Doctoral Thesis

Study of Lipid Components in Bovine Frozen Sperm

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The Author

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Chapter 1

General Introduction

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Reproduction is the process of producing new generation that are more or less similar to it's ancestors. In the nature, there are two types of reproduction- sexual and asexual. In the sexual reproduction two types of gametes (sperm cell and oocyte) are fused together to produce the diploid zygote in the process of fertilization (Frits & Gadella, 2000). The spermatozoa are produced by the process of spermatogenesis inside the male gonads (testicles) via meiotic division in the seminiferous tubules (Barth & Oko, 1989).

For the last 50 years, in the modern reproductive biotechnology, semen cryopreservation is being used as assisted reproductive technology (ART) for genetic improvement, control of venereal diseases and facilitates the management of beef and dairy cattle (Khalil et al., 2017). Although in frozen semen technology, almost 50% sperm is died during cryopreservation due to cryoinjury or cryodamage and thawing of the semen, artificial insemination (AI) with frozen semen is predominantly used in animal husbandry sectors especially in dairy cattle production to improve the genetic potentiality and to increase the productivity (Perumal, 2018).

1.1 Spermatogenesis and spermiogenesis

Male germ cells start to develop from a self-renewing stem cell pool, in the seminiferous tubules of the testes throughout life from puberty to old age and the complete process of germ cell development is termed as spermatogenesis. Spermatogenesis is a complex process which produce large numbers of normal spermatozoa that is required for male fertility. Spermatogenesis commences in the fetus at puberty after a long preparatory period of "prespermatogenesis". There are three major stages of spermatogenesis; spermatogoniogenesis, maturation of spermatocytes and spermiogenesis i.e cytodifferentiation of spermatids. Depending on the position in the basal part of the germinal epithelium, their morphology and stainability of nuclei spermatogonia are varied into three types: A pale type-, A dark type- and B type-spermatogonia (Clermont, 1966). Both pale and dark A type spermatogonia are necessary for intact spermatogenesis and A type spermatogonia belong to the stem cell pool of spermatogenesis whereas B type-spermatogonia represent the starting of germ cell development up to spermatids.

The meiosis stage of spermatogenesis is revealed by the changes in chromatin configuration in the nucleus after the last spermatogonial division and the cells in this stage are called spermatocytes. Due to the two divisions of meiosis process, cells before the first meiotic division are named primary spermatocytes and before the second meiotic division secondary spermatocytes. The primary spermatocytes are the largest germ cells of the germinal epithelium. In the basal

compartment of the germinal epithelium meiosis of spermatocytes begins with the leptotene stage of prophase cell division.

The round spermatid, which evolves from the second meiotic division, go through a series of complex cytological incidents which transmute it into the spermatozoon (de Kretser and Kerr, 1994). Several events like doubling of DNA, condensation of chromosomes, pairing of homologuous chromosomes and finally the "crossing over" take place during the prophase. After division, the germ cells turned into secondary spermatocytes. Secondary spermatocytes divide quickly to the spermatids without DNA replication. Two maturation divisions of each spermatocyte result in four haploid cells, the spermatids and these distinguish into mature spermatids through a process called spermiogenesis. During the spermiogenesis three processes take place; condensation of the nuclear chromatin in volume to about one tenth of an immature spermatid, development of the enzyme filled acrosome cap by the golgi apparatus and its attachment to the nucleus and expansion of flagellum structures and their implantation to the nucleus (Holstein & Roosen-Runge,1981). The spermatids develop such a configuration which enables them to depart the germinal epithelium during a complex process, called spermiation. After the spermiogenesis the cells are released from the germinal epithelium and during this moment the free cells are known as spermatozoa.

1.2 Sperm Maturation

After completing spermatogenesis and spermiogenesis in the testis, the spermatozoa are not capable of fertilizing an oocyte. The immature sperm in testis do not have a moving ability and maturation process in epididymis is required for sperm mobility. The epididymal sperm maturation includes the attainment of the forward motility and the fertilizing capability (Cooper & Yeung, 2006). Epididymis is the male reproductive organ, anatomically characterized as three different regions as the head (caput), the body (corpus), and the tail (cauda), responsible for the sperm transport, concentration, storage, and maturation, (Frenette et al., 2004). Each epididymal segment demonstrated great variability from one epididymal segment to the other in fluid composition (Dacheux, 2002; Dacheux & Belghazi, 2006) as well as the pattern of gene expression (Kirchhoff, 1999; Rodriguez et al., 2001).

Spermatozoa are transported through the caput and corpus epididymal regions by nonstop peristaltic contractions created in the smooth muscles present in the wall of the duct, whereas the cauda is maintained calm unless it can be stimulated to contract, and cauda is the main region

responsible for the sperm storage and survival (Jones & Murdoch, 1996). During this journey, spermatozoa undergo many biochemical alterations including changes in the plasma membrane protein and lipid composition (phospholipids composition, cholesterol/phospholipids ratio), relocalization of surface antigens, removal, and modifications of surface proteins (Cuasnicu et al., 2002; Sullivan, 1999), structural modifications of the cytoplasmic perinuclear theca (PT) (Mujica et al., 2003), and the ability to respond to hypoosmotic stress (Sahin et al., 2009). Biochemical and morphological modifications during sperm maturation include the addition of epididymal proteins to the maturating sperm, and some molecular reorganizational changes at the sperm membrane (Cooper, 1998). Furthermore, changes in membrane fluidity as a consequence of cholesterol efflux and protein dissociation from raft domain seems to be also a mechanism that occurs both in the maturational processes occurring at the time of ejaculation (Girouard et al., 2008).

Sperm undergo changes in their protein, lipid, and sugar content during epididymal shipment. The mechanisms involved in the transfer of epididymal proteins to the sperm are not fully understood. The epididymal epithelium consist of different epithelial cell types, notably principal cells, narrow cells (found only in the initial segment), clear cells and basal cells (Hermo et al., 2002). Each of these cell categories has a certain structure and function that fluctuates depending on their localization along the epididymis (Breton et al., 2016). Additionally, interaction between these different cellular types creates a luminal microenvironment suitable for sperm maturation and storage in the epididymis (Shum et al., 2011). Principal cells secrete proteins by both merocrine and apocrine mechanisms to the epididymal lumen (Hermo et al., 2002). Merocrine secretion includes membrane fusion between Golgi-derived vesicles and the cell plasma membrane permitting the release of the vesicle contents to the extracellular space (Farkas, 2015). In apocrine secretion epididymosomes are believed to be released by principal cells into the lumen of the epididymis (Sullivan et al., 2007). Epididymosomes are tiny membranous vesicles (25–300 nm in diameter) that contain different proteins, lipids, and non-coding RNAs (Belleannee et al., 2013, Martin-DeLeon, 2015). Proteins transmitted from epididymosomes to sperm can either be incorporated into the plasma membrane of sperm (Kirchhoff et al., 1996) or to intracellular structures (Eickhoff et al., 2001, Frenette et al., 2005). Epididymosome may transfer protein to sperm by fusion between sperm and these membrane vesicles through ADAM7, a member of transmembrane protein family, has biochemical characteristics of an integral membrane protein (Oh et al. 2009).

1.3 Changes of Sperm during Ejaculation

During ejaculation, the seminal plasma components are mixed with the spermatozoa. In the bovine, the seminal vesicles produce a family of proteins called bovine seminal plasma or BSP proteins which contain two fibronectin type II domains in tandem (Seidah et al., 1987, Calvete et al., 1996) that bind to choline phospholipids (Desnoyers & Manjunath, 1992) on the sperm membrane and excite their phospholipid and cholesterol efflux (Therien et al., 1998).

Bovine cauda epididymal spermatozoa have proteins associated to rafts (P25b and AK1) and non-rafts domains (aldose reductase and MIF) and these raft domain proteins are displaced to non - rafts domains as early as 15 min and 30 min after ejaculation respectively (Girouard et al., 2008). These changes are accompanied by a decrease in the cholesterol content in ejaculated sperm comparedd with cauda epididymal spermatozoa (Caballero et al., 2011) and PDC-109, a protein responsible for the cholesterol efflux in the bovine sperm at the ejaculation (Esch & Ling, 1983). Niemann-Pick C2 (NPC2), also found in the seminal plasma of various species capable of dissociating the P25b from the raft domains after 4 hours of incubation (Okamura et al., 1999; Fouchecourt et al., 2000). The ejaculated spermatozoa plasma membrane reorganization could have an important role in the correct positioning of molecules involved with the attachment to the oviductal epithelium and female gamete interactions and AK activity is important for biosynthesis of ATP and then for flagellar motility (Schoff et al., 1989).

During ejaculation, the composition of epididymal sperm is altered, the proteome of ejaculated sperm varies from epididymal sperm in ram (Pini et al., 2016) and boar (Perez et al., 2018). Additionally, ejaculated sperm of ram contain lower concentrations of phospholipids and cholesterol than testicular sperm (Scott et al., 1967). Although variations between seminal and epididymal fluid are involve in freezing resistance in different specieses (Varisli et al., 2009), epididymal sperm was found to be more resistant than ejaculated sperm to osmotic stress, cold shock, and cryopreservation process in ram (Tsikis et al., 2018), bulls (Cunha et al., 2016), boars (Perez et al., 2019), and stallions (Braun et al., 1994). The luminal fluid microenvironment protects the undermining processes related with sperm capacitation during sperm storage in the cauda of the epididymis (Fraser et al., 1990, Verma, 2001).

In mammals, for *in vivo* fertilization sperm motility is crucial for sperm movement from the ejaculation sites to the oviduct and the motility patterns are continuously changed after ejaculation to fertilization in the female tract (Suarez & Pacey,2006). Motility patterns are reliant on flagellar

motion and the linear motility pattern is induced by the symmetrical flagellar motion (Curtis et al., 2012). The linear motility, is essential for sperm migration from the cervix to the uterus and then to oviduct, characterized by low lateral amplitude and high straight-line velocity (Shalgi et al., 1992; Ishijima,2015). In oviduct during fertilization process, hyperactivation is prerequisite for capacitation of sperm (Suarez, 2008), where curvilinear velocity is high as well as high lateral amplitude (Stauss et al., 1995).

During capacitation, sperm go through a change in the motility pattern called hyperactivation and become competent to undergo a physiological secretory event known as acrosome reaction (AR) also known as acrosomal exocytosis (AE) (Yanagimachi, 1970). Hyperactivation is crucial for successful fertilization because it simplifies the sperm release from the oviductal reservoir and the penetration through the cumulus oophorus and the zona pellucida (ZP), the extracellular matrix enveloping the egg (Demott and Suarez, 1992). It was demonstrated that sperm separated from the cauda of epididymis, which is ready to ejaculate demonstrate progressive motility, which is characterized by high velocities and symmetrical, low-amplitude flagellar bends (Fraser, 1977). On the contrary, sperm isolated from the oviduct the fertilization site, show hyperactivated motility characterized by more asymmetrical, higher-amplitude flagellar bends, leading to more vigorous and less progressive ‘‘whiplash’’ motility (Suarez & Osman, 1987) which is required for fertilization (Yanagimachi,1970). Another report on mice demonstrated that sperm in the caput of epididymis showed irregular flagellar beat with little progression whereas in the proximal corpus region circular movement patterns in reduced linearity (LIN) and straightness (STR) were reflected (Soler et al., 1994). In conclusion, epididymis sperm easily induce capacitation whereas ejaculated sperm show linear motility pattern.

1.4 Sperm Motility Pattern and ATP Production

Sperm motility pattern is very peculiar characteristic which varies from ejaculation to fertilization. Motility patterns are dependent on flagellar motion, and it was reported that the linear motility pattern was induced by the symmetrical flagellar motion (Ho et al., 2002; Curtis et al., 2012). The linear motility is prerequisite in the migration process of sperm from the cervix to the uterus and then to oviduct, characterized by low lateral amplitude and high straight-line velocity (Shalgi et al., 1992; Ishijima,2015). Another motility pattern known as hyperactivated motility characterized by asymmetrical, irregular flagellar beat with high lateral amplitude and high curvilinear

velocity (Stauss et al., 1995). In oviduct during fertilization process, hyperactivation is basic requirement for capacitation of sperm (Suarez, 2008).

Mammalian spermatozoa particularly use adenosine triphosphate (ATP) to maintain the intracellular milieu (Mishro & Ramya, 2012) and for cellular processes such as motility, capacitation, hyperactivation and the acrosome reaction, all are required for successful fertilization (Mannowetz et al., 2012, Mukai et al., 2012). ATP, the leading chemical energy in cells, is generated mostly from two pathways: glycolysis in the cytosol and oxidative phosphorylation (OXPHOS) in the mitochondria (Du Plessis et al., 2015). The amount of ATP produced by glycolysis versus OXPHOS added to the total amount of ATP in the cell varies depending on the cell type, the growth stage, and the microenvironment (Zheng, 2012, Jose et al., 2011). Though, a spermatozoon can survive entirely on glycolytic energy (Spiropoulos et al., 2002), but it necessitates OXPHOS for differentiation and maturation (Nakada et al., 2006, Ford, 2006). The selected metabolic pathway chosen by spermatozoa relating to energy production is extremely species specific (Storey, 2008). For example, glycolysis was required for capacitation, motility hyperactivation, and oocyte-fusion in human (Hereng et al., 2011) hamster (Dravland & Meizel, 1981) and macaque sperm (VandeVoort & Overstreet, 1995). On contrary, glucose hindered capacitation in guinea pig (Hyne & Edwards,1985), bovine (Parrish et al., 1989) and dog sperm (Albarracın et al., 2004), where in vitro capacitation was carried out in glucose free medium.

Mitochondrial ATP is generated through transformation of ADP by the reduction of electron carriers (Amaral et al., 2013). It is reported that under low glucose conditions, most cells can move on the glycolytic pathway to mitochondrial oxidative phosphorylation (OXPHOS) to produce ATP (du Plessis et al., 2015), for instance, report on boar sperm study (Zhu et al., 2019). The electron transfer chain (ETC) comprises of several complexes that carry electrons attained from the oxidation of NADH and FADH2 moiety of succinate dehydrogenase, and ultimately decreased the final acceptor oxygen to water (Bratic & Trifunovic, 2010).

In reality, Ldh-c knock out mice model revealed that for hyperactivated motility the glycolysis was crucial (Odet et al., 2008). Rotenone, an inhibitor of complex I, dropped the sperm mitochondrial activity and ATP levels with progressive motility and straight-line velocity but not lateral amplitude and total motility in boar (Zhu et al., 2019), stallion (Plaza Davila et al.,2015) and human (Barbonetti et al.,2010). Additionally, rotenone suppressed the sperm capacitation, acrosome reaction and fertilization rate in hamster (Rogers et al., 1977) and stallion (Plaza Davila et

al.,2015), implying that mitochondrial ATP production is vital for sperm linear motility and hyperactivation. It has been reported that acetyl CoA is generated by mitochondrial β-oxidation from fatty acids (Aon et al., 2014). In human sperm it was described that a large proportion of the metabolic proteome (24%) comprised enzymes were engaged in lipid metabolism, including enzymes for β-oxidation (Amaral et al., 2013). Excitingly, the mitochondrial β-oxidation is an active regulator for sperm motility because inhibition of β-oxidation using etomoxir led to a decrease of sperm motility (Amaral et al., 2013). Moreover, it was reported in bulls that palmitic acid and oleic acid advanced sperm progressive linear motion and viability when added to the extender (Kiernan et al., 2013). Another report on boar sperm stated that unsaturated fatty acids enhanced progressive motility and viability during in vitro incubation (Kelso et al., 1997), suggesting that mitochondrial β-oxidation is very essential for sperm progressive motility and survivability.

1.5 Changes of Sperm during Cryopreservation

In the present world artificial insemination (AI) of animals and assisted reproductive technology (ART) in human routinely use cryopreserved spermatozoa (Kopeika et al., 2015; Yeste, 2016) although first semen cryopreservation record dates back approximately 250 years, when Lazaro Spallanzani (1776) attempted to preserve spermatozoa by cooling it in snow (Royere et al.,1996). Crucial factor in sperm cryobiology is that sperm are small cells with a substantial surface area (John Morris et al., 2012; Morris, 2006). These physical characteristics makes them less vulnerable to potential damage which is engaged by the viscosity and glass transition temperature of the intracellular cytosol in sperm cells (Isachenko et al., 2003). Although cryodamage in spermatozoa varies among different species (Ozkavukcu et al., 2008; Yeste, 2016), common modifications are acrosome disintegration and partial deletion of the outer acrosomal membrane with reduction of acrosomal content that are attached to physical freezing events of sperm (Barthelemy et al., 1990). On the Other hand, damage to the lipid membrane structure caused by osmotic changes, prominent to tension changes in water canal proteins and ionic leakage in plasma membranes and resulting in morphological changes (Sa-Ardrit et al., 2006).

The sperm plasma membrane is the first structure affected by cryopreservation (Bailey et al., 2000). It has been reported that sperm cryopreservation induced in enlarge plasma membrane fluidity–permeability, overproduction of reactive oxygen species (ROS), reduction of acrosome integrity, impairment of mitochondrial membrane potential and lower sperm motility in bull

(Khalil et al., 2018, Yoon et al., 2015), buffalo (Rasul et al., 2001, Kumar et al., 2016), buck (Ahmad et al., 2014, Chauhan et al., 1994), ram (Nur et al., 2010, García-Álvarez et al., 2009) and red deer (Fernández-Santos et al., 2006). The sperm plasma membrane of ruminants contains high levels of unsaturated phospholipids and low levels of cholesterol that declines the resistance of sperm to the freezing–thawing process (Darin-Bennett et al., 1977). During freezing process, phospholipids of sperm membrane undergo a reallocation throughout the membrane, and some of them switch from liquid to gel state quicker than others due to structural differences, subsequently a lipid phase separation occurred (Grötter et al., 2019). As a result, some sperm surface proteins as well as membrane proteins are lost or translocated and the lipid–protein interactions are disturbed which is required for a proper membrane activity (Lemma, 2011).

1.6 Known Fact and Unknown Maiden Discovery of Present Study

In the present time this is well established that sperm are very unique male reproductive unit that has a distinctive membrane configuration and unusual nucleus, mitochondria, cytoplasm & cytoskeleton structure compared to somatic cells. These characteristics are formed during spermatogenesis in testis. Different maturational phases occur in the epididymis. Epididymal sperm are more effective in capacitation than fresh sperm immediately after ejaculation. For the effective use of potentialities of breeding animal specially in cattle industry artificial insemination (AI) is used for the last 5/6 decades using frozen sperm. Cryopreservation although can keep the sperm for long time future use even the animal is dead, but unfortunate it deteriorates the quality of sperm. There are so many studies regarding the cryodamage of bovine sperm for example, Khalil et al. (2018) stated that sperm cryopreservation is responsible to enhance plasma membrane fluidity–permeability, excessive production of reactive oxygen species (ROS), decline of acrosome integrity, deficiency of mitochondrial membrane potential and lower sperm motility in bull (Khalil et al., 2018). Though some researcher reported on prevention of cryodamage by supplying cholesterol or other cryoprotectant during freezing of bovine sperm (Purdy & Graham, 2004) but unfortunately, there are lack of published reports on recovery of bovine frozen sperm after thawing and washing using an appropriate media. Many researchers described that fatty acids in thawing media can improve sperm motility, acrosome reaction and fertility in case of boar sperm (Hossain et al., 2007). Again, some researcher stated that sperm can utilize energy substrate supplied in the thawing media of boar sperm (Zhu et al., 2020). Sperm are changed during maturation and ejaculation stages. As far author knowledge, nobody described what is the actual

molecular mechanism of sperm to use the fatty acid as energy source and cholesterol to recover the damages of sperm during freezing and thawing process.

Therefore, the present study was undertaken to understand the components of LM (LM) in bovine frozen sperm membrane with the amount of fatty acids on sperm membrane and their role in thawing media to improve the quality of bovine frozen sperm were investigated. Moreover, the individual effect of cholesterol and fatty acids combined in the LM on bovine frozen sperm were also analyzed. To clear the precise hypothesis, three (3) individual experiments were conducted.

In Experiment 1, it was hypothesized that sperm contain lipid components in variable quantity in its membrane and sperm may uptake fatty acids to their membrane from LM during thawing. Another assumption was LM in thawing media of bovine frozen sperm can improve sperm quality like motility, membrane integrity, survivability and ultimately invitro fertilization (IVF) rate. To clear the hypothesis, computer assisted semen analysis (CASA), Gas chromatography and mass spectrometry (GC-MS), and flow cytometry (FCM) with different stains were performed using bovine frozen sperm.

In Experiment 2, it was assumed that sperm can transport incorporated the fatty acid to the mitochondria for energy production to keep the linear motility pattern during incubation. To clarify the assumption, CASA, GC-MS, FCM with different stains, oxygen consumption rate (OCR) and immunofluorescence (IF) were performed using bovine frozen sperm.

In Experiment 3, it was supposed that sperm can recover their membrane integrity using the cholesterol supplied in thawing media and improve quality parameters like sperm motility and sperm membrane integrity. To make understand the hypothesis, CASA, FCM with different stains, oxygen consumption rate (OCR) was performed using bovine frozen sperm.

Chapter 2

Experiment 1

Study on variation of lipid components in bovine frozen sperm and their role on accelerating motility and quality parameters as mixture

Contents

2.1 Introduction

Sperm are the biological and functional male reproductive unit and have a unique membrane configuration and unusual nuclear, mitochondrial, cytoplasmic, and cytoskeletal structures compared to those of somatic cells (Flesch & Gadella, 2000). These characteristics are formed during spermatogenesis in the testis. However, the plasma membrane structure of sperm is not fully mature when they are released from the testis, and the membrane structure is further changed during the maturation process in the epididymis (Eddy et al., 1994). The biochemical changes in the sperm plasma membrane in the epididymis include release, modification, and adsorption of proteins and lipids (Frits & Gadella, 2000). Although the role of these surface alterations has not been clearly described, it has been investigated that some adsorbed proteins are involved in sperm-oocyte binding during fertilization (Flesch & Gadella, 2000). Another report indicated that the expression of membrane receptors, e.g., C-met receptors, varies due to changes in the lipid structure of the sperm membrane (Depuydt et al., 1996).

The lipid composition of the sperm membrane significantly affects the functional characteristics of spermatozoa (Zalata et al., 1998). This composition is different among species, but normally it contains approximately 70% phospholipids, 25% neutral lipids and 5% glycolipids (on a molar basis) (Mann et al., 1981). Biophysical studies reported that just after leaving the testes, sperm cells stop the synthesis of plasma membrane lipids and proteins, which means that components of the plasma membrane cannot be newly synthesized (Frits & Gadella, 2000). During epididymal maturation, the phospholipid (PL) composition of the membrane may change due to a high content of unsaturated fatty acids in epididymis fluid (Hall et al., 1991). These changes increase membrane fluidity because lipids in the sperm plasma membrane regulate the permeability of the sperm cell membrane (Frits & Gadella, 2000). The increased permeability returns to a low level after sperm are mixed with seminal plasma. The change of membrane permeability is explained by different stability of cholesterol that is taken to the sperm membrane being different from that of seminal plasma, imposing a conformational order of lipids in the membrane (Leahy & Gadella, 2015). Therefore, the sperm membrane would be changed by different lipid components under epididymal and semen conditions (Towhidi & Parks, 2012).

In the relationship between exogenous fatty acids and sperm quality, an experiment on a feeding trial of essential fatty acids in rats implied that membrane-associated fatty acids are important for

sperm motility, sperm viability and the fusion process between spermatozoa and oocytes (Lenzi et al., 1996; Lewis SE & Maccarrone, 2009). It was observed that the incubation of human sperm with glycerophospholipids improves sperm viability, motility, and resistance to oxidizing agents like H_2O_2 (Ferreira et al.,2018). In the boar sperm study, it was reported that fatty acids, especially arachidonic acid, play an important role in sperm movement and a combination of arachidonic and oleic acid is important for inducing acrosomal reaction (AR) (Hossain et al., 2007). In the Brown Swiss bull sperm study, it was shown that the optimal level of n-3 FA (10 ng mL−1) combined with the highest level of vitamin E (0.4 mM) in sperm handling extender changed the membrane fatty acid composition and improved the freezability of sperm (Towhidi & Parks, 2012).

Against the positive effects of exogenous fatty acids, sperm plasma and acrosome membranes are damaged during the freezing and thawing process. The process increases oxidative stress in sperm, which changes sperm membrane permeability and might lead to reducing the spermatozoa functional integrity and decreasing its fertilizing ability (Gillan & Maxwell, 1999). However, to the best of our knowledge, the changes in fatty acid components in the sperm structure during sperm freezing and thawing process is unrevealed. Moreover, the relationship of different fatty acid components of the sperm membrane with the freeze-thaw process and sperm motility is still unknown in bovine species. Therefore, firstly we focused on the variations in membrane fatty acid components among the individuals of bovine frozen sperm from different animals. Secondly, we tried to correlate the relationship between sperm motility with the different membrane incorporated fatty acids of bovine frozen sperm. Finally, we studied on sperm quality parameters like motility, membrane integrity, hexokinase activity and mitochondrial activity by adding a LM to the basic thawing media.

2.2 Materials and methods

2.2.1 Materials

Routine chemicals were obtained from FUJIFILM Wako Pure Chemical Industries, Ltd., (Osaka, Japan) and Nakarai Chemical Co. (Osaka, Japan). Frozen straws of bovine sperm (Holstein Friesian) were kindly gifted from Japan and Livestock improvement association of Japan. INC (Maebashi, Japan).

2.2.2 Sperm preparation and incubation

Frozen bull semen was thawed in water at 37 °C and washed by centrifugation twice (600 g, 37 °C, 4 min) with 6 mL of Bracket-Oliphant (BO) medium (Table-1) (Brackett et al. 1978) using the heated and refrigerated centrifuge machine (Kubota 5910, Japan). The sperm pellet was resuspended with BSA containing BO medium without LM (0.1%) (L0288, Sigma-Aldrich, USA) that were used in thawing media, and then the sperm were incubated without LM for 30 min at 37 °C under a humidified atmosphere of 5% CO2 in the air.

2.2.3 Sample preparation for gas chromatography-mass spectrometry analysis

Fatty acids in sperm were extracted and methylated by the Fatty Acid Methylation Kit (06482-04, Nacalai Tesque Inc. Japan), and then were purified by the Fatty Acid Methyl Ester Purification Kit (06483-94, Nacalai Tesque Inc. Japan) according to manufacturer's instructions. The methylated samples (3.0 mL) were dried, and then dissolved in 50 µL of the eluting solution. The samples were subjected to GC-MS.

2.2.4 Gas chromatography-mass spectrometry analysis

Agilent 7890A GC System coupled to JMS-T 100 GCv mass detector, an Agilent 7693A autosampler was injected with 1.0 µL aliquot of samples. HP-5 capillary column (60 m × 0.32 mm i.d. × 0.25 µm film thickness; 19091J-413, Agilent Technologies, Santa Clara, CA) was used to analyze the samples and reference standards (10, 1.0, 0.1, 0.01, 0.001mM). Helium was used as the carrier gas at a constant flow rate of 2.0 mL/min. The temperature for the GC oven varied starting at 100 °C to gradually increasing up to 300 °C at the rate of 10 °C / min and held at 300 °C for 20 min. Ionization was performed using electron ionization (EI) at electron energy of 70 eV. The operation of mass spectrum was carried out in the scan mode (mass scanning range of 29-800 m/z). NIST MS Search v. 2.0 was used to detect and identify the fatty acids.

2.2.5 Detection of Sperm Motility by Computer-Assisted Sperm Analysis (CASA) System

Sperm motility was evaluated using CASA according to our previous study (Zhu et.al. 2019). A 10 µL of the sample was placed in a pre-warmed counting chamber to take the CASA reading after incubation of sperm at different time intervals. Sperm tracks (0.5s, 45 frames) were captured at 60

Hz according to our previous study (Zhu et.al. 2019), using a CASA system (HT CASA-CerosII; Hamilton Thorne, MA, United States). Minimum three replications were used for each sample and more than 200 trajectories were recorded.

2.2.6 Mitochondrial Activity

Mitochondrial activity of sperm was measured using MitoPT® JC-1 Assay Kit (911, Immuno Chemistry Technologies, LLC.) according to Zhu et.al. 2019. Briefly, sperm samples were incubated with 200 µL of 1x working solution at 37ºC for 30 min in dark. The mitochondrial activity was analyzed by flow cytometry using a filter with a bandwidth of 574/26 nm (Attune® NxT Acoustic Focusing Cytometer, Invitrogen) and measured as mean fluorescence intensity (MFI) of JC-1 orange aggregates. A total of 20,000 sperm events were analyzed.

2.2.7 Membrane integrity

The sperm membrane integrity was measured using a LIVE/DEAD Sperm Viability Kit (L7011; Thermo Fisher Scientific) according to manufacturer protocol. Briefly, Sperm were stained with SYBR-14/PI and incubated with 200 µL 1x working solution at 37ºC for 10 min in dark. The staining was analyzed by flow cytometer (Attune® NxT Acoustic Focusing Cytometer, Invitrogen) with Excitation/ Emission= 515/545 nm for SYBR-14 fluorescence, Excitation/ Emission= 675/715 nm for PI fluorescence. A total of 20,000 sperm events were analyzed. There were four quadrants on the specific events gated area. Q1 (-/+) indicating the percentage of SYBR-14 containing sperm cells, Q2 (+/+) indicating the percentage of both SYBR-14 and PI containing sperm cells, Q3 (+/-) indicating the percentage of PI containing sperm cells and Q4 (-/-) indicating the percentage of sperm cells neither containing PI nor SYBR-14.

2.2.8 Annexin v staining

The sperm membrane apoptosis was measured using an Annexin v conjugate: Alexa Fluor®647 Kit (A23204; Invitrogen, Molecular probes) according to manufacturer protocol. Briefly, Sperm were stained with Alexa Fluor®647 /PI and incubated with 200 µL 1x working solution at 37ºC for 30 min in dark. The staining was analyzed by flow cytometer (Attune® NxT Acoustic Focusing Cytometer, Invitrogen) with Excitation/ Emission= 663/677 nm for Alexa Fluor®647 fluorescence, Excitation/ Emission= 561/587 nm for PI fluorescence. A total of 20,000 sperm events were analyzed. There were four quadrants on the specific events gated area. Q1 (-/+)

indicating the percentage of necrotic sperm cells, Q2 (+/+) indicating the percentage of early necrotic sperm cells, Q3 (+/-) indicating the percentage of apoptotic sperm cells and Q4 (-/-) indicating the percentage of live sperm cells.

2.2.9 Statistical analysis

Statistical analyses of data from three or four replicates for comparison were carried out by either Student's t-test or one-way ANOVA followed by Student's t-test (Statview; Abacus Concepts, Inc., Berkeley, CA).

2.3 Results

2.3.1 Variations of membrane fatty acids by GC-MS

The variations of fatty acid components of the sperm membrane were analyzed by GC-MS using frozen semen samples from ten different bulls (Table- 2). Both saturated and unsaturated fatty acids were detected at a significant ($P<0.05$) level of difference in different bull's straws. Among the saturated fatty acids, palmitic acid (C16:0), margaric acid (C17:0), stearic acid (C18:0) and docosanoic acid, commonly known as behenic acid (C22:0), were present in all bull straws. Myristic acid (C14:0), pentadecylic acid (C15:0), and arachidic acid (C20:0) were present in all bull straws except in those of bulls (Bull ID 656 & 729); (Bull ID 445, 612, 656 & 729), and (Bull ID 445, 612, 656,729 & 250), respectively. In the case of unsaturated fatty acids, docosahexaenoic acid (C22:6) was detected in all bull's straws. Linoleic acid (C18:2) and arachidonic acid (C20:4) were present in all bull's straws except those of bulls (Bull ID 445, 612, 656 & 729) and (Bull ID 408 & 656), respectively.

2.3.2 Relationship between sperm total motility and different fatty acids

There were positive correlations with some fatty acids and total sperm motility after 30 min of incubation, and others were negatively correlated (Figure 1 & 2). Total sperm motility was calculated by CASA derived motility tracks after 30 min of incubation at 37ºC condition. Myristic acid and stearic acid showed a positive correlation with the total motility of sperm (Y-axis of the graph), whereas docosanoic acid (behenic acid) was negatively correlated, although all correlations were statistically non-significant at $P= 0.28$, $P= 0.06$ and $P= 0.10$, respectively.

2.3.3 Effect of LM on sperm membrane fatty acids

To understand whether sperm selectively uptake fatty acids from exogenous source or not, sperm were thawed with 0.1 % (v/v) of LM containing fatty acids (2 µg/mL arachidonic acid and 10 µg/mL each linoleic, linolenic, myristic, palmitic and stearic acid) and 0.22 mg/mL cholesterol. Fourteen kinds of fatty acids were detected by GC-MS (Figure 3 & 4) and 6 of them were significantly ($P < 0.05$) increased in sperm thawed with LM as compared with sperm without LM condition (Figure 5).

2.3.4 Sperm motility parameters with the LM

Sperm motility parameters were calculated by CASA-derived motility tracks after 30-min incubation. BCF, VCL, VSL and TM were significantly ($P<0.05$) higher in sperm thawed with 0.1% (v/v) LM condition (0.1% LM) compared to sperm thawed without LM condition (control) and other doses of LM (Figure 6 & 7). Lateral amplitude (ALH) was not significantly ($P<0.05$) changed in all conditions except 1% LM condition. Therefore, 0.1% LM was selected as optimum dose for further experiments.

Scatter plot was made considering the parameters of individual sperm data that were significantly increased by LM treatment, BCF (Y Axis of graph) and VSL (X axis of graph) in figure 8; BCF (Y Axis of graph) and VCL (X axis of graph), VSL (Y Axis of graph) and VCL (X axis of graph) in figure 9 to understand the effect of LM on individual sperm (Figure 8 & 9). In each figure, the lines were made in scatter plot considering the mean value of each motility parameter in control group of sperm. The percentage of sperm in the upper right quadrant of scatter plot was significantly ($P<0.05$) higher in LM treatment group rather than control group in both figures.

2.3.5 LM affect sperm mitochondrial activity

Mitochondrial membrane activity was analyzed by JC-1 staining. Mean fluorescence intensity (MFI) of the sperm incubated for 30 min at 37ºC thawed in the LM condition significantly ($P<0.05$) differed from the sperm incubated for the same time and under the same conditions but underwent thawing without LM condition (Figure 10-A). Sperm showed a significantly lower MFI in without LM condition than with LM condition.

2.3.6 LM affect sperm membrane integrity

The sperm membrane integrity was analyzed by SYBR-14/PI staining. The intact membrane integrity of the sperm incubated for 30 min at 37ºC thawed in the LM condition, stained by SYBR-14 showed significantly ($P<0.05$) higher percentages in the LM condition than without LM condition thawing (Figure 10-B). Damages of sperm membrane integrity stained by PI was significantly ($P<0.05$) lower in the LM condition group than in the group without LM condition thawing (Figure 10-B).

2.3.7 LM suppress sperm apoptosis

Live, apoptotic, necrotic, and early necrotic cells were analyzed by Annexin V staining. The number of live sperm cells was significantly ($P<0.05$) higher in the LM condition group than without LM condition thawing (Figure 10-C). The numbers of necrotic and early necrotic sperm cells were significantly ($P<0.05$) lower in the LM group than without LM condition thawing (Figure 10-C).

2.4 Discussion

During the freezing and/or thawing process, the levels of some fatty acids, such as the PUFAs C18:2, C20:3, C20:4, and docosahexaenoic acid (C22:6 n-3; DHA) significantly decreased, and the levels of saturated fatty acids (SFAs) C:16 and C:18 increased in human sperm (Alvarez & Storey,1992). The increase of the fatty acids might be explained by the incorporation or contamination of them from egg-yolk based freezing extender to sperm. However, the reduction of them during the freezing and thawing process is induced by the release of polyunsaturated fatty acids from the plasma membrane (Aitken,1995). The alteration of the membrane is thought to be the lipid peroxidation process induced by ROS (Reactive oxygen species) that is generated in sperm during both the freezing and thawing process (Alvarez et al., 1987). It has also been known that oxidative stress decreases sperm survival and fertility (Aitken,1995). In the present study, 10 lots of cryopreserved bull semen were used for GC-MS analysis, and among the saturated fatty acids, except docosanoic acid (behenic acid) (C22:0), a positive correlation between the level of fatty acids and total sperm motility was observed as similar to those in human sperm study (Martinez et al., 2013). Although we did not determine whether the low levels of sperm were caused by the freezing and/or thawing process or were dependent on the individual's samples

before freezing, the different components of fatty acids would affect the sperm motility of frozen-thawed bull sperm.

Moreover, it was reported in bulls that palmitic acid and oleic acid advanced sperm progressive linear motion and viability when added to the extender (Kiernan et al., 2013). Another report on boar sperm stated that unsaturated fatty acids enhanced progressive motility and viability during in vitro incubation (Kelso et al., 1997). The treatment with unsaturated fatty acids enhances ATP (Adenosine triphosphate) production and increases intracellular Ca2+ and cAMP (Cyclic adenosine monophosphate) concentrations, which in turn induces the acrosome reaction (AR) in boar spermatozoa (Hossain et al., 2007). The process of induction of AR is called as capacitation, and sperm undergo a change in the motility pattern called as hyper activation during the capacitation process (Yanagimachi,1970). Moreover, Abavisani et al. (2013) reported that the addition of Ω-3 PUFAs (Poly unsaturated fatty acids) directly to semen extenders was not effective in protecting the bull sperm membrane (Abavisani et al., 2013). It was shown that a long-chain SFA (palmitic acid) can preserve progressive linear motility and viability whilst keeping ROS levels at a minimum level in boar sperm (Kiernan et al., 2013). On the other hand, the very long-chain SFAs C22:0, and C24:0 were inversely related to the total antioxidant capacity (TAC), which is directly correlated with motion parameters after thawing of human sperm (Martinez et al., 2012). Some studies on bovine species (Neill & Masters,1972) and ovine species (Neill & Masters,1973) reported that sperm cells are able to take up lipid components or fatty acids from the surrounding environment during incubation (Cerolini et al., 2001). We used a LM in the thawing media with the hypothesis that sperm could selectively uptake some kinds of fatty acids from a LM containing both saturated and unsaturated fatty acids to improve their motility. In our GC-MS study, the status of different fatty acids was significantly ($P<0.05$) higher in the LM condition rather than without LM condition (Figure 5). The most up taken saturated fatty acid was myristic acid (C14: 0), and in the case of unsaturated fatty acids, linoleic acid (C18:2) was most significant. It has been described that fatty acids were incorporated into the lipids of sperm cell membranes within two hours of addition to cell cultures (Neill & Masters,1971). From the perspective of fatty acid composition, it was reported that diglycerides contained myristic acid (C14: 0) as the major component of bull sperm lipids (Neill & Masters,1972). It is suggesting that myristic acid (C14: 0) was incorporated into sperm membrane lipids and improved sperm motility and other parameters. Boar sperm study described that the addition of exogenous fatty acids (oleic acid and

palmitic acid) to the dilution medium improved sperm quality by utilizing them as energy substrates for ATP production via β-oxidation (Zhu et al., 2020).

The metabolic activity of the sperm including motility, vigor, and wave motion decreases with the damage of the sperm membrane due to cold shock followed by increasing the permeability that causes the loss of intracellular components (Amann et al., 1987, Graham et al.,1996). Cholesterol controls membrane structure by decreasing cold shock sensitivity through extending the phase transition of the sperm membranes (Quinn, 1989), thus decreasing lipid from accumulating into particular domains within the membrane (Drobnis et al., 1993) and increasing membrane stability at lower temperature (Purdy et al., 2005). Therefore, cryo-damage occur at freeze thaw process can be reduced by supplying cholesterol in freezing diluents (Wessel and Ball, 2004). Additionally, it was described that cholesterol loaded methyle β-cyclodextrin in extender advanced survivability of stallion (Combes et al., 2000; Graham, 1998) and bull spermatozoa (Amorim et al., 2009) after cryopreservation.

In conclusion it can be said that, as LM is composed of both saturated and unsaturated fatty acids with cholesterol thus, it affects the sperm motility pattern, membrane structure. This is actually preliminary findings about the effect of LM on bovine frozen sperm. Mechanism of sperm energy production for linear motility and retain of sperm membrane integrity is still unknown. Therefore, our next study will focus on molecular mechanism of sperm energy production using fatty acids in thawing media and sperm membrane potentials using cholesterol separately.

Abstract

Sperm is very unique in their membrane structure and cryopreserved sperm is very sensitive to cold shock damage. Cryodamage affect sperm motility and membrane structure highly. In this study it was hypothesized that Lipid Mixture (LM) in thawing media maybe beneficial for bovine frozen sperm to keep good motility with sound membrane structure by maintaining their membrane lipid composition healthy. To clarify the hypothesis CASA, GC-MS and FCM with different stains were performed. In this study it was revealed that LM thawed bovine frozen sperm showed significantly ($P<0.05$) good motility parameters compared to control group. It was observed that fatty acids from LM was up taken by LM thawed sperm compared to control and there was a positive correlation between fatty acids amount and total sperm motility. From this study it was also uncovered that membrane integrity, mitochondrial activity and live sperm was significantly ($P<0.05$) higher in LM thawed sperm compared to without LM thawed sperm used as control. Therefore, it can be concluded that LM in thawing media is beneficial for bovine frozen sperm to keep their motility with other quality parameters in healthy status.

Table -1: Modified Bracket-Oliphant (BO) medium for thawing and incubation of sperm

Component	Amount
NaCl	65.5 mg/mL
KCl	3.0 mg/mL
$CaCl_2$-$2H_2O$	3.3 mg/mL
NaH_2PO_4-$2H_2O$	1.28 mg/mL
$MgCl_2$-$6H_2O$	1.06 mg/mL
$NaHCO_3$	3.104 mg/mL
Pyruvic acid	0.1376 mg/mL
Streptomycine	0.05 mg/mL
Glucose	2.502 mg/mL
Caffeine	0.9 mg/mL
Heparin	0.015 mg/mL
BSA	5.0 mg/mL

Table -2 Different fatty acid amount (pmol/sperm) calculated by GC-MS from different stocks of bull semen straw. Different superscripts differ significantly at $P < 0.05$.

Bull ID	MA (C14:0)	PA (C15:0)	PA (C16:0)	MA (C17:0)	SA (C18:0)	AA (C20:0)	BA (C22:0)	LA (C18:2)	AA (C20:4)	DHA (C22:6)
408	116.16^{e}	69.66^{d}	5628.22^{h}	28.33^{h}	1256.43^{f}	12.32^{e}	4965.64^{e}	91.41^{f}	0.00^{i}	4525.84^{g}
445	53.58^{f}	0.00^{g}	14483.84^{c}	59.08^{f}	65.98^{j}	0.00^{f}	8122.40^{a}	0.00^{g}	128.55^{f}	13661.29^{b}
612	11.71^{h}	0.00^{g}	11595.12^{e}	41.19^{g}	145.88^{h}	0.00^{f}	5428.30^{d}	0.00^{g}	31.52^{h}	10337.20^{d}
656	0.00^{i}	0.00^{g}	3007.02^{j}	72.77^{e}	86.33^{i}	0.00^{f}	729.35^{j}	0.00^{g}	0.00^{i}	799.70^{j}
729	0.00^{i}	0.00^{g}	3930.35^{i}	24.85^{i}	151.97^{g}	0.00^{f}	3568.45^{g}	0.00g	71.38^{g}	2166.90^{h}
423	934.76^{d}	1438.17^{a}	20348.23^{a}	1662.61^{b}	2280.13^{c}	852.70^{a}	6600.56^{b}	1717.67^{a}	1418.16^{b}	14866.99^{a}
389	3160.63^{a}	31.14^{f}	14501.66^{b}	1938.53^{a}	1806.41^{d}	829.97^{b}	4064.14^{f}	1173.42^{e}	816.55^{c}	8911.47^{e}
950	1452.53^{b}	621.39^{c}	10764.49^{f}	1175.23^{d}	2838.80^{b}	13.74^{d}	934.84^{i}	1292.40^{d}	673.60^{d}	804.42^{i}
250	18.76^{g}	935.91^{b}	12072.47^{d}	5.73^{j}	1714.67^{e}	0.00^{f}	5533.74^{c}	1334.86^{c}	1568.16^{a}	10946.61^{c}
148	1228.41^{c}	62.77^{e}	7377.45^{g}	1226.70^{c}	3505.65^{a}	44.22^{c}	2595.73^{h}	1475.46^{b}	554.88^{e}	462.31^{f}

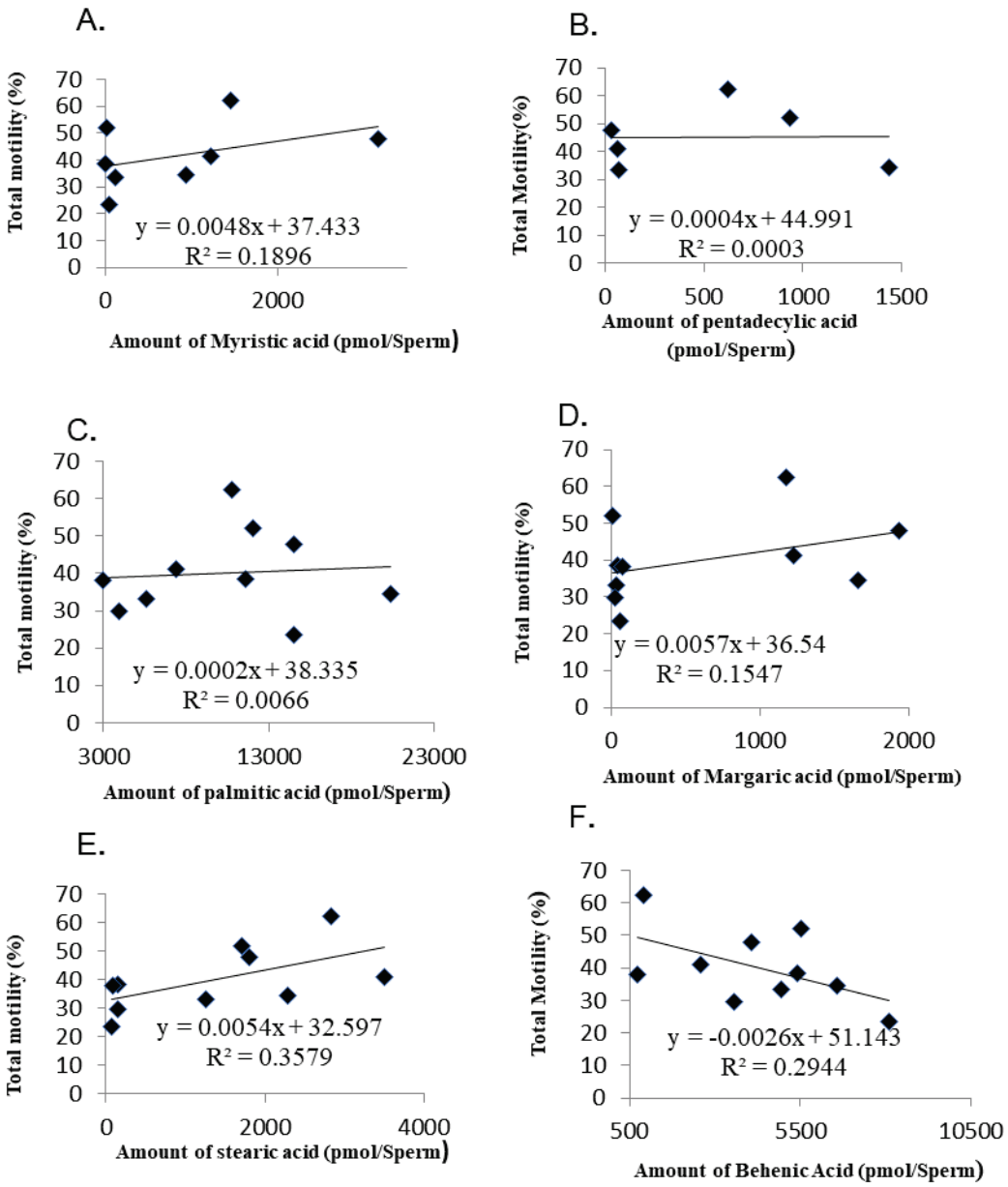


Figure -1: Correlation between different saturated fatty acids with total sperm motility. Here, X-axis indicates the amount of different fatty acids, and the Y-axis indicates the total sperm motility of different bull straws.

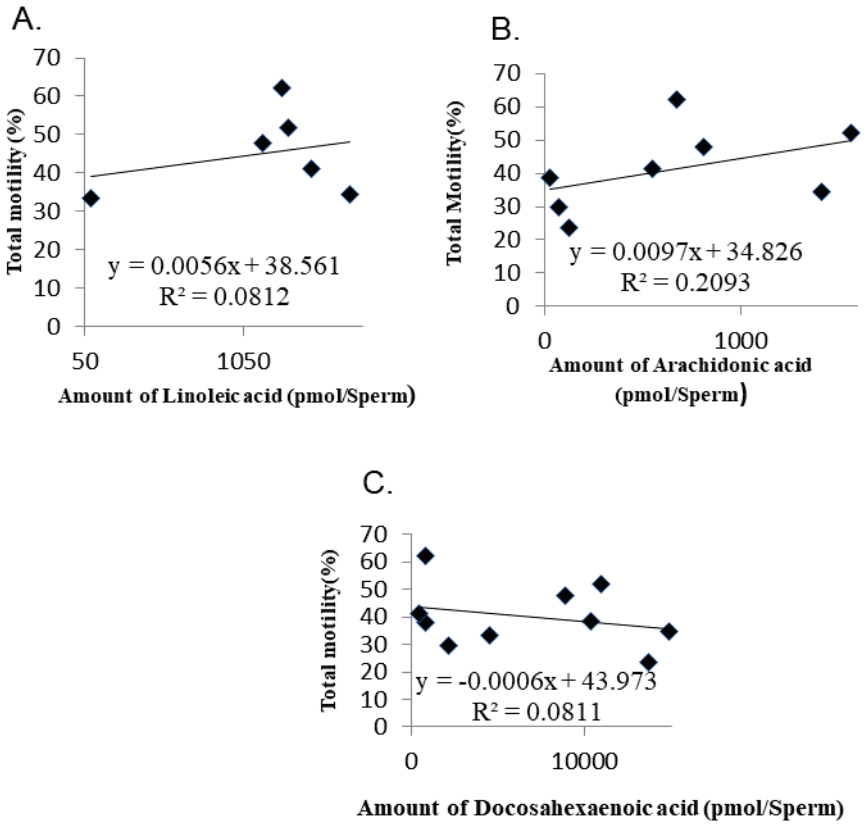


Figure -2: Correlation between different unsaturated fatty acids with the total sperm motility. Here, X-axis indicates the amount of different fatty acids, and the Y-axis indicates the total sperm motility of different bull straws.

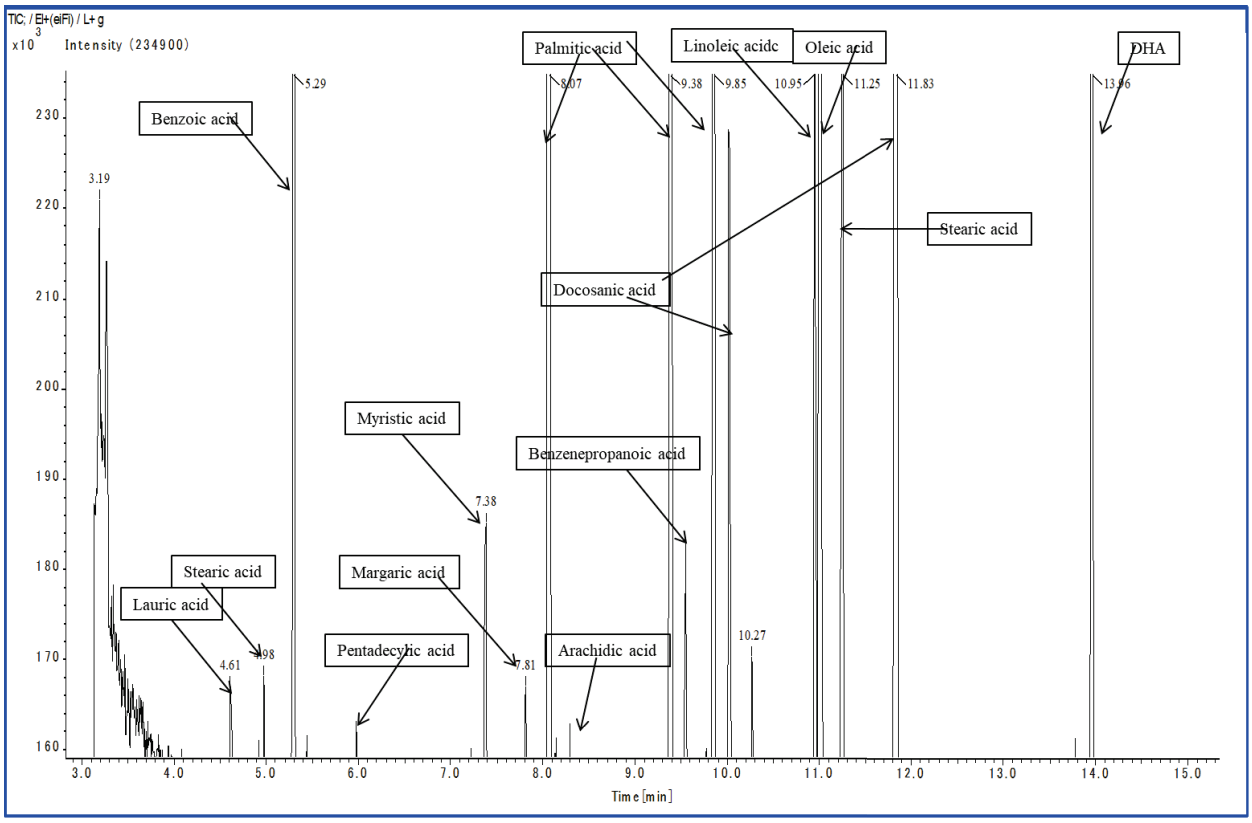


Figure -3: Chromatogram of different fatty acid components present in sperm thawed with lipid mixture found by GC-MS.

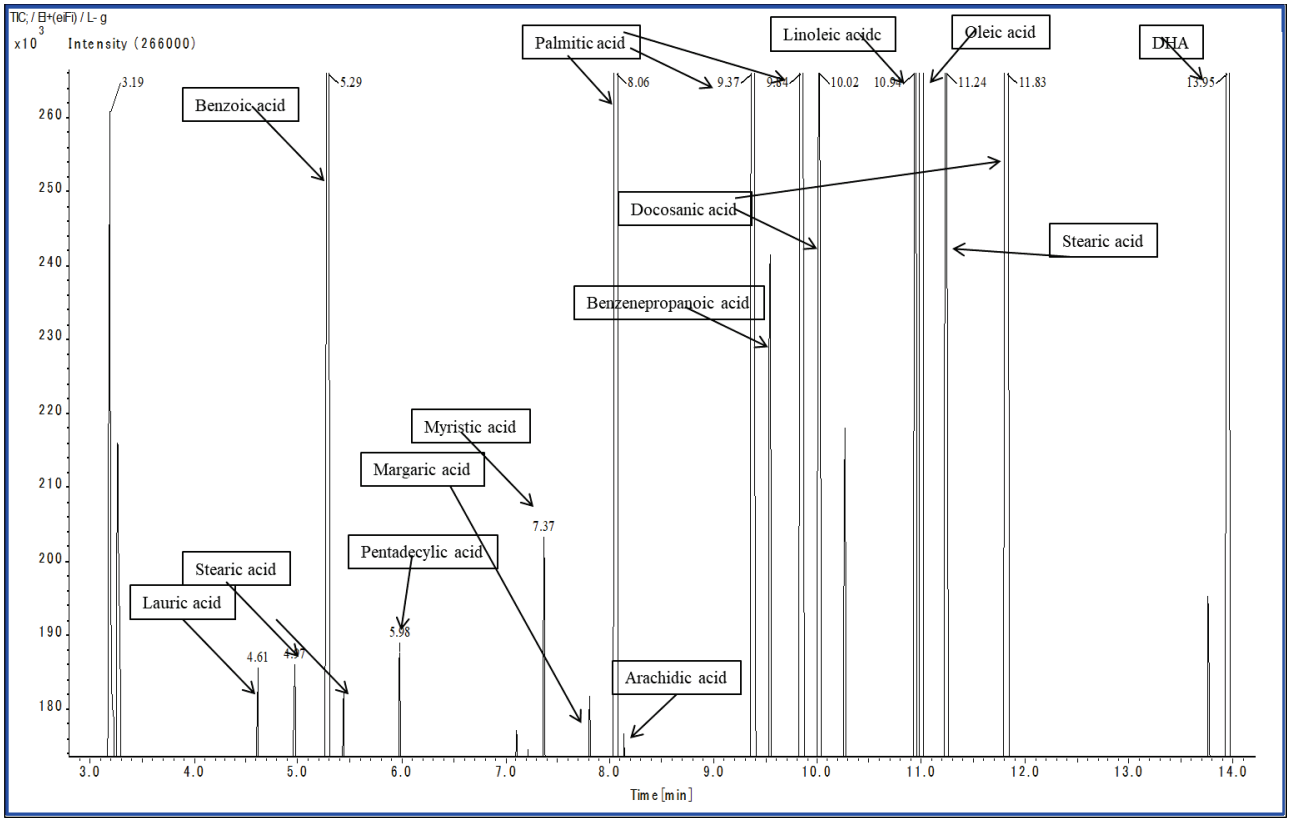


Figure - 4: Chromatogram of different fatty acid components present in sperm thawed without lipid mixture found by GC-MS.

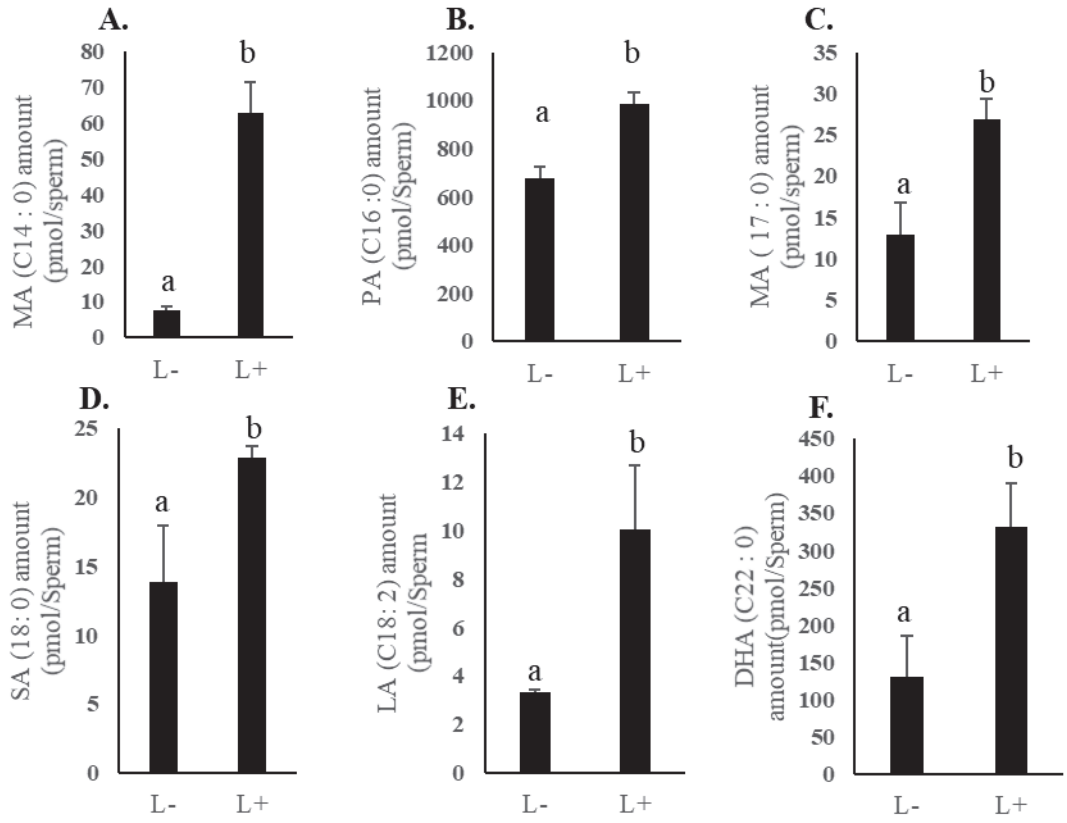


Figure -5: Total fatty acids amount (pmol/ Sperm) after thawing the sperm in lipid mixture (L+) compared to control (L-) found by GC-MS study.

Here, L+ means thawing sperm with lipid mixture and L- means thawing sperm without lipid mixture condition. Y axis indicating the amount of total fatty acid.

Values are specified (Mean±SD) with three replications. Different superscripts differ significantly at $P < 0.05$.

Here, (A.) MA (C14 :0) = Myristic acid; (B.) PA (C16 :0) = Palmitic acid; (C.) MA (C17 :0) = Margaric acid (D.) SA (C18 :0) = Stearic acid (E.) LA (C18 :2) = Linoleic acid; (F.) DHA (C22 :6) = Docosahexaenoic acid

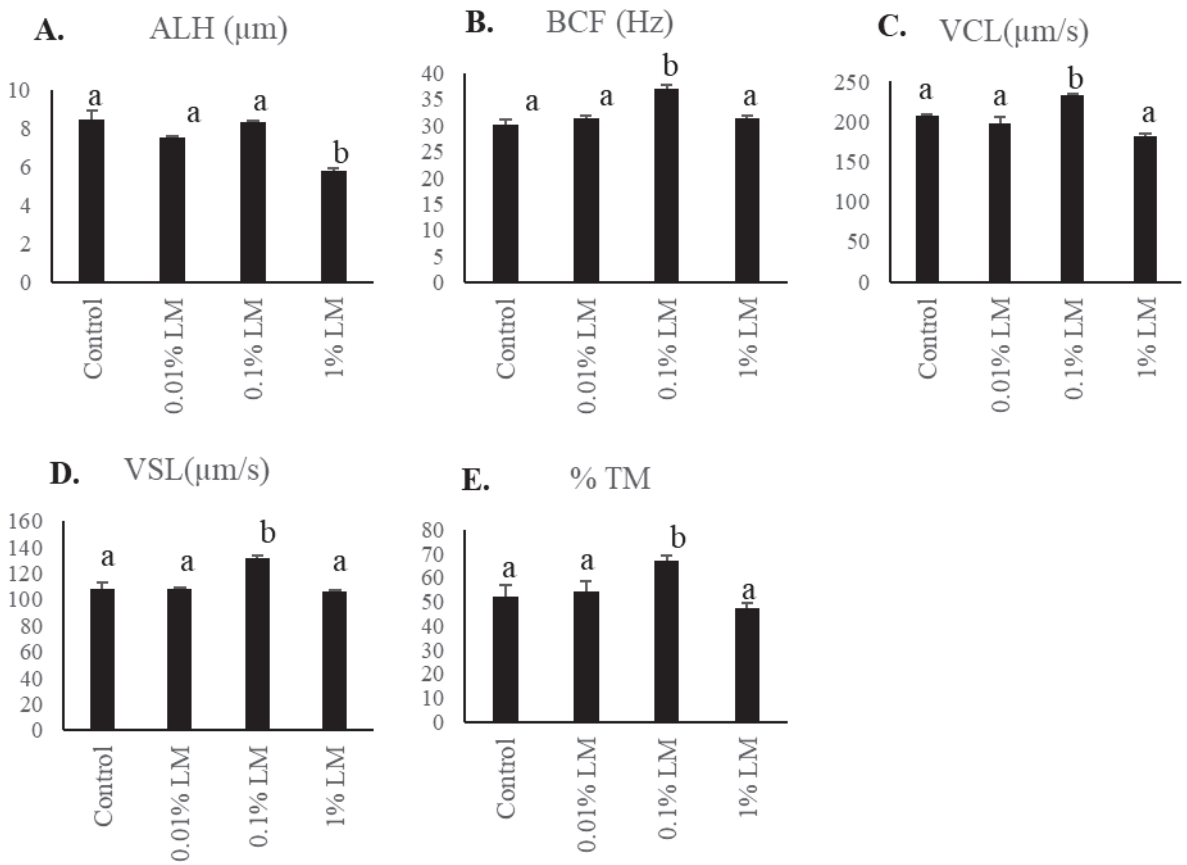


Figure - 6: Lipid mixture improves sperm motility pattern. Here, LM means thawing sperm with lipid mixture and control means thawing sperm without lipid mixture condition.

Values are specified (Mean±SD) with three replications. Different superscripts differ significantly at $P < 0.05$.

ALH indicating amplitude of lateral head displacement

BCF indicating beat cross frequency

VCL indicating curvilinear velocity

VSL indicating straight line velocity

TM indicating total motility

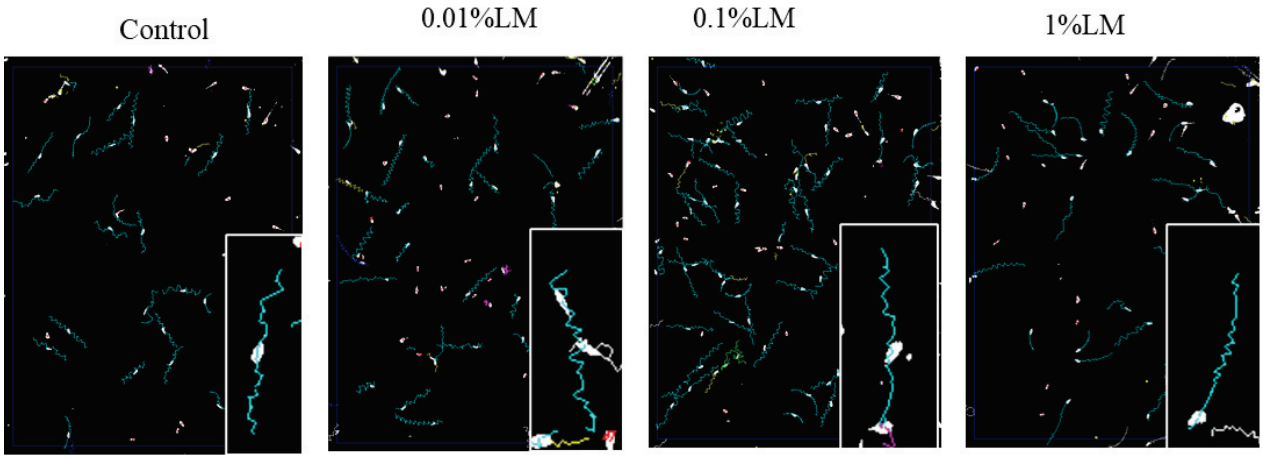
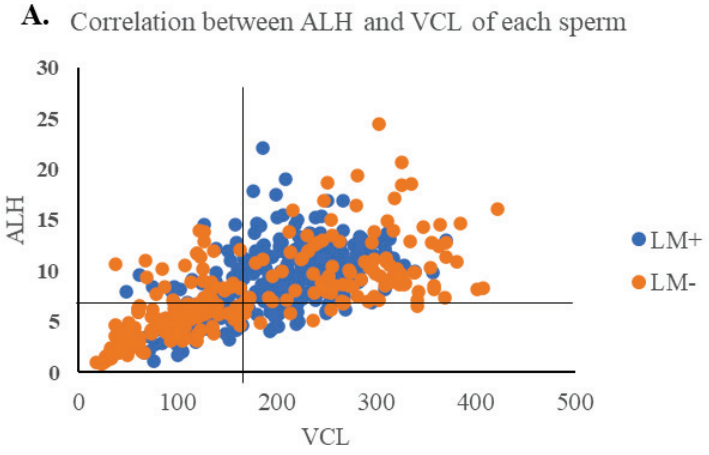
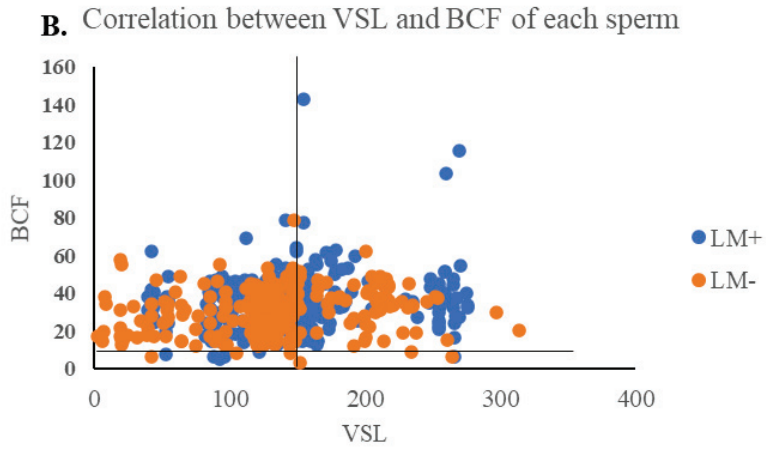


Figure -7: CASA motility track of different doses of lipid mixture (LM). Here, 0.1% LM shows Highest linear motility track compared to other tracks.



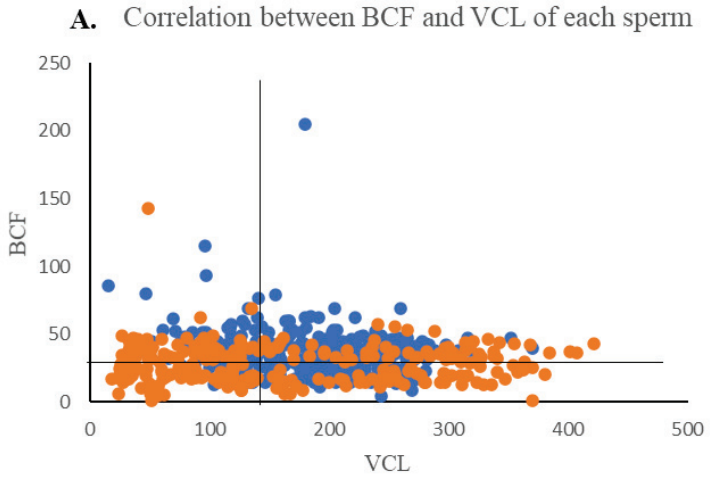
Sperm(%) (Mean ±SE)	
LM	
13 ± 4	60 ± 5[a]
14 ± 1.52[a]	13.33 ± 2.33
Sperm(%) (Mean ±SE)	
Control	
13.33 ± 1.45	35.33 ± 1.33[b]
40.00 ± 1.73[b]	10.66 ± 1.86



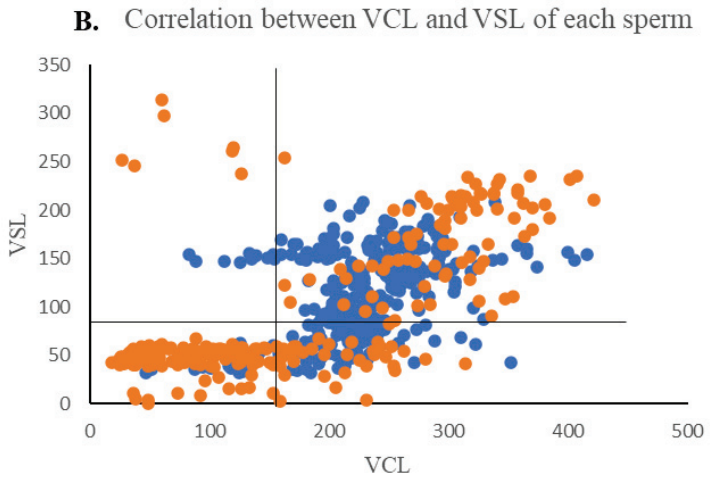
Sperm(%) (Mean ±SE)	
LM	
18 ± 3.21	51 ± 6.65[a]
14 ± 1.15[a]	17 ± 3.78
Sperm(%) (Mean ±SE)	
Control	
20.67 ± 2.33	26.67 ± 2.03[b]
33.33 ± 3.33[b]	14.33 ± 1.76

Figure - 8: Correlation between ALH and VCL (A.) and BCF and VSL(B.) of each sperm treated with Lipid mixture (LM+) in thawing media compared with control (LM-). The lines in scatter plot were made considering the mean value of each motility parameter in control group of sperm.

In each correlation figure the second quadrant (ii) region, indicating the lower value of both parameters compared to mean value of control and third quadrant (iii) region, indicating the higher value of both parameters compared to mean value of control are significantly ($P<0.05$) different in LM treated group compared to control.



Sperm(%) (Mean ±SE)	
LM+	
22 ± 1.72	44.67 ±1.76[a]
10.67 ± 1.76[a]	22.67 ± 2.40
Sperm(%) (Mean ±SE)	
Control	
31 ± 2.08	20.67 ± 1.45[b]
25.33 ± 1.20[b]	20.67 ± 2.33



Sperm(%) (Mean ±SE)	
LM+	
5.33 ± 0.89	61.33 ± 1.76[a]
6.33 ± 0.89[a]	26 ± 1.15
Sperm(%) (Mean ±SE)	
Control	
5 ± 1.15	26.67 ± 1.45[b]
51.67 ± 2.40[b]	12 ± 1.73

Figure - 9: Correlation between BCF and VCL (A.) and VCL and VSL (B.) of each sperm treated with Lipid mixture (LM+) in thawing media compared with control (LM-). The lines in scatter plot were made considering the mean value of each motility parameter in control group of sperm.

In each correlation figure the second quadrant (ii) region, indicating the lower value of both parameters compared to mean value of control and third quadrant (iii) region, indicating the higher value of both parameters compared to mean value of control are significantly ($P<0.05$) different in LM treated group compared to control.

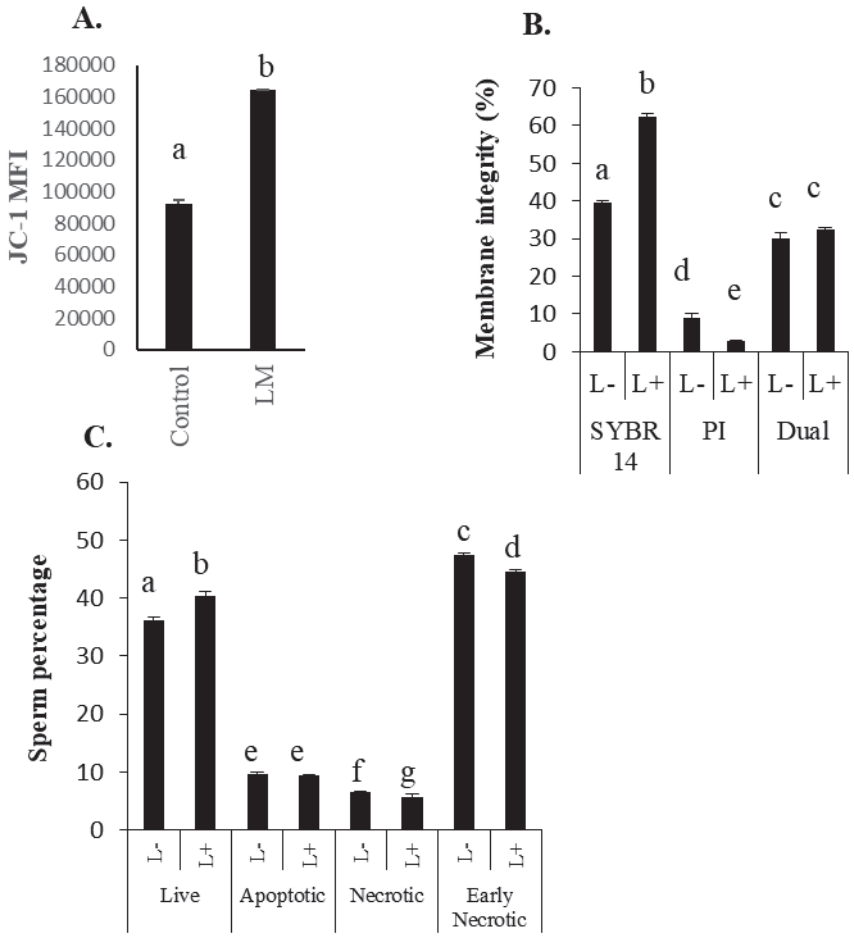


Figure -10: Lipid mixture improves sperm quality. Here, LM means thawing sperm with lipid mixture and control means thawing sperm without lipid mixture condition.

Values are specified (Mean±SD) with three replications. Different superscripts in various parameters differ significantly at $P < 0.05$ in One way ANOVA with t-test.

A. Mean fluorescence intensity (MFI) observed by JC-1 stain for mitochondrial activity

B. Membrane integrity detected by SYBR-14/PI stain

C. Live sperm (%) measured by Annexin V stain

Chapter 3

Experiment 2

Study on effect of fatty acids on sperm linear motility through mitochondrial ATP production

Contents

3.1 Introduction

Each living cell need energy for both growth and function and sperm, the male gametes, are not exception. Sperm motility is induced by ATP production (Brokaw, 1972) and indispensable for sperm migration to the oocyte for successful fertilization (Suarez & Pacey, 2006). Motility patterns are reliant on flagellar motion and the linear motility pattern is induced by the symmetrical flagellar motion (Ho et al., 2002; Curtis et al., 2012). The linear motility, is essential for sperm migration from the cervix to the uterus and then to oviduct, characterized by low lateral amplitude and high straight-line velocity (Shalgi et al., 1992; Ishijima,2015). In oviduct during fertilization process, hyperactivation is prerequisite for capacitation of sperm (Suarez, 2008), where curvilinear velocity is high as well as high lateral amplitude (Stauss et al., 1995).

For sperm motility, two main metabolic pathways are required for ATP production namely glycolysis which is occurred in sperm tail and mitochondrial oxidative phosphorylation (OXPHOS) is activated in midpiece of sperm (Du Plessis et al., 2015). Generally, glycolysis is carried out in the cytoplasm where pyruvate is produced from glucose and then it enters the mitochondria to be oxidized to acetyl CoA which combines with oxaloacetate to start the tricarboxylic acid (TCA) cycle (Zheng, 2012). However, in sperm tail as mitochondria are inadequate, so mitochondria ATP production is not highly increased although high dose of glucose rapidly induces temporary hyperactivation status (Suarez et al., 1991). Indeed, Ldh-c knock out mice model demonstrated that for hyperactivated motility the glycolysis was essential (Odet et al., 2008). Rotenone, an inhibitor of complex I, declined the sperm mitochondrial activity and ATP levels with progressive motility and straight-line velocity but not lateral amplitude and total motility in boar (Zhu et al., 2019), stallion (Plaza Davila et al., 2015) and human (Barbonetti et al., 2010). Further reports revealed that rotenone suppressed the sperm capacitation, acrosome reaction and fertilization rate in hamster (Rogers et al., 1977) and stallion (Plaza Davila et al., 2015), indicating that mitochondrial ATP production is crucial for sperm linear motility and hyperactivation.

Remarkably, it was reported in boar sperm decreasing the glucose level in extender induced linear motility and boosted OXPHOS, again completely removal of glucose from the medium failed to maintain the high linear motility and mitochondrial activity (Zhu et al., 2019). The glucose is used

as substrate not only in glycolysis but also in pentose phosphate pathway (PPP) to generate NADPH, which impacts on redox homeostasis to maintain sperm motility (Stincone et al., 2015; Gruning et al., 2014) suggesting that the pyruvate transformed from glucose would not be main sources of acetyl CoA to activate OXPHOS in sperm. It has been reported that acetyl CoA is produced by mitochondrial β-oxidation from fatty acids (Aon et al., 2014). The oxygen availability and the composition of metabolic substrates causes variation in metabolic pathways of cells (Gohil et al., 2010; Potter et al., 2016).

Interestingly, LM study (Chapter 2) showed that the saturated fatty acids have a positive correlation between the level of fatty acids and total sperm motility, similar result was observed in human sperm study (Martinez et al., 2013). From GC-MS study of previous chapter, the status of different fatty acids was significantly ($P<0.05$) higher in the LM condition and the most up taken fatty acid was myristic acid (C14: 0), a long chain saturated fatty acid. It was reported that a long-chain SFA (palmitic acid) can preserve progressive linear motility and viability whilst keeping ROS levels at a minimum level in boar sperm (Kiernan et al., 2013). Additionally, it was reported that the addition of Ω-3 PUFAs (Poly unsaturated fatty acids) directly to semen extenders was not effective in protecting the bull sperm membrane (Abavisani et al., 2013). Alternatively, the very long-chain SFAs C22:0, and C24:0 were inversely related to the total antioxidant capacity (TAC), which is directly correlated with motion parameters after thawing of human sperm (Martinez et al., 2012). Several reports on bovine species (Neill & Masters,1972) and ovine species (Neill & Masters,1973) revealed that sperm cells are able to take up lipid components or fatty acids from the surrounding environment during incubation (Cerolini et al., 2001). From the perspective of fatty acid composition, it was described that diglyceride contained myristic acid (C14: 0) as the major component of bull sperm lipids (Neill & Masters,1972) suggesting that myristic acid (C14: 0) can incorporated into sperm lipids and might improve sperm motility parameters.

In human sperm it is reported that a large proportion of the metabolic proteome was involved in lipid metabolism, including enzymes for β-oxidation (Amaral et al., 2013). Excitingly, the mitochondrial β-oxidation is an active regulator for sperm motility because inhibition of β-oxidation using etomoxir led to a decrease of sperm motility (Amaral et al., 2013). Moreover, it was reported in bulls that palmitic acid of saturated fatty acid class improved sperm progressive linear motion and viability when supplemented to the extender (Kiernan et al., 2013), suggesting

that mitochondrial β-oxidation is very essential for sperm progressive motility and survivability where exogenous fatty acids are the main source of energy.

However, the mechanism by which saturated fatty acid promotes sperm progressive motility and viability is still ambiguous. Therefore, in this present study, it was assumed that saturated fatty acid might contribute to ATP production via mitochondrial β-oxidation to protect sperm in vitro. To clarify the hypothesis sperm motility was focused with exogenous different saturated fatty acids to the thawing media by CASA and the level of the different incorporated fatty acids was analyzed by GC-MS in bovine frozen sperm. Finally, sperm mitochondrial activity and oxygen consumption rate was studied by the treatment with saturated fatty acid.

3.2 Materials and methods

3.2.1 Materials

Routine chemicals were obtained from FUJIFILM Wako Pure Chemical Industries, Ltd., (Osaka, Japan) and Nakarai Chemical Co. (Osaka, Japan). Frozen straws of bovine sperm were kindly gifted from Japan and Livestock improvement association of Japan. INC (Maebashi, Japan).

3.2.2 Sperm preparation and incubation

Frozen bull semen was thawed in water at 37 °C and washed by centrifugation twice (600 g, 4 min) with 6 mL of Bracket-Oliphant (BO) medium (Table-1) (Brackett et al. 1978) using the heated and refrigerated centrifuge machine (Kubota 5910, Japan). The sperm pellet was resuspended with BSA containing BO medium without 50 nM of myristic acid (MA) (130-03432, FUJIFILM Wako Pure Chemical Industries, Ltd., Japan), palmitic acid (PA) (165-00102, FUJIFILM Wako Pure Chemical Industries, Ltd., Japan,), and behenic acid (BA) (216941-5G, Sigma- Aldrich, USA), that were used in thawing media, and then the sperm were incubated for 30 min at 37 °C under a humidified atmosphere of 5% CO2 in the air. Before using these FAs in thawing media, they were dissolved in 99.5% ethanol (09-0770-3, Sigma- Aldrich, USA) and diluted with BO media to make the 50 nM final concentration.

3.2.3 Sample preparation for gas chromatography-mass spectrometry analysis

Fatty acids in sperm were extracted and methylated by the Fatty Acid Methylation Kit (06482-04, Nacalai Tesque Inc. Japan), and then were purified by the Fatty Acid Methyl Ester Purification Kit (06483-94, Nacalai Tesque Inc. Japan) according to manufacturer's instructions. The methylated

samples (3.0 mL) were dried, and then dissolved in 50 µL of the elution solution. The samples were subjected to GC-MS.

3.2.4 Gas chromatography-mass spectrometry analysis

Agilent 7890A GC System coupled to JMS-T 100 GCv mass detector, an Agilent 7693A autosampler was injected with 1.0 µL aliquot of samples. HP-5 capillary column (60 m × 0.32 mm i.d. × 0.25 µm film thickness; 19091J-413, Agilent Technologies, Santa Clara, CA) was used to analyze the samples and reference standards. Helium was used as the carrier gas at a constant flow rate of 2.0 mL/min. The temperature for the GC oven varied starting at 100 °C to gradually increasing up to 300 °C at the rate of 10 °C / min and held at 300 °C for 20 min. Ionization was performed using electron ionization (EI) at electron energy of 70 eV. The operation of mass spectrum was carried out in the scan mode (mass scanning range of 29-800 m/z). NIST MS Search v. 2.0 was used to detect and identify the fatty acids.

3.2.5 Detection of Sperm Motility by Computer-Assisted Sperm Analysis (CASA) System

Sperm motility was evaluated using CASA according to our previous study (Zhu et al., 2019). A 10µL of the sample was placed in a pre-warmed counting chamber to take the CASA reading after incubation of sperm at different time intervals. Sperm tracks (0.5s, 45 frames) were captured at 60 Hz according to our previous study (Zhu et al., 2019), using a CASA system (HT CASA-CerosII; Hamilton Thorne, MA, United States). Minimum three replications were used for each sample and more than 200 trajectories were recorded.

3.2.6 Mitochondrial Activity

Mitochondrial activity of sperm was measured using MitoPT® JC-1 Assay Kit (911, Immuno Chemistry Technologies, LLC.) according to previous study (Zhu et al., 2019). Briefly, sperm samples were incubated with 200 µL of 1x working solution at 37ºC for 30 min in dark. The mitochondrial activity was analyzed by flow cytometry using a filter with a bandwidth of 574/26 nm (Attune® NxT Acoustic Focusing Cytometer, Invitrogen) and measured as mean fluorescence intensity (MFI) of JC-1 orange aggregates. A total of 20,000 sperm events were analyzed.

3.2.7 Oxygen Consumption Assay

Cellular oxygen consumption rate (OCR) was monitored in real time using a Seahorse Bioscience Extracellular Flux Analyzer (XF HS Mini, Agilent, Santa Clara, CA). For flux analyzer, $NaHCO_3$-Free HTF medium was made according to previous study (Balbach et al., 2020). Frozen bovine sperm were thawed as above. After washing by $NaHCO_3$-Free HTF medium, cell numbers were quantified and diluted to 3,000,000 sperms/180 µL of $NaHCO_3$-Free HTF medium. Assay plates were coated with concanavalin A (0.5 mg/mL, Fujifilm Wako Chemicals, Osaka, Japan) overnight the day before the assay. Each well was then seeded with 180 µL of $NaHCO_3$-Free HTF medium containing 3,000,000 sperm. The assay was performed in 6 min cycles of mix (3 min) and measure (3 min) as per manufacturer's recommendations. Cells were treated as indicated with myristic acid (final concentration: 5, 50 or 500 nM) and/or etomoxir (final concentration: 40 nM).

3.2.8 Flow cytometry for quantifying the intake of palmitic acid:

To detect the fluorescence intensity in each sperm by flow cytometry, frozen-thawed bovine sperm were incubated with fluorescence labeled palmitic acid (BODIPY ^{TM} FL C_{16}, D3821 Invitrogen, USA) in a dose dependent manner (1 µM, 10 µM, 100 µM). After washing, the fluorescence level was analyzed by flow cytometry using a filter with a bandwidth of 530/30 nm (Attune® NxT Acoustic Focusing Cytometer, Invitrogen) and measured as the intensity of the average value. A total of 20,000 sperm events were analyzed.

3.2.9 Immunofluorescence

Sperm were thawed and washed by BSA-free BO medium and mounted on glass slides and air-dried. The sperm were fixed with 100% methanol for 10 min at room temperature. Sperm were probed with the 1:100 diluted primary antibody (Anti-CD36 antibody; NB 110-59724, Novus Biologicals, USA and anti-GOT2 antibody; NBP2-16708, Novus Biologicals). After washing by PBS (-), the antigens were visualized with Cy3-conjugated goat anti-mouse IgG (1:100, Sigma), FITC-conjugated goat anti-rabbit IgG (1:100, sigma) and DAPI (VECTESHIELD Mounting Medium with DAPI, Vector Laboratories, Burlingame, CA, USA). Digital images were captured using a Keyence BZ-9000 microscope (Keyence Co., Osaka, Japan).

3.2.10 Statistical analysis

Statistical analyses of data from three or four replicates for comparison were carried out by either Student's t-test or one-way ANOVA followed by Student's t-test (Statview; Abacus Concepts, Inc., Berkeley, CA).

3.3 Results:

3.3.1 Sperm motility parameters with fatty acids

Sperm motility parameters were calculated by CASA-derived motility tracks after 30-min incubation. In the sperm treated with MA or PA, BCF, VCL, VSL and TM were significantly ($P < 0.05$) higher than the control (F-) group (Figure 11). The values of VCL, VSL and TM were highest in MA group. Scatter plot was made considering the parameters of individual sperm data that were significantly increased by fatty acid treatment, BCF (Y Axis of graph) and VSL (X axis of graph), between BCF (Y Axis of graph) and VCL (X axis of graph), and between VSL (Y Axis of graph) and VCL (X axis of graph) in MA group (Figure 13 & 14) and PA group (Figure 15 & 16) respectively. The lines were made in scatter plot considering the mean value of each motility parameter in control group of sperm. The percentage of the number of sperm in the upper right quadrant of scatter plot was significantly ($P<0.05$) higher in fatty acid treatment group rather than control group in both fatty acid's figures (Figure 13,14,15,16).

3.3.2 Changes of different fatty acid level during incubation

To know whether the exogenous fatty acids of sperm were incorporated to sperm and then used as energy sources during 30-min incubation to increase the sperm velocity or not. GC-MS study showed that the significant increase levels of myristic acid (MA), palmitic acid (PA) and stearic acid (SA) but not behenic acid (BA) were observed in the treated sperm with each fatty acid (Figure 17). Interestingly, the increasing levels were not maintained during incubation and the levels were significantly ($P<0.05$) decreased to the basal levels. However, BA had no significant changes during incubation (Figure 17).

3.3.3 Fatty acids affect sperm mitochondrial activity

Mitochondrial membrane activity was analyzed by JC-1 staining. Mean fluorescence intensity (MFI) of the sperm incubated for 30 min at 37ºC thawed with the different fatty condition

significantly ($P<0.05$) differed from the sperm incubated for the same time and under the same conditions but underwent thawing without fatty condition (Figure 18). Sperm showed a significantly lower MFI in without fatty acid condition and BA condition than with MA and PA condition.

3.3.4 Localization of fatty acid transporter in sperm

When frozen-thawed sperm were incubated with fluorescence labeled palmitic acid, the fluorescence signals were selectively observed in midpiece of sperm. The fluorescence intensity in each sperm detected by FACS was significantly ($P<0.05$) increased in dose dependent manner (Figure 19-A, B & C). Furthermore, fatty acid transporters (CD36 and GOT2) were localized in the joint region between head and midpiece of sperm (Figure 19-D).

3.3.5 Effect of fatty acid in sperm metabolic changes

To quantify the metabolic changes of mitochondrial activity, real-time changes were monitored in mitochondrial β-oxidation using a Seahorse Bioscience Extracellular Flux Analyzer (XF HS Mini, Agilent, Santa Clara, CA). Firstly, the oxygen consumption rate (OCR) was traced at different concentration of MA (Figure 20-A & B). The significant increase of OCR was induced by the injection of 50 nM of MA. Etomoxir, an inhibitor of β-oxidation significantly suppressed the MA-induced OCR rate (Figure 20-C & D).

3.4 Discussion

Sperm motility pattern are changed from ejaculation up to fertilization process. In vivo fertilization, the motility pattern of sperm is altered from linear motility in the uterus to zigzag motility in the oviduct during the journey of sperm in female reproductive tracts (De Lamirande et al., 1997). It is generally accepted that the sperm motility is regulated by ATP produced either from glycolytic pathway in cytoplasm or OXPHOS in mitochondria or both (Storey, 2008; Mukai & Travis, 2012). It was revealed in boar sperm study that mitochondrial activity was increased under low glucose condition, and the ATP produced in mitochondria was associated with a high-speed linear motility (Zhu et al., 2019), which is characterized by high straight-line velocity (VSL) and low lateral amplitude (ALH) (Shalgi et al., 1992), indicating that sperm linear motility is directly associated with mitochondrial activity.

As LM is composed of both saturated and unsaturated fatty acids and cholesterol, in previous chapter it was described that the levels of saturated long fatty acid including myristic acid (C14); palmitic acid (C16); margaric acid (C17) and stearic acid (C18) were increased in sperm after the treatment with LM. Excitingly, present study showed that after the treatment of each fatty acid the increasing levels of myristic acid (MA), palmitic acid (PA) and stearic acid (SA) but not behenic acid (BA), were not maintained during incubation and the levels were significantly ($P<0.05$) decreased to the basal levels. However, BA had no significant changes during incubation (Figure 17). It is suggesting that during incubation period the long chain fatty acids (MA, PA, SA) were incorporated to sperm and used as energy sources to keep motility sound.

It was reported that unsaturated fatty acids reduced quality of frozen thawed bull sperm in citrate extender (Kandelousi et al., 2013). Similarly, other experiments revealed that unsaturated fatty acids supplementation lowered sperm viability; motility and morphology (Abavisani et al., 2013). Therefore, in this study saturated fatty acids were added to sperm thawing medium to determine whether exogenous saturated fatty acids would be incorporated into frozen-thawed bull sperm and improve the motility of frozen-thawed sperm or not. The addition of long-chain saturated fatty acids (C14) but not ultralong one (C22) to thawing medium significantly ($P<0.05$) induced linear motility pattern. It has been also reported that the long chain saturated fatty acid (C16) can preserve progressive linear motility and viability whilst keeping ROS levels at a minimum level in boar sperm (Kiernan et al., 2013). On the other hand, the very long-chain saturated fatty acids (C22:0 and C24:0) were inversely related to the total antioxidant capacity (TAC), which is directly correlated with motion parameters after thawing of human sperm (Martinez et al., 2012).
In this study with fatty acid, the progressive linear motility was improved in bovine frozen sperm with high straight-line velocity (Figure11). The high curvilinear velocity (VCL) was also observed in fatty acid treatment compared to control (Figure 11); however, ALH was not increased, suggesting that hyperactivation was not induced by the treatment with fatty acid because both of ALH and VCL are increased whereas VSL is decreased in hyper-activated sperm (Zhu et al., 2019). Individual sperm effect of fatty acid on sperm motility parameters also revealed that fatty acid affected significantly ($P< 0.05$) individual sperm VSL and VCL (Figure 13,14,15,16), indicating that linear motility pattern was induced after the treatment of fatty acid. Therefore, it is suggesting that saturated fatty acids would be utilized in the mitochondria of sperm for the production of ATP.

Although fatty acids are essential nutrients for cellular functions (McArthur et al., 1999), but how they are transported across the plasma membrane in sperm, is not well understood. There was evidence that the uptake of protein-facilitated fatty acids is the key pathway in metabolic tissues including liver, adipose tissue, and muscle (Hajri et al., 2002; Ehehalt et al., 2006). CD36 was a major participant in metabolic tissues including the proteins involved in fatty acids uptake (Pepino et al., 2014; Su & Abumrad, 2009). The CD36 forms heterodimer with plasma membrane fatty acid binding protein (FABPpm/GOT2), which transports the vital fatty acid in the heart of human (Gimeno et al., 2003). Another study on liver cell of rat revealed that FABPpm/GOT2 was located in the plasma membrane and has been detected as fatty acid transporters to regulate the uptake of long chain fatty acids (Schwieterman et al., 1988). Immunofluorescence study showed that both CD36 and GOT2 are localized in the joint of head and midpiece of sperm (Figure 19) where actually mitochondria are located. Moreover, the fluorescence-tagged palmitic acid was observed in sperm midpiece, indicating that saturated fatty acid was up taken to sperm midpiece via a CD36-GOT2 dependent manner for using ATP production in mitochondria.

Mitochondrial ATP is generated through transformation of ADP by the reduction of electron carriers (Amaral et al., 2013). The electron transfer chain (ETC) comprises of several complexes that carry electrons attained from the oxidation of NADH and FADH2 moiety of succinate dehydrogenase, and ultimately decreased the final acceptor oxygen to water (Bratic & Trifunovic, 2010). It has been reported that exogenous fatty acids can be used as energy substrate for sperm. For example, boar sperm study reported that the addition of exogenous fatty acids (oleic acid and palmitic acid) to the dilution medium improved sperm quality by utilizing them as energy substrates for ATP production via β-oxidation (Zhu et al., 2020). In this study, myristic acid showed good result in mitochondrial β-oxidation (Figure 20), suggesting that long chain saturated fatty acids in thawing media of frozen bovine sperm can be used as energy substrate for ATP production in mitochondria in sperm midpiece to induce and maintain their linear motility.

It can be concluded that saturated fatty acids were transported to the sperm mitochondria through fatty acid transporters, where they acted as energy substrate for mitochondrial β-oxidation to produce energy for the sperm to keep linear motility. Thus, to increase and keep sperm fertilization ability, exogenous fatty acid is a beneficial factor to induce and keep sperm linier motility. It may possible that thawing sperm with saturated fatty acids containing insemination medium will boost

sperm linear motility in the female genital tracts to improve fertilization as a simple and low-cost tactic.

Although present study was deeply concerned with motility parameters, especially the motility pattern of sperm induced by fatty acid treatment with mitochondrial activity as energy production unit alongside the fatty acid transporter of sperm molecular mechanism, activities of cholesterol another vital part of LM on the sperm membrane is still unknown. Further studies on the actual mechanism of cholesterol on the sperm membrane will be discussed in the next chapter.

Abstract

Sperm motility patterns are dependent on energy substrate and their effective utilization in energy producing segments of sperm. For successful fertilization sperm linear motility is very crucial and this is induced by mitochondrial ATP production. Present study was undertaken to clarify whether bovine sperm could uptake fatty acids (FAs) in thawing media and produce ATP in mitochondria for maintaining linear motility. Bovine frozen semen was thawed in FAs contained in thawing media and the sperm motility was analyzed by commuter assisted sperm analysis (CASA). The kinetic changes of FAs level in sperm were detected by GC-MS. The mitochondrial activity of sperm treated with FAs was analyzed as fluorescence intensity of JC-1 staining and oxygen consumption rate (OCR). FA transporter (CD36 and GOT2) was observed by whole-mounted immunofluorescence. Sperm linear motility was significantly ($P<0.05$) increased by the thawing with FA. Moreover, saturated fatty acids (SFAs) were predominant in sperm thawed with LM described in previous study. Interestingly, present study revealed that long chain saturated fatty acids (LCSFA) (C14, C16, C18) were decreased during 30 min incubation, except very long chain saturated fatty acid (C22). Another crucial factor revealed from this study that the bovine frozen sperm possessed FA transporter in midpiece where the fluorescence signals were detected after the treatment with fluorescence-tagged FA. The treatment with FA activated electron transportation in mitochondria through β-oxidation. Sperm linear motility is prompted by FAs in thawing media of bovine frozen sperm might provide a new approach for upgrading the artificial insemination technique of both livestock animals and human infertility care.

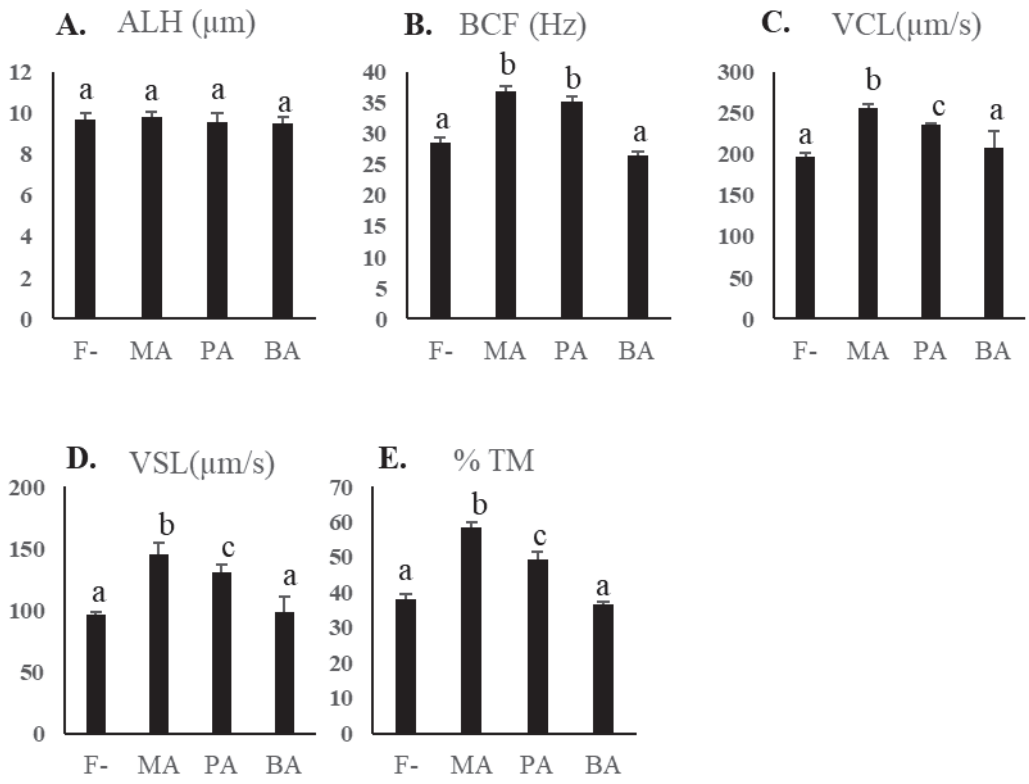


Figure -11: Fatty acids improve sperm motility parameter.

Values are specified (Mean±SD) with three replications. Different superscripts differ significantly at $P < 0.05$.

Here, MA= Myristic Acid (C14:0), PA= Palmitic Acid (C16:0), BA= Behenic Acid (C22:0) and F- = Without any fatty acid.

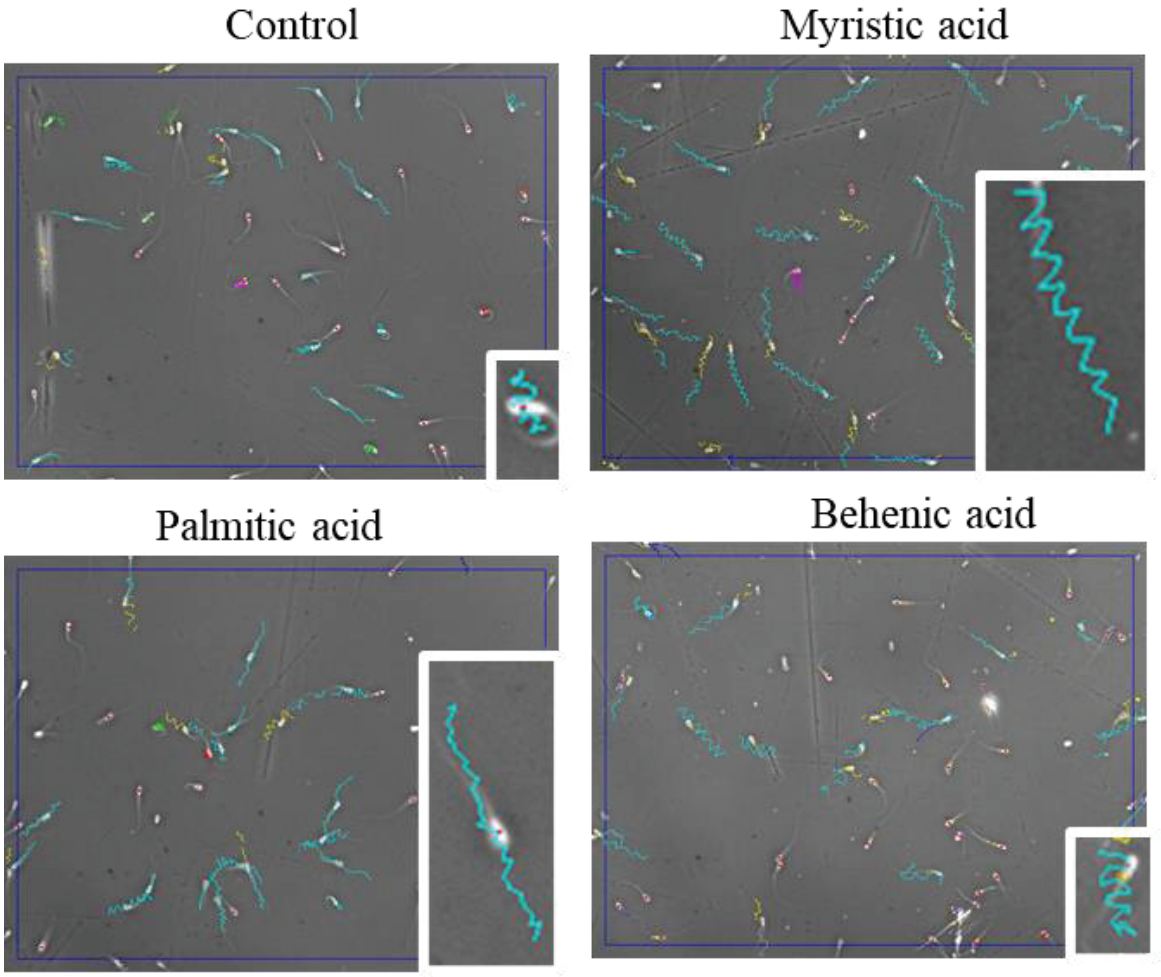
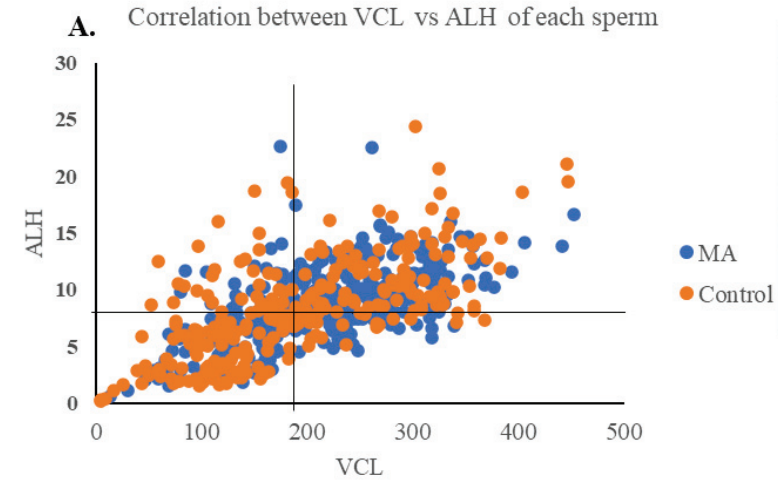
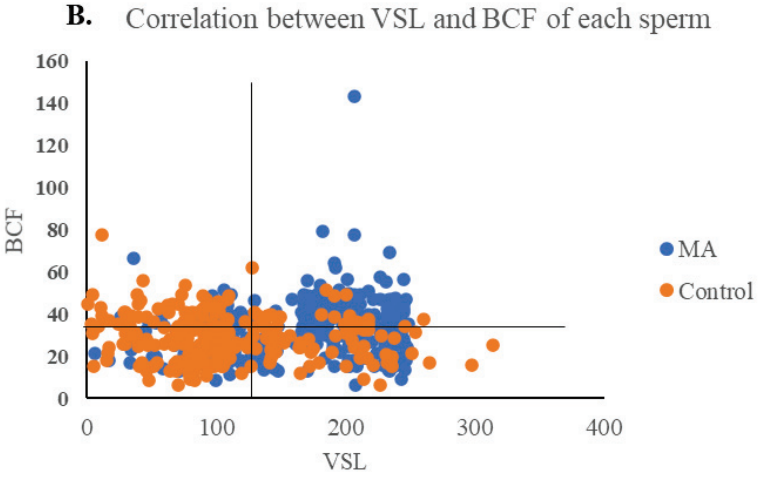


Figure -12: CASA derived motility track of different fatty acids at 50 nM concentration



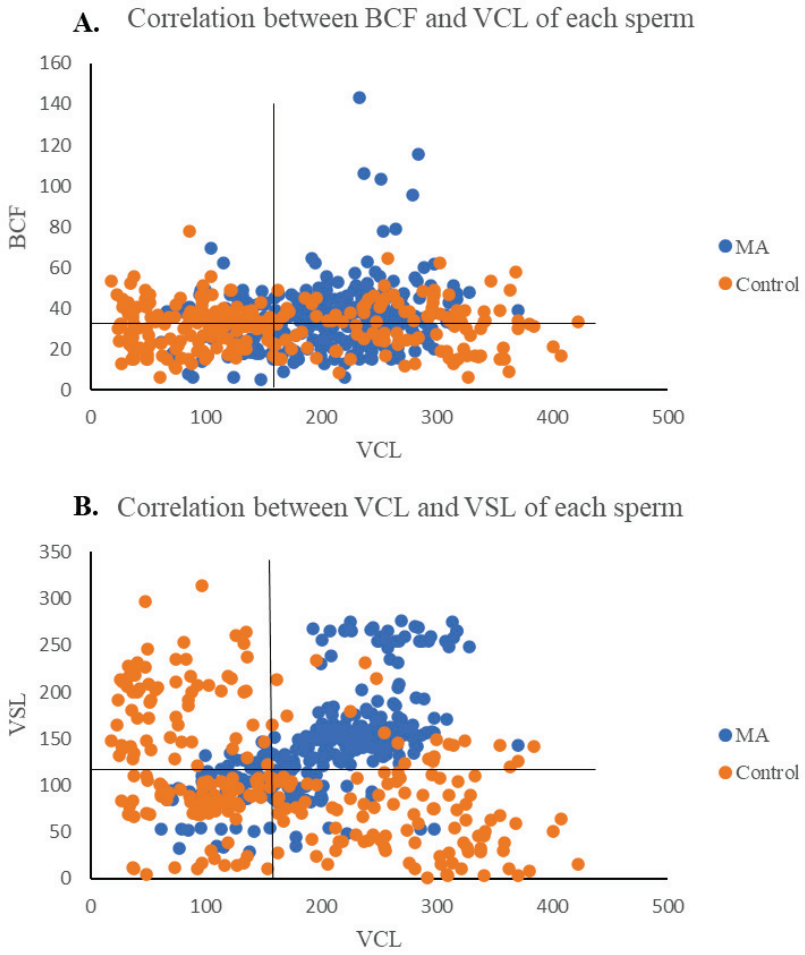
Sperm(%) (Mean ±SE)	
MA	
12.67 ± 2.67	51.67 ± 2.4[a]
22.33 ± 2.72[a]	14.0 ± 1.73
Sperm(%) (Mean ±SE)	
Control	
12.67 ± 1.20	32.67 ± 2.84[b]
42.67 ± 2.72[b]	13 ± 2.0



Sperm(%) (Mean ±SE)	
MA	
9 ± 1.52	55.33 ± 6.56[a]
13.67 ± 1.85[a]	21.33 ± 5.69
Sperm(%) (Mean ±SE)	
Control	
27 ± 5	23 ± 4.58[b]
35 ± 4.72[b]	15.33 ± 3.71

Figure -13: Correlation between ALH and VCL (A.) and BCF and VSL(B.) of each sperm treated with Myristic Acid (MA) in thawing media compared with control. The lines in scatter plot were made considering the mean value of each motility parameter in control group of sperm.

In each correlation figure the second quadrant (ii) region, indicating the lower value of both parameters compared to mean value of control and third quadrant (iii) region, indicating the higher value of both parameters compared to mean value of control are significantly ($P<0.05$) different in MA treated group compared to control.

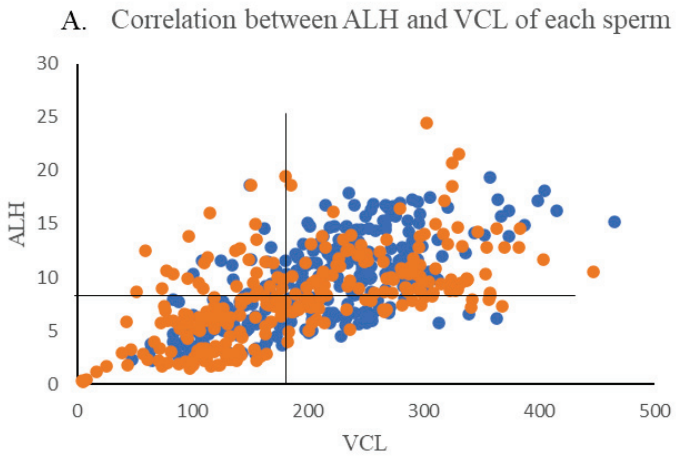


Sperm(%) (Mean ±SE)	
MA	
11 ± 1.52	51.67 ± 2.02[a]
12 ±1.15[a]	25.67± 1.76
Sperm(%) (Mean ±SE)	
Control	
30.33 ± 1.45	21.67 ± 0.89[b]
30 ± 1.73[b]	19 ± 1.15

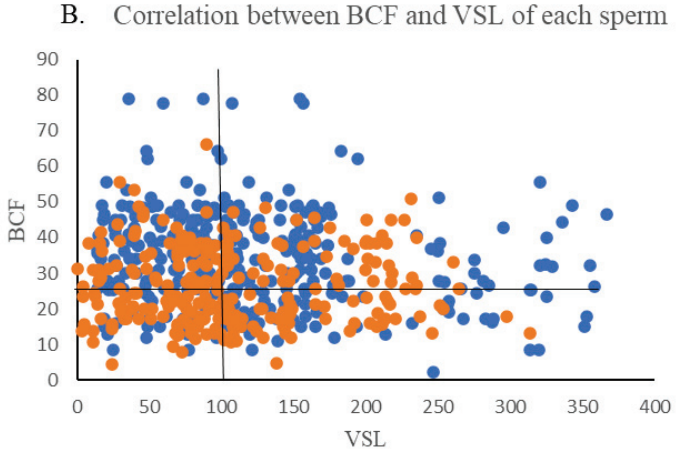
Sperm(%) (Mean ±SE)	
MA	
10 ± 1.15	67.33 ± 1.20[a]
11.67 ± 0.89[a]	8.67 ± 1.20
Sperm(%) (Mean ±SE)	
Control	
30 ± 1	10.33 ± 0.89[b]
30 ± 1.15[b]	30 ± 1.73

Figure -14: Correlation between BCF and VCL (A.) and VCL and VSL(B.) of each sperm treated with Myristic Acid (MA) in thawing media compared with control. The lines in scatter plot were made considering the mean value of each motility parameter in control group of sperm.

In each correlation figure the second quadrant (ii) region, indicating the lower value of both parameters compared to mean value of control and third quadrant (iii) region, indicating the higher value of both parameters compared to mean value of control are significantly ($P<0.05$) different in MA treated group compared to control.



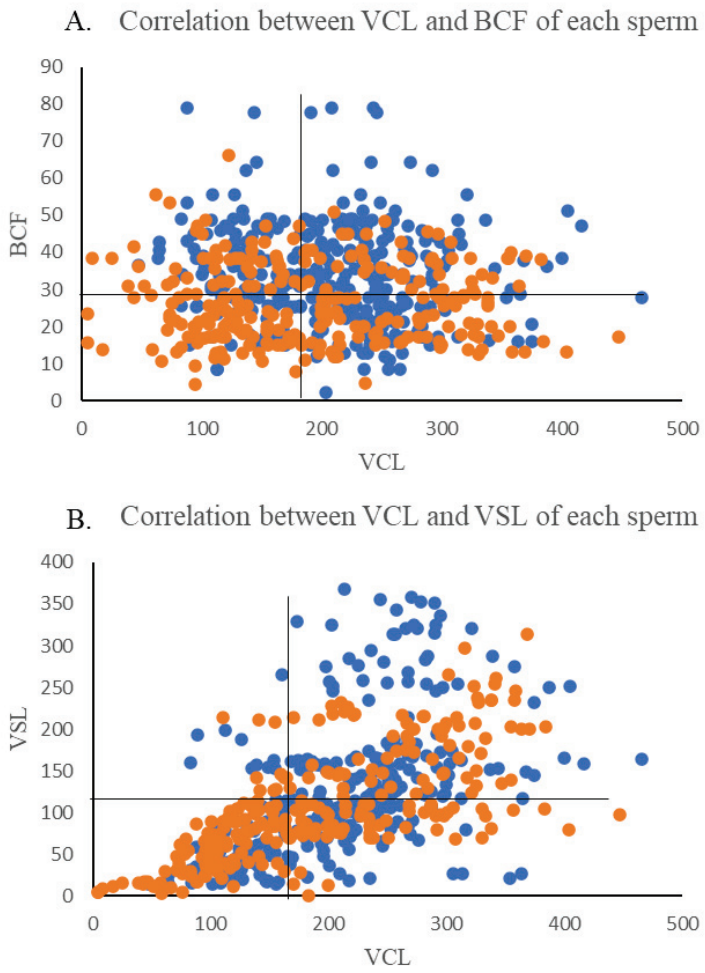
Sperm(%) (Mean ±SE)	
PA	
8.33 ± 0.88	50.33 ± 2.96[a]
24.67 ± 5.45[a]	16.33 ± 2.84
Sperm(%) (Mean ±SE)	
Control	
16 ± 3.05	34 ± 2[b]
37.33 ± 3.17[b]	12.67 ± 2.18



Sperm(%) (Mean ±SE)	
PA	
23.67 ± 6.76	39.33 ± 6.43[a]
19.67 ± 3.33[a]	17.67 ± 3.92
Sperm(%) (Mean ±SE)	
Control	
24.33 ± 3.71	22.67 ± 4.66[b]
36.33 ± 3.84[b]	16.67 ± 4.33

Figure -15: Correlation between ALH and VCL (A.) and BCF and VSL(B.) of each sperm treated with Palmitic Acid (PA) in thawing media compared with control. The lines in scatter plot were made considering the mean value of each motility parameter in control group of sperm.

In each correlation figure the second quadrant (ii) region, indicating the lower value of both parameters compared to mean value of control and third quadrant (iii) region, indicating the higher value of both parameters compared to mean value of control are significantly ($P<0.05$) different in PA treated group compared to control.



Sperm(%) (Mean ±SE)	
PA	
25.0±2.30	40.33±1.45[a]
9.67±1.20[a]	24.33±0.67
Sperm(%) (Mean ±SE)	
Control	
24.67± 2.33	23.33± 0.89[b]
28.33 ± 1.20[b]	23.33±1.20

Sperm(%) (Mean ±SE)	
PA	
8.67 ± 1.20	42.0 ± 0.58[a]
27.67±1.45[a]	22.67 ± 1.45
Sperm(%) (Mean ±SE)	
Control	
12.0±1.67	30.0± 1.15[b]
40.67 ± 2.33[b]	17.67±1.20

Figure -16: Correlation between BCF and VCL (A.) and VCL and VSL(B.) of each sperm treated with Palmitic Acid (PA) in thawing media compared with control. The lines in scatter plot were made considering the mean value of each motility parameter in control group of sperm.

In each correlation figure the second quadrant (ii) region, indicating the lower value of both parameters compared to mean value of control and third quadrant (iii) region, indicating the higher value of both parameters compared to mean value of control are significantly ($P<0.05$) different in PA treated group compared to control.

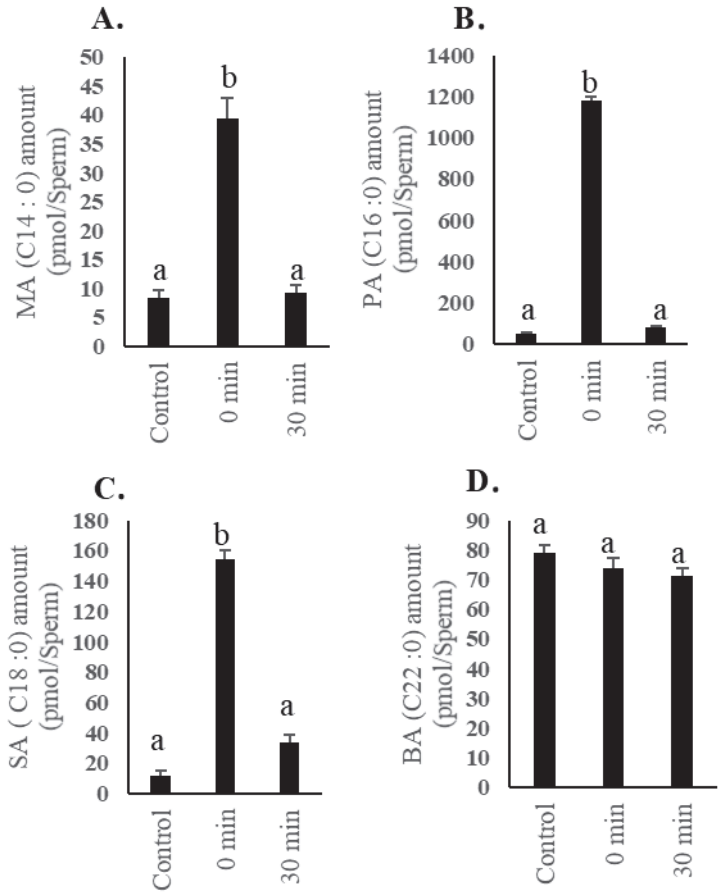


Figure -17: Total fatty acids amount measured by GC-MS after thawing the sperm in respective fatty acids and incubation for 30 minutes with BSA at 37˚C. Y axis indicating the amount of fatty acids (pmol/ Sperm).

Values are specified (Mean±SD) with three replications. Different superscripts differ significantly at $P < 0.05$.

Here, Control = Without any fatty acids; (A) MA= Myristic acid; (B) PA= Palmitic acid; (C) SA= Stearic acid; and (D) BA= Behenic (Docosanoic acid) acid.

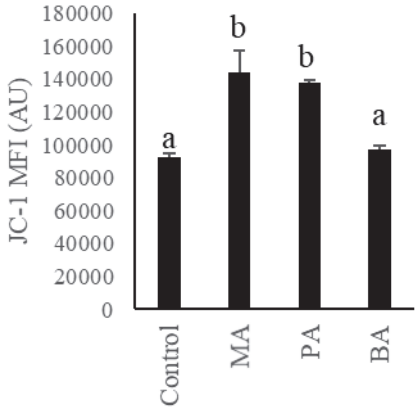


Figure -18: Fatty acids changes sperm mitochondrial activity.

Values are specified (Mean±SD) with three replications. Different superscripts differ significantly at $P < 0.05$.

Here, MA= Myristic Acid (C14:0), PA= Palmitic Acid (C16:0), BA= Behenic Acid (C22:0), and control = Without fatty acids.

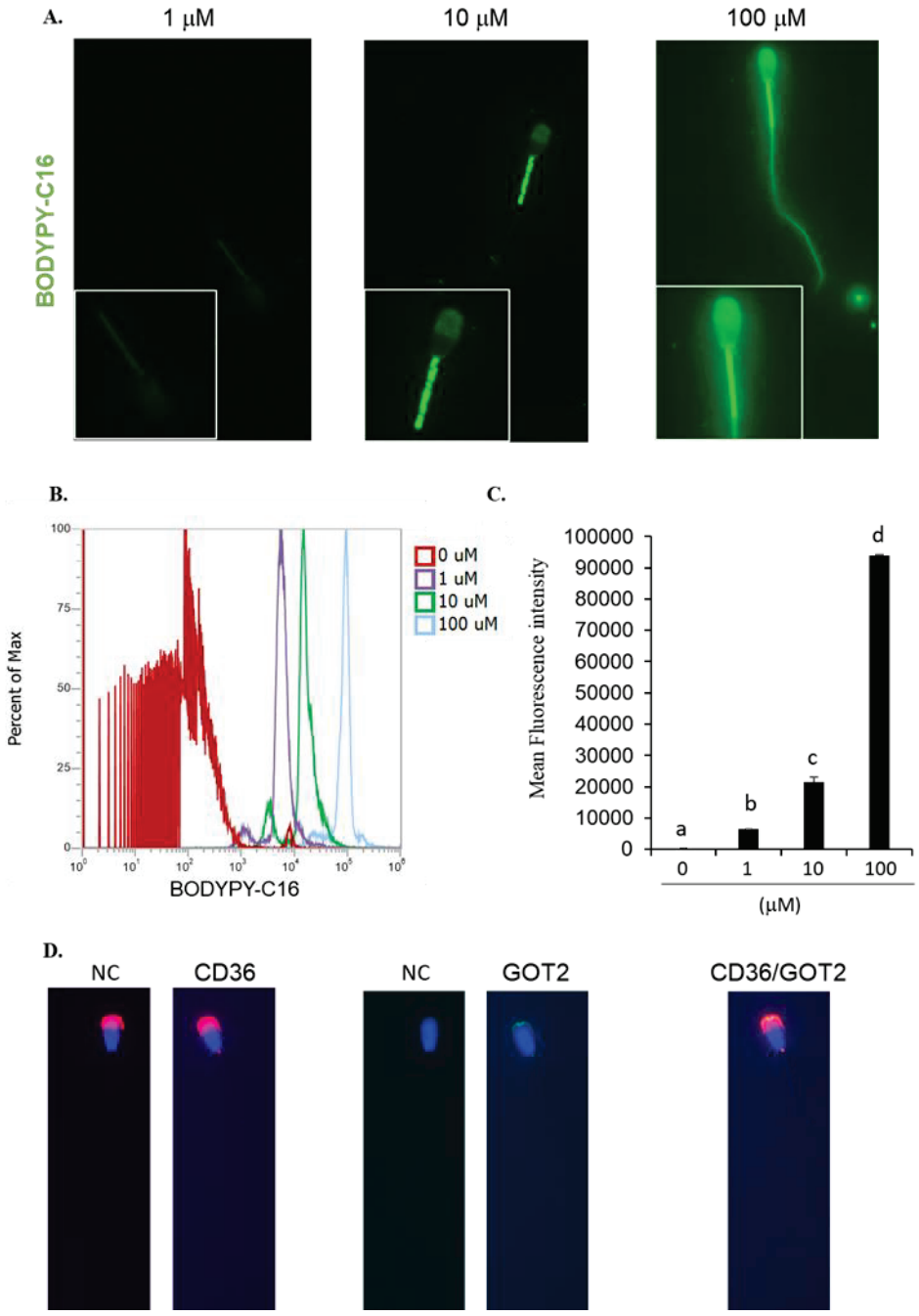


Figure 19: Fluorescence intensity of sperm and localization of fatty acid transporter.

A. Fluorescent signal of FITC palmitic acid,
B. overlay of different doses of FITC palmitic acid
C. Mean fluorescence intensity of different doses of FITC palmitic acid and
D. Localization of fatty acid transporters

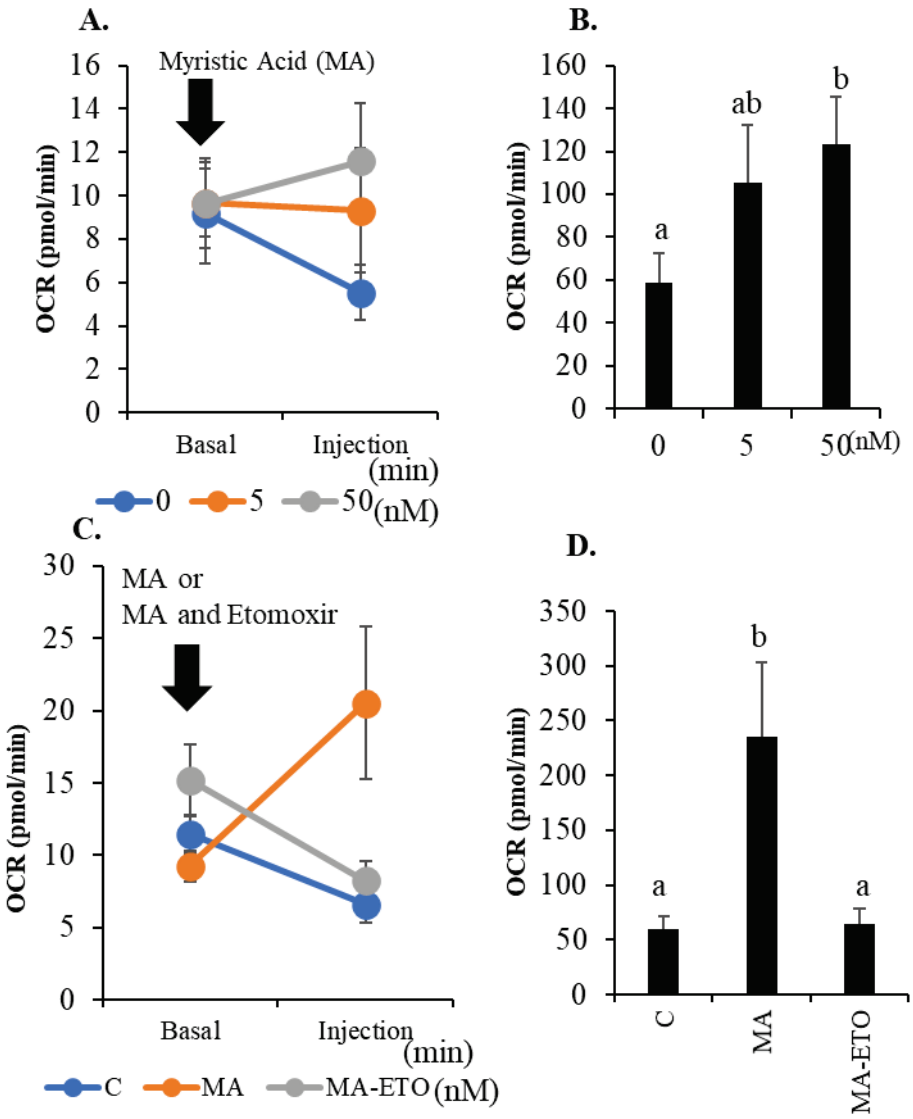


Figure -20: Metabolic effect of MA or Etomoxir, the inhibitor of β-oxidation, on frozen bovine sperm.

Values are specified (Mean±SD) with three replications. Different superscripts differ significantly at $P < 0.05$.

A. Trace of OCR before/after the treatment of several concentration of myristic acid (MA)

B. Increase ratio of OCR after the treatment of MA

C. Trace of OCR before/after the treatment of 50 nM MA or MA + Etomoxir

D. Increase ratio of OCR after the treatment of 50 nM MA or MA + Etomoxir

Chapter 4

Experiment 3

Study on effect of cholesterol on recovery of mitochondrial potential to keep linear motility in bovine frozen sperm

Contents

4.1 Introduction

Sperm are greatly polarized and specialized male reproductive unit that have a unique membrane configuration and unusual nuclear, mitochondrial, cytoplasmic, and cytoskeletal structures comparedd to those of somatic cells (Kaka et al., 2015). The sperm membranes consist of phospholipids and sphingolipids in large proportions, along with cholesterol (Cincik et al., 2007). Plasma membrane structure of sperm is not fully mature when they are released from the testis, and it is further changed during the maturation process in the epididymis (Eddy et al., 1994). The change of membrane permeability is explained by different cholesterol stability, also changed in the sperm plasma membrane by imposing conformational order on lipids in the seminal plasma during ejaculation (Leahy & Gadella, 2015). Furthermore, changes in membrane fluidity as a consequence of cholesterol efflux and protein dissociation from raft domain seems to be also a mechanism that occurs both in the maturational processes occurring at the time of ejaculation (Girouard et al., 2008) and in capacitation (Sleight et al., 2005; Cross, 2004).

Mammalian spermatozoa can be damaged during the semen cryopreservation process (Watson, 2000). During cryopreservation sperm damage arise due to cold shock, low-temperature exposure, osmotic stress, oxidative stress, and combinations of these circumstances (Purdy, 2006). It has been proposed that the sperm plasma membrane is the leading site of damage induced by cryopreservation and is one of the major causes to reduced motility and fertility of sperm during cryopreservation (Moore et al., 2005; Chakrabarty et al., 2007). Cold shock induces plasma membrane lipid phase transitions, which can be reduced by increasing the proportion of cholesterol within the plasma membrane (Drobnis et al., 1993). In addition, cryopreservation induces cholesterol depletion from plasma membrane, which in turn causes membrane destabilization (Bailey et al., 2008). Damages occur in spermatozoa due to cryopreservation includes the metabolism and mitochondrial bioenergetic processes of sperm (Oberoi et al., 2014).
Surprisingly, in LM study (Chapter 2) it was observed that mitochondrial activity, membrane integrity and rescue from cell apoptosis was promisingly higher in LM treated sperm compared to control. Additionally, in the fatty acid study (Chapter 3) it was described that saturated fatty acids are used for energy sources in mitochondria, which impacted on linear motility pattern through

mitochondrial ATP production. However, LM contains both fatty acids and cholesterol, hypothesizing that cholesterol would be incorporated to frozen-thawed sperm to recover the sperm motility. To clarify the hypothesis firstly the sperm motility parameters were examined by CASA using cholesterol in thawing media. After that, the localization of incorporated cholesterol specific signals on sperm were analyzed by fluorescence tagged cholesterol. Finally, the mitochondria metabolic activity was detected using flux analyzer system.

4.2 Materials and methods

4.2.1 Materials

Routine chemicals were obtained from FUJIFILM Wako Pure Chemical Industries, Ltd., (Osaka, Japan) and Nakarai Chemical Co. (Osaka, Japan). Frozen straws of bovine sperm were kindly gifted from Japan and Livestock improvement association of Japan. INC (Maebashi, Japan).

4.2.2 Sperm preparation and incubation

Frozen bull semen was thawed in water at 37 °C and washed by centrifugation twice (600 g, 4 min) with 6 mL of Bracket-Oliphant (BO) medium (Table-1) (Brackett et al. 1978) using the heated and refrigerated centrifuge machine (Kubota 5910, Japan). The sperm pellet was resuspended with BSA containing BO medium without Cholesterol Lipid Concentrate (250X) (0.1%) (Gibco™ 12531018) that were used in thawing media, and then the sperm were incubated for 30 min at 37 °C under a humidified atmosphere of 5% CO2 in the air.

4.2.3 Detection of Sperm Motility by Computer-Assisted Sperm Analysis (CASA) System

Sperm motility was evaluated using CASA according to our previous study (Zhu et.al. 2019). A 10µL of the sample was placed in a pre-warmed counting chamber to take the CASA reading after incubation of sperm at different time intervals. Sperm tracks (0.5s, 45 frames) were captured at 60 Hz according to our previous study (Zhu et.al. 2019), using a CASA system (HT CASA-CerosII; Hamilton Thorne, MA, United States). Minimum three replications were used for each sample and more than 200 trajectories were recorded.

4.2.4 Mitochondrial Activity

Mitochondrial activity of sperm was measured using MitoPT® JC-1 Assay Kit (911, Immuno Chemistry Technologies, LLC.) according to Zhu et.al. 2019. Briefly, sperm samples were

incubated with 200 µL of 1x working solution at 37ºC for 30 min in dark. The mitochondrial activity was analyzed by flow cytometry using a filter with a bandwidth of 574/26 nm (Attune® NxT Acoustic Focusing Cytometer, Invitrogen) and measured as mean fluorescence intensity (MFI) of JC-1 orange aggregates. A total of 20,000 sperm events were analyzed.

4.2.5 Membrane integrity

The sperm membrane integrity was measured using a LIVE/DEAD Sperm Viability Kit (L7011; Thermo Fisher Scientific) according to manufacturer protocol. Briefly, Sperm were stained with SYBR-14/PI and incubated with 200 µL 1x working solution at 37ºC for 10 min in dark. The staining was analyzed by flow cytometer (Attune® NxT Acoustic Focusing Cytometer, Invitrogen) with Excitation/ Emission= 515/545 nm for SYBR-14 fluorescence, Excitation/ Emission= 675/715 nm for PI fluorescence. A total of 20,000 sperm events were analyzed. There were four quadrants on the specific events gated area. Q1 (-/+) indicating the percentage of SYBR-14 containing sperm cells, Q2 (+/+) indicating the percentage of both SYBR-14 and PI containing sperm cells, Q3 (+/-) indicating the percentage of PI containing sperm cells and Q4 (-/-) indicating the percentage of sperm cells neither containing PI nor SYBR-14.

4.2.6 Annexin v staining

The sperm membrane apoptosis was measured using an Annexin v conjugate: Alexa Fluor®647 Kit (A23204; Invitrogen, Molecular probes) according to manufacturer protocol. Briefly, Sperm were stained with Alexa Fluor®647 /PI and incubated with 200 µL 1x working solution at 37ºC for 30 min in dark. The staining was analyzed by flow cytometer (Attune® NxT Acoustic Focusing Cytometer, Invitrogen) with Excitation/ Emission= 663/677 nm for Alexa Fluor®647 fluorescence, Excitation/ Emission= 561/587 nm for PI fluorescence. A total of 20,000 sperm events were analyzed. There were four quadrants on the specific events gated area. Q1 (-/+) indicating the percentage of necrotic sperm cells, Q2 (+/+) indicating the percentage of early necrotic sperm cells, Q3 (+/-) indicating the percentage of apoptotic sperm cells and Q4 (-/-) indicating the percentage of live sperm cells.

4.2.7 Oxygen Consumption Assay

Cellular oxygen consumption rate (OCR) was monitored in real time using a Seahorse Bioscience Extracellular Flux Analyzer (XF HS Mini, Agilent, Santa Clara, CA). For flux analyzer, $NaHCO_3$-

Free HTF medium was made according to previous study (Balbach et al., 2020). Frozen bovine sperm were thawed with 0.1% cholesterol as above. After washing by $NaHCO_3$-Free HTF medium, cell numbers were quantified and diluted to 3,000,000 sperms/180 µL of $NaHCO_3$-Free HTF medium. Assay plates were coated with concanavalin A (0.5 mg/mL, Fujifilm Wako Chemicals, Osaka, Japan) overnight the day before the assay. Each well was then seeded with 180 µL of $NaHCO_3$-Free HTF medium containing 3,000,000 sperm. The assay was performed in 6 min cycles of mix (3 min) and measure (3 min) as per manufacturer's recommendations. Two inhibitors were used separately in this study namely oligomycin (04876, Sigma, USA) and FCCP (Carbonyl cyanide-p-trifluoro methoxy phenylhydrazone) (C2920, Sigma, USA). Final concentration was 5 µM for these inhibitors. ATP production rate was calculated according to previous guideline (Romero et al., 2018).

4.2.8 Flow cytometry for quantifying the intake of cholesterol:

To detect the fluorescence intensity in each sperm by flow cytometry, frozen-thawed bovine sperm were incubated with fluorescence labeled cholesterol (TopFluor® Cholesterol 810255, Nanocs, NY, USA) in a dose dependent manner (1.0%, 5.0% and 10.0%). After washing, the fluorescence level was analyzed by flow cytometry using a filter with a bandwidth of 530/30 nm (Attune® NxT Acoustic Focusing Cytometer, Invitrogen) and measured as the intensity of the average value. A total of 20,000 sperm events were analyzed. Digital images were captured using a Keyence BZ-9000 microscope (Keyence Co., Osaka, Japan). Trypan blue (0.4%) (355-25, Nakarai Chemicals Ltd. Japan) was used as quenching media to detect the actual region of cholesterol uptake.

4.2.9 Statistical analysis

Statistical analyses of data from three or four replicates for comparison were carried out by either Student's t-test or one-way ANOVA followed by Student's t-test (Statview; Abacus Concepts, Inc., Berkeley, CA).

4.3 Results:

4.3.1 Sperm motility parameters with cholesterol

Sperm motility parameters were calculated by CASA-derived motility tracks after 30-min incubation. In the sperm treated with 0.1% cholesterol (CHL), BCF, VCL, VSL and TM were significantly ($P < 0.05$) higher than the control (F-) group and other concentrations of CHL treated

group (Figure 21). The values of VCL, VSL and TM were highest in 0.1% CHL treated group. In CASA motility track, stretched zigzag motility pattern was observed in 0.1% CHL treated group compared to other concentrations of CHL treated group and control group (Figure 22). Scatter plot was made considering the parameters of individual sperm data that were significantly increased by 0.1% CHL treatment, BCF (Y Axis of graph) and VSL (X axis of graph), between BCF (Y Axis of graph) and VCL (X axis of graph), and between VSL (Y Axis of graph) and VCL (X axis of graph) in 0.1% CHL (Figure 23 & 24). The lines were made in scatter plot considering the mean value of each motility parameter in control group of sperm. The percentage of the number of sperm in the upper right quadrant of scatter plot was significantly ($P<0.05$) higher in 0.1% CHL treatment group rather than control group (Figure 23,24).

4.3.2 Cholesterol affect sperm membrane integrity

The sperm membrane integrity was analyzed by SYBR-14/PI staining. The intact membrane integrity of the sperm incubated for 30 min at 37ºC thawed under the presence of 0.1 % (v/v) cholesterol, stained by SYBR-14 showed significantly ($P<0.05$) higher percentages than without cholesterol (Figure 25-A). Damages of sperm membrane integrity stained by PI was significantly ($P<0.05$) lower in the cholesterol condition group than in the group without cholesterol condition thawing (Figure 25-A).

Live, apoptotic, necrotic, and early necrotic cells were analyzed by Annexin V staining. The number of live sperm cells was significantly ($P<0.05$) higher in the cholesterol condition group than without cholesterol condition thawing (Figure 25-B). The numbers of necrotic and early necrotic sperm cells were significantly ($P<0.05$) lower in the cholesterol group than without cholesterol condition thawing (Figure 25-B).

4.3.5 Localization of cholesterol signals in sperm

When frozen-thawed sperm were incubated with fluorescence labeled cholesterol, the fluorescence signals were selectively observed in midpiece of sperm. The fluorescence intensity in each sperm detected by FACS was significantly ($P<0.05$) increased in dose dependent manner (Figure 26-A, B). Furthermore, incorporated cholesterol was localized in the midpiece and head of sperm (Figure 26-C).

4.3.3 Cholesterol affect sperm mitochondrial activity

Mitochondrial membrane activity was analyzed by JC-1 staining. Mean fluorescence intensity (MFI) of the sperm incubated for 30 min at 37ºC thawed in the cholesterol condition significantly ($P<0.05$) differed from the sperm incubated for the same time and under the same conditions but underwent thawing without cholesterol condition (Figure 27-E). Sperm showed a significantly lower MFI in without cholesterol condition than with cholesterol condition.

To quantify the metabolic changes of mitochondrial activity, real-time changes of mitochondria metabolic activity were monitored using a Seahorse Bioscience Extracellular Flux Analyzer (XF HS Mini, Agilent, Santa Clara, CA). The significant increase of OCR was induced 0.1% CHL used in thawing of sperm. Oligomycin, an inhibitor of oxidative phosphorylation significantly ($P<0.05$) suppressed the CHL-induced OCR rate (Figure 27-A), whereas FCCP accelerated the OCR rate (Figure 27-C & D). Most interestingly, the ATP production rate was significantly ($P<0.05$) increased in CHL thawed group of sperm compared to the control (Figure 27-B).

4.4 Discussion

Sperm motility is thought to be a strong prognostic marker of male fertility potential along with all the semen parameters and it is controlled by its complex, structural, and molecular signaling mechanisms (Dcunha et al., 2020). *In vivo* fertilization of mammalian animals is required to maintain linear motility of sperm during migration process through the uterus to oviduct for penetrating the oocyte and ATP generation is indispensable for flagellar movement to preserve this type of motility pattern (Shalgi et al., 1992, Suarez & Pacey, 2006). Most commonly it is believed that the sperm motility is controlled by ATP produced either from glycolytic pathway in cytoplasm or OXPHOS in mitochondria or both (Storey, 2008; Mukai & Travis, 2012). It was uncovered in boar sperm study that mitochondrial activity was increased under low glucose condition, and the ATP produced in mitochondria was correlated with a high-speed linear motility (Zhu et al., 2019), which is described by high straight-line velocity (VSL) and low lateral amplitude (ALH) (Shalgi et al., 1992), suggesting that sperm linear motility is directly related with mitochondrial activity.

Kinematic parameters, such as straight-line velocity (VSL) and curvilinear velocity (VCL), have prognostic value in predicting the fertilization potential of spermatozoa (Tan et al., 2014; Joshi et al., 1996). Sperm motility is supposed to have a strong correlation with assisted reproductive technology (For example, AI and IVF) success and pregnancy outcome, and it was reported that values for VAP, VSL, and VCL were significantly higher in samples that results more than 50% fertilization, representing positive correlation between progressive motility and fertilization ability (Donnelly et al., 1998).

In this study with cholesterol, the progressive linear motility was improved in bovine frozen sperm with high straight-line velocity (Figure 21). The high curvilinear velocity (VCL) was also observed in cholesterol treatment compared to control (Figure 21); however, ALH was not increased, suggesting that hyperactivation was not induced by the treatment with cholesterol because both of ALH and VCL are increased whereas VSL is decreased in hyper-activated sperm (Zhu et al., 2019). Individual sperm effect of cholesterol on sperm motility parameters also revealed that cholesterol affected significantly ($P< 0.05$) individual sperm VSL and VCL (Figure 23, 24), indicating that linear motility pattern was induced after the treatment of cholesterol. Therefore, it is suggesting that cholesterol would be incorporated and utilized in the mitochondria of sperm for the production of ATP.

It was described that cryo-damage occur at freeze thaw process can be reduced by supplying cholesterol in freezing diluents (Wessel and Ball, 2004). Moreover, it was stated that membrane cholesterol to phospholipid ratio (C:P) influences the sperm sensitivity to cold shock damage (Holt, 2000). Sperm possessing elevated C:P ratios for example, rabbit and human sperm; 0.88 and 0.99 respectively, are more resistant to the "cold shock" damage compared to sperm having low C:P ratios for instances, boar, ram, and bull sperm; 0.35, 0.37 and 0.45 respectively (Davis, 1981, White, 1993). Prevention of the cryo-capacitation enhances the durability of the sperm cells after thawing which can be achieved by adding cholesterol in sperm during freezing (Moce et al., 2010). Additionally, it was described that cholesterol loaded methyle β-cyclodextrin in extender advanced survivability of stallion (Combes et al., 2000; Graham, 1998) and bull spermatozoa (Amorim et al., 2009) after cryopreservation suggesting that cholesterol can be up taken by sperm to recover membrane damage and motility.

Interestingly, in this cholesterol study it was observed that the sperm membrane integrity (Figure-25) and mitochondrial activity (Figure-27) was significantly ($P<0.05$) increased in cholesterol

treated group compared to control. Additionally, it was discovered in this study that cholesterol was localized in the mid piece and head region of sperm (Figure-6). As cholesterol was up taken by sperm in the midpiece region so new story raised from this study that up taken cholesterol may be used as mitochondrial membrane structure recovery to maintain sperm linear motility pattern. In fact, cholesterol showed good result in real ATP production as well as increasing the potential of mitochondria ATP production (Figure 7), suggesting that cholesterol in thawing media of frozen bovine sperm can improve the structure of mitochondria in sperm midpiece to induce and maintain ATP production for linear motility.

In concluding remarks, it can be said that cholesterol is up taken to the sperm mitochondria where they acted as components of not only cell membrane but also mitochondria membrane structure to produce energy for the sperm to keep linear motility. Thus, to increase and keep sperm fertilization ability, exogenous cholesterol is an advantageous factor to induce and keep sperm linear motility which is very maiden in the discovery of sperm research. It may possible that thawing sperm with cholesterol containing insemination medium will boost sperm linear motility in the female genital tracts to improve fertilization as a simple and low-cost approach.

Abstract

Mitochondria are an important source of energy required for sperm motility. Abnormalities of mitochondrial membrane integrity are vitally related with abnormal sperm motility. For successful fertilization sperm linear motility is very important and this is induced by mitochondrial ATP production. Present study was undertaken to clarify whether bovine sperm could uptake cholesterol (CHL) in thawing media and recover the mitochondrial membrane structure which was distorted in cryopreservation of sperm. Bovine frozen semen was thawed in CHL contained in thawing media and the sperm motility was analyzed by commuter assisted sperm analysis (CASA). The cholesterol uptake signal was observed by fluorescence intensity in the mid piece and head region of sperm after the treatment with fluorescence-tagged CHL. The mitochondrial activity of sperm treated with CHL was analyzed as fluorescence intensity of JC-1 staining and oxygen consumption rate (OCR). OCR was higher in CHL treated group due to up taken CHL was used to renovate the altered mitochondrial structure. Sperm linear motility was significantly ($P<0.05$) increased by the thawing with CHL. Sperm linear motility is prompted by CHL in thawing media of bovine frozen sperm might provide a new vision for modernizing the artificial insemination technique of both livestock animals and human infertility care.

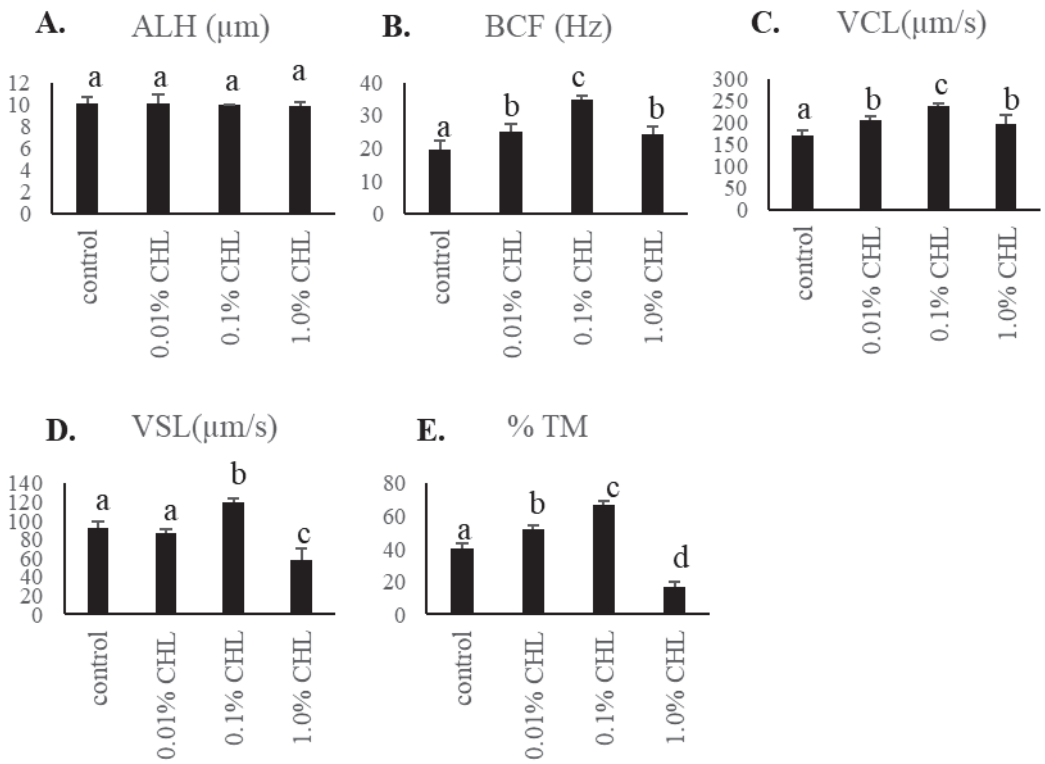


Figure -21: CASA motility parameters of sperm incubated with different doses of cholesterol for 30 min

Values are specified (Mean±SD) with three replications. Different superscripts differ significantly at $P < 0.05$.

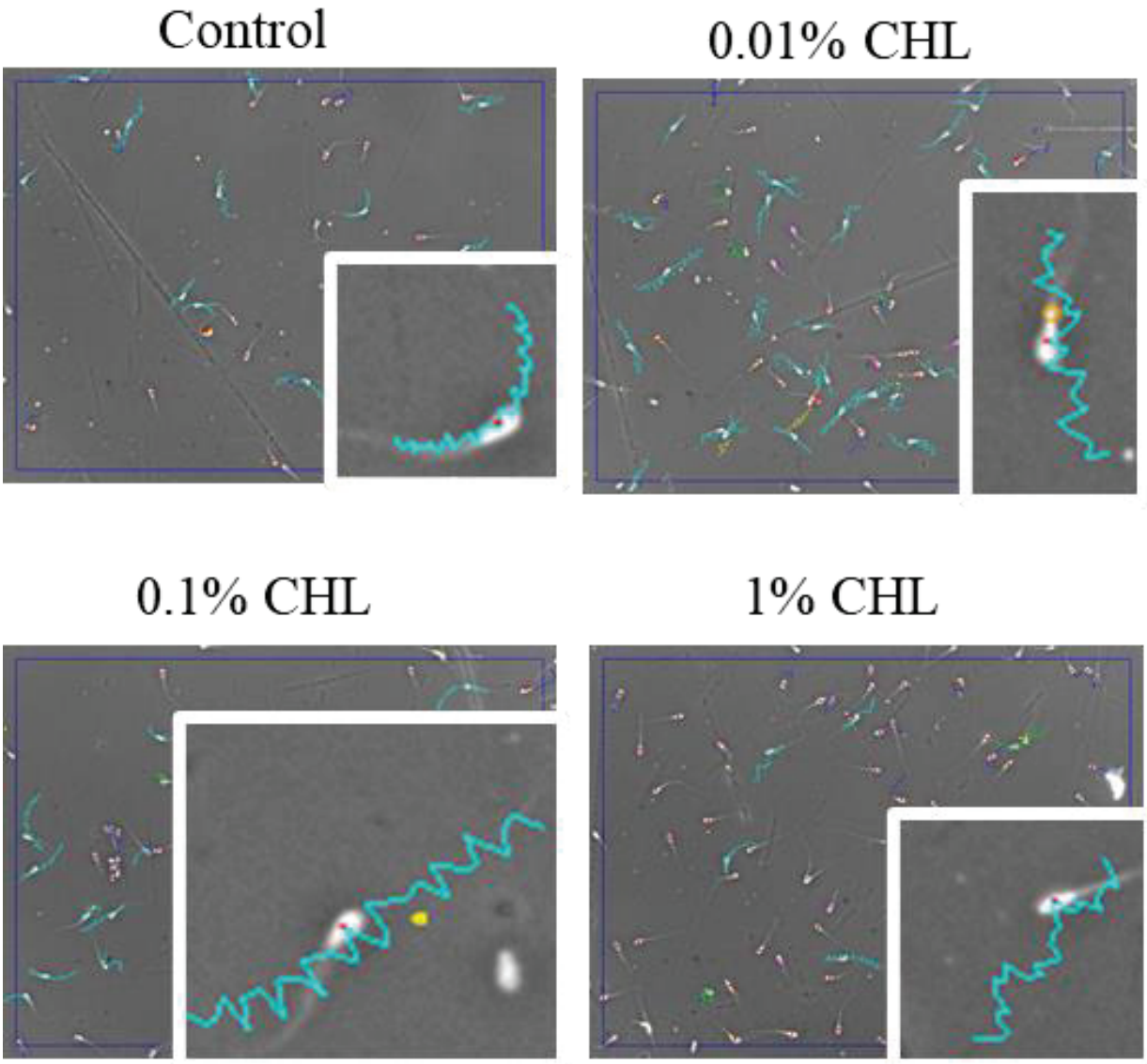
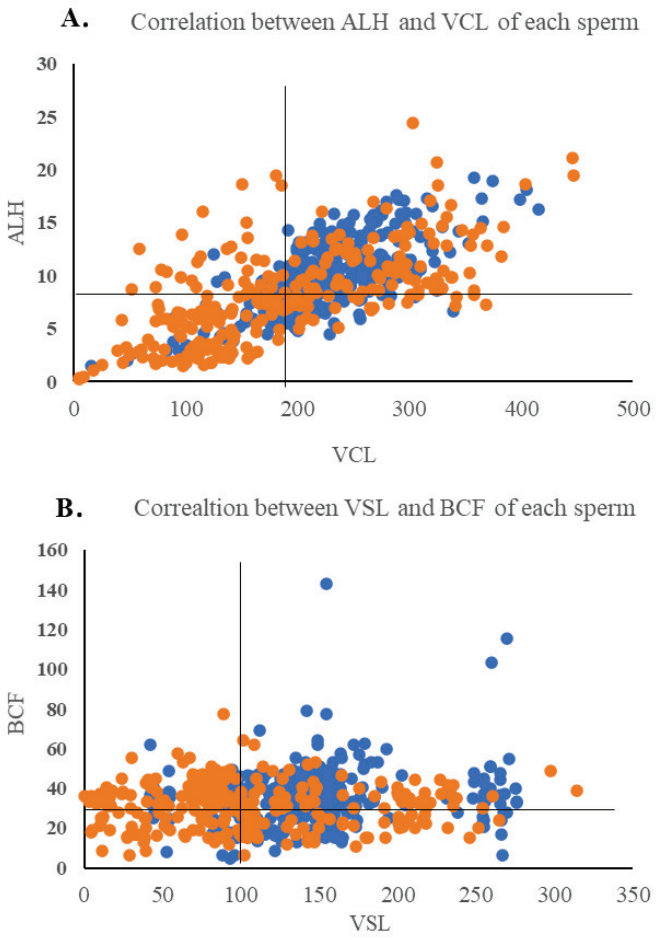


Figure -22: CASA motility track at different doses of cholesterol (CHL)



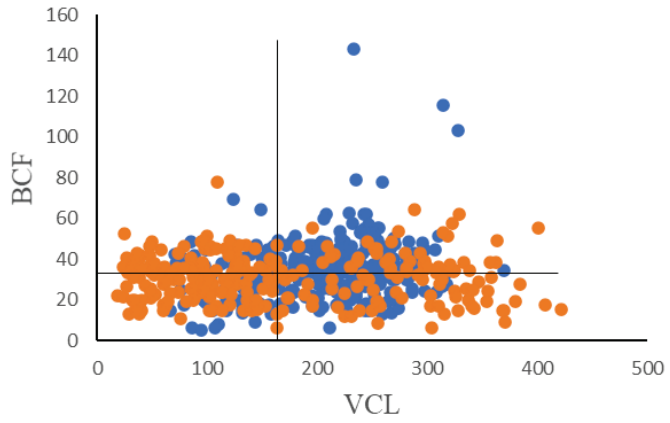
Sperm(%) (Mean ±SE)	
CHL	
5.67 ± 2.02	67.67 ± 1.45[a]
13.00 ± 2.51	14.33 ± 2.84
Sperm(%) (Mean ±SE)	
Control	
13.33 ± 1.85	33.00 ± 2.51[b]
45.67 ± 2.33	8.33 ± 1.33

Sperm(%) (Mean ±SE)	
CHL	
15.67 ± 2.33	45 ± 5.13[a]
20.67 ± 5.33	18.33 ± 5.36
Sperm(%) (Mean ±SE)	
Control	
21 ± 2.08	25 ± 3.60[b]
31.67 ± 6.88	16.33 ± 2.33

Figure -23: Correlation between ALH and VCL (A.) and BCF and VSL(B.) of each sperm treated with Cholesterol (CHL) in thawing media compared with control. The lines in scatter plot were made considering the mean value of each motility parameter in control group of sperm.

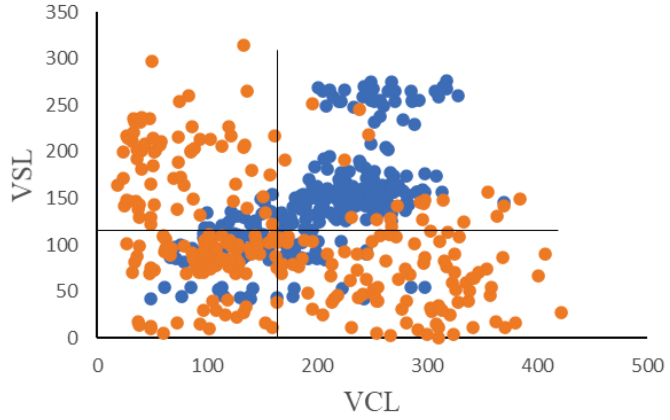
In each correlation figure the second quadrant (ii) region, indicating the lower value of both parameters compared to mean value of control and third quadrant (iii) region, indicating the higher value of both parameters compared to mean value of control are significantly ($P<0.05$) different in CHL treated group compared to control.

A. Correlation between VCL and BCF of individual sperm



Sperm(%) (Mean ±SE)	
CHL	
11.33 ± 1.20	52.67± 1.20[a]
10.33 ± 1.20[a]	23.67 ± 0.89
Sperm(%) (Mean ±SE)	
Control	
30 ± 1.52	22 ± 1.52[b]
26.67 ± 1.33[b]	20.67 ± 1.20

B. Correlation between VCL and VSL of each sperm



Sperm(%) (Mean ±SE)	
CHL	
11.33 ± 1.33	64.67 ± 1.45[a]
11.33 ± 0.89[a]	11.67 ± 2.02
Sperm(%) (Mean ±SE)	
Control	
25.67 ± 2.40	10.67 ± 1.20[b]
27.67 ± 1.45[b]	28.33 ± 1.20

Figure -24: Correlation between BCF and VCL (A.) and VCL and VSL(B.) of each sperm treated with Cholesterol (CHL) in thawing media compared with control. The lines in scatter plot were made considering the mean value of each motility parameter in control group of sperm.

In each correlation figure the second quadrant (ii) region, indicating the lower value of both parameters compared to mean value of control and third quadrant (iii) region, indicating the higher value of both parameters compared to mean value of control are significantly ($P<0.05$) different in CHL treated group compared to control.

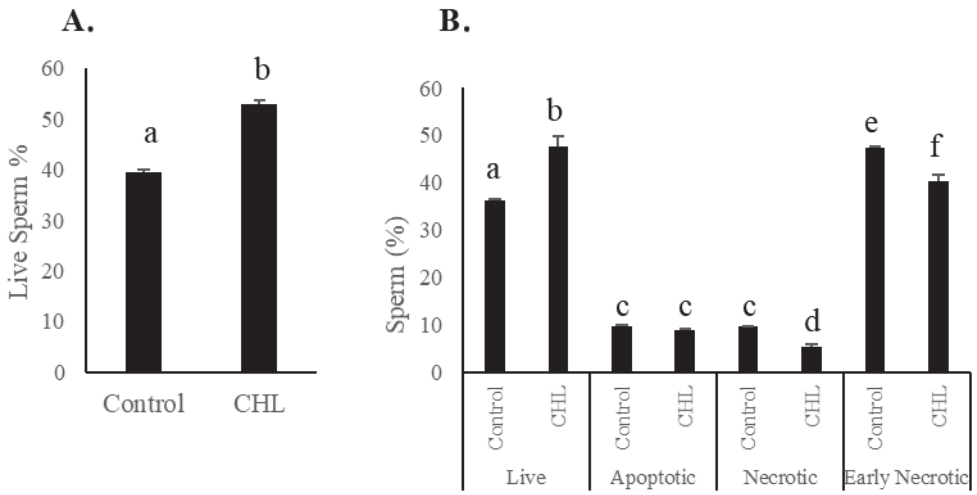


Figure -25: Cholesterol improves sperm survivability.

Values are specified (Mean±SD) with three replications. Different superscripts with various parameters differ significantly at $P < 0.05$ in One way ANOVA with t-test.

(A) Membrane integrity (B) Annexin v Staining

Here, CHL = Cholesterol (0.1%) and control = Without cholesterol.

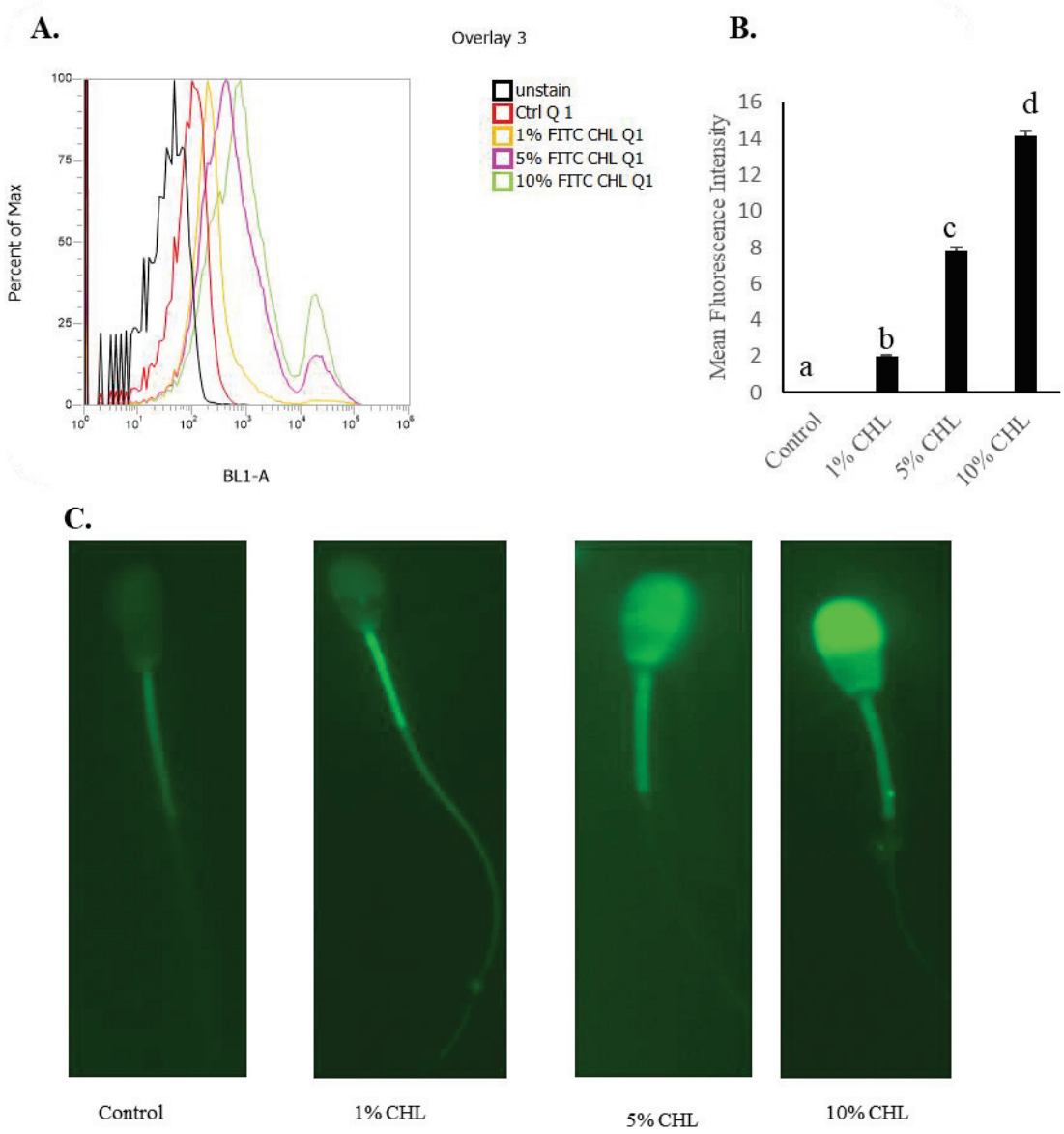


Figure-26: Fluorescence intensity of sperm and localization of cholesterol uptake

Values are specified (Mean±SD) with three replications. Different superscripts differ significantly at $P < 0.05$.

A. Overlay of fluorescence intensity at different doses of fluorescence tagged cholesterol
B. Mean fluorescence intensity at different doses of fluorescence tagged cholesterol
C. Localization of cholesterol uptake signal in sperm

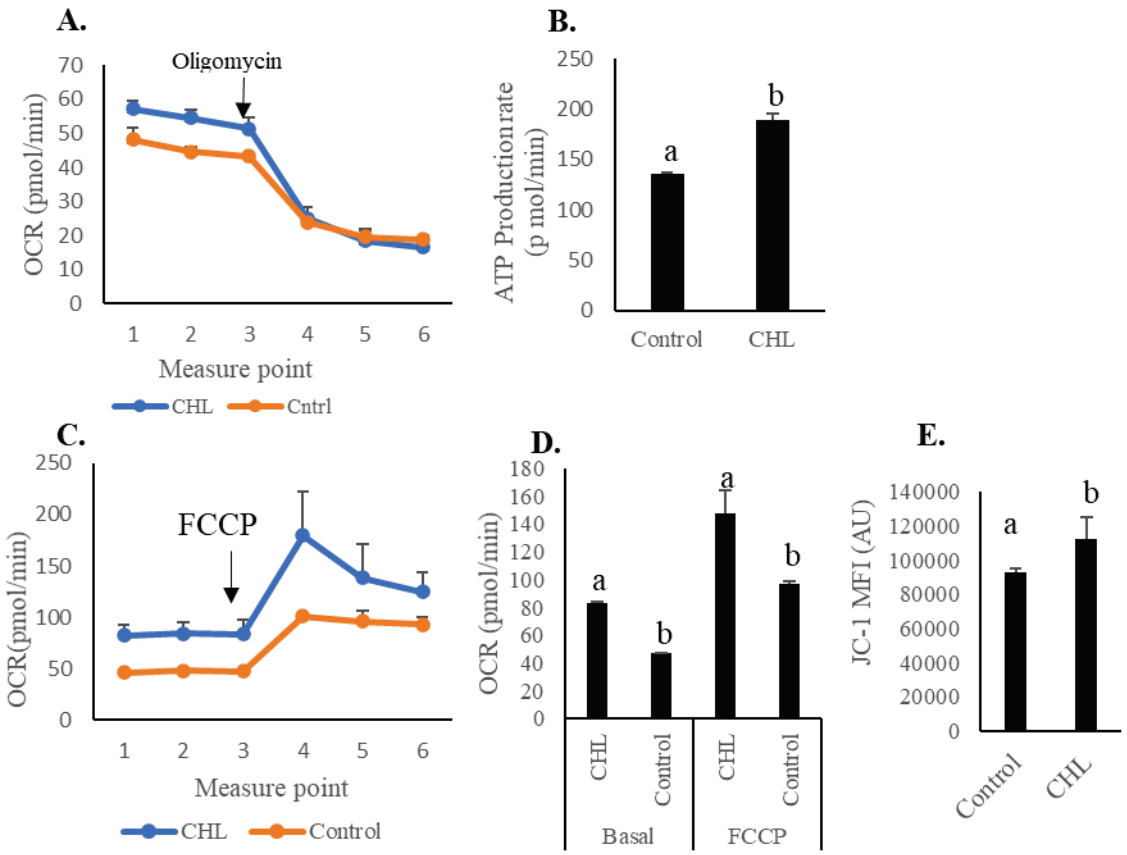


Figure-27: Effect of Cholesterol on oxygen consumption rate (OCR) of sperm. Values are specified (Mean±SD) with three replications. Different superscripts differ significantly at $P < 0.05$.

A indicating the effect of oligomycin injection

B indicating the ATP production rate of sperm

C &D indicating the effect of FCCP injection

E indicating the mitochondrial activity of sperm

Chapter 5

General Discussion

(Page No. 68-72)

Frozen sperm are being used highly in Artificial Insemination (AI), vital reproductive technology that has been utilized to advance livestock farming and have an important, positive impact on cattle production especially, and product quality allowing for accelerated genetic progress and selection (Medeiros et al., 2002). Sperm cryopreservation procedures are not always effective because a huge number of sperm undergo damage which leads to the loss of fertility following freezing and thawing processes (Bailey et al., 2000). The main cause of cellular injury in cryopreservation is the damage endured by the plasma membrane due to cold shock that is associated with the lipid composition of the membrane bilayer (Watson, 1995). Mammalian sperm maintain linear motility during fertilization, to migrate through the uterus and reach the oviduct for penetrating the oocyte (Shalgi et al., 1992). During the journey ATP production is crucial for flagellar movement (Suarez & Pacey, 2006). In this critical situation of cryopreserved sperm to recover membrane integrity and supply adequate energy production for migration from insemination site to fertilization site aiming successful fertilization, thawing of sperm is utmost important with an optimum media from where sperm can get both energy substrates and membrane components is really a challenge.

In this study underlying mechanism of LM on keeping linear motility and sperm quality of bovine frozen sperm (Experiment 1) have become clear. The molecular mechanism of fatty acids to produce energy through mitochondrial β oxidation and localization of fatty acid transporter to the mitochondria of sperm (Experiment 2) is the maiden report. Although there are some studies regarding on effect of fatty acids on sperm motility and quality parameters, but nobody described the process of fatty acid transport to the sperm mitochondria and energy production mechanism. Moreover, the actual cholesterol uptake and localization on sperm mid piece and head region is entirely a new story regarding sperm membrane potential and mitochondrial membrane potentiality for ATP production (Experiment 3).

It was reported that the cold shock during freeze-thaw process injures mitochondria (Pena et al., 2009), plasma and acrosome membranes (Meyers, 2005) of spermatozoa which may lead to decreasing the spermatozoa functional integrity and declining its fertilizing ability (Gillan & Maxwell, 1999). Moreover, it was observed that after freezing of the spermatozoa acrosome reaction was reduced, distorted mitochondria increased, chromatin damage and DNA breaks was enhanced (Khalil et al., 2017). In this study, it was observed that LM not only improved the motility parameters of sperm but also the membrane potentials of bovine frozen sperm. Therefore,

this study might be effectively applied in improving the success rate of AI in livestock industry by diluting frozen sperm in LM containing thawing media as low- cost approach.

Research on bovine species (Neill & Masters,1972) and ovine species (Neill & Masters,1973) reported that sperm cells can take up lipid components or fatty acids from the surrounding environment during incubation (Cerolini et al., 2001). It has been described that fatty acids were incorporated into the lipids of sperm cell membranes within two hours of addition to cell cultures (Neill & Masters,1971). Present study revealed the molecular mechanism of exogenous fatty acid uptake and effect on the sperm quality in cryopreserved semen technology (Experiment 2).

LM is composed of both saturated and unsaturated fatty acids with cholesterol. The effect of fatty acids already discussed so far. In this present study it was revealed that cholesterol in thawing media of bovine frozen sperm was beneficial for improving the linear motility pattern and membrane potentials of sperm mitochondria. Amusingly, the cholesterol was up taken with the treatment of fluorescence tagged cholesterol and located in the mid piece and head region of sperm (Experiment 3). Additionally, these up taken cholesterol was used to recover the mitochondrial modified structure due to cryopreservation of sperm and thereby ATP production rate was significantly increased in cholesterol treated group compared to control (Experiment 3). Therefore, this study is beneficial to frozen sperm technique to recover sperm membrane integrity and mitochondrial potentiality to maintain linear motility of cryopreserved sperm.

Artificial insemination (AI) is the most regularly used biotechnology in livestock industry in various developed and developing countries. In AI industry, the main constraint to the genetic impact of sires is decreased sperm motility and functions during cryopreservation (Woelders et al., 1997; Celeghini et al., 2008). It was reported that 20-30 million sperm per straw have nowadays been used as a standard for dairy industry and 10-15 million progressive motile sperm per dose were necessary for achieving desirable conception rate in French midi straws of cattle (Sullivan, 1970; Allen and Seidel,1996). The artificial insemination technique is commercially applied worldwide to breed cattle, however, the technique is still not efficient, as the large sperm numbers per cow in estrus is required for getting high reproductive performance. The substantial sperm numbers are a vital limitation for artificial insemination application not only in cattle but also in human infertility treatment. Because in human artificial insemination, 1×10^7 or more motile sperm are required (Van Voorhis et al., 2001), in vitro fertilization or intracytoplasmic sperm injection (ICSI) are usually selected in the case of oligospermia. From the above-mentioned references, it is

very clear that in any type of reproductive technology sperm quality with good motility is the prerequisite to get optimum success rate. The novel findings of the present study are directly related to sperm quality specifically linear motility which is very crucial for desired fertilization to get optimum conception rate. In another view, it can be said that the new information of this study can minimize the sperm content of frozen straws as LM treatment in thawing media can recover the cryodamage after thawing and washing. When sperm content can be reduced in single straw then the number of straws can be increased from single collection and ultimately it will be beneficial to make profitable farming. In a single sentence, the inventory of the current study might provide a new strategy for improving the artificial insemination technique of both livestock animals and human infertility care.

The human connection to livestock is also absolutely intimate. Domestic animals are crucial to the survival of populations living in variable environments (FAO, 2009), and were historically central to the formation of societies and cultures (Smil, 2009). According to the most recent (around 2000) World Census of Agriculture, of the approximately 308 million farmers asked in 83 countries across the world, 23.4% reported that they own at least one cow or head of cattle (FAO, 2013). While the number of livestock owned typically increases with farm size, livestock are also important for smaller farms with lower income levels (FAO, 2009). AI using cryopreserved sperm improves desired characteristics more rapidly through intensive genetic selection and has significant advantages, such as enabling the use of superior quality sires even the animal is dead or injured, and greatly reducing the risk of introducing venereal diseases into the herd (Fontes et al., 2020; Vishwanath, 2003). It is a tool for improving reproductive performance and genetic quality of livestock.

It was investigated that the livestock sector affects at least 58% (10 out of the 17) of the SDGs (Sustainable Development Goals) and 16% (28 out of the 169) of the SDG targets (Mehrabi et al., 2020). Globally, livestock products contribute 34 percent of protein and 17 percent of calorie intake of diets and animal-source foods are energy and protein-dense and sources of many other essential nutrients, (FAO/ WHO,2019). Animal food products provide livelihoods for millions of people (Pica-Ciamarra et al., 2011), deliver essential protein and micronutrients for the world's poor (Frelat et al., 2016; Cordain et al., 2002) and provide a vital mechanism for reducing inhibiting and wasting globally (Grace et al., 2018).

There is an emergency to improve the efficiency and sustainability of producing animals for food

to face the demand of ever-increasing world population. Increasing the fertility of livestock, especially cattle, around the world is important for overcoming this problem. Improved understanding of mechanisms and challenges of reproductive technologies are vital for improving the viability of the livestock industry. Among such reproductive technologies, artificial insemination (AI) is a significant technology that has been utilized to advance livestock farming, allowing for promoting genetic progress and selection (Medeiros et al., 2002) where successful semen cryopreservation improves the efficiency and success rate of AI. It was reported that conception rate after artificial insemination of cattle were in the range of 60–73% with an average 71% (Ricardo et al., 2004). Sperm cryopreservation procedures are not always efficient because a large number of sperm suffer physiological damage which leads to the loss of fertility following freezing and thawing (Bailey et al., 2000). Techniques and novel findings of the present study can improve the cryodamage of sperm during thawing and ultimately the success rate of AI.

Considering above all discussion it is very clear that LM in thawing media is very helpful to recover the sperm linear motility pattern with other parameters. Furthermore, LM is beneficial to rescue the sperm damage after thawing and washing of bovine frozen sperm. However, an increased number of sperm linear motility for IVF or AI is the aim of animal reproduction. In this study, LM in thawing media of bovine frozen sperm did neither any abnormalities in sperm rather improved their quality with increased linear motility pattern. The findings of this study will be useful for in vitro fertilization and AI decision in both human and animal fertility improvement. Additionally, exogenous LM treatment in thawing of frozen sperm could be successfully applied in livestock production program, which will improve the non -return rate and pregnancy with an augment in ensuring profitable farming.

In conclusion, this study unveiled the mechanism of LM to improve the linear motility pattern and membrane integrity of bovine frozen sperm. Two parts in LM fatty acids and cholesterol contribute separately to rescue the quality of bovine frozen sperm after thawing in a LM containing media. Interestingly, this study uncovers the energy metabolism of bovine frozen sperm thawed in LM or fatty acid containing thawing media with the molecular strategy of fatty acid uptake to the sperm mitochondria from the membrane. Moreover, cholesterol helps to recover the membrane integrity and mitochondrial membrane potentials declined during cryopreservation process and contribute to regain the linear motility pattern through accelerate ATP production rate. This study invented new information for reproductive science to maintain linear motility of sperm after thawing and

washing which is prerequisite for in vivo fertilization. Therefore, the new story developed from the present study is strongly believed to be fruitful to the future research in AI technology for livestock and human infertility care as inexpensive technique. New approach of thawing frozen sperm with LM containing media from the present study will increase the conception rate definitely and ultimately the production of livestock will increase which may be beneficial to overcome poverty, increase income and attain the SDGs of the United Nations (UN).

Chapter 6

Summary

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Mammalian sperm migrate through uterus to oviduct by maintaining linear motility pattern during in vivo fertilization process. For the migration process sperm require adequate energy to reach the oocyte for fertilization. Sperm gets maximum energy from mitochondria ATP production. The quality of sperm deteriorates in the process of cryopreservation. Sperm membrane integrity, acrosomal integrity and mitochondrial abnormality are the main physical negative effect of sperm cryopreservation. Therefore, the present study was aimed to investigate the recovery of bovine frozen sperm quality after thawing and washing. To achieve the aim, firstly effect of LM in thawing media of bovine frozen sperm were studied. After that, molecular mechanism of mitochondrial ATP production by using fatty acids were investigated. Finally, the sperm membrane potentiality with mitochondrial membrane potentials of sperm with cholesterol were studied.

1. LM (LM) in thawing media is beneficial to keep sperm motility with other quality parameters in healthy status of bovine frozen sperm

Cryodamage affect sperm motility and membrane structure highly. In Experiment 1, it was hypothesized that LM in thawing media maybe beneficial for bovine frozen sperm to keep good motility with sound membrane structure by maintaining their membrane lipid composition healthy and there may be a correlation between lipid components and sperm motility. To clear the hypothesis CASA, GC-MS and FCM with different stains were performed using bovine frozen sperm. It was observed that fatty acids from LM was up taken by LM thawed sperm compared to control and there was a positive correlation between fatty acids amount and total sperm motility. It was also observed that LM thawed bovine frozen sperm showed significantly good motility parameters compared to control group. Moreover, it was also uncovered that membrane integrity, mitochondrial activity and live sperm percentage was significantly higher in LM thawed sperm compared to without LM thawed sperm used as control. Although, it is known that lipid components can improve sperm motility, but this is the maiden report considering the variability in the amount of lipid components in sperm membrane of bovine frozen sperm and their correlation to sperm motility according to author best of knowledge.

2. Sperm linear motility is induced by saturated fatty acid being used as energy substrate in the mitochondria of bovine frozen sperm

Successful fertilization depends on sperm linear motility and motility patterns are dependent on energy substrate and their effective utilization in energy producing segments of sperm. In Experiment 2, it was hypothesized that bovine sperm could uptake fatty acids (FAs) in thawing media and produce ATP in mitochondria for maintaining linear motility. To clear this hypothesis; bovine frozen sperm was thawed in FAs containing thawing media and the sperm motility was analyzed by commuter assisted sperm analysis (CASA). The kinetic changes of FAs level in sperm were detected by GC-MS. The mitochondrial activity of sperm treated with FAs was analyzed as fluorescence intensity of JC-1 staining and oxygen consumption rate (OCR). FA transporter (CD36 and GOT2) was observed by whole-mounted immunofluorescence. It was observed that sperm linear motility was significantly increased by the thawing with FA. Interestingly, it was revealed that long chain saturated fatty acids (LCSFA) (C14, C16, C18) were decreased during 30 min incubation, except very long chain saturated fatty acid (C22). Moreover, another crucial factor was observed that the bovine frozen sperm possessed FA transporter in midpiece where the fluorescence signals were detected after the treatment with fluorescence-tagged FA. Furthermore, it was discovered that the treatment with FA activated electron transportation in mitochondria through β-oxidation. These findings highlight the molecular mechanism of fatty acid uptake from sperm membrane to mitochondria and ATP production through β-oxidation process.

3. Cholesterol in thawing media regain mitochondrial potential which accelerates ATP production in mitochondria that induces linear motility in bovine frozen sperm

For successful fertilization sperm linear motility is very important and this is induced by mitochondrial ATP production. In Experiment 3 it was supposed that cholesterol may be up taken by sperm and used to remake the mitochondrial membrane potential along with sperm membrane potential which was damaged during cryopreservation process. To clarify the hypothesis bovine frozen sperm was thawed in CHL contained thawing media and the sperm motility was analyzed by commuter assisted sperm analysis (CASA). The cholesterol uptake signal was observed by fluorescence intensity in the mid piece and head region of sperm after the treatment with fluorescence-tagged CHL. The mitochondrial activity of sperm treated with CHL was analyzed as fluorescence intensity of JC-1 staining and oxygen consumption rate (OCR). OCR was higher in

CHL treated group due to up taken CHL was used to renovate the altered mitochondrial structure. ATP production rate was also significantly higher in CHL thawed group compared to control. Sperm linear motility was significantly ($P<0.05$) increased by the thawing with CHL. Sperm linear motility is prompted by CHL in thawing media of bovine frozen sperm might provide a new vision for modernizing the artificial insemination technique of both livestock animals and human infertility care.

In conclusion, this study unveiled the mechanism of LM to improve the linear motility pattern and membrane integrity of bovine frozen sperm. Two parts in LM fatty acids and cholesterol contribute separately to rescue the quality of bovine frozen sperm after thawing in a LM containing media. Interestingly, this study uncovers the energy metabolism of bovine frozen sperm thawed in LM or fatty acid containing thawing media with the molecular strategy of fatty acid uptake to the sperm mitochondria from the membrane. Moreover, cholesterol helps to recover the membrane integrity and mitochondrial membrane potentials declined during cryopreservation process and contribute to regain the linear motility pattern through accelerate ATP production rate. This study invented new information for reproductive science to maintain linear motility of sperm after thawing and washing which is prerequisite for fertilization. Therefore, the new story developed from the present study is strongly believed to be fruitful to the future research in AI technology for livestock and human infertility care as inexpensive technique. New approach of thawing frozen sperm from the present study will increase the conception rate definitely and ultimately the production of livestock will increase which may be beneficial to overcome poverty, increase income and attain the SDGs of the United Nations (UN).

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