Roles of Energy Production Pathways in Sperm Motility Regulation in Chickens

January 2020

RANGGA SETIAWAN

Roles of Energy Production Pathways in Sperm Motility Regulation in Chickens

A Dissertation Submitted to

the Graduate School of Life and Environmental Sciences,

the University of Tsukuba

in Partial Fulfillment of the Requirements

for the Degree of Doctor of Philosophy

(Doctoral Program in Biosphere Resource Science and Technology)

RANGGA SETIAWAN

Table of Contents

Abstract

Sperm require exogenous signalling and energy substrates to regulate fertilization competence. Despite of extreme importance of glycolysis and oxidative phosphorylation for ATP generation in sperm, a primary mechanism of ATP-generating pathways is species-specific, leaving characteristics of energy management pathways unclear in birds. This study demonstrates how chicken sperm utilize exogenous metabolic substrates to generate ATP production and support sperm motility and fertility.

As described in chapter II, this study characterized expression and localization of several transporter proteins including plasma membrane calcium ATPase isoform 2 and 4 (PMCA2 and PMCA4), and glucose transporter 1 (GLUT1). Detection of these proteins localizing to later spermatids in testis and the midpiece along with sperm flagellum, suggesting these proteins are expressed at late spermiogenesis and play a role in sperm flagellar motility.

As described in chapter III, this study reveals the functional importance of GLUT1 in chicken sperm motility. Of note, female reproductive tract fluids in chicken contain a high concentration of glucose, suggesting the importance of glucose for sperm motility during their traverse in the female reproductive tract. However, the glucose metabolic pathways are poorly characterized in chicken sperm. Using specific antibodies and ligand, localization experiments found that GLUT1 was specifically localized to the midpiece. Sperm motility analysis showed that glucose supported sperm flagellar movements during incubation up to 80 min. However, this was abolished by the addition of a GLUT1 inhibitor, concomitant with a substantial decrease in glucose uptake and ATP production, followed by elevated mitochondrial activity in response to glucose addition. More potent

inhibition of ATP production and mitochondrial activity was observed in response to treatment with GLUT inhibitor and uncouplers of oxidative phosphorylation. Because mitochondrial inhibition only reduced a subset of sperm movements, we investigated the localization of the glycolytic pathway and showed GAPDH and hexokinase I at the midpiece and principal piece of the spermatozoa. Together, GLUT1 is specifically localized to the midpiece and plays the main role in glucose uptake to generate ATP production for flagellar motility of chicken sperm.

Chapter IV reveals the primary mechanism of ATP -generating pathways for regulation of sperm function. Sperm provide metabolic energy via glycolysis and oxidative phosphorylation to support sperm motility and penetrability. Although oxidative phosphorylation is considered to be efficient in ATP production, the utilization of ATP for specific sperm function varies among species. Therefore, this study was carried out to reveal the roles of ATP glycolysis and oxidative phosphorylation on sperm motility and penetrability. Sperm motility analysis showed that the addition of either glucose or pyruvate alone, or their combination enhanced sperm movement characteristics. The increasing effects of these metabolic substrates were also found on sperm penetrability, with more potent increase under glucose addition. However, the inhibition of glycolysis and oxidative phosphorylation abolished the increasing effects, with dramatic decrease in the inhibition of oxidative phosphorylation. Similar changes were observed in ATP content. Although the oxidative phosphorylation pathway important in ATP production, is associated with reactive oxygen species (ROS) production, the addition of glucose or pyruvate did not increase ROS level. Instead, the addition of the metabolic substrates appeared to change intracellular pH (pHi) associated with sperm flagellar activity. Since pentose phosphate pathway (PPP) is a semi-

intermediate and parallel pathway to glycolysis, we investigated a possession of the pathway in sperm by immunodetection of G6PD, a unique enzyme in PPP, demonstrating its absence in chicken sperm. Our results provide strong evidence that sperm motility and penetrability rely on ATP generated from both glycolysis and oxidative phosphorylation, with a high dependency on glucose.

In conclusion, chicken sperm generate ATP via glycolysis and oxidative phosphorylation by glucose uptake via GLUT1, which results in supporting sperm motility and penetrability. These results reveal new insights into the mechanisms involved in ATP production pathways as well as regulation of sperm fertilization ability and contribute to the development of assisted reproductive technologies (ART) in birds.

Keywords: Glucose, GLUT1, ATP, sperm motility, sperm penetrability, chicken

List of Abbreviations

2-NBDG	2-(*N*- [7-nitrobenz-2-oxa-1,3-diazol-4-yl]-amino)-2-deoxy-D-glucose
3-MCPD	3-monochloro-1,2-propanediol
AA	antimycin A
Acetyl Co-A	acetyl coenzyme A
ALH	amplitude lateral-head displacement
ATP	adenine triphosphate
BCECF-AM	2',7'-bis. (2-carboxyethyl)-5(and-6)carboxyfluorescein acetoxymethylester
BCF	beat cross frequency
BF	bright field
cAB	chicken antibody
CCCP	carbonyl cyanide *m*-chlorophenyl hydrazine
DAPI	4',6-diamidino-2-phenylindole
DCFDA	2',7' –dichlorofluorescin diacetate
F30; F50	fasentin 30 μM; fasentin 50 μM
Fasentin IC	fasentin-induced cell death
G6PD	glucose-6-phosphate dehydrogenase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
Glu	glucose
GLUT	glucose transporter
GLUT1	glucose transporter isoform 1
HK1	hexokinase I
IgG	immunoglobulin
IPVL	inner perivitelline layer
JC-1	5,5', 6,6'-tetrachloro-1,1'3,3'-tetraethylbenzimi-dazolylcarbocyanine iodide

kDa	kilo Dalton
LIN	linearity
mmHg	millimeter of mercury
MMP	mitochondrial membrane potential
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
Oxphos	oxidative phosphorylation
PBS	phosphate buffer saline
pHi	intracellular pH
PMCA	plasma membrane calcium ATPase
PPP	pentose phosphate pathway
Pyr	pyruvate
ROS	reactive oxygen species
RT	room temperature
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMAS	sperm motility analysis system
SST	sperm-storage tubules
STR	straightness
TBHP	tert-butyl hydroperoxide
TES	*N*-Tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid
VAP	average path velocity
VCL	curvilinear velocity
VSL	straight-line velocity

Chapter I

I.1 General Introduction

According to Food and Agriculture Organization's (FAO), one of every 10 people on this planet deal with undernourishment which measures the proportion of people who are unable to consume enough food for an active and healthy life. Proteins, vitamin, minerals, water, and fats are essential nutrients for human survival. Out of them, protein, a macronutrient that has to be fulfilled in a relatively large amount for body need is a vital component of every cell which plays critical roles in structure and function of the body. Protein is also crucial to synthesize enzymes, hormones, and other body chemicals. Lack in the source of protein is one of the major causes of various health problems.

It is generally accepted that animal protein is of better quality than plant-based protein. The difference between animal-based and plant-based protein is based on the amino acid contents. For example, the essential amino acid lysine is less available in plant-based proteins but can be easily obtained from animal source food (Walker *et al*. 2005). In developing countries, the amount of animal-based protein consumption is approximately 6 to 12 g/person/day which is almost less than half of the average world's protein intake (15 g/person/day), while in high-income countries are approximately 30 g/person/day) (Sans and Combris 2015). This is what led to some studies projecting that the consumption of animal protein tends to increase with per capita income (Schroeder *et al*. 1996, Henchion *et al*. 2014), that might increase livestock demand. The total demand for livestock product is projected to more than double by 2030 in developing countries (FAO 2003). Therefore, it is urgent to develop our ability to produce livestock efficiently.

Poultry meat and eggs are among the most common animal-source foods most widely consumed at a global level, across a wide diversity of traditions, cultures, and religions. FAO reported that poultry meat accounts for 30% of global meat consumption, making them a key to food security and nutrition. As regards other kinds of poultry, chicken is the most common type of poultry raised in the world due to its several benefits compared to other livestock. Classified as white meat, the nutritional value of chicken meat is extremely protein-dense, with 23 grams of protein per 100 grams chicken breast (Mudalal *et al*. 2014) and most affordable compared to other meats. It also contains fat less than 3 g/100 g or less than half of red meat (5 to 7 g/100 g) (Farrel 2008). These facts make poultry meat to be the most consumed meat protein in the world compared to other meats.

To respond to the high demand for poultry products such as meat, egg and feather, remarkable improvements in chicken productivity have been achieved through laboratory-based research into technical applications. Selection and breeding program identifying superior traits based on performance and phenotype have been practised since ages. Adapted from maize breeding concept in the 1980s, the two-, three-, or four-way concept in poultry has directed the poultry for production of high-yielding broiler and layer strains (Saxena and Kolluri 2018). However, this method requires a large population to permit the full exploitation of genetic variation and to prevent inbreeding effects. New technology on chicken genome sequencing and the genetic variation map, developed in 2004, have the most significant impact on commercial broiler and layer breeding programmes, allowing the breeders to select multiple traits more accurate, even on difficult-to-measure traits such as disease resistance and sex-linkage traits, and low heritability (Pym 2008).

Since reproductive technology encompasses the application of the selective breeding program, it acts as the main chord in sustainable livestock production. Various techniques have been developed to obtain livestock productivity from genetically superior or desired animals, such as artificial insemination, cryopreservation of sperm or embryo, sex determination, nuclear transfer, and cloning. Among these techniques, artificial insemination using fresh or frozen semen is familiar and has been used for a wide variety of species, especially in dairy and beef farm. However, this technique is still not reliable for use in the poultry industry.

Poultry sperm has unique morphological and physiological characteristics which might affect semen preservation. Unlike mammalian sperm, poultry sperm has a cylindrical head, approximately 0.5 μm in diameter size, and less cytosolic content which might reduce their ability to move cryoprotectant inside the head, thus lowering survivability in the freezing process (Thurston and Hess 1987, Donoghue and Wishart 2000). Moreover, length of the tail sperm is quite long approximately 82 μm (Grigg and Rodge 1949), making the sperm more susceptible to freezing damage. Likewise, the female reproductive tract has unique physiological ability to store fertile sperm for long periods of time. Sperm storage tubules (SST) located to the distal half of the oviduct can prolong sperm survivability and fertilizability up to 2-15 weeks in poultry, including turkeys, quails, ducks, and chickens (Bakst *et al*. 1994, Bakst 2011). These facts showed physiological differences between avian and mammalian, which might contribute as the essential information to the effectivity of sperm preservation in poultry. Despite many efforts to demonstrate sperm preservation, limited attention has been directed the cellular mechanisms of how and why sperm functional competence is sustained such long period.

After ejaculation, sperm must move forward along the female reproductive tract and reach the site of fertilization. During this migration, sperm encounter different compartment of the genital tract with changes in various biochemical and mechanical properties. In mammalian, sperm have to swim in a highly viscous medium in the cervix, avoid immunological detection in the uterus, pass through the utero-tubal junction, bind to cells in the oviductal reservoir and be released in time to reach the oocyte to complete their journey, thus only a small number of sperm deposited reach and fertilize the egg (Druart 2012). However, the natural mechanism of sperm transport is different in avian. For example, the female possesses a unique property in the oviduct that SST can store sperm for a prolonged time and also are thought to function as the sperm selection site. Besides, hen's reproductive tract fluid contain tremendous glucose content by 21-40 mM depending on oviposition phase (Dupuy and Blesbois 1996), which is in contrast to the situation in mammals by 0.61 mM (Harris *et al*. 2005). These conditions may lead to different energy expenditure required for maintaining survivability and fertilizing ability of chicken sperm.

Sperm require a continuous supply of adenosine triphosphate (ATP) as energy to support various sperm functions, particularly for motility. Sperm motility is highly dependent on the ATP availability and accounts for approximately 70% ATP consumption (Bohnensack and Halangk 1986). Since sperm are highly streamlined, less amount of organelles and cytoplasmic volume, their transcription and translation events are inactive, which lead to the limitation of enzymatic processes (Travis *et al*. 2001). In addition, different environmental conditions of the female reproductive tract region with regards to the availability of metabolite substrates and oxygen, it is not thought to be an easy task for the sperm to survive. Therefore, sperm have flexible use of metabolic

substrates in a different region of the female reproductive tract in order to provide energy (Miki 2007, Storey 2008).

There are two major metabolic pathways which generate ATP as the form of cellular energy, i.e. glycolysis and oxidative phosphorylation (Oxphos). In mammalian spermatozoa, these metabolic pathways occur in distinct regions of the cell. Glycolysis mainly takes place in the fibrous sheath of the flagellum and smaller quantities in the head, while Oxphos occurs in the mitochondria which is highly packed in the midpiece. Numerous studies have emphasised the importance of Oxphos in sperm bioenergetics since the complete oxidative breakdown of glucose via pyruvate and acetyl Co-A are capable of generating high yield molecules of ATP, relegating to glycolysis as a secondary role (Ruiz-Pesini *et al*. 1998, Marchetti 2002, Gallon *et al*. 2006). However, the primary mechanism of ATP production is different from species to species. For example, boar and stallion sperm motility are highly dependent on high respiration rates and cannot be supported only by glycolysis alone, human and murine sperm motility rely on ATP from glycolysis, and sperm motility of guinea pig and bull rely on both high respiration and glycolysis (Tourmente *et al*. 2015). To date, the primary mechanism of ATP production in avian sperm is not documented yet, making energy management strategy of avian sperm elusive.

Glucose is the most reliable metabolic substrates for energy production in most cells. In sperm, glucose is derived either from the extracellular environment/medium or from intracellular stores of glycogen (Ballester *et al*. 2000). Despite the importance of glucose in cellular energy production, its roles in sperm functions are species-specific. In human, glucose supports sperm motility and capacitation (Williams and Ford 2001), but it inhibits sperm capacitation in bull (Storey 2008).

Glucose across a concentration gradient of the plasma membrane is accomplished by glucose transporter (GLUTs) through a facilitated diffusion mechanism. GLUT isoforms consist of 14 members in which of each isoform has a different affinity to glucose. Among GLUTs member, GLUT1 is the main GLUT isoform and has high affinity to glucose which plays a central role in maintaining basal glucose uptake. In mammalian spermatozoa, it has been reported to be localized into the principal piece and acrosomal region (Angulo *et al*. 1998). Considering that the high level of glucose content in the hen's reproductive tract fluid, and the high affinity of GLUT1 to glucose, we hypothesise GLUT1 is essential in glucose uptake for ATP production-underlying sperm functions.

After reaching fertilization site, sperm must undergo physiological changes and interact with extracellular matrix of the egg to perform acrosomal reaction. In addition to glucose, modulation of intracellular calcium ions ($[Ca^{2+}]i$) is essential events during capacitation and acrosome reaction. Calcium is essential for the regulation of kinase activity, phosphatases, gene activation, and protein translation. Several studies showed the increase of $[Ca^{2+}]i$ is required for sperm capacitation (Visconti *et al*. 1995, Luconi *et al*. 1996). However, a sustained increase of $[Ca^{2+}]i$ has dramatic consequences on cellular behaviour, eventually leading to cell death (Farber 1990, Bouillot *et al*. 2018). It is required for short periods of time at high concentrations to maintain a physiological calcium gradient. Therefore, the excessive $[Ca^{2+}]i$ contents need to be pumped out to the extracellular.

Plasma membrane calcium ATPase (PMCA) is known having the major task for $[Ca^{2+}]i$ clearance. Out of four isoforms, PMCA4 is the main isoform with 90% expression (Wennemuth *et al*. 2003, Okunade *et al*. 2004). A previous study found that sperm are

unable to undergo hyperactivated motility in PMCA4 knock-out mice (Schuh *et al*. 2004). Moreover, PMCA4 is highly expressed and localized to the acrosome plasma membrane and the principal piece of the tail, suggesting the involvement of PMCA4 in multiple sperm functions (Okunade *et al*. 2004, Schuh *et al*. 2004). Several studies have also reported that Ca^{2+}-dependent capacitation is associated with cholesterol efflux that leads to reorganization of membrane lipid and protein formation (Cross 2004, Shadan *et al*. 2004, Thaler *et al*. 2006). Our previous study on proteomic analysis found a variety of ion transporters/channels in detergent-resistant membrane (DRM), including PMCA (Ushiyama *et al*. 2017). Although the functional importance of PMCAs is important in Ca^{2+} clearance, but the presence of this membrane protein is still unknown in chicken sperm. Therefore, together with GLUT1 as one of membrane proteins, this study characterized localization of PMCAs and GLUT1 as a foundation to unravel their functional properties in chicken sperm.

I.2 Major objectives of this study

Sperm are highly dependent on extracellular metabolic and signalling substrates to power sperm fertilizing ability. However, the primary mechanisms of flagellar motility regulation vary among species and poorly understood in chicken sperm. Understanding the mechanisms of energy generation might provide insight a basic knowledge on the alternative attempt to increase sperm fertility. This understanding might also give a fundamental theory for the development of semen preservation in chicken. These motivated me to characterize localization and function of membrane transporter with particularly focus on the involvement of GLUT1 on glucose uptake in providing energy for chicken sperm functions.

Chapter II

Localization of plasma membrane calcium ATPase 2, and 4 (PMCA2 and PMCA4) and glucose transporter 1 (GLUT1) in chicken sperm

II.1 Introduction

Since the global demand for poultry meat and eggs tend to increase exponentially in the next decades, many efforts have been made to develop chicken production systems to maximize production ability. Recent developments in the field of poultry reproductive technology have led to the importance of fertility as the main chord in sustainable chicken production. For example, sperm cryopreservation and artificial insemination have been widely used for genetic make-up and efficiency use of the male genetic resource. However, a significant problem with this kind of applications is the fertility rates remain unreliable for use in commercial production. This is mostly because it is still unclear the natural state of mechanisms by which chicken sperm undergo deterioration of fertilizing ability underlying sperm preservation.

For the successful fertilization, sperm require to travel in the female reproductive tract and undergo physiological changes before reaching the female egg. Moreover, after reaching the target, the sperm must interact with the egg's extracellular matrix and perform acrosomal exocytosis. In addition to glucose, calcium is considered playing roles in most of these processes. It is well known that the elevation of intracellular calcium ($[Ca^{2+}]i$) plays a vital role as a second messenger in the physiology of cells. In sperm, $[Ca^{2+}]i$ responsible for flagellar movement as well as activation of signalling pathways leading to acrosome exocytosis that pre-requisite to fertilize the oocyte (Zhang *et al*. 2009). Previous studies documented that the increase of basal $[Ca^{2+}]i$ level ranged from

25-75 nM to 500 nM leads to membrane fusion and acrosome reaction (Brewis *et al*. 2000, Breitbart 2002). The functional importance of $[Ca^{2+}]i$ is also supported by the evidence that the fertilizing ability of cryopreserved mammalian sperm is impaired by extreme elevation of $[Ca^{2+}]i$ due to the compositional changes in sperm membrane during cryopreservation (Robertson *et al*. 1990, Zhao and Buhr 1995). Even though the elevation of $[Ca^{2+}]i$ is essential for sperm functions, but the sustained elevation of $[Ca^{2+}]i$ is toxic for the cell (Farber 1990). Therefore, the modulation of calcium influx/efflux should be required for sperm to attain an optimum calcium level that excites specific functions.

Because of extreme cytotoxicity, $[Ca^{2+}]i$ is strictly regulated by several channel and transporters. Plasma membrane calcium ATPase (PMCAs), transmembrane proteins acts as a primary channel involved in ATP-dependent expulsion $[Ca^{2+}]i$, providing a fine-tuning $[Ca^{2+}]i$ for sperm functions. PMCAs consist of four different isoforms, where PMCA1 and PMCA4 are expressed in several tissues including testis, while PMCA2 and PMCA3 are expressed in very limited tissues (Strehler and Zacharias 2001), suggesting the functional distinction between isoforms. PMCA1 and PMCA4 are highly conserved in sperm from different species (Strehler and Zacharias 2001, Okunade *et al*. 2004). In murine sperm, PMCA4 has been found in the sperm tail (Schuh *et al*. 2004), while in bull sperm, it is enriched in the midpiece (Post *et al*. 2010). Although some researches have been carried on the functional importance of PMCA in sperm from different animals, no single study has been focused on avian sperm.

In addition, energy is a crucial factor to support sperm functions, which are abundantly provided from extracellular metabolic substrates. As a metabolic substrate, glucose has been reported playing roles in various biochemical reactions of sperm, such as on motility of goat sperm (Fukuhara and Nishikawa 1973), dog sperm (Rigau *et al*.

2001), capacitation of human sperm (Williams and Ford 2001), capacitation and acrosome reaction of hamster sperm (Dravland and Meizel 1981). In aves, glucose is tremendously found in the female reproductive tract fluids of chicken, suggesting the importance of glucose on sperm during travel in the tract.

Due to its high polarity and large size, glucose cannot freely traverse the lipid membrane layer of the cell. Instead, glucose molecules enter the cell facilitated by glucose transporters (GLUTs). Out of 14 GLUT member, GLUT1 is ubiquitously distributed of the transporter isoform and has a very high affinity to glucose. In mammalian sperm, it is located to the principal piece and acrosome region, although the functions remain unknown. Our previous proteomic analysis found that GLUT1 is exclusively located to the membrane rafts, a microdomain of plasma membrane which is associated with sperm functions in chicken. Considering the expulsion of high level $[Ca^{2+}]i$ requires ATP against ion concentration gradient, the presence of GLUT1 as a glucose transporter in providing energy might have effects on the function of PMCAs. However, no information related to those transmembrane proteins is available in chicken sperm. Therefore, it was investigated the subcellular location of PMCA2, PMCA4 and GLUT1 as a foundation to dissect the functional mechanisms of chicken sperm.

II.2 Materials and Methods

Localization of PMCA2, PMCA4, and GLUT1 in chicken testis

We firstly deparaffinized and rehydrated paraffin-embedded testis in 100% xylene, 100% ethanol, and 70% ethanol for 15, two, and one minute, respectively. Heat-induced epitope retrieval for 10 minutes in 0.01 M citrate buffer, pH 6.0 was performed and followed by cooling down at room temperature (RT). Blocking solution (10% of goat

serum in PBS) incubated the slides for one hour at RT. The slides were then incubated in PMCA2, PMCA4, or GLUT1 polyclonal antibody [1:50 rabbit serum immunoglobulin (IgG) antibody in PBS] at 4 ºC for overnight. After three times washing in PBS, anti-rabbit IgG Alexa Fluor 488 antibody (1:200) was applied to incubate the slides for one and half hour at RT. Coverslips were mounted on to the glass slides using VECTASHIELD mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). Histological sections of each sample were used as negative controls, substituting primary antibody with PBS. All procedures after antigen retrieval were performed in a humid chamber to avoid the slides to dry out.

Localization of PMCA2, PMCA4 and GLUT1 in chicken sperm

Sperm were allowed to settle on anti-liquid bordered coverslips at 39 ºC for 30 minutes, followed by fixation in 4% paraformaldehyde for 15 minutes at RT. The sperm samples were then washed and permeabilised in 0.5% Triton X-100 for 1 min. Blocking solution (10% goat serum in PBS) was applied on to the samples for 1 hour at RT. The samples were then incubated in a primary antibody (chicken PMCA2, PMCA4, or GLUT1 polyclonal antibody 1:150 PBS) at 4 ºC for overnight. After three times washing in PBS, the samples were then incubated in goat anti-rabbit IgG Fluor 488 antibody (1:200). VECTASHIELD mounting medium with 4'6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) mounted the coverslips on to glass slides. Sperm samples without a primary antibody were used as negative controls. All the procedures were performed in a humid chamber.

Immunoblotting

Sperm were washed with PBS for 5 min at 1000 g. The resulting pellet was then resuspended in PBS containing protease inhibitors (Free EDTA protease inhibitors

cocktail, Roche, Basel, Swiss), followed by dounce-homogenisation and sonication. After centrifugation, the resulting pellet was incubated with 4% of 2,2,2 trichloroacetic acid for 15 min on ice chamber. Samples were centrifuged at 1000 g for 15 min, and the precipitated proteins were obtained from the resulting pellet. The precipitated protein was resuspended in water, and the pH was neutralized with 1 M NaOH until the colour changed to purple.

Samples of 70 µg of sperm protein or 1×10^7 sperm were processed for SDS-PAGE analysis. Transfer, blocking, and immunodetection of PMCA2, PMCA4, and GLUT1 were performed as previously described (Asano *et al*. 2009). Antibody against PMCA2, PMCA4, or GLUT1 and anti-rabbit IgG conjugated with HRP were diluted 1:3000 and 1:10,000, respectively. Immunoreactivity was detected by chemiluminescence.

II.3 Results

Localization of PMCA2, PMCA4, and GLUT1 in chicken testis

This study used a specific antibody for each protein transmembrane PMCA2, PMCA4, and GLUT1 to identify their expression using fluorescence-labelled anti-rabbit IgG as secondary antibodies in tissue sections obtained from adult chicken testis (Fig. 1). The immunohistochemistry analysis demonstrated PMCA2, PMCA4, and GLUT1 were homogenously expressed at the edge of adluminal seminiferous tubules. There was a weak immunoreaction in the basal seminiferous tubules. No signals were found in negative control that was only treated with fluorescent secondary antibody.

Expression and localization of PMCA2, PMCA4, and GLUT1 in chicken spermatozoa

Immunoblot analysis using chicken sperm showed detection of PMCA2, PMCA4, and GLUT1 at the predicted molecular weight (Fig. 3). Immunostaining showed

localization of PMCA2, PMCA4, and GLUT1 at the midpiece region of the flagellum (Fig. 2). This is consistent with a previous study that PMCA is abundant in the flagellum that mediates chemotactic behaviour of ascidian sperm (Yoshida *et al*. 2018), while GLUT1 in this study has different localization from that of human sperm which is located in the acrosomal region and principal and end piece of the tail (Bucci *et al*. 2010). No signals were observed in control sperm that were only treated with a fluorescent secondary antibody. These results showed that PMCA2, PMCA4, and GLUT1 was expressed and specifically localized at the midpiece of the flagellum of chicken sperm.

II.4 Discussion

PMCA is known as a transmembrane protein which has the main task in Ca^{2+} clearance (Wennemuth *et al*. 2003). The protein consists of four isoforms, PMCA1-4, encoded by four different genes and have tissue-specific expression. PMCA1 and 4 are expressed in most tissues and act as housekeeping isoform, whereas PMCA2 and 3 are more restricted, mostly in the brain and striated muscle (Brini 2009). Okunade *et al*. (2004) reported that PMCA4 is more abundant isoform in mouse testes compared to PMCA1, making PMCA4 is the major isoform in the tissue, even though PMCA1 can compensate its absence. In contrast to housekeeping isoforms, a previous study observed in mice that PMCA2 acts as the primary controller of milk calcium concentration, in which 60% of milk calcium decline are due to the absence of PMCA2 (Reinhardt *et al*. 2004). In the present study, we found that PMCA2 and PMCA4 were detected in chicken seminiferous tubules from the basal to the edge adluminal compartment of seminiferous tubules (Fig.1). This localization indicates that both PMCA2 and PMCA4 might be synthesized and embedded into the sperm membrane during spermatogenesis. The

similarities between PMCA2 and PMCA4 protein localization indicate the requirement of Ca^{2+} clearance at late spermatogenic cells.

In addition, glucose is a vital source of energy to support not only testicular development and function but also sperm cells development and quality (Williams and Ford 2001). In the present study, we observed the immunoreactivity of GLUT1 at the edge of adluminal seminiferous tubules, suggesting the involvement of GLUT1 in spermatogenesis in chicken. However, the presence of GLUT1 varies from species to species. For example, GLUT1 was expressed in the peritubular myoid cells of rat testis (Kokk *et al*. 2004), but not expressed in the testes of dog, human, and mice (Kokk *et al*. 2004, Hahn *et al*. 2017). Considering the fluorescent intensity was emitted at the edge of adluminal seminiferous tubules for all membrane proteins in this study, we hypothesised that all these proteins might be synthesized and embedded into sperm plasma membrane during late spermatogenic cells. At this stage, spermatids undergo extensive remodelling, whereby their cytoplasm content is removed by approximately 70% during spermiation (Sprando and Russell 1987), resulting in the cell more compact and dense. Moreover, numerous proteins have also been localized to specific sites during spermiation (O'Donnell 2014).

Our immunoblotting and immunostaining results using a specific antibody showed that PMCA2, PMCA4, and GLUT1 were expressed at their predicted molecular weight and detected at the midpiece. The localization of PMCA4 is the same with that in bull sperm, showing co-localization with a lipid raft marker in the midpiece (Post *et al*. 2010), and is direct vicinity to the high concentration Ca^{2+} stores in mammalian sperm (Costello *et al*. 2009). Information related to PMCA2 is still limited in mammalian or avian sperm. However, the expression of PMCA2 is associated with sperm motility parameters, such

as percentage of motile sperm, curvilinear velocity (VCL), straight-line velocity (VSL), and average path velocity (VAP) in cryopreserved sturgeon sperm (Xi *et al*. 2018). Considering PMCA2 and PMCA4 were localized to the midpiece wherein mitochondria are located, these proteins might be involved in energy generation since ATP is generated by an increase in $[Ca^{2+}]i$ via Ca^{2+} sensitive dehydrogenases and NADH production (Ren and Xia 2010). Concomitant to these results, GLUT1 as a channel for glucose uptake was also located in the midpiece of chicken sperm. It is well accepted that glucose is the main source for ATP production for sperm (Miki 2007, Storey 2008, Bucci *et al*. 2011), which might be connected to function of PMCA. However, the involvement of these proteins regulating sperm function in chicken remains unknown. Therefore, the functional significance of these findings needs to be studied.

In conclusion, the present study showed that chicken testes expressed PMCA2, PMCA4, and GLUT1 at the edge of adluminal seminiferous tubules. The localization of these membrane proteins were detected to the midpiece of chicken sperm. This study provide a foundation to unravel cellular mechanisms by which calcium ions and energy production possibly regulate chicken sperm functions.

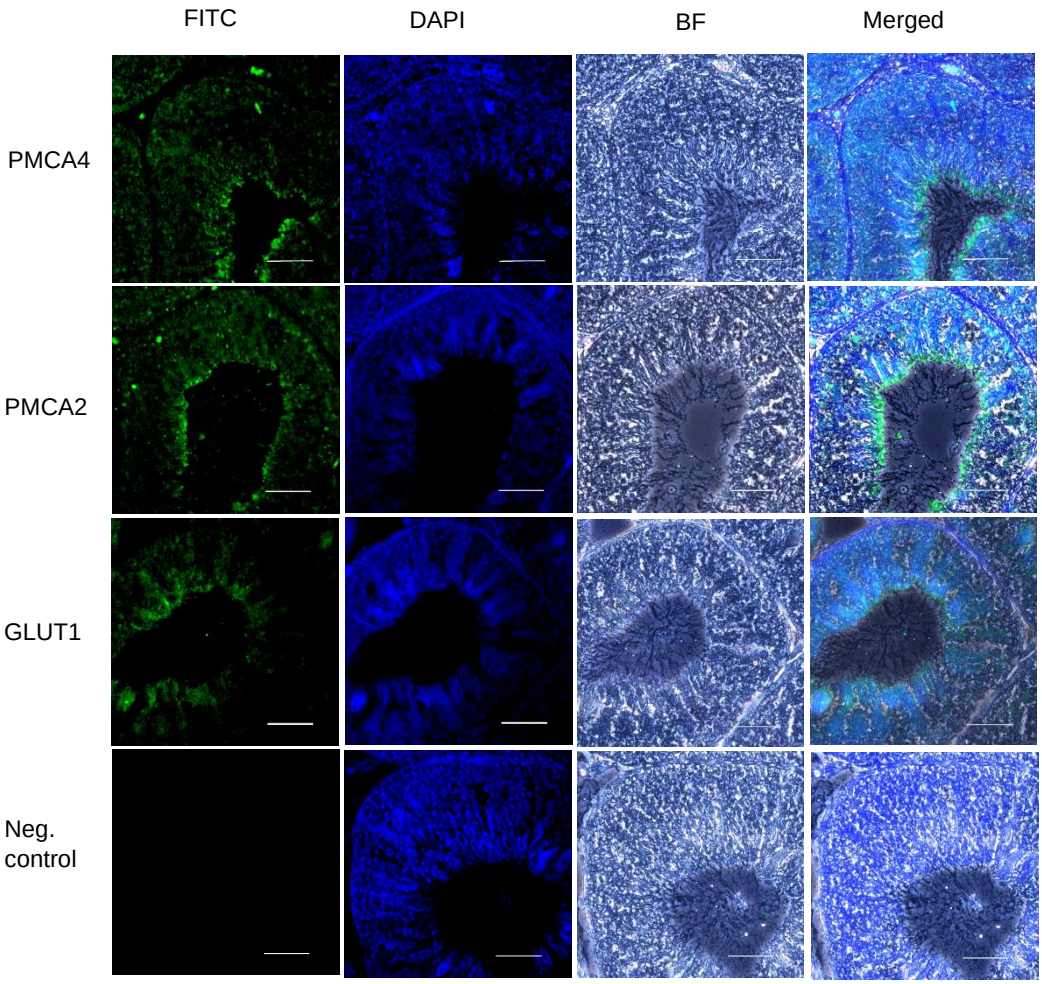


Fig. 1. Immunohistochemistry in paraffin-embedded chicken testes using polyclonal antibody against PMCA2, PMCA4, and GLUT1.

Immuno-positive signals using chicken-specific antibody against PMCA2, PMCA4, and GLUT1 showed the localization of PMCA2, PMCA4, and GLUT1 in the adluminal seminiferous tubules of chicken testis (*green*). Nuclei were fluorescence-labelled using DAPI (*blue*). No signals were detected in sample labelled with secondary antibody used as control. Bars: 50 µm.

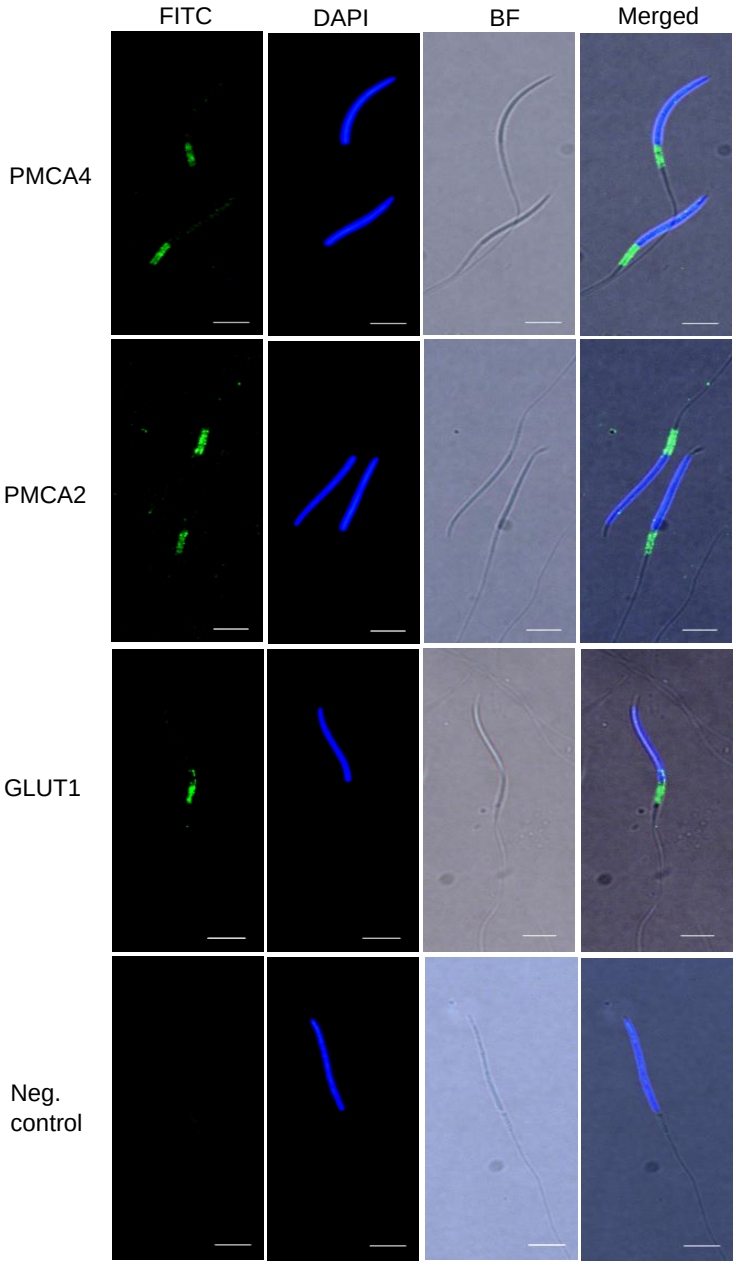


Fig. 2. Immunocytochemistry of PMCA2, PMCA4, and GLUT1 chicken sperm.

PMCA2, PMCA4, and GLUT1 were strongly identified in the midpiece of the spermatozoa (*green*). Nuclei were fluorescence-labelled using DAPI (*blue*). No signals were detected in control labelled with secondary antibody. Bars: 5 µm.

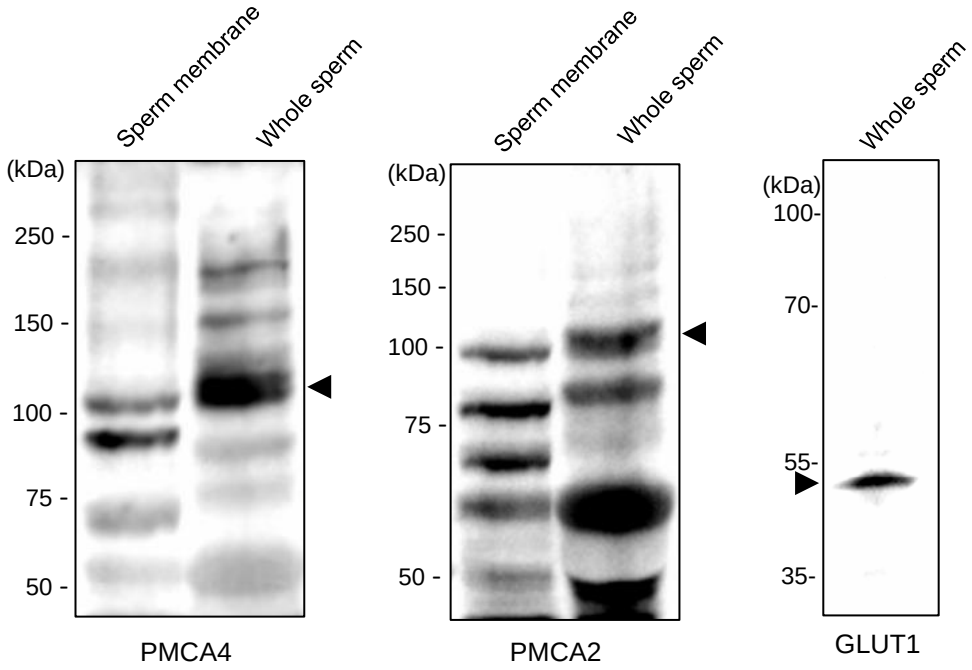


Fig. 3. SDS-PAGE and Western Blotting analysis of PMCA2, PMCA4, and GLUT1 in chicken sperm.

Protein of sperm membranes were extracted with 4% trichloroacetic acid on ice and subjected to immunoblotting. Sperm (1×10^7) were utilized as control. Immuno-reactivity of PMCA2, PMCA4, and GLUT1 were expressed in sperm at predicted molecular weight, including the expression of PMCA2 and PMCA4 in sperm membrane.

Chapter III

Localization and function of GLUT1 glucose transporter in chicken sperm (*Gallus gallus domesticus*): relationship between ATP production pathways and flagellar motility

III.1 Introduction

Sperm acquire energy from nutrient molecules present in extracellular environments for a variety of functions, such as flagellar motility. Adenosine triphosphate (ATP) is the principal form of energy. It is generated via glycolysis and oxidative phosphorylation in the flagellum, which comprises the midpiece and principal piece. Glucose is the predominant substrate for glycolysis in female reproductive tract fluids in mice (Gardner and Leese 1990), pigs (Nichol *et al*. 1992), and cows (Carlson *et al*. 1970). However, the effect of glucose on sperm varies between species. For example, glucose stimulates capacitation-associated changes including hyperactivated motility (Fraser and Quinnn 1981) and activation of signal transduction pathways (Travis *et al*. 2001). However, capacitation and successful fertilization are inhibited by the presence of glucose in bovine (Parrish *et al*. 1989) and guinea pig sperm (Hyne and Edwards 1985). Birds maintain unusually high concentrations of glucose in uterine fluid (van Eck and Vertommen 1984, Dupuy and Blesbois 1996). Although capacitation is not recognised in birds, our recent study and others found that glucose stimulates a signal transduction pathway and flagellar motility in chicken sperm, resulting in an elevated fertilization potential (McLean *et al*. 1997, Ushiyama *et al*. 2019). Despite reports of a close relationship between glucose and sperm motility in some species, the molecular mechanisms involved in glucose uptake remain poorly understood in birds.

Facilitative diffusion of glucose into sperm along a concentration gradient is catalysed by specific carriers, known as glucose transporters (GLUTs). The GLUT family comprises 14 glucose transport proteins with high structural similarity and sequence homology. Multiple GLUT isoforms, including GLUT1, 2, 3, 4, and 8, are suggested to mediate glucose transport in sperm (Burant *et al*. 1992, Haber *et al*. 1993, Angulo *et al*. 1998, Schurmann *et al*. 2002). Studies into GLUT expression in mammalian sperm have established that these GLUT isoforms are localized at differential cellular compartments that require ATP to function (Bucci *et al*. 2011, Dias *et al*. 2014). The different affinities to glucose between the isoforms (Devaskar and Mueckler 1992) suggests specific roles for these isoforms. Our study and others recently reported that GLUT3 is present in sperm and shows differences in localization between murine and chicken sperm (Simpson *et al*. 2008, Ushiyama *et al*. 2019). GLUT1 is a major GLUT isoform in mammalian sperm and is localized to the acrosome region and principal piece of the flagellum in human, rat, and bull sperm (Angulo *et al*. 1998). In addition, GLUT1 is a low-Km, high-affinity GLUT believed to play a central role in maintaining basal glucose uptake in many mammalian cells (Devaskar and Mueckler 1992). Taken together, this implies the functional roles of this isoform in mammalian sperm. However, the function of GLUT1 in mammalian and avian sperm remains unclear. This is partly because targeted deletion of GLUT1 results in embryonic lethality in mice (Heilig *et al*. 2003).

In sperm, ATP is generated from glucose via glycolysis and oxidative phosphorylation, which occur in different regions of the cell. Numerous mammalian sperm studies have demonstrated that oxidative phosphorylation occurs in the mitochondria, which are highly packed into the midpiece of the flagellum, while glycolysis occurs in the principal piece that occupies the major part of flagellum (Mukai

and Okuno 2004, Storey 2008). Most of the enzymes necessary for glycolysis, such as hexokinase I and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), possess sperm-specific isoforms that are mainly associated with the fibrous sheath in the principal piece due to N-terminal domain anchor (Bunch *et al*. 1998, Travis *et al*. 1998). Due to its higher efficiency of ATP production, oxidative phosphorylation is considered to be the main provider of ATP for sperm motility, although some studies have shown that there is wide variation among the primary mechanisms for ATP production in mammalian species (Storey 2008). For example, glycolysis plays a major role in ATP production for flagellar movement in murine and human sperm (Williams and Ford 2001, Mukai and Okuno 2004). On the other hand, ATP production is also critical for maintenance of motility and fertilization ability in avian sperm (Wishart and Palmer 1986, McLean *et al*. 1997), potentially via a metabolic signalling pathway (Nguyen *et al*. 2014). However, the ATP production pathways along the flagellum are poorly characterised in avian sperm.

The present study demonstrated that GLUT1 is specifically localized to the midpiece of the flagellum and contributes to glucose uptake and ATP production via both glycolysis and oxidative phosphorylation for flagellar motility. Due to the differences in mammalian data and a lack of knowledge of the subcellular regions involved in glycolysis in avian sperm, we performed immunodetection for hexokinase I and GAPDH and showed that these enzymes were localized to both the midpiece and principal piece of the chicken sperm flagellum. Our results provide new insight into the glucose metabolic pathways involved in supporting flagellar motility and suggest the involvement of the midpiece in both glycolysis and oxidative phosphorylation in avian sperm.

III.2 Materials and Methods

Reagents and animals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. Fasentin and broad spectrum GLUTs inhibitor (GLUTi; glucose transporter inhibitor II) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Recombinant GLUT1 ligand tagged with green fluorescent protein (GFP) was purchased from Metafora Biosystems (Paris, France). 2-(*N*- [7-nitrobenz-2-oxa-1,3-diazol-4-yl]-amino)-2-deoxy-D-glucose (2-NBDG) and 5,5',6,6'-tetrachloro-1,1'3,3'-tetraethylbenzimi-dazolylcarbocyanine iodide (JC-1) were obtained from Invitrogen (Waltham, MA, USA) and Cayman Chemical (Ann Arbor, MI, USA), respectively. "Cellno" ATP assay reagent was from TOYO B-Net Co., Ltd (Tokyo, Japan). Polyclonal antiserum against chicken GLUT1 was raised in two rabbits at Eurofins Genomics (Tokyo, Japan) using the antigenic epitope [Lys^{476}–Gln^{489}] specific for GLUT1. Polyclonal and monoclonal antibodies against hexokinase I and GAPDH were purchased from Proteintech (Rosemont, IL, USA) and FUJIFILM Wako Pure Chemicals (Osaka, Japan), respectively.

Semen was collected from fertile male Rhode Island Red roosters using the dorsal-abdominal massage method (Burrows and Quinn, 1935). Pooled semen from at least four roosters was centrifugally washed twice with phosphate buffered saline (PBS) to exclude secretory fluids, including seminal plasma. All animal work was performed under the approval of the Institutional Animal Care and Use Committee of the University of Tsukuba (approval no. 18-349).

Localization

For immunostaining, sperm were fixed with 4% paraformaldehyde for 15 min at room temperature (RT), permeabilised in 0.5% Triton X-100 for 1 min, and blocked in 10% goat serum for 1 h. Sperm were incubated with anti-GLUT1 (1:150), anti-hexokinase I (1:200), or anti-GAPDH (1:200) at 4 °C overnight followed by incubation with either anti-rabbit or anti-mouse IgG Alexa Fluor 488 (1:200) for 1 h at RT. Coverslips were mounted using VECTASHIELD mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) on glass slides.

Labeling with GLUT1 ligand tagged with GFP (GLUT1–GFP) was performed by fixing sperm with 1% paraformaldehyde, followed by incubation with GLUT1–GFP (1:25) at 4 °C overnight and mounting on glass slides as described above.

Immunoblotting

Sperm were resuspended in PBS containing a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland), douce-homogenized, and total protein was measured using a Bradford protein assay. Samples of 70 μg of sperm protein were processed for SDS–PAGE analysis. Transfer, blocking, and immunodetection of GLUT1 was performed as previously described (Asano *et al*. 2009). Anti-GLUT1 antibody and anti-rabbit IgG conjugated with HRP were diluted 1:3000 and 1:10,000, respectively. Chemiluminescence was used to detect the immunoreactivity.

Sperm incubation and motility

Sperm (1×10^7) were incubated in *N*-Tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES)–NaCl medium (20 mM TES, 150 mM NaCl, pH 7.4) containing 5 mM Ca^{2+} at 39 °C with 0–50 μM fasentin, 10 μM antimycin A (AA), or 10 μM carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) in the presence or absence of 20 mM glucose for 0, 40, or 80 min. AA and CCCP are respiratory chain inhibitors and a proton ionophore,

respectively, and were used to inhibit mitochondrial oxidative phosphorylation. Sperm motility profiles were analysed using a sperm motility analysis system (SMAS; DITECT, Tokyo, Japan). At least 200 sperm per sample were used to examine the following movement characteristics: motility (%), straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), average path velocity (VAP, μm/s), linearity (LIN, VSL/VCL), straightness (STR, VSL/VAP), amplitude of lateral head displacement (ALH, μm), and beat-cross frequency (BCF, Hz).

Glucose uptake

A glucose uptake assay using a fluorescent glucose analog was performed as described previously (Ushiyama *et al*. 2019). In brief, sperm (1×10^7) were incubated in the presence or absence of 60 μM 2-NBDG with 0, 30, or 50 μM fasentin in TES–NaCl solution containing 5 mM Ca^{2+} for 40 min at 39 ℃, then centrifuged at 1000 × *g* for 5 min and resuspended. Fluorescence intensity was measured using a Multimode Detector DTX800 (Beckman Coulter, CA, USA) at wavelengths of 488 nm (excitation) and 530 (emission). The subcellular localization of 2-NBDG was viewed using a Leica DMI 4000B microscope (Leica Microsystems, Wetzlar, Germany). Images were captured using identical exposure times using different fasentin concentrations.

Mitochondrial activity

Mitochondrial activity was assessed using JC-1, a lipophilic fluorescent probe that reversibly changes its fluorescence from green (monomeric form) to orange (aggregate form) as the mitochondrial membrane potential (MMP) increases. Sperm (2×10^7) were incubated with 0, 30, or 50 μM fasentin, 1 μM GLUTi, 10 μM AA, or 10 μM CCCP in TES-NaCl solution containing 5 mM Ca^{2+}, with or without 20 mM glucose at 39 ℃ for 40 min. Sperm were centrifuged at 1000 × *g* for 5 min and resuspended in 100 μL of

TES–NaCl solution containing 5 μL of JC-1. After incubation at 39 °C for 15 min, samples were centrifugally washed twice and resuspended in assay buffer. The fluorescence intensity in each sample (2×10^6) was analysed using a Multimode Detector DTX800 with excitation and emission wavelengths set at 535 and 590 nm for the aggregate form and 485 and 530 nm for the monomeric form. The fluorescence intensity ratio (590/530 nm) was used to evaluate the MMP.

ATP quantification

ATP content was quantified using "Cellno" ATP assay reagent (TOYO B-Net, Tokyo, Japan). Sperm (2×10^7) were incubated with 0, 30, or 50 μM fasentin, 1 μM GLUTi, 10 μM AA, and 10 μM CCCP in the presence or absence of 20 mM glucose in TES–NaCl solution containing 5 mM Ca^{2+} at 39 °C for 40 min. After washing with TES–NaCl solution, sperm (6×10^4) were solubilised in ATP assay reagent at 23 °C for 10 min and the luminescence signal was measured using a Multimode Detector DTX800.

Statistical analysis

Multiple comparisons were carried out using one-way ANOVA, followed by Tukey's HSD, except for utilization of two-way ANOVA to determine the effect of glucose supplementation and fasentin concentration on motility profile. Differences were considered significant when $P < 0.05$.

III.3 Results

Expression and localization of GLUT1

Immunoblot analysis using chicken sperm showed detection of GLUT1 at the predicted molecular weight (Fig. 4A). Immunostaining showed localization of GLUT1 at the midpiece region of the flagellum (Fig. 4B). We also found specific localization of

GLUT1 at the midpiece when sperm were labeled with GLUT1–GFP, a recombinant GLUT1 ligand. No signals were observed in control sperm that were only treated with a fluorescent secondary antibody. These results showed that GLUT1 was expressed and specifically localized at the midpiece of the flagellum of chicken sperm.

Effects of glucose on sperm motility

To examine the effects of glucose on sperm motility, sperm were subjected to SMAS analysis immediately (0 min) or after incubation with or without 20 mM glucose for 40 and 80 min. The sperm motility parameters at 0 min were as follow: motility, 96.0 %; VSL, 27.8 μm/s; VCL, 110.4 μm/s; VAP, 44.1 μm/s; LIN, 0.3 (VSL/VCL); STR, 0.7 (VSL/VAP); ALH, 1.7 μm; and BCF, 7.9 Hz (Table 1). When sperm were incubated without glucose supplementation, motility (40 min, 83.9 %; 80 min, 76.3%), VSL (40 min, 20.1 μm/s; 80 min, 16.8 μm/s), VCL (40 min, 75.4 μm/s; 80 min, 70.7 μm/s), VAP (40 min, 29.7 μm/s; 80 min, 24.2 μm/s), and ALH (40 min, 1.3 μm; 80 min, 1.2 μm) decreased in a time-dependent manner ($P < 0.05$). No changes were observed for LIN (40 min, 0.28 VSL/VCL; 80 min, 0.26 VSL/VCL), STR (40 min, 0.72 VSL/VAP; 80 min, 0.72 VSL/VAP), and BCF (40 min, 8.4 Hz; 80 min, 7.4 Hz). In contrast, addition of 20 mM glucose showed no decrease in motility (40 min, 93.4%; 80 min, 88.8%) and VAP (40 min, 38.9 μm/s; 80 min, 37.1 μm/s) up to 40 min post-incubation, or VSL (40 min, 28.0 μm/s; 80 min, 26.1 μm/s) and LIN (40 min, 0.36 VSL/VCL; 80 min, 0.32 VSL/VCL) up to 80 min post-incubation. Higher motility, VSL, and VAP were detected after 40 min post-incubation following glucose supplementation.

Similar to the observations without glucose, there was no change in BCF throughout the incubation period with glucose supplementation (40 min, 11.1 Hz; 80 min, 10.6 Hz). These results indicate that glucose supports sperm flagellar motility. Further we examined

the effect of mitochondrial inhibition on glucose-dependent motility using AA, an electron transport inhibitor. The result was suggestive of involvement of mitochondrial oxidation in the glucose-mediated motility regulation (Fig. 8).

Role of GLUT1 in sperm motility

To examine the glucose-dependent role of GLUT1 supporting sperm motility, sperm were incubated with 0, 30, or 50 μM fasentin, a GLUT1 inhibitor, for 40 min in the presence or absence of 20 mM glucose, followed by SMAS analysis. In the absence of glucose, there were no differences in any parameters among fasentin concentrations (motility, 75.8%–83.0%; VSL, 17.8–18.7 μm/s; VCL, 74.2–86.5 μm/s; VAP, 22.8–29.0 μm/s; LIN, 0.2–0.3; STR, 0.7–0.8; ALH, 1.4–1.5 μm; BCF, 5.9–8.3 Hz) (Table 2). Consistent with the findings present in Table 1, incubation with glucose improved sperm motility (91.8 %), VSL (28.0 μm/s), and VAP (39.6 μm/s) in the absence of fasentin ($P <$ 0.05). In contrast, these effects were abolished by the addition of 30 or 50 μM fasentin (motility, 83.7% or 82.0%; VSL, 18.5 or 18.7 μm/s; VAP, 25.6 or 27.5 μm/s, respectively). Furthermore, motility, VSL, VCL, and VAP significantly decreased with increasing concentrations of fasentin, suggesting GLUT1 inhibition by fasentin. These results suggest that GLUT1 plays an important role in supporting flagellar motility in chicken sperm.

Glucose uptake assay

To determine the role of GLUT1 in glucose uptake in chicken sperm, the fluorescent analog, 2-NBDG, was used to visualize sperm incubated with different concentrations of fasentin. We found that treatment with both 30 and 50 μM fasentin reduced glucose uptake (Fig. 5A and B). Together with the GLUT1 localization data, these results strongly

suggest that GLUT1 contributes glucose uptake in the midpiece of the flagellum and suggests the involvement of GLUT1 in mitochondrial activity.

Role of GLUT1 in ATP production

To examine the role of GLUT1 in chicken sperm ATP production, cellular ATP content was quantified in sperm incubated with fasentin, GLUTi, AA, or CCCP, with or without 20 mM glucose. Incubation with 20 mM glucose increased the ATP content (mean increase, 80%), although fasentin decreased the ATP content in a dose-dependent manner (22%–33%) (Fig. 6A). No difference was observed between 50 μM fasentin and no glucose, suggesting that GLUT1 plays a major role in ATP production. GLUTi, AA, and CCCP dramatically reduced the ATP content (mean decreases of 93.1%, 88.4%, and 82.4 %, respectively, compared with glucose alone), but no differences were observed between them. Considering the specific localization of GLUT1 in the midpiece of the flagellum, together with clearance of glucose-dependent motility regulation by fasentin, these results reinforce that glucose uptake via GLUT1 plays a major role in ATP production. When we incubated sperm without glucose, lower ATP contents were observed in fasentin, GLUTi, AA and CCCP treatments than control with no addition, suggesting that small remnant of glycosable substrates may be present in sperm suspension or cytoplasm prior to incubation.

To examine the effect of GLUT1 on mitochondrial activity, the MMP was assessed in sperm incubated with fasentin, GLUTi, AA, or CCCP, in the absence or presence of 20 mM glucose. Glucose supplementation dramatically increased the MMP (Fig. 6B); however, it was decreased by fasentin treatment in a dose-dependent manner, resulting in no difference in MMP between presence and absence of glucose under fasentin treatments. AA and CCCP were used as negative controls, and showed a lower MMP compared with

50 μM fasentin. Taken together, these results demonstrate that GLUT1 plays a major role in stimulation of oxidative phosphorylation in response to glucose uptake.

Our results indicated that oxidative phosphorylation is the predominant source of ATP in chicken sperm. Therefore, movement was analysed in sperm treated with uncouplers of mitochondrial oxidative phosphorylation. The results showed a significant decrease in motility, VSL, VCL, and VAP compared with treatment with glucose alone (Fig. 6C). Despite the potent inhibition of VSL, VCL, and VAP, more than 60% of sperm were still motile. In addition, a stark difference was seen in the extent of reduction in response to AA and CCCP between ATP content and motility (mean decrease, 29.0% and 31.2% compared with glucose alone), suggesting the involvement of both glycolysis and oxidative phosphorylation in flagellar motility in chicken sperm.

Localization of glycolytic enzymes

Several glycolytic enzymes have been identified in mammalian sperm that mainly localize to the principal piece of the flagellum, suggesting this is the primary site for glycolysis (Miki *et al*. 2004). However, expression and localization of glucose metabolic pathways has not been demonstrated in avian sperm; therefore, we aimed to identify the localization of hexokinase I and GAPDH, major glycolytic enzymes, in chicken sperm. Immunoblotting for GAPDH and hexokinase I showed they could be detected at the predicted molecular weights (Fig. 7A).

GAPDH and hexokinase I were localized to both the midpiece and principal piece of the flagellum (Fig. 7B). In addition, hexokinase I showed patch-like localization at the sperm head region despite it being largely devoid of GAPDH. Taken together, these results suggest the glycolytic pathway occurs in both the midpiece and principal piece of chicken sperm.

III.4 Discussion

Sperm have a high requirement for ATP as an energy source to maintain flagellar motility. Despite the importance of glucose transport for ATP production and fertility in chicken sperm, the regulatory mechanisms involved in glucose-dependent sperm motility remains poorly understood. We found that GLUT1 is specifically localized to the midpiece of the flagellum and contributes to flagellar motility by ATP production via glycolysis and oxidative phosphorylation. Our results provide new insight into sperm function, such as flagellar motility, and suggest dual roles of the midpiece in the glucose metabolic pathways in avian sperm.

Our immunoblotting and immunostaining results using a specific antibody showed GLUT1 was detected in the midpiece of the flagellum. This was confirmed by labelling sperm with GLUT1–GFP, a specific ligand tagged with GFP. This recombinant ligand was originally generated from a binding domain of human T cell leukemia virus to GLUT1, and has been widely used to determine expression and localization of GLUT1 (Manel *et al*. 2003, Kinet *et al*. 2007). Taken together, our results strongly suggest the specific localization of GLUT1 at the midpiece in chicken sperm. A previous localization study showed that the midpiece of the flagellum was largely devoid of GLUT1, while it was localized to the acrosomal region and principal piece in several mammalian species (Angulo *et al*. 1998). This suggests a functional distinction of GLUT1 between mammalian and avian sperm.

A previous study in chicken sperm demonstrated the importance of glucose transport in sperm motility and fertility (Wishart 1982, McLean *et al*. 1997). Consistent with this, our SMAS analysis showed that glucose supplementation supports sperm motility. It was previously shown that several GLUTs were associated with the flagellar

regions in mammalian sperm (Bucci *et al*. 2011). In addition, we recently reported that GLUT3 is localized to the entire flagellum as well as acrosomal regions in chicken sperm (Ushiyama *et al.* 2019). However, it remains unclear which GLUTs support flagellar motility. Fasentin binds to the intramembrane channel of GLUT1, resulting in the inhibition of glucose uptake (Wood *et al*. 2008). We identified effective fasentin concentrations on motility profile and found the concentration-dependent inhibition. This combined with the fact that fasentin IC_{50} is 68 μM, made us to choose 30 and 50 μM fasentin concentrations, resulting in almost completely abolished sperm motility dependent on extracellular glucose. Similarly, uptake of 2NBDG decreased following treatment with fasentin. Furthermore, these concentrations showed no cytotoxicity at least in chicken sperm motility, which is in agreement with a previous study performed using cultured cells (Wood *et al.* 2008). Taken together, our results suggest that GLUT1 plays a major role in glucose transport to power flagellar motility.

In mammalian sperm, glucose-mediated acceleration of flagellar motility depends on ATP production via oxidative phosphorylation in mitochondria and glycolysis in the principal piece (Ford 2006). In agreement with this, we found that GLUT1 inhibition abolished elevation of ATP production and mitochondrial activity in response to glucose supplementation in chicken sperm. In addition, more potent inhibition was observed in ATP production when sperm were treated with GLUTi, but it disappeared in mitochondrial activity. Considering that this inhibitor blocks not only GLUT1, but also GLUT3, GLUT4, and GLUT9-mediated transport when used at high concentrations (Wang *et al*. 2012), these results suggest an important role of GLUT1 in glucose metabolic pathways as well as the potential involvement of other GLUTs. Supporting this, we and others previously demonstrated that GLUT3 is present along the length of the

flagellum in chicken and murine sperm (Simpson *et al*. 2008). Furthermore, flagellar localization of the GLUT9 isoform was also reported in murine sperm (Sung and Moley 2007), although no information is available in avian sperm. Considering the restricted localization of chicken GLUT1 to the midpiece of the flagellum, together with varied affinity of glucose transporters to glucose (Devaskar and Mueckler 1992), these findings suggest differential roles of glucose transporters in ATP production pathways.

The present study showed that disruption of mitochondrial ATP production using AA or CCCP, uncouplers of mitochondrial oxidative phosphorylation, reduced cytoplasmic ATP and sperm movement, concomitant with mitochondrial activity. These findings are consistent with a previous study that showed a strong correlation between mitochondrial ATP production and motility in chicken sperm (Froman *et al*. 1999). Surprisingly, around 60% of sperm were still motile after mitochondrial inhibition in chickens. Studies in mammalian sperm have shown different roles of ATP produced via different metabolic pathways (Travis *et al*. 2001, Mukai and Okuno 2004). For example, in mice, CCCP treatment had no effect on motility or ATP levels, but diminished mitochondrial activity following glucose supplementation (Goodson *et al*. 2012). Furthermore, studies in guinea pig sperm showed that glucose stimulates motility and ATP production, even with inhibition of oxidative phosphorylation, although it retarded the acrosome reaction inherent with the capacitation process (Jane Rogers and Yanagimachi 1975, Mujica *et al*. 1991). Despite a wealth of knowledge about mammalian sperm, the localization and functional roles of glucose metabolic pathway remain poorly characterised in avian sperm. Our results showed localization of GAPDH and hexokinase I at the midpiece and principal piece of the flagellum, suggesting the glycolytic pathway operates along the entire length of the flagellum in chicken sperm. Several glycolytic

enzymes, including GAPDH and hexokinase I, have sperm-specific isoforms directly or indirectly tethered to the fibrous sheath exclusively present in the principal piece of the flagellum (Welch *et al*. 1992, Westhoff and Kamp 1997), resulting in the distinction of its localization from the somatic isoform. Despite the similarity in the flagellar structure between mammals and birds, antibodies were not able to distinguish tissue-specific isoforms due to commonality of the epitope region between isoforms. Recent studies in bull sperm using tissue-specific antibodies against GAPDH showed that somatic GAPDH was localized to the midpiece, but the sperm-specific isoform was confined to the principal piece (Feiden *et al*. 2008). It was also reported that the midpiece contains hexokinase I isoforms (Travis *et al*. 1998, Nakamura *et al*. 2008). These findings suggest that chicken sperm might possess both somatic and sperm-specific isoforms. Of note, we found faint patch-like distribution of hexokinase I in sperm head, largely devoid of GAPDH. Hexokinase I is also involved in pentose phosphate pathway (PPP), a semi-independent and parallel pathway to glycolysis. Previous studies in mammalian sperm showed that active PPP is localized to the midpiece and sperm head, and plays a role in multistage of fertilization (Bolton 1970, Urner and Sakkas 2005). Our results suggest a future investigation for functional nature of PPP in avian sperm.

Although oxidative phosphorylation is more efficient than glycolysis for ATP production, in most mammalian sperm, glycolysis is exclusively responsible for supporting capacitation-associated changes, such as protein tyrosine phosphorylation and hyperactivated motility in some species (Travis *et al*. 2001, Williams and Ford 2001). Considering that avian sperm, unlike mammalian sperm, do not require capacitation for functional changes to acquire the competency to fertilize, our results provide a foundation for investigation into ATP production pathways in vertebrates.

In summary, the present study demonstrated that GLUT1 is specifically localized to the midpiece of the flagellum in chicken sperm and plays a major role in ATP production and flagellar motility supported by glucose uptake. Furthermore, our results suggest that chicken sperm rely on both the glycolysis and oxidative phosphorylation to support flagellar motility. These findings provide new insight into the glucose metabolic pathways in avian sperm, and highlight their distinction from mammalian sperm.

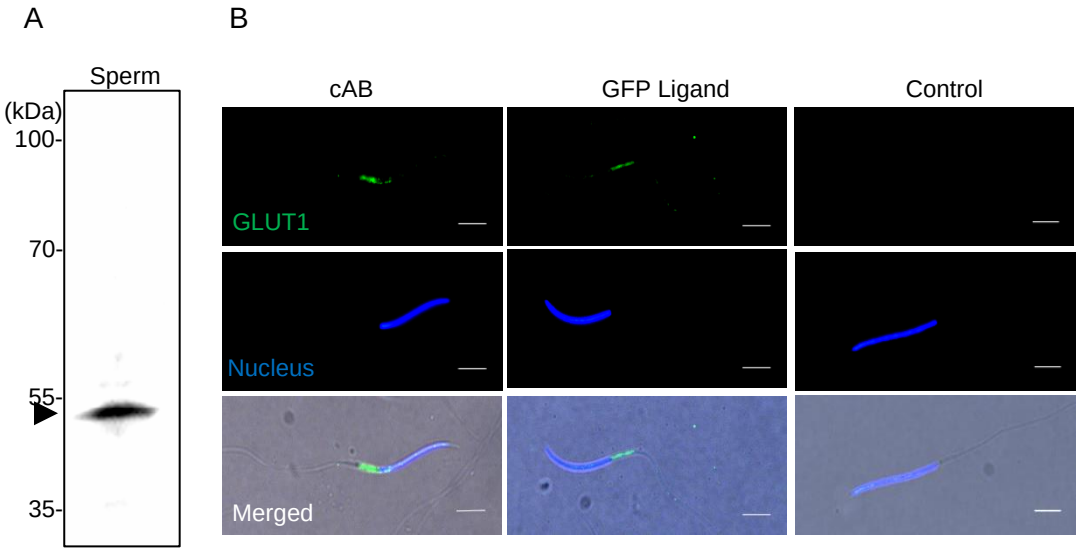


Fig. 4. Expression and localization of GLUT1 in chicken sperm.

Immunoblot using chicken GLUT1-specific antibody (cAB) showed the presence of GLUT1 at the predicted molecular weight (A). Sperm were fixed and subjected to immunostaining with cAB or labelling with GLUT1 ligand fused with GFP (GFP ligand). GLUT1 was found to be localized at the midpiece (B). No signals were detected in sperm labelled with secondary antibody used as control. Scale bars represent 5 μm (n=3).

Table 1. Changes in sperm motion parameters during incubation for 0 – 80 min under 0 – 20 mM glucose supplementation.

	0 min	40 min		80 min	
	Control	Control	Glucose	Control	Glucose
Motility (%)	95.98 ± 0.64^{a}	83.89 ± 1.93^{c}	93.37 ± 0.53^{ab}	76.30 ± 0.81^{d}	88.84 ± 0.65^{bc}
VSL (μm/sec)	27.75 ± 0.70^{a}	20.08 ± 1.16^{b}	28.04 ± 0.89^{a}	16.77 ± 0.41^{b}	26.13 ± 1.01^{a}
VCL (μm/sec)	110.38 ± 3.40^{a}	75.41 ± 2.70^{cd}	87.34 ± 1.82^{bc}	70.73 ± 3.48^{d}	93.10 ± 2.43^{b}
VAP (μm/sec)	44.12 ± 1.25^{a}	29.72 ± 1.63^{c}	38.93 ± 0.89^{ab}	24.21 ± 0.55^{c}	37.08 ± 1.15^{b}
LIN (VSL/VCL)	0.27 ± 0.00^{ab}	0.28 ± 0.02^{ab}	0.36 ± 0.02^{a}	0.26 ± 0.02^{b}	0.32 ± 0.02^{ab}
STR (VSL/VAP)	0.66 ± 0.01^{b}	0.72 ± 0.02^{ab}	0.76 ± 0.02^{a}	0.72 ± 0.01^{ab}	0.76 ± 0.01^{a}
ALH (μm)	1.73 ± 0.04^{a}	1.31 ± 0.08^{b}	1.37 ± 0.03^{b}	1.19 ± 0.07^{b}	1.40 ± 0.06^{b}
BCF (Hz)	7.91 ± 0.15	8.43 ± 1.04	11.12 ± 0.66	7.43 ± 0.81	10.59 ± 0.64

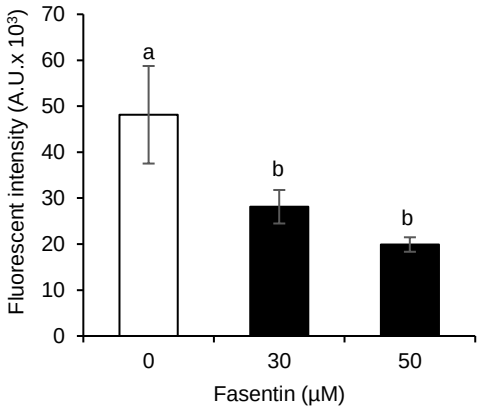
Straight-line velocity (VSL, μm/sec), curvilinear velocity (VCL, μm/sec), average path velocity (VAP, μm/sec), linearity (LIN, VSL/VCL), straightness (STR, VSL/VAP), amplitude of lateral head displacement (ALH, μm), beat-cross frequency (BCF, Hz). Data are expressed as mean ± SEM (n=5). $^{a\text{-}d}P < 0.05$ in same row.

Table 2. Motility parameters of sperm incubated for 40 min under presence of 0 – 50 μM fasentin with 0 – 20 mM glucose.

		Fasentin (μM)		
		0	30	50
Motility (%)	Control	82.96 ± 2.21^{A}	75.75 ± 4.57^{A}	83.00 ± 3.58^{A}
	Glucose	$91.81 \pm 1.14^{a, B}$	$83.65 \pm 1.17^{ab, A}$	$81.93 \pm 2.16^{b, A}$
VSL (μm/sec)	Control	18.72 ± 1.95^{A}	17.98 ± 1.61^{A}	17.81 ± 0.91^{A}
	Glucose	$27.96 \pm 1.01^{a, B}$	$18.46 \pm 1.73^{b, A}$	$18.68 \pm 3.92^{b, A}$
VCL (μm/sec)	Control	86.47 ± 7.85^{A}	86.16 ± 9.99^{A}	74.18 ± 2.34^{A}
	Glucose	$110.37 \pm 8.25^{a, B}$	$73.81 \pm 7.83^{b, A}$	$77.19 \pm 15.08^{b, A}$
VAP (μm/sec)	Control	27.55 ± 2.75^{A}	26.22 ± 2.57^{A}	24.27 ± 0.96^{A}
	Glucose	$39.63 \pm 1.39^{a, B}$	$25.55 \pm 2.12^{b, A}$	$27.51 \pm 5.09^{b, A}$
LIN (VSL/VCL)	Control	0.24 ± 0.01^{A}	0.25 ± 0.02^{A}	0.25 ± 0.01^{A}
	Glucose	0.28 ± 0.01^{A}	0.28 ± 0.02^{A}	0.25 ± 0.01^{A}
STR (VSL/VAP)	Control	0.72 ± 0.02^{A}	0.73 ± 0.02^{A}	0.74 ± 0.01^{A}
	Glucose	0.74 ± 0.02^{A}	0.75 ± 0.02^{A}	0.71 ± 0.02^{A}
ALH (μm)	Control	1.41 ± 0.08^{A}	1.58 ± 0.18^{A}	1.36 ± 0.04^{A}
	Glucose	1.65 ± 0.09^{A}	1.40 ± 0.18^{A}	1.29 ± 0.04^{A}
BCF (Hz)	Control	6.78 ± 0.65^{A}	7.38 ± 1.08^{A}	6.05 ± 0.75^{A}
	Glucose	6.98 ± 0.33^{A}	7.99 ± 1.15^{A}	7.89 ± 1.50^{A}

Straight-line velocity (VSL, μm/sec), curvilinear velocity (VCL, μm/sec), average path velocity (VAP, μm/sec), linearity (LIN, VSL/VCL), straightness (STR, VSL/VAP), amplitude of lateral head displacement (ALH, μm), beat-cross frequency (BCF, Hz). Data are expressed as mean ± SEM (n=5-6). $^{a\text{-}b}P < 0.05$ in same row. $^{AB}P < 0.05$ in comparison between control and glucose addition.

(A)



(B)

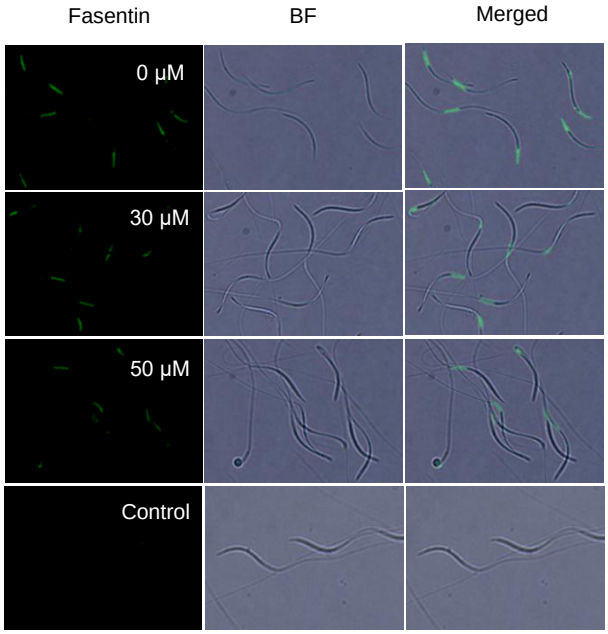


Fig. 5. Glucose uptake in sperm incubated with 0–50 μM fasentin.

Chicken sperm were incubated with 0–50 μM fasentin and 60 μM 2-NBDG in TES–NaCl + 5 mM CaCl2 prior to glucose uptake assay. Fasentin treatment inhibited glucose uptake (A). Signals for 2-NBDG were exclusively found in the midpiece of the flagellum and were reduced in response to higher concentrations of fasentin (B). No fluorescent signals were found in the negative control. Data are expressed as mean ± SEM (n = 5). Scale bars represent 10 μm. $^{a–b}P < 0.05$.

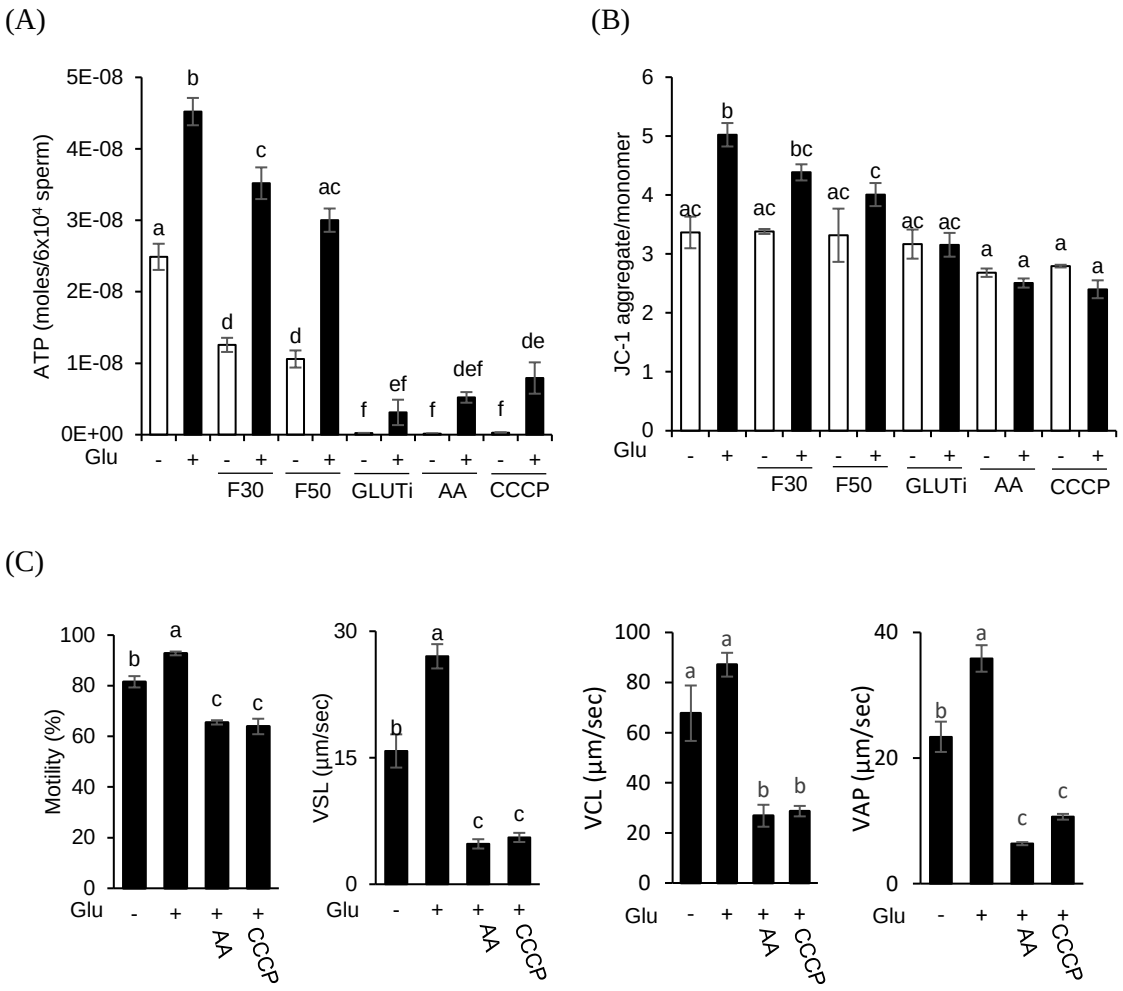


Fig. 6. Changes in ATP content, mitochondrial activity, and sperm motility profile in response to various inhibitors.

Sperm were incubated with 30 or 50 μM fasentin (F30 and F50), 1 μM glucose transporter inhibitor (GLUTi), 10 μM antimycin A (AA), or 10 μM carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP), with 0 or 20 mM glucose supplementation for 40 min. ATP was quantified (A), and mitochondrial activity was measured using JC-1 fluorescence (B). ATP content was increased with glucose supplementation but decreased when F30 and F50 were added. GLUTi, AA, and CCCP dramatically reduced the ATP content. Data are expressed as mean ± SEM (n = 4). Mitochondrial activity was also higher with glucose

supplementation but was inhibited by the addition of F50 and GLUTi. AA and CCCP reduced mitochondrial activity. Data are expressed as mean ± SEM (n = 6). SMAS analysis using sperm incubated with glucose alone, or AA or CCCP together with glucose (C), showed reduced motility, VSL, VCL, and VAP. Data are expressed as mean ± SEM (n = 5). $^{a–c}P < 0.05$.

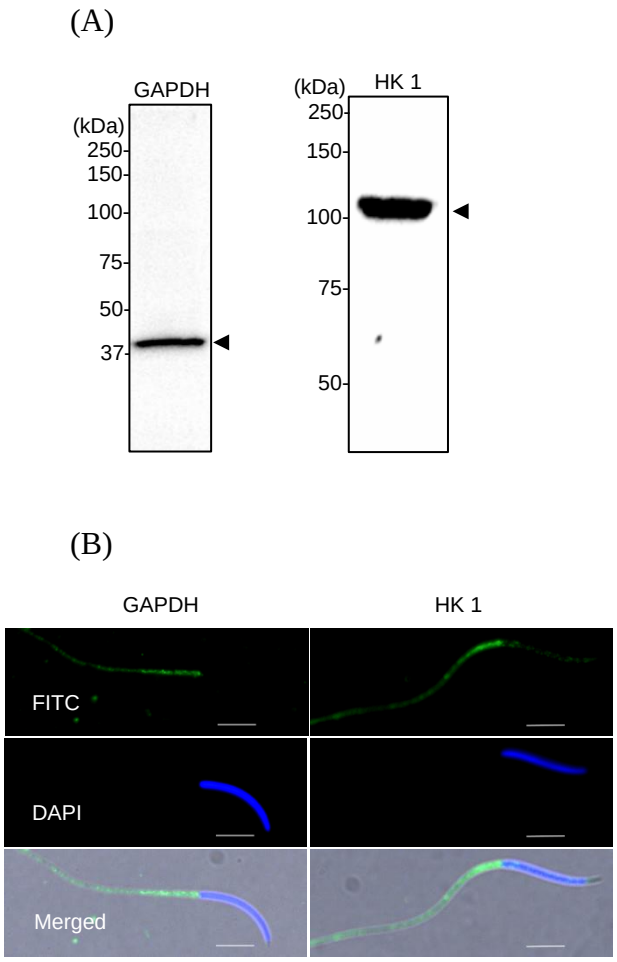


Fig. 7. Expression and localization of GAPDH and hexokinase 1 (HK1) in chicken sperm.

GAPDH and HK1 were expressed at the predicted molecular sizes (A). GAPDH and HK1 were both localized to the flagellum, with clear enrichment in the midpiece (*green*) (B). Faint, dotted distribution of HK1 was observed in the sperm head, whereas GAPDH was not present. Nuclei were fluorescent-labelled using DAPI (*blue).* Scale bars represent 5 μm (n = 5).

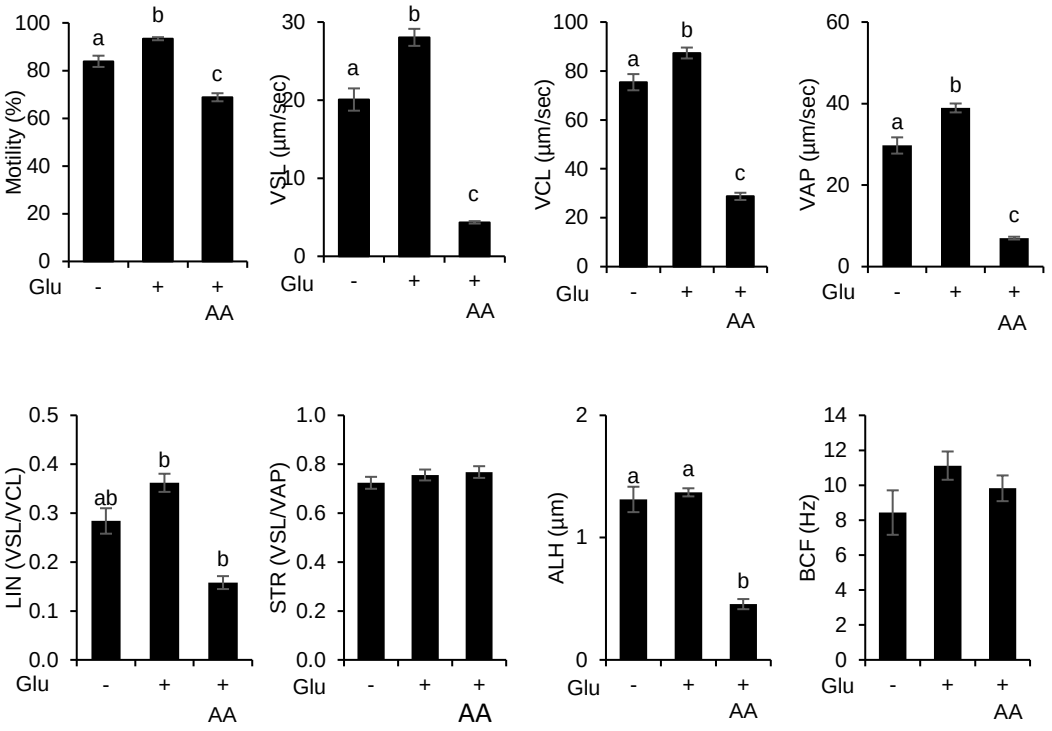


Fig. 8. Changes in sperm motility parameters in response to glucose and mitochondrial inhibition.

Effect of mitochondrial inhibition on glucose-dependent motility was examined with sperm incubated with antimycin A (AA) under glucose supplementation. Sperm motility parameters were increased with glucose supplementation but dramatically decreased when Antimycin A were added. $^{a\text{-}c}P<0.05$. (n=5).

Chapter IV

Relative dependency of flagellar movement and sperm penetrability on ATP production pathways in chicken sperm

IV.1 Introduction

Understanding sperm energy metabolism is a crucial factor in improving the efficacy of assisted reproductive technologies. In particular, maintaining sperm fertilizing ability in artificial insemination requires to manage sperm metabolism closely linked to their functionality.

Sperm produce ATP to maintain sperm motility and sperm survival via glycolysis and oxidative phosphorylation, which are localized to different regions of the cells. Oxidative phosphorylation takes place in the mitochondria, which are tightly packed in the midpiece. Many studies have provided evidence that oxidative phosphorylation produces higher levels of ATP, facilitating sperm motility than that from glycolysis (Ferramosca *et al*. 2012, Tourmente *et al*. 2015). Aerobic metabolism creates reactive oxygen species (ROS) as a by-product. In the physiological level, ROS regulate intracellular signalling pathway, which is essential for physiological mechanisms such as maturation, capacitation, hyperactivation, acrosome reaction, and fertilization (De Lamirande *et al*. 1997, Du Plessis *et al*. 2015). However, the excess of ROS without antioxidant imbalance induces lipid peroxidation, deoxyribonucleic acid (DNA) damage, and apoptosis, which is associated with reduced sperm function and infertility (Shen and Ong 2000, Yumura *et al*. 2009).

On the other hand, being compartmentalized in the principal piece, glycolysis is vital for fully supporting tyrosine phosphorylation and hyperactivated motility. Several

glycolytic enzymes of sperm-specific isoforms such as hexokinase 1 (HK1S) (Nakamura *et al*. 2008), pyruvate kinase (PKS), and glyceraldehyde 3-phosphate dehydrogenase (GAPDS) (Feiden *et al*. 2008) have been detected in the fibrous sheath of the principal piece where dynein ATPases convert chemical energy of ATP into mechanical work on microtubules, resulting changes in motility pattern (hyperactivation) and penetration of zona pellucida (Krisfalusi *et al*. 2006, Young *et al*. 2016). However, the primary ATP provider for sperm function varies among species. For example, bull and guinea pig depend on both high glycolysis and respiration, boar and horse sperm are highly dependent on respiration rate and cannot support motility with glycolysis alone, while human sperm rely mainly on glycolysis (Tourmente *et al*. 2015).

Our previous study observed that chicken sperm are likely to have a unique pattern concerning the mechanism of ATP production. The sperm are not able to maintain sperm velocities and have remarkably decrease ATP level when treated with uncoupler agents suggesting oxidative phosphorylation plays a vital role as ATP provider in flagellar movement. In addition, we found that chicken sperm posses Hexokinase I and Glyceraldehyde 3-phosphate dehydrogenase, glycolytic enzymes located to the midpiece and principal piece, suggesting glycolysis exists in chicken sperm metabolism. However, the mechanisms of both ATP providers regulating chicken sperm functions are poorly understood. These prompted us to investigate primary ATP production pathways regulating sperm functions. To begin to understand the mechanisms of energy metabolism, we performed detailed analysis regarding the effect of glucose, pyruvate supplementation, and their combination on sperm functional parameters, particularly on sperm motility and penetrability. Since we observed differences in the abilities of both ATP providers to support sperm functional changes, we compared ATP production and sperm functional

parameters incubated with either glycolysis or oxidative phosphorylation inhibitor. We also determined changes of intracellular pH (pHi) and reactive oxygen species (ROS) in response to metabolism substrates.

IV.2 Materials and Methods

Reagents and animals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise mentioned. (±) -3-Chloro-1,2-propanediol (3-MCPD) and antimycin A were purchased from Sigma (St. Louis, MO, USA). "Cellno" ATP assay and ATP standard reagents were obtained from TOYO B-Net Co., Ltd (Tokyo, Japan). 2',7' – dichlorofluorescein diacetate (DCFDA) cellular ROS detection assay kits were obtained from Abcam (Tokyo, Japan). 2',7'-bis. (2-carboxyethyl)-5(and-6)carboxyfluorescein acetoxymethyl ester (BCECF-AM) intracellular ratiometric pH indicator reagents were purchased from Abcam (Tokyo, Japan).

Semen was collected from fertile Rhode Island Red roosters using the dorsal-abdominal massage method (Burrows and Quinn 1935). Pooled semen from at least four roosters were washed twice by centrifugation at 1,000 g for 5 min in *N*-Tris(hydroxymethil)methyl-2-aminoethane sulfonic acid (TES-NaCl) to remove secretory fluids before used in experiments. All animal work was performed under the approval of the Institutional Animal Care and Use Committee of the University of Tsukuba (approval no. 18-349).

Sperm motility analysis

Sperm (1×10^7/ml) were incubated in TES-NaCl medium (20 mM TES, 150 mM NaCl, pH 7.4) containing 5 mM Ca^{2+} at 39 ^{0}C for 40 min with the presence, absence, or

combination of 20 mM glucose and 0.5 mM pyruvate. Sperm motility parameters measured using a sperm motility analysis system (SMAS; DITECT, Tokyo, Japan) consisted of motility (%), straight-line velocity (VSL, μm/sec), curvilinear velocity (VCL, μm/sec), average path velocity (VAP, μm/sec), linearity (LIN, VSL/VCL), straightness (STR, VSL/VAP), amplitude of lateral head displacement (ALH, μm), and beat-cross frequency (BCF, Hz).

ATP measurement

Sperm (2×10^7/ml) were incubated with 20 mM glucose, 0.5 mM pyruvate, 0.5 mM 3-MCPD, or 10 μM antimycin A in TES-NaCl solution containing 5 mM Ca^{2+} at 39 ^{0}C for 40 min. After washing, 6×10^4 sperm were solubilised in ATP assay reagent at 23 ^{0}C for 10 min, and the luminescence signal was measured using a Multimode Detector DTX800.

Penetration assay of sperm

Penetrating capability of sperm was assessed as described previously (Ushiyama *et al*. 2019). In brief, sperm (1×10^7/500 μl) were incubated with 5 mM Ca^{2+} Tes-NaCl containing different combination of 20 mM glucose and 0.5 mM pyruvate under the supplementation of 0.5 mM 3-monochloro-1,2-propanediol (3-MCPD) as glycolysis inhibitor or 10 μM antimycin A (AA) as oxphos inhibitor at 39 ^{0}C for 40 min, followed by co-incubation with a piece of 1 cm^2 inner perivitelline layer (IPVL) for 10 min at 39 ^{0}C. After three times washing with PBS, IPVL was fixed with 4% paraformaldehyde for 15 min at RT. IPVL was then transferred onto a glass slide and photographed under a microscope (Leica Microsystems, Wetzlar, Germany). The number of holes per 0.29 mm^2 IPVL was calculated.

Reactive oxygen species (ROS) measurement

Sperm (10^7/ml) were incubated with presence or absence the combination between 20 mM glucose and 0.5 mM in 5 mM Ca^{2+} Tes-NaCl at 39 ^{0}C for 40 min. After washing with 1× buffer, the sperm were stained with 20 µM DCFDA in 1× buffer for 35 min at 37 ^{0}C in the dark. After washing with 1× buffer, the fluorescence signal of 5×10^6 was measured using a Multimode Detector DTX800 with the excitation/emission wavelength 485/535 nm. Duplicate tubes were provided as positive control by substituting the desired treatments with 55 µM tert-butyl hydroperoxide (TBHP), an oxidative stress inducer in 1× supplemented buffer.

Immunoblotting and immunostaining of G6PD in chicken sperm

Sperm (10^7/ml) were processed for SDS-PAGE analysis. Transfer, blocking, and immunodetection of glucose-6-phosphate dehydrogenase (G6PD) was conducted as previously described (Asano *et al*. 2009). Anti-G6PD antibody and anti-rabbit IgG conjugated with HRP were diluted 1:5000 and 1:10,000, respectively. The immunoreactivity was detected using chemiluminescence.

For immunostaining, sperm were fixed with 4% paraformaldehyde for 15 min at room temperature (RT), permeabilised with 0.5% Triton X-100 for 1 min, and blocked with 10% goat serum for 1 hour. Sperm were incubated with anti-G6PD (1:150) at 4 ^{0}C overnight followed by incubation with anti-rabbit IgG Alexa Fluor 488 (1:200) for 1 h at RT. Coverslips were mounted using VECTASHIELD mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) on a glass slide.

Intracellular pH (pHi) measurement

Sperm were incubated with 5 µM BCECF-AM (2'7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester) in the presence or absence of 20 mM

glucose or 0.5 mM pyruvate 5 Ca^{2+} Tes-NaCl at 39 ^{0}C for 40 min. After incubation, sperm were centrifuged at 1000 g for 5 min, and the pellet was resuspended in the same medium, followed by incubation at 39 ^{0}C for 10 min. The fluorescence of 2×10^6 was measured at excitation wavelengths of 450 nm (F1) and 500 nm (F2) and an emission wavelength of 535 nm. Intracellular pH was determined from the ratio of BCECF fluorescence (F2/F1) calibrated using K^+ nigericin.

For pHi calibration curve, sperm were incubated with 5 µM BCECF-AM and 5 µM nigericin in a KCl medium (130 mM KCl, 10 mM NaCl, 1 mM $MgSO_4$, and 10 mM Na-MOPS) at different pH values (6.4, 7.0, 7.2, 7.4, and 7.8) at 39 ^{0}C for 40 min. After incubation, sperm were centrifuged at 1000 g for 5 min, and the pellet was resuspended in the same media and incubated at 39 ^{0}C for 10 min. The fluorescence intensity was measured as described above.

IV.3 Results

Sperm motility characteristics under different levels of pyruvate

To examine pyruvate concentration supporting sperm motility, sperm were incubated with 0, 0.5, and 1 mM for 40 min, followed by SMAS analysis. There were no significant effects of 0 – 1 mM pyruvate addition on motile percentage (85.3% - 86.0%), LIN (0.2), and BCF (4.2 – 6.1 Hz) (Table 3). However, other parameters were significantly increased by the addition of 0.5 or 1 mM pyruvate (VSL, 24.1 or 22.2 µm/s; VCL, 139.4 or 97.3 µm/s; VAP, 40.1 or 33.3 µm/s; STR of 0.5 mM pyruvate, 0.7; and ALH of 0.5 mM pyruvate, 2.2) ($P < 0.05$). Higher velocities properties, such as VSL, VCL, and VAP were detected at 0.5 mM pyruvate supplementation, suggesting pyruvate

plays an important role as a metabolic substrate and the level could be optimum in supporting flagellar motility in chicken sperm.

Effects of different concentration of 3-MCPD on sperm motility characteristics

To see the effects of pyruvate on sperm motility, sperm motility characteristics were measured using SMAS analysis after incubation with 0 – 1 mM 3-MCPD for 40 min. The characteristics of 3-MCPD-free sperm were as follow: motility, 89.0%; VSL, 25.7 μm/s; VCL, 105.5 μm/s; VAP, 40.0 μm/s; LIN, 0.3; STR, 0.7; ALH, 1.5 μm, and BCF, 8.3 Hz (Table 4). The use of 3-MCPD in dose-dependent manner significantly decreased motility parameters as follow: motility, (0.1 mM, 79.2%; 0.5 mM, 82.4%; 1 mM, 78.5%), VSL, (0.1 mM, 19.0 μm/s; 0.5 mM, 9.4 μm/s; 1 mM, 8.8 μm/s), VCL, (0.1 mM, 71.5 μm/s; 0.5 mM, 44.2 μm/s; 1 mM, 41.4 μm/s), VAP, (0.1 mM, 29.8 μm/s; 0.5 mM, 17.7 μm/s; 1 mM, 18.3 μm/s), and ALH, (0.1 mM, 1.1 μm; 0.5 mM, 0.8 μm; 1 mM, 0.8 μm). There were no differences between 0.5 and 1 mM on all parameters, suggesting the potent inhibition on glycolysis.

Roles of glycolysis and oxidative phosphorylation on sperm motility

To determine the roles of glycolysis and oxidative phosphorylation in chicken sperm motility, sperm were incubated with metabolic substrates (in the presence or absence of of 20 mM glucose and 0.5 mM pyruvate, hereinafter namely as Glu+ Pyr+, Glu+Pyr-, Glu-Pyr+, and Glu-Pyr-) under presence or absence glycolysis and oxidative phosphorylation inhibitors (0.5 mM 3-MCPD and 10 μM antimycin A (AA)) for 40 min, followed by SMAS analysis. There were no differences in the group with or without metabolic substrates addition on LIN (0.2 – 0.3), STR (0.6-0.7) and BCF (5.9 – 6.1 Hz) (Fig. 9). However, the addition of metabolic substrates significantly increased on other parameters, such as motile percentage from 84.2% in Glu-Pyr- group to 91.5% in

Glu+Pyr-, 88.9% in Glu-Pyr+, and 92.7% in Glu+Pyr+; VSL from 11.6 µm/s (Glu-Pyr-) to 24.7 µm/s (Glu+Pyr-), 21.1 µm/s (Glu-Pyr+), and to 25.0 µm/s (Glu+Pyr+); VCL from 59.1 µm/s (Glu-Pyr-) to 114.3 µm/s (Glu+Pyr-), 87.7 µm/s (Glu-Pyr+), and to 102.7 µm/s (Glu+Pyr+); VAP from 19.9 µm/s (Glu-Pyr-) to 37.7 µm/s (Glu+Pyr-), 29.8 µm/s (Glu-Pyr+), and to 35.4 µm/s (Glu+Pyr+); and ALH from 1.1 µm (Glu-Pyr-) to 1.8 µm (Glu+Pyr-), 1.4 µm (Glu-Pyr+), and to 1.6 µm (Glu+Pyr+).

The supporting effects of glucose and pyruvate supplementation were decreased when 3-MCPD and AA were applied on all motility parameters, except on LIN, STR and BCF. The decreasing effects of inhibitors were found on motile percentage, VSL, VCL, VAP, and ALH. Percentage of motile sperm in Glu+Pyr+ group decreased to 86.9% (in the presence of 3-MCPD) and 80.8% (in the presence of AA) from 92.7% (control); in Glu+Pyr- decreased to 82% (3-MCPD) and 79.8% (AA) from 91.5% (control); in Glu-Pyr+ decreased to 86.1% (3-MCPD) and 68.9% (AA) from 88.9% (control); in Glu-Pyr- decreased to 81.3% (3-MCPD) and 72.4% (AA) from 84.2% (control). The dramatic decreasing was observed on velocities parameters. The VSL in Glu+Pyr+ group decreased to 11.5 µm/s (3-MCPD) and 8.9 µm/s (AA) from 25 µm/s (control); in Glu+Pyr- group decreased to 7.3 µm/s (3-MCPD) and 7.5 µm/s (AA) from 24.7 µm/s (control); in Glu-Pyr+ decreased to 9.2 µm/s (3-MCPD) and 4.8 µm/s (AA) from 21.1 µm/s (control); in Glu-Pyr- decreased to 7.2 µm/s (3-MCPD) and 4.7 µm/s (AA) from 11.6 µm/s (control). The VCL in Glu+Pyr+ group decreased to 38.9 µm/s (3-MCPD) and 34.6 µm/s (AA) from 102.7 µm/s (control); in Glu+Pyr- group decreased to 33.5 µm/s (3-MCPD) and 30.5 µm/s (AA) from 114.3 µm/s (control); in Glu-Pyr+ decreased to 36.0 µm/s (3-MCPD) and 22.4 µm/s (AA) from 87.7 µm/s (control); in Glu-Pyr- decreased to 30.9 µm/s (3-MCPD) and 20.8 µm/s (AA) from 59.1 µm/s (control). The VAP in

Glu+Pyr+ group decreased to 19.6 µm/s (3-MCPD) and 14.3 µm/s (AA) from 35.4 µm/s (control); in Glu+Pyr- group decreased to 13.2 µm/s (3-MCPD) and 11.4 µm/s (AA) from 37.7 µm/s (control); in Glu-Pyr+ decreased to 17.3 µm/s (3-MCPD) and 5.3 µm/s (AA) from 29.8 µm/s (control); and in Glu-Pyr- decreased from 19.9 µm/s (control) to 12.8 µm/s (3-MCPD) and 5.0 µm/s (AA).

Despite the use of either 3-MCPD or AA increased BCF, but they decreased ALH. These both parameters are important for the bending tail movement of sperm flagellum since frequency pattern is highly dependent on amplitude pattern which characterize vigorous movement. Consistent with this, the progressive movement of sperm is characterized by a change in VSL and VAP, while vigorous movement of sperm is characterized by a change of VCL, ALH, and BCF (Kato *et al*. 2002). The ALH in Glu+Pyr+ group decreased to 0.6 µm (in both 3-MCPD and AA) from 1.6 (control); in Glu+Pyr- group decreased from 1.8 (control) to 0.6 µm (3-MCPD) and 0.5 µm (AA); in Glu-Pyr+ decreased to 0.6 µm (3-MCPD) and 0.3 µm (AA) from 1.4 (control); and in Glu-Pyr- decreased to 0.5 µm (3-MCPD) and 0.3 µm (AA) from 1.1 (control). Together, these results indicated that glycolysis and oxidative phosphorylation play roles in supporting flagellar motility in chicken sperm.

Roles of glycolysis and oxidative phosphorylation on IPVL-penetrating sperm

To examine the roles of glycolysis and oxidative phosphorylation on sperm penetrability, sperm were co-incubated with presence and absence of 20 mM glucose, 0.5 mM pyruvate, 0.5 mM 3-MCPD or 10 µM AA at 39 ^{0}C for 40 min, followed by incubating with ± 1 cm IPVL for 15 min at 39 ^{0}C. The sperm penetrability was assessed by counting the IPVL holes in the size of 0.29 mm^{2}. The addition of metabolic substrates, either glucose, pyruvate, or their combination were significantly increased the number of IPVL

holes (number of holes/0.29 mm^2); Glu+Pyr+ (99.8), Glu+Pyr- (104.0), Glu-Pyr+ (42.8) compared to the substrates-free group; Glu-Pyr- (32.6) (Fig. 10.). A significant difference was also found between glucose and pyruvate alone (Glu+Pyr- VS Glu-Pyr+). No significant difference was found between Glu+Pyr+ and Glu+Pyr-.

The increasing effect by these metabolic substrates was abolished when either 3-MCPD or AA was applied. Under 3-MCPD addition, the number of IPVL holes ranged from 29.8 to 35.6 holes/0.29 mm^2 in all metabolic substrates-added groups and it was significantly different from the substrates-free group (Glu-Pyr-, 12.6 holes/0.29 mm^2). The sperm penetrability was remarkable decreased when AA was applied. The number of IPVL holes under the presence of AA were 8.6 holes/0.29 mm^2 in Glu+Pyr+ group, 2.2 (Glu+Pyr-), 1.6 (Glu-Pyr+), and 2.0 (Glu-Pyr-). These results might indicate that oxidative phosphorylation primarily acts as a basic need of energy supply for sperm to penetrate the IPVL, while glycolysis acts as an energy booster to power up the penetrability. However, pyruvate alone did not support it suggesting complexity of energy source for IPVL penetration.

Roles of glycolysis and oxidative phosphorylation in ATP production

To determine the roles of glycolysis and oxidative phosphorylation in chicken sperm ATP production, intracellular ATP content was measured in sperm incubated with 0.5 mM 3-MCPD, or 10 µM AA, with or without 20 mM glucose or/and 0.5 mM pyruvate. The ATP content of sperm incubated with glucose, pyruvate, or their combination were 74% - 96% higher than that of sperm incubated without substrates addition (Fig. 11), suggesting glucose and pyruvate contribute to ATP production via glycolysis and respiration. No difference was observed among metabolic substrates-added groups. The ATP content was reduced when 3-MCPD or AA was applied. The decreasing ATP

content due to 3-MCPD utilization compared to control in each metabolic substrate group was as follow: decreased by 71.8% in Glu+Pyr+; 93.3% in Glu+Pyr-; 65.9% in Glu-Pyr+; and 72.0% in Glu-Pyr-. The use of AA dramatically reduced ATP content compared to control in each metabolic substrate group; the decreasing as follow: by 96.7% in Glu+Pyr+; 98.2% in Glu+Pyr-; 99.8% in Glu-Pyr+; and 99.6% in Glu-Pyr-, indicating that respiration is the primary pathway of ATP production in chicken sperm.

No detection of pentose phosphate pathway (PPP) in chicken sperm

Pentose phosphate pathway has been identified to localize in mammals to the post acrosomal region and tail and involved in sperm capacitation (Luna *et al*. 2016). However, expression and localization of PPP in chicken sperm has not been documented yet; therefore, this study was subjected to identify G6PD, a cytosolic enzyme involved in pentose phosphate pathway, using immunoblotting and indirect immune-fluorescence. The results showed that no G6PD detected at the predicted molecular weight (Fig. 12A). Although fluorescence signals were detected in subcellular compartment of the sperm (Fig. 12B), those cannot be considered as G6PD localization since no expression at the predicted molecular weight and could be nonspecific bindings shown in immunoblotting results.

Effects of glucose and pyruvate on reactive oxygen species (ROS)

To see the possible occurrence of ROS in chicken sperm metabolism, sperm were incubated with 55 μM TBHP, with or without 20 mM glucose, or 0.5 mM pyruvate. The result showed that TBHP, an exogenous inducer of oxidative stress, significantly induced ROS in chicken sperm (Fig. 13A). No significant differences were found in ROS when metabolic substrates added (Fig. 13B).

Roles of glucose and pyruvate on intracellular pH

To measure intracellular pH due to glucose and pyruvate supplementation in chicken sperm, sperm were incubated with the combination of 20 mM glucose and 0.5 mM pyruvate for 40 min in 39 ^{0}C in the presence of 5 µM BCECF-AM. After calibration to KCl media with different pH values containing 5 µM nigericin (Fig. 14A), significant differences of pHi between treatment groups were determined (Fig. 14B). The addition of metabolic substrates significantly decreased compared pHi to the metabolic substrates-free group. Glucose addition combined with pyruvate showed the lowest pHi. Considering the effects of metabolic substrates on sperm flagellar characteristics and sperm penetrability together with the increase of ATP production, these results reinforce that glycolysis and oxidative phosphorylation play important roles in motility and fertilization of chicken sperm.

IV.4 Discussion

Energy metabolism is a vital factor to support sperm functions. Activation of motility, protein modification such as phosphorylation, capacitation, and acrosome reaction, are sperm competencies that are highly dependent on the availability of energy in order to complete the task of fertilization. Sperm provide ATP as energy form via glycolysis and mitochondrial oxidative phosphorylation. Although the majority of ATP sources originated from oxidative phosphorylation, the primary ATP providers for sperm varies among species. For example, Miki *et al*. (2004) reported that glycolysis is the primary ATP provider for sperm motility in mice. They showed that *GAPDH*-knocked out mice lack progressive motility and produce only 10% of ATP compared to the wild type mice, although the mitochondrial condition between knock out and wild type mice is similar. We found that sperm functions in chicken sperm rely on ATP generated from

both glycolysis and oxidative phosphorylation. However, the roles of ATP providers on specific functions, such as sperm motility leading to successful egg penetration in chicken, remain unknown. Therefore, this study investigated the relative dependency of sperm motility and penetrability on ATP glycolysis and oxidative phosphorylation. Our results provide new insights on the cellular mechanism of energy metabolism in chicken sperm function and suggest the differences with mammalian.

It has been known that the generation of ATP relies on the availability of exogenous metabolic substrates. Glucose, a significant source for ATP production via glycolysis, is converted into pyruvate to generate more ATP in the respiration pathway. It has been reported that female reproductive tract fluid in chicken is enriched with pyruvate (0.1 – 0.3 mM) and high content of glucose (±20 mM) (Brady *et al*. 1978, Dupuy and Blesbois 1996), which might implicate sperm metabolism during transport in the female reproductive tract. Singh *et al*. (2011) demonstrated the addition of 1 mM pyruvate increased sperm metabolic in quail. Consistent with this, our SMAS analysis showed that the addition of glucose, pyruvate, or their combination support sperm motility. However, it remains unclear how these substrates generate ATP subsequently supporting sperm functions. 3-MCPD inhibits enzymes involved in glycolysis and reduces the motility of sperm (Jones 1983), while antimycin A (AA) inhibits mitochondrial electron transport chain complex III which can deplete mitochondrial ATP production (Ma *et al*. 2011). We found that treatment with 0.5 mM 3-MCPD and 10 µM AA abolished the increasing effect of either glucose or pyruvate, suggesting both glycolysis and oxidative phosphorylation play roles in providing energy for sperm motility.

The present study also investigated the effect of glucose and pyruvate supplementation on the ability of IPVL-penetrating sperm. The sperm penetrability was

slightly different in response to the addition of glucose and pyruvate. Although both glucose and pyruvate increase sperm ability, glucose supplementation is likely to more potent than pyruvate. Consistent with this result, the penetrating ability of human sperm is dependent on glucose (Jane Rogers and Perreault 1990). We have presumed that pyruvate is important for sperm penetrability by utilizing ATP respiration and that glucose support respiration by providing adequate pyruvate, which eventually generates respiration. These results were reinforced by the use of glycolysis (3-MCPD) and respiration (AA) inhibitors. The increasing effects of either glucose, pyruvate, or their combination supplementation on sperm penetrability was decreased by approximately 67% or 96.6% when glycolysis or respiration inhibitor, respectively, was applied. These results might imply that oxidative phosphorylation is the primary mechanism for ATP-dependent sperm penetrability, while glycolysis plays an important role in providing pyruvate for oxidative phosphorylation. These findings are consistent with previous studies reporting a strong correlation between mitochondrial ATP content and sperm progressivity in chicken sperm (Froman *et al*. 1999, Kamali Sangani *et al*. 2017).

Although mitochondrial oxidation produces high ATP content, previous studies have reported that mitochondrial oxidation is a significant source of ROS generation (Chen *et al*. 2003, Koppers *et al*. 2008). Under physiological conditions, sperm require ROS for regular functions, such as hyperactivated motility (O'Flaherty *et al*. 2006), capacitation and acrosome reaction via tyrosine phosphorylation (Aitken *et al*. 1995). However, under pathological conditions resulted from the excessive of ROS, ROS leads to oxidative stress that can cause DNA damage, extensive membrane damage via lipid peroxidation; eventually, impair sperm functions (Wagner *et al*. 2018). Other adverse effects of ROS are the depletion of intracellular ATP, axonemal damage, and increased

defects in the midpiece, thus decrease in sperm motility (Bansal and Bilaspuri 2011). Our study found that the addition of metabolic substrates, either glucose or pyruvate alone as well as their co-addition did not change the level of ROS. Considering the addition of glucose or pyruvate support sperm motility and penetrability, the level of ROS in this study might be in the physiological condition that supports sperm functions.

It is well accepted that glycolytic pathway is the first step in glucose metabolism to produce ATP, but pentose phosphate pathway (PPP), intermediate glycolysis has not been unequivocally elucidated in sperm. The main function of PPP is to generate NADPH which is required for reductive reactions (Urner and Sakkas 2005). A functional PPP has been demonstrated to play a role in capacitation of human sperm (Miraglia *et al*. 2010), in fertilization of mouse sperm (Urner and Sakkas 1999), and sperm motility of goat (Qiu *et al*. 2016). However, the presence of PPP was not observed in bull, dog, and ram spermatozoa (Scott *et al*. 1962). Our present study using a specific antibody against glucose 6-phosphate dehydrogenase (G6PD), a rate-limiting enzyme of PPP showed that PPP was not detected in chicken sperm. This is consistent with a previous study that pentose phosphate pathway does not appear to exist in chicken and turkey spermatozoa (Sexton 1974).

The present study showed that the addition of either glucose or pyruvate alone, or its combination decreased intracellular pH. Considering the addition of these metabolic substrates supporting sperm motility by increasing sperm velocities and ALH as well as sperm penetrability, these results suggest an important role of glucose and pyruvate in regulating pHi for enhancing flagellar movement to supports IPVL penetration. These findings are consistent with a previous study that showed glucose not only yield ATP but also is associated with intracellular pH, which eventually enhances flagellar beat of sperm

(Mannowetz *et al*. 2012). Studies in mammalian sperm have shown different roles of pHi on sperm functions. For example, the increasing of pHi mediates Ca^{2+} influx that alters flagellar bending patterns in hyperactivated motility associated with higher acrosome responsiveness of bovine sperm (Cross 2007, Marquez and Suarez 2007), and the acidic condition does not support capacitation and acrosome reaction of guinea pig sperm (Hyne and Garbers 1981). We found that the co-supplementation of glucose and pyruvate showed the lowest pHi level. This might suggest that the endogenous pyruvate produced from glycolysis is adequate to generate mitochondrial respiration, while the excess of pyruvate due to additional exogenous pyruvate might be converted into lactate, thus lowering pHi. The excess of pyruvate is converted into lactate when the capacity of mitochondrial respiration is exceeded (Hughes 2004).

In summary, the present study demonstrated that both glycolysis and oxidative phosphorylation provide energy for chicken sperm motility and penetrability. Furthermore, ATP generated from oxidative phosphorylation act as a primary energy for sperm, while ATP glycolysis act as an energy booster for sperm functions. These findings provide new insights into energy metabolism for chicken sperm functions and might provide the foundation to develop techniques to sustain sperm fertility.

Table 3. Motility characteristics of sperm incubated for 40 min under the presence of 0 – 1 mM pyruvate

	Pyruvate (mM)		
	Cntrl	0.5	1
Motility (%)	85.3 ± 0.82	84.8 ± 1.32	86.0 ± 1.80
VSL (μm/sec)	14.6 ± 0.92[a]	24.1 ± 1.91[b]	22.2 ± 0.10[b]
VCL (μm/sec)	78.7 ± 2.28[a]	139.4 ± 4.73[b]	97.3 ± 4.37[c]
VAP (μm/sec)	22.9 ± 1.20[a]	40.1 ± 2.89[b]	33.3 ± 0.57[b]
LIN (VSL/VCL)	0.2 ± 0.01	0.2 ± 0.01	0.2 ± 0.01
STR (VSL/VAP)	0.7 ± 0.01[ab]	0.7 ± 0.01[a]	0.7 ± 0.02[b]
ALH (μm)	1.4 ± 0.04[a]	2.2 ± 0.04[b]	1.6 ± 0.10[a]
BCF (Hz)	4.2 ± 0.31	5.5 ± 0.81	6.1 ± 0.24

Straight-line velocity (VSL, μm/sec), curvilinear velocity (VCL, μm/sec), average path velocity (VAP, μm/sec), linearity (LIN, VSL/VCL), straightness (STR, VSL/VAP), amplitude of lateral head displacement (ALH, μm), beat-cross frequency (BCF, Hz). Data are expressed as mean ± SEM (n=3). [a-b] *P < 0.05.*

Table 4. Changes in flagellar movement characteristics of sperm during incubation for 40 min under presence of 0 – 1 mM 3-MCPD

	3-MCPD (mM)			
	Cntrl	0.1	0.5	1
Motility (%)	89.0 ± 0.90[a]	79.2 ± 1.08[b]	82.4 ± 1.70[b]	78.5 ± 1.75[b]
VSL (μm/sec)	25.7 ± 1.25[a]	19.0 ± 1.62[b]	9.4 ± 0.65[c]	8.8 ± 0.59[c]
VCL (μm/sec)	105.5 ± 3.05[a]	71.5 ± 2.90[b]	44.2 ± 1.60[c]	41.4 ± 1.30[c]
VAP (μm/sec)	40.0 ± 1.35[a]	29.8 ± 1.82[b]	17.7 ± 0.90[c]	18.3 ± 0.80[c]
LIN (VSL/VCL)	0.3 ± 0.02	0.3 ± 0.03	0.2 ± 0.02	0.2 ± 0.02
STR (VSL/VAP)	0.7 ± 0.02	0.7 ± 0.02	0.6 ± 0.01	0.6 ± 0.05
ALH (μm)	1.5 ± 0.03[a]	1.1 ± 0.05[b]	0.8 ± 0.05[c]	0.8 ± 0.02[c]
BCF (Hz)	8.3 ± 0.50	9.5 ± 1.06	9.0 ± 0.45	11.6 ± 1.09

Straight-line velocity (VSL, μm/sec), curvilinear velocity (VCL, μm/sec), average path velocity (VAP, μm/sec), linearity (LIN, VSL/VCL), straightness (STR, VSL/VAP), amplitude of lateral head displacement (ALH, μm), beat-cross frequency (BCF, Hz).

Data are expressed as mean ± SEM (n=3). [a-c] $P < 0.05$.

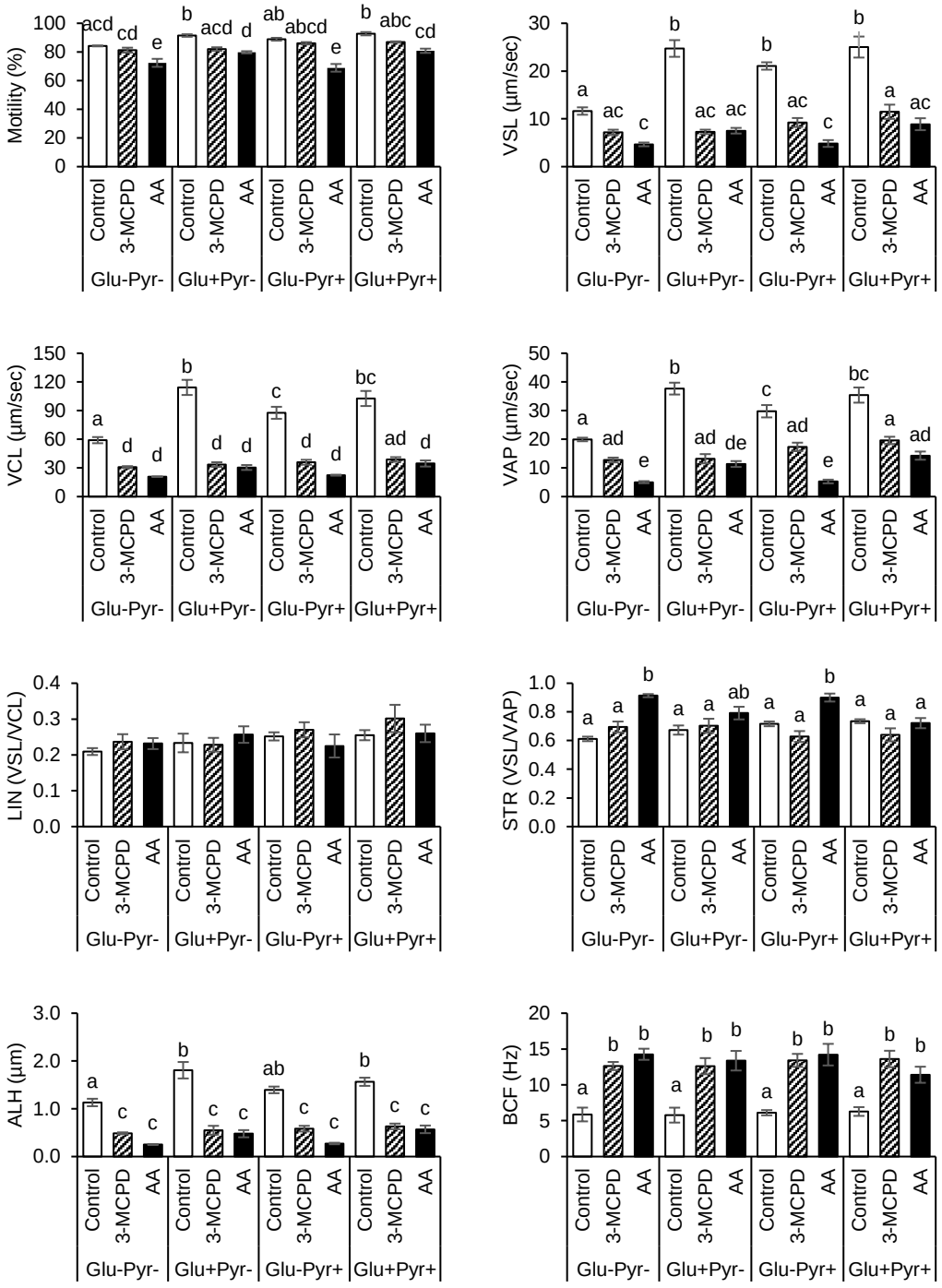
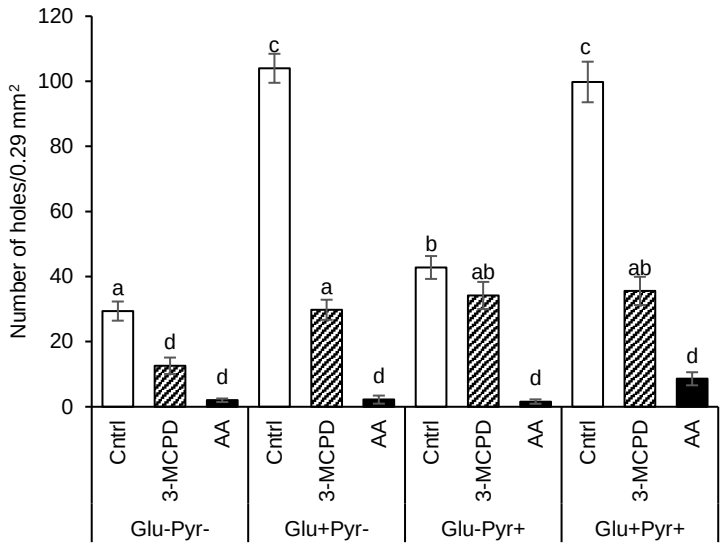


Fig. 9. Changes in sperm motility profiles in response to glycolysis and oxidative phosphorylation substrates and inhibitors.

Sperm were incubated with the combination of 20 mM glucose and 0.5 mM pyruvate under the presence of 0.5 mM 3-monochloro-1,2-

propanediol (3-MCPD) or 10 μM antimycin A (AA). The addition of either glucose, pyruvate, or their combination increased flagellar movement parameters compared to the group without metabolic substrates supplementation, although the glucose addition alone is better than that of pyruvate on VCL and VAP. However, the inhibition of respiration and glycolysis using AA and 3-MCPD decrease the parameters, particularly on velocities parameters (VSL, VCL, and VAP), ALH and increase BCF. Straight-line velocity (VSL, μm/sec), curvilinear velocity (VCL, μm/sec), average path velocity (VAP, μm/sec), linearity (LIN, VSL/VCL), straightness (STR, VSL/VAP), amplitude of lateral head displacement (ALH, μm), beat-cross frequency (BCF, Hz), the presence or absence of glucose (Glu+/-), the presence or absence of pyruvate (Pyr+/-). Data are expressed as mean ± SEM (n=6). $^{a\text{-}e}$ $P < 0.05$.

A



B

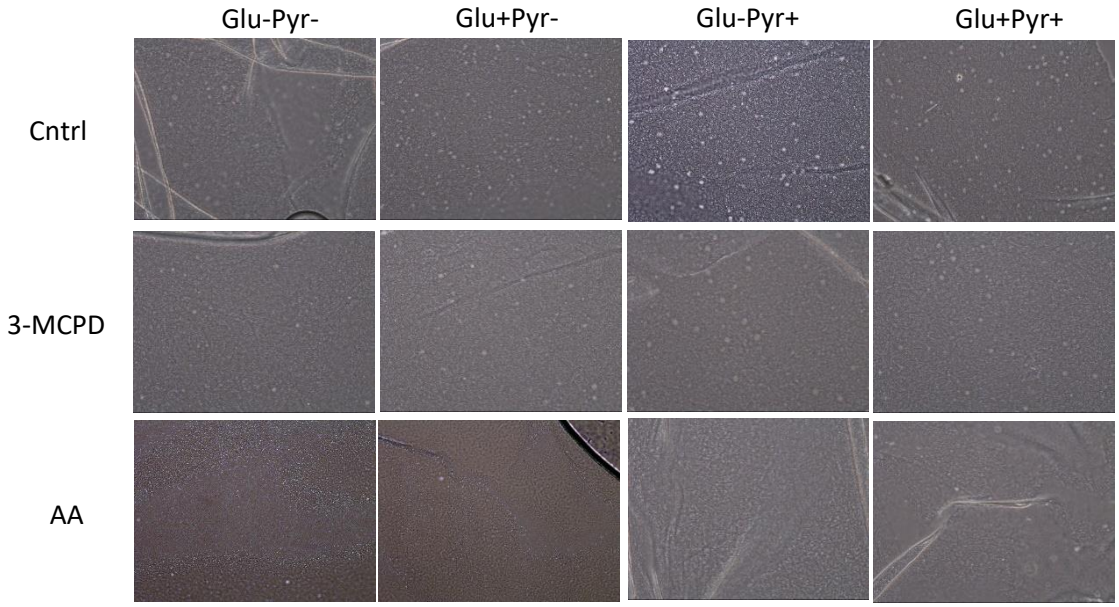


Fig. 10. Roles of glycolysis and oxidative phosphorylation on IPVL-sperm penetrability.

The addition of glucose together with pyruvate increase number of IPVL holes compared to group of sperm incubated without the addition of the substrate. However, the number of holes from group of sperm incubated with glucose alone

was higher than that of pyruvate alone and was equal to the group of glucose-pyruvate supplementation. However, 3-MCPD and AA decreased the number of holes, with AA showed a tremendous decreasing in all combination of glucose and pyruvate groups (A). Images of IPVL holes in response to metabolic substrates and inhibitors-added sperm (B). Data are expressed as mean ± SEM (n=5). $^{a\text{-}d}$ $P < 0.05$.

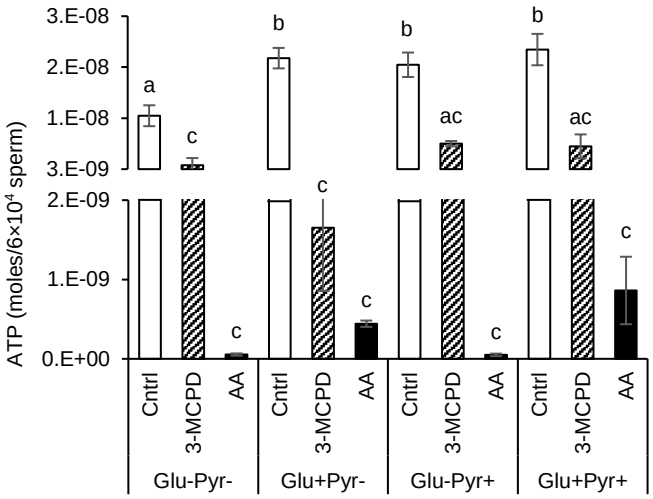


Fig. 11. Changes in ATP concentration in response to metabolic substrates supplementation and various inhibitors.

Sperm were incubated with metabolic substrates (20 mM glucose and 0.5 mM pyruvate) and in glycolysis and respiration inhibitors (0.5 mM 3-MCPD and 10 µM AA, respectively) for 40 min. ATP concentration was increased with metabolic substrates supplementation but was decreased when 3-MCPD and AA were added. AA dramatically reduced ATP concentration. Data are expressed as mean ± SEM (n=4). $^{a\text{-}c}P < 0.05$.

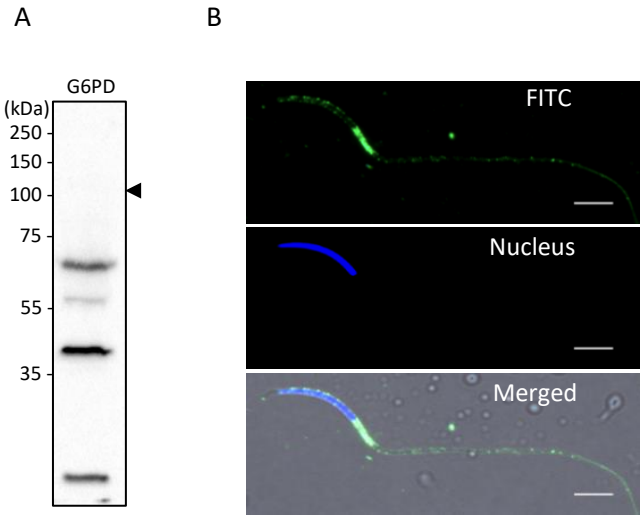


Fig. 12. Expression and localization of G6PD in chicken sperm.

Immunoblot using specific antibody against G6PD showed the absence of G6PD at the predicted molecular weight (at 108 kDa) (A). Signals detected in G6PD-immunostained sperm represented unspecific bindings (*green*) (B). Nuclei were fluorescence-labelled using DAPI (blue). Scale bars represent 5 µm (n=3).

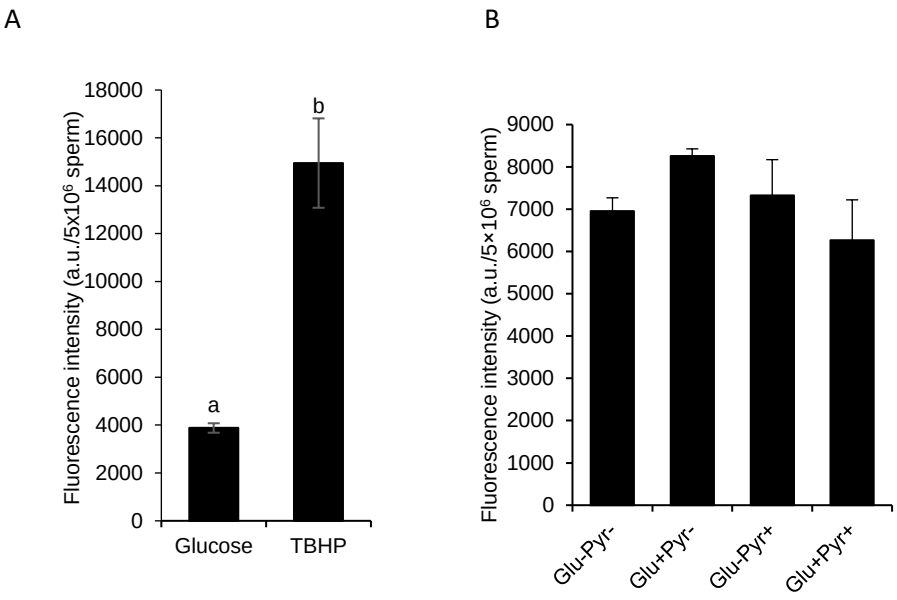


Fig. 13. Reactive oxygen species (ROS) levels of sperm incubated with the combination of metabolic substrates (glucose and pyruvate supplementation).

Sperm were incubated with 20 mM glucose, oxidizing agent (55 µM TBHP), and 0.5 mM pyruvate for 40 min. Sperm were subjected to ROS induction (A), and oxidative stress of sperm in metabolic substrates supplementation (B) were measured using Cellrox reagent. No significant different of oxidative stress in metabolic substrates-added sperm. Data are expressed as mean ± SEM (n=4). $^{a\text{-}b}$ $P < 0.05$.

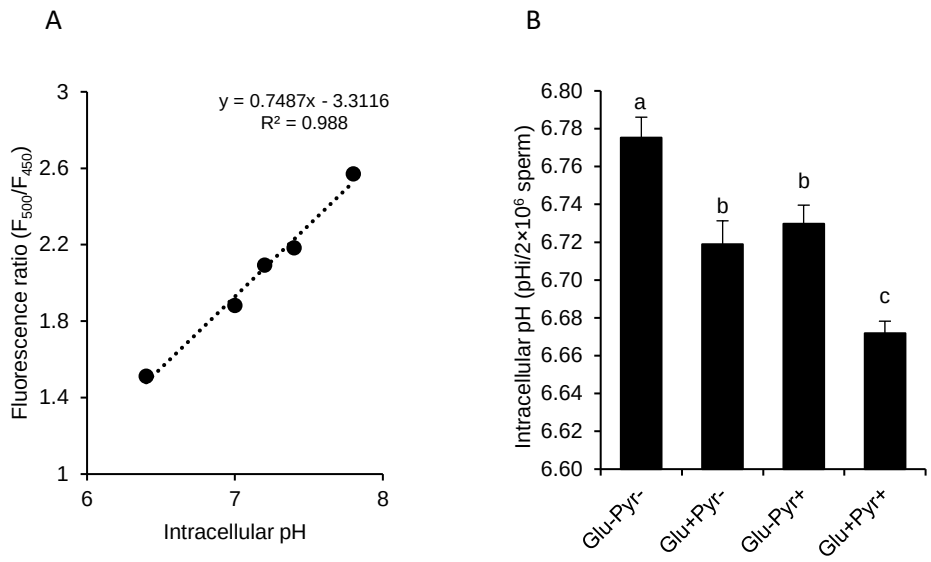


Fig. 14. Changes in intracellular pH in response to glucose and pyruvate supplementation.

Sperm were incubated in KCl medium with different pH values (6, 7.0, 7.2, 7.4, and 7.8) as calibration media (A). Intracellular pH was decreased when glucose and pyruvate were added (B). Data are expressed as mean as mean ± SEM (n=3). $^{a\text{-}c}P < 0.05$.

Chapter V

V.1 General Discussion

Sperm are formed in the seminiferous tubules of the testis by a complex process known as spermatogenesis. The process involves morphological transformation where round germ cells undergo division, differentiation and meiosis to form haploid cells, and elongated spermatids (Leahy and Gadella 2011). Once formed in the seminiferous tubules, immature sperm traverse through the testicles to reside in the epididymis. In the epididymis, they undergo a series of changes including maturation where membrane and nuclear remodelling such as insertion or/and extraction of various proteins, neutral and polar lipids occur that result sperm differentiation and acquisition of sperm motility (Van Tilburg *et al*. 2013). Moreover, spermatozoa density increases by shedding the excess of cytoplasm with the maturation process (Cui *et al*. 2016), and reside in the cauda epididymis in mammalian sperm until the moment of ejaculation.

Although cauda epididymis sperm are motile, they are unable to fertilize the egg. Sperm require to undergo a complex series of events in the female reproductive tracts that transform them from non-fertilizing to potentially fertilizing sperm, referred to as capacitation (Austin 1952). The capacitated sperm are then able to respond to the signals from the egg and undergo acrosome reaction, an exocytotic event leading to the enzymes that allow the sperm to penetrate the egg. Also, sperm flagellum undergo changes involving the alteration of the progressive movement patterns from low amplitude flagellar bending to hyperactivated movement with large amplitude flagellar bending. The advantages of the hyperactivation are to aid the sperm to move from the oviductal epithelium to fertilization site and allow sperm to bounce off rather than stick to the

epithelium (Demott and Suarez 1992). It also provides the motive thrust during penetration zona pellucida of the egg (Stauss *et al*. 1995).

To sustain viability and functions during migration to fertilization site, sperm are highly dependent on the availability of energy. The progressive movement of sperm as a result of thrust generated by flagellum is directly dependent on a continuous ATP supply and account for 70% of total ATP consumption (Bohnensack and Halangk 1986). The availability of ATP is also used in maintaining ion homeostasis. For example, the active transport of the excess of intracellular calcium $[Ca^{2+}]i$ across concentration gradient via plasma membrane calcium ATPase (PMCA) requires an amount of energy (Dragileva *et al*. 1999, Schuh *et al*. 2004).

Sperm are highly polarised and morphologically different from somatic cells. These differences lead to different cellular mechanisms and functions. For example, sperm head contains the paternal genetic and has to interact with the extracellular matrix of the egg, while sperm flagellum is responsible for providing cell movement. Since spermatozoa having less amount of cytosolic and transcriptionally inactive, their ability to synthesize proteins is limited (Travis *et al*. 2001). Also, during their travel in the female reproductive tract, they encounter different environmental conditions with regards to the availability of exogenous metabolic substrates and oxygen. Therefore, sperm are thought to have adaptive metabolic processes to overcome the limitations above depending on the condition in the female reproductive tract, metabolic substrate and oxygen content.

Sperm exhibit a considerable respiratory activity in the presence of oxygen that is usually associated with motility. A previous study reported that sperm are able to maintain a high rate of respiration in the range of oxygen concentration from that in air down to a critical oxygen concentration of 0.6% or equal to an O_2 partial pressure of 4 to 5 mmHg.

At this point, sperm reduce their respiratory rate and entirely cease below 1 mmHg O_2 partial pressure or correspond to 0.13% O_2 (Nevo 1965). Of note, external oxygen concentration (normal air) is 20% O_2 (corresponding to 150 mmHg), while 5% O_2 usually used in *in vitro* fertilization correspond to 38 mmHg, and the physiological oxygen concentration in female reproductive tract ranging from 2-8% O_2 or 11-60 mmHg (Fischer and Bavister 1993, Ng *et al*. 2018). Sperm, however, are still able to survive by maintaining metabolism anaerobically in the presence of glucose or other glycolyzable substrates (Mann 1946).

Several metabolic substrates have been reported to be enriched in female reproductive tract fluids, including glucose, lactate, and pyruvate. Uterine glucose, lactate, and pyruvate level in bovine ranging from 3.78-4.5 mM, 5.35-6.66 mM, and 0.09-0.12 mM, respectively (Hugentobler *et al*. 2008), while in mouse oviduct fluid the mean concentration of glucose, lactate, and pyruvate is approximately 3.4 mM, 4.79 mM, and 0.37 mM, respectively (Leese 1988). These metabolic substrates are likely to play important roles for sperm during migration up to the fertilization site in the female reproductive tract.

Glucose is the predominant substrate in female reproductive tract for generating ATP glycolysis of sperm. However, the effect of glucose on sperm is different from species to species. In mice, it stimulates capacitation-associated hyperactivated motility of sperm (Gardner and Leese 1990) but inhibits capacitation and successful fertilization in guinea pig and bovine sperm (Hyne and Edwards 1985, Parrish *et al*. 1989). In birds, the uterine fluid is enriched in glucose content by 20-40 mM depending on oviposition events (Dupuy and Blesbois 1996) and the oviduct contains 35 mmHg of O_2 partial press (Nevo 1965). These might also indicate the importance of glucose for sperm function.

Our recent study found that glucose supplementation increases flagellar movements characteristics. The increasing effect was found in the motile percentage, VSL, VCL, and VAP up to 80 min incubation, suggesting glucose supports sperm flagellar movements. Due to high polarity, glucose requires a specific transporter, known as glucose transporters (GLUTs) to enter the cell membrane along a concentration gradient.

Out of 14 GLUT isoforms, GLUT1 is the main GLUT isoform that is responsible in maintaining basal glucose uptake. This present study found that glucose transporter 1 (GLUT1) is specifically located on the midpiece of chicken sperm. The localization is different from that of mammalian sperm, which is located to the acrosomal region and principal piece (Angulo *et al*. 1998). The present results also provide new insights over GLUT expressions in chicken sperm, since our previous results found that GLUT3 are expressed and located to the acrosome region and entire flagellum (Ushiyama *et al*. 2019). As described in Chapter II, our immunohistochemistry results showed that GLUT1 together with other plasma membrane proteins, PMCA2 and PMCA4 were expressed and located to the edge of adluminal seminiferous tubules, suggesting these proteins are synthesized and embedded into the sperm plasma membrane at the late spermatogenic cells. Supporting with this, a previous study reported that numerous proteins are localized to specific sites during spermiation (Vogl *et al*. 2013).

Since many calcium channels in sperm, such as calmodulin-dependent protein kinase II (CaMKII), cation channel of sperm (catsper), and voltage-gated Ca^{2+} (Ca_V) (Darszon *et al*. 2011, Lishko and Mannowetz 2018), PMCA2 and PMCA4 are likely to have minor effects on calcium-associated sperm function in chicken. Therefore, chapter III of this study was focused on the functional of GLUT1 in sperm functions. Using fasentin, a specific inhibitor which specifically binds to the intramembrane channel of

GLUT1 (Wood *et al*. 2008), the function of GLUT1 in glucose uptake was abolished, leading to depleting sperm motility. The decreasing of glucose uptake and sperm motility were associated with changes in mitochondrial activity and ATP concentration. Using oxidative phosphorylation inhibitors antimycin A (AA) which blocks electron transport chain complex III (Ma *et al*. 2011) and mitochondrial uncoupler carbonyl cyanide m-chlorophenyl hydrazine (CCCP), the total ATP of sperm was decreased by 88.4% and 82.4%, respectively. The decreasing was accompanied with low mitochondrial activity. Thereby, reduced mitochondrial activity and ATP levels of GLUT1-inhibited sperm indicate that the energy supply required for sperm motility was impaired.

It is well known that glucose is the primary metabolic substrate for glycolysis. In mice, glycolysis-mediated glucose uptake support motility, even with inhibition of oxidative phosphorylation (Goodson *et al*. 2012). However, the localization and functional roles of glucose metabolic pathway are poorly determined in avian sperm. Our study localized two different glycolytic enzymes, hexokinase I and GAPDH at the midpiece and principal piece of chicken sperm, suggesting the glycolytic pathway takes part in energy metabolism along the entire of chicken sperm flagellum. Several studies found that glycolytic enzymes have sperm-specific isoforms and were attached to the fibrous sheath, a proteinous structure of the principal piece (Welch *et al*. 1992, Westhoff and Kamp 1997).

The roles of glycolysis and oxidative phosphorylation on specific sperm functions were further investigated in chapter IV. Glycolysis and oxidative phosphorylation were stimulated by the addition of glucose and pyruvate, respectively. The addition of either glucose or pyruvate alone, or their combination increased sperm motility characteristics, such as motile percentage, velocities, and amplitude of head lateral displacement (ALH)

compared to those of sperm incubated without metabolic substrates. The increasing effects were also found in their ATP content. Sperm incubated with metabolic substrates (either glucose or pyruvate alone, or its combination) have ATP content 74-96% higher than that of substrates-free sperm, suggesting these metabolic substrates are essential for energy metabolism of sperm. However, the increasing effects were abolished when either a glycolysis inhibitor 3-monochloro-1,2-propanediol (3-MCPD) or mitochondrial respiration antimycin A (AA) was applied.

The present study also investigated the ATP-generating pathways on sperm penetrability. Although both glucose and pyruvate significantly increased sperm penetrability, the addition of glucose dramatically increased sperm penetration. Considering glycolytic enzymes (HK1 and GAPDH) were localized in the midpiece and whole of sperm tail (Setiawan *et al*., accepted), the addition of glucose might generate ATP glycolysis which provides energy locally and enhances the flagellar movement in penetrating the IPVL. This finding was similar to a previous study that glucose support penetration ability of human sperm (Jane Rogers and Perreault 1990). To determine the relative dependency of sperm penetrability on ATP-generating pathways, we inhibited glycolysis and oxidative phosphorylation pathway using 3-MCPD and AA, respectively. Although the inhibition of glycolysis significantly decreased sperm penetrability, the inhibition of oxidative phosphorylation almost abolished the ability in all metabolic substrates-added sperm, suggesting ATP-generating respiration pathway is the primary ATP provider for sperm penetration, while ATP-generating glycolysis exists to power up the sperm function.

The addition of glucose as a metabolic substrate has been widely used in sperm cryopreservation in mammals. Glucose increase sperm motility during 6 h of post thawed

incubation (Ataur Rahman *et al*. 2018), long-term storage of chilled dog sperm (Ponglowhapan *et al*. 2004), and long-term liquid storage of goat sperm (Qiu *et al*. 2016). The latter study found that glucose metabolism generates not only glycolysis pathway but also pentose phosphate pathway (PPP), which reduces oxidative stress and provides glycolysis with more intermediate products such as fructose-6-phosphate. In frozen turkey sperm, glucose sustains better motility and vitality, although no further information on sperm fertility (Kuzlu and Taskin 2017). A previous study reported interesting findings that glucose does not inhibit cryo-preserved spermatozoa (Cormier and Bailey 2003), although it inhibits capacitation of fresh bull spermatozoa (Parrish *et al*. 1989). These facts showed the importance of glucose in semen extender for maintaining quality of sperm preservation.

In conclusion, our study is the first to demonstrates that chicken spermatozoa possess GLUT1 localized to the midpiece, which plays a role in ATP production and flagellar motility supported by glucose uptake. These results suggested the importance of glucose to support sperm motility and penetrability, thus may offer new insights into cellular mechanisms of energy metabolism for the improvement of assisted reproductive technologies in poultry.

Acknowledgements

Firstly, I would like to express my sincere gratitude to my advisor Dr. Atsushi Asano for the continuous encouragement and support of my PhD study and research. His valuable guidance helped me all the time of research. I would also like to thank Dr. Atsushi Tajima, Dr. Naoto Ishikawa, Dr. Hitoshi Miyazaki, and Dr. Taylor DeMar for giving encouragement and sharing insightful suggestions. They all have played a significant role in polishing my research writing skills.

I gratefully acknowledge the funding towards my PhD study from Indonesia Endowment Fund for Education (LPDP) so that I only focus on the studies without any significant obstacle on the way. I also wish to express my gratitude the Faculty of Animal Husbandry, Padjadjaran University, for supporting me to pursue this study.

I would always remember my fellow labmates, especially Ai Ushiyama and Chathura Priyadarshana as a source of friendship as well as useful advice and collaboration. They have provided me with their precious effort and time during my research pursuit.

At last but not least gratitude towards my family; my parents and sisters who remembered me in their prayers for the ultimate achievement. My deepest gratitude also goes to my supportive and loving wife, Indira. Her encouragement and willingness to provide management of our household activities during my study are much appreciated and duly noted.

References

Aitken RJ, Paterson M, Fisher H, Buckingham DW, Van Duin M. Redox regulation of tyrosine phosphorylation in human spermatozoa and its role in the control of human sperm function. *J Cell Sci* 1995;108:2017–2025.

Angulo C, Rauch MC, Droppelmann A, Reyes AM, Slebe JC, Delgado-López F, Guaiquil VH, Vera JC, Concha II. Hexose transporter expression and function in mammalian spermatozoa: Cellular localization and transport of hexoses and vitamin C. *J Cell Biochem* 1998;71(2):189–203.

Asano A, Selvaraj V, Buttke DE, Nelson JL, Green KM, Evans JE, Travis AJ. Biochemical characterization of membrane fractions in murine sperm: Identification of three distinct sub-types of membrane rafts. *J Cell Physiol* 2009;218(3):537–548.

Ataur Rahman M, Park SH, Yu IJ. Dog sperm cryopreservation in glucose-fructose or sucrose supplemented glycerol-free tris: Effect of post-thaw incubation time on gene expression related to apoptosis and motility. *Cryo-Letters* 2018;39(1):45–52.

Austin CR. The capacitation of the mammalian sperm. *Nature* 1952;170(4321):326.

Bakst MR. Physiology and endocrinology symposium: Role of the oviduct in maintaining sustained fertility in hens. *J Anim Sci* 2011;89(5):1323–1329.

Bakst MR, Wishart G, Brillard J-P. Oviducal sperm selection, transport, and storage in poultry. *Poult Sci Rev* 1994;5:117–143.

Ballester J, Fernández-Novell JM, Rutllant J, García-Rocha M, Palomo MJ, Mogas T, Peña A, Rigau T, Guinovart JJ, Rodríguez-Gil JE. Evidence for a functional glycogen metabolism in mature mammalian spermatozoa. *Mol Reprod Dev* 2000;56(2):207–219.

Bansal AK, Bilaspuri GS. Impacts of oxidative stress and antioxidants on semen functions.

Vet Med Int 2011;2011:1–7.

Bohnensack R, Halangk W. Control of respiration and of motility in ejaculated bull spermatozoa. *BBA - Bioenerg* 1986;850(1):72–79.

Bolton AE. Presence of the dehydrogenases of the pentose phosphate pathway in boar spermatozoa. *Reproduction* 1970;21(2):353–354.

Bouillot S, Reboud E, Huber P. Functional consequences of calcium influx promoted by bacterial pore-forming Toxins. *Toxins (Basel)* 2018;10(10):387.

Brady LJ, Romsos DR, Brady PS, Bergen WG, Leveille GA. The effects of fasting on body composition, glucose turnover, enzymes and metabolites in the chicken. *J Nutr* 1978;108:648–657.

Breitbart H. Role and regulation of intracellular calcium in acrosomal exocytosis. *J Reprod Immunol* 2002;53(1–2):151–159.

Brewis IA, Morton IE, Mohammad SN, Browes CE, Moore HD. Measurement of intracellular calcium concentration and plasma membrane potential in human spermatozoa using flow cytometry. *J Androl* 2000;21(2):238–249.

Brini M. Plasma membrane Ca^{2+}-ATPase: From a housekeeping function to a versatile signaling role. *Pflugers Arch Eur J Physiol* 2009;457(3):657–664.

Bucci D, Isani G, Spinaci M, Tamanini C, Mari G, Zambelli D, Galeati G. Comparative immunolocalization of GLUTs 1, 2, 3 and 5 in boar, stallion and dog spermatozoa. *Reprod Domest Anim* 2010;45(2):315–322.

Bucci D, Rodriguez-Gil JE, Vallorani C, Spinaci M, Galeati G, Tamanini C. GLUTs and mammalian sperm metabolism. *J Androl* 2011;32(4):348–355.

Bunch DO, Welch JE, Magyar PL, Eddy EM, O'Brien DA. Glyceraldehyde 3-phosphate dehydrogenase-S protein distribution during mouse spermatogenesis. *Biol Reprod*

1998;58(3):834–841.

Burant CF, Takeda J, Brot-Laroche E, Bell GI, Davidson NO. Fructose transporter in human spermatozoa and small intestine is GLUT5. *J Biol Chem* 1992;267(21):14523–14526.

Burrows WH, Quinn JP. A method of obtaining spermatozoa from the domestic fowl. *Poult Sci* 1935;14(4):251–254.

Carlson D, Black DL, Howe GR. Oviduct secretion in the cow. *J Reprod Fertil* 1970;22(3):549–552.

Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ. Production of reactive oxygen species by mitochondria: Central role of complex III. *J Biol Chem* 2003;278(38):36027–36031.

Cormier N, Bailey JL. A differential mechanism is involved during heparin- and cryopreservation-induced capacitation of bovine spermatozoa. *Biol Reprod* 2003;69(1):177–185.

Costello S, Michelangeli F, Nash K, Lefievre L, Morris J, Machado-Oliveira G, Barratt C, Kirkman-Brown J, Publicover S. Ca^{2+}-stores in sperm: Their identities and functions. *Reproduction* 2009;138(3):425–437.

Cross NL. Reorganization of lipid fafts during capacitation of human sperm. *Biol Reprod* 2004;71(4):1367–1373.

Cross NL. Effect of pH on the development of acrosomal responsiveness of human sperm. *Andrologia* 2007;39(2):55–59.

Cui Z, Sharma R, Agarwal A. Proteomic analysis of mature and immature ejaculated spermatozoa from fertile men. *Asian J Androl* 2016;18(5):735–746.

Darszon A, Nishigaki T, Beltran C, Treviño CL. Calcium channels in the development,

maturation, and function of spermatozoa. *Physiol Rev* 2011;91:1305–1355.

Demott RP, Suarez SS. Hyperactivated sperm progress in the mouse oviduct. *Biol Reprod* 1992;46(5):779–785.

Devaskar SU, Mueckler MM. The mammalian glucose transporters. *Pediatr Res* 1992;31(1):1–13.

Dias TR, Alves MG, Silva BM, Oliveira PF. Sperm glucose transport and metabolism in diabetic individuals. *Mol Cell Endocrinol* 2014;396(1):37–45.

Donoghue AM, Wishart GJ. Storage of poultry semen. *Anim Reprod Sci* 2000;62(1–3):213–232.

Dragileva E, Rubinstein S, Breitbart H. Intracellular Ca^{2+}-Mg^{2+}-ATPase regulates calcium influx and acrosomal exocytosis in bull and ram spermatozoa. *Biol Reprod* 1999;61(5):1226–1234.

Dravland E, Meizel S. Stimulation of hamster sperm capacitation and acrosome reaction in vitro by glucose and lactate and inhibition by the glycolytic inhibitor α-chlorohydrin. *Gamete Res* 1981;4(6):515–523.

Druart X. Sperm interaction with the female reproductive tract. *Reprod Domest Anim* 2012;47:348–352.

Dupuy V, Blesbois E. The effects of age on the composition of uterine fluid of broiler breeder hens and on maintenance of quality of fowl spermatozoa when stored in uterine fluid or in a synthetic medium. *Theriogenology* 1996;45(6):1221–1234.

van Eck JH, Vertommen M. Biochemical changes in blood and uterine fluid of fowl following experimental EDS'76 virus infection. *Vet Q* 1984;6(3):127–134.

FAO. World agriculture: towards 2015/2030 an FAO perspective. *Earthscan Publ Ltd* 2003;158–175.

Farber JL. The role of calcium ions in toxic cell injury. *Environ Health Perspect* 1990;84:107–111.

Farrel D. Poultry development Review - The role of poultry in human nutrition. *FAO* 2008;34.

Feiden S, Wolfrum U, Wegener G, Kamp G. Expression and compartmentalisation of the glycolytic enzymes GAPDH and pyruvate kinase in boar spermatogenesis. *Reprod Fertil Dev* 2008;20(6):713–723.

Ferramosca A, Provenzano SP, Coppola L, Zara V. Mitochondrial respiratory efficiency is positively correlated with human sperm motility. *Urology* 2012;79(4):809–814.

Fischer B, Bavister BD. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. *J Reprod Fertil* 1993;99(2):673–679.

Ford WCL. Glycolysis and sperm motility: Does a spoonful of sugar help the flagellum go round? *Hum Reprod Update* 2006;12(3):269–274.

Fraser LR, Quinnn PJ. A glycolytic product is obligatory for initiation of the sperm acrosome reaction and whiplash motility required for fertilization in the mouse. *J Reprod Fertil* 1981;61(1):25–35.

Froman DP, Feltmann AJ, Rhoads ML, Kirby JD. Sperm mobility: A primary determinant of fertility in the domestic fowl (*Gallus domesticus*). *Biol Reprod* 1999;61(2):400–405.

Fukuhara R, Nishikawa Y. Effects of various substrates on respiration, glycolysis and motility of goat spermatozoa. *Nihon Chikusan Gakkaiho* 1973;44(5):271–274.

Gallon F, Marchetti C, Jouy N, Marchetti P. The functionality of mitochondria differentiates human spermatozoa with high and low fertilizing capability. *Fertil Steril* 2006;86(5):1526–1530.

Gardner DK, Leese HJ. Concentrations of nutrients in mouse oviduct fluid and their effects on embryo development and metabolism in vitro. *J Reprod Fertil* 1990;88(1):361–368.

Goodson SG, Qiu Y, Sutton KA, Xie G, Jia W, O'Brien DA. Metabolic substrates exhibit differential effects on functional parameters of mouse sperm capacitation. *Biol Reprod* 2012;87(3):75.

Grigg GW, Rodge AJ. Electron microscopic studies of spermatozoa: I. The morphology of the spermatozoon of the common domestic fowl (*Gallus domesticus*). *Aust J Biol Sci* 1949;2(3):271–286.

Haber RS, Weinstein SP, O'Boyle E, Morgello S. Tissue distribution of the human GLUT3 glucose transporter. *Endocrinology* 1993;132(6):62538–62543.

Hahn KR, Jung HY, Yoo DY, Kim JW, Kim YH, Jo YK, Kim GA, Chung JY, Choi JH, Hwang IK, Jang G, Yoon YS. Immunohistochemical localization of glucose transporter 1 and 3 in the scrotal and abdominal testes of a dog. *Lab Anim Res* 2017;33(2):114–118.

Harris SE, Gopichandran N, Picton HM, Leese HJ, Orsi NM. Nutrient concentrations in murine follicular fluid and the female reproductive tract. *Theriogenology* 2005;64(4):992–1006.

Heilig CW, Saunders T, Brosius FC, Moley K, Heilig K, Baggs R, Guoll LR, Conner D. Glucose transporter-1-deficient mice exhibit impaired development and deformities that are similar to diabetic embryopathy. *Proc Natl Acad Sci U S A* 2003;100(26):15613–15618.

Henchion M, McCarthy M, Resconi VC, Troy D. Meat consumption: Trends and quality matters. *Meat Sci* 2014;98(3):561–568.

Hugentobler SA, Humpherson PG, Leese HJ, Sreenan JM, Morris DG. Energy substrates in bovine oviduct and uterine fluid and blood plasma during the oestrous cycle. *Mol Reprod Dev* 2008;75(3):496–503.

Hughes D. Lactate: What does it really tell us? *J Physiol* 2004;558(1):363–368.

Hyne R V., Edwards KP. Influence of 2-deoxy-D-glucose and energy substrates on guinea-pig sperm capacitation and acrosome reaction. *J Reprod Fertil* 1985;73(1):59–69.

Hyne R V., Garbers DL. Requirement of serum factors for capacitation and the acrosome reaction of guinea pig spermatozoa in buffered medium below pH 7.81. *Biol Reprod* 1981;24(2):257–266.

Jane Rogers B, Perreault SD. Importance of glycolysable substrates for in vitro capacitation of human spermatozoa. *Biol Reprod* 1990;43:1064–1069.

Jane Rogers B, Yanagimachi R. Retardation of guinea pig sperm acrosome reaction by glucose: The possible importance of pyruvate and lactate metabolism in capacitation and the acrosome reaction. *Biol Reprod* 1975;13(5):568–575.

Jones AR. Antifertility actions of α-chlorohydrin in the male. *Aust J Biol Sci* 1983;36(4):333–350.

Kamali Sangani A, Masoudi AA, Vaez Torshizi R. Association of mitochondrial function and sperm progressivity in slow- and fast-growing roosters. *Poult Sci* 2017;96(1):211–219.

Kato M, Makino S, Kimura H, Takao OTA, Furuhashi T, Nagamura Y, Hirano K. In vitro evaluation of acrosomal status and motility in rat epididymal spermatozoa treated with α-chlorohydrin for predicting their fertilizing capacity. *J Reprod Dev* 2002;48(5):461–468.

Kinet S, Swainson L, Lavanya M, Mongellaz C, Montel-Hagen A, Craveiro M, Manel N, Battini JL, Sitbon M, Taylor N. Isolated receptor binding domains of HTLV-1 and HTLV-2 envelopes bind GLUT-1 on activated CD4+ and CD8+ T cells. *Retrovirology* 2007;4:31.

Kokk K, Veräjänkorva E, Wu XK, Tapfer H, Põldoja E, Pöllänen P. Immunohistochemical detection of glucose transporters class I subfamily in the mouse, rat and human testis. *Medicina (Kaunas)* 2004;40(2):156–160.

Koppers AJ, De Iuliis GN, Finnie JM, McLaughlin EA, Aitken RJ. Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *J Clin Endocrinol Metab* 2008;93(8):3199–3207.

Krisfalusi M, Miki K, Magyar PL, O'Brien DA. Multiple glycolytic enzymes are tightly bound to the fibrous sheath of mouse spermatozoa. *Biol Reprod* 2006;75(2):270–278.

Kuzlu M, Taskin A. The effect of different extenders on the sperm motility and viability of frozen Turkey semen. *Indian J Anim Res* 2017;51(2):235–241.

De Lamirande E, Jiang H, Zini A, Kodama H, Gagnon C. Reactive oxygen species and sperm physiology. *Rev Reprod* 1997;2(1):48–54.

Leahy T, Gadella BM. Sperm surface changes and physiological consequences induced by sperm handling and storage. *Reproduction* 2011;142(6):759–778.

Leese HJ. The formation and function of oviduct fluid. *J Reprod Fertil* 1988;82(2):843–856.

Lishko P V., Mannowetz N. CatSper: a unique calcium channel of the sperm flagellum. *Curr Opin Physiol* 2018;2:109–113.

Luconi M, Krausz C, Forti G, Baldi E. Extracellular calcium negatively modulates

tyrosine phosphorylation and tyrosine kinase activity during capacitation of human spermatozoa. *Biol Reprod* 1996;55(1):207–216.

Luna C, Serrano E, Domingo J, Casao A, Pérez-Pé R, Cebrián-Pérez JA, Muiño-Blanco T. Expression, cellular localization, and involvement of the pentose phosphate pathway enzymes in the regulation of ram sperm capacitation. *Theriogenology* 2016;86(3):704–714.

Ma X, Jin M, Cai Y, Xia H, Long K, Liu J, Yu Q, Yuan J. Mitochondrial electron transport chain complex III is required for antimycin A to inhibit autophagy. *Chem Biol* 2011;18(11):1474–1481.

Manel N, Kim FJ, Kinet S, Taylor N, Sitbon M, Battini JL. The ubiquitous glucose transporter GLUT-1 is a receptor for HTLV. *Cell* 2003;115(4):449–459.

Mann T. Studies on the metabolism of semen; fructose as a normal constituent of seminal plasma; site of formation and function of fructose in semen. *Biochem J* 1946;44:97.

Mannowetz N, Wandernoth PM, Wennemuth G. Glucose is a pH-dependent motor for sperm beat frequency during early activation. *PLoS One* 2012;7(7):1–15.

Marchetti C. Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm. *Hum Reprod* 2002;17(5):1257–1265.

Marquez B, Suarez SS. Bovine sperm hyperactivation is promoted by alkaline-stimulated Ca^{2+} influx. *Biol Reprod* 2007;76(4):660–665.

McLean DJ, Jones LG, Froman DP. Reduced glucose transport in sperm from roosters (*Gallus domesticus*) with heritable subfertility. *Biol Reprod* 1997;57(4):791–795.

Miki K. Energy metabolism and sperm function. *Soc Reprod Fertil Suppl* 2007;65:305–325.

Miki K, Qu W, Goulding EH, Willis WD, Bunch DO, Strader LF, Perreault SD, Eddy EM, O'Brien DA. Glyceraldehyde 3-phosphate dehydrogenase-S, a sperm-specific glycolytic enzyme, is required for sperm motility and male fertility. *Proc Natl Acad Sci U S A* 2004;101(47):16501–16506.

Miraglia E, Lussiana C, Viarisio D, Racca C, Cipriani A, Gazzano E, Bosia A, Revelli A, Ghigo D. The pentose phosphate pathway plays an essential role in supporting human sperm capacitation. *Fertil Steril* 2010;93(7):2437–2440.

Mudalal S, Babini E, Cavani C, Petracci M. Quantity and functionality of protein fractions in chicken breast fillets affected by white striping. *Poult Sci* 2014;93(8):2108–2116.

Mujica A, Moreno-Rodriguez R, Naciff J, Neri L, Tash JS. Glucose regulation of guinea-pig sperm motility. *Reproduction* 1991;92(1):75–87.

Mukai C, Okuno M. Glycolysis plays a major role for adenosine triphosphate supplementation in mouse sperm flagellar movement. *Biol Reprod* 2004;71(2):540–547.

Nakamura N, Shibata H, O'Brien DA, Mori C, Eddy EM. Spermatogenic cell-specific type 1 hexokinase is the predominant hexokinase in sperm. *Mol Reprod Dev* 2008;75(4):632–640.

Nevo AC. Dependence of sperm motility and respiration on oxygen concentration. *J Reprod Fertil* 1965;9:103–107.

Ng KYB, Mingels R, Morgan H, Macklon N, Cheong Y. In vivo oxygen, temperature and pH dynamics in the female reproductive tract and their importance in human conception: A systematic review. *Hum Reprod Update* 2018;24(1):15–34.

Nguyen TMD, Alves S, Grasseau I, Métayer-Coustard S, Praud C, Froment P, Blesbois E. Central role of 5-AMP-activated protein kinase in chicken sperm functions. *Biol*

Reprod 2014;91(5):121.

Nichol R, Hunter RHF, Gardner DK, Leese HJ, Cooke GM. Concentrations of energy substrates in oviductal fluid and blood plasma of pigs during the peri-ovulatory period. *J Reprod Fertil* 1992;96(2):699–707.

O'Donnell L. Mechanisms of spermiogenesis and spermiation and how they are disturbed. *Spermatogenesis* 2014;4(2):e979623.

O'Flaherty C, de Lamirande E, Gagnon C. Positive role of reactive oxygen species in mammalian sperm capacitation: triggering and modulation of phosphorylation events. *Free Radic Biol Med* 2006;41(4):528–540.

Okunade GW, Miller ML, Pyne GJ, Sutliff RL, O'Connor KT, Neumann JC, Andringa A, Miller DA, Prasad V, Doetschman T, Paul RJ, Shull GE. Targeted ablation of plasma membrane Ca^{2+}-ATPase (PMCA) 1 and 4 indicates a major housekeeping function for PMCA1 and a critical role in hyperactivated sperm motility and male fertility for PMCA4. *J Biol Chem* 2004;279(32):33742–33750.

Parrish JJ, Susko-Parrish JL, First NL. Capacitation of bovine sperm by heparin: Inhibitory effect of glucose and role of intracellular pH. *Biol Reprod* 1989;41(4):683–699.

Du Plessis SS, Agarwal A, Halabi J, Tvrda E. Contemporary evidence on the physiological role of reactive oxygen species in human sperm function. *J Assist Reprod Genet* 2015;32(4):509–520.

Ponglowhapan S, Essén-Gustavsson B, Linde Forsberg C. Influence of glucose and fructose in the extender during long-term storage of chilled canine semen. *Theriogenology* 2004;62:1498–1517.

Post H, Schwarz A, Brandenburger T, Aumüller G, Wilhelm B. Arrangement of PMCA4

in bovine sperm membrane fractions. *Int J Androl* 2010;33(6):775–783.

Pym R. Poultry genetics and breeding in developing countries. *Food Agric Organ United Nations Poult Dev Rev* 2008;1–3.

Qiu JH, Li YW, Xie HL, Li Q, Dong HB, Sun MJ, Gao WQ, Tan JH. Effects of glucose metabolism pathways on sperm motility and oxidative status during long-term liquid storage of goat semen. *Theriogenology* 2016;86(3):839–849.

Reinhardt TA, Lippolis JD, Shull GE, Horst RL. Null mutation in the gene encoding plasma membrane Ca^{2+}-ATPase isoform 2 impairs calcium transport into milk. *J Biol Chem* 2004;279(41):42369–42373.

Ren D, Xia J. Calcium signaling through CatSper channels in mammalian fertilization. *Physiol* 2010;25(3):165–175.

Rigau T, Farré M, Ballester J, Mogas T, Pea A, Rodríguez-Gil JE. Effects of glucose and fructose on motility patterns of dog spermatozoa from fresh ejaculates. *Theriogenology* 2001;56(5):801–815.

Robertson L, Bailey JL, Buhr MM. Effects of cold shock and phospholipase A2 on intact boar spermatozoa and sperm head plasma membranes. *Mol Reprod Dev* 1990;26(2):143–149.

Ruiz-Pesini E, Diez C, Lapena AC, Perez-Martos A, Montoya J, Alvarez E, Arenas J, Lopez-Perez MJ. Correlation of sperm motility with mitochondrial enzymatic activities. *Clin Chem* 1998;44(8 part 1):1616–1620.

Sans P, Combris P. World meat consumption patterns: An overview of the last fifty years (1961-2011). *Meat Sci* 2015;109:106–111.

Saxena VK, Kolluri G. Selection methods in poultry breeding: From genetics to genomics. *Appl Genet Genomics Poult Sci* 2018;19–32.

Schroeder TC, Barkley AP, Schroeder KC. Income growth and international meat consumption. *J Int Food Agribus Mark* 1996;7(3):15–30.

Schuh K, Cartwright EJ, Jankevics E, Bundschu K, Liebermann J, Williams JC, Armesilla AL, Emerson M, Oceandy D, Knobeloch KP, Neyses L. Plasma membrane Ca^{2+} ATPase 4 is required for sperm motility and male fertility. *J Biol Chem* 2004;279(27):28220–28226.

Schurmann A, Koling S, Jacobs S, Saftig P, Krau S, Wennemuth G, Kluge R, Joost H-G. Reduced sperm count and normal fertility in male mice with targeted disruption of the ADP-ribosylation factor-like 4 (Arl4) gene. *Mol Cell Biol* 2002;22(8):2761–2768.

Scott TW, White IG, Annison EF. Glucose and acetate metabolism by ram, bull, dog and fowl spermatozoa. *Biochem J* 1962;83:398–404.

Sexton TJ. Oxidative and glycolytic activity of chicken and turkey spermatozoa. *Comp Biochem Physiol -- Part B Biochem* 1974;48(B):59–65.

Shadan S, James PS, Howes EA, Jones R. Cholesterol efflux alters lipid raft stability and distribution during capacitation of boar spermatozoa. *Biol Reprod* 2004;71(1):253–265.

Shen HM, Ong CN. Detection of oxidative DNA damage in human sperm and its association with sperm function and male infertility. *Free Radic Biol Med* 2000;28(4):529–536.

Simpson IA, Dwyer D, Malide D, Moley KH, Travis A, Vannucci SJ. The facilitative glucose transporter GLUT3: 20 Years of distinction. *Am J Physiol - Endocrinol Metab* 2008;265(2):E242-253.

Singh RP, Sastry KVH, Pandey NK, Shit N, Agrawal R, Singh KB, Mohan J, Saxena VK,

Moudgal RP. Characterization of lactate dehydrogenase enzyme in seminal plasma of Japanese quail (*Coturnix coturnix japonica*). *Theriogenology* 2011;75:555–562.

Sprando RL, Russell LD. Comparative study of cytoplasmic elimination in spermatids of selected mammalian species. *Am J Anat* 1987;178(1):72–80.

Stauss CR, Votta TJ, Suarez SS. Sperm motility hyperactivation facilitates penetration of the hamster zona pellucida. *Biol Reprod* 1995;53(6):1280–1285.

Storey BT. Mammalian sperm metabolism: Oxygen and sugar, friend and foe. *Int J Dev Biol* 2008;52(5–6):427–437.

Strehler EE, Zacharias DA. Role of alternative splicing in generating isoform diversity among plasma membrane calcium pumps. *Physiol Rev* 2001;81(1):21–50.

Sung TK, Moley KH. The expression of GLUT8, GLUT9a, and GLUT9b in the mouse testis and sperm. *Reprod Sci* 2007;14(5):445–455.

Thaler CD, Thomas M, Ramalie JR. Reorganization of mouse sperm lipid rafts by capacitation. *Mol Reprod Dev* 2006;73(12):1541–1549.

Thurston RJ, Hess RA. Ultrastructure of spermatozoa from domesticated birds: comparative study of turkey, chicken and guinea fowl. *Scanning Microsc* 1987;1(4):1829–1838.

Van Tilburg MF, Rodrigues MAM, Moreira RA, Moreno FB, Monteiro-Moreira ACO, Cândido MJD, Moura AA. Membrane-associated proteins of ejaculated sperm from Morada Nova rams. *Theriogenology* 2013;79(9):1247–1261.

Tourmente M, Villar-Moya P, Rial E, Roldan ERS. Differences in ATP generation via glycolysis and oxidative phosphorylation and relationships with sperm motility in mouse species. *J Biol Chem* 2015;290(33):20613–20626.

Travis AJ, Foster JA, Rosenbaum NA, Visconti PE, Gerton GL, Kopf GS, Moss SB.

Targeting of a germ cell-specific type 1 hexokinase lacking a porin-binding domain to the mitochondria as well as to the head and fibrous sheath of murine spermatozoa. *Mol Biol Cell* 1998;9(2):263–276.

Travis AJ, Jorgez CJ, Merdiushev T, Jones BH, Dess DM, Diaz-Cueto L, Storey BT, Kopf GS, Moss SB. Functional relationships between capacitation-dependent cell signaling and compartmentalized metabolic pathways in murine spermatozoa. *J Biol Chem* 2001;276(10):7630–7636.

Urner F, Sakkas D. A possible role for the pentose phosphate pathway of spermatozoa in gamete fusion in the mouse. *Biol Reprod* 1999;60(3):733–739.

Urner F, Sakkas D. Involvement of the pentose phosphate pathway and redox regulation in fertilization in the mouse. *Mol Reprod Dev* 2005;70(4):494–503.

Ushiyama A, Priyadarshana C, Setiawan R, Miyazaki H, Ishikawa N, Tajima A, Asano A. Membrane raft-mediated regulation of glucose signaling pathway leading to acrosome reaction in chicken sperm. *Biol Reprod* 2019;100(6):1482–1491.

Ushiyama A, Tajima A, Ishikawa N, Asano A. Characterization of the functions and proteomes associated with membrane rafts in chicken sperm. *PLoS One* 2017;12(11):e0186482.

Visconti PE, Bailey JL, Moore GD, Pan D, Olds-Clarke P, Kopf GS. Capacitation of mouse spermatozoa: I. Correlation between the capacitation state and protein tyrosine phosphorylation. *Development* 1995;121(4):1129–1137.

Vogl AW, Young JS, Du M. New insights into roles of tubulobulbar complexes in sperm release and turnover of blood-testis barrier. *Int Rev Cell Mol Biol* 2013;303:319–355.

Wagner H, Cheng JW, Ko EY. Role of reactive oxygen species in male infertility: An updated review of literature. *Arab J Urol* 2018;16(1):35–43.

Walker P, Rhubart-Berg P, McKenzie S, Kelling K, Lawrence RS. Public health implications of meat production and consumption. *Public Health Nutr* 2005;8(4):348–356.

Wang D, Chu PC, Yang CN, Yan R, Chuang YC, Kulp SK, Chen CS. Development of a novel class of glucose transporter inhibitors. *J Med Chem* 2012;55(8):3827–3836.

Welch JE, Schatte EC, O'Brien DA, Eddy EM. Expression of a glyceraldehyde 3-phosphate dehydrogenase gene specific to mouse spermatogenic cells. *Biol Reprod* 1992;46(5):869–878.

Wennemuth G, Babcock DF, Hille B. Calcium clearance mechanisms of mouse sperm. *J Gen Physiol* 2003;122(1):115–128.

Westhoff D, Kamp G. Glyceraldehyde 3-phosphate dehydrogenase is bound to the fibrous sheath of mammalian spermatozoa. *J Cell Sci* 1997;110(15):1821–1829.

Williams AC, Ford WCL. The role of glucose in supporting motility and capacitation in human spermatozoa. *J Androl* 2001;22(4):680–695.

Wishart GJ. Maintenance of ATP concentrations in and of fertilizing ability of fowl and turkey spermatozoa in vitro. *J Reprod Fertil* 1982;66(2):457–462.

Wishart GJ, Palmer FH. Correlation of the fertilising ability of semen from individual male fowls with sperm motility and ATP content. *Br Poult Sci* 1986;27(1):97–102.

Wood TE, Dalili S, Simpson CD, Hurren R, Mao X, Saiz FS, Gronda M, Eberhard Y, Minden MD, Bilan PJ, Klip A, Batey RA, Schimmer AD. A novel inhibitor of glucose uptake sensitizes cells to FAS-induced cell death. *Mol Cancer Ther* 2008;7(11):3546–3555.

Xi MD, Li P, Du H, Qiao XM, Liu ZG, Wei QW. Disaccharide combinations and the expression of enolase3 and plasma membrane Ca^{2+} ATPase isoform in sturgeon

sperm cryopreservation. *Reprod Domest Anim* 2018;53(2):472–483.

Yoshida K, Shiba K, Sakamoto A, Ikenaga J, Matsunaga S, Inaba K, Yoshida M. Ca^{2+} efflux via plasma membrane Ca^{2+}-ATPase mediates chemotaxis in ascidian sperm. *Sci Rep* 2018;8(1):16622.

Young SAM, Miyata H, Satouh Y, Aitken RJ, Baker MA, Ikawa M. CABYR is essential for fibrous sheath integrity and progressive motility in mouse spermatozoa. *J Cell Sci* 2016;129(23):4379–4387.

Yumura Y, Iwasaki A, Saito K, Ogawa T, Hirokawa M. Effect of reactive oxygen species in semen on the pregnancy of infertile couples. *Int J Urol* 2009;16(2):202–207.

Zhang J, Xiao P, Zhang X. Phosphatidylserine externalization in caveolae inhibits Ca^{2+} efflux through plasma membrane Ca^{2+}-ATPase in ECV304. *Cell Calcium* 2009;45(2):177–184.

Zhao Y, Buhr MM. Cryopreservation extenders affect calcium flux in bovine spermatozoa during a temperature challenge. *J Androl* 1995;16(3):278–285.