Lysophosphatidic acid signalling in human sperm viability

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August 2022

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Masters of Science

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Abbreviations

AA	Arachidonic Acid
AKT	Protein Kinase B
ATP	Adenosine Triphosphate
BAD	BCL-2-Associated Death Promoter
BF	Bright Field
BSA	Bovine Serum Albumin
BWW	Biggers, Whitten and Whittingham Medium
Cys	Cysteine
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
ECL	Enhanced Chemiluminescence
EGFR	Epidermal Growth Factor Receptor
Fluo	Fluorescent
HBS	HEPES Buffered Saline
HOST	Hypo-osmotic Swelling Test
HRP	Horseradish Peroxidase
IgG	Immunoglobulin G
LPA	Lysophosphatidic Acid
LPAR	Lysophosphatidic Acid Receptor
MJ33	1-hexadecyl-3-(trifluoroethyl)-sn-glycero-2-phosphomethanol Lithium Salt
OAG	1-Oleoyl-2-acetyl-sn-glycerol
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PEN	D-Penicillamine
PI	Phosphatase Inhibitors
PI3K	Phosphatidylinositol-3-kinase
pH	Potential Hydrogen
PKA	Protein Kinase A
PKC	Protein Kinase C
PLA_2	Phospholipase A_2

PLC	Phospholipase C
PP	Protein Phosphatases
PRDX	Peroxiredoxin
PS	Phosphatidylserine
RTK	Receptor Tyrosine Kinases
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TTBS	Tris-Buffered Saline with 0.1% Tween 20
WHO	World Health Organization

Contribution

All experiments were done by the MSc candidate Haoyu Liao. Dr. Cristian O'Flaherty conceived of and designed the research. Haoyu Liao contributed to the experimental design.

Original contributions to scientific knowledge:

- The LPA signalling regulates human sperm viability by activating the PI3K/AKT pathway.
- The inhibition of the PKC leads to the decrease of PI3K and AKT-S phosphorylations in human spermatozoa and causes apoptotic-like changes in human spermatozoa.
- The addition of OAG overrides the inhibition of PLC and prevents the decrease of PI3K and AKT-S phosphorylations.

Acknowledgements

The completion of my MSc. Experimental Medicine degree is an important moment in my life. This accomplishment would be impossible without the support and help of numerous people. Therefore, I would like to take this opportunity to express my appreciation to them.

Firstly, I would like to extend my sincerest gratitude to my research supervisor, Dr. Cristian O'Flaherty. Even though my previous study is not in the reproduction field and I am not good at English, thank you so much for giving me a chance to be welcomed as a member of your laboratory. Under your unconditional patience, guidance and suggestion, I have had many opportunities to attend conferences and seminars to improve my writing and presentation.

I would also like to thank Dr. Connie (Tita) San Gabriel. Your fantastic knowledge of spermatozoa was beneficial in the progress of my projects. Your kind suggestions assist the troubleshooting of my experiments. To Ms. Marie France Lusignan, I appreciate you for helping me familiarize myself with using the microscope. I had gained much knowledge when I discussed with you two every time. My projects would not be completed without your help.

Next, I would like to thank my Research Committee members, Dr. Shafaat Rabbani, Dr. Terry Hebert, and Dr. Carlos Morales, for providing insightful comments that inspired and enhanced my study's related knowledge. Thank all of you for being there and listening to my presentation despite your busy schedules.

To Edrian, the first and the best friend at this lab since I started my master's degree life, I appreciate you for treating me so kindly. No matter what kind of questions I had, you were always patient in answering me. This is the greatest heartening for me, whose mother tongue is not French and English. I will not forget forever every chat and some little trips

we have.

To Steven, I have always admired your productivity and your efficiency in your research. Thank you for assisting me with the lab work when I was busy. To John and Diego, I wish you good luck with everything.

I look forward to meeting you all whenever in the future.

Finally, I would like to take this opportunity to thank my family, friends and my undergraduate supervisors. Under their support and motivation, I can keep it up and finish my MSc. degree.

Thank you all!

Abstract

Lysophosphatidic acid (LPA) signalling is essential to maintain germ cell viability during spermatogenesis. We previously demonstrated that inhibiting peroxiredoxin 6 (PRDX6) phospholipase A_2 activity impairs sperm cell viability. Exogenous addition of LPA bypassed the inhibition of PRDX6 $iPLA_2$ activity and maintained the active PI3K/AKT pathway. An active LPA signalling pathway and its regulation by kinases in mature spermatozoa are unknown. Thus, we hypothesize that LPA signalling is active in spermatozoa and regulated by protein kinases. The main goal of this thesis was to characterize the LPA signalling pathway in human spermatozoa and establish its role in maintaining sperm viability. Our objectives were: 1) to determine the presence of LPA receptors (LPAR) in human spermatozoa and whether the LPAR signalling activates the PI3K/AKT pathway; and 2) to study the regulation of the LPA signalling by protein kinases and phospholipase C (PLC).

LPAR1 and LPAR6 were localized on the plasma membrane, and the LPAR1 was also found in the Triton-insoluble fraction of human spermatozoa by subcellular fractionation and immunoblotting using specific antibodies. LPAR1, 3, 5, and 6 were found in the tail and midpiece of the non-permeabilized spermatozoon determined by immunocytochemistry. LPAR1 was also detected in the post-acrosomal and acrosome regions in methanol-permeabilized spermatozoa. LPAR3 and LPAR5 were found in the equatorial segment and acrosome region, respectively. Immunoblotting experiments revealed that phospho-PI3K and phospho-AKT substrates were found in cytosolic and Triton-insoluble fractions. Interestingly, most of the phospho-AKT substrates were found in the Triton-insoluble fraction. Sperm viability (determined by Hypoosmotic Swelling Test), phospho-PI3K and phospho-AKT substrates were inhibited by Ki16425, Chelerythrine, and U73122, inhibitors of LPAR1-3, PKC, and PLC, respectively. We found that adding 1-oleoyl-2-acetyl-sn-glycerol (OAG), a cell-permeable analog of diacylglycerol (DAG), prevented the loss of

sperm viability and maintained the phosphorylation status of PI3K.

In conclusion, these results suggest the presence of an active LPA pathway regulated by PKC, receptor-type PTK and PLC, which activate the PI3K/AKT pathway to prevent apoptotic-like changes and maintain sperm viability in human spermatozoa. This research will help decipher the causes of sperm dysfunction associated with male infertility and design novel treatments to help infertile men achieve fatherhood.

Résumé

La signalisation de l'acide lysophosphatidique (LPA) est importante pour maintenir la viabilité des cellules germinales pendant la spermatogenèse. Nous avons précédemment démontré que l'inhibition de l'activité de la phospholipase A_2 de la peroxyredoxine 6 (PRDX6) altère la viabilité de spermatozoïdes. L'addition exogène de LPA a contourné l'inhibition de l'activité PRDX6 $iPLA_2$ et a maintenu active la voie de signalisation de PI3K/AKT. Actuellement, la présence d'une voie de signalisation active de LPA et sa régulation par des kinases dans les spermatozoïdes matures est inconnue. Ainsi, nous présumons que la signalisation LPA est active dans les spermatozoïdes et régulée par des protéines kinases. Le but principal de cette thèse était de caractériser la voie de signalisation de LPA dans les spermatozoïdes humains. Nos objectifs étaient les suivants : 1) déterminer la présence de récepteurs LPA (LPAR) dans les spermatozoïdes humains et si la signalisation LPAR active la voie PI3K/AKT ; et 2) étudier la régulation de la signalisation LPA par les protéines kinases et la phospholipase C (PLC).

LPAR1 et LPAR6 ont été localisés sur la membrane plasmique et le LPAR1 a également été trouvé dans la fraction Triton-insoluble des spermatozoïdes humains par fractionnement subcellulaire, et immunoblotting utilisant des anticorps spécifiques. LPAR1, 3, 5, 6 ont été trouvés dans la queue et le midpiece du spermatozoon par immunocytochemistry. En outre, LPAR1 a été également détecté dans la région acrosomal et acrosome de poteau dans les spermatozoïdes méthanol-perméabilisés. LPAR3 et LPAR5 ont été trouvés dans le segment équatorial et la région acrosome, respectivement. Les expériences immunoblotting ont indiqué que les substrats phospho-PI3K et phospho-AKT ont été trouvés dans les fractions cytosoliques et Triton-insolubles. Intéressant, la plupart des substrats de phospho-AKT ont été trouvés dans la fraction Triton-insoluble. La viabilité de sperme (déterminée par essai hypoosmotic de gonflement), les substrats de phospho-PI3K et de phospho-AKT ont été

empêchés par Ki16425, Chelerythrine, et U73122, inhibiteurs de LPAR1-3, de PKC, et de PLC, respectivement. Nous avons constaté que l'addition de 1-oleoyl-2-acetyl-sn-glycérol (OAG), un analogue cellule-perméable de diacylglycerol (DAG), a empêché la perte de viabilité de sperme et a maintenu le statut de phosphorylation de PI3K.

En conclusion, ces résultats suggèrent la présence d'une voie active de LPA qui est principalement réglée par PKC, récepteur-type PTK et PLC qui activent la voie PI3K/AKT pour empêcher apoptotic-comme des changements et maintenir la viabilité de sperme dans les spermatozoïdes humains. Cette recherche aidera à déchiffrer les causes du dysfonctionnement du sperme associé à l'infertilité masculine et à concevoir de nouveaux traitements pour aider les hommes infertiles à atteindre la paternité.

Introduction

Male Infertility

Infertility is a significant health and social problem that affects men worldwide. Almost 8-12% of couples suffer from complications related to infertility (de Kretser 1997, Kumar and Singh 2015). Moreover, the male factor is the cause of approximately half of the infertility cases (Boivin, Bunting et al. 2007, Kumar and Singh 2015, Longo, Forleo et al. 2019). Male infertility is a multifactorial disorder with various phenotypes, from normal sperm count and morphology to low or absent sperm counts and the presence of unmatured sperm cells because of abnormal spermatogenesis (de Kretser 1997). Many conditions, such as varicocele, hypogonadism, urogenital tract infection, cryptorchidism, ageing, drugs, lifestyle and trauma, can affect male fertility (Sharpe 2000, Cavallini 2006, Harris, Fronczak et al. 2011). Varicocele is the dilation of the pampiniform plexus veins that produces an increase in intratesticular temperature and influences the process of spermatogenesis, which relates to male infertility (Pastuszak and Wang 2015). The failure of testes to descend into the scrotum properly is defined as cryptorchidism, and it is associated with male infertility and testicular cancer (Fawzy, Hussein et al. 2015). In addition, 30-40% of male infertility cases cannot be explained (idiopathic infertility) (Figure 1) (Jungwirth, Giwercman et al. 2012, Longo, Forleo et al. 2019).

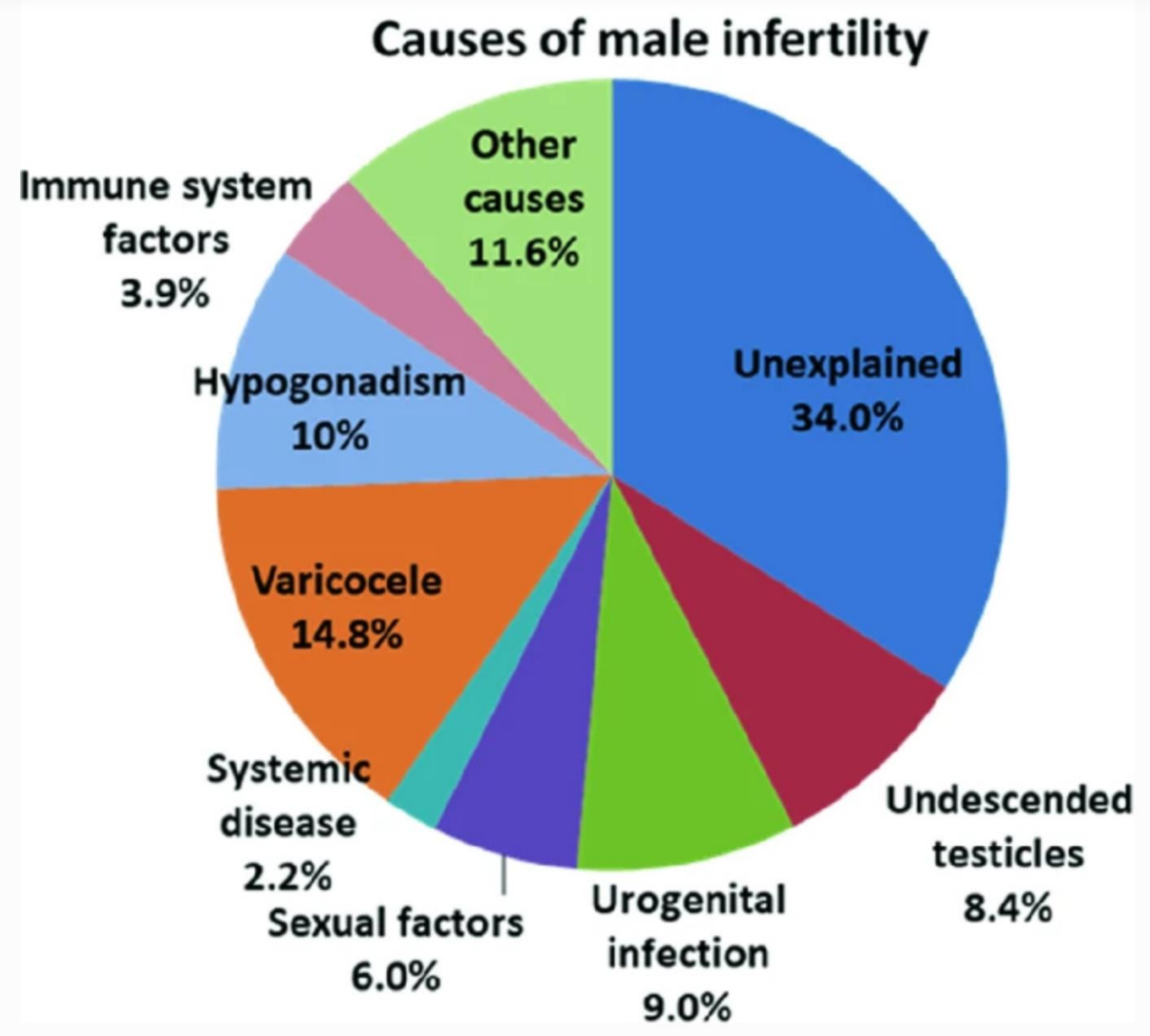


Figure 1 : Percentage of the male infertility causes.
The figure was adapted from(Longo, Forleo et al. 2019).

In vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are often options for treating infertile couples. Still, the success rate of these methods is approximately 30-40% in obtaining a live birth (Zini and Chokron 2004, Merchant, Gandhi et al. 2011, Said and Land 2011). By selecting the spermatozoa based on morphology and motility to perform these artificial reproductive technologies (ARTs), the conceived child may have a higher risk of defects than the children conceived naturally (Hansen, Kurinczuk et al. 2002, Zini and Sigman 2009, Zheng, Chen et al. 2018).

Sperm Morphology

Spermatozoa are the end product of spermatogenesis proceeding in the seminiferous tubules of the testes. Spermatozoa are highly differentiated haploid cells in structure and function through the acrosome formation, nuclear condensation, flagellar development, and shedding of the excess of cytoplasm during the process of spermiogenesis. The main goal of the spermatozoa is to deliver the paternal genome to the oocyte through the process of fertilization, to produce the embryo that will originate a new individual (Yanagimachi 1994, Toshimori and Eddy 2015). The spermatozoon comprises the head and the tail (flagellum), which connect by the neck or connecting piece (Figure 2). The head of a spermatozoon contains the nucleus, acrosome, a small amount of cytoplasm and the cytoskeleton. The sperm DNA is highly condensed due to the presence of protamines. Protamines, replace the histones and play a vital role in the chromatin condensation of spermatozoa because of their small size and the high level of cysteine to form disulfide bonds. Due to the compactness of spermatozoal chromatin, protamine prevents the spermatozoa from DNA damage caused by toxicants and oxidative stress (Steven Ward and Coffey 1991, Gimenez-Bonafé, Ribes et al. 2002, Akmal, Aulanni'am et al. 2016). The sperm head can be divided into the acrosome, the equatorial segment, and the post-acrosomal region. In these regions, the plasma membrane has different components needed for each region's special function. The acrosome is a vesicle derived from the Golgi apparatus. It contains hydrolytic enzymes necessary to assist the spermatozoa in penetrating the cumulus cells and zona pellucida of the oocyte (Yanagimachi 1994, Toshimori and Eddy 2015). During the acrosome reaction, controlled exocytosis, the outer acrosomal membrane fuses with the plasma membrane to release hydrolytic enzymes such as acrosin and hyaluronidase. The plasma membrane on the equatorial segment fuses with the oolemma to allow the fertilization of the oocyte

(Yanagimachi 1994, Gadella and Evans 2011, Toshimori and Eddy 2015, Vazquez-Levin and Verón 2018).

The flagellum comprises the midpiece (containing the mitochondria), principal piece and end piece, and it is involved in sperm movement. The mitochondrial sheath is the helical and highly wrapped structure in the midpiece enclosing sperm mitochondria. The mitochondria produce or generate the essential energy for movement and phosphorylations via the tricarboxylic acid (TCA) cycle and the oxidative phosphorylation or respiratory chain (Osellame, Blacker et al. 2012, Amaral, Lourenço et al. 2013). The sperm axoneme is composed of 9+1 pairs of microtubules formed by α- and β-tubulin monomers (Ishikawa 2017). A cross-section of a representative segment of the principal piece demonstrates that the axoneme is surrounded by nine outer dense fibers inside the fibrous sheath (Toshimori and Eddy 2015). The fibre sheath contains different enzymes, including those belonging to the glycolysis, necessary to generate energy and protein kinase A anchoring proteins. The end-piece is only composed of the axoneme.

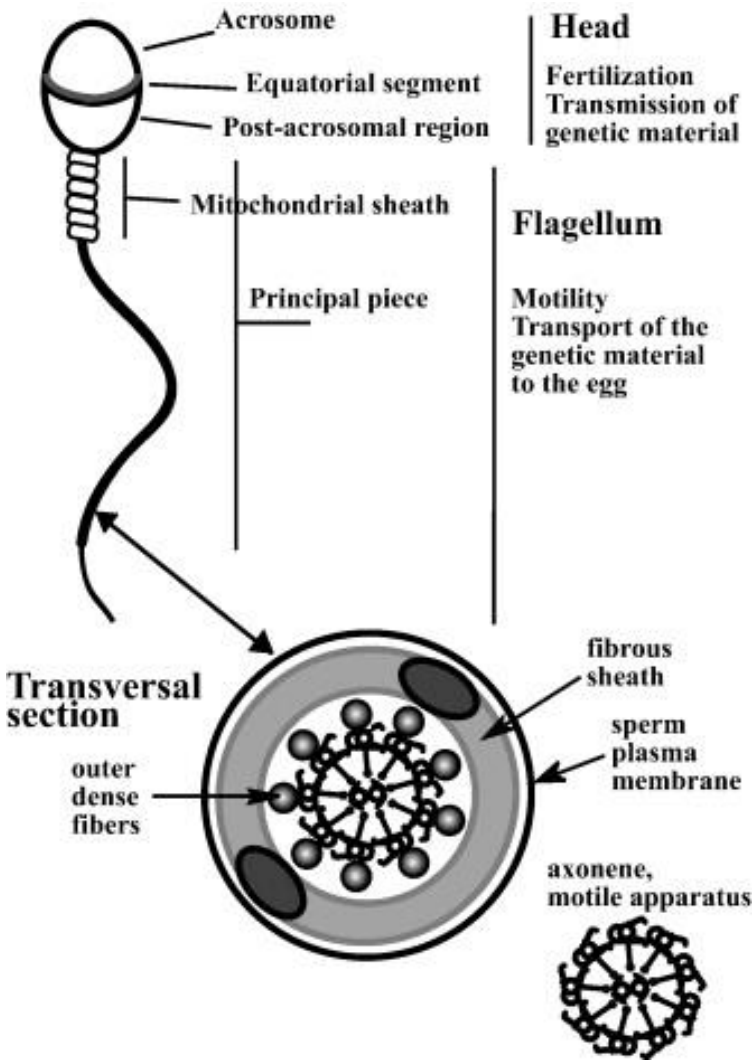


Figure 2: The general structure of the mammalian spermatozoon.
The figure was taken from (de Lamirande and O'Flaherty 2008).

Spermatogenesis, Maturation and Capacitation

Spermatogenesis is the process by which the male germ cells ultimately differentiate from spermatogonia into spermatozoa (Hunter, Anand-Ivell et al. 2012, Linn, Ghanem et al. 2021). The process of spermatogenesis, which takes place in the seminiferous tubules of the testes, can be divided into three steps: spermatocytogenesis, spermiogenesis, and spermiation (O'Donnell, Nicholls et al. 2011, Hunter, Anand-Ivell et al. 2012, Sharma and Agarwal 2013).

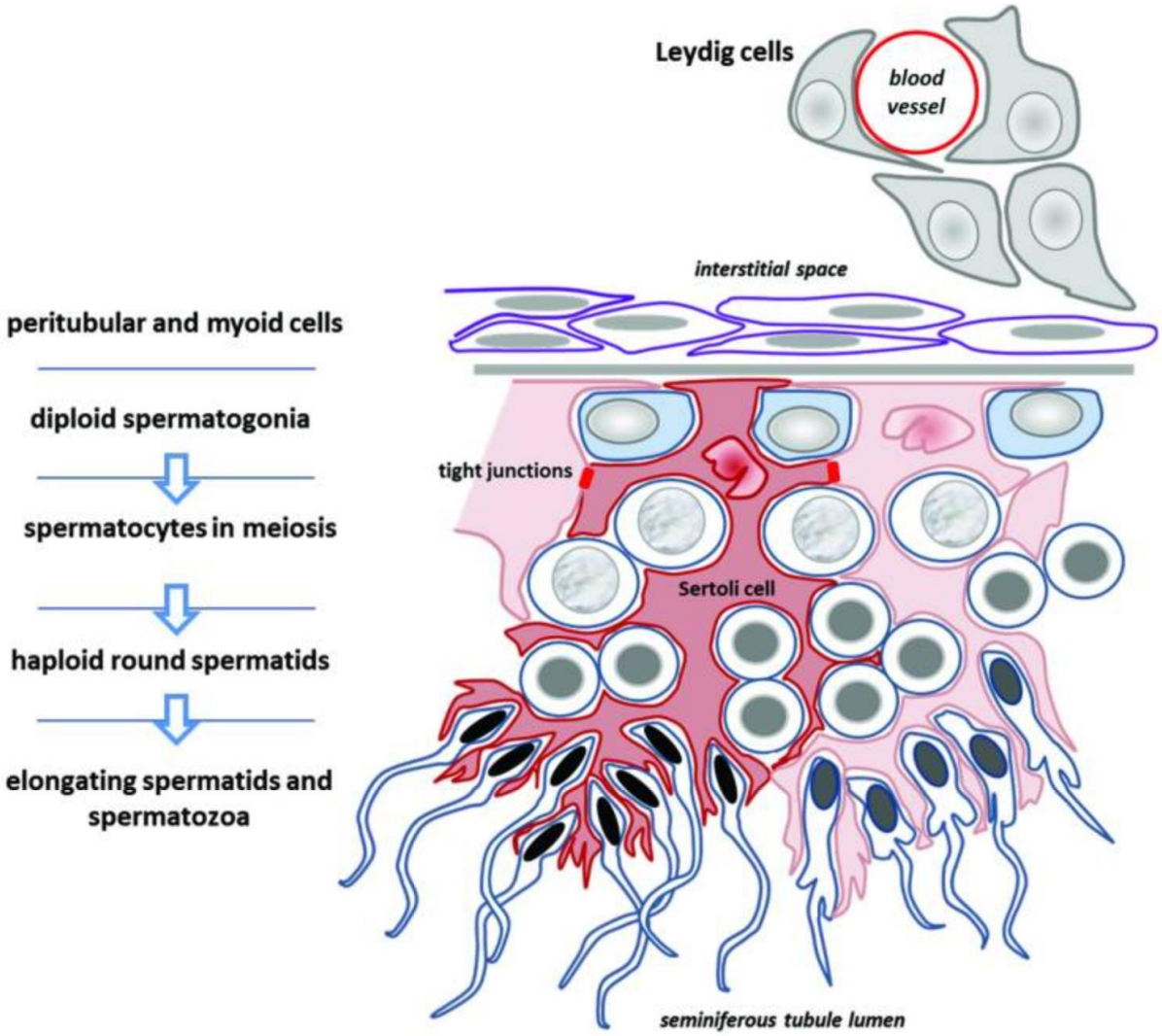


Figure 3: A diagram to illustrate the essential structures during spermatogenesis
The figure was taken from (Hunter, Anand-Ivell et al. 2012)

Firstly, the spermatogonia increase the cell numbers and develop into diploid spermatocytes via mitosis during spermatocytogenesis (Johnson, Varner et al. 2000, Nishimura and L'Hernault 2017). After cell division of meiosis I, the spermatocytes turn into haploid secondary spermatocytes (Figure 3). Then, through meiosis II, the haploid secondary spermatocytes develop into haploid spermatids, which become the spermatozoa (Hunter, Anand-Ivell et al. 2012, Feng, Bowles et al. 2014, Nishimura and L'Hernault 2017). Leydig cells, found in the interstitium of the testes, secret the testosterone to drive spermatogenesis (Clermont 1963, Sharpe, Maddocks et al. 1990). During spermiogenesis,

the round spermatids' shape changes, enlarging the tail and removing the excess cytoplasm in the head by Sertoli cells (McLachlan, O'Donnell et al. 2002). And the acrosome is from the Golgi apparatus (McLachlan, O'Donnell et al. 2002, Toshimori and Eddy 2015). After spermiogenesis, there is spermiation, the process by which the fully differentiated spermatozoa are released into the lumen of seminiferous tubules and then transported to the epididymis (Griswold 1998, Robaire and Hinton 2015). In the epididymis, the spermatozoa acquire the ability to move and the fertilizing ability. Besides absorbing excess cytoplasm, the Sertoli cells protect and support the germ cells during spermatogenesis (Griswold 1998, Griswold and McLean 2006).

At ejaculation, mature spermatozoa are mixed with secretions from seminal vesicles and prostate containing nutrients and antioxidant enzymes to protect the spermatozoa (Yanagimachi 1994, Robert and Gagnon 1999). Then, the spermatozoa reside in the oviduct of the female reproductive tract to undergo capacitation, a complex biochemical process to prepare the spermatozoon to recognize and fertilize the oocyte (Yanagimachi 1994, de Lamirande, Leclerc et al. 1997, de Lamirande and O'Flaherty 2012, Alwaal, Breyer et al. 2015).

Sperm capacitation is a reversible phenomenon that takes approximately 4-6 hours in humans (Yanagimachi 1994, O'Flaherty, de Lamirande et al. 2006, de Lamirande and O'Flaherty 2012). During this process, low levels of ROS are generated that are essential to trigger and regulate several signalling pathways, including PKA, PKC, ERK, PI3K/AKT pathways that ultimately will activate tyrosine kinases and thus, increase tyrosine phosphorylation in the spermatozoon (O'Flaherty, de Lamirande et al. 2006, de Lamirande and O'Flaherty 2012). The regulation of phosphorylations will promote other changes, such as efflux of cholesterol and an influx of calcium ions in the sperm head and flagellum, increasing membrane fluidity to allow the acrosome reaction (Yanagimachi 1994, de

Lamirande and O'Flaherty 2012). The acrosome reaction, which requires the influx of calcium ions and low levels of ROS, a release of hydrolytic enzymes, allows the spermatozoon to penetrate the zona pellucida and fuse with the oolemma (Yanagimachi 1994, Fraser 1998, de Lamirande and O'Flaherty 2012).

Sperm survival

The survival of the spermatozoon is essential to ensure viability during the entire life of the cell, from its formation during spermatogenesis, its maturation in the epididymis and finally, during its residence in the female reproductive tract to undergo capacitation and fertilize the oocyte. During spermatogenesis, abnormal spermatozoa undergo apoptotic-like changes that tag them to be eliminated by Sertoli cells (Ramos-Ibeas, Pericuesta et al. 2013). The apoptotic-like changes in spermatozoa are poorly understood. They are different from the apoptosis occurring in somatic cells because of the triggering factors and the absence of expression of proteins associated with apoptosis (Aitken, Baker et al. 2015). After the spermatozoon leaves the testes, it could also undergo an apoptotic-like cascade, including the loss of motility, lipid peroxidation, caspase activation, phosphatidylserine externalization, and oxidative DNA damage (Wang, Sharma et al. 2003, Ramos-Ibeas, Pericuesta et al. 2013, Aitken, Baker et al. 2015). These damages could be related to the abnormal production of ROS in mitochondria that drive the oxidative damage in lipids, proteins and DNA in spermatozoa (Aitken and Baker 2013). Lipid peroxidation dysregulates mitochondria electron transport, increasing ROS levels, exacerbating the oxidative stress and generating more lipid peroxidation and oxidative damage in spermatozoa (Alvarez and Aitken 2012, Aitken, Baker et al. 2015, Fernandez and O'Flaherty 2018). Besides this, the high levels of ROS activate the caspases by disrupting mitochondrial membranes and releasing cytochrome c (Agarwal, Makker et al. 2008).

Typically, phosphatidylserine is not found on the outer layer of the sperm plasma membrane (Barroso, Morshedi et al. 2000). After the activation of caspases and the elevated level of mitochondrial ROS in the apoptosis-like cascade, the spermatozoa display the phosphatidylserine translocation (Agarwal, Makker et al. 2008, Aitken and Baker 2013). The last event of this cascade is DNA damage. Because of spermatozoa structure, H_2O_2 is the only apoptotic product that can move from the sperm midpiece to the nucleus, probably using aquaporin 8, which is present in human spermatozoa (Pellavio and Laforenza 2021). DNA damage oxidatively induced is associated with forming 8-hydroxy-2'-deoxyguanosine (8OHdG) (De Iuliis, Thomson et al. 2009, Aitken and Koppers 2011, Aitken, Baker et al. 2015). The spermatozoa with apoptotic-like changes are removed by phagocytosis without producing pro-inflammatory cytokines in the male or female reproductive tract (Koppers, Mitchell et al. 2011, Aitken and Baker 2013).

The sperm viability can be commonly evaluated by the hypo-osmotic swelling test (Ramu and Jeyendran 2013). Under the hypo-osmotic stress exposure, the spermatozoa will attempt to reach osmotic equilibrium. The most susceptible part of the sperm to hypo-osmotic stress is the tail, and hence the sperm tail's different swelling pattern can be an indicator of the viability of the sperm. Using microscopy to observe the swelling pattern of the sperm tail and determine the sperm viability. Once there is damage to the outer membrane, the tail will not swell but be straight (Jeyendran, Van der Ven et al. 1984, Ramu and Jeyendran 2013). Using fluorescent dyes, such as Sytox Green and Propidium Iodide, is another way to assess sperm viability. Based on the damage to the outer membrane, those fluorescent molecules enter the spermatozoa and bind to the compacted DNA in the sperm head. It can be quantified via flow cytometry (Martínez-Pastor, Mata-Campuzano et al. 2010, Moawad, Fernandez et al. 2017).

The primary prosurvival signalling in human spermatozoa is the PI3K/AKT pathway

(Koppers, Mitchell et al. 2011). Inhibition of the phosphorylation of PI3K or AKT leads spermatozoa to apoptotic-like changes, such as sperm motility and viability decrease, an increase in lipid peroxidation and DNA damage (fragmentation and oxidation), etc. The dephosphorylation of AKT prevented the activation of its downstream substrates, such as BAD (Koppers, Mitchell et al. 2011). The level of lipid oxidation, one of the steps in the apoptotic-like cascade, is regulated by $iPLA_2$ activity of Peroxiredoxin 6 (PRDX6), which is the primary antioxidant defence in human spermatozoa. The $iPLA_2$ activity maintains sperm viability by activating the PI3K/AKT pathway by releasing fatty acids and lysophospholipids, such as Lysophosphatidic acid (LPA), Arachidonic Acid (AA) (Murakami, Nakatani et al. 1997, Fernandez, Yu et al. 2019). It has recently been pointed out that LPA is a prosurvival factor during spermatogenesis in the mouse (Ye, Skinner et al. 2008). Other studies have also reported leptin and prolactin exert the pro-survival mechanism via phosphatidylinositol 3-kinase (PI3K) / protein kinase B (AKT) pathway on spermatozoa (Aquila, Rago et al. 2008, Pujianto, Curry et al. 2010).

LPA signalling

Lysophosphatidic acid (LPA) is a bioactive lysophospholipid that plays an essential role in multiple cellular mechanisms, such as cell proliferation and migration (Xiang, Lu et al. 2020). It widely exists in almost all mammalian cell types and is biosynthesized via two major routes (Aoki, Inoue et al. 2008, Choi, Herr et al. 2010). One is converting membrane phospholipid into phosphatidic acid by phospholipase D, then turning it into LPA species via phospholipase A1/A2(PLA1/2). The second way is using PLA1/2 cleaves membrane phospholipid into lysophospholipids, which metabolizes to LPA species by phospholipase D (Aoki, Inoue et al. 2008). LPA elicits a broad range of cellular mechanisms by acting on G protein-coupled receptors (GPCRs) on the plasma membrane (Xiang, Lu et al. 2020).

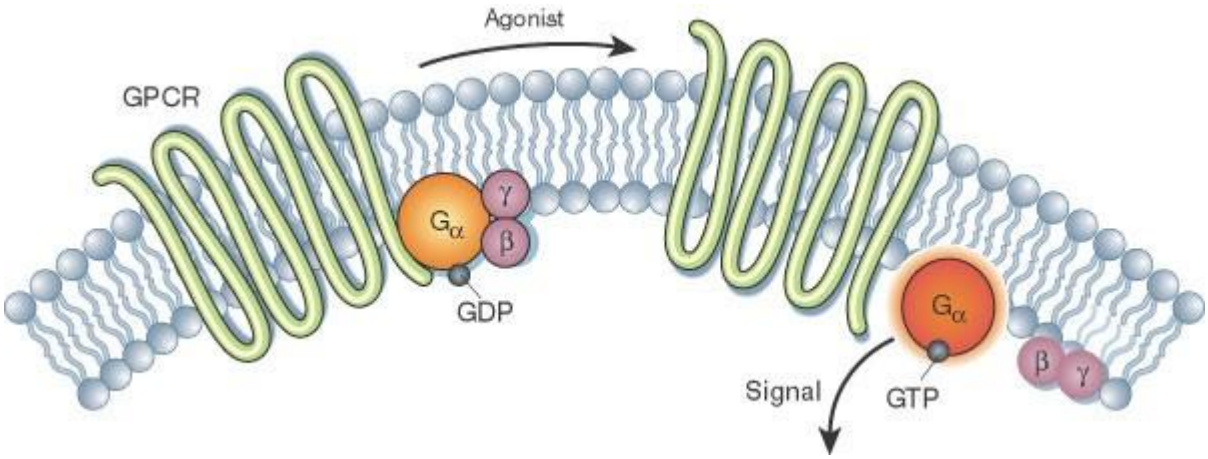


Figure 4: Activation of G protein.

The figure was adapted from (Li, Ning et al. 2002)

There are six currently known lysophosphatidic acid receptors (LPARs). Like other members of the GPCR family members, LPARs contained seven rhodopsin-like transmembrane domains. All LPARs exert their signal through Gα subunit families (Gα12/13, Gαq/11, Gαi/o and Gαs) to activate different downstream pathways. When the LPAR (GPCR) binds its ligand, the G protein is activated by exchanging the GDP bound to the G protein for a GTP. Gα with GTP can dissociate from Gβ and Gγ and induce intracellular signalling (Figure 4) (Li, Ning et al. 2002). Gα12/13 regulates the cell migration and cell invasion through RHO, Gαq/11 activates PLC to mediate Ca^{2+} homeostasis and Gαs increases cAMP concentration by inducing adenylyl cyclase. Lastly, Gαi/o keeps the survival signalling through the PI3K/AKT pathway (Figure 5).

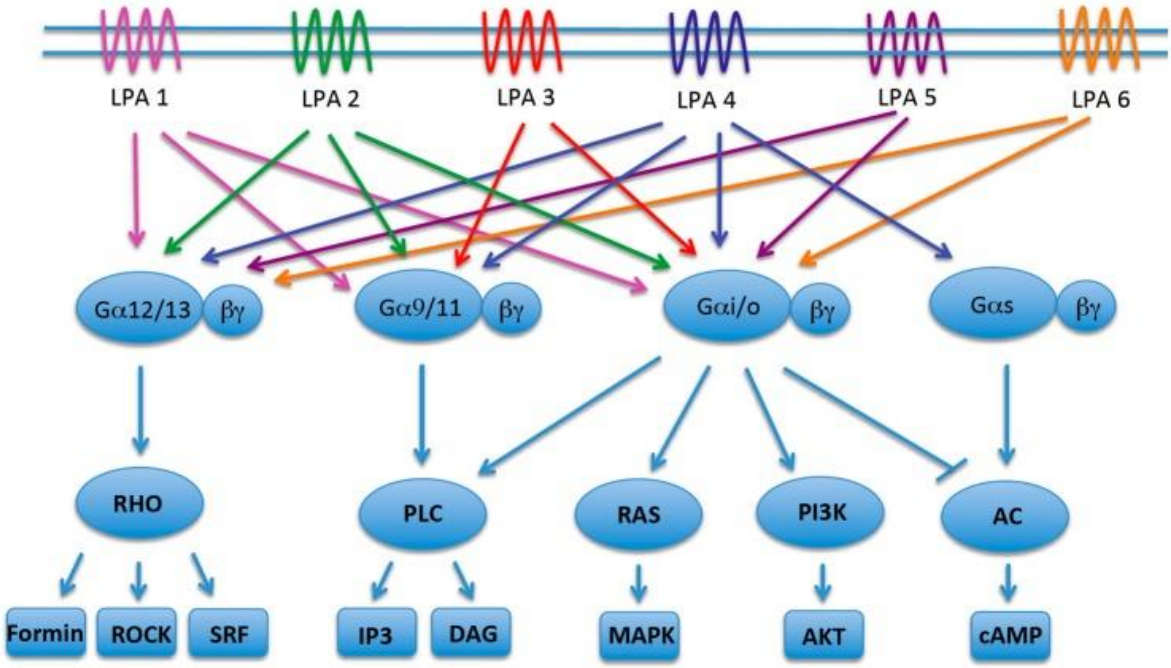


Figure 5: Cell surface LPAR and the downstream signalling pathway.
The figure was referred from(Riaz, Huang et al. 2016).

Based on their homology, LPARs can be divided into two groups. LPAR1-3 is identified as an endothelium differentiation gene (EDG) receptor which has 50-57% amino acid identity, while LPAR4-6 is referred to as a non-EDG receptor, which has 35-55% amino acid identity with each other (Riaz, Huang et al. 2016, Xiang, Lu et al. 2020). LPAR1-4 are expressed in the human testes, and LPAR1-3 are highly expressed in mice testes (Ye, Skinner et al. 2008, Yung, Stoddard et al. 2014). It has been previously demonstrated that the absence of LPAR1-3 in mice is associated with loss of germ cells and the reduction of sperm production (Ye, Skinner et al. 2008). Aging exacerbates the negative impact of the lack of LPAR1-3 in mouse spermatozoa (Ye, Skinner et al. 2008). However, whether the LPARs are present and the role of LPAR signalling in human spermatozoa are unknown.

Hypothesis and Objective

It has been reported that PRDX6 iPLA2 activity produces phospholipids and LPA and the sperm viability can be rescued when PRDX6 iPLA2 activity is inhibited (Fernandez, Yu et al. 2019). However, the presence of an active LPA signalling pathway in human spermatozoa is currently unknown. Thus, I hypothesized in this thesis that the LPA signalling is active in spermatozoa and regulated by protein kinases.

The objectives were: 1) to determine the presence of LPA receptors (LPAR) in human spermatozoa and whether the LPAR signalling activates the PI3K/AKT pathway; 2) to study the regulation of the LPA signalling by protein kinases and phospholipase C (PLC).

Materials and Methods

MATERIALS

Percoll was purchased from GE Healthcare (Baie d'Urfe, QC, Canada). Mouse monoclonal Anti-tubulin, rabbit polyclonal anti p-PI3K, and rabbit polyclonal anti-p-AKT substrate antibodies were purchased from Cell Signaling (Beverly, MA, USA). Donkey polyclonal anti-rabbit (H+L) and goat polyclonal anti-mouse (H+L) antibodies, both conjugated with horseradish peroxidase, were purchased from Jackson ImmunoResearch Laboratories, Inc (West Grove, Pennsylvania, USA). The enhanced chemiluminescence (ECL) Kit and goat polyclonal anti-rabbit (H+L) conjugated with AlexaFluor 555 were purchased from Thermo Fisher Scientific (Saint-Laurent, Québec, Canada). Rabbit polyclonal Anti-EDG2 (LPAR1), EDG7 (LPAR3), LPAR5 and LPAR6 antibodies, Ki16425 ≥98% (HPLC), U-73122, OAG were purchased from Sigma Aldrich (Oakville, ON, Canada). Other chemicals used were of at least reagent grade.

HUMAN SPERM PREPARATION

Semen samples were collected from healthy volunteers (20-30 years old) by masturbation after three days of abstinence period at the Royal Victoria Hospital (Montreal, QC, Canada) and incubated for 30 min at 37°C to allow liquefaction. Then, samples were placed on top of a 4-layer Percoll gradient (from bottom to top: 95%-65%-40%-20%) and centrifuged for 30 min at 3,500 rpm and 20°C. The highly motile sperm fraction found in the 95% layer and 65%-95% interface was obtained and sperm concentration was determined using a Neubauer chamber. Then, sperm suspension were diluted in Biggers, Whitten and Whittingham Medium, pH 7.95 (BWW medium; 91.5 mM NaCl, 4.6 mM KCl, 1.7 mM

$CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 5.6 mM D-glucose, 0.27 mM sodium pyruvate, 44 mM sodium lactate, 20 mM HEPES) (Biggers, Whitten et al. 1971) to 250 or 50 x 10^6 cell/mL were in BWW medium. The aliquots with 250 x 10^6 cell/mL were used for cellular fractionation and those at 50 x 10^6 cell/mL for the other experiments.

Sperm samples (50 x 10^6 sperm/mL) were incubated in BWW with or without the following inhibitors for 3.5 hours at 37°C: 50 & 100 μM H89 (PKA inhibitor), 50 & 100 μM chelerythrine (PKC inhibitor), 150 & 300 μM PD98059 (MEK inhibitor), 0.5 & 1 μM PP2 (Src, Non-Receptor Tyrosine Kinase inhibitor), 50 & 100 μM Tyrphostin A47 (EGFR, Receptor Tyrosine Kinase inhibitor), 2 & 5 & 10 & 20 μM–Ki16425, an inhibitor of LPAR1-3, to determine the regulation of the LPA signalling by kinases and the presence of LPARs in human spermatozoa. Spermatozoa were incubated for 3.5 h at 37°C with 10 μM of U-73122, an inhibitor of PLC to determine whether the LPAR signalling involves PLC activity to maintain sperm viability. Spermatozoa incubated with the PLC inhibitor were also incubated with 1-Oleoyl-2-acetyl-sn-glycerol (OAG), the PLC product, to bypass the inhibition and maintain the LPA signalling. The kinase inhibitors, Ki16425, U-73122 and OAG were dissolved in DMSO, which final concentration that never exceeded 1% (v/v) to prevent loss of motility (O'Flaherty, de Lamirande et al. 2005).

CELLULAR FRACTIONATION AND LOCALIZATION OF LPAR IN SPERMATOZOA

Spermatozoa at 250 x 10^6 cell/mL in BWW, supplemented with proteinases inhibitors, were fractionated in cytosolic, Triton-soluble, and Triton-insoluble fractions as done previously (O'Flaherty and de Souza 2011). Briefly, sperm samples were frozen at -20°C for 15 mins, and then thawed at room temperature to break the sperm membrane and allow the release of the cytosolic content. Then, samples were centrifuged at 12,000 xg at 4°C for 5

mins, and the supernatant (cytosol-enriched fraction) was collected. The remaining pellet was resuspended in HBS with 0.2% Triton-X100 and incubated for 10 mins on ice. The Triton-soluble fraction was collected from the supernatant after centrifuging the chilled sample at 12,000 xg at 4°C for 5 mins. The rest of the pellet (Triton-insoluble) was resuspended in HBS, and sonicated (three times for 15 sec each time at 30% output) with a Sonic Vibracell sonicator (Sonics and Materials Inc, Newtown, CT, USA). The cytosolic, Triton-soluble, and Triton-insoluble fraction were supplemented with the sample buffer with 100mM dithiothreitol (DTT) and phosphatase inhibitors (PI) (0.1mM Sodium Vanadate, 20mM Glycerol phosphate and 5mM Sodium Fluoride) then boiled for 5 mins at 95°C. Samples were centrifuged at 21,000 xg at 20°C for 5 mins. Then the supernatant of samples was loaded into 10% acrylamide gel.

SDS-PAGE AND IMMUNOBLOTTING

After 3.5 hours of incubation, samples treated with the different inhibitors were supplemented with sample buffer containing 100Mm DTT, 0.1mM Sodium Vanadate, 20mM Glycerol phosphate, and 5mM Sodium Fluoride, and then boiled for 5 mins at 95°C. Samples were centrifuged at 21,000 xg at 20°C for 5 mins. Then the supernatant of samples was loaded into 10% acrylamide gel. Sperm proteins were electrophoresed in 25 mM Tris base, 192 mM glycine, 0.1% SDS) and electrotransferred onto nitrocellulose membranes using transfer buffer (192mM glycine and 25 mM Tris, pH 8.3), containing 20% methanol, for 45 mins with constant voltage (100 volts). Then, the membranes were blocked in 5% skim milk in 2 mM Tris (pH 7.8)-buffered saline and 0.1% Tween 20 (TTBS) 1X for 1 hour at 20°C. The membranes were washed in TTBS 1X, and incubated overnight at 4°C in either polyclonal anti-p-PI3K antibody and anti-p-AKT substrates antibodies (1:2,000 and 1:1,000

in antibody buffer (TBS 1X, 0.1% Tween 20, 25 mg/ml BSA and ddH_2O), respectively). The following day, the membranes were washed with TTBS 1X. Then, they were incubated with the donkey anti-rabbit secondary antibody (1:2,500 diluted in TTBS) for 45 mins at room temperature and then washed with TTBS 1X. Finally, the membranes were soaked in ECL solution for 5 mins before detecting Positive immunoreactive bands by Amersham Imager 600 (GE Healthcare, USA). The quantification normalized each band's intensity to the respective intensity of tubulin (loading control) to obtain the relative intensity of each band.

SPERM VIABILITY DETERMINATION

Sperm viability was determined by the hypo-osmotic swelling test (HOS) with modifications (WHO 2010). Briefly, sperm samples were gently mixed with 150 µL hypo-osmotic (HOS) solution (1.5 mM fructose and 1.5 mM sodium citrate) after treatment and incubated at 37°C for 30 mins which slightly modified the previous method (Ramu and Jeyendran 2013, WHO 2021). Then, 10 µl of the samples were placed onto Superfrost plus slides (Fisher Scientific, Montreal, QC, Canada), covered with a coverslip and the edges sealed to avoid evaporation. Two hundred spermatozoa were analyzed from pictures taken with a Leica DFC 450C microscope at 200X magnification with Leica Application Suite X (LASX) software (Leica Microsystems, Wetzlar, Germany) to determine the percentage of viable spermatozoa (Figure 6, patterns b-g) and non-viable spermatozoa (Figure 6, pattern a). Only viable spermatozoa show different degrees of curly tail or presence of a droplet.

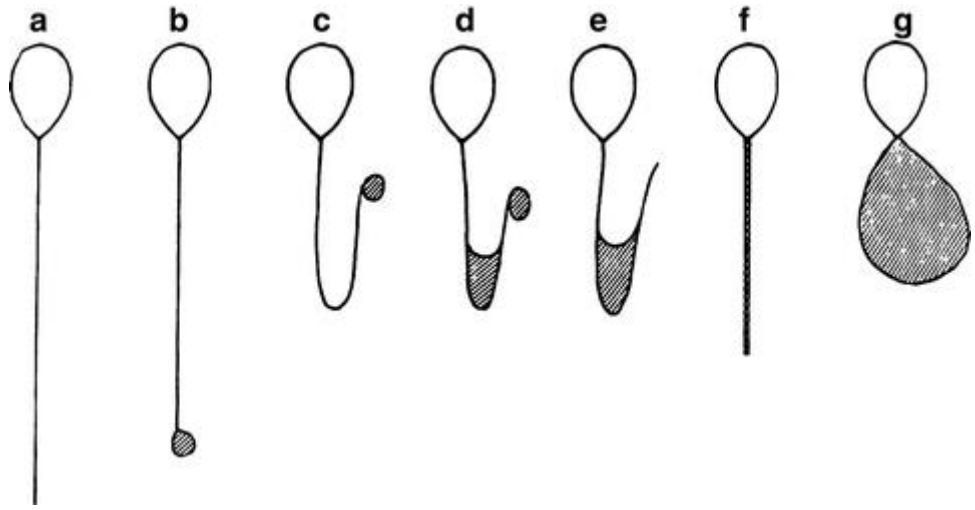


Figure 6: Schematic of morphological changes of human spermatozoa exposed under hypo-osmotic stress.

a) Sperm without altered morphology. b-g) Different morphology of the sperm tail swelling. The figure was adapted from (Jeyendran, Van der Ven et al. 1984).

IMMUNOCYTOCHEMISTRY

A 10 µL aliquot from each sperm sample was smeared onto the Superfrost plus slides (Fisher Scientific, Montreal, QC, Canada) and permeabilized with 100% methanol for 10 mins at -20°C. A group of samples were not permeabilized to determine the labelling on the plasma membrane. Then, samples were dried at room temperature. A circle was drawn onto each slide using ImmedgePen. Samples were rehydrated with PBS supplemented with or without Triton-X100 (PBS-T)/(PBS) and blocked with 5% goat serum in PBS-T/PBS for 30 mins at 20°C. Slides were washed with PBS-T/PBS and incubated overnight at 4°C with anti-LPAR1, 3, 5 or 6 antibodies (1:50, 1:10, 1:10, 1:10 dilution in 1% goat serum in PBS-T/PBS, respectively) and anti-P-PI3K (1:100 dilution in 1% goat serum in PBS-T) or anti-P-AKT substrates antibodies (1:100 dilution in 1% goat serum in PBS-T). The following day, samples were washed and incubated with goat anti-rabbit (H+L) antibody conjugated with AlexaFluor 555 (1:2,000 dilution in PBS-T + 1%BSA) for 1 hour at 20°C. After washing with PBS-T, ProLong Antifade with DAPI was added to the samples, and they were covered

and the edges sealed to avoid evaporation. Negative controls were prepared by omitting the primary antibody to confirm the absence of non-specific binding. Pictures showing LPARs labelling were taken using a Carl Zeiss Axiophot microscope (Oberkochen, Germany) at 1,000X magnification. Leica DFC 450C microscope at 400X magnification with Leica Application Suite X (LASX) software (Leica Microsystems, Wetzlar, Germany) was used to take pictures showing P-PI3K and P-AKT substrates labelling.

STATISTICAL ANALYSIS

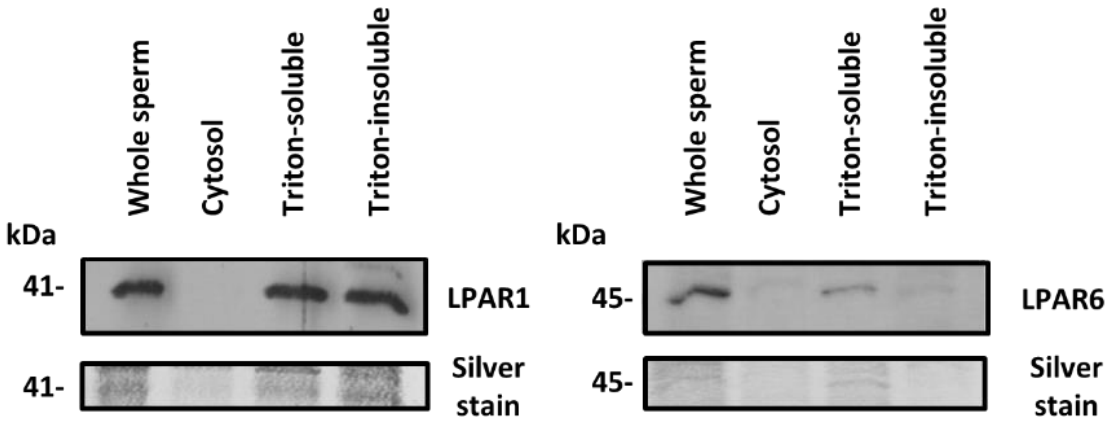
All graphical results were present as mean ± SEM, and we used one-way ANOVA and Dunnet test to determine the statistical differences among the groups ($p \leq 0.05$).

Results

The localization of different LPARs

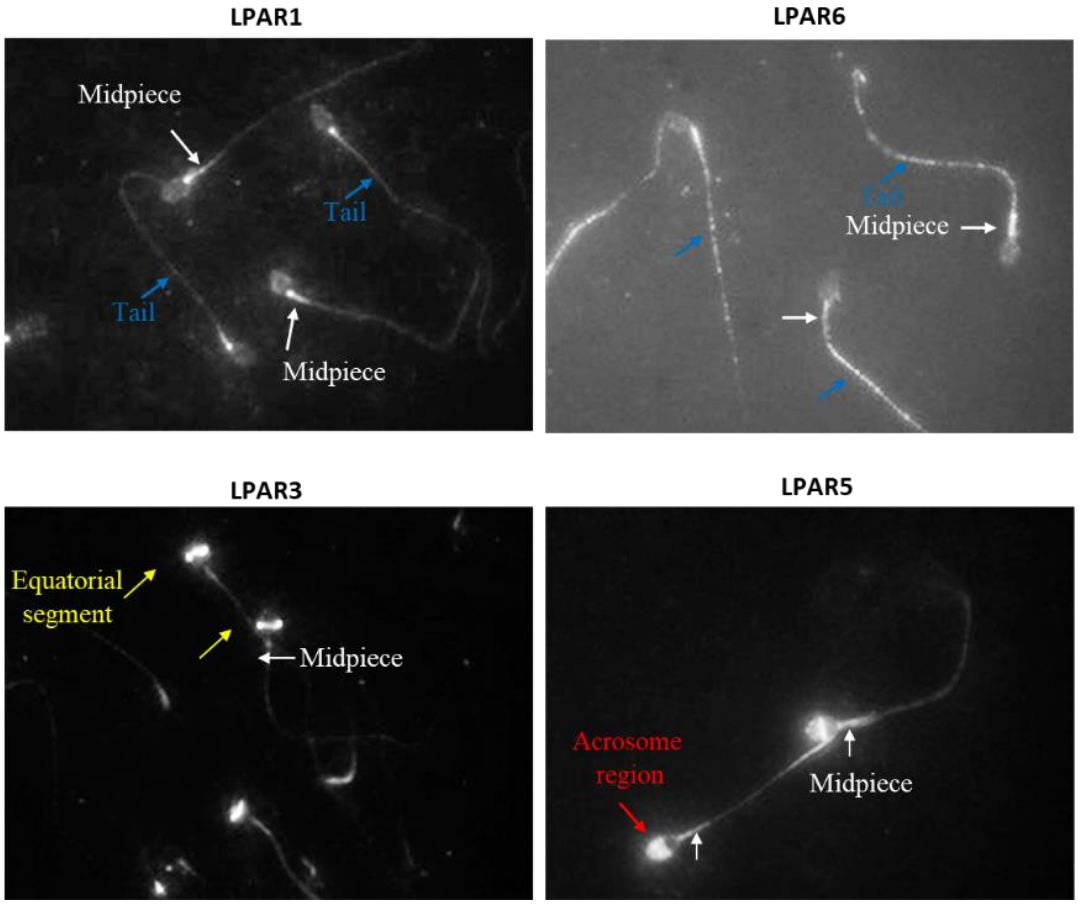
One of our objectives was to determine whether LPA receptors are present in human spermatozoa. The subcellular fractionation experiments revealed that LPAR1 and LPAR6 are present in the Triton-soluble fraction that contains proteins associated with the sperm plasma membrane (Figure 7A). Interestingly, LPAR1 was detected in the Triton-insoluble fraction. Using immunocytochemistry, we determined the exact localization of LPARs in human spermatozoa. LPAR1 was found in non-permeabilized and permeabilized human spermatozoa. LPAR6 only showed the labelling in non-permeabilized human spermatozoa (Figure 7B, 7C). These results are in accordance with those obtained by subcellular fractionation and immunoblotting (Figure 7A). We also determined the localization of LPAR3 and LPAR5 by immunocytochemistry. LPAR1, 3, 5, and 6 labellings were observed in the midpiece and tail of human spermatozoa (Figure 7B). Moreover, LPAR3 and LPAR5 labelling was found in the equatorial segment and acrosome region, respectively, in non-permeabilized human spermatozoa. LPAR1 was detected in the post-acrosome region and acrosome region in permeabilized human spermatozoa (Figure 7C).

A



B

Non-permeabilized



C Permeabilized D Secondary antibody alone

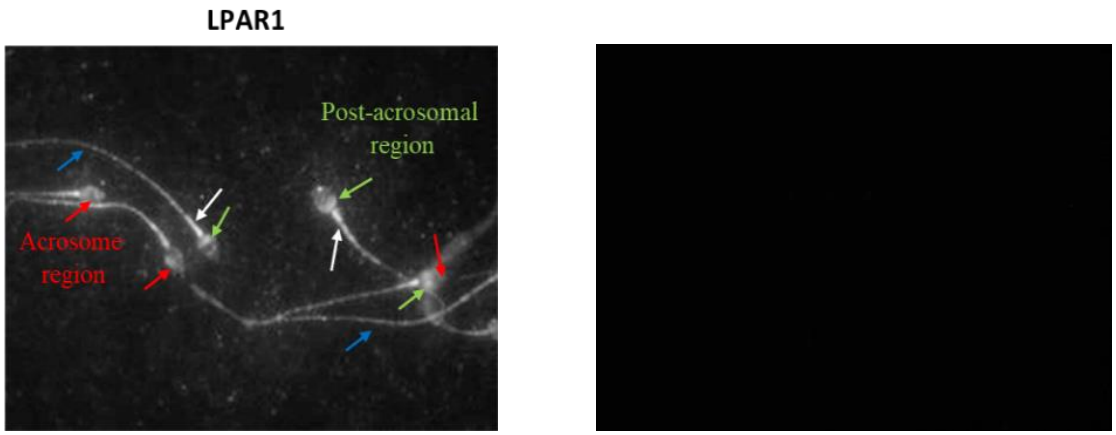


Figure 7: LPARs are localized in different places in the sperm cell and most abundantly found in Triton-soluble protein of midpiece and tail.

A) Spermatozoa immunoblotted with anti LPARs' antibody. 250 x 10^6 cell/mL, 10 µL was loaded for each lane (n=3). B) Immunocytochemistry images of non-permeabilized human sperm. C) Immunocytochemistry images of permeabilized human sperm. Images were taken using (n=3).

The localization of P-PI3K and P-AKT substrates

The subcellular fractionation of human spermatozoa revealed that the phosphorylated PI3K (P-PI3K) is present in the cytosolic and Triton-insoluble fractions (Figure 8A). The majority of P-PI3K was shown in cytosolic fractions. The immunocytochemistry experiments demonstrated that the p-PI3K is located in the neck and equatorial segment of the sperm head in non-permeabilized sperm (Figure 8D, left). This labelling was only seen in the neck of the permeabilized spermatozoa (Figure 8D, right).

On the other hand, P-AKT substrates were displayed in all cytosolic, Triton-soluble and -insoluble fractions (Figure 8B). These phosphorylated substrates were seen in the entire tail of non-permeabilized and permeabilized spermatozoa (Figure 8E).

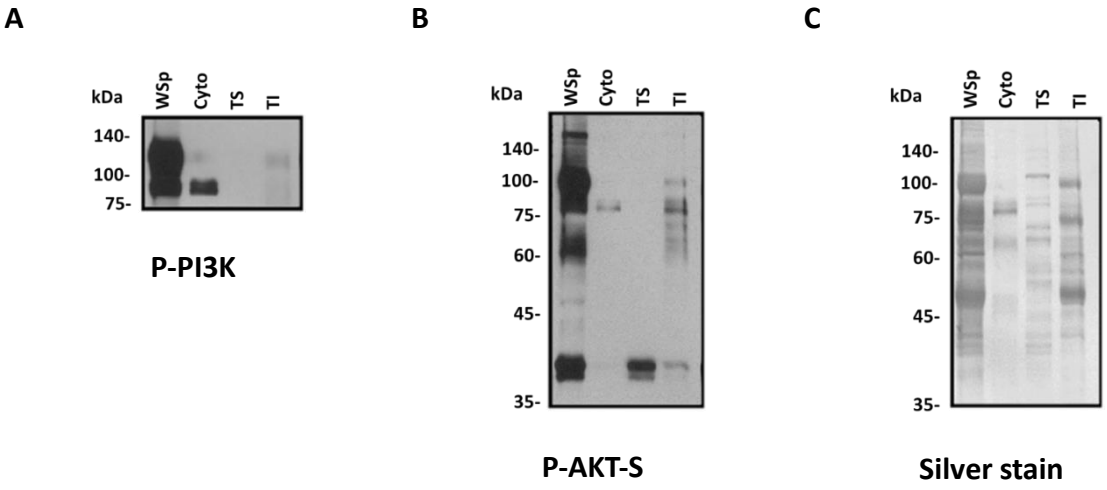


Figure 8: P-PI3K and P-AKT substrates in human spermatozoa.

Spermatozoa immunoblotted with A) anti-P-PI3K and B) anti-P-AKT substrates antibody. 250 x 10^6 cell/mL, 10 µL was loaded for each lane (n=3). C) The silver staining of the membrane. WSp, whole sperm fractions Cyto, cytosol fraction; TS, Triton-soluble fraction; TI, Triton-insoluble fraction.

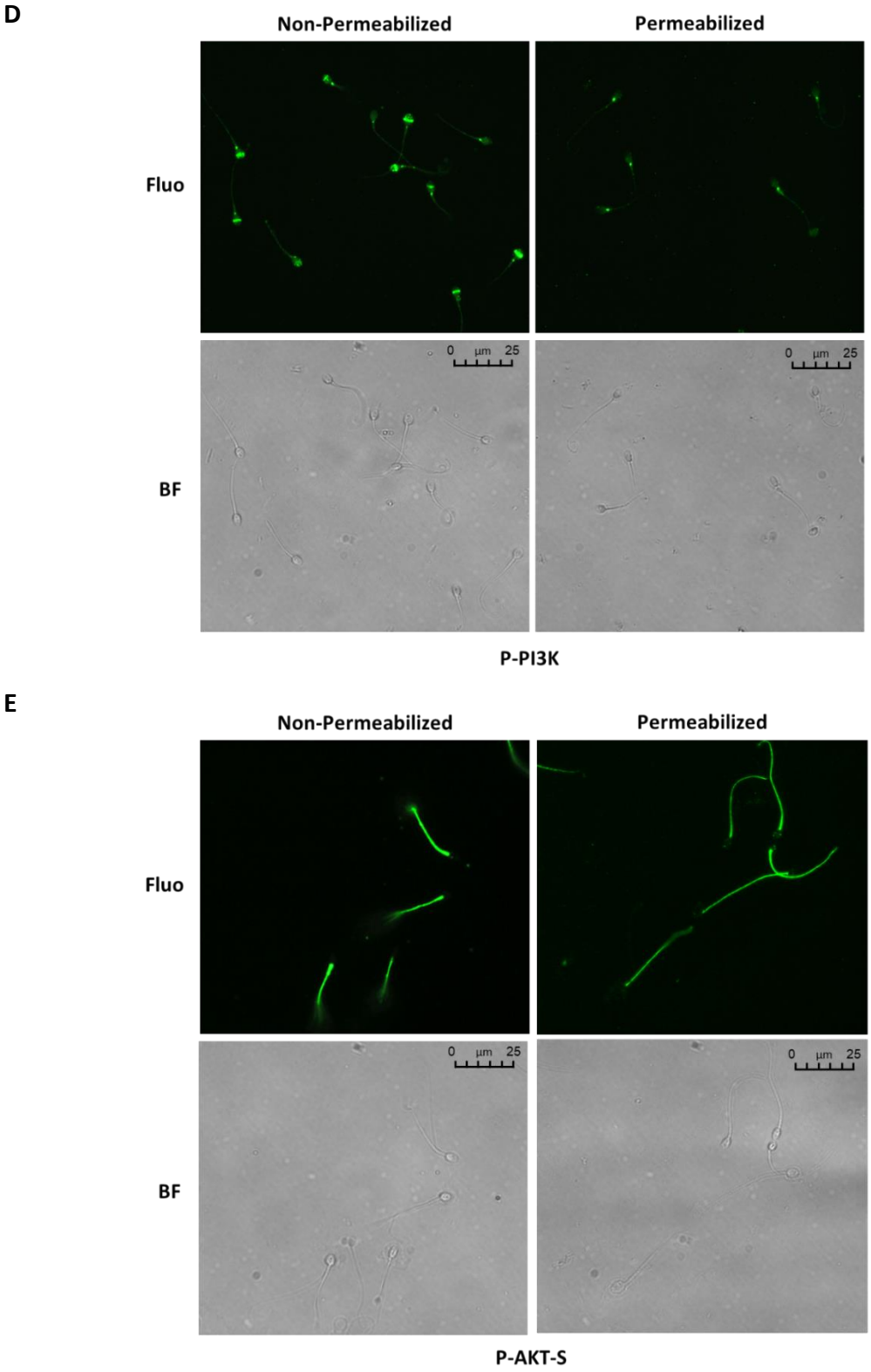


Figure 8 : P-PI3K and P-AKT substrates in human spermatozoa.

Immunocytochemistry images showing the subcellular distribution for D) P-PI3K and E) P-AKT substrates. (n=3) Cells were stained for the specific antibody. The Upper is fluorescence (Fluo), and the bottom is the bright field (BF).

The LPAR activity regulates the PI3K/Akt pathway to maintain viability in human spermatozoa

The percentage of viable spermatozoa decreased dose-dependently with increasing concentrations of Ki6425, an inhibitor of LPARs (Figure 9A). The phosphorylation of PI3K/AKT pathway is associated with sperm survival. We observed that the phosphorylation of PI3K and AKT substrates also decreased dose-dependently with increasing concentrations of Ki16425 (Figure 10 and Figure 11). Last but not least, P-PKC-S expression of human spermatozoa administrated 10μM, and 20μM Ki16425 has a lower level compared with control (Figure 9B).

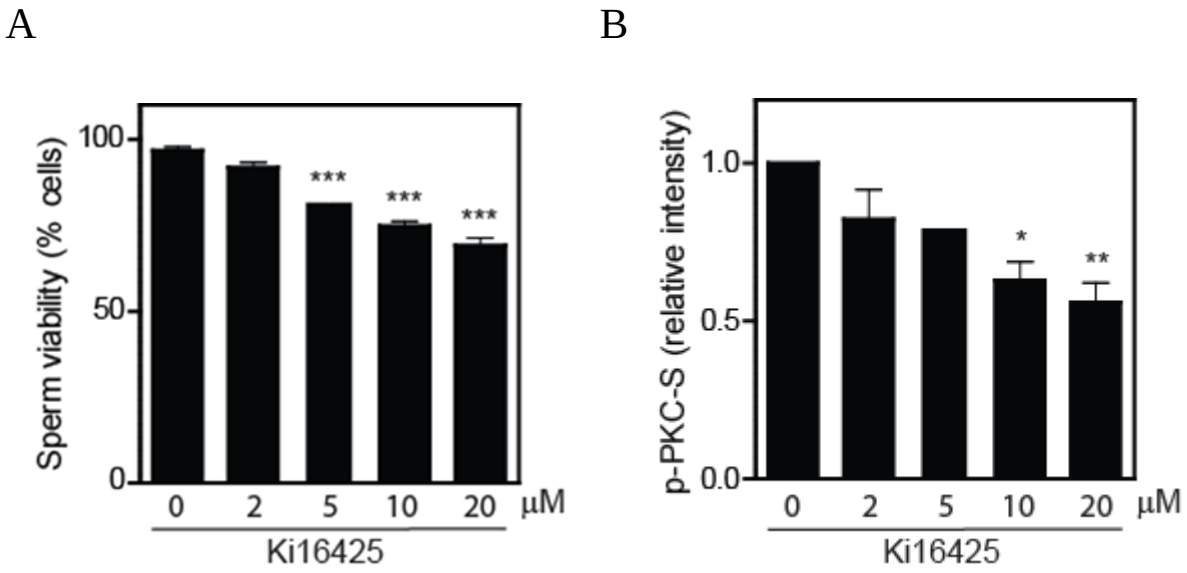


Figure 9: The effect of Ki16425 causes the cell death of human spermatozoa and the impact of Ki16425 on P-PKC-S.

A)Spermatozoa were treated with Ki16425 for 3.5 hours at 37˚C, and added hypo-osmotic swelling buffer to distinguish whether the spermatozoa were dead or alive (see method) (n=4; *** p<= 0.001, ANOVA and Dunnet test). B) The relative intensity of P-PKC-S was obtained by normalizing each band's intensity to the respective intensity of tubulin (n=4; *<=0.05 **<=0.01, ANOVA and Dunnet test).

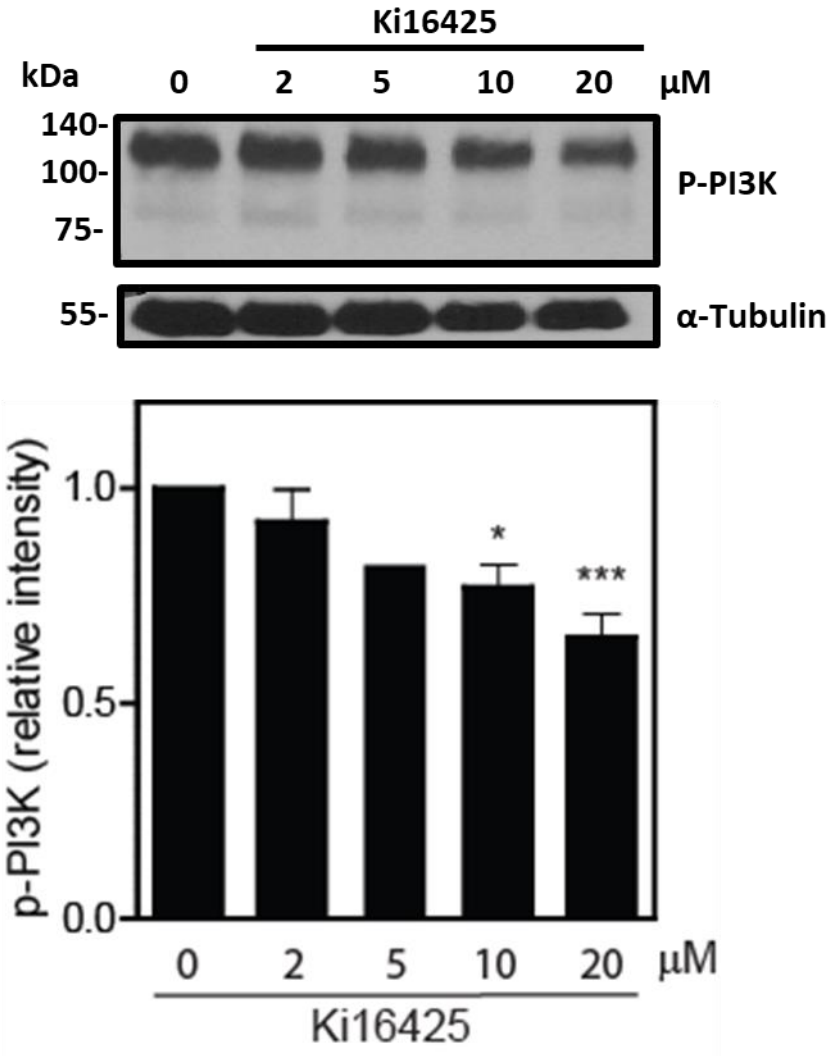


Figure 10: The effect of Ki16425 on PI3K phosphorylation of human spermatozoa.
Spermatozoa were treated with increasing concentrations of Ki16425 for 3.5 hours at 37°C. Sperm proteins were electrophoresed-electrotransferred and immunoblotted with the anti-P-PI3K antibody. The membrane was stripped and reblotted with the anti-Tubulin antibody to confirm equal loading for each lane. The relative intensity of P-PI3K was obtained by normalizing each band's intensity to the respective intensity of tubulin (n=4 ; *<=0.05, *** p<= 0.001, ANOVA and Dunnet test).

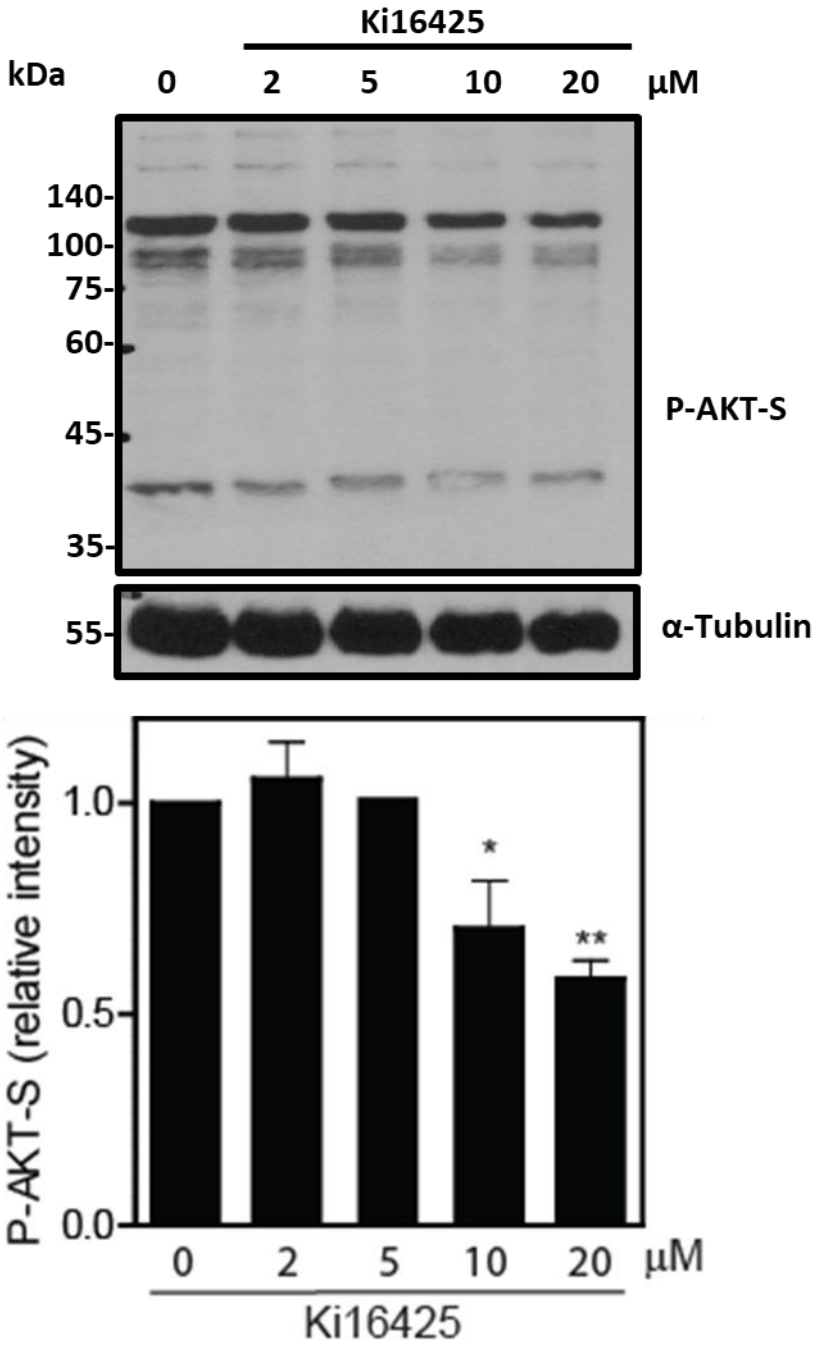


Figure 11: The effect of Ki16425 on AKT substrates phosphorylation of human spermatozoa.

Spermatozoa were treated with increasing concentrations of Ki16425 for 3.5 hours at 37°C. Sperm proteins were electrophoresed-electrotransferred and immunoblotted with the anti-P-AKT-S antibody. The membrane was stripped and reblotted with the anti-Tubulin antibody to confirm equal loading for each lane. The relative intensity of P-AKT-S was obtained by normalizing each band's intensity to the respective intensity of tubulin (n=4; *<=0.05 **<=0.01, ANOVA and Dunnet test).

Regulation of PI3K and AKT substrates phosphorylation by kinases in human spermatozoa

To further explore the pathway involved in sperm survival, spermatozoa were incubated with H89, chelerythrine, PD98059, PP2 and Tyrphostin A47, the inhibitor of PKA, PKC, MEK, non-RTK and RTK, to determine what kinases regulate this pathway. As shown in Figure 12, sperm viability was decreased by chelerythrine and Tyrphostin A47 compared to untreated controls. Noteworthy, chelerythrine promoted the strongest effect on sperm viability. Tyrphostin A47 alone only partially decreased in higher concentrations.

Besides investigating the sperm viability, we also examined the level of the phosphorylation of PI3K and AKT substrates of spermatozoa treated with the kinase inhibitor. P-PI3K and P-AKT substrates were significantly decreased by chelerythrine and Tyrphostin A47 compared to untreated controls. Chelerythrine promoted the strongest effect on sperm viability (Figures 13 and 14).

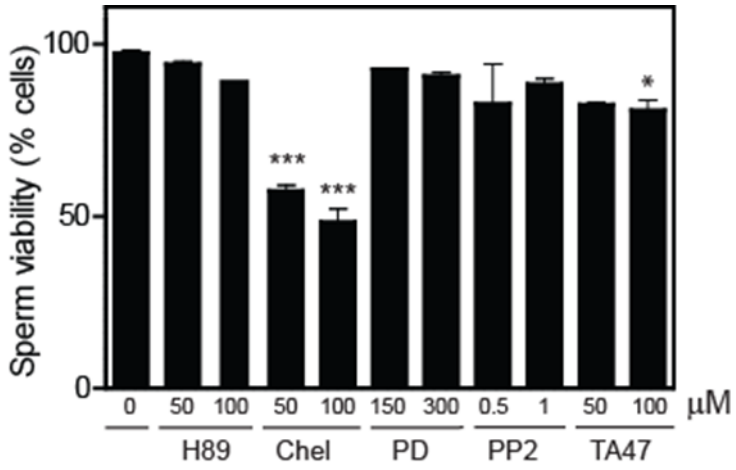


Figure 12: The effect of kinase inhibitor caused the cell death of human spermatozoa. Spermatozoa were treated with kinase inhibitor for 3.5 hours at 37°C, and added hypo-osmotic swelling buffer to distinguish whether the sperm dead or live (see method) (n=3, * means significant difference within the same strain. ANOVA and Dunnet test; * p<= 0.05; *** p<= 0.001). Chel, chelerythrine; PD, PD98059; TA47, Tyrphostin A47.

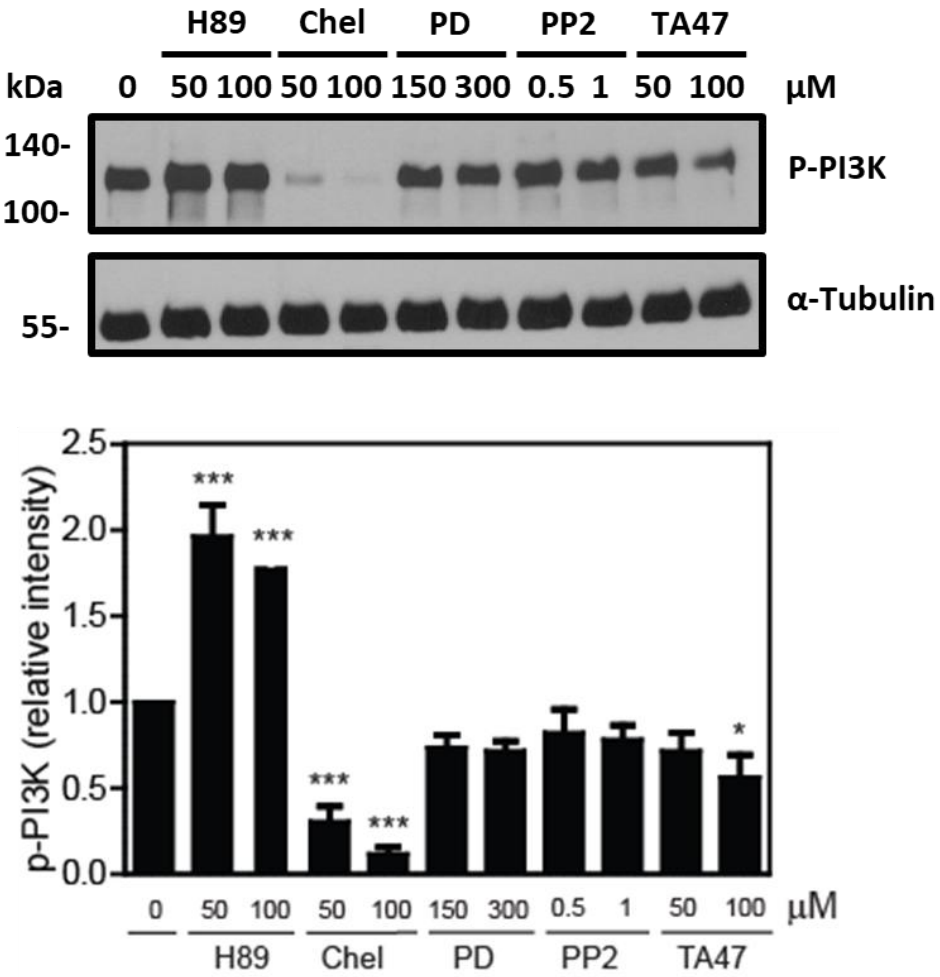


Figure 13: The different kinase inhibitors led to the effect on PI3K phosphorylation of human spermatozoa.

Levels of P-PI3K in spermatozoa incubated with H89, chelerythrine, PD98059, PP2 and Tyrphostin A47 for 3.5 hours at 37°C. Sperm proteins were electrophoresed, electrotransferred and immunoblotted with the anti-P-PI3K antibody. The membrane was stripped and reblotted with the anti-Tubulin antibody to confirm equal loading for each lane. The relative intensity of P-PI3K was obtained by normalizing each band's intensity to the respective intensity of tubulin (n=4; *<=0.05, *** p<= 0.001, ANOVA and Dunnet test). Chel, chelerythrine; PD, PD98059; TA47, Tyrphostin A47.

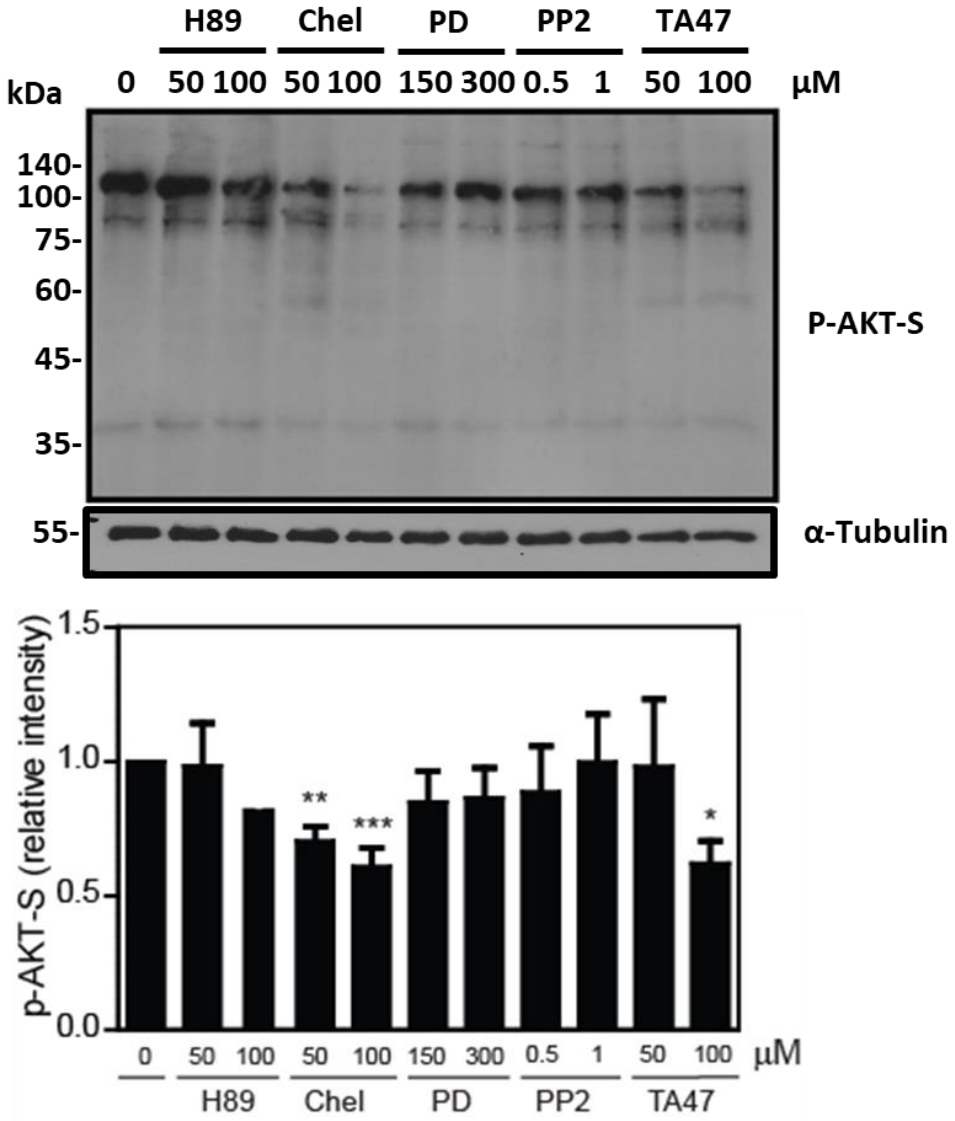


Figure 14: The different kinase inhibitors led to the effect on AKT substrates phosphorylation of human spermatozoa.

Levels of P-AKT substrates were determined in spermatozoa incubated with H89, chelerythrine, PD98059, PP2 and Tyrphostin A47 for 3.5 hours at 37°C. Sperm proteins were electrophoresed, electrotransferred and immunoblotted with the anti-P-AKT-S antibody. The membrane was stripped and reblotted with the anti-Tubulin antibody to confirm equal loading for each lane. The relative intensity of P-AKT-S was obtained by normalizing each band's intensity to the respective intensity of tubulin (n=4; *<=0.05 **<=0.01 *** p<= 0.001, ANOVA and Dunnet test). Chel, chelerythrine; PD, PD98059; TA47, Tyrphostin A47.

Finally, to study whether the upstream effectors of PKC may are involved in the sperm

survival pathway, we incubated spermatozoa with 10 μM U-73122, the inhibitor of PLC, and with or without 2 μM OAG, the analog of DAG, for 3.5 hours at 37°C. Spermatozoa treated with U-73122 showed lower viability (Figure 15). This reduction was prevented by the addition of OAG to the U-73122-treated spermatozoa.

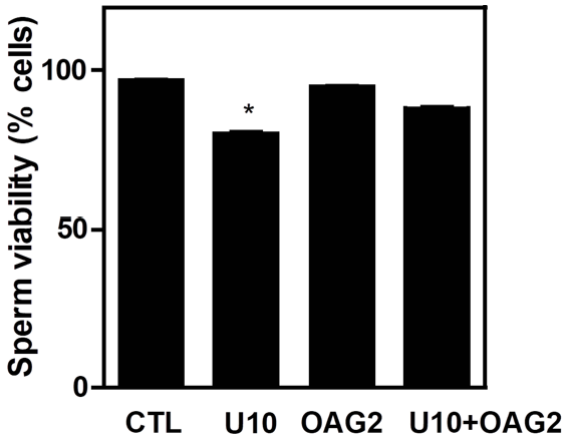
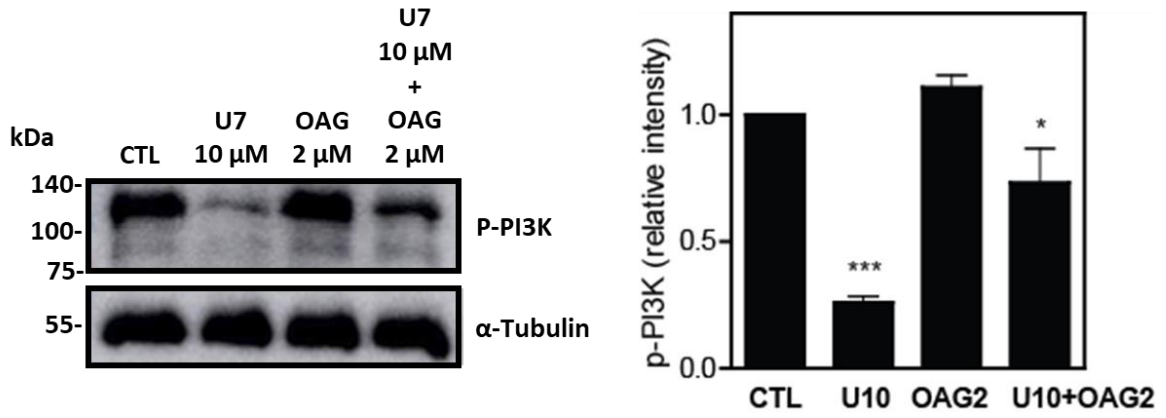


Figure 15: OAG prevents the loss of human sperm viability when U-73122 is present. Spermatozoa were incubated with U-73122 and OAG for 3.5 hours at 37°C and added hypo-osmotic swelling buffer to distinguish whether the spermatozoa were dead or alive (see method) (n=3 * means significant difference within the same strain. ANOVA and Dunnet test; * p<= 0.05).

The U-73122 treatment drastically reduced PI3K and AKT substrates phosphorylation (Figure 16). Moreover, OAG treatment prevented the inhibition of these phosphorylations by U-73122 treatment.

A



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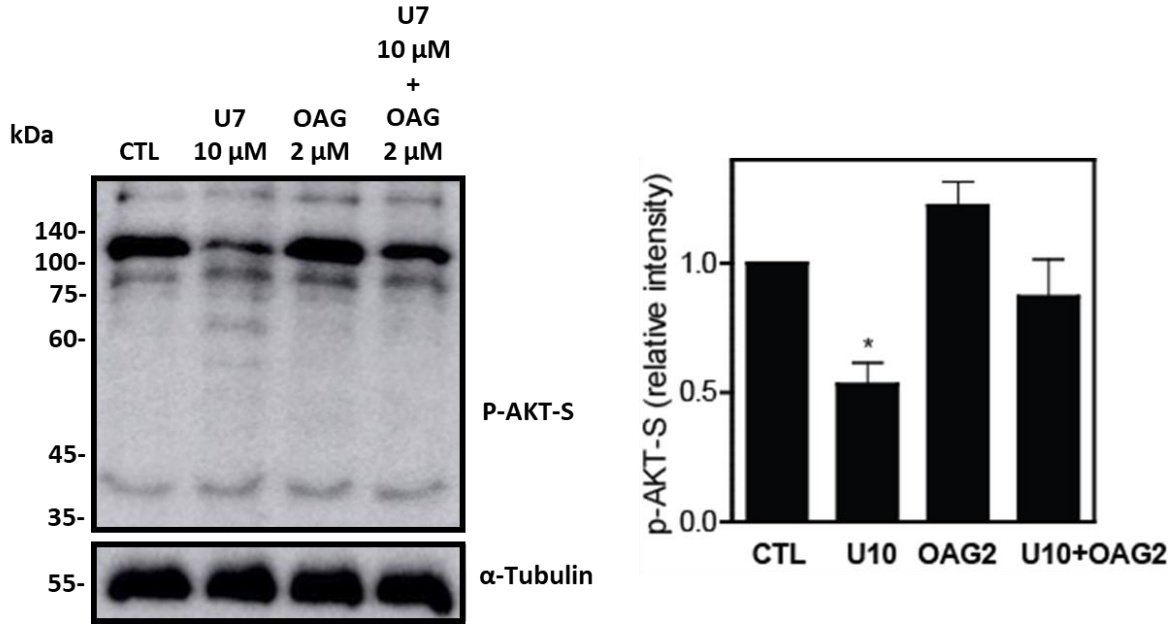

Figure 16: OAG prevents the loss of PI3K and AKT substrates phosphorylation when U-73122 is present.

Levels of P-PI3K and P-AKT-S in spermatozoa incubated with U-73122 and OAG for 3.5 hours at 37°C. Sperm proteins were electrophoresed, electrotransferred and immunoblotted with the anti-PI3K/P-AKT-S antibody. The membrane was stripped and reblotted with the anti-Tubulin antibody to confirm equal loading for each lane. The relative intensity of A)P-PI3K and B) P-AKT-S was obtained from normalizing each band's intensity to the respective intensity of tubulin (n=4, * means significant difference within the same strain. ANOVA and Dunnet test; * p<= 0.05; *** p<= 0.001).

Discussion

As foreign cells, mammalian spermatozoa face numerous challenges before the fertilization in the female reproductive tract, such as the interactions of sperm with immune cells or secretions of the oviduct, and the combination between the sperm surface molecules with receptors in the epithelium of the oviduct (Suarez and Pacey 2006, Ma, Pan et al. 2016, Suarez 2016). Since the transportation of spermatozoa from the vagina to the upper part of oviduct takes a few minutes to 6 hr. The waiting time for the spermatozoa to arrive at the ova in the oviduct is a couple of hours (Chang 1951). Based on that information, the spermatozoa must reside in the female reproductive tract and be kept alive. The mammalian spermatozoa must undergo capacitation in the oviduct to acquire the ability to penetrate and fertilize the oocyte.

Nevertheless, the life span of the human spermatozoa is only 4 hours once they are fully capacitated (Giojalas, Rovasio et al. 2004). The sperm viability is maintained to ensure the completion of capacitation by tight interaction between spermatozoa and the oviduct's epithelium. Indeed, it has been reported that the interaction between spermatozoa and oviduct epithelium effectively declines Ca^{2+} in the spermatozoon and then maintains sperm survival because of the inhibition of capacitation (Baker, Hetherington et al. 2004, Suarez and Pacey 2006, Suarez 2016).

In addition, the elevated ROS leads to DNA oxidation and lipid peroxidation, which are markers of oxidative stress and impair human sperm function (Alvarez, Touchstone et al. 1987, Morielli and O'Flaherty 2015). PRDX6 $iPLA_2$ activity prevents the effects of oxidative stress on DNA integrity and viability of human spermatozoa by providing LPA and AA and removing oxidized lipids from sperm membranes (Fernandez and O'Flaherty 2018, Fernandez, Yu et al. 2019). Mouse testes have LPA receptors 1-3, and their absence is related to the reduction in sperm production (Ye, Skinner et al. 2008).

For the first time, we demonstrated the presence of active LPAR1, LPAR3, LPAR5, and LPAR6 in the human spermatozoa. Most of them are expressed on the tail and midpiece region on non-permeabilized sperm, which means their location is on the plasma membrane (Figure 7). The expression of LPAR3 and LPAR5 on the equatorial segment and acrosome region suggests that they may play roles in fusing with oocytes. In the CD8+ T-cells, the granule exocytosis can be inhibited by LPAR5 (Mathew, Kremer et al. 2019). On the other hand, the prevailing view is that LPAR1 is localized on the cell plasma membrane, where it transduces extracellular LPA signalling (Gobeil, Bernier et al. 2003, Moughal, Waters et al. 2004, Waters, Saatian et al. 2006). Moreover, LPAR1 is expressed in the nucleus of PC12 (pheochromocytoma of the rat adrenal medulla), HBEC cells (human bronchial epithelial cells), rat liver cells and HTC4 cells (rat hepatoma cells). Under the stimulation of nerve growth factor or LPA, the amount of LPAR1 in the nucleus increases and LPAR1 on the plasma membrane declines (Waters, Saatian et al. 2006). These findings suggest that LPAR1 may be trafficking from plasma membrane to nucleus and participate in the intranuclear LPA signalling during fertilization. The nuclear LPAR1, which is novel and found in pig cerebral microvascular endothelial cells, is functional. It is related to calcium signalling, PI3K/AKT activation, and subsequent inflammatory gene transcription (Gobeil, Bernier et al. 2003). LPAR1 was shown in permeabilized sperm by ICC and the Triton-insoluble fraction by Western blotting. This evidence supports the idea that LPAR1 is also localized in the human spermatozoal nucleus. However, further studies are required to identify the functions of the nuclear localization of LPAR1 on human spermatozoa.

The localization of P-PI3K (Figure 8) corresponded with the previous research (Aitken and Baker 2013), which is shown in the head. P-AKT substrates position is in tail and midpiece, which is in accordance with the localization of the AKT. AKT

substrates, downstream of AKT, activated by phosphorylation, control more than one cellular function, such as proliferation, survival, metabolism, etc. (Manning and Cantley 2007). We detected P-AKT substrates with molecular masses of approximately 40 kDa (Figure 8B). In previous studies involving prostate cancer cells undergoing apoptosis, Par-4 (40 kDa) was identified as a pro-apoptotic protein (Goswami, Burikhanov et al. 2005). AKT inhibits apoptosis via phosphorylating Par-4, essential for cancer cell survival. Therefore, we can suggest that Par-4 might participate in the mechanisms to prevent the apoptotic-like changes in human spermatozoa. Par-4 is found in seminal plasma that has protective properties for spermatozoa (Untergasser, Rumpold et al. 2001), but the localization in spermatozoa remains elusive. Therefore, further studies are needed to confirm the involvement of Par-4 in the pro-survival pathway of human spermatozoa.

Results presented in this thesis also established the importance and the effect of LPAR signalling in maintaining the human spermatozoal survival. The treatment with Ki16425 on human spermatozoa promotes lower percentages of sperm viability compared to the control group (Figure 9). Even if Ki16425, the competitive antagonist of LPARs, has a different inhibitory potency for LPARs, being the most sensitive LPAR1 and LPAR3, but not LPAR2 (Ohta, Sato et al. 2003), we still found the LPAR signalling has the positive correlation with P-PI3K and P-AKT substrates. Impairment of sperm viability and decreasing P-PI3K and P-AKT substrates due to the inhibition of LPAR1-3 indicates that LPAR signalling activities are essential to maintaining male fertility.

LPAR signalling participates in a broad range of cellular mechanisms, depending on the activation of the downstream pathways via activation of various G proteins., such as Rho, which influences cell proliferation and migration (Xiang, Dusaban et al. 2013),

MAPK, which is related with mitogenic signalling (Fang, Yu et al. 2000) and PI3K associated with survival signalling (Ye, Ishii et al. 2002). Additional evidence supports the concept that the activation of PI3K/AKT can be triggered by different signalling pathways, induced via LPAR or cooperating with LPAR action. The cAMP-PKA activation by Gs protein-coupled receptors of PI3K signalling is related to growth and survival in mouse 3T3 fibroblasts (Cosentino, Di Domenico et al. 2007), and LPAR enhances the activity of PI3K/AKT by the cooperative action of activated RTK (e.g. EGFR) and non-RTK (e.g. Src) in cancer cells (Mittal, Pavlova et al. 2011).

Based on what has been explained above, it is possible that LPAR regulates the phosphorylation and activation of the PI3K/AKT pathway, which is important for sperm survival through different downstream effectors. The spermatozoa treated with chelerythrine and Tyrphostin A47 have lower viability (Figure 12) and low levels of P-PI3K and P-AKT substrates (Figure 13. 14) compared to the control group, confirming the interaction of PKC and EGFR with the sperm survival signalling through the PI3K-AKT pathway. The fact that chelerythrine drastically prevents the phosphorylation of PI3K and AKT substrates and decrease viability, and the lower level of phosphorylation of PKC substrates due to inhibition of LPAR activity with Ki16425 (Figure 9B); altogether, these finding strongly support that PKC is an important regulator of the LPAR-PI3K-AKT pathway. In human spermatozoa, EGFR signalling also shows the cross-talks with LPAR signalling to maintain human sperm viability (Figure 12-14). Further research is needed to elucidate the molecular mechanisms associated with the interaction between EGFR and LPAR in human spermatozoa.

Diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), which are the second messengers of phospholipase C (PLC), have been shown to initiate further signal transduction pathways by PKC (Suh, Park et al. 2008). Similarly, the inhibition of PLC

activity leads to a decrease in sperm viability (Figure 15) and PI3K and AKT substrates phosphorylation (Figure 16). Exogenous addition of the analog of DAG, OAG, prevented the inhibition of U73122 on PLC, which led to a decrease in PI3K phosphorylation (Figure 16). These results suggest that exogenous OAG bypasses the inhibition of the PLC activity and confirm the participation of PLC/DAG signalling in sperm survival. This result and the prevention of sperm viability loss and sustained phosphorylation in U73122-treated spermatozoa incubated with OAG (Figures 15 and 16) demonstrate that the PLC activity is required to maintain the phosphorylation of PI3K signalling to ensure sperm viability.

Conclusion and Future Directions

The experiments conducted in this study prove the presence of LPAR in the human spermatozoa and also provide evidence that LPAR signalling directly activates the PI3K/AKT pathway. The inhibition of PKC or PLC on human spermatozoa results suggest that LPAR signalling may affect the pro-survival mechanism indirectly. Thus, the LPAR is important to protect sperm from apoptotic-like changes. Figure 17 presents a potential pro-survival mechanism regulated by LPAR signalling in human spermatozoa.

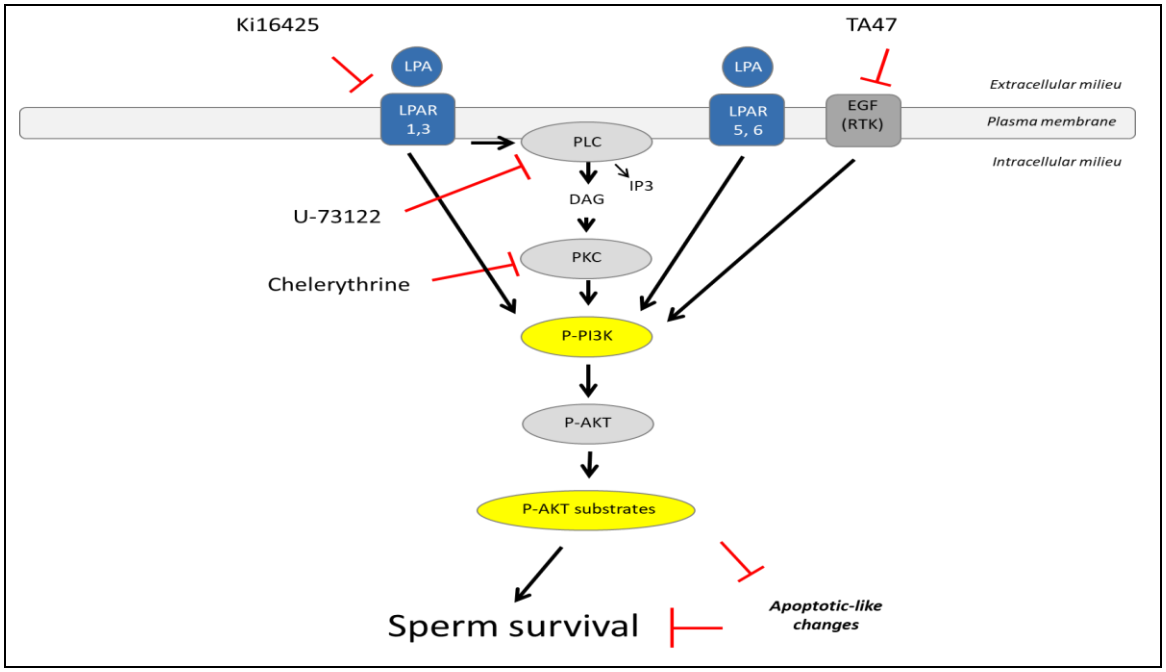

Figure 17: The proposed schematic shows the LPAR and pro-survival PI3K/AKT pathway in human spermatozoa.

LPAR1, 3, 5, 6 activity directly contributes to maintaining sperm survival through PI3K/AKT pathway. The inhibition of LPAR1-3 by Ki16425 leads to apoptotic-like changes observed in dying spermatozoa. LPAR1 and LPAR3 can also activate PLC-PKC signalling to induce the downstream PI3K/AKT pathway through different G-proteins and promote apoptotic-like changes, leading to cell death. Due to the inhibition of PLC, it is known that the positive effect of DAG in activating PLC-PKC signalling is necessary to maintaining cell viability. In addition, EGFR participates the survival mechanism on human spermatozoa.

Further studies are required to elucidate the mechanism of EGFR interdependent pro-survival pathways present in spermatozoa in this project. Moreover, studies are needed to identify the species of LPA produced by PRDX6 that are involved in the maintenance of sperm viability in humans. Moreover, the identification by phosphoproteomics strategies of phosphorylated AKT substrates involved in the LPAR signalling will help to understand the molecular mechanisms to maintain sperm viability and would help to establish molecular markers to determine the health of a given sperm sample from infertile patients and design treatment to ensure sperm viability during ARTs such as in vitro fertilization or artificial insemination.

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