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Role(s) of Sperm Membrane Associated α-L-Fucosidase in Fertilization

By

Kamonrat Phopin

A Dissertation Presented to the Graduate and Research Committee

of Lehigh University

in Candidacy for the Degree of

Doctor of Philosophy

in

Molecular Biology

Lehigh University

January 12, 2012

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Approved and recommended for acceptance as a dissertation in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Kamonrat Phopin

Role(s) of sperm membrane associated α-L-fucosidase in fertilization

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Acknowledgements

First of all, I would like to extend special thanks to my research advisor, Dr. Barry Bean, for all of his support, time, guidance and understanding throughout my doctorate study at Lehigh University. I appreciate all of his contributions to make my Ph.D. experience productive and stimulating. I have been very fortunate to have him as my advisor. Thank you very much for what you have done for me.

I would like to extend my thanks to members of my dissertation committee: Dr. Linda Lowe-Krentz, Dr. Robert Skibbens, and Dr. Stephen Somkuti, for their time, support, and valuable suggestions. I also would like to thank Dr. Jack Alhadeff for providing alpha-L-fucosidase antibody and purified human liver fucosidase, Dr. Inmaculada Robina for 18a inhibitor, Dr. Jennifer Venditti for teaching me enzyme assay, Jen Golley for technical assistance in the animal facility, and Nitwara Wikan for statistical analysis.

Best regards to my colleagues and friends Mackenzie Bartlett, Elijah Douglass, Jaclyn Taroni, Nevada Heft, John Fong, and Quynh Ton. In addition, I would like to give my special thanks to my special friends, Pijit Jiemvarangkul and Wutigri Nimlamool. Thank you all for your assistances in experiments and friendship. With the help from all of you, I have been enjoying this work.

I have to give special thanks to the Royal Thai Government for the support and the scholarship to study at Lehigh University.

Most importantly, my deepest gratitude goes to my family in Thailand for their unflagging love, support, and understanding throughout my life, especially

my mother, Sudarat Phopin, my father, Montree Phopin and my lovely sisters, Nipapat Phopin and Natrada Phopin, and the rest of my extended family. This research would not have been possible without them. Thank you.

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List of Abbreviations

α – alpha

AR – acrosome reacted sperm

BrA23187 – Bromo ionophore A23187

BSA – bovine serum albumin

CEC – cauda epididymal contents

CRM – cumulus removal medium

DFJ – deoxyfuconojirimycin

ECL– enhanced chemiluminescence

EqS – sperm equatorial segment

ECM – extracellular matrix

hCG – human chorionic gonadrotropin

HSM – human sperm medium

IACUC – Institutional Animal Care and Use Committee

IVF – *in vitro* fertilization

Ki – dissociation constant for enzyme-inhibitor complex

4-MU – 4-methylumbelliferone

4-MU-Fuc – 4-methyl-umbelliferyl-α-L-fucopyranoside

GalNAc – N-acetyl-D-galactosamine

GlcNAc – N-acetyl-D-glucosamine

OECs – oviduct epithelial cells

PBS – phosphate buffered saline

PMSG – pregnant mare serum gonadotropin

PVDF – polyvinylidene fluoride

2-PN – 2-pronuclear

PVS – perivitelline space

RT – room temperature

TB – tight binding or tightly bound

ZP – zona pellucida

Abstract

Sperm associated alpha-L-fucosidases have been found to function in fertilization in numerous organisms. Here, we used a mouse model for studying the role of alpha-L-fucosidase during fertilization and early embryo development. Firstly, we investigated the localization, distribution, crypticity, and stability of alpha-L-fucosidase in mouse cauda epididymal sperm and cauda fluid. Alpha-L-fucosidase activity was detected using the fluorogenic substrate 4-MU-FUC. Of the total α-L-fucosidase activity recovered in the cauda epididymal contents, 80% was found in the cell-free cauda fluid and about 10% was found in sperm cells. Interestingly, after capacitation, cell-associated enzyme activity increased by about three fold, consisting mainly of soluble enzyme along with residual cell-bound fucosidase activity within capacitated cells. Comparable increases in enzyme activity following permeabilization of sperm confirmed the presence of soluble cryptic α-L-fucosidase within the sperm structure. Moreover, membrane associated enzyme activity was still detectable in acrosome-reacted cells. Alpha-L-fucosidase activity of both cauda fluid and sperm at 37 ˚C, 5% CO_2, was relatively stable and detectable up to 72 h.

Secondly, α-L-fucosidase enzyme assay was performed on extracts of mouse oocytes and early embryos and the existence of α-L-fucosidase glycoprotein was studied using western analysis. The studies showed that there is no detectable α-L-fucosidase enzyme activity or glycoprotein in mouse oocytes and blastocysts. On the other hand, ten-day embryos revealed the activity of α-L-

fucosidase. The presence of α-L-fucosidase in mouse sperm but not in mouse oocytes suggests that this enzyme might be delivered by sperm to eggs.

Thirdly, we investigated the role of sperm associated α-L-fucosidase in sperm-egg zona binding and membrane adhesion. Mouse sperm were pretreated with 5 mM DFJ or anti-fucosidase antibody. Another experiment was conducted by pretreating oocytes with purified human liver fucosidase. We found that 5 mM DFJ cannot inhibit sperm-egg zona or membrane binding. Interestingly, anti-fucosidase antibody and purified human liver fucosidase showed significant reduction of the number of sperm bound to oocytes. The binding results indicate that the fucosidase glycoprotein plays a role in sperm-egg zona and membrane binding steps; this interaction is primarily associated with fucosidase structural binding, and does not involve the catalytic site of α-L-fucosidase.

Fourthly, the role of sperm associated α-L-fucosidase during sperm-egg fusion and sperm penetration was evaluated using 5 mM DFJ, anti-fucosidase antibody, and purified human liver fucosidase. 5 mM DFJ did not reduce the number of sperm fused per egg, but again anti-fucosidase antibody and purified human liver fucosidase significantly decreased the number of sperm fused to oocytes. Therefore, α-L-fucosidase may play a role during sperm-egg membrane fusion. More probably, fusion and penetration by sperm is rate limited by the prior membrane recognition and tight binding steps that involve the fucosidase glycoprotein.

Finally, the role of sperm associated α-L-fucosidase in post-fusion events and/or early embryo development was examined by using intracytoplasmic

sperm injection and incubation of 2-pronuclear (2-PN) embryos with 5 mM DFJ. Intracytoplasmic sperm injection was conducted by injecting a single sperm that had been pretreated with 5 mM DFJ into each oocyte. No significant difference in 2-cell embryo development was observed in 5 mM DFJ pretreated sperm group compared to the untreated control. Another experiment was performed by culturing 2-pronuclear (2-PN) embryos in the presence of 5 mM DFJ. The results showed that 5 mM DFJ did not inhibit embryo development from 2-PN to blastocysts. Both experiments suggest that α-L-fucosidase does not play a role in post fusion events and/or early embryo development in mice. Taken together, the roles of α-L-fucosidase discovered in this work might contribute to the success of fertilization. A Lack of α-L-fucosidase might lead to infertility, so understanding the roles of this enzyme could lead to development of improved infertility treatments.

Chapter 1

Introduction

1.1 Fertilization and roles of carbohydrate in fertilization

Fertilization takes place in a series of discrete steps. The sperm interacts with the egg on five separate stages: firstly, the sperm interacts with the cumulus cells and the hyaluronic acid extracellular matrix (ECM) of the egg. Secondly, several sperm attach to the egg's zona pellucida (ZP). The binding of the sperm to specific ZP glycoproteins induces the sperm to undergo the acrosome reaction, the exocytosis of the acrosomal vesicle on the head of the sperm and then enzymes released from the acrosome allow the sperm to penetrate the ZP to gain access to the perivitelline space. Along with exocytosis of the acrosome, previously hidden membranous surfaces of the sperm are exposed. Thirdly, once a sperm penetrates the zona pellucida, it binds to and fuses with the plasma membrane of the oocyte. Fourthly, following fusion of the fertilizing sperm with the oocyte, the sperm head is incorporated into the egg cytoplasm. The nuclear envelope of the sperm disperses, and the chromatin rapidly loosens from its tightly packed state in a process called decondensation and remodeling of the sperm pronucleus. Fifthly, karyogamy, the paring and fusion of sperm and oocyte pronuclei occurs (Evans 1999, Alberts 2002).

The cell-cell recognition of fertilization requires carbohydrates as mediating molecules. There are numerous lines of evidence that support the roles of carbohydrate mediating fertilization. Several carbohydrates, including D-

mannose, mannan, D-galactose, N-acetyl-D-galactosamine (GalNAc), N-acetyl-D-glucosamine (GlcNAc), L-fucose, fucoidan, sialic acid, dextran sulfate, heparin, and heparan sulfate are involved in or influence sperm-zona binding (Tanghe et al. 2004a, Song et al. 2007). In addition, carbohydrate components of the sperm plasma membrane and/or oolemma may be involved in sperm-oocyte fusion. Several studies on sperm-oolemma interactions have shown the participation or modulation by D-mannose, D-galactose, GalNAc, GlcNAc, L-fucose, fucoidan, dextran sulfate, fibronectin, and vitronectin in human (Gabriele et al. 1998), bovine (Tanghe et al. 2004a), and hamster (Ponce et al. 1994) systems. Furthermore, GlcNAc and fucose are also involved in sperm binding to bovine oviduct epithelial cells (OECs) via sperm surface modification (Lefebvre et al. 1997, Kon et al. 2008).

1.2 L-fucose and roles of L-fucose in reproduction

L-fucose (6-deoxy-L-galactose) is a deoxyhexose that is a common component of many N- and O-linked glycans and glycolipids produced by mammalian cells. Fucose-containing glycans play significant roles in blood transfusion reactions, selectin mediated leukocyte-endothelial adhesion, host-microbe interactions, and numerous ontogenic events. Fucose frequently exists as a terminal modification of glycan structures, fucosylated glycans (Becker and Lowe 2003). Well-known fucosylated glycans include ABO blood group antigens.

Many reports have shown that L-fucose is a component of gametes and plays roles in gamete recognition. L-fucose it is incorporated into cumulus cells, perivitelline space (PVS), and zona pellucida (ZP) during oocyte maturation (Flechon et al. 2003). Fucose residues were detected in the zona pellucida and cortical granules of oocytes, using Aleuria aurantia lectin (AAA) as a probe (El-Mestrah and Kan 2002, Jiménez-Movilla et al. 2004). A recent study suggests that epitopes containing fucose in the Lewis-like structure are involved in the human gamete interaction (Focarelli et al. 2003). In 2004, Tanghe et al. showed that L-fucose decreased the penetration rate of sperm into oocytes in bovine in vitro fertilization (IVF) (Tanghe et al. 2004b). Moreover, the presence of L-fucose or fucoidan in the IVF droplets during insemination of zona free mouse oocytes decreased sperm egg fusion (Boldt et al. 1989). L-fucose has been also found to inhibit sperm binding to bovine oviductal explants (Lefebvre et al. 1997).

1.3 Mammalian alpha-L-fucosidases

α-L-fucosidase is an acidic glycosidase found in a variety of organisms including bacteria (Cobucci-Ponzano et al. 2005), ascidians (Matsumoto et al. 2002), mollusks (Focarelli et al. 1997), and mammals (Johnson and Alhadeff 1991). Mammalian α-L-fucosidases are lysosomal multimeric glycoproteins of approximately 53 kDa exhibiting optimal activity between pH values of 4 and 6.5 (Alhadeff et al. 1999). Mammalian α-L-fucosidases are involved in the hydrolytic degradation of fucose-containing molecules and their deficiency results in fucosidosis, the lethal accumulation of fucosylated glycosphingolipids and

glycoproteins in the central and peripheral nervous system (Michalski and Klein 1999).

The structural gene for α-L-fucosidase (FUCA1) has been assigned to human chromosome 1. It consists of eight exons dispersed over 23 kb of genomic DNA (Kretz et al. 1992). A second locus, FUCA2 is associated with inherited quantitative variation in enzyme activity in plasma, has been assigned to chromosome 6 (Ng et al. 1976). In addition, in humans there is an α-L-fucosidase pseudogene on chromosome 2. This pseudogene is 80% identical to fucosidase cDNA but it does not contain introns and is thought to have no protein coding potential (Carritt and Welch 1987, Kretz et al. 1992). Homologous mutation of the FUCA1 gene results in fucosidosis in humans (Tiberio et al. 1995, Willems et al. 1999, Turkia et al. 2008). Fucosidosis has also been found in English Springer Spaniels, however; homozygous mutation of the homologous gene results in a viable condition with male infertility and impaired sperm maturation (Veeramachaneni et al. 1998). For mice, the genes of the fucosidase family include Fuca1 and Fuca2. Fuca1 has been located on chromosome 4 and consists of eight exons dispersed over 19.56 kb of genomic DNA (NCBI database). Another locus, Fuca2 has been assigned to chromosome 10 (NCBI database). A knockout mouse for Fuca2 gene may be available; and might be useful for future studies of α-L-fucosidase.

1.4 Sperm associated alpha- L-fucosidases

Sperm associated α-L-fucosidases have been found in many species including rats (Hancock et al. 1993, Aviles et al. 1996, Abascal et al. 1998), *Drosophila* (Intra et al. 2009, Intra et al. 2006), ascidians (Matsumoto et al. 2002), *Unio elongatulus* (Focarelli et al. 1997), toads (Martínez et al. 2000), Bulls (Jauhiainen and Vanhaperttula 1986), fishes (Venditti et al. 2009), Syrian hamsters (Venditti et al. 2010) and humans (Alhadeff et al. 1999, Khunsook et al. 2002, Khunsook et al. 2003). Several studies showed evidence supporting roles of sperm associated α-L-fucosidases in reproductive processes. In the ascidian, the binding of sperm is mediated by a sperm α-L-fucosidase and complementary L-fucosyl residues of glycoproteins in the vitelline coat (Matsumoto et al. 2002). In *Drosophila*, immunofluorescence labeling of sperm showed that the enzyme is present in the sperm plasma membrane (acrosome and tail). Therefore, the enzyme appears to be involved in sperm-egg recognition by interacting with its glycoside ligands on the oocyte surface (Intra et al. 2006). Characterization of the sperm associated fucosidase of human has been a focus of our laboratory (Alhadeff et al. 1999, Khunsook et al. 2002, Khunsook et al. 2003, Venditti et al. 2007, Venditti and Bean 2009). By contrast with the acid hydrolase optima of the lysosomal isoforms, the human seminal plasma and sperm membrane associated isoforms show strong enzymatic activity broadly across the neutral pH range. The α-L-fucosidase is relatively enriched within the equatorial segment of human sperm (Venditti et al. 2007). Sperm-associated α-L-fucosidase is stable up to 72 hours, consistent with a possible role in sperm–egg interaction (Venditti

and Bean 2009). From our previous lab study, inhibiting α-L-fucosidase activity of capacitated hamster sperm does not inhibit tight binding (TB) of sperm to the zona pellucida (ZP). However, interestingly, they found that pre-treatment of capacitated hamster sperm with 5 mM DFJ inhibits development of oocytes to 2-cell embryos (Venditti et al. 2010).

Previous studies in this and other laboratories implicate the sperm associated fucosidase in fertilization and/or early embryo development. It is the primary goal of the investigation reported here to identify the specific timing of the sperm enzyme in these key reproductive events. For our experiments, Deoxyfuconojirimycin (DFJ) was used as a tool to study the role of α-L-fucosidase during fertilization and embryogenesis. DFJ, a non-hydrolysable molecule, has been previously shown to be a highly specific and high affinity competitive inhibitor of α-L-fucosidase (Winchester et al. 1990). This inhibitor is a powerful tool for these investigations. For instance, starting with freshly fertilized embryos, DFJ can be introduced at sequential time points in order to determine the temporal window during embryogenesis when sperm fucosidase function is essential.

1.5 Aims of Study

In particular, sperm associated α-L-fucosidase has been shown to be important in the events surrounding fertilization in many species. Fucose-containing molecules and the enzyme α-L-fucosidase are involved in numerous species. The mouse has been a key model organism as well as a versatile laboratory model for investigation of the cellular and molecular events surrounding fertilization, but there has been no systematic investigation of the roles of fucosidases in this species. The project was designed to provide an over view of fucosidases in mouse reproduction, and to assess the functional roles of sperm fucosidases in fertilization. Specifically, the objectives of this study were (1) to evaluate the presence of α-L-fucosidase in cauda fluid and mouse sperm cells, (2) to evaluate the subcellular distribution of α-L-fucosidase in mouse sperm, (3) to evaluate the stability of α-L-fucosidase in mouse sperm and fluid, (4) to measure α-L-fucosidase enzyme activity in extracts of mouse oocytes and early embryos, (5) to detect α-L-fucosidase glycoprotein in extracts of mouse oocytes and embryos, (6) to evaluate the role of sperm associated α-L-fucosidase in the binding of mouse sperm to the zona pellucida of the mouse oocyte, (7) to evaluate the role of sperm associated α-L-fucosidase in sperm-egg membrane to membrane adhesion, (8) to evaluate the role of sperm associated α-L-fucosidase in sperm-egg membrane fusion, and sperm penetration into the ooplasm, (9) to evaluate the role of sperm associated α-L-fucosidase in post-fusion events and early embryogenesis.

The presence of α-L-fucosidase in mouse cauda fluid and sperm and the subcellular distribution of α-L-fucosidase in mouse sperm were examined by

measuring α-L-fucosidase activity in mouse cauda fluid and sperm before and after capacitation with 1.5% BSA and acrosome reaction with 0.01 mM BrA23187 using the α-L-fucosidase specific fluorogenic substrate 4-MU-Fuc. Other than the enzyme distribution, α-L-fucosidase stability can inform us about its potential for delayed function during fertilization. In this research, the stability of α-L-fucosidase in mouse sperm and fluid was determined by conducting α-L-fucosidase assay using the fluorogenic substrate 4-MU-Fuc in mouse cauda fluid and sperm cells incubated at 37 ^{0}C under 5% CO_2, ambient air, or room temperature up to 72 h.

Knowing that the sperm contain fucosidase, it is important to know if the fucosidase might be a unique resource that is brought to the fertilization area, or if there is also fucosidase present in the oocytes themselves. In order to evaluate this possible uniqueness, we evaluated the presence of α-L-fucosidase enzyme activity and glycoprotein in extracts of mature mouse oocytes and early embryos using the α-L-fucosidase specific fluorogenic substrate 4-MU-Fuc and the western analysis, respectively.

Appreciating the absence of fucosidase in oocytes, we further evaluated the role of sperm associated α-L-fucosidase in sperm-egg zona binding, membrane adhesion, and fusion by a mouse *in vitro* fertilization system, which was conducted by using capacitated control, 5 mM DFJ, anti-fucosidase antibody pretreated sperm, or oocytes that had been pretreated with purified human liver α-L-fucosidase. To examine the wider range of sperm α-L-fucosidase during fertilization, we further investigated the roles of this enzyme in post fusion events

and early embryo development using intracytoplasmic sperm injection and incubation of 2-pronuclear (2-PN) embryos. Intracytoplasmic sperm injection was conducted by injecting a single untreated or DFJ pretreated sperm directly into a zona intact oocyte, followed by incubation at 37 ^{0}C under 5% CO_2 for 24 h. Incubation of 2-pronuclear (2-PN) embryos with DFJ was performed by incubating freshly harvested 2-PN embryos in culture medium with or without 5 mM DFJ at 37 ^{0}C under 5% CO_2 for 5 days. These experiments provided direct evidence of the functions of sperm associated α-L-fucosidase during fertilization in mice.

Chapter 2

Materials and Methods

Part I. Distribution of α-L-fucosidase in mouse sperm

2. 1 Animal housing

Institutional Animal Care and Use Committee (IACUC) protocol # 90, "Role(s) of sperm membrane associated α-L-fucosidase in fertilization and early embryogenesis" was approved. Adult (8 to 10 weeks) CF1 male and female mice were obtained from Charles River Laboratories (Wilmington, MA), housed individually and allowed free access to food and water. The animals were kept on a 14:10 light:dark cycle, with lights on at 8:00 AM and off at 10:00 PM, daylight saving time.

2. 2 Collection of cauda epididymal contents (CEC)

Male mice were sacrificed under CO_2 sedation and asphyxiation according to IACUC approved protocols. Fresh cauda epididymes were retrieved by post-mortem dissection, placed in an organ culture dish with 1 mL Human Sperm Medium (HSM) (Suarez et al. 1986), cut around 5 times, and incubated for 15 minutes at 37 ˚C, 5% CO_2 to release cauda fluid and sperm (CEC, cauda epididymal contents). CEC was centrifuged at 500 g at room temperature (RT) for 5 minutes (min) to pellet sperm cells. The first supernatant from this treatment was reserved and referred to as **CEC supernatant**. The sperm cells were

resuspended in 1 mL of HSM and centrifuged again. The resulting pellet was resuspended in HSM to a final cell concentration of 10–30 million sperm per mL, referred to as **uncapacitated sperm**.

2. 3 Mouse sperm capacitation for enzyme assay

CEC was collected as described above. After the second washing step of the uncapacitated cells, sperm cells were concentrated to 5X denser than their original suspension in CEC by resuspending the cells in a total volume of 200 µL of HSM. Capacitation was achieved by incubation of CEC in a microfuge tube containing 1.5% bovine serum albumin (BSA, Sigma A7906). The suspension was gently mixed and incubated 1-1.5 h at 37 ˚C, 5% CO_2. After incubation, cells were washed in 500 µL HSM by centrifugation for 5 min, 500 g, and the resulting pellet was resuspended to 10–30 million sperm per mL, this suspension is called **capacitated sperm**. Then, hyperactivation of sperm was observed and α-L-fucosidase activity was measured using 10 µL samples of uncapacitated, capacitated mixture, the capacitated supernatant, and the capacitated pellet following the assay protocol below.

2. 4 Induction of the acrosome reaction in mouse sperm for enzyme assay

After capacitation, sperm in HSM were supplemented with the bromo ionophore (BrA23187) (Molecular Probes, Eugene, OR) to give a final concentration of 0.01 mM ionophore, followed by incubation for 30 min at 37 ˚C,

5% CO_2 (Venditti et al. 2010). The entire **acrosome reacted mixture**, the **acrosome reacted supernatant**, and **the acrosome reacted cells** were separately assayed to detect the α-L-fucosidase distribution following the acrosome reaction.

2. 5 Enzyme assay for α-L-fucosidase and distribution of the enzyme in CEC, capacitated sperm and acrosome reacted sperm

CEC, cauda supernatant, uncapacitated, capacitated and acrosome reacted sperm were evaluated for α-L-fucosidase activity. Enzyme assays were performed using a Turner Biosystems TBS-380 fluorometer with miniature cuvettes and the fluorogenic substrate 4-methylumbelliferyl-α-L-fucopyranoside (4-MU-FUC, Biosynth). Each enzyme assay contained 40 μL (0.67 mM) of 4-methyl-umbelliferyl-α-L-fucopyranoside (4-MU-FUC), 10 μL HSM (in some experiments inhibitors were introduced within this portion), and 10 μL of the α-L-fucosidase source or control (Venditti et al. 2007). Enzyme samples were added at a 0 time point, and increasing fluorescence was measured at 10 or 20 min intervals, pH 7-7.4 during incubation at 22 ˚C for about an hour without addition of termination reagents. Enzyme activities were calculated from the slope of the fluorescence curve for each specimen. Comparison with fluorescence standards for known concentrations of 4-methyl-umbelliferone (4-MU, Sigma M1381) allowed calculation of nMoles of 4-MU product produced. Data are reported here as average α-L-fucosidase activity per 10^6 cells or mL of solution.

2. 6 Preparation of cauda fluid and sperm for stability assay

Six cauda epididymes harvested from three mice were placed in an organ culture dish with 1.5 mL HSM and cut around 5-10 times. Afterward, the dish was incubated at 15 min, 37 ˚C, 5% CO_2 to allow sperm to swim out. CEC was centrifuged at 500 g at RT for 5 min to pellet sperm cells. The supernatant from this treatment was referred as **cauda fluid**. The sperm cells were resuspended in 1.5 mL of HSM and centrifuged again to remove residual supernatant enzyme. The washing step was repeated twice before final resuspension of cells in 500 µL of HSM, these were referred to as **cauda sperm**. Cauda fluid was transfered into three microfuge tubes with 170 µL each and incubated under three conditions: (1) 37 ˚C, 5% CO_2, high humidity, (2) 37 ˚C, ambient air, and (3) room temperature (RT). The fluid was diluted 1:20 in HSM before performing an enzyme assay. Washed cauda sperm were divided into three microfuge tubes containing 150 µL each and incubated under the same three conditions as mentioned above. Sperm samples were permeabilized with 0.01% Triton X-100 and incubated 7 min at their initial incubation condition before performing enzyme assay. Throughout the time of the incubation, sterile, neutralized, distilled water pre-equilibrated to each incubation condition was added to each stock tube to replace water lost as a result of evaporation.

2. 7 Enzyme assay for α-L-fucosidase stability of cauda fluid and sperm

α-L-Fucosidase activity was quantified as described above. All assays were conducted in duplicate for 30 min at pH = 7.0. For stability studies, samples

of cauda fluid and sperm were taken at incubation times 0, 24, 48 and 72 h, and subjected to 30 min assays for α-L-fucosidase. In addition, the pH of all samples was measured after performing enzyme assay at 72 h. Average fluorescence at each discrete time-point was compared to t = 0 to determine % enzyme activity present. Data were plotted as time versus average % relative α-L-fucosidase activity.

2. 8 Inhibition of enzyme activity in CEC supernatant

CEC supernatant was used as an enzyme source for inhibition profiling based upon the CEC distribution results. Small aliquots of supernatant were pre-treated at various concentrations with deoxyfuconojirimycin (DFJ, Biomol) a potent competitive inhibitor (Winchester et al. 1990), and a novel pyrrolidine 3, 4-diol derivative (compound 18a) (Moreno-Clavijo et al. 2009) for 10 min before the enzyme assay. Enzyme activities under various inhibitor concentrations were compared to the activity of uninhibited controls to quantify the extent of inhibition.

Part II. Distribution of α-L-fucosidase in mouse oocytes

2. 9 Superovulation

To superovulate, female mice were administered 7.5 IU pregnant mare serum gonadotropin (PMSG, Sigma G4877) dissolved in 0.1 mL sterile water, followed by 0.1 mL of 7.5 IU hCG (human chorionic gonadotropin, Sigma C1063) dissolved in sterile water 48 h later. Both drugs were administered through

intraperitoneal injection using a 30 gauge needle. Oocytes were collected from oviducts about 16 h after hCG injection (Barraud-Lange *et al*. 2007).

2. 10 Collection of oocytes

Oocytes were harvested from superovulated female mice following post mortem surgery. The oviducts were removed from each animal and placed into a dish containing a 150 µL droplet of M2 medium (Sigma, M7167). The swollen portion of the oviducts were held with forceps in the droplet of medium and pricked with a fine gauge needle, allowing the cumulus mass to spill into the surrounding buffer. The cumulus masses were released into a 400 µL droplet of pre-equilibrated M2 containing 40 U/mL hyaluronidase (Sigma, 96F-8240). In addition, the droplet was gently pipetted up and down using a glass pipette to mechanically dislodge oocytes from the cumulus masses. Then, oocytes were washed 3 times by serial dilutions through 400 µL droplets of M2 (Kola and Sumarsono 1995).

2. 11 *In vitro* culture of blastocysts

Mouse embryo collection was obtained from 8-10 week female mice by superovulation induced by sequential (48 h apart) injection of 7.5 IU pregnant mare serum gonadotropin (PMSG) and 7.5 IU human chorionic gonadotropin (hCG). After hCG injection, females were caged with fertile males and examined for the presence of copulation plugs on the following morning. To obtain zygotes

at the blastocyst stage, embryos were recovered by flushing the uterus of females 96 h post hCG injection with M2 medium.

2. 12 Oocyte and blastocyst extraction for enzyme assay and western blot analysis

Oocytes or blastocysts were collected from superovulated female mice as described above. Extracts were prepared as follows. 50-600 mouse oocytes or blastocysts were sonicated in 40 μL of 0.01%TritonX-100/PBS containing 1 μL of protease inhibitors cocktail (Sigma, P2714). For 10 day embryos, around 5 embryos were harvested and homogenized in 40 μL using a Dounce homogenizer followed by sonication in 0.5% TritonX-100/PBS on ice. Protein concentrations were measured with the BioRad protein assay kit.

2. 13 Enzyme assay for alpha-L-fucosidase in mouse oocytes and early embryos

After preparing extracts, alpha-L-fucosidase activity of mouse oocytes and blastocysts was quantified using the fluorometric method, previously described in section 2.5, in a final volume of 60 μL (Venditti et al. 2007). Enzyme assays were continued for up to 90 min at room temperature without addition of termination reagents. Standard curves were generated using 4-methyl-umbelliferone (Sigma, M1381). Enzyme activity was measured as [nM] of 4-MU produced per minute per oocyte (or unit protein) at room temperature. Data were plotted as time (min) versus [nM] product.

2. 14 Western blot analysis

30 µL of extracted proteins from section 2.12 was denatured by heating to 100℃ for 10 min in 6 µL of 5X sample buffer (BioRad). SDS-PAGE was performed using a vertical gel apparatus (BioRad) with 5% stacking and 8% separating gels. Ten microliters of BioRad protein standard or 15 µL of each sample were loaded and subjected to electrophoresis for 1.5-2 h at 100 V. After separation, proteins were electrotransferred onto polyvinylidene fluoride (PVDF) membrane at 100 V for 2 h. Membranes were exposed to 5% skim milk powder in TBST (0.1% Tween) for 1 h. Primary polyclonal goat-anti-human liver alpha-L-fucosidase antibody was diluted 1:100 in 5% skim milk in TBST and incubated with shaking overnight at 4 ^{o}C. After that, the membrane was washed 3 times with TBST for 5 min and then incubated with 10 µg/mL of biotinylated rabbit anti-goat secondary antibody in 5% skim milk in TBST for 1 h. The blot was again washed 3 times with TBST for 5 minutes and then incubated with the ABC reagent (Pierce) for horseradish peroxidase detection prepared in 5% skim milk in TBST for 30 min. The blot was washed 3 times with TBST for 5 min and proteins were detected using the Enhanced Chemiluminescence (ECL) detection system following the manufacturer's instructions (BioRad) by exposure of Kodak film (Venditti et al. 2009, Worrad et al. 1994).

Part III. Role of mouse α-L-fucosidase in sperm-oocyte zona pellucida binding

2. 15 Mouse sperm collection for *in vitro* fertilization

Sperm were collected from the cauda epididymis following post mortem surgery under approved IACUC protocol #90. Two cauda epididymes were collected from one male, cut 5 times and placed in an organ culture dish with 500 μL of Whitten's medium. The contents were collected by gently squeezing the cauda epididymes with forceps and incubating them at 37 ^{o}C, 5% CO_2 for 10-15 min (to allow sperm to swim out). This sperm suspension is called the **cauda epididymal contents** (CEC).

2. 16 Mouse sperm capacitation for *in vitro* fertilization

CEC was collected as described above. Capacitation was achieved by incubation of 250 μL of CEC in an organ culture dish containing 250 μL of Whitten's medium supplemented with 10 mg/mL BSA. The suspension was gently mixed and incubated 2-3 h at 37℃, 5% CO_2.

2. 17 Preparation of Media and Droplets

Cumulus removal medium (CRM)

CRM was prepared by mixing 375 μL M2 medium and 25 μL of 640 U/mL hyaluronidase (Sigma, 96F-8240) to bring a final concentration of 40 U/mL hyaluronidase.

Oocyte culture medium

Oocyte culture medium was prepared by supplementing global medium (LifeGlobal, LGGG) with 4 mg/mL bovine serum albumin (BSA).

in vitro fertilization droplet

Each IVF droplet was prepared by pipetting 50 μL of oocyte culture medium (4 mg/mL BSA/global medium) into an organ culture dish. Then, the droplet was overlaid with 580 μL of sterile mineral oil (Sigma, M8410) and incubated at 37 ^oC with 5% CO_2 overnight prior use.

2. 18 *in vitro* fertilization

Ten oocytes were placed into an organ culture dish containing 50 μL of an IVF droplet prepared as described in 2.17. For sperm pretreatment, 50 μL of capacitated sperm were incubated 30 min with 5 mM DFJ or 1:25 polyclonal goat-anti-human liver alpha-L-fucosidase antibody (final concentration). 5 mM DFJ or 1:25 polyclonal goat-anti-human liver alpha-L-fucosidase antibody was also included in the IVF droplet for the test group. For controls, HSM or goat IgG was used instead of DFJ or polyclonal goat-anti-human liver alpha-L-fucosidase antibody. For some experiments, oocytes were pre-treated with PBS (control), 1:10 or 1:5 purified human liver alpha-L-fucosidase in the IVF droplet for 30 min at 37 ^oC with 5% CO_2 before insemination with capacitated sperm. Sperm motility was evaluated microscopically prior to insemination. To each IVF droplet, 2 μL of

untreated, DFJ or alpha-L-fucosidase antibody pretreated capacitated sperm was added into the IVF droplet (approximately 500 sperm per oocyte). Gametes were co-incubated for various lengths of time at 37℃, 5% CO_2, high humidity.

2. 19 Evalution of sperm-oocyte zona pellucida binding

10 oocytes were placed into an organ culture dish containing 50 μL of global medium (LifeGlobal) with 4 mg/mL BSA. For some experiments, oocytes were pre-treated with 1:10 purified fucosidase for 1 h prior to sperm insemination. Control, DFJ, or anti-fucosidase pre-treated capacitated mouse sperm were incubated with 3 μg/mL of Hoechst 33342 for 15 min before insemination into each droplet containing 10 oocytes. After 30 and 60 min gamete co-incubation, oocytes were placed into a fresh 100 μL drop of PBS and pipetted up and down three times using a glass pipette to dislodge sperm bound loosely to the zona pellucida. Oocytes were fixed by placing them into a 100 μL droplet of 4% paraformaldehyde for 10 min, washed 3 times in PBS, and transferred to a glass slide in a drop of mounting media (KPL). A coverslip, supported by a mixture of petroleum jelly and mineral oil (4:1) was gently placed on top of the drop to flatten the oocytes (Bean et al. 1991). Slides were observed using a Nikon Eclipse TE 2000-u fluorescence microscope (Nikon, Japan), and data were plotted as average number of sperm tightly bound to ZP per oocyte versus length of gamete co-incubation for each group. A two-tailed t-Test was performed to determine if a significant difference between the control and 5 mM DFJ, 1:25

anti-fucosidase, or 1:10 purified fucosidase groups existed (considered significant at p-value <0.05).

Part IV. Role of mouse α-L-fucosidase in sperm-oocyte plasma membrane interaction

2. 20 Removal of zona pellucida

After digesting cumulus masses, oocytes were transferred into a 400 μL of acid Tyrode's solution (Sigma, T1788) on a plastic dish. Digestion and dissolution of the zona pellucida were performed within 1-2 min with monitoring under the dissecting microscope to avoid under or over treatment. Then zona-free oocytes were washed three times by transferring oocytes into 400 μL fresh new drops of M2 to remove remnant acid Tyrode's solution.

2. 21 Evalution of sperm-oocyte plasma membrane binding

After removing the zona pellucida, 10 oocytes were placed into an organ culture dish containing 50 μL of global medium (LifeGlobal) with 4 mg/mL BSA. For some experiments, oocytes were incubated with 1:10 purified human liver fucosidase for 1 h prior to insemination. DFJ, anti-fucosidase pre-treated or untreated capacitated mouse sperm were incubated with 3 μg/mL of Hoechst 33342 for 15 min before insemination into each droplet containing 10 oocytes. Purified human liver fucosidase, DFJ or anti-fucosidase antibody remained in the mixtures throughout the experiments. After 30 and 60 min gamete co-incubation,

oocytes were placed into a fresh 100 μL drop of PBS and pipetted up and down three times using a glass pipette to dislodge sperm bound loosely to the egg plasma membrane. Oocytes were fixed by placing them into a 100 μL droplet of 4% paraformaldehyde for 10 min, washed 3 times in PBS, and transferred to a glass slide in a drop of mounting media (KPL). A coverslip, supported by a mixture of petroleum jelly and mineral oil (4:1) was gently placed on top of the drop to flatten the oocytes (Bean et al. 1991). Slides was observed using a Nikon Eclipse TE 2000-u fluorescence microscope (Nikon, Japan), and data were plotted as average number of sperm tightly bound to ZP per oocyte versus length of gamete co-incubatioon for each group. A two-tailed t-Test was performed to determine if a significant difference between the control and 5 mM DFJ, 1:25 anti-fucosidase, or 1:10 purified human liver fucosidase groups existed (considered significant at p-value <0.05).

Part V. Role of mouse α-L-fucosidase in sperm-oocyte plasma membrane penetration

2. 22 Evalution of sperm-oocyte plasma membrane penetration

The methods as described for Part II and III were used except the zona-free oocytes were preloaded with 3 μg/mL of Hoechst 33342 for 15 min. Then, the oocytes were washed 3 times in M2 medium before being placed into each IVF droplet. For some experiments, oocytes were pretreated with purified human liver fucosidase (1:5). Purified human liver fucosidase, DFJ or anti-fucosidase

antibody remained in the mixtures during insemination. After insemination with DFJ, anti-fucosidase pre-treated, or untreated sperm, the gametes were co-incubated for 4 h at 37 ℃, 5% CO_2. Following 4 h incubation, the zona-free oocytes were observed for the average number of sperm fused and/or penetrated per oocyte under a Nikon Eclipse TE 2000-u fluorescence microscope (Nikon, Japan). Data were plotted as the average number of sperm fused per oocyte versus duration of gamete co-incubation for each group. A two-tailed t-Test was performed to determine if a significant difference between the control and 5 mM DFJ, 1:25 anti-fucosidase antibody, or 1:5 purified fucosidase groups existed (significant at p-value <0.05).

Part VI. Role of sperm associated α-L-fucosidase in post-fusion events and/or embryogenesis

2. 23 Intracytoplasmic sperm injection assay

For each assay, several droplets were prepared in an organ culture dish. The first droplet (10 μL) contained the capacitated sperm suspension (control) or 5 mM DFJ pretreated (for 15 min) sperm in M2 medium. The second droplet (10 μL) contained 10% PVP in M2 medium. The remaining 5 droplets contained one oocyte per droplet of M2 medium. The first and second droplets were fused by using a small pipette tip to slow down the motility of sperm. These droplets were covered with pre-equilibrated, sterile mineral oil (overnight, at 37 ℃, 5% CO_2). The dish was placed on the stage of a Nikon Diaphot-TMB inverted microscope

equipped with a Narishige MM188 micromanipulator. A single spermatozoan was picked up, tail first, in an injection pipette (Origio, MIC-35-30) and positioned close to the pipette opening. The injection pipette was successively placed within each 10 μL drop containing an oocyte. The oocyte was held against the holding pipette (Origio, MPH-MED-30) using gentle suction, with the polar body oriented at 12 o'clock and the bevel of the injection pipette at 6 o'clock. The sperm was positioned near the pipette opening. The injection pipette was pushed steadily against the zona pellucida (ZP) until it pierced the ZP and was situated well within the oocyte. The sperm was then expelled into the ooplasm with a minimum amount of medium and the injection pipette was withdrawn from the oocyte. Then, oocytes were washed three times in M2 medium and they were cultured in 50 μL drops of 4mg/mL BSA/global medium under sterile mineral oil about 24 h. IVF drops were monitored to observe fertilization. Fertilization success was scored based upon the development of the embryo to the 2-cell stage.

2. 24 Post-fusion α-L-fucosidase association assay

in vivo collection of 2-pronuclear mouse embryos

Mouse embryos were collected from 8-10 week female mice by superovulation induced by sequential (48 h apart) injection of 7.5 IU pregnant mare serum gonadotropin (PMSG) and 7.5 IU human chorionic gonadotropin (hCG). After hCG injection, females were mated with fertile males and examined for the presence of copulation plugs on the following morning. To obtain zygotes

at the two-pronuclear (2-PN) stage, embryos were recovered by flushing the oviducts 17-20 h (2-PN) post hCG injection with M2 medium (Kaplan et al. 1995).

Evaluation of 2-PN embryo development

2-PN mouse embryos were harvested from mated female mice. After washing in droplets of M2 medium three times, embryos were transferred into a 50 μL drop of 4 mg/mL BSA/global medium (control) or a 50 μL drop of 4 mg/mL BSA/global medium with 5 mM DFJ in an organ culture dish. Each dish was placed into 5% CO_2, 37 ℃, 24 – 96 h. The development of the embryos t o the 2-cell stage and beyond was evaluated microscopically.

Chapter 3

Results

3. 1 Distribution of α-L-fucosidase in mouse cauda epididymal contents (CEC)

Based upon many reports, α-L-fucosidase has been found to be involved in fertilization in many species. Before studying α-L-fucosidase roles in mouse fertilization, it was appropriate to characterize the basic existence of the enzyme in mouse CEC and sperm. To evaluate the presence of α-L-fucosidase in cauda fluid and mouse sperm cells, α-L-fucosidase enzyme assay was conducted by using the α-L-fucosidase specific fluorogenic substrate 4-MU-Fuc. Data were collected as increasing fluorescence over time as summarized in Figure 1. Low speed centrifugation of CEC, removal of CEC supernatant, and resuspension of uncapacitated cells were done to determine α-L-fucosidase distribution within CEC suspensions. The large majority of enzyme activity was found in the CEC supernatant. Almost 80% of initial CEC enzyme activity was recovered in the supernatant, and after 2 washes about 10% of activity was recovered in uncapacitated sperm. Approximately 10% of the activity was lost. The total wet volume of cells corresponded to about 1/100 of the CEC, so these values reflect a relative enrichment of the enzyme in mouse sperm.

3. 2 Inhibition of enzyme activity in CEC supernatant

Given evidence that our assay for α-L-fucosidase revealed activity in sperm cells, it became appropriate to 1) confirm the specificity of the assay, and 2) characterize inhibitors that could be used in subsequent evaluation of the biological roles of the enzyme. The specific competitive inhibitors DFJ and 18a were used to confirm that product generation was indeed attributable to the existence of α-L-fucosidase in mouse CEC. The experiment also defined conditions for future inhibitor experiments. Mouse CEC supernatants were pre-treated with inhibitors 18a and DFJ at various concentrations prior to initiation of enzyme assays to determine efficacy of α-L-fucosidase inhibition. Figure 2 demonstrates enzyme activity inhibition with various concentrations of DFJ and 18a. Enzyme activity was reduced to 65% at 10 nM of 18a and continuously decreased to 55%, 20%, and 5% at 1 μM, 100 μM, and 1 mM, respectively. Nearly full inhibition (around 1% activity) was found at 10 mM and 20 mM of 18a. On the other hand, DFJ reduced the enzyme activity to 30% at 10 nM concentration and achieved full inhibition at 100 μM and higher concentrations.

3. 3 α-L-fucosidase activity in capacitated and acrosome reacted sperm

The detectable α-L-fucosidase in mouse sperm and fluid during capacitation and acrosome reaction supports the possibility that this enzyme is involved in fertilization including sperm-egg interaction. Hence, we further evaluated the subcellular distribution of α-L-fucosidase in mouse sperm after capacitation and acrosome reaction by measuring α-L-fucosidase activity in

mouse cauda fluid and sperm after capacitation with 1.5% BSA and treatment to induce acrosome reaction with 0.01 mM BrA23187. After capacitation with BSA, α-L-fucosidase activity was measured in the capacitated mixture, capacitated supernatant and capacitated cells, as summarized in Figure 3 and 4. α-L-fucosidase activity was measured at 2.4 units in uncapacitated cell, 6.27 units in capacitated mixture, 1.66 units in capacitated cells (Fig. 3). In capacitated supernatant, α-L-fucosidase activity was detected at 98 units (Fig. 4). After capacitation, the activity of α-L-fucosidase in the capacitated mixture increased around 2.6 times compared to uncapacitated cells. This increase of the enzyme activity in the capacitated mixture shows that the enzyme has been released to the soluble fraction. These findings are consistent with the significant decrease of fucosidase activity in the capacitated cells compared to the capacitated mixture. On the other hand, there is no statistically significant difference found for capacitated cells compared to uncapacitated cells.

The acrosome reaction was induced by treatment of capacitated sperm with 0.01 mM BrA23187. PNA-TRITC staining showed that the percentage of acrosome reacted sperm after treatment was about 60-70%. α-L-fucosidase activity in acrosome reacted mixture, supernatant and cells was measured. Figure 3 and 4 show the results of enzyme activity in acrosome reacted samples. 1.88 units of the enzyme activity was found in the acrosome reacted mixture and 0.8 units of the enzyme activity was recovered in acrosome reacted cells (Fig. 3). In the acrosome reacted supernatant, 22.34 units of the enzyme activity was

detected (Fig. 4). Acrosome reacted cells retained about 48% of the fucosidase activity that was present in the capacitated cell suspension, and the difference between these cell suspensions was significant. The difference between the acrosome reacted mixture and the acrosome reacted cellular component was also significant and reproducible, showing that some cell-associated fucosidase of capacitated cells is released to the soluble supernatant during incubation to induce acrosome reaction.

3. 4 Stability of cauda fluid and sperm α-L-fucosidase

Since mammalian fertilization can occur at a considerable time following ejaculation, persistence of enzyme function for later roles in reproduction was expected and predicted. To determine the stability of α-L-fucosidase in mouse sperm and fluid, we performed α-L-fucosidase assay using the fluorogenic substrate 4-MU-Fuc in mouse cauda fluid and sperm cells incubated at 37 ºC under 5% CO_2, ambient air, or room temperature up to 72 h. Cauda fluid and sperm cells were sampled for quantification of α-L-fucosidase activity at time = 0, 24, 48, and 72 h during 3 incubation conditions: 1) 37 ˚C, 5% CO_2, 2) 37 ˚C, ambient air, and 3) RT. Results documenting stability of the enzyme in cauda sperm under those conditions are presented in Figure 5. At 37 ˚C, 5% CO_2, 78%, 56%, and 31% of initial enzyme activity was detected in sperm cells after 24, 48, and 72 h of incubation, respectively. On the other hand, at 37 ˚C, ambient air, the activity decreased dramatically within 24 h and was undetectable after 24 h. Additionally, 52%, 16%, and 4% of initial enzyme activity was detected in cauda

sperm following 24, 48, and 72 h at RT, respectively. Figure 6 illustrates the results of the enzyme activity in cauda fluid at various conditions. At 37 ˚C, 5% CO_2, 84% and 81% of initial enzyme activity was detected in fluid after 24 and 48 h of incubation. The activity still remained at a high level, around 75%, at 72 h. However, at 37 ˚C, ambient air, the activity decreased dramatically with 24 h and was undetectable after 24 h incubation. Moreover, the enzyme activity was found around 61%, 45%, and 32% of original enzyme activity after 24, 48, and 72 h at RT, respectively. Based on these results, the enzyme was highly stable at 5% CO_2, 37 ˚C in both cauda fluid and sperm.

3. 5 Detection of α-L-fucosidase activity in mouse oocytes and embryos using fluorometric enzyme assays

To understand the roles of α-L-fucosidase in fertilization, not only is the distribution of the enzyme in mouse sperm important, but also the possible existence of the enzyme in mouse oocytes. In hamster, α-L-fucosidase was found to be undetectable in oocytes (Venditti et al. 2010). To measure whether α-L-fucosidase enzyme is detectable in oocytes and early embryos, we determined α-L-fucosidase activity in extracts of mouse oocytes and embryos using the α-L-fucosidase specific fluorogenic substrate 4-MU-Fuc. Oocytes or blastocysts were harvested from superovulated female mice by injecting PMSG and HCG as detailed in the section of materials and methods. After digesting cumulus masses, 50-600 oocytes or blastocysts were sonicated in 40 μL of 0.01% TritonX-100/PBS on ice. 10 day embryos (5 embryos) were harvested and

homogenized in 40 μL using a Dounce homogenizer followed by sonication in 0.5% TritonX-100/PBS on ice. An enzyme assay was performed using the fluorogenic substrate (4-MU-FUC). Enzyme assays were continued for 90 min. No detectable α-L-fucosidase activity was found in mouse oocytes from 6 independent experiments using 22, 27, 44, 48, 125, and 135 oocytes, respectively. In the same way, α-L-fucosidase activity was not found in blastocysts from 3 independent experiments using 15, 88, and 126 blastocysts, respectively. Conversely, we found α-L-fucosidase in the extract of 10 day embryos. At 30 min, the relative α-L-fucosidase activity per embryo present was 16.6, and at 90 min was 30.8. These results indicate that oocytes and blastocysts contain no detectable endogenous α-L-fucosidase but this enzyme was expressed after blastocysts stage.

3. 6 Detection of α-L-fucosidase glycoprotein in mouse oocytes and embryos using western analysis

Catalytic action of α-L-fucosidases could not be detected in mouse oocytes or blastocysts. To confirm and extend this observation, we undertook detection of α-L-fucosidase glycoprotein in mouse oocytes and embryos using western blot analysis. 600 oocytes or 600 blastocysts were extracted as described in section 3.5. 30 μL of each extract was loaded in 2 lanes (15 μL each) of 5% stacking and 8% separating gels; then subjected to electrophoresis for 1.5-2 h at 100 V. Following polyclonal goat-anti-human liver alpha-L-fucosidase antibody incubation, the results showed that no detectable α-L-

fucosidase glycoprotein was found in mouse oocytes and blastocysts as shown in Figure 7. Anti-α-tubulin antibody was used as an internal control. Mouse oocyte and blastocyst extracts showed α-tubulin band at 50 kDa (Fig. 7). Purified human liver α-L-fucosidase was used as a positive control (Mr. 51 and 56 kDa). The western blot results confirm the previous results that there was no α-L-fucosidase found in oocytes and blastocysts.

3. 7 Role of α-L-fucosidase in sperm-zona binding

Based on the results in section 3.3, the crypticity of α-L-fucosidase in mouse sperm during capacitation and acrosome reaction raised the possibility that this enzyme may be involved in sperm-egg zona binding. Here, we evaluated whether sperm associated α-L-fucosidase is involved in sperm-egg zona binding. Three methods were used to study roles of α-L-fucosidase in sperm-zona binding in mice. Firstly, 5 mM DFJ pretreated mouse sperm were inseminated into IVF droplets containing ten oocytes. Figure 8 presents average tightly bound (TB) sperm per oocyte of unpretreated (control) and DFJ pretreated sperm at 60 min incubation (37 ºC, 5% CO_2). The average TB sperm per oocyte were 27.35 (20 oocytes) for control group and 27.21 (23 oocytes) for pretreated sperm group. The images of sperm (labeled with Hoechst 33342) tightly bound to zona pellucida of oocytes in control and DFJ pretreated sperm were taken by Nikon eclipse TE 2000-u fluorescence microscope (Nikon, Japan) as shown in Figure 11A. The results show that there is no significant difference of sperm-zona binding between control and DFJ pretreated sperm.

Secondly, anti-fucosidase antibody was used to block the α-L-fucosidase of mouse sperm before insemination into IVF droplets containing freshly harvested oocytes. At 30, 60, and 120 min, the average TB sperm per oocyte were 12 (14 oocytes), 29.6 (14 oocytes), and 34.75 (16 oocytes) for the control group, respectively. By contrast, for the anti-fucosidase-pretreated sperm group, the corresponding numbers of TB sperm were 7 (22 oocytes), 5.5 (24 oocytes), and 12 (20 oocytes) (Fig. 9). Figure 11B shows the binding of sperm to zona pellucida of oocytes in IgG control and anti-fucosidase antibody groups. Based upon the results, anti-fucosidase-pretreated sperm show significant decrease in the ability to bind to zona pellucida of mouse oocytes around 41.7%, 81.4% and, 65.5% for 30, 60, and 120 min incubation, respectively.

Finally, mouse oocytes were pretreated with purified human liver α-L-fucosidase to block the target site of sperm associated α-L-fucosidase on oocyte prior to sperm insemination. As shown in Figure 10, the average TB sperm per oocyte were 31.04 (23 oocytes) for control group and 8.28 (29 oocytes) for purified α-L-fucosidase pretreated oocytes. 73.3% decrease of sperm bound to oocyte was found when oocytes had been treated with purified α-L-fucosidase. Microscopic images of sperm-zona binding in purified fucosidase pretreated oocytes illustrate the reduction of sperm numbers per oocyte (Fig. 11C). The zona binding results suggest that sperm associated α-L-fucosidase plays a role during sperm-oocyte zona binding.

3. 8 Role of α-L-fucosidase in sperm-oocyte membrane interaction

In previous results, we found that α-L-fucosidase activity was detected after acrosome reaction. This finding is consistent with immunofluorescence studies where α-L-fucosidase was predominantly localized in the equatorial segment suggesting the involvement of the enzyme during sperm-egg membrane interaction. To evaluate whether sperm associated α-L-fucosidase is required for sperm-egg membrane adhesion, DFJ, anti-fucosidase antibody, and purified α-L-fucosidase were used during membrane adhesion of sperm to eggs that had been stripped of their zona pellucidae. Mouse sperm that had been pretreated with 5 mM DFJ were inseminated in IVF droplets containing ten zona-free oocytes. Then, sperm-oocyte plasma membrane binding was observed at 15, 30 and 60 min as shown in Figure 12. The average TB sperm per oocyte were 47 (10 oocytes), 51 (11 oocytes), and 30 (13 oocytes) for control group, and 46 (22 oocytes), 48 (24 oocytes), and 22 (20 oocytes) for DFJ pretreated mouse sperm at 15, 30, and 60 min, respectively (Fig. 12). The images of sperm tightly bound to the membrane of oocytes in control and DFJ pretreated sperm were shown in Figure 15A. The results indicate that DFJ does not inhibit sperm-egg membrane interaction.

On the other hand, when sperm were pretreated by anti-fucosidase (Fig.13), the average TB sperm per oocyte were significantly decreased around 43% and 38.5% at 30 and 60 min compared to control. 3.5 TB sperm per oocyte (22 oocytes) at 30 min and 8.56 TB sperm per oocyte (27 oocytes) at 60 min

were found in anti-fucosidase pretreated sperm. 6.14 TB sprem per oocyte (22 oocytes) and 13.92 TB sperm per oocyte (24 oocytes) were observed from control group at 30 and 60 min, respectively (Fig. 13). Figure 15B shows the decrease of sperm number bound to oocyte plasma membrane in IgG control and anti-fucosidase antibody groups.

Purified α-L-fucosidase was used to pretreat zona-free oocytes prior to sperm insemination. The average TB sperm per oocyte were 32.86 (22 oocytes) for the control group and 10.83 (23 oocytes) for purified α-L-fucosidase pretreated oocytes (Fig. 14). Sperm-egg plasma membrane adhesion in purified fucosidase pretreated oocytes was reduced significantly compared to untreated group (Fig. 15C). Interestingly, 67% decrease of sperm bound to oocytes was found after treating oocytes by purified α-L-fucosidase at 60 min. These observations show that sperm associated α-L-fucosidase plays a role during sperm-oocyte membrane interaction.

3. 9 Role of α-L-fucosidase in sperm-oocyte membrane fusion and penetration

Based on findings that sperm associated α-L-fucosidase is mainly localized within the equatorial segment after acrosome reaction (Phopin et al. Submitted manuscript), it is possible that this enzyme may be involved in membrane fusion and/or sperm penetration within the oocyte. To evaluate the role of sperm associated α-L-fucosidase in sperm-egg membrane fusion, we

pretreated sperm with DFJ and anti-fucosidase antibody and zona-free oocytes with purified α-L-fucosidase. Sperm-egg fusion and penetration were observed after 4 h. Figure 16 shows average sperm fused per oocyte of unpretreated (control) and DFJ pretreated sperm at 60 and 240 min. The average sperm fused per oocyte were 2.79 (14 oocytes) and 3.13 (15 oocytes) for control and 3.07 (15 oocyte) and 2.5 (16 oocytes) for pretreated sperm group. The results indicate that DFJ does not block sperm-egg membrane fusion and penetration.

Mouse sperm were pretreated with anti-fucosidase and inseminated into IVF droplets containing ten zona-free oocytes. Following 4 h, the average number of sperm fused per oocyte was 1.57 (56 oocytes) in control group and 0.69 (59 oocytes) in the anti-fucosidase-pretreated sperm group (Fig. 17). Figure 19A shows the decrease in sperm numbers fused to oocytes in IgG control and anti-fucosidase antibody groups. Based upon the results, anti-fucosidase-pretreated sperm showed significant decrease in the number of sperm fused and penetrated to mouse oocytes around 56%.

Zona-free oocytes were pretreated with purified α-L-fucosidase prior to sperm insemination. The average number of sperm fused per oocyte were 2.1 (40 oocytes) for the control group and 1.57 (44 oocytes) for the purified α-L-fucosidase pretreated oocytes (Fig. 18). Sperm-egg fusion in purified fucosidase pretreated oocytes was reduced compared to untreated group (Fig. 19B). A 25.2% decrease of sperm fused to oocytes was observed after treating zona-free

oocytes with purified α-L-fucosidase. The results from anti-fucosidase are consistant with those of purified α-L-fucosidase consistent with a role of sperm associated α-L-fucosidase during sperm-egg membrane penetration.

3. 10 Role of α-L-fucosidase in post-fusion events

Because α-L-fucosidase was found to have roles during sperm-egg interaction and fusion, it is possible that this enzyme may be involved in later steps of fertilization. To test the role of α-L-fucosidase for post fusion and/or early embryo development, we performed two experiments to study this enzyme action beyond fusion event. The first experiment, intracytoplasmic sperm injection, was to bypass sperm-egg binding and fusion steps by directly injecting DFJ pretreated or untreated sperm into an oocyte incubated at 37 ^{o}C under 5% CO_2 for 24 h. Secondly, to study possible roles of α-L-fucosidase during embryo development from 2-PN to blastocyst stages, 2-pronuclear (2-PN) embryos were cultured with or without 5 mM DFJ at 37 ^{o}C under 5% CO_2 for 5 days.

3.10.1 Evaluation of intracytoplasmic sperm injection

An intracytoplasmic sperm injection technique was performed to inject a single untreated sperm (control) or DFJ pretreated sperm into a mouse oocyte. The percentage of 2-cell embryo development was observed as shown in Figure 20. 66.82% development to the stage 2-cell embryo was detected in control (20 oocytes) and 56.9% development to the stage 2-cell embryo was detected in DFJ pretreated sperm (21 oocytes). The results show no significant difference in

development to the stage 2-cell embryo between untreated and DFJ pretreated sperm, indicating that DFJ cannot block 2-cell embryo development. Moreover, α-L-fucosidase might not have any function during development to the 2-cell embryo.

3.10.2 Evaluation of 2-PN embryo development

2-PN embryos were harvested from superovulated female mice and incubated in IVF droplets containing 5 mM DFJ or without DFJ (control). The percentage of embryo development was observed from day 1 to day 5. Embryos were developed from 2-PN to 2-cell and beyond at 94.32% for control (2-PN = 142) and 86.5% for DFJ treatment (2-PN = 147) as summarized in Figure 21. For development to the blastocyst stage, 77.15% and 50.77% of embryo development were found in control and DFJ treatment, respectively (Fig. 21). These results showed no significant difference in early embryo development between the control and DFJ groups. Although the blastocyst development showed a decrease in the number of embryos that developed in the DFJ group, there is no statistically significant decrease compared to the development in control group. The results indicate that DFJ does not significantly inhibit mouse embryo development from 2-PN to blastocyst stage.

Chapter 4

Discussion

4.1 Distribution of α-L-fucosidase in mouse sperm

The present investigation shows that the majority of α-L-fucosidase in male mouse CEC is in the supernatant. This abundant soluble isoform in mouse CEC is analogous to the α-L-fucosidase present in the seminal plasma of humans and other organisms (Khunsook et al. 2002). A considerable amount of enzyme activity remains associated with the uncapacitated sperm of the CEC. These cell-bound α-L-fucosidases are composed of at least two isoforms with the molecular mass ratio of ~56 kDa and ~49 kDa, respectively (Phopin et al. Submitted manuscript). These data are reminiscent of α-L-fucosidases of human semen, in which seminal fluid fucosidase contains a single reactive band with the molecular mass ratio of ~56 kDa while sperm membrane-associated fucosidase contains a major protein band of ~51 kDa (Alhadeff et al. 1999, Khunsook et al. 2003). Moreover, it has been reported that rat epididymal sperm contains two immunoreactive bands of 54 and 50 kDa (Avilés et al. 1996). This current study demonstrates that almost 80% of α-L-fucosidase activity remains in the supernatant of the CEC after centrifugation, and about 10% of measurable activity is associated with the sperm cells (Phopin et al. Submitted manuscript). This is most likely a result of cell loss during cell washing steps and the enzyme may have lost some activity over time during9i experiments. The total wet volume of cells corresponded to about 1/100 of the CEC, so these values reflect a

relative enrichment of the enzyme in mouse sperm. While there is abundant fucosidase activity in the cauda fluid of mice, it is also relatively concentrated in sperm cells considering the small volume that they occupy. In addition, as also demonstrated here, the additional amount of α-L-fucosidase is cryptically sequestered within mouse sperm, and is detectable upon permeabilization or acrosomal exocytosis.

Seminal plasma or cauda fluid enzyme has also been found in hamster (Venditti et al. 2010), bulls (Moura et al. 2006), and gerbil (unpublished observations). Moura et al. have studied the relationship between fertility scores and proteins in cauda epididymal fluid of bulls. They found that the level of α-L-fucosidase increased around 2.3 fold in cauda epididymal fluid of high-fertility compared to low-fertility bulls (Moura et al. 2006). Thus, it suggests that α-L-fucosidase may be one of the cauda fluid proteins that could be used as a powerful biomarker for diagnosis of fertility. In addition, It has been proposed that the large amount of α-L-fucosidase in the soluble fraction may aid the passage of sperm through the reproductive ducts, whereas the enzyme associated with sperm cells may have a more direct interaction with the target oocyte (Khunsook et al. 2002, Khunsook et al. 2003, Venditti and Bean 2009, Venditti et al. 2010).

4.2 Inhibition of α-L-fucosidase in mouse cauda fluid

Competitive inhibitors DFJ and 18a were used to study the enzyme activity inhibition in mouse sperm supernatant. DFJ has been shown to be a

potent inhibitor of α-L-fucosidase (Winchester et al. 1990), and was previously shown to be a potent inhibitor of human and hamster seminal fucosidases (Winchester et al. 1990, Venditti et al. 2007, Venditti et al. 2010). In our standard assay condition, DFJ significantly inhibits the activity of α-L-fucosidase at concentrations of 10 nM or higher for mouse supernatant enzyme sources as shown in Figure 2. We have also demonstrated that compound 18a (a novel pyrrolidine 3,4-diol derivative α-L-fucosidase inhibitor) significantly inhibits the activity of mouse α-L-fucosidase and nearly completely knocks out activity at concentrations of 10 mM and above (Phopin et al. Submitted manuscript). Based upon the inhibition results, DFJ shows more potent inhibition than that of 18a. These results are consistent with the *Ki* values of DFJ (*Ki* = 1×10^{-8} M) (Winchester et al. 1990) and 18a (*Ki* = 0.08 M)(Moreno-Clavijo et al. 2009). Considering the known specificities of inhibition for both of these inhibitors (Winchester et al. 1990, Moreno-Clavijo et al. 2009), these results confirm that our enzyme assay does accurately reflect specific function of α-L-fucosidase and not any other source of product generation.

4.3 Crypticity of α-L-fucosidase after sperm capacitation and acrosome reaction

Capacitation is an event that naturally occurs to sperm on interaction with the female genital tract. Redistribution of lipids, carbohydrates and membrane proteins occurs to alter motility and prepare the cell to undergo the acrosome reaction. To achieve artificial capacitation, mouse sperm were incubated with

1.5% BSA for 1-1.5 h. Capacitation has been shown to change α-L-fucosidase staining on the human sperm cell membrane (Venditti et al. 2007). The data presented herein show that capacitation also unmasks a cryptic store of α-L-fucosidase within the cells. A significant increase in activity (2.6 times) is observed in the capacitated mixture. Most of this activity increase is found in the capacitated supernatant, although, α-L-fucosidase activity associated with the capacitated cells is persistent and stable through multiple incubations and manipulations. (Phopin et al. Submitted manuscript). The significant increase of fucosidase activity in the capacitated mixture compared to the uncapacitated cells and the significant decrease of the activity in the capacitated sperm compared to the capacitated mixture indicate that most cell associated α-L-fucosidase is released into the soluble fraction.

These observations suggest that there are at least two distinct populations of sperm associated α-L-fucosidase. Firstly, there is a pool of soluble enzyme, primarily within the acrosomal compartment, which can be released from uncapacitated cells upon permeabilization. Some of this soluble enzyme may also be liberated during capacitation. In our "capacitated" suspension, there may still be a subpopulation of cells that are still progressing through capacitation, and may still be able to release their soluble components. During natural reproductive processes, the released fucosidase might function to facilitate progression of sperm through the female reproductive tract. Secondly, there is a population of sperm cell-associated fucosidase that is not readily extractable due to its intimate

association with other structures within the sperm cell. During the morphological reorganization that takes place during capacitation and the acrosome reaction, these structurally bound fucosidase molecules become progressively restricted to and localized within the sperm equatorial segment. As an integral component of the complex equatorial segment, the α-L-fucosidase is positioned for possible engagement in sperm-oocyte interactions and/or triggering of processes of early embryogenesis.

A parallel model for the relationship proposed above has been reported for another sperm protein. As reported by Cohen et al. protein DE, a member of the CRISP family, consists of two populations within sperm. One of them is loosely bound to sperm and released from the sperm cells during capacitation, acting as a "decapacitating factor". A second population of sperm associated protein DE becomes an integral membrane protein tightly associated with sperm after capacitation. This remaining protein DE migrates to the equatorial segment as the acrosome reaction occurs, and participates in sperm-egg fusion (Cohen et al. 2008).

In our experiments, the acrosome reaction was induced by treatment of capacitated sperm with 0.01 mM BrA23187 for 30 min at 37 ˚C, 5% CO_2. α-L-fucosidase enzyme assays showed that after the acrosome reaction, enzyme activity in the acrosome reacted mixture was relatively constant compared to capcitated cells, a significant decrease in enzyme activity was found in the

acrosome reacted cells, indicating that some loosely bound α-L-fucosidase is released into the soluble fraction. However, cell-associated α-L-fucosidase still remained in the acrosome reacted cells, suggesting a function of this enzyme during gamete interaction. Moreover, immunofluorescence studies showed that after the acrosome reaction, α-L-fucosidase appears at the equatorial segment (Phopin et al. Submitted manuscript).This result is consistent with enzyme activity assay which shows detectable activity of α-L-fucosidase within acrosome-reacted sperm, confirming the existence of α-L-fucosidase in the sperm. A previous study in our lab also showed that after the acrosome reaction, α-L-fucosidase is noticeably detected at the equatorial segment and post acrosomal region of human sperm (Venditti et al. 2007). Exocytosis of the acrosomal membrane during the acrosome reaction might expose some of the enzyme, originally stored at the outer acrosomal membrane underlying the plasma membrane. The life history of the sperm α-L-fucosidase supports the possibility that this enzyme might play additional roles in sperm penetration, membrane fusion, and early embryo development.

4.4 Stability of α-L-fucosidase in mouse sperm

Our stability studies show that α-L-fucosidase activity of both cauda fluid and sperm is stable at 37 ˚C, 5% CO_2. On the other hand, at 37 ˚C, ambient air, the activity is undetectable after 24 h of incubation (Phopin et al. Submitted manuscript). These results are consistent with the stability results of human sperm associated α-L-fucosidase activity (Venditti and Bean 2009). A basic shift

in pH may influence the decrease of the enzyme activity as observed from the pH of samples: 7 – 7.5 at 37 ˚C, 5% CO_2 and around 9 at ambient air. Previous study in our lab on purified human membrane associated α-L-fucosidase revealed that the optimal condition for the enzyme activity is at acidic condition at pH 4.0 – 6.0 with 80% of the maximal activity at pH 7.0 (Khunsook et al. 2003). Moreover, the enzyme activity in both fluid and sperm at 5% CO_2, 37 ˚C is still detectable after 72 h, showing that enzyme activity might still be present under conditions where fertilization is delayed after ejaculation.

Based on the results of distribution, crypticity, and stability of α-L-fucosidase in mouse sperm, α-L-fucosidase is mostly found in fluid, suggesting that this enzyme might assist passage of sperm through the female reproductive tract. The enzyme is also detected in sperm cells and shows crypticity after capacitation and the acrosome reaction. Sperm-associated α-L-fucosidase might be involved in sperm-egg interaction and subsequent processes. The stability of the enzyme under physiologically relevant conditions (5% CO_2, 37 ˚C) spans the timeframe of fertilization events. This confirms the possible action of α-L-fucosidase during gamete interaction and early embryogenesis. However, the specific mechanism of this enzyme during fertilization is still unknown and merits further investigation.

4.5 The existence of α-L-fucosidases in mouse oocytes and embryos

Mouse oocytes or embryos were harvested from superoverated female mice and were extracted in 0.01%TritonX-100/PBS with sonication. α-L-fucosidase enzyme assay was performed using flurogenic substrate (4-MU-FUC). Additionally, western blot analysis was employed to confirm the existence of α-L-fucosidase glycoprotein in oocytes and embryos. No detectable α-L-fucosidase activity was found in oocytes and blastocysts. Moreover, there is no α-L-fucosidase glycoprotein detected by western blotting. The results are consistent with the previous work in hamster oocytes (Venditti et al. 2010). On the other hand, we detected the α-L-fucosidase activity in 10 day embryos. There is some evidence showing that α-L-fucosidase glycoprotein has a molecular mass ratio around 50 kDa (Avilés et al. 1996, Khunsook et al. 2003, Alhadeff et al. 1999). The existence of α-L-fucosidase activity in mouse sperm but not in the oocytes suggests the possibility that this enzyme may be delivered by sperm to the oocytes for activation of fertilization and embryogenesis. In contrast, the detectable α-L-fucosidase activity in 10 day embryos might play an important role during embryo development from 10 days to beyond.

4.6 α-L-fucosidase during sperm-oocyte zona pellucida and membrane binding

Zona intact oocytes were placed in organ culture dishes and inseminated with control, DFJ pretreated or anti-fucosidase pretreated sperm. For some experiments, oocytes were pretreated with purified α-L-fucosidase to block the

binding sites of α-L-fucosidase in the egg zona pellucida. Treatment with 5 mM DFJ did not result in the inhibition of the sperm tightly binding to the zona pellucida of the oocytes. Conversely, anti-fucosidase antibody and purified α-L-fucosidase significantly decreased the binding of sperm to egg zona pellucida.

Sperm-egg plasma membrane binding assay was performed using zona free mouse oocytes. 5 mM DFJ did not inhibit sperm binding to zona free oocytes. In contrast, anti-fucosidase antibody and purified α-L-fucosidase treatments significantly reduced of sperm binding to zona free eggs. The average TB sperm per oocyte were significantly decreased around 43% and 38.5% at 30 and 60 min compared to control for anti-fucosidase antibody and a 67% decrease was observed after treating zona free oocytes by purified α-L-fucosidase at 60 min. These findings provide strong evidence for a role of α-L-fucosidase during sperm-egg zona and membrane binding.

For our experiments, we used purified human liver α-L-fucosidase to challenge and confirm the results from experiments with anti-fucosidase antibody. Since the FUCA1 gene is very conserved throughout several different species and the major difference responsible for different functions of isoforms is modification (glycosylation). However, it is possible that the structure of α-L-fucosidases contains conserved sequences necessary for interacting with specific targets on the oocyte. This possibility parallels a report in which cysteine-rich secretory protein (CRISP) was characterized. Generally, CRISP is

known to function in ion channel regulation. Additionally, it has been reported that this protein family plays roles in fertilization. The study showed that rat CRISP1 plays roles in zona binding and fusion steps and mouse CRISP2 functions during the fusion step (Cohen et al. 2008, Busso et al. 2007). Studies using recombinant CRISP1 and CRISP2 polypeptide sequences in inhibiting gamete interaction were consistent with studies where antibodies against CRISP1 or 2 were used (Cohen et al. 2008, Busso et al. 2007). Particularly, signature 2 sequence (S2) was found to be very conserved between rat CRISP1 and mouse CRISP2, and is the region involved in gamete interaction (Cohen et al. 2008). S2 regions of rat CRISP1 and mouse CRISP2 are different in only two amino acid residues. This may result in minor differences in their functions during steps of fertilization. Like CRISP studies, purified human liver α-L-fucosidase was used in this study to establish that binding of this glycoprotein to targets on the oocyte can compete with binding of sperm that carry the homologous glycoprotein as an integral element of their structure.

Interestingly, while the experiments on anti-fucosidase antibody and purified human liver α-L-fucosidase showed consistent inhibition of sperm binding to the zona pellucida and membrane of eggs, the opposite results were observed for the DFJ treated group. Anti-fucosidase antibody does not inhibit catalytic activity of the enzyme, but specifically binds to α-L-fucosidase on the sperm head and interferes with the availability of the glycoprotein, resulting in reduction of sperm-egg zona and membrane binding. On the other hand, if α-L-fucosidase

plays a role during zona and membrane binding, the signature sites for enzyme binding should be found in the zona pellucida and plasma membrane. The decrease of sperm bound to purified α-L-fucosidase pretreated zona intact and zona free oocytes suggests the availability of binding sites for this enzyme in mouse oocyte zona pellucida and plasma membrane, supporting the role of α-L-fucosidase during zona and membrane binding steps. However, DFJ, α-L-fucosidase competitive inhibitor, does not interfere in binding assays. DFJ competitively binds to the active site of α-L-fucosidase which inhibits the enzyme activity. Based on our data with mice, the binding of sperm associated α-L-fucosidase to the zona pellucida and plasma membrane of oocytes requires a structural interaction and not mainly active site function.

Consistently, α-L-fucosidase has been reported to be involved in fertilization at the step of binding in ascidians. HrFuc'ase antibody against α-L-fucosidase has been shown to inhibit fertilization in a dose-dependent manner, suggesting the existence of α-L-fucosidase in the outer surface of ascidian sperm and its important role during fertilization at the binding step mediated by sperm α-L-fucosidase and complementary L-fucosyl residues of glycoproteins in the vitelline coat (Matsumoto et al. 2002). Intra and colleagues have studied the immunolocalization pattern of α-L-fucosidase in *Drosophila* sperm. They revealed that α-L-fucosidase is predominantly localized in the sperm plasma membrane overlying the acrosome and the tail. Moreover, α-L-fucose terminal residues are present on the chorion with a strongly polarized localization on the micropyle,

suggesting that this enzyme might be involved in sperm-egg recognition in *Drosophila* by interacting with α-L-fucose residues on the micropyle of the oocyte surface (Intra et al. 2006). Additionally, sperm associated α-L-fucosidase has been also demonstrated to be involved in sperm-egg interaction in *Unio elongatulus* (Focarelli et al. 2001). For hamsters, although fucosidase has been shown to have no role in the zona binding step, it has been reported to be involved in sperm oocyte membrane-membrane interaction, fusion, and/or some early stages of embryogenesis (Venditti et al. 2010). Functions of hamster sperm fucosdase in membrane-membrane interaction and fusion correlate with our studies where mouse sperm fucosidase has been investigated. From these observations, it is clear that some reproductive roles of fucosidase are conserved among different species and some functions are different from one species to another. Considerably, these reports support the notion that the sperm fucosidase has been an important focus for natural selection during speciation.

4.7 α-L-fucosidase during sperm-oocyte membrane penetration

Zona-free oocytes were used to investigate a role of α-L-fucosidase during sperm-egg fusion, which includes binding and penetration of sperm into the ooplasm. The experiments were conducted in three groups: 5 mM DFJ pretreated sperm, anti-fucosidase antibody pretreated sperm, and purified human liver α-L-fucosidase pretreated oocytes. No statistically significant difference was observed in DFJ-pretreated sperm during the fusion step. In contrast, a 56% decrease of sperm fused to oocytes was found in the presence anti-fucosidase

antibody. A consistent finding was observed for sperm fusion with purified human liver α-L-fucosidase pretreated oocytes. Considered together with the results for tight binding, these observations support a single most likely interpretation of the results. The fucosidase glycoprotein has a direct role where α-L-fucosidase molecules on sperm bind and fuse to targets on the outer surface of the plasma membrane of oocytes. These target sites may or may not contain fucose residues. Both tight binding to the oolemma and penetration into the ooplasm are enabled by binding of the fucosidase glycoprotein, and do not require completion of enzyme activity.

We would expect that the fucosidase molecules engaged in this binding are localized within the equatorial segment of acrosome reacted (AR) sperm. Our data are consistent with this expectation since it is known that the acrosome-induced sperm population is, in fact, a very mixed population. Within this population, cells that have not yet completed AR are not available for antibody binding, yet they might progress through AR during the incubation. Thus we should not expect to observe complete inhibition of tight binding or penetration.

Similarly for the case of pretreatment of the oocytes with purified human liver fucosidase we observed statistically significant inhibition. Again the extent of inhibition was not extensive, but that should not be expected since the binding of the glycoprotein may be of low affinity, and the oocytes are alive and may be

dynamic in refreshing their membranous surfaces, particularly since the zonas have been removed.

There have been some reports showing that L-fucose and/or α-L-fucosidase participate in the steps of membrane adhesion, fusion, and penetration. For cattle, the percentage of the sperm penetration into oocytes was decreased significantly in the presence of L-fucose and fucoidan. 46.8% and 98.2% of inhibition of bovine sperm penetration were found in 50 mM L-fucose and 1 mg/ml fucoidan, respectively (Tanghe et al. 2004b). Additional examples were described in the introduction (sections 1.2, 1.3, and 1.4). A study in hamster using DFJ revealed that sperm associated fucosidase participates in sperm-oocyte membrane-membrane interaction, fusion, and/or some early stages of embryo development (Venditti et al. 2010).

4.8 α-L-fucosidase in post-fusion events and early embryo development

Intracytoplasmic sperm injection technique was performed with a single untreated sperm (control) or 5 mM DFJ pretreated sperm. We found that there is no significant difference between control and DFJ pretreated sperm groups, suggesting that enzymatic activity of α-L-fucosidase does not play a role during embryo development from oocytes to 2-cell embryos.

In addition, an experiment was designed to detect possible roles of α-L-fucosidase during embryogenesis. 2-PN embryos were incubated in embryo

culture droplets containing 5 mM DFJ or without DFJ (control). DFJ does not significantly inhibit embryo development from 2-PN to blastocyst stage. This finding suggests that α-L-fucosidase has no role during embryo development from 2PN to blastocyst stage. We have also found that readily measurable fucosidase activity is present in 10-day embryos. It is evident that α-L-fucosidase is not an important protein for mouse early embryo development. There is no detectable α-L-fucosidase in those cells.

4.9 Conclusion

This research confirms the existence of α-L-fucosidase in mouse cauda epididymal fluid and sperm, documents many of its characteristics, and identifies some of its biological roles. α-L-fucosidase activity was detected in mouse sperm, but not found in mouse oocytes. The cell bound mouse α-L-fucosidase glycoprotein, but not its enzyme activity, fosters the binding of sperm to the zona pellucida and to the plasma membrane of mouse oocytes. These results confirm our general hypotheses, concerning participation of α-L-fucosidase in gamete interaction during mouse fertilization. The sperm α-L-fucosidase glycoprotein is also implicated in the process of sperm penetration into the ooplasm, probably as a consequence of its inhibition of tight binding to the oolemma. On the other hand, the α-L-fucosidase appears not have essential catalytic functions during early embryogenesis in mice, although it is readily detectable in 10-day mouse embryos.

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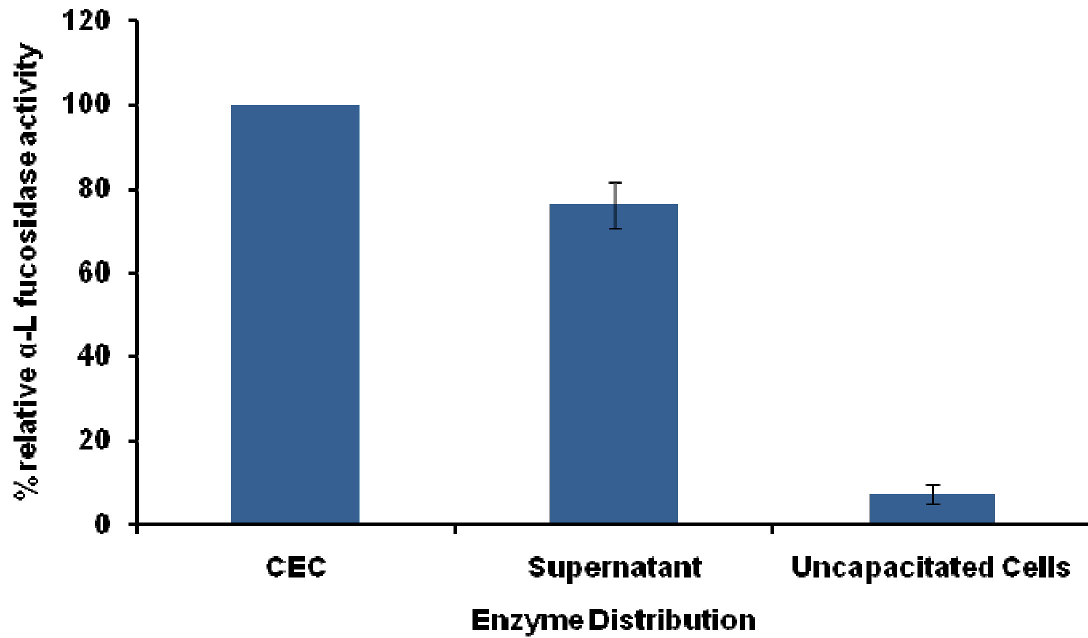

Figure 1. α-L-fucosidase distribution in mouse CEC. Enzyme activity in CEC, supernatant, and uncapacitated sperm was measured by using fluorogenic substrate 4-MU-Fuc. The percent relative α-L-fucosidase activity was calculated for each sample. Data represent averages from five independent experiments. Error bars represent standard error of the mean.

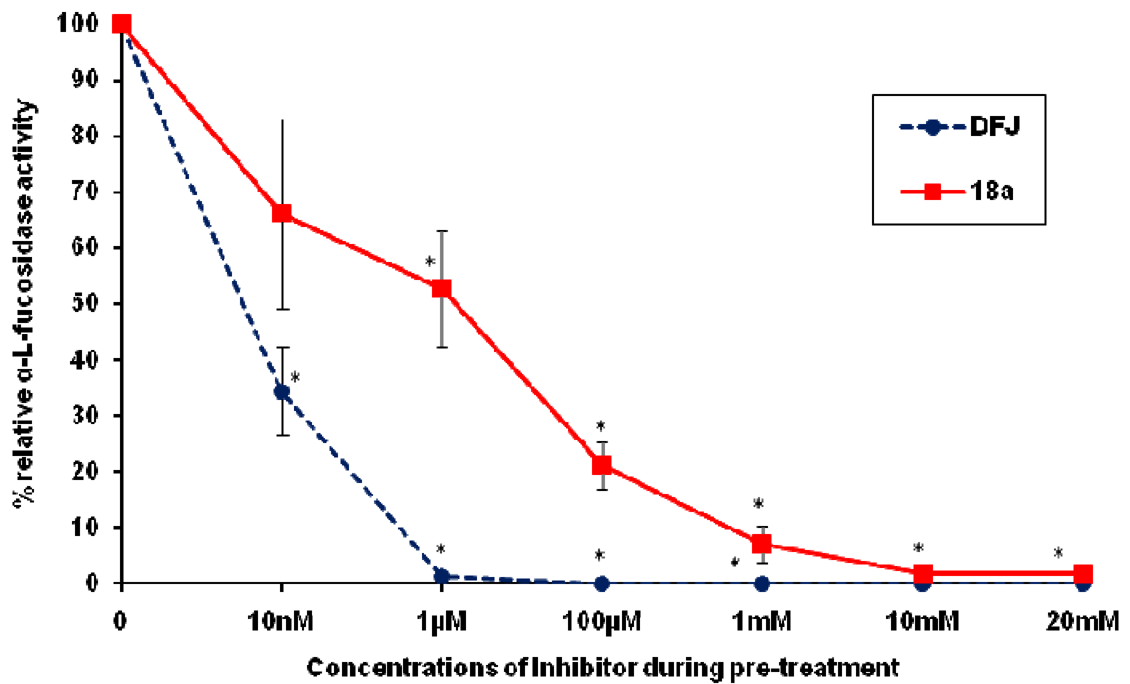

Figure 2. Comparison of α-L-fucosidase inhibition in mouse CEC supernatants. CEC supernatants were pretreated with various concentrations of DFJ or 18a competitive α-L-fucosidase inhibitors for 10 min as shown on the X-axis. Enzyme activity of each sample was measured by using fluorogenic substrate 4-MU-Fuc. The percent relative α-L-fucosidase activity was calculated in each sample. Asterisks indicate p-values <0.05 compared to untreated controls (over 3-6 replicates). Error bars represent standard error of the mean.

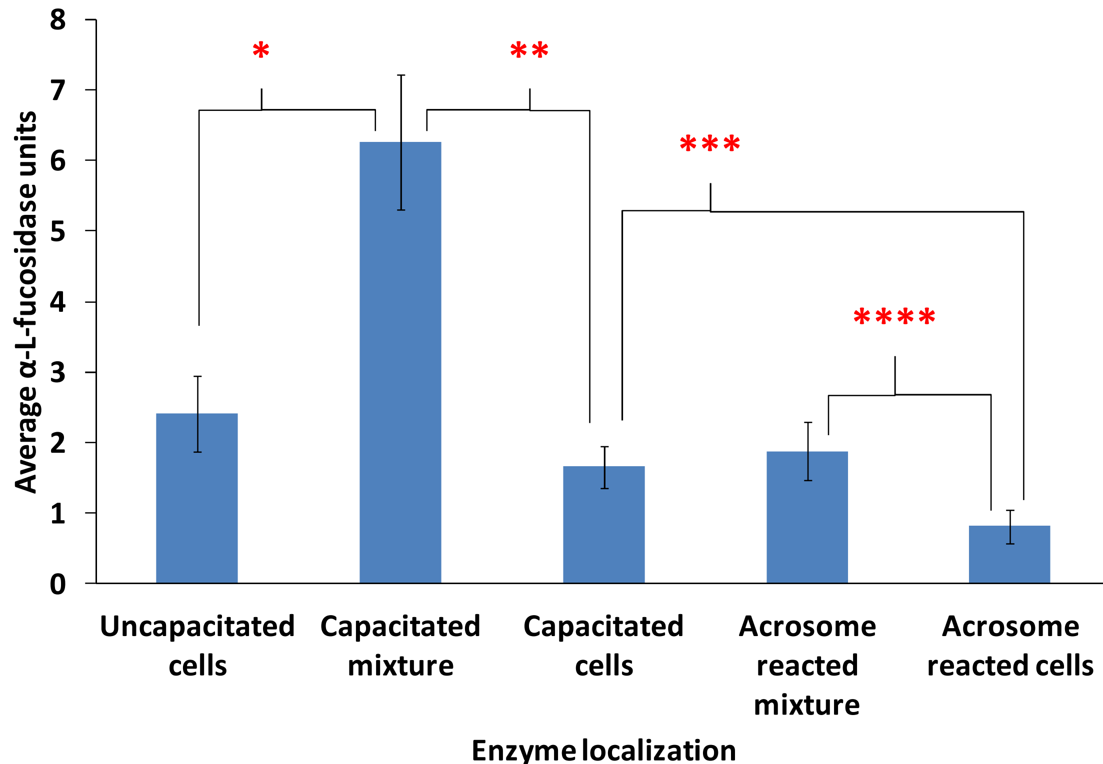

Figure 3. α-L-fucosidase activity in sperm cells after the capacitation and the acrosome reaction. Enzyme activity was measured in mouse sperm cells before and after treatment with 1.5% BSA, 1.5 h for the capacitation or 0.01 mM BrA23187, 30 min for the acrosome reaction. Average α-L-fucosidase units were calculated in all samples. Capacitated mixture shows significant increase in enzyme activity compared to uncapacitated sperm (*p-value <0.05, over 13 replicates). Capacitated cells show significant decrease in enzyme activity compared to uncapacitated sperm (** p-value <0.05, over 13 replicates). Acrosome reacted cells show significant decrease in enzyme activity compared to capacitated cells and acrosome reacted mixture (***p-value <0.05, **** p-value <0.05, over 13 replicates). Error bars represent standard error of the mean.

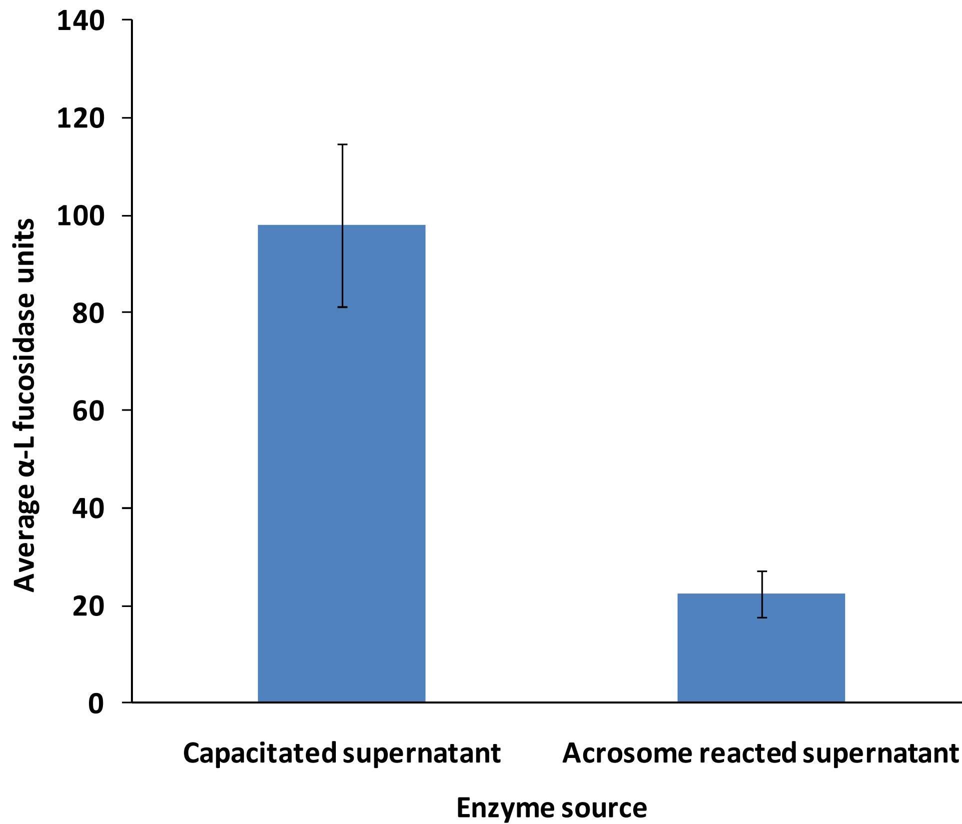

Figure 4. α-L-fucosidase activity in supernatant after the capacitation and treatment to induce acrosome reaction (over 13 replicates). Enzyme activity was measured in supernatant before and after treatment with 1.5% BSA, 1.5 h for the capacitation or 0.01 mM BrA23187, 30 min for the acrosome reaction. Average α-L-fucosidase units were calculated in each sample. Error bars represent standard error of the mean.

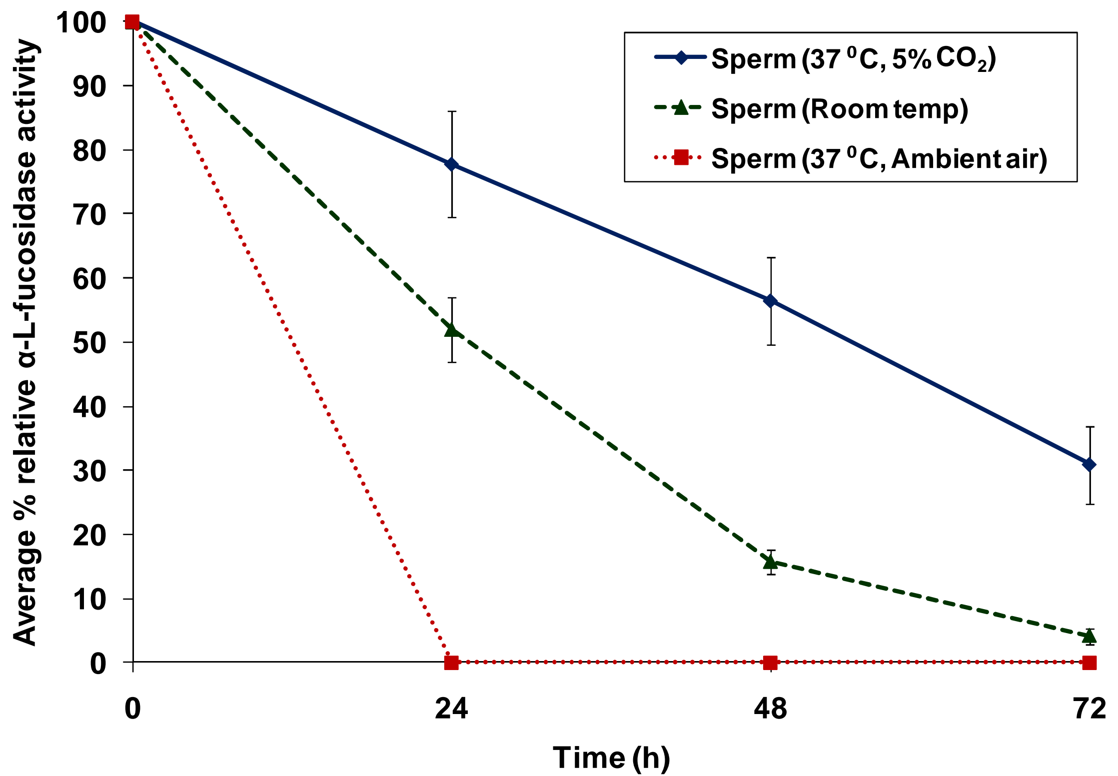

Figure 5. Stability of cauda sperm α-L-fucosidase at 37 ˚C, 5% CO_2, 37 ˚C, ambient air, and room temperature. Data represent averages from three independent experiments. Error bars represent standard error of the mean.

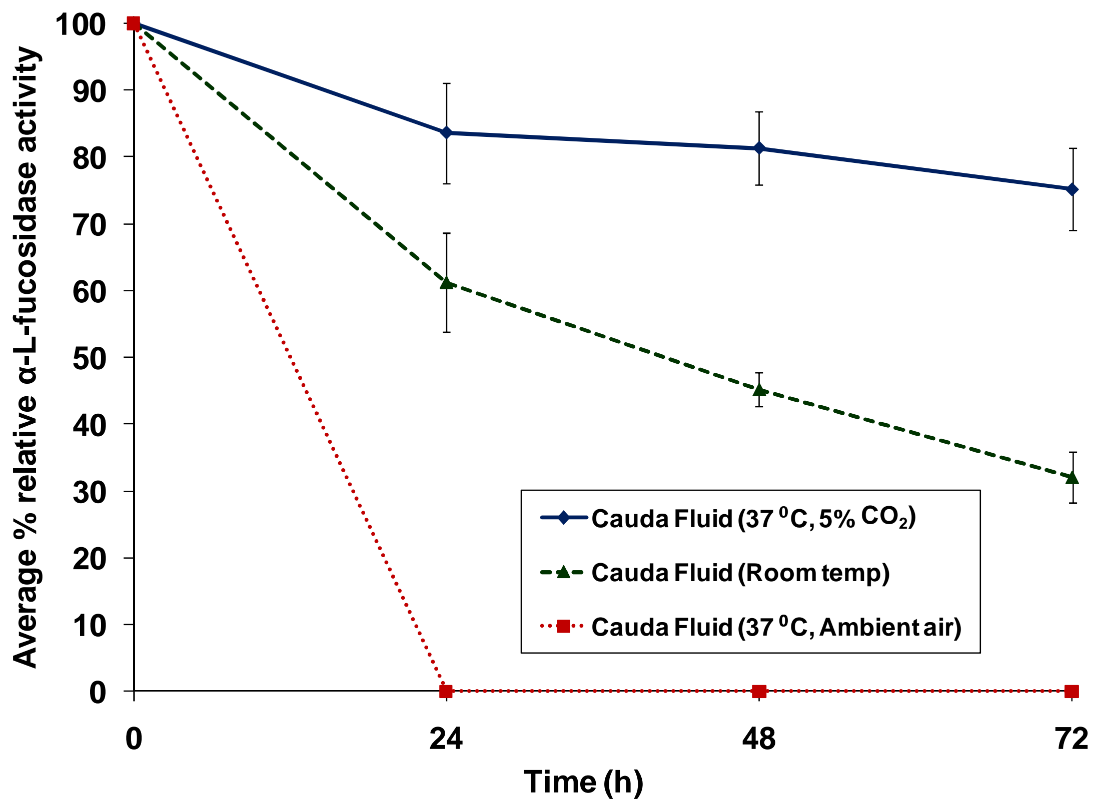

Figure 6. Stability of cauda fluid α-L-fucosidase at 37 ˚C, 5% CO_2, 37 ˚C, ambient air, and room temperature. Data represent averages from three independent experiments. Error bars represent standard error of the mean.

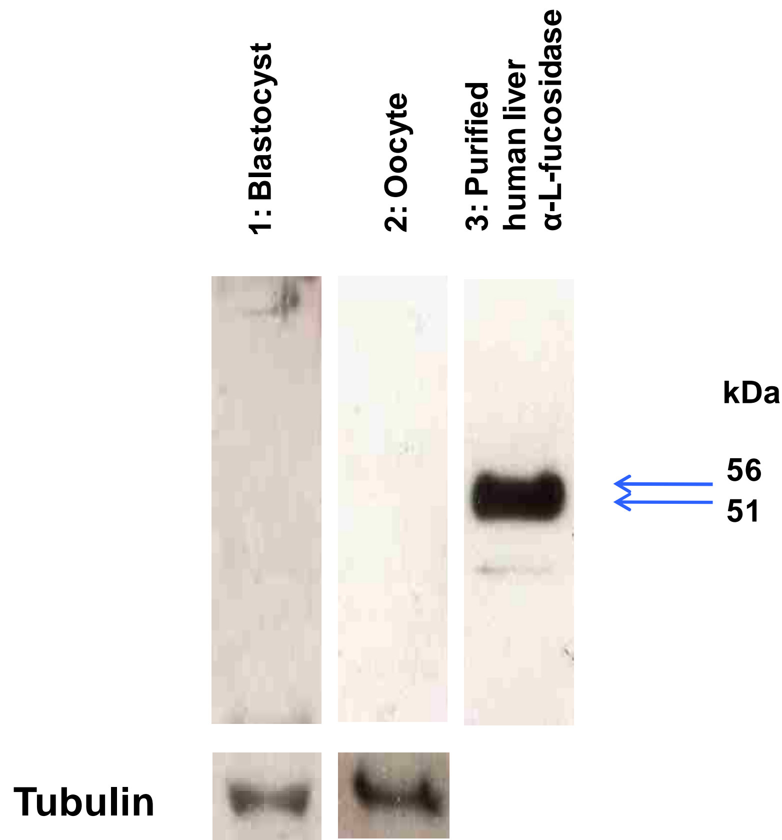

Figure 7. Western blot analysis of mouse oocyte and blastocyst contents for α-L-fucosidase. Lane 1: mouse blastocysts (200 blastocyts), lane 2: mouse oocytes (200 oocytes), and lane 3: purified human liver α-L-fucosidase. Anti-tubulin was used as an internal control. Mouse blastocyst and oocyte extracts showed no detectable α-L-fucosidase glycoprotein. Purified human liver used as a positive control showed two immunoreactive bands at 51 and 56 kDa.

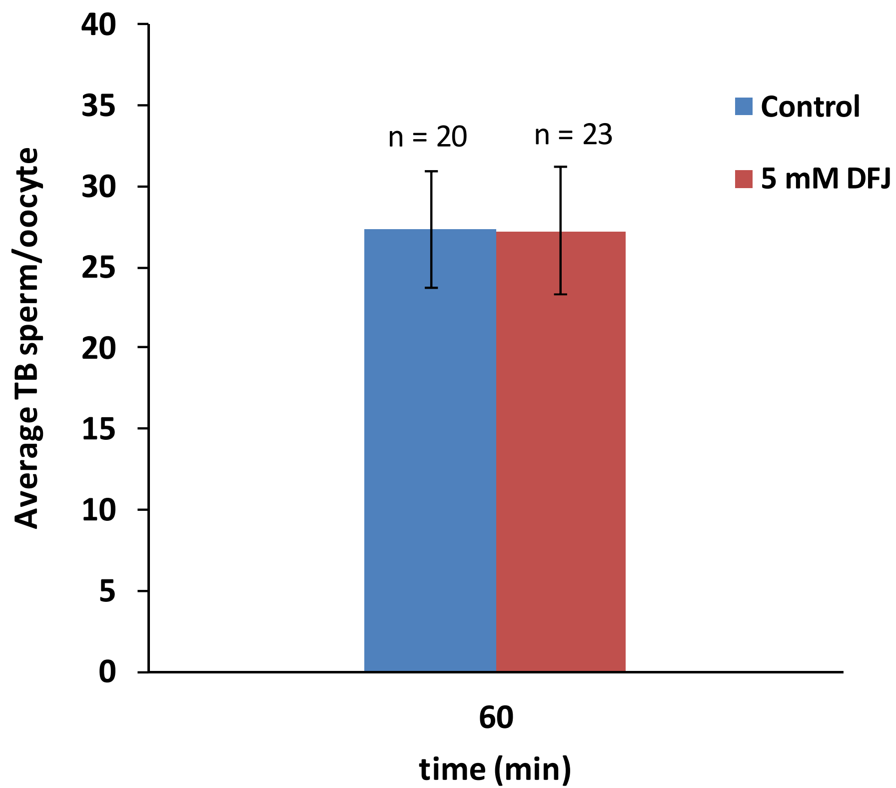

Figure 8. Average TB sperm to oocyte zona pellucida of untreated and 5 mM DFJ pretreated sperm at 60 min. Mouse oocytes were inseminated with either control or 5 mM DFJ pretreated capacitated sperm. The number of TB sperm to the ZP was quantified at 60 min post insemination for each oocyte. Error bars represent standard error of the mean.

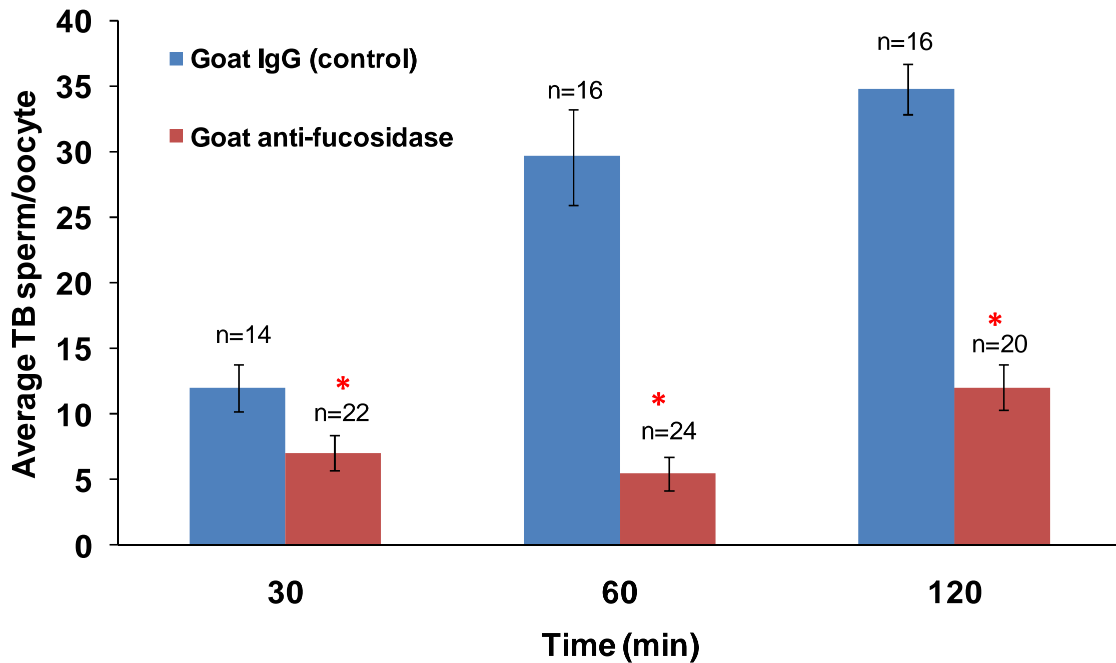

Figure 9. Average TB sperm to oocyte zona pellucida of untreated and anti-fucosidase pretreated sperm at 30, 60, and 120 min. Mouse oocytes were inseminated with either IgG control or anti-fucosidase pretreated capacitated sperm. The number of TB sperm to the ZP was quantified at 30, 60, and 120 min post insemination for each oocyte. Asterisks indicate p-values <0.05 compared to control. Error bars represent standard error of the mean.

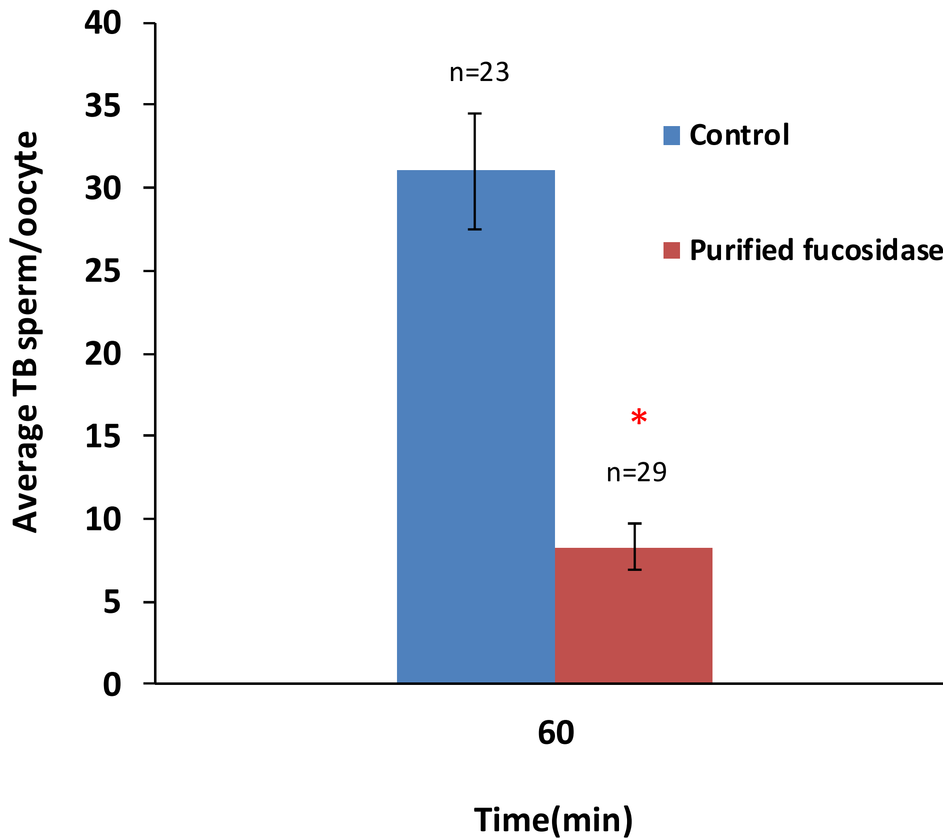

Figure 10. Average TB sperm to oocyte zona pellucida of untreated and purified human liver α-L-fucosidase pretreated oocytes at 60 min. Mouse oocytes were pretreated with either control or purified human liver fucosidase prior to insemination with capacitated sperm. The number of TB sperm to the ZP was quantified at 60 min post insemination for each oocyte. An asterisk indicates p-value <0.05 compared to control. Error bars represent standard error of the mean.

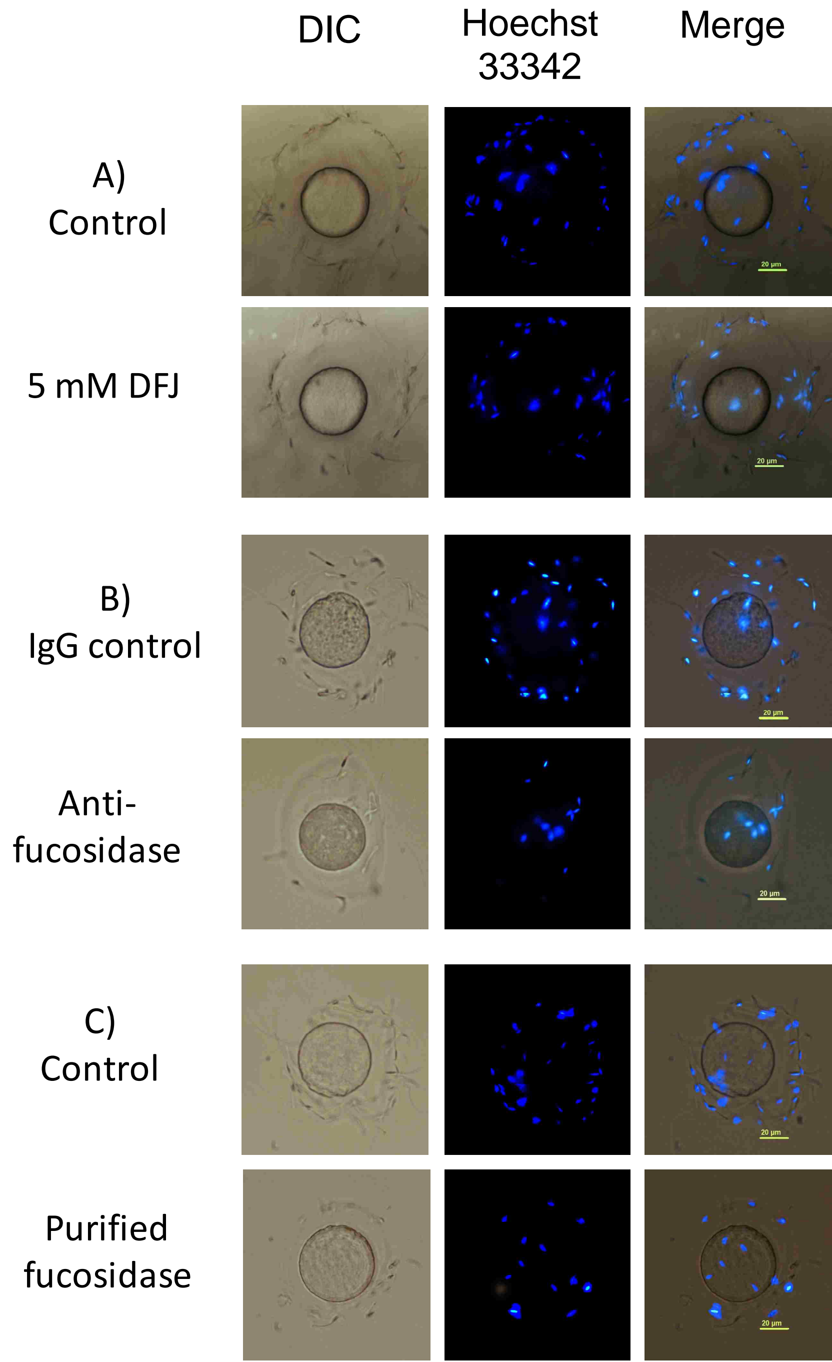

Figure 11. Binding of sperm labeled with Hoechst 33342 (blue) are shown tightly bound to the zona pellucida of oocytes at 1 h post insemination: A) 5 mM DFJ pretreated sperm, B) Anti-fucosidase pretreated sperm and C) Purified human liver fucosidase pretreated oocytes (scale bar = 20 μm). (* tightly bound: see section 2.19)

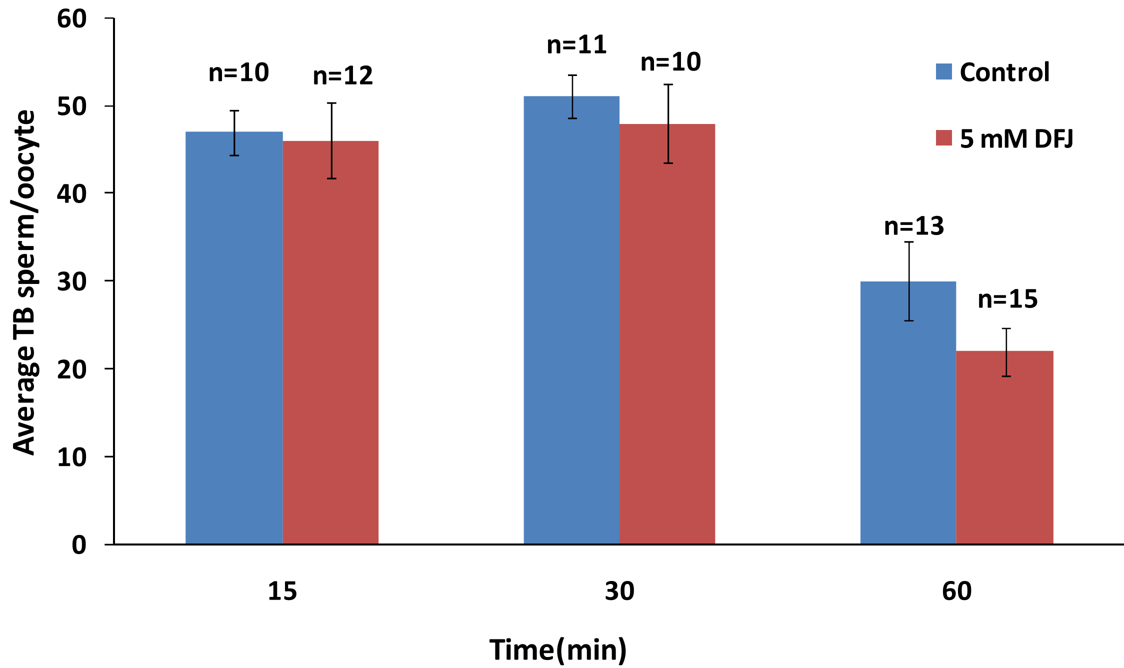

Figure 12. Average TB sperm to oocyte membrane of untreated and 5 mM DFJ pretreated sperm at 15, 30, and 60 min. Zona free mouse oocytes were inseminated with either control or 5 mM DFJ pretreated capacitated sperm. The number of TB sperm to the oocyte plasma membrane was quantified at 15, 30, and 60 min post insemination for each oocyte. Error bars represent standard error of the mean.

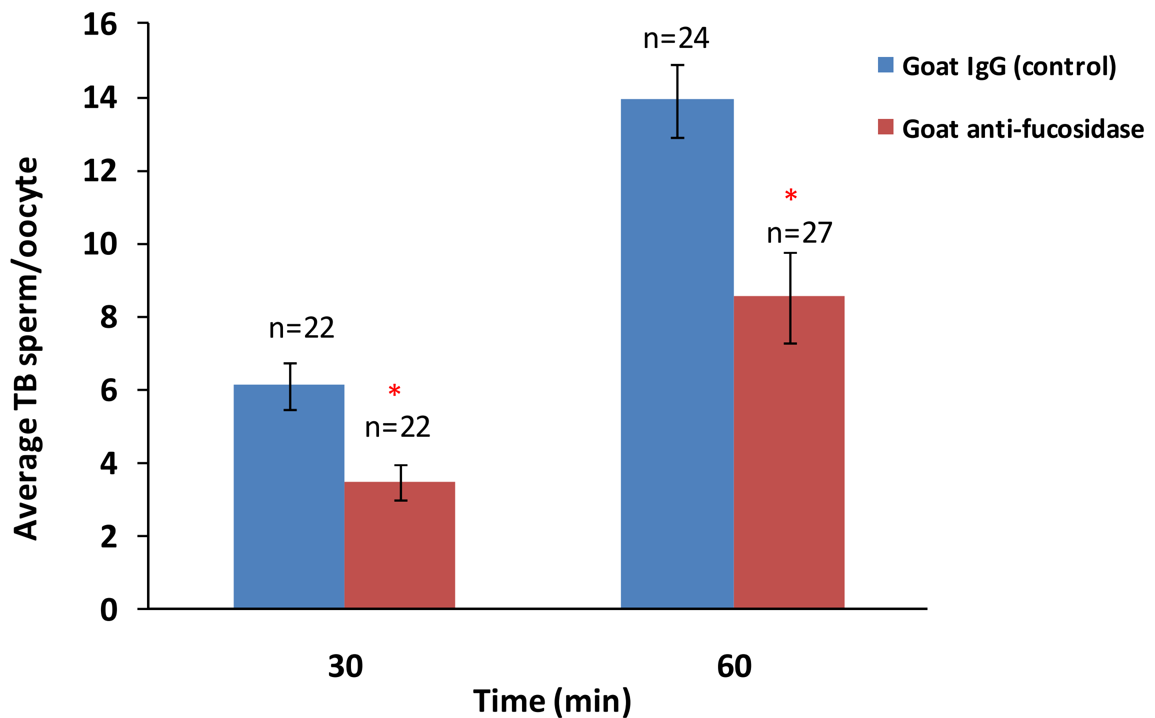

Figure 13. Average TB sperm to oocyte membrane of untreated and anti-fucosidase pretreated sperm at 30 and 60 min. Zona free mouse oocytes were inseminated with either IgG control or anti-fucosidase pretreated capacitated sperm. The number of TB sperm to the oocyte plasma membrane was quantified at 30 and 60 min post insemination for each oocyte. Asterisks indicate p-values <0.05 compared to control. Error bars represent standard error of the mean.

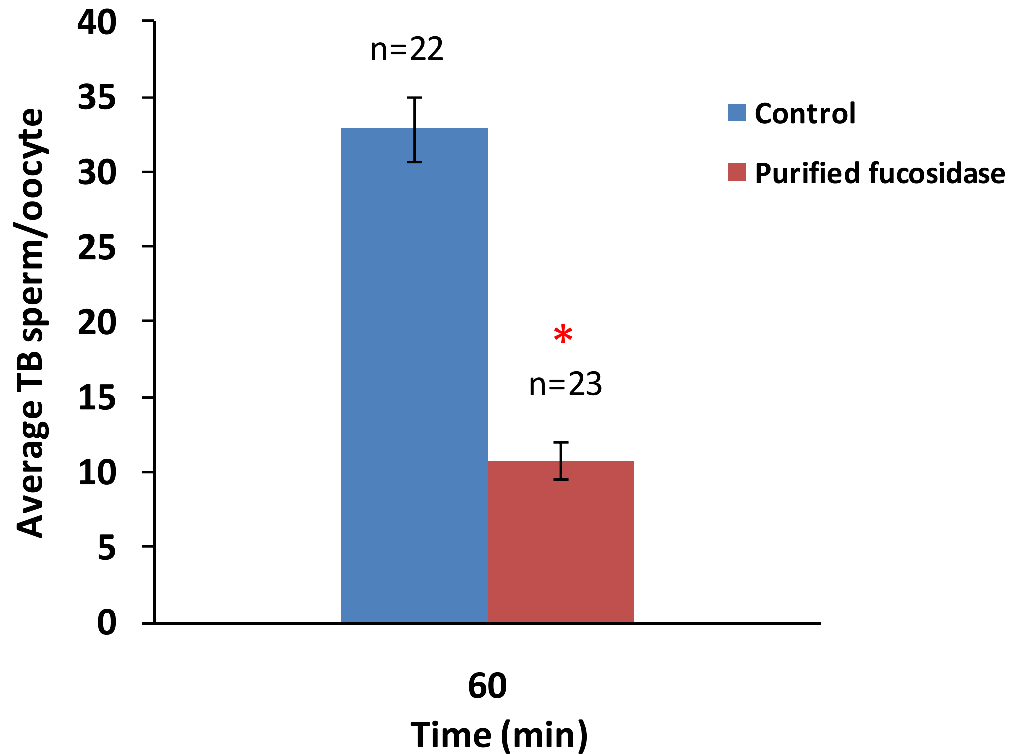

Figure 14. Average TB sperm to oocyte membrane of untreated and purified human liver α-L-fucosidase pretreated oocytes at 60 min. Zona free mouse oocytes were pretreated with either control or purified human liver fucosidase prior to insemination with capacitated sperm. The number of TB sperm to the oocyte plasma membrane was quantified at 60 min post insemination for each oocyte. An asterisk indicates p-value <0.05 compared to control. Error bars represent standard error of the mean.

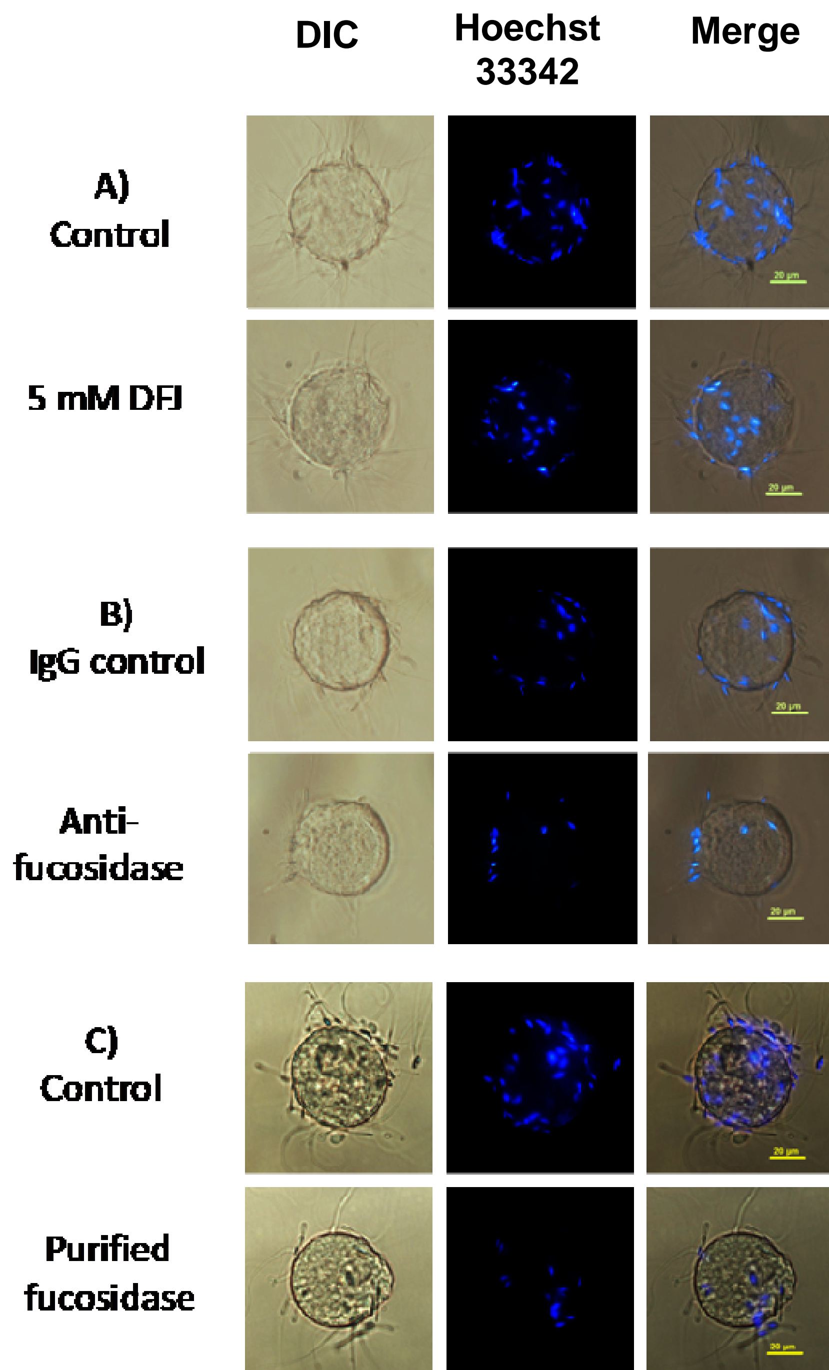

Figure 15. Sperm labeled with Hoechst 33342 (blue) are shown tightly bound to plasma membrane of the oocyte 1 h post insemination: A) 5 mM DFJ pretreated sperm, B) Anti-fucosidase pretreated sperm and C) Purified human liver fucosidase pretreated oocytes (scale bar = 20 μm). (* tightly bound: see section 2.21)

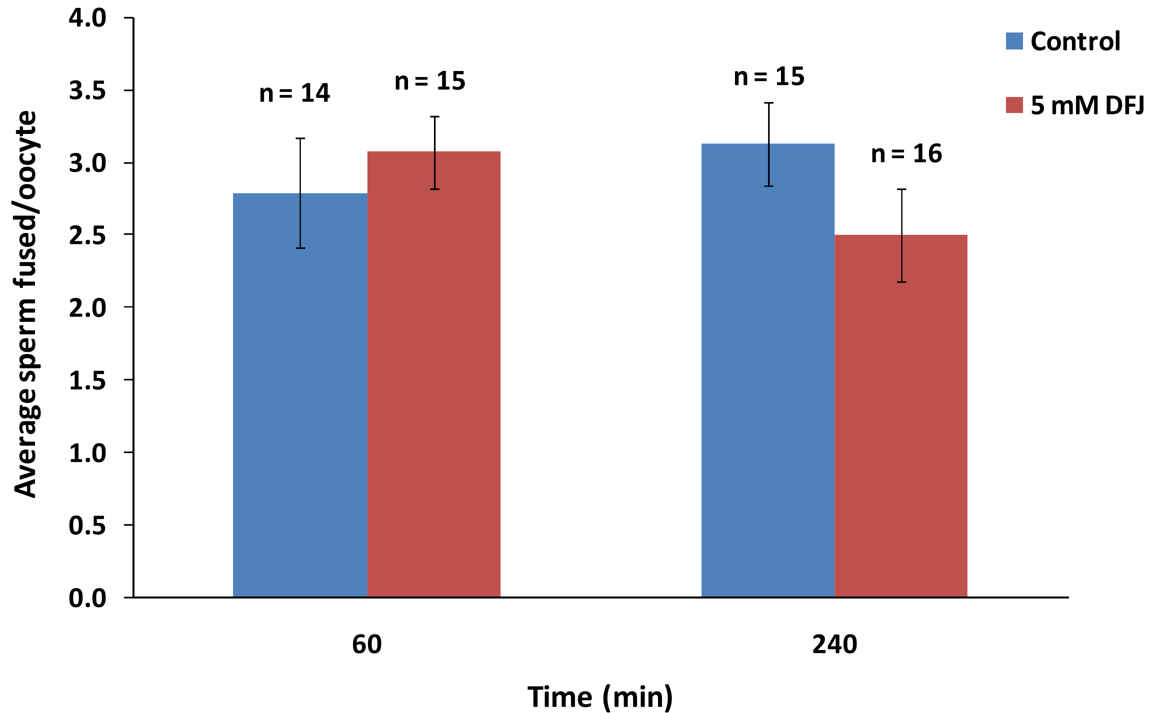

Figure 16. Average sperm fused to oocyte membrane of untreated and 5 mM DFJ pretreated sperm at 60 and 240 min. Zona free mouse oocytes were inseminated with either control or 5 mM DFJ pretreated capacitated sperm. The number of sperm fused per oocyte was quantified at 60 and 240 min post insemination for each oocyte. Error bars represent standard error of the mean.

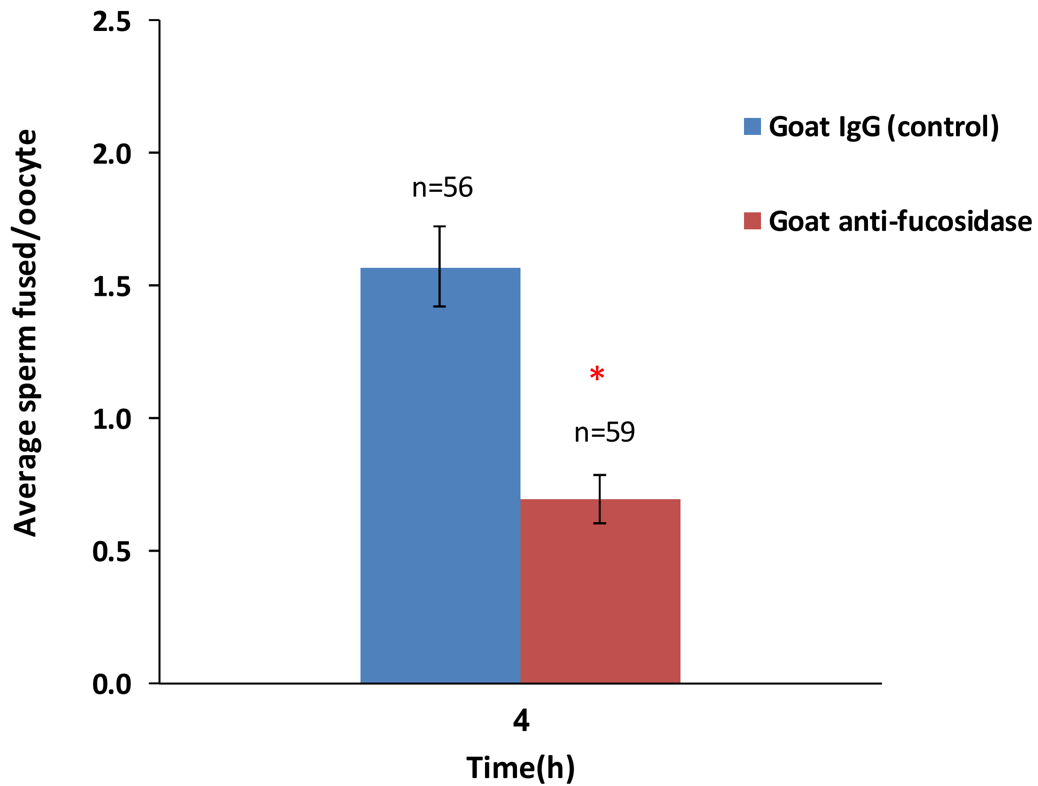

Figure 17. Average sperm fused to oocyte membrane of untreated and anti-fucosidase pretreated sperm at 4 h. Zona-free mouse oocytes were inseminated with either IgG control or anti-fucosidase pretreated capacitated sperm. The number of sperm fused per oocyte was quantified at 4 h post insemination for each oocyte. An asterisk indicates p-value <0.05 compared to control. Error bars represent standard error of the mean.

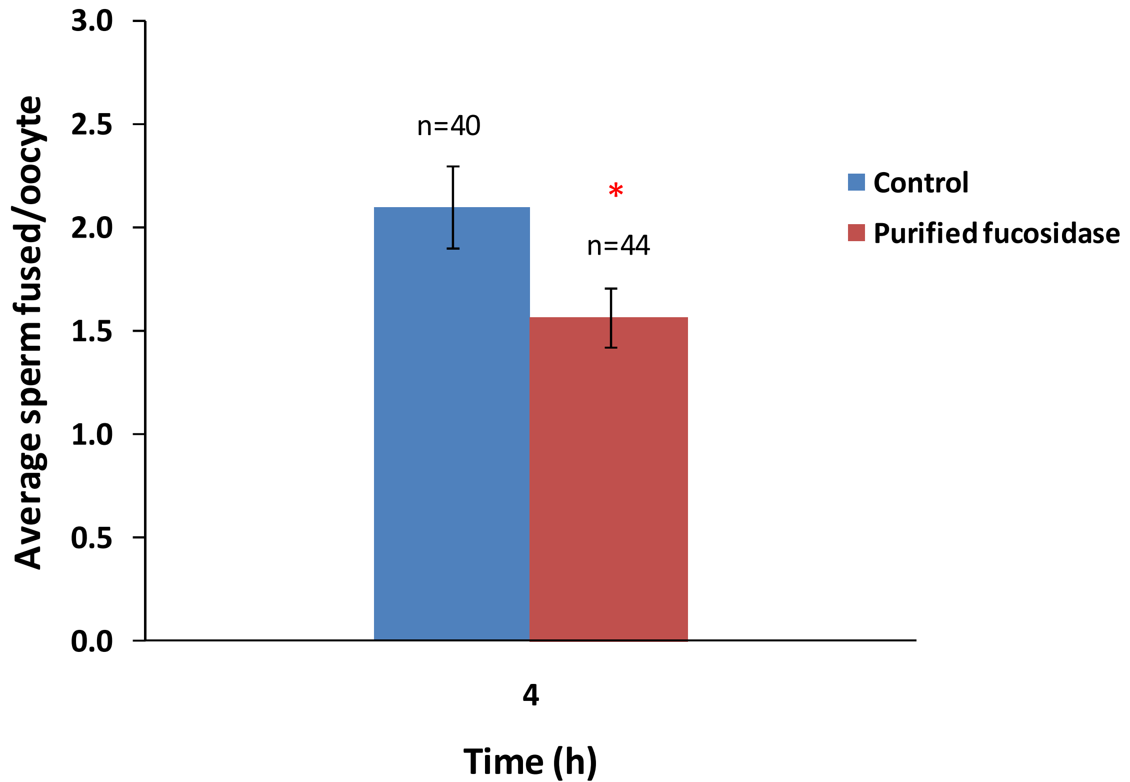

Figure 18. Average sperm fused to oocyte membrane of untreated and purified human liver α-L-fucosidase pretreated oocytes at 4 h. Zona-free mouse oocytes were pretreated with either control or purified human liver fucosidase prior to insemination with capacitated sperm. The number of sperm fused per oocyte was quantified at 4 h post insemination for each oocyte. An asterisk indicates p-value <0.05 compared to control. Error bars represent standard error of the mean.

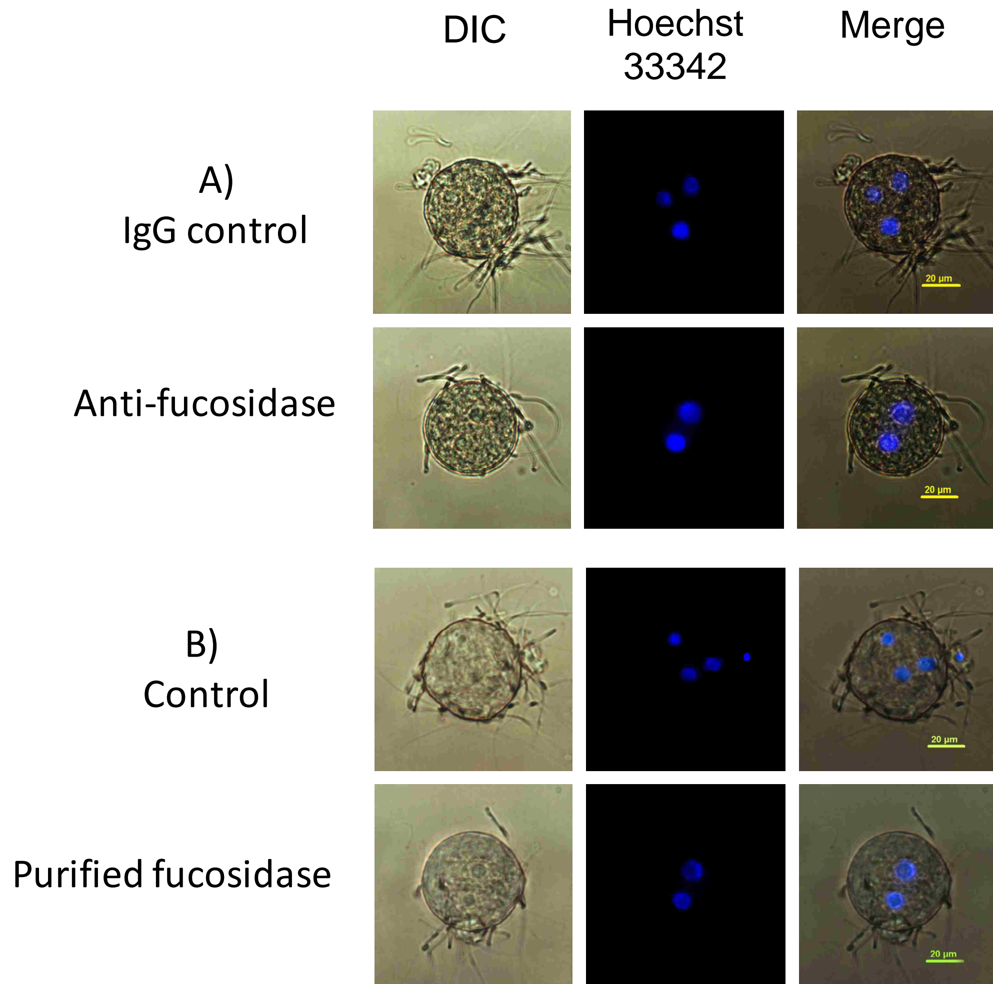

Figure 19. Penetration of sperm into oocytes pre-loaded with Hoechst 33342 (blue) 4 h after insemination: A) Anti-fucosidase pretreated sperm and B) Purified human liver fucosidase pretreated oocytes (scale bar = 20 µm).

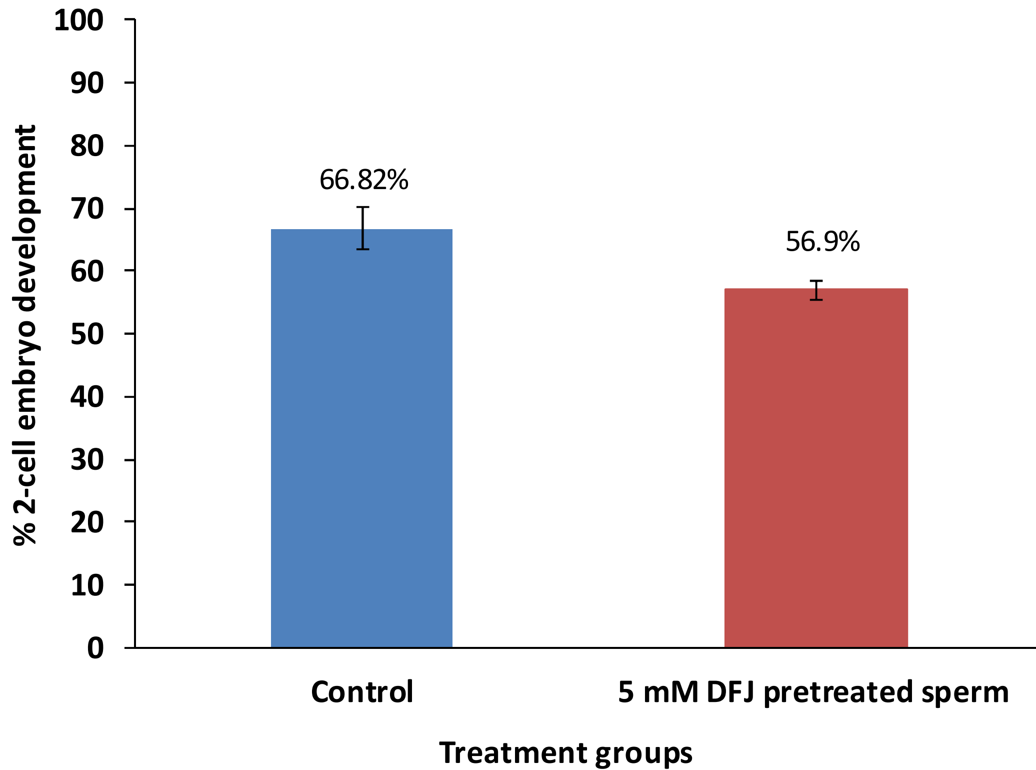

Figure 20. Percentage of 2-cell embryo development of intracytoplasmic sperm injection for untreated (20 oocytes) and 5 mM DFJ pretreated (21 oocytes) sperm. Control or 5 mM DFJ pretreated sperm was injected into an oocyte incubated at 37 ºC, 5% CO_2. The percentage of development to 2-cell embryos was quantified at 24 h post injection for all oocytes. Pretreatment of sperm with 5 mM DFJ showed no significant reduction of development to 2-cell embryos. Error bars represent standard error of the mean.

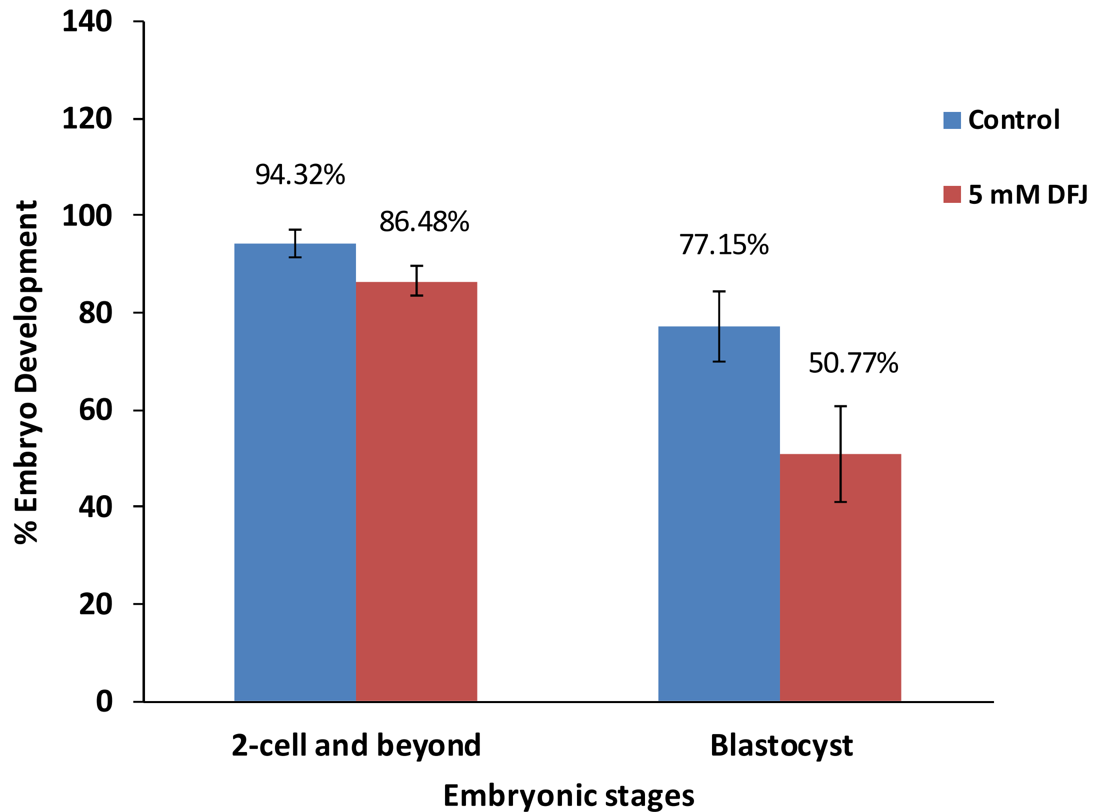

Figure 21. Percentage of 2-PN embryos that develop to 2-cell and blastocyst stages of control (142 embryos) and 5 mM DFJ treatment (147 embryos). 2-PN embryos were incubated in IVF droplets containing either 5 mM DFJ or control medium at 37 ºC, 5% CO_2. The percentage of development to 2-cell embryos and beyond was determined at 1 – 5 days. Inclusion of 5 mM DFJ in the IVF medium did not significantly inhibit development of 2-PN to blastocyst embryos. Error bars represent standard error of the mean.

Curriculum Vitae

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2006-2011	Royal Thai Government Scholarship for Ph.D. study.
2003	ISHAM Travel Award for “15th Congress of the International Society for Human and Animal Mycology (ISHAM)”, May 25-29, 2003. San Antonio,Texas, USA.

2002 National Science and Technology Development Agency (NSTDA) Scholarship for Master degree study.

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Publications

1. **Phopin K.**, W. Nimlamool, M. Bartlett, & B. Bean. Distribution, Crypticity, Stability and Localization of Alpha-L-Fucosidase of Mouse Cauda Epididymal Sperm., Submitted Manuscript.

2. **Phopin K**. (2003) "The ribosomal DNA restriction fragment length polymorphism (RFLP) patterns of isolated *Candida* spp. from King Chulalongkorn Memorial Hospital." Oct; ISBN 974-17-3836-6.

Conference Abstracts

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1. **Phopin, K**., W. Nimlamool, M. Bartlett, & B. Bean, 2011. Distribution, crypticity, and stability of alpha-L-fucosidase in mouse cauda epididymal sperm and fluid. J. Andrology 32, in press [abstract #31]

2. Nimlamool, W., **Phopin, K**., & B.Bean, 2011. Dynamic relocalization of alpha-L-fucosidase of mouse sperm during spermatogenesis and the acrosome reaction. J. Andrology 32, in press [abstract #30]

3. Kammarnjassadakul P, **Phopin K**, Kanbe T and, Chindamporn A., The Antibody Patterns Against Phosphomanoprotein of *C. albicans* in HIV-Seropositive Individual in Thailand, The 15th Congress of the International Society for Human and Animal Mycology (ISHAM), May 25-29, 2003. San Antonio, Texas, USA.

4. Thaweephon T, **Phopin K**, Yumyaun P, Chindamporn A., Chromosomal Size Polymorphism in *Candida albicans* Isolated from HIV-infected Patients, "1st Asian Congress of Pediatric Infectious Diseases (ACPID), Nov. 10-13, 2002. Thailand.

References

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