP and motility, do not appear to be affected by aging. Although some samples showed significant differences in sperm motility parameters when the semen was collected at three different time points in the lives of sire bulls in the second experiment. However, in the current study, which was designed using commercial frozen semen provided from a local AI center, we had limitations in

systematically matching the ages of the sire bulls. Further strict research may be required to clarify the relationship between the age of the bulls at the time of semen collection and MDCN/MC in more details.

In conclusion, MDCN and MC of commercial frozen-thawed spermatozoa differ among sire bulls and have significantly negative correlations with HMMP and a majority of sperm motility parameters, probably due to significant positive correlations with ROS. It should be noted that the MDCN and MC of commercially available frozen-thawed bull spermatozoa do not vary overall with the age of the sire from which the semen was collected, whereas they change with age in some individuals and may also affect sperm motility. Furthermore, the current simple method of measuring sperm MC by fluorescence intensity under flow cytometry is a sufficiently reliable sperm evaluation method, since there is a significant correlation with MDCN.

CHAPTER 5

GENERAL DISCUSSION

Artificial insemination (AI) using frozen-thawed bull semen has been the most widely used and has had the greatest impact on the genetic improvement of both dairy and beef cattle, as compared to other domestic animal species. However, the conception and pregnancy rates after AI in developed countries including Japan have been on a steady decline. Rapid genetic improvement of cattle could be thought to have caused this decline, it is considered necessary to develop advanced feeding management techniques and highly accurate estrus detection technologies suited to high-performance cattle. Furthermore, since no major improvements have been attempted in the process of freezing and thawing semen for a long time, the reconsidering techniques during the freezing and thawing process and examination of factors considered necessary for selecting bull sperm suitable for freezing and thawing would greatly contribute to improving the conception rate of frozen-thawed bovine sperm. Although low fertility of frozen-thawed spermatozoa is associated with many factors and aspects, higher quality parameters, especially motility indicators, of frozen bull semen after thawing play a central role in the success rate following AI, since they are found to have significantly positive correlations with bull fertility. On the other hand, several potentially important parameters associated with sperm mitochondria, such as mitochondrial membrane potential (MMP) or intracellular reactive oxygen species (ROS) presence, remarkedly affect the motility and viability of frozen-thawed bull spermatozoa, thus subsequent effect on sperm fertilizability. In fact, MMP is well known to be strongly positively correlated with the viability, motility, and fertilizability of spermatozoa, while an excessive ROS level can impair the plasma membrane, mitochondria homeostasis, motility, and consequent penetrability. It seems that more motile and viable frozen-thawed bull spermatozoa and better fertility can be achieved by controlling the intracellular ROS in spermatozoa. Therefore, further comprehensive investigations of the motility and other vital quality parameters as well as intracellular ROS of frozen-thawed bull spermatozoa under different conditions and their correlations are needed to further improve the current situation of the poor quality characteristics of bull semen after thawing.

Although there may be numerous potential factors influencing bull semen frozen in straws during the freezing-thawing procedure, it may be valuable to reconsider if there is

any space to reanalyze the effect of the thawing process on the quality of frozen-thawed bull semen to overcome existing disadvantages under conventional thawing methods and further improve the motility and other important parameters of frozen-thawed spermatozoa. Thawing is a vital process for frozen semen, to bring spermatozoa from the inactively frozen status to the physiological temperature reactivating metabolism for their key functions. However, the changes during the freezing-thawing process could deleteriously alter the status of the biological membrane of spermatozoa, and consequently may reduce the motility and viability of post-thawed spermatozoa. Up to now, most studies have just focused on the conventional parameters such as motility and viability of frozen-thawed bull spermatozoa as main indicators for the quality of frozen semen, while evaluation of other numerous pivotal parameters of post-thawed bull spermatozoa associated with semen quality and bull fertility has still been limited. In the first study, not only the motility and viability but also high MMP, acrosome integrity, ROS level, and PLCZ1 distribution were assessed to investigate the effects of thawing of frozen bull spermatozoa at different thawing temperatures. We observed that the time it took for the temperature change inside the straw to warm up from -196 to 15°C was nearly twice as faster when the frozen straw was thawed in 70°C water compared with 37 or 39°C. Regarding thawing speeds, many studies have demonstrated that spermatozoa inside frozen straw need to pass through the dangerous temperature range during thawing as soon as possible to reduce the deleterious injury to the organelles. Mammalian spermatozoa are well known to be susceptible to not only the harmful temperature zone with the deleterious recrystallization of intracellular ice (-60 to 0°C) but also detrimental shock temperature ranges (< 15°C). Therefore, when frozen semen was thawed at 37 and 39°C, as compared with thawing at 70°C, spermatozoa inside the straw seemed to expose longer to the dangerous temperature range and also to the low temperatures outside the physiological range.

To date, although it has still been reported that the viability and acrosome integrity of bull spermatozoa did not differ between thawing at 60 and 37°C, other reports have demonstrated that rapid thawing at higher temperatures for shorter durations maintained higher viability and motility of frozen bull, buffalo, horse and pig spermatozoa, as compared with conventional thawing temperatures. In the present study, in addition to the conventional post-thawed parameters such as motility and viability, the percentages of spermatozoa with high MMP and high ROS level were also significantly improved under rapid thawing protocol (RT70) as compared to conventional thawing methods (CT37 and CT39), although

there were no differences in acrosome integrity and PLCZ1 distribution among three thawing conditions. These results suggest that conventional thawing protocols may induce more oxidative stress related to ROS production in the spermatozoa, rather than rapid thawing method. Interestingly, although intracellular ROS production has been reported to be induced even during and/or after the thawing process of frozen spermatozoa, it is also possible from the study results that intracellular ROS level increases during sperm mortality, rather than that ROS elevation induced sperm mortality. It was also confirmed similar better results at 0 and 3 hours after thawing when frozen semen straws from a total of 4 bulls were thawed at RT70, as compared with those of CT39, although there were differences among bulls, which is in line with several recent observations. The findings related to acrosome status and PLCZ1 distribution suggest that the biological membrane and/or integrity at acrosome region seem to be similarly maintained in spermatozoa under three thawing conditions. Therefore, rapid thawing (RT70) should be applied to improve viability, motility and mitochondrial health of frozen bull spermatozoa rather than conventional thawing at 37 or 39°C. The positive effect of rapid transient thawing could be due to shorter exposure to temperatures from -196°C to the physiological temperature range, consequently less damage of the biological membrane associated with mitochondrial health and less oxidative stress associated with ROS production in spermatozoa.

Furthermore, mitochondria of spermatozoa, especially mitochondrial content (MC) and mtDNA copy number (MDCN), are universally known as the principal multitasking organelles for energy production and other numerous key physiological functions, thus significantly associating with the quality characteristics of frozen-thawed bull spermatozoa. However, the contradictory variations of mitochondrial content and mtDNA copy number per spermatozoon, as well as their relationships with semen quality have still been reported among different studies. In the second study, the MDCN, MC, HMMP, ROS and motility indicators of frozen-thawed spermatozoa were evaluated to determine their differences and relationships among spermatozoa from multiple sires and collected at various times during the lives of sire bulls. The results indicated that MC in frozen-thawed bull spermatozoa not only had highly negative correlations with HMMP and a majority of sperm motility parameters but also had significant positive correlations with MDCN and ROS. To our knowledge, no report has demonstrated the accuracy of the MC evaluation by fluorescence intensity with specific dyes through comparative assessments with other methods, although MTG has been used to determine MC in mammalian spermatozoa in several previous

studies. Therefore, the present study is the first to indicate that this simple method of measuring sperm MC by fluorescence intensity under flow cytometry appears to be a potently reliable sperm evaluation method. On the other hand, the current study revealed that both MDCN and MC not only had significantly negative correlations with HMMP and a majority of motility parameters in frozen-thawed bull spermatozoa but also had positive correlations with ROS. These findings are in contrast to the recently reported study in frozen bull spermatozoa from two groups of different semen quality separated by the AI performance, but are consistent with numerous previous reports in stallion and boar spermatozoa as well as clinical studies in humans. Since ROS are strongly associated with HMMP and DNA integrity, therefore, in bull spermatozoa with higher MDCN and MC, it is quite possible that oxidative stress due to excessive ROS production can significantly reduce sperm HMMP and motility, which is consistent with a previous report in human spermatozoa of patients with severe asthenozoospermia (spermatozoa typically characterized by low motility parameters) showing the relation between increased MDCN and elevated ROS level. It has also been demonstrated that less mitochondrial and mtDNA contents do not impact the mitochondrial activity and conventional motion characteristics, but MDCN- and MC-rich bull spermatozoa may have further reduced MMP due to severe oxidative stress under the presence of intracellular ROS, consequently leading to lower sperm motility. As a result, it seems reasonable to conclude that MDCN and MC are inversely correlated with the motility of frozen-thawed bull spermatozoa, similar to what has been reported in other species, rather than that only bovine sperm require a reverse special interpretation. Interestingly, bull spermatozoa with relatively larger copy numbers and contents may be immature during spermatogenesis and accompanied by severe status of oxidative stress from ROS presence since they are needed to drastically reduce through this process.

Recently, although it has been demonstrated that motility and fertility of bull spermatozoa could decrease with the age of sires, our current results showed that bull's age (1.3-14.3 years old) at semen collection did not correlate generally with all sperm motility parameters examined. There were also no correlations between bull's age at semen collection and MDCN, MC or HMMP, whereas both MDCN and MC differed at different time points in the lives of a few individuals and may also affect sperm motility. Besides, there were no significant differences in both MDCN and MC even when the age of bulls at semen collection was classified into three groups (< 5 years old, 5-10 years old and ≥ 10

years old). Our results on the relationship of bull's age with MDCN or MC are consistent with previous reports from a large number of males of various ages in humans and stallions. Therefore, within the range of semen collection ages of the four Japanese Black bulls examined in the present study, the sperm MDCN and MC as well as the HMMP and motility seem to be not influenced by aging. From these results, it is concluded that MDCN and MC of commercial frozen-thawed bull spermatozoa significantly differ among bulls, and have considerably inverse correlations with HMMP and sperm motility parameters, probably due to oxidative stress resulted in the presence of ROS, indicating that these appear to be potential predictors to evaluate the sire bulls' spermatozoa. It should be noted that MDCN and MC of commercially available frozen-thawed bull spermatozoa do not change overall according to the age in bull life, but significant changes occur in several individuals and consequently reduce sperm motility.

Overall, the results of the first series of the current studies indicate that there are rooms to further improve the viability and motility of frozen bull spermatozoa by controlling temperature during the thawing process, especially by allowing it to pass as quickly as possible through areas outside the physiological temperature range to minimize damage of the biological membrane associated with mitochondrial health and oxidative stress associated with ROS production in spermatozoa. In particular, this study showed that thawing temperature also affected sperm mitochondrial membrane potential, intracellular ROS and motility. However, further research will be needed to further clarify the details of the effects of thawing rate and exposure to non-physiological temperatures on sperm mitochondria. In addition, the findings of the second series of studies revealed that a positive correlation existed between the amount of sperm mitochondrial DNA/mitochondrial content and the amount of ROS, which in turn negatively affects the HMMP and motility indicators. The current study proposes that mitochondrial content will be a useful information in evaluating the reproductive activity of sire bulls. Finally, the findings of two studies in this dissertation indicate that it is still possible to provide more motile and viable frozen-thawed spermatozoa through further improvement of thawing conditions of frozen spermatozoa and selection of bulls using mitochondrial content as an indicator, and that fertility after artificial insemination can still be improved through modifying sperm thawing process and the bull selection conditions for less oxidative stress from intracellular ROS generation.

CHAPTER 6

RESEARCH SUMMARY

The low conception and pregnancy rates following AI with frozen-thawed bull semen are still the main concerns in the dairy and beef industry, although this technique has been demonstrated to provide many practical advantages and genetic improvements in cattle production. Although a steady decline in the fertility of frozen-thawed bull semen is associated with many factors and aspects, the poor quality parameters, especially motility, of bull semen frozen in straws after thawing significantly contribute to the failure rate after AI, since sperm motility parameters are found to have positive correlations with bull fertility. Low motility and other quality characteristics of frozen-thawed bull spermatozoa are associated with many potential factors, such as the freezing procedure, type of extenders and cryoprotectants, thawing process, sire individuality, semen-collection ages of bull life, seasonal changes or sperm-specific important features, etc. Some main factors of these areas (thawing process, sire individuality and age at semen collection of bull life), with indefinitely final conclusions due to inconsistent results and/or limited assessments, were addressed in the current research by using commercial frozen-thawed semen straws of Japanese Black bulls.

First, effectiveness of rapid thawing by using various post-thawed parameters of frozen bull spermatozoa was evaluated. Results indicated that the rapid transient thawing procedure, RT70, maintained the better viability, HMMP, ROS, and motility of frozen bull spermatozoa than conventional thawing methods (CT39 or CT37), despite no differences in acrosome integrity and PLCZ1 distribution among three thawing conditions. The present study pointed out that better post-thawed maintenance could come from pass faster not only through the critical harmful temperature range (from -60 to 0°C) but also the dangerous shock temperatures below the physiological range (< 15°C) during thawing at RT70, thus making less injury of the biological membrane associated with mitochondrial health.

Furthermore, relationships between mtDNA copy number (MDCN) or mitochondrial content (MC) and motility indicators of frozen-thawed bull spermatozoa were investigated. Findings revealed that mtDNA copy number and mitochondrial content of frozen-thawed bull spermatozoa significantly differed among sires, and had highly negative correlations with HMMP and sperm motility parameters, probably due to remarkably positive

correlations with ROS, revealing that these appear to be potent indicators to assess the sire sperm quality. The contents of mtDNA and mitochondria of frozen-thawed bull spermatozoa seem to not vary overall with the age of the sire, whereas significant changes with age in some individuals can be appeared and may consequently affect sperm motility. The simple method of measuring sperm mitochondrial content by fluorescence intensity under flow cytometry appears to be a reliable sperm evaluation method, since its levels are significantly associated with not only the conventional characteristics (HMMP and motility parameters) but also mtDNA copy number and ROS.

In general, the results of the first series of the current studies indicate that there are rooms to further improve the viability, motility and mitochondrial health of frozen bull spermatozoa by controlling temperature during the thawing process, especially by allowing it to pass as quickly as possible through zones outside the physiological temperature range. Besides, since the results of the second series of studies revealed that a positive correlation existed between the amount of sperm mitochondrial DNA/mitochondrial content and the amount of ROS, which in turn negatively affects the motility. Therefore, the findings of studies in this dissertation suggest that it is still possible to provide more motile frozen-thawed spermatozoa through further improvement of thawing conditions of frozen sperm and selection of bulls using mitochondrial content as an indicator, and that fertility after artificial insemination can still be improved through modifying sperm thawing process and the bull selection conditions.

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