Role of citrate in the production of reactive oxygen species during human sperm capacitation

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December 2023

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

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ABSTRACT

Sperm capacitation involves a series of biochemical and morphological changes, which are necessary for the spermatozoon to fertilize the oocyte. The process of sperm capacitation requires the production of low levels of reactive oxygen species, an increase in tyrosine phosphorylation, and sufficient levels of different energy metabolites such as citrate. Human spermatozoa are exposed to high concentrations of citrate from the seminal plasma, yet the role of citrate in sperm capacitation is largely unknown. Extracellular citrate can be imported into the cytosol of the spermatozoon via sodium-coupled solute carrier proteins, and mitochondrial citrate can be exported to the cytosol via the mitochondrial citrate transport protein (CIC). This cytosolic citrate is used as a substrate by ATP-citrate lyase (ACLY) to produce acetyl-CoA and oxaloacetate. Oxaloacetate can then be converted by malate dehydrogenase into malate, which is subsequently converted by the malic enzyme (ME) to yield pyruvate and NADPH. This resulting NADPH produced from citrate metabolism may be used to produce nitric oxide ($NO^{\bullet}$) via nitric oxide synthase (NOS). We hypothesize that cytosolic citrate supports human sperm capacitation through $NO^{\bullet}$ production.

Highly motile human spermatozoa were incubated in capacitating conditions in the presence and absence of citrate with no other energy metabolites present in the medium, and levels of sperm capacitation were determined by quantifying levels of tyrosine phosphorylation through immunoblotting. The mitochondrial CIC, cytosolic ACLY, and ME were then inhibited to establish their involvement in capacitation, and total sperm motility and viability were assessed. To determine the role of citrate in $NO^{\bullet}$ production during capacitation, levels of capacitation were determined in spermatozoa incubated with an inhibitor of NOS, and levels of $NO^{\bullet}$ were determined by fluorescence microscopy following the incubation with citrate and the ACLY inhibitor.

Citrate supported the process of sperm capacitation when added to the capacitation medium, and CIC, ACLY, and ME inhibition prevented FCSu-induced capacitation with no effect on total sperm motility or viability. Capacitation levels decreased by adding the NOS inhibitor L-NAME to the capacitation media, and $NO^{\bullet}$ production was reduced following ACLY inhibition.

These results demonstrate that citrate supports sperm capacitation in a process mediated by CIC, ACLY, and ME activity, resulting in $NO^{\bullet}$ production. This research will help understand citrate regulation in sperm capacitation and ameliorate current treatment strategies for male infertility.

Key Words: sperm, capacitation, citrate, reactive oxygen species (ROS), nitric oxide ($NO^{\bullet}$), male fertility

RÉSUMÉ

La capacitation des spermatozoïdes implique une série de changements biochimiques et morphologiques, qui sont nécessaires pour que le spermatozoïde féconde l'ovocyte. Le processus de capacitation des spermatozoïdes nécessite la production de faibles niveaux d'espèces réactives de l'oxygène, une augmentation de la phosphorylation de la tyrosine et des niveaux suffisants de différents métabolites énergétiques tels que le citrate. Les spermatozoïdes sont exposés à des concentrations élevées de citrate provenant du plasma séminal. Cependant, le rôle du citrate dans la capacitation des spermatozoïdes est largement inconnu. Le citrate extracellulaire est importé dans le cytosol du spermatozoïde par des protéines porteuses de soluté couplées au sodium, et le citrate mitochondrial est exporté vers le cytosol par la protéine de transport de citrate mitochondrial (CIC). Ce citrate cytosolique est utilisé comme substrat par l'ATP-citrate lyase (ACLY) pour produire de l'acétyl-CoA et de l'oxaloacétate. L'oxaloacétate peut ensuite être converti par le malate déshydrogénase en malate, qui est ensuite converti par l'enzyme malique (ME) pour produire du pyruvate et du NADPH. Ce NADPH résultant du métabolisme du citrate peut être utilisé pour produire de l'oxyde nitrique ($NO^{\bullet}$) par l'oxyde nitrique synthase (NOS). Nous émettons l'hypothèse que le citrate cytosolique soutient la capacitation du sperme humain par la production de $NO^{\bullet}$.

Les spermatozoïdes humains hautement motiles ont été incubés dans des conditions capacitantes en présence et en absence de citrate sans aucun autre métabolite énergétique, et les niveaux de capacitation ont été déterminés en quantifiant les niveaux de phosphorylation de la tyrosine par immunobuvardage de type Western. Le CIC mitochondrial, l'ACLY cytosolique et le ME ont ensuite été inhibés pour caractériser leurs implications dans la capacitation, et la motilité totale et la viabilité des spermatozoïdes ont été évaluées. Pour déterminer le rôle du citrate dans la

production de NO• pendant la capacitation, les niveaux de capacitation ont été déterminés dans les spermatozoïdes incubés avec un inhibiteur de NOS, et les niveaux de NO• ont été déterminés par microscopie à fluorescence après l'incubation avec le citrate et l'inhibiteur ACLY.

Le citrate a soutenu le processus de capacitation des spermatozoïdes lorsqu'il est ajouté au milieu de capacitation, et l'inhibition CIC, ACLY et ME a empêché la capacitation induite par FCSu sans effet sur la motilité totale ou la viabilité des spermatozoïdes. Les niveaux de capacitation ont diminué en ajoutant l'inhibiteur de NOS L-NAME au milieu de capacitation, et la production de NO• a été réduite suite à l'inhibition d'ACLY.

Ces résultats démontrent que le citrate soutient la capacitation des spermatozoïdes dans un processus médiatisé par l'activité CIC, ACLY et ME, entraînant une production de NO•. Cette recherche aidera à comprendre la régulation du citrate dans la capacitation des spermatozoïdes et à améliorer les stratégies de traitement de l'infertilité masculine.

Mots clés : sperme, capacitation, citrate, espèces réactives de l'oxygène (ROS), oxyde nitrique (NO•), fertilité masculine

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my research supervisor and mentor, Dr. Cristian O'Flaherty, who gave me the opportunity to pursue my passion for scientific research. His guidance helped shape me as a student and gave me the tools needed for my future endeavours.

I would also like to thank my graduate committee members, Dr. Bernard Robaire, Dr. Jacquetta Trasler, and Dr. Anne McKinney, whose insights and advice were invaluable towards the advancement of my project. In addition, I would like to express my appreciation toward Dr. Jean-François Trempe, who not only served as a replacement advisor, but has also served as a role-model for me since I began my graduate studies.

A special thank you to Haoyu (Caleb) Liao and Steven Serafini, for teaching me all the lab techniques necessary to finalize this project, and for patiently answering all my questions. To Chika, for being an absolute pleasure to work with (I wish you the best of luck with your exciting new project!). To Marie-France, for always being there to give words of encouragement, and Joanna for always giving me a warm welcome in the morning and brightening up the lab environment.

Finally, I would like to thank my parents, my sister Sarah, and my better half Sabrina for offering constant support and motivation. I truly couldn't have done it without you.

CONTRIBUTIONS

All experiments presented in this thesis were performed by the M.Sc. candidate Diego Loggia, and were designed with the help of Dr. Cristian O'Flaherty.

Distinct contributions to scientific knowledge include:

1) Citrate supports the process of human sperm capacitation through a biochemical mechanism involving nitric oxide synthesis
2) The inhibition of mitochondrial citrate transport, ATP-citrate lyase, malic enzyme, and nitric oxide synthase decreases citrate-mediated sperm capacitation

LIST OF ABBREVIATIONS

6PGD	6-phosphogluconate dehydrogenase
AC	Adenylyl cyclase
ACLY	ATP-citrate lyase
AKAP	Protein A-kinase anchoring protein
AKT	Protein Kinase B
ART	Assisted reproductive technology
ATP	Adenosine Triphosphate
BPA	Bromopyruvic acid
BSA	Bovine serum albumin
BWW-NES	Biggers-Whitten-Whittingham medium (no energy substrates)
BWW-REG	Biggers-Whitten-Whittingham medium (regular)
CASA	Computer assisted semen analysis
c-AMP	Cyclic adenosine monophosphate
CAT	Carnitine/acylcarnitine translocase
CIC	Mitochondrial citrate carrier
CO_2	Carbon dioxide
CoA	Coenzyme A
CPT1	Carnitine palmitoyltransferase I
CPT 2	Carnitine palmitoyltransferase II
CTCF	Corrected total cell fluorescence
DAF-2 DA	Diaminofluorescein-2 diacetate
DAPI	4′,6-diamidino-2-phenylindole

DTT	Dithiothreitol
ERK	Extracellular signal-regulated kinase
FCSu	Fetal cord serum ultrafiltrate
G6PD	Glucose-6-phosphate dehydrogenase
GPX	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HBS	HEPES buffered saline
HOS	Hypo-osmotic swelling
ICSI	Intracytoplasmic sperm injection
$iPLA_2$	Calcium-independent phospholipase A_2
IDPc	Cytosolic $NADP^+$-dependent isocitrate dehydrogenase
IMM	Inner mitochondrial membrane
IUI	Intrauterine insemination
IVF	In vitro fertilization
L-NAME	L-N^G-Nitro arginine methyl ester
LPCAT	Lysophosphatidylcholine acyl transferase
MDH	Malate dehydrogenase
ME	Malic enzyme
MEK	Mitogen-activated protein kinase kinase
MPC	Mitochondrial pyruvate carrier
NaCT	Sodium-citrate cotransporter
NADPH	Nicotinamide adenine dinucleotide phosphate
$NO^{\bullet}$	Nitric oxide

NOS	Nitric oxide synthase
$O_2^{\bullet-}$	Superoxide anion
OMM	Outer mitochondrial membrane
$ONOO^-$	Peroxynitrite
P-ACLY	Phospho-ATP-citrate lyase
P-MEK	Phospho-mitogen-activated protein kinase kinase
P-Tyr	Phospho-tyrosine
P-PKA	Phospho-protein kinase A
PDC	Pyruvate dehydrogenase complex
PI3K	Phosphatidylinositol-3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PTK	Protein tyrosine kinase
ROS	Reactive oxygen species
SLC	Solute carrier
SOD	Superoxide dismutase
TRX	Thioredoxin
TTBS	Tris buffered saline with Tween 20
WHO	World Health Organization
ZP	Zona pellucida
ZP1	Zona pellucida glycoprotein 1
ZP3	Zona pellucida glycoprotein 3
ZP4	Zona pellucida glycoprotein 4

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1. INTRODUCTION

1.1 Male Infertility

Human infertility is a complex and multifaceted medical condition which can be characterized as the inability to achieve pregnancy after one year of unprotected sexual intercourse (WHO, 2018). It has been reported that nearly half of infertility cases can be traced to the male, stemming from underlying issues such as varicocele, hypogonadism, or urogenital infections (Fallara et al., 2021; Leslie et al., 2023). Male-factor infertility can present itself clinically through low or no sperm counts (oligospermia or azoospermia), abnormal sperm morphology (teratozoospermia), and low sperm motility (asthenozoospermia) (Grimes & Lopez, 2007; Leslie et al., 2023). However, male infertility can be caused or amplified by different factors such as pollutants, drugs, and aging, making the diagnosis and treatment of the medical condition very difficult. It is estimated that around 34% of infertile men suffer from idiopathic infertility, where the cause of their medical condition is unknown (Figure 1) (Longo et al., 2019).

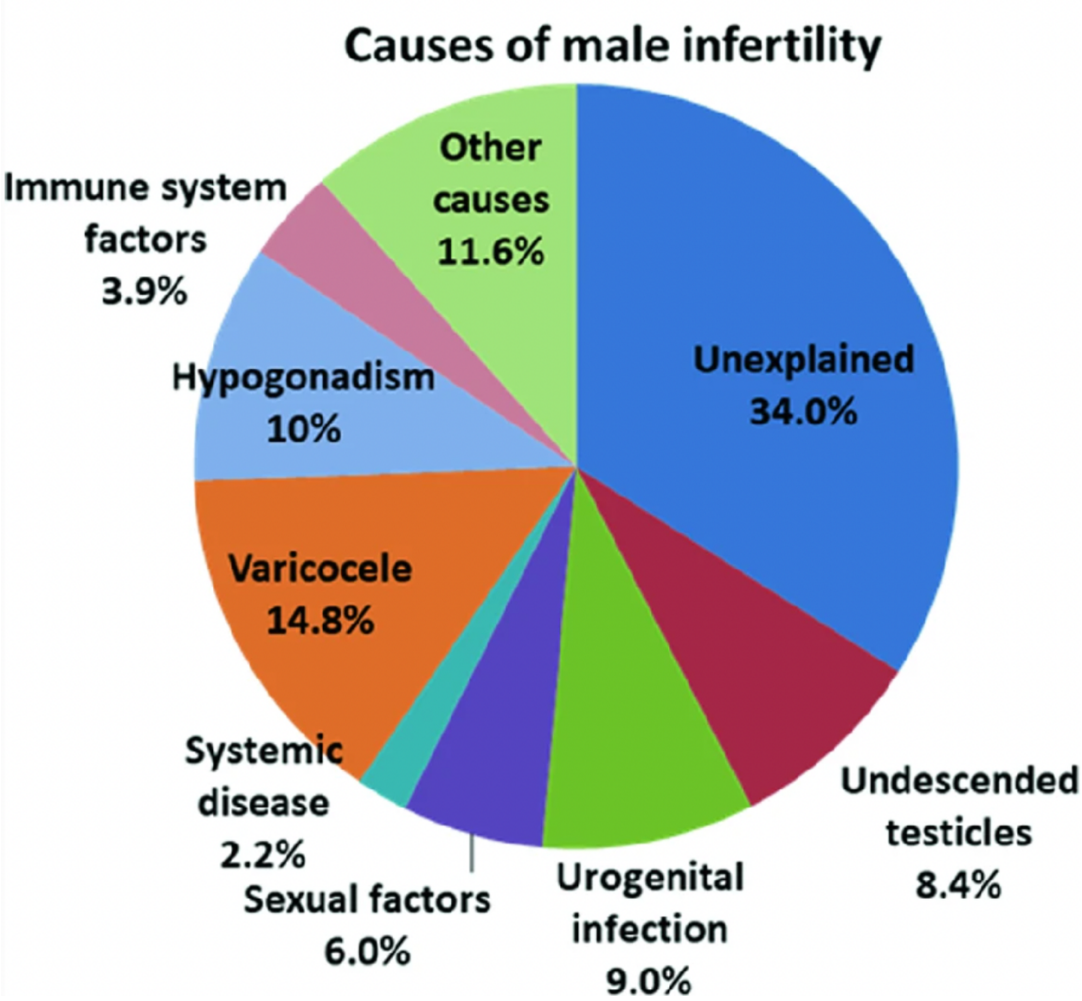


Figure 1. Percentage of male infertility causes. There are numerous causes for male infertility. In 34% of cases, male infertility cannot be explained.
This figure was adapted from Longo et al., 2019.

The implications of infertility are multidimensional, as the diagnosis of infertility has been shown to negatively affect mental health in both males and females (Fisher & Hammarberg, 2012; Rooney & Domar, 2018). In cases of male-factor infertility, symptoms of depression, anxiety, and lower self-esteem were reported (Biggs et al., 2023). Moreover, the World Health Organization (WHO) notes that a couple's decision to have a child and start a family encompasses an essential human right, and that infertility may abrogate this right (WHO, 2023).

The gravity of the issue of infertility is further highlighted when considering the rate at which global infertility is growing. The prevalence of infertility has been steadily increasing, as the percentage of couples who suffer from this condition has risen from about 8% in the 1980s to 15% today (Bushnik et al., 2012; Comhaire & WHO, 1987; WHO, 2023). In addition, there has been a 51.6% decrease in mean sperm count between 1973 and 2018 (Levine et al., 2023). Such a decrease in male fertility may be partially explained by the increased average age of parents, several lifestyle factors such as smoking and alcohol use, and increased long-term exposure to toxins in the environment (Jafari et al., 2021; Kaltsas et al., 2023; Petraglia et al., 2013). Of further concern is the recent report from the World Health Organization demonstrating that the infertility rates among high-, middle-, and low-income countries are similar, stressing that the issue of infertility is a global crisis (WHO, 2023).

1.2 Assisted Reproduction Techniques and their Limitations

Alternatives to traditional fertilization are a possibility with the advent of assisted reproduction technologies (ARTs). The most commonly used ARTs include intrauterine insemination (IUI), in-vitro fertilization (IVF), and intracytoplasmic sperm injection (ICSI). During an IUI, a prepared sample of highly active spermatozoa is inserted directly into the uterus using a thin, flexible catheter. The IUI procedure is generally the least expensive and invasive for the female, but comes with a lower success rate compared to IVF or ICSI (Cohlen et al., 2018). On the other hand, IVF involves a series of carefully scheduled hormone injections, which the female must take for oocyte maturation to occur. Eggs are then collected from the ovary and fertilized *in vitro* using specialized culture media. The resulting embryos are finally transferred into the uterus using a catheter to establish a pregnancy. IVF has certain advantages when compared to IUI, as it offers the option to

perform genetic testing on the embryo and to store other embryos through cryopreservation. In addition, IVF has a higher success rate when compared to IUI, with a reported value of 30-40% (Smith et al., 2015; Wade et al., 2015). However, one cycle of IVF can cost upwards of $10,000 and is very invasive for the female, making it a less than ideal procedure. The ICSI procedure also involves ovarian stimulation and egg retrieval, but differs from IVF as the spermatozoon is injected directly into a mature egg (Balli et al., 2022; Jain & Singh, 2023). ICSI has a similar cost and success rate when compared to IVF, but comes with an additional risk of damaging the egg upon sperm injection, which occurs in roughly 5-10% of retrieved eggs (Woodland & Carroll, 2022). In addition, increased risks of genetic abnormalities and congenital malformations have been reported in offspring conceived with ICSI when compared to conventional insemination, which may be explained in part by underlying defects in the spermatozoon (Alukal & Lamb, 2008; Coates et al., 2015; Wennerholm et al., 2000). There is currently no possibility to assess the DNA quality of the spermatozoon injected in ICSI. Consequently, an abnormal paternal genome can promote these abnormalities in offspring, which may appear at early or later stages in life.

The limitations of conventional ARTs, namely the high invasiveness, cost, and low success rates, have expectedly veered many infertile couples away from considering them as sensible options. Thus, there is currently an unmet need to ameliorate current alternatives to fertilization.

1.3 Sperm Morphology

Mature spermatozoa are highly specialized and compartmentalized terminally differentiated cells with unique functional properties. Mammalian spermatozoa consist of three functionally distinct compartments: the head, midpiece, and tail (Figure 2). The head can be described as a smooth, oval-shaped structure around 3-5 μm in length (Menkveld et al., 2011). This structure

contains a small amount of cytoplasm and a large nucleus, which holds tightly compacted chromatin containing the paternal genome that will ultimately be delivered to the offspring. Importantly, 40-70% of the anterior portion of the sperm head is covered by the acrosome. The acrosome is an acidic vacuole derived from the Golgi apparatus, which contains hydrolytic enzymes necessary to dissolve the zona pellucida in the process of fertilization (Berruti & Paiardi, 2011; WHO, 2021). The acrosome of the sperm head is separated from the post-acrosomal region by the equatorial segment, which contains proteins involved in sperm-egg fusion (Wolkowicz et al., 2008). At the base of the sperm head lies the midpiece, a compartment of the spermatozoon containing an abundance of mitochondria. The mitochondrial sheath of the midpiece forms a helical structure, which surrounds nine outer dense fibers and the axoneme (a structure composed of microtubule pairs formed by alpha and beta tubulin). The mitochondria that reside in the midpiece serve as efficient producers of ATP through the citric acid cycle and oxidative phosphorylation, which is required for providing energy to the cell as well as supplying phosphate groups, which the spermatozoon uses to phosphorylate proteins (Piomboni et al., 2012; Urner & Sakkas, 2003). Connected to the midpiece by a structure known as the annulus resides the sperm tail. The tail of the spermatozoon comprises a structure roughly 45 μm in length which can be divided into the principal piece and end piece (Menkveld et al., 2011). The principal piece is the longest portion of the sperm tail and is integral for sperm propulsion and motility. This structure contains the fibrous sheath, a unique cytoskeletal structure surrounding nine outer dense fibers and the axoneme (Eddy et al., 2003; Leung et al., 2023). The fibrous sheath serves in the maintenance of the structural integrity of the sperm tail and in the scaffolding of proteins involved in various signaling pathways that regulate sperm maturation and energy production (e.g., glycolytic enzymes) (Eddy et al., 2003). As the tail gets thinner along the distal end of the spermatozoon, it

is terminated with the end piece, which does not contain the fibrous sheath but only the axoneme (Lehti & Sironen, 2017). The entire spermatozoon is enclosed by a plasma membrane containing phospholipids rich in polyunsaturated fatty acids, and other structural components such as cholesterol (Lenzi et al., 1996).

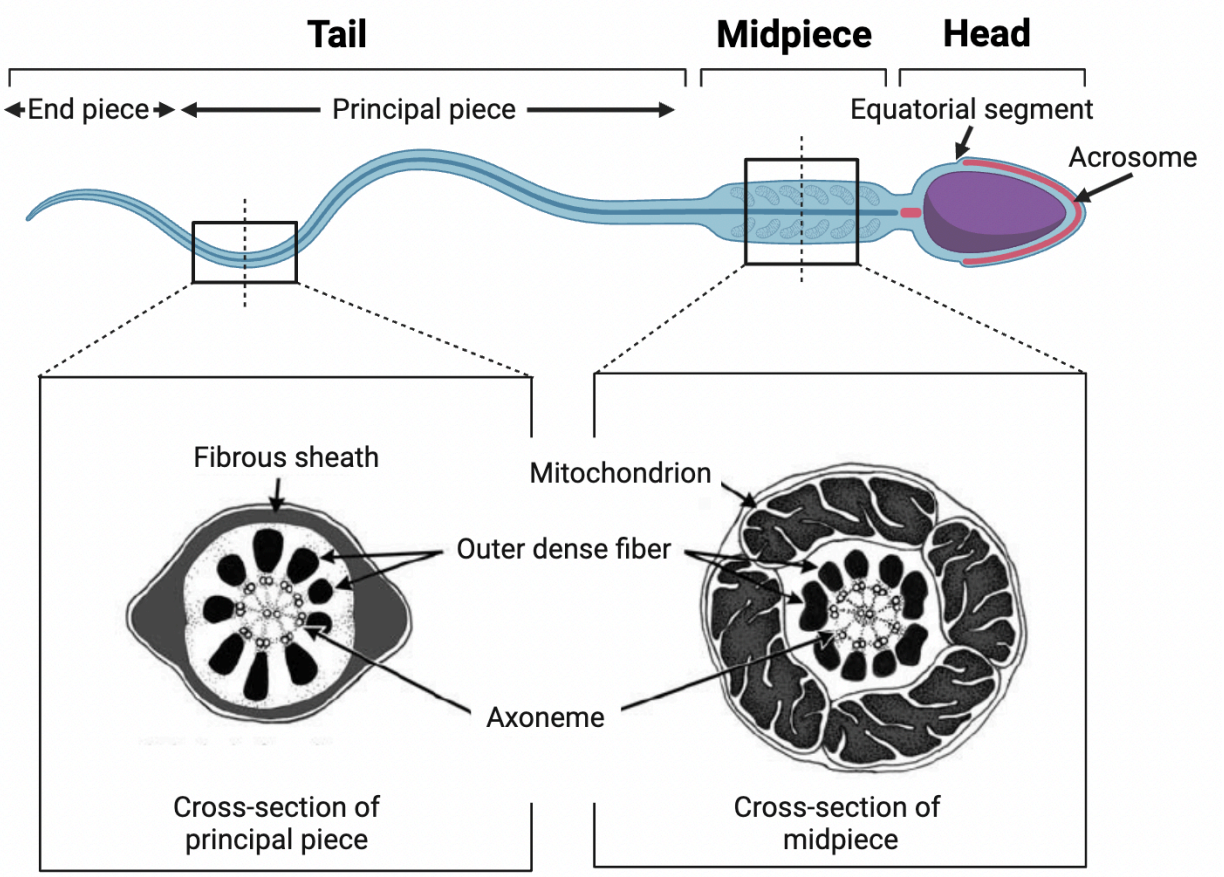


Figure 2. Morphology of mammalian spermatozoa. Mature mammalian spermatozoa are made up of a head, midpiece, and flagellum (tail). At the anterior portion of the head resides the acrosome, which is separated from the post-acrosomal region by the equatorial segment. The sperm midpiece contains a mitochondrial sheath, which forms a helical structure around nine outer dense fibers and the axoneme. The principal piece of the sperm flagellum contains the fibrous sheath, which surrounds the outer dense fibers and axoneme, whereas the end piece contains only the axoneme surrounded by the plasma membrane.
This figure was adapted from Suarez, 2010, and generated using BioRender.

1.4 Spermatogenesis and Epididymal Sperm Maturation

Before considering the process of fertilization, we must review the process of spermatogenesis, an aptly named cellular developmental process occurring within the seminiferous tubules of the testes, whereby the differentiation of male germ cells results in the production of spermatozoa. Spermatogenesis is highly regulated and involves the activity of Sertoli cells, which are stimulated by FSH to provide nutrition and support for sperm development, and Leydig cells, which are stimulated by LH to produce testosterone, which supports spermatogenesis (de Kretser et al., 1998; O'Shaughnessy, 2014; Oduwole et al., 2021). Spermatogenesis is a complex process that can be divided into three steps: spermatocytogenesis, spermiogenesis, and spermiation (de Kretser et al., 1998; Sharma & Agarwal, 2011). In spermatocytogenesis, stem cells known as spermatogonia undergo mitosis to become diploid primary spermatocytes, which then undergo meiosis I and II to generate haploid round spermatids. The following step, spermiogenesis, involves a series of events to form a spermatozoon, including spermatid elongation, chromatin compaction, and the formation of key structures such as the acrosome and tail. Importantly, the replacement of most histones with protamines and the compaction of chromatin during spermiogenesis renders the spermatozoon transcriptionally and translationally inactive (Gardiner-Garden et al., 1998; Wang et al., 2019). Spermiogenesis also involves the loss of most of the spermatozoon's cytoplasm, along with the loss of cytoplasmic organelles such as the endoplasmic reticulum, peroxisomes, and ribosomes, making the spermatozoon a highly specialized cell (O'Donnell, 2015). Following spermiogenesis, spermatozoa can be released into the lumen of the seminiferous tubules in the process of spermiation. Spermatozoa then travel through the rete testis and into the epididymis, where they further mature to gain motility and other factors required for fertilization. The maturation of spermatozoa within the epididymis is a complex process involving cargo-containing exosomes

known as epididymosomes, which are released by the epidydimal epithelium (James et al., 2020). Through these epididymosomes, the sperm plasma membrane is modified, and the spermatozoon acquires vital proteins that are needed for their fertilizing ability (James et al., 2020; Paul et al., 2021). It should be noted that up to half of the spermatozoa that exit the testis can die and be reabsorbed by the epithelium, but the surviving spermatozoa can be stored in the epididymis for days (Sharma & Agarwal, 2011).

1.5 Sperm Transport, Capacitation and Acrosome Reaction

The objective of the spermatozoon is to fertilize the oocyte to successfully transfer its haploid genome to the offspring. When the male is sexually stimulated, muscle contractions allow the spermatozoa to travel from the epididymis into the vas deferens, followed by the urethra. Upon ejaculation, these spermatozoa are mixed with seminal plasma originating from the epididymis and accessory glands (such as the prostate and seminal vesicles), generating semen. The seminal plasma is a complex fluid which not only serves as a vehicle for sperm transport, but also provides the spermatozoon with sufficient nutrition and protection, which is needed in the harsh vaginal environment (Szczykutowicz et al., 2019). Seminal plasma also contains several substances that regulate sperm function, such as energy metabolites (e.g., citrate, pyruvate, and lactate, which provide sufficient energy for motility) and proteins (e.g., decapacitation factors and semenogelin which prevent premature capacitation) (de Lamirande, 2007; Rodríguez-Martínez et al., 2011; Talluri et al., 2017). Once deposited inside the anterior vagina, the spermatozoon uses is flagellum to propel itself across its first barrier, the cervix, before entering the uterus. The movement of spermatozoa within the uterus is facilitated by uterine peristaltic contractions, which guide the cells toward its second barrier, the uterotubal junction (Kunz et al., 1997; Sakkas et al., 2015). The

barriers of the cervix and uterotubal junction serve as gates of passage for spermatozoa, leading small numbers of predominantly normal spermatozoa toward the fertilization site (Druart, 2012; Krzanowska, 1974; Sakkas et al., 2015). Once the spermatozoon has passed the uterotubual junction into the fallopian tube (also referred to as the oviduct in other mammalian species), the spermatozoon is guided to move toward the egg by a gradient of chemoattractants that are secreted from the oocyte in a process known as chemotaxis (Kaupp et al., 2008; Teves et al., 2009). In addition, evidence suggests that spermatozoa move in a temperature gradient towards the warmer fertilization site in a process known as thermotaxis (Bahat & Eisenbach, 2006; Xiao et al., 2022). However, freshly ejaculated spermatozoa do not have the ability to fertilize the oocyte and must first undergo a series of complex and time-dependent events in the female reproductive tract known as capacitation and the acrosome reaction (Jin & Yang, 2017).

Sperm capacitation was originally characterized in the early 1950s, primarily by Colin Russell Austin and Min Chue Chang, as a series of reversible morphological and biochemical changes that are required for the spermatozoon to recognize and fertilize the oocyte (Austin 1951; Austin 1952; Chang, 1951). Capacitation in human spermatozoa takes roughly 4-10 hours, requires a temperature of 37^{o}C, and occurs in the fallopian tubes in a minority of ejaculated cells (Figure 3) (Marín-Briggiler et al., 2002; Puga Molina et al., 2018). More specifically, the spermatozoa bind to the oviductal epithelium at the level of the isthmus, where they form a reservoir, allowing them to undergo capacitation for the amount of time required. It is important to note that premature sperm capacitation is averted through elements found in the seminal plasma. More specifically, decapacitation factors prevent capacitation primarily through the stabilization of the sperm membrane and semenogelin, zinc, and other antioxidant enzymes interfere with reactive oxygen species (ROS) production required for capacitation to occur (Begley & Quinn, 1982; de Lamirande

& Lamothe, 2010). Once the spermatozoon enters the female reproductive tract, the removal of these decapacitation factors, semenogelin, and zinc, as well as the high levels of bicarbonate and calcium present, allow the initiation of capacitation (Delgado-Bermudez et al., 2022; Fraser & Adeoya-Osiguwa, 2005).

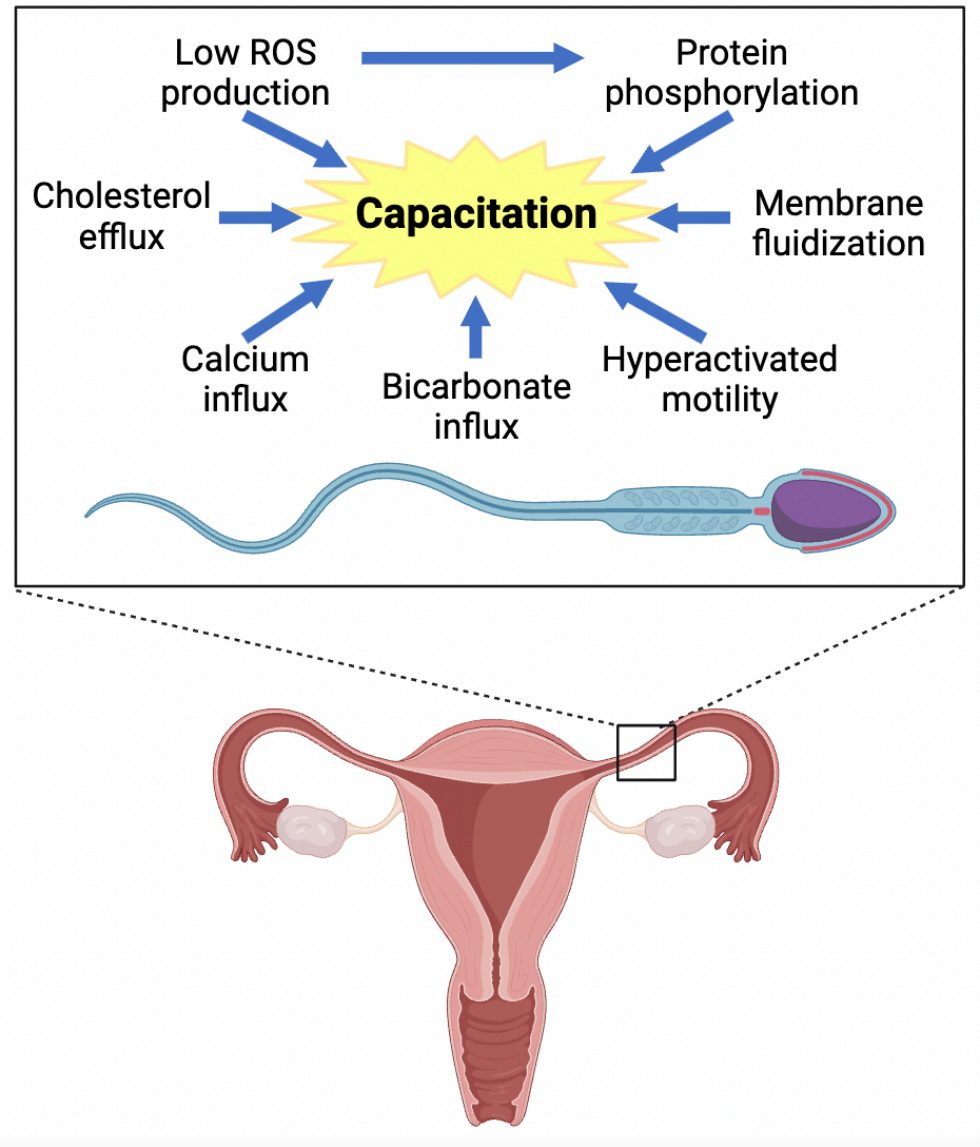


Figure 3. Diagrammatic representation of human sperm capacitation at the level of the isthmus. Human sperm capacitation involves a series of biochemical and morphological changes, including (but not limited to): low ROS production which can initiate protein phosphorylation events, membrane fluidization, efflux of cholesterol from the plasma membrane, bicarbonate and calcium influx, and cholesterol efflux. This figure was generated using BioRender.

Capacitation is a process that can be characterized as a series of time-dependent events, including the acquisition of hyperactivated motility (due to calcium and bicarbonate influx), membrane fluidization (due to cholesterol efflux), and the increase in intracellular pH (Gangwar & Atreja, 2015; Jin & Yang, 2017). Notably, the acquisition of sperm hyperactivation gives the spermatozoon the force required to detach from the oviduct and continue its journey to the oocyte.

The process of capacitation is guided by the low and controlled production of reactive oxygen species (ROS) such as the superoxide anion ($O_2^{\bullet -}$) and nitric oxide ($NO^{\bullet}$), and the phosphorylation of proteins on serine, threonine, and tyrosine residues (de Lamirande & Gagnon, 1993; de Lamirande & O'Flaherty, 2012; de Lamirande et al., 1997; de Lamirande et al., 2009; Leclerc et al., 1996; O'Flaherty et al., 1999; O'Flaherty et al., 2003; Zini et al., 1995). It is important to note that the low levels of ROS that are generated during capacitation have been reported to increase tyrosine phosphorylation events by triggering a series of signalling pathways including cAMP/PKA, PKC, PI3K/AKT, and ERK (Figure 4) (de Lamirande & O'Flaherty, 2008; Leclerc et al., 1996; Leclerc et al., 1997; O'Flaherty et al., 2006). During capacitation, the most prominent tyrosine-phosphorylated proteins in human spermatozoa are protein A-kinase anchoring proteins (AKAPs) located at the level of the fibrous sheath. These include AKAP82 (~80 kDa), its precursor pro-AKAP82, and FSP95 (~100 kDa) (Carrera et al., 1996; Mandal et al., 1999; Naz & Rajesh, 2004). It is through the regulation of these tyrosine phosphorylation events that the spermatozoon modulates the many changes associated with capacitation, such as the acquisition of hyperactivation and membrane fluidity. Thus, tyrosine phosphorylation is one of the most important events to occur during capacitation, and assessing levels of tyrosine phosphorylation in human spermatozoa remains one of the most useful tools in evaluating human sperm capacitation.

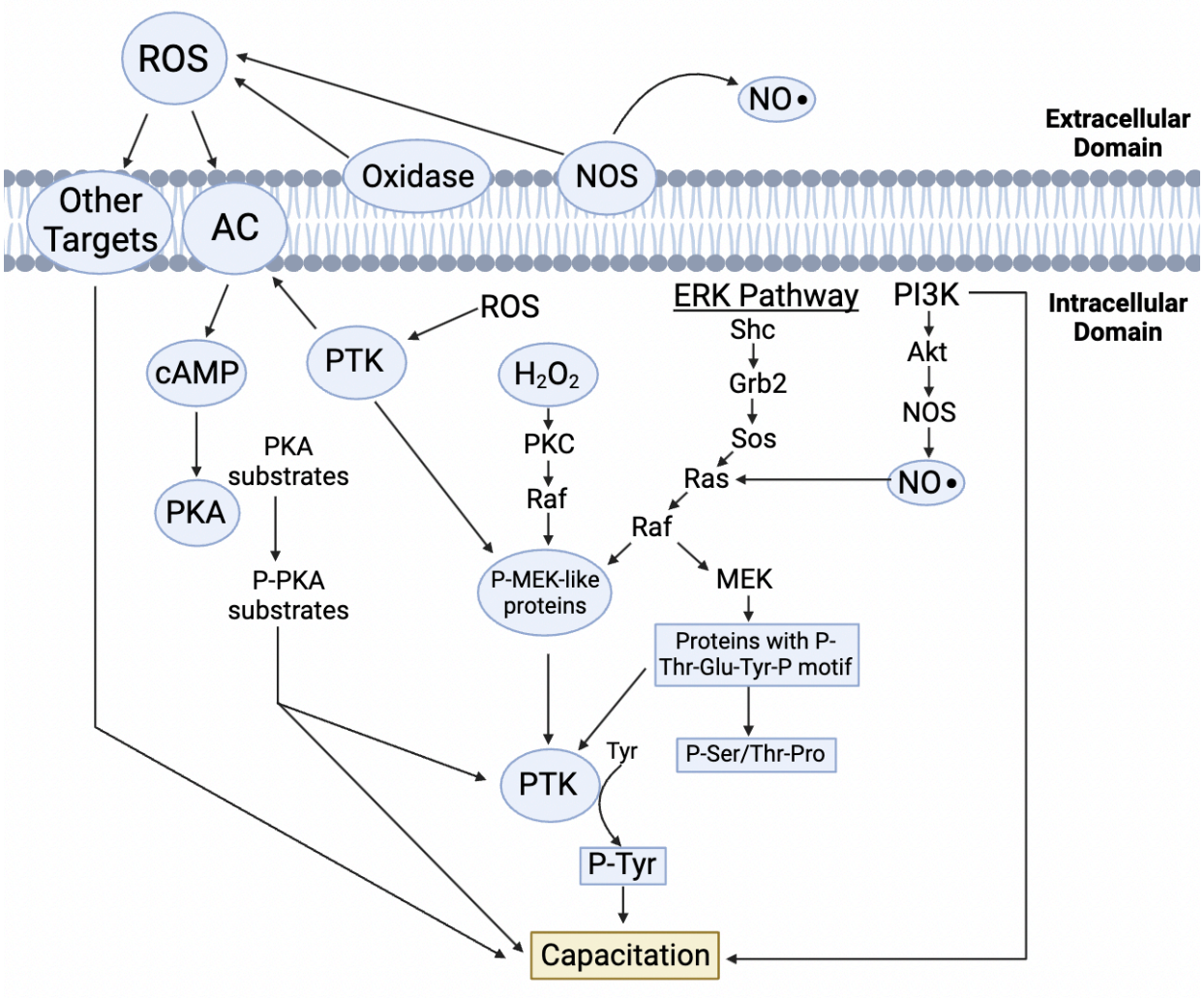


Figure 4. Regulation of phosphorylation events in human sperm capacitation.
Reactive oxygen species (ROS) such as the superoxide anion ($O_2^{\bullet -}$) and nitric oxide ($NO^{\bullet}$) are produced by sperm oxidases and nitric oxide synthase (NOS). Stimulation of protein tyrosine kinases (PTK) and adenylyl cyclase (AC) by ROS leads to an increase in cAMP, resulting in the activation of the catalytic subunits of protein kinase A (PKA). This causes an increase in phospho-PKA (P-PKA) substrates, which leads to tyrosine phosphorylation (P-Tyr) events that drive sperm capacitation through a second PTK. Hydrogen peroxide (H_2O_2) can also stimulate protein kinase C (PKC), promoting the phosphorylation of Raf which regulates phospho-mitogen-activated protein kinase kinase (P-MEK)-like proteins that are involved in tyrosine phosphorylation in capacitation. The extracellular signal-regulated kinase (ERK) pathway and phosphoinositide 3-kinase (PI3K) signaling-mediated nitric oxide production also play a role in tyrosine phosphorylation, through the stimulation of Ras and Raf. In addition, both ERK and PI3K pathways can mediate the phosphorylation of proteins with the Thr/Glu/Tyr motif.
This figure was adapted from de Lamirande & O'Flaherty, 2008, and generated using BioRender.

The following step in fertilization, the acrosome reaction, is a calcium- and progesterone-dependent event which is initiated by the binding of zona pellucida (ZP) glycoproteins known as ZP1, ZP3, and ZP4 to the capacitated human spermatozoa (Breitbart, 2002; Gupta & Bhandari, 2011). The acrosome reaction involves the release of hydrolytic enzymes of the acrosome, allowing the spermatozoon to penetrate the thick glycoprotein matrix surrounding the oocyte known as the zona pellucida. The particular enzymes released include the corona-penetrating enzyme hyaluronidase, and the trypsin-like protease acrosin (Brucker & Lipford, 1995; Patrat et al., 2000). Notably, only capacitated spermatozoa are able to undergo the acrosome reaction, yet both events are required for fertilization to occur. It has been reported that spermatozoa that fail to undergo capacitation or the acrosome reaction are associated with male infertility (Aitken, 2006; Buffone et al., 2005; O'Flaherty, 2020).

1.6 Sources of ROS in Human Sperm Capacitation

Human spermatozoa produce several ROS, including the superoxide anion ($O_2^{\bullet-}$), nitric oxide ($NO^{\bullet}$), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$), all with varying half-lives, reactivity, and cell permeability (Table 1) (de Lamirande & O'Flaherty, 2012). To produce ROS, mammalian spermatozoa utilize sperm oxidases as well as nitric oxide synthase (NOS) (de Lamirande & O'Flaherty, 2012; O'Flaherty et al., 2006). Sperm oxidases transfer electrons from NAD(P)H to molecular oxygen (O_2), generating $O_2^{\bullet-}$, which can then be dismutated to H_2O_2. NOS, on the other hand, uses NADPH and O_2 to convert L-arginine to L-citrulline, forming $NO^{\bullet}$. The $NO^{\bullet}$ that is formed in this process can also react with $O_2^{\bullet-}$ to form $ONOO^-$. It should be noted that the L-citrulline that is formed by NOS can be recycled back into L-arginine by argininosuccinate lyase and argininosuccinate synthase, which can then be used to generate more $NO^{\bullet}$ (Goodwin et

al., 2004; Haines et al., 2012). The activity of argininosuccinate synthase is the rate-limiting step in this process (Goodwin et al., 2004).

Type of ROS	$O_2^{\bullet-}$	H_2O_2	$NO^{\bullet}$	$ONOO^-$
Origin	One electron reduction of molecular oxygen	$O_2^{\bullet-}$ dismutation	Conversion of L-arginine to L-citrulline via nitric oxide synthase	Reaction of $O_2^{\bullet-}$ with $NO^{\bullet}$
Half-life	1 ms	Minutes to hours	1-7 s	Minutes
Reactivity	Low	Low to medium	Low	High
Cell permeant	No	Yes	Yes	No
Specific scavenger	Superoxide dismutases (SODs)	Catalase, glutathione peroxidases (GPXs), thioredoxins (TRXs), peroxiredoxins (PRDXs)	None	Glutathione peroxidases (GPXs), peroxiredoxins (PRDXs)

Table 1. Types of ROS in human spermatozoa. Human spermatozoa possess numerous reactive oxygen species (ROS) including the superoxide anion ($O_2^{\bullet-}$), nitric oxide ($NO^{\bullet}$), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$). Each ROS has a unique origin, and activity within the spermatozoon.
This figure was adapted from de Lamirande & O'Flaherty, 2012.

Although the involvement of ROS generation in sperm capacitation is evident, the mechanism and localization of intracellular ROS production during sperm capacitation remain largely unknown. Studies have suggested that oxidases at the level of the plasma membrane play a major role in extracellular ROS generation during capacitation, since superoxide dismutase (SOD; an impermeable scavenger of $O_2^{\bullet-}$) and catalase (CAT; a scavenger of H_2O_2) inhibit sperm capacitation (de Lamirande & Gagnon, 1995; de Lamirande & Lamothe, 2009; O'Flaherty, 2015). Yet, cytosolic oxidases may also play a role in ROS generation during capacitation, and remain to be further explored (O'Flaherty, 2015). In the context of $NO^{\bullet}$ generation, all isoforms of the NOS enzyme are found in human spermatozoa including endothelial, neuronal, and inducible NOS (de Lamirande et al., 2009; Herrero et al., 1996; O'Bryan et al., 1998). NOS enzymes may be localized at both the intracellular and extracellular levels, thus $NO^{\bullet}$ generation in the process of human sperm capacitation may not limited to one compartment. The permeability of $NO^{\bullet}$ must also be considered, as it strengthens its ability to act at different levels, both intracellularly and extracellularly. It should be noted that although the mitochondria are typically associated with ROS generation in somatic cells, studies have demonstrated that mitochondrial ROS generation likely does not play a role in human sperm capacitation. This has been shown as mitochondrial toxins such as carbonyl cyanide m-chlorophenyl hydrazone (CCCP; an uncoupler of oxidative phosphorylation) or rotenone (an inhibitor of complex I of the electron transport chain) did not prevent ROS production in spermatozoa (Aitken et al., 1997; O'Flaherty, 2015).

1.7 ROS and Sperm Function

ROS are highly reactive chemicals conventionally associated with their toxic effects, as the generation of high levels of ROS can damage vital cellular organelles and disrupt normal cellular

processes. This oxidative stress can be reflected through lipid peroxidation and DNA damage, as well as sperm morphological defects leading to poor motility (Aitken, 2017; Fernandez & O'Flaherty, 2018; Kurkowska et al., 2020; Sanocka & Kurpisz, 2004). However, there is growing evidence that low concentrations of ROS are required, as they serve as cell signalling molecules that are necessary for normal cell function. Spermatozoa are no exception to this phenomenon, as high ROS levels are detrimental, whereas low ROS levels are imperative for their function (de Lamirande & O'Flaherty, 2008; de Lamirande & O'Flaherty, 2012; O'Flaherty & Matsushita-Fournier, 2017; Zini et al., 1997). To help combat this excessive oxidative stress, the spermatozoon is equipped with various antioxidant systems. This includes the action of various isoforms of SOD, glutathione peroxidase (GPX), thioredoxin (TRX), and peroxiredoxins (PRDX) found in spermatozoa (O'Flaherty, 2014). PRDX6 in particular has been revealed to be a unique regulator of ROS action in spermatozoa, as it is the only peroxiredoxin isoform that has both peroxidase activity and an intrinsic calcium-independent phospholipase A_2 ($iPLA_2$) activity (Figure 5) (O'Flaherty, 2018). Other sperm PRDX isoforms contain only the peroxidase activity (which removes H_2O_2, other hydroperoxides, and $ONOO^-$), but the $iPLA_2$ activity of PRDX6 gives it the ability to combat lipid peroxidation at the level of the plasma membrane (Fisher, 2017; O'Flaherty, 2018). Once the peroxidized phospholipid is removed, the lysophosphatidylcholine acyl transferase (LPCAT) activity of PRDX6 enables the replacement with a non-oxidized phospholipid (Fisher et al., 2016).

It has been previously reported that the seminal plasma of 30-40% of infertile men contains increased ROS levels and deficiencies in the aforementioned antioxidant systems (Agarwal et al., 2014; Iwasaki & Gagnon, 1992; Mannucci et al., 2022; Takeshima et al., 2020; Yumura et al., 2017). For instance, levels of PRDXs (particularly PRDX6) have been reported to be low in

spermatozoa from infertile men, which was correlated with high levels of lipid peroxidation (an indicator of oxidative stress) (Gong et al., 2012). This is consistent with the finding that *Prdx6*$^{-/-}$ mouse spermatozoa exhibit low fertilizing ability, with impaired motility and severe DNA damage (Ozkosem et al., 2016). Altogether, it should be emphasized that the balance of ROS in human spermatozoa is essential for its fertilizing ability. Sufficient ROS production is required in certain signaling pathways, yet excessive ROS may impair certain functions of the spermatozoon.

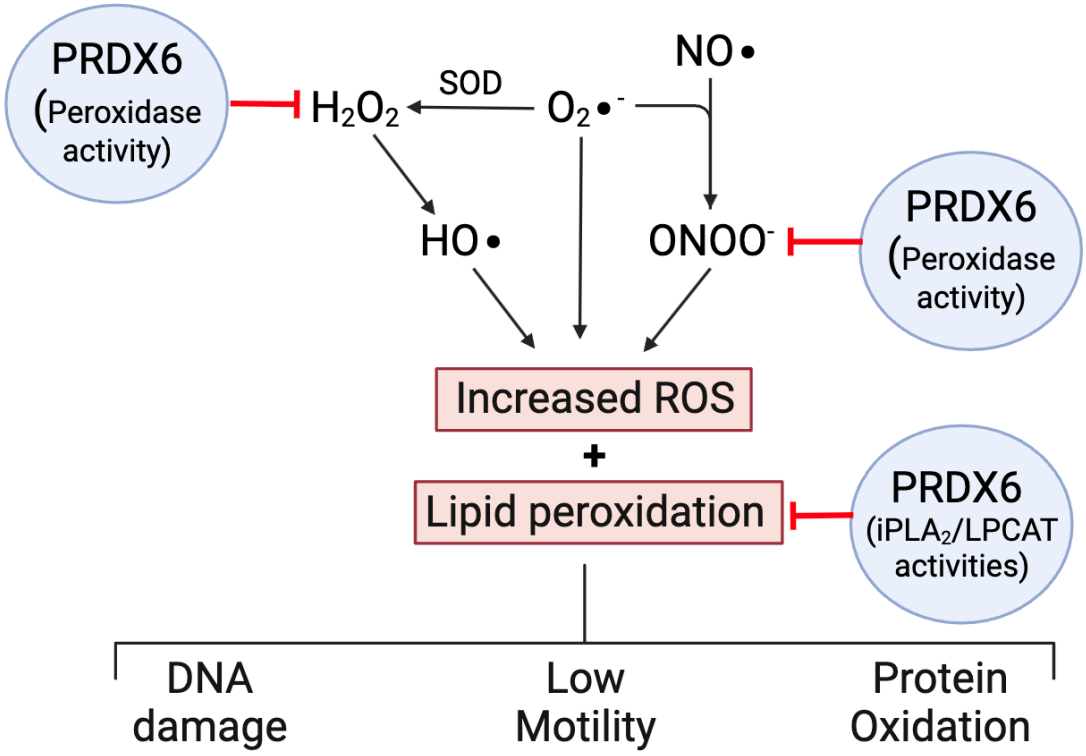


Figure 5. ROS-mediated damage and regulation via PRDX6 in mammalian spermatozoa. Peroxiredoxin 6 (PRDX6) plays a key role in protecting spermatozoa against oxidative stress that may impair the DNA, motility, and protein function of the spermatozoon. The peroxidase activity of PRDX6 is required in the removal of harmful reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$). The additional calcium-independent phospholipase A_2 ($iPLA_2$) activity of PRDX6 is necessary to protect against lipid peroxidation at the level of the sperm plasma membrane, and the LPCAT activity allows for the replacement of the oxidized phospholipid with a non-oxidized phospholipid.
This figure was adapted from O'Flaherty, 2018, and generated using BioRender.

1.8 Citrate Abundance and Implications in Human Spermatozoa

Citrate is an organic carboxylic acid whose principal role is to regulate energy production in the cell. Human spermatozoa are exposed to very high concentrations of citrate in the seminal plasma, originating primarily from the prostate (Costello & Franklin, 1991). Levels of citrate in the seminal plasma of fertile men have been reported to be in the range of 5-50 mM (Costello & Franklin, 1991; Zöpfgen et al., 2000). However, seminal citrate levels have been reported to be notably lower in some infertile men, including idiopathic cases (Gruhl et al., 2023; Gupta et al., 2011, Hamamah et al., 1993). For instance, average levels of citrate in the seminal plasma of idiopathic oligoasthenoteratozoospermic patients were shown to be decreased by about 50% when compared to normozoospermic patients (Mumcu et al., 2020). Surprisingly, despite the fact that human spermatozoa are exposed to such high concentrations of citrate through seminal plasma, and there seems to be a correlation between citrate and infertility, there are surprisingly few studies demonstrating how citrate metabolism and male fertility intersect.

1.9 Energy Metabolism in Spermatozoa

Much like in somatic cells, spermatozoa require a supply of ATP, which provides the energy necessary to ensure cell survival and motility, as well as phosphate groups needed for phosphorylation events in various cell signaling pathways. This ATP is produced through glycolysis (the cytosolic breakdown of glucose), as well as downstream mitochondrial events: the citric acid cycle and oxidative phosphorylation. However, these processes do not have the same yield of ATP, as glycolysis yields only 2 molecules of ATP per mole of glucose, whereas the citric acid cycle and oxidative phosphorylation produce about 36 molecules of ATP per glucose. Although the complete oxidation of glucose clearly generates much more ATP when compared to

glycolysis alone, the main source of energy in spermatozoa has been a topic of major contention (known as the "sperm energy debate") (Amaral, 2022). Some research supports the idea that spermatozoa gain ATP exclusively through glycolysis, since the ATP produced by mitochondria in the midpiece cannot reach the distal end of the sperm tail by simple diffusion (du Plessis et al., 2015). However, this seems unlikely due to the vast abundance of active mitochondria in the midpiece of the spermatozoon and the ability of both mouse and human spermatozoa to consume oxygen to produce energy (Eliasson, 1971; Tourmente et al., 2015). Thus, it is possible that ATP transfer shuttles exist to assist in the transport of ATP from the midpiece to the tail to maintain sperm motility and function (Ford, 2006).

In the process of glycolysis, glucose is first broken down at the expense of 2 molecules of ATP, in a series of steps known as the "investment" phase. The steps that require ATP include the enzyme hexokinase and phosphofructokinase-1, the rate-limiting step of glycolysis (Chaudry & Varacallo, 2023). In later reactions, 4 molecules of ATP are generated, along with 2 molecules of pyruvate. This is known as the "payoff" phase. Once pyruvate is generated, it can enter the mitochondrion to be oxidized into acetyl-CoA via the pyruvate dehydrogenase complex (PDC) to initiate the citric acid cycle and aerobic respiration.

The citric acid cycle (also known as the Krebs or tricarboxylic acid cycle) begins as mitochondrial acetyl-CoA is converted to citrate by citrate synthase (Alabuladhem & Bordoni. 2022; Arnold & Finley, 2023). Once citrate is synthesized, a series of redox reactions leads to the generation of 2 molecules of ATP, as well as 6 molecules of NADH and 2 molecules of $FADH_2$ (per molecule of glucose). The reducing equivalents NADH and $FADH_2$ can pass their electrons to the electron transport chain in order to generate more ATP via oxidative phosphorylation. Once glycolysis, the citric acid cycle, and oxidative phosphorylation have all occurred, a maximum

theoretical yield of 38 ATP molecules are produced per glucose, giving the spermatozoon the supply of energy required for various cell processes (Cooper, 2000; Miki 2007; Visconti, 2012).

1.10 Cytosolic Citrate Metabolism

Although citrate metabolism is typically associated with the citric acid cycle in the mitochondrion, cytosolic citrate metabolism also plays a large role in regulating cellular processes. To enter the cytosol, extracellular citrate can be transported via solute carrier proteins such as the sodium-citrate cotransporter (NaCT) (Pajor, 2014; Pizzagalli et al., 2021). In addition, mitochondrial citrate can enter the cytosol via the mitochondrial citrate carrier (SLC25A1/CIC) (Figure 6) (Iacobazzi & Infantino, 2014; Zara et al., 2022). The CIC is a transmembrane protein on the inner mitochondrial membrane, which belongs to a large family of membrane transport proteins known as solute carriers (SLCs) (Sun et al., 2010; Zara et al., 2022). Citrate traverses the CIC in exchange for malate and enters the intermembrane space of the mitochondria. From there, citrate can passively diffuse into the cytosol via an anion-selective channel on the outer mitochondrial membrane, and malate can re-enter the citric acid cycle within the mitochondrion.

Once it has entered the cytosol, citrate can be converted by an ATP- and coenzyme A (CoA)-dependent enzyme known as ATP-citrate lyase (ACLY), to yield acetyl-CoA and oxaloacetate (Iacobazzi & Infantino, 2014; Zara et al., 2022). The resulting cytosolic acetyl-CoA can then be used for many cellular processes, such as de novo fatty acid synthesis (providing long-term energy storage) and protein acetylation (affecting protein functions) (Drazic et al., 2016; Shi, 2015). The oxaloacetate, on the other hand, can be converted by cytosolic malate dehydrogenase (MDH), using NADH as a cofactor, to yield malate and NAD^+. Malate is then used by the malic enzyme (ME), using $NADP^+$ as a cofactor, to generate NADPH, pyruvate, and CO_2. The resulting pyruvate

can then re-enter the mitochondrion via the mitochondrial pyruvate carrier (MPC) to continue energy production. Importantly, the NADPH generated through this pathway can play a role in fatty acid elongation, and may potentially be used by the aforementioned NOS enzyme to produce $NO^{\bullet}$ (Salati & Goodridge, 1996). However, this link between NADPH generated from citrate metabolism and $NO^{\bullet}$ production has never been demonstrated, and the role of this potential source of intracellular $NO^{\bullet}$ in human sperm capacitation remains unclear.

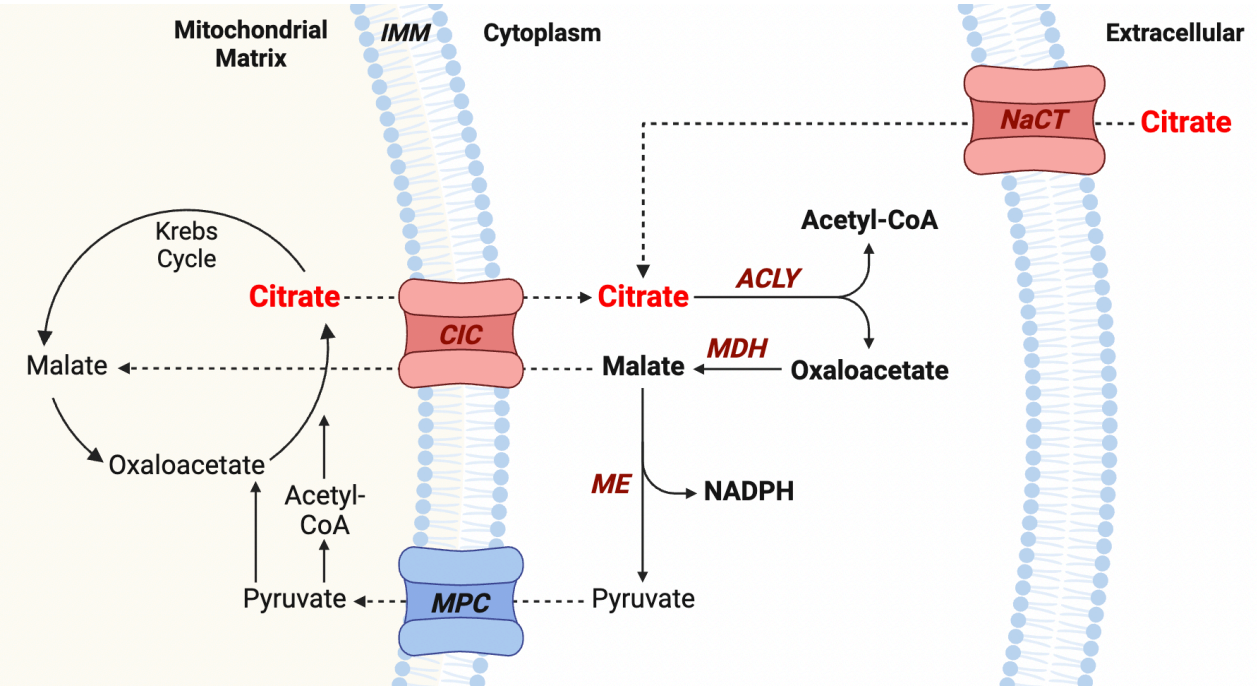


Figure 6. Cytosolic citrate metabolism. Extracellular citrate can enter the cytosol via solute carriers such as the sodium-citrate cotransporter (NaCT), and mitochondrial citrate can be transported into the cytosol via the mitochondrial citrate transporter (CIC) on the inner mitochondrial membrane (IMM; outer mitochondrial membrane not depicted). Once citrate enters the cytosol, a series of biochemical reactions involving ATP-citrate lyase (ACLY), malate dehydrogenase (MDH), and malic enzyme (ME), leads to the production of NADPH. The resulting pyruvate that is generated from ME can re-enter the mitochondrion via the mitochondrial pyruvate carrier (MPC) to continue energy production.
This figure was adapted from Zara et al., 2022, and generated using BioRender.

2. HYPOTHESIS AND OBJECTIVES

It is known that citrate is an energy metabolite that is very abundant in the seminal plasma of fertile men, and its levels are decreased in some cases of idiopathic infertile men. However, the role of citrate in human sperm capacitation is largely unknown. We hypothesize that cytosolic citrate can support human sperm capacitation by producing intracellular nitric oxide ($NO^{\bullet}$). Our objectives are to determine whether the mitochondrial citrate transporter (CIC), ATP-citrate lyase (ACLY), and the malic enzyme (ME) are necessary for intracellular $NO^{\bullet}$ production during human sperm capacitation.

3. MATERIALS AND METHODS

3.1 Materials

Percoll was purchased from GE Healthcare (Baie d'Urfe, QC, Canada). Nitrocellulose membranes (pore size, 0.22 mm) were purchased from Osmonics, Inc. (Westborough, MA, USA) and the Pierce™ ECL Western Blotting Substrate was purchased from ThermoScientific (Rockford, IL, USA). The primary antibodies anti-phosphotyrosine and anti-phospho-ATP-citrate lyase, were supplied by Cell Signaling Technology, Inc. (Beverly, MA, USA). The peroxidase-conjugated secondary antibodies, goat anti-mouse IgG and donkey anti-rabbit IgG, were supplied by Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Fetal cord serum was provided by the birthing center of the McGill University Health Center. Trisodium citrate dihydrate was purchased from BioShop (Burlington, ON, Canada). Mitochondrial citrate transport protein (CIC) inhibitor, SB204990, and bromopyruvic acid (BPA) were purchased from MilliporeSigma (Burlington, MA, USA). L-N^G-Nitro arginine methyl ester (L-NAME) was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Diaminofluorescein-2 diacetate (DAF-2DA) was supplied by Calbiochem (San Diego, CA, USA). Other chemicals used were of reagent grade or higher.

3.2 Human Sperm Preparation

Healthy donors (21-30 years old) provided semen samples on the same day as the experiment after at least 3 days of sexual abstinence, which were incubated at 37°C for 30 minutes to allow liquefaction. The liquified semen sample was centrifuged at 2300 x g at room temperature for 30 minutes over a four-layer Percoll gradient, consisting of 0.1 mL 95%, 2 mL 65%, 2 mL 40%, and 2 mL 20% Percoll (bottom-to-top), made with isotonic HEPES buffered saline (HBS; 500 mM

HEPES, 280 mM fructose, 2.3 M NaCl, 80 mM KCl, 10 mM $MgCl_2$) and deionized water. Percoll gradient centrifugation was performed in order to separate the sperm cells with the best morphology and motility from the seminal plasma, abnormal sperm cells, and other cells such as leukocytes (Kovalski et al., 1992). Highly motile spermatozoa from the 95% fraction and the 65-95% interface were recovered, and an aliquot was used to determine sperm concentration.

The recovered highly motile human spermatozoa were diluted to 150 x 10^6 or 250 x 10^6 cells per mL in regular Biggers, Whitten, Whittingham medium (BWW-REG; 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, pH 7.95) (Biggers et al., 1971). In experiments that required the absence of energy substrates, spermatozoa were instead prepared in BWW medium with no energy substrates (BWW-NES), omitting the addition of sodium pyruvate, sodium lactate, and D-glucose. Aliquots of the prepared sperm samples were then diluted to 30 x 10^6 or 50 x 10^6 cells per mL (either in BWW-REG or BWW-NES), and incubated for 3.5 hours at 37ºC in the absence and presence of the following compounds: trisodium citrate (5, 10, 15, and 20 mM), mitochondrial citrate transport inhibitor (0.5 mM), SB204990 (30 μM), bromopyruvic acid (100 μM), and L-NAME (1 mM). In each experiment, 10% fetal cord serum ultrafiltrate (FCSu) was used as an inducer of capacitation.

3.3 SDS-PAGE and Immunoblotting

Following human sperm preparation, each sample was supplemented with sample buffer containing 100 mM dithiothreitol (DTT) and phosphatase inhibitors (0.1 mM sodium vanadate, 20 mM glycerol phosphate, and 5 mM sodium fluoride). Samples were then heated at 97ºC for 5 minutes, followed by centrifugation at 21000 x g for 5 minutes at room temperature. The

supernatant of each of the samples was then loaded into a 10% polyacrylamide gel, and electrophoresed in electrophoresis buffer (25 mM Tris, 182 mM Glycine, 0.1% SDS, and deionized water). The amperage for gel electrophoresis was set at 25 mA for every gel used. Once electrophoresis was complete, the protein was electrotransferred onto a nitrocellulose membrane at 100 volts for 45 minutes using transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol). Once complete, the membranes were blocked for 1 hour with 5% skim milk in Tris-buffered saline with Tween 20 (TTBS 1X). The milk was removed, and the membranes were washed 2-3 times with TTBS 1X alone. The nitrocellulose membranes were then incubated overnight at 4^{o}C with either an anti-phosphotyrosine or anti-phospho-ATP-citrate lyase primary antibody (1:10,000 and 1:500, respectively) in antibody buffer (TBS 1x, 0.1% Tween 20, 25 mg/mL BSA, and deionized water). The primary antibody was removed, and the membranes were washed 5 times for 10 minutes in TTBS 1X. This was followed by incubation of the membranes for 1 hour in either goat anti-mouse or donkey anti-rabbit secondary antibodies for anti-phosphotyrosine or anti-phospho-ATP-citrate lyase, respectively (diluted at 1:2,500 in TTBS 1X). Once the secondary antibody was removed, the membranes were washed 5 times for 10 minutes in TTBS 1X and soaked in ECL solution (luminol enhancer and peroxide) for 5 minutes. Imaging was done using the Amersham Imager 600 supplied by GE Healthcare (Baie d'Urfe, QC, Canada). The relative intensity of each protein band was determined using ImageJ software (Bethesda, MD, USA), which was then normalized to the corresponding band on the silver-stained membrane, and normalized to the respective control sample.

3.4 Silver Staining

Once SDS-PAGE and immunoblotting were completed, the nitrocellulose membrane was washed 3 times for 10 minutes in deionized water, and placed inside a separate container along with silver stain solution (recipe from Jacobson & Kårsnäs, 1990). The silver stain solution, which contains silver nitrate, allows silver to bind to the chemical sidechains of amino acids (more specifically, carboxyl and sulfhydryl groups), and thus acts as a good measure of total protein content on the nitrocellulose membrane. Once a maximum signal was obtained, the membrane was washed with deionized water to remove excess silver stain solution. 2-3 drops of Farmer's reducer (0.05% sodium carbonate, 0.15% potassium hexacyanoferrate, and 0.3% thiosulfate) was added to the water to enhance staining, followed by drying of the membrane and imaging.

3.5 Sperm Motility and Viability

Sperm motility was determined using computer-assisted semen analysis (CASA). In this assay, 3 mg/mL BSA was added to each sample tube. The samples were mixed very well and loaded onto a Makler chamber with a cover slip placed on top. The motility of each sperm sample was then assessed using the HT-IVOS II CASA system set at 37^{o}C (purchased from Hamilton Thorne (Beverly, MA, USA)). The values of total motility and progressive motility for at least 200 spermatozoa were then saved for analysis. Total motility was defined as the number of motile sperm divided by total number of sperm x 100%, while progressive motility was defined as sperm with time-average velocity (VAP) $\geq$ 25 μm/s and straightness of trajectory (STR) $\geq$ 80 % divided by total sperm x 100% (Sloter et al., 2006).

Sperm viability was determined using the hypo-osmotic swelling (HOS) test, which evaluates the functional integrity of spermatozoa based on their ability to maintain equilibrium between the

cell and the environment (WHO, 2021). In this assay, the 50 µL sperm samples were incubated with 150 µL of hypo-osmotic swelling solution (75 mM fructose and 25 mM sodium citrate dihydrate) for 30 minutes at 37°C. Ten µL of the sample was then placed onto a Superfrost microscope slide supplied by Fisher Scientific (Ottawa, ON, Canada), and covered with a coverslip. A minimum of 200 spermatozoa were counted using the images captured with the HT-IVOS II CASA sperm analyzer (set at 20X magnification), and the percentage of viable spermatozoa (Figure 7, patterns b-g), was determined.

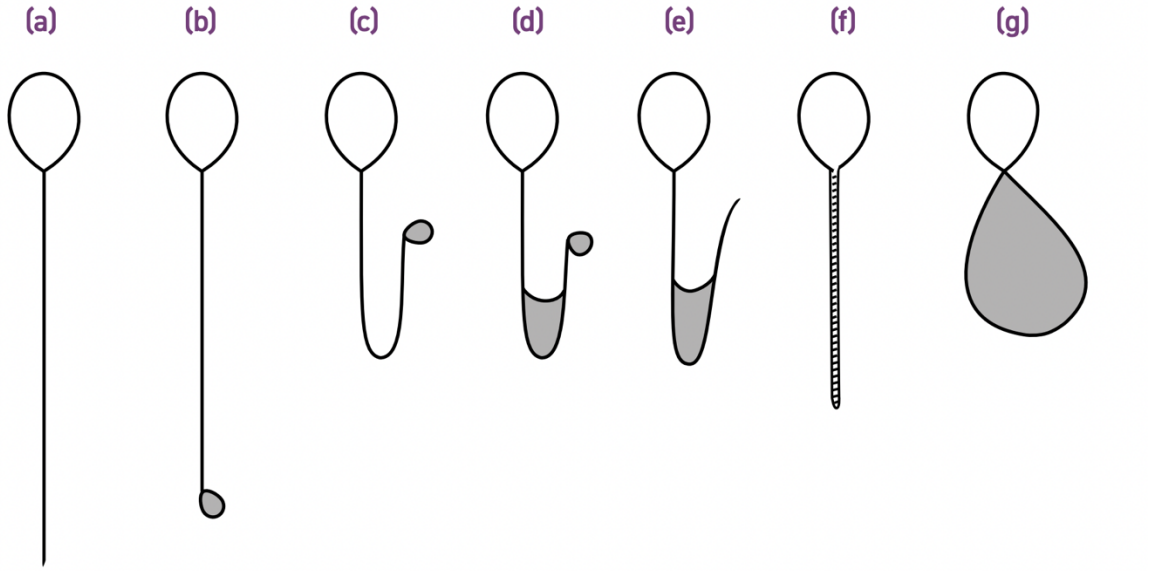


Figure 7. Graphic representation of morphological changes of spermatozoa incubated in hypo-osmotic swelling solution. Pattern (a) represents no change in sperm morphology. Patterns (b) through (g) represent changes in the tail of the spermatozoon, where swelling is represented by a darkened area.
This figure was adapted from WHO, 2021.

3.6 Nitric Oxide Determination by Fluorescence Microscopy

Spermatozoa from the 95% Percoll fraction (250 x 10^6 cells per mL) were incubated for 30 minutes at 37 °C with 10 µM of diaminofluorescein-2 diacetate (DAF-2 DA; a sensitive and specific fluorescent probe for intracellular $NO^{\bullet}$) (de Lamirande & Lamothe, 2009). DAF-2 DA is

diacetylated, making it cell permeable. Once it enters the cell, the hydrolyzation of DAF-2 DA by an intracellular esterase generates DAF-2, which can react with $NO^{\bullet}$ to produce fluorescent DAF-2T. It should be noted that the amount of fluorescence observed corresponds with levels of intracellular nitric oxide, making it appropriate to quantitatively assess nitric oxide production in spermatozoa.

Aliquots of the prepared sperm samples containing DAF-2 DA were then diluted to 50 x 10^6 cells per mL and treated with 10 mM of trisodium citrate and SB204990, with FCSu as an inducer of capacitation. The samples were then incubated for 3.5 hours at 37 ^{o}C, followed by centrifugation for 5 minutes at 600 x g. The supernatant was removed, and the pellet was resuspended in HBS 1X containing 2% paraformaldehyde.

Each sample tube was gently mixed, and a 10 μL aliquot was pipetted onto a Superfrost microscope slide. A drop of ProLong Antifade with 4′,6-diamidino-2-phenylindole (DAPI) was added to the samples, which were then smeared along the microscope slide and covered with a cover slip. The slides containing each sample were then placed under a Zeiss Axiophot microscope at the 40X and 100X objectives, and fluorescence intensity was analyzed with a 450-490 nm bandpass excitation filter. Images for at least 200 spermatozoa were saved for analysis, and levels of $NO^{\bullet}$ in each sample were expressed by determining average corrected total cell fluorescence (CTCF).

3.7 Statistical Analysis

All results were presented as mean ± standard error, and were analyzed using ANOVA and Tukey's test. A $P<0.05$ value was regarded as statistically significant. The normality of the data distribution was confirmed using the Shapiro-Wilk test.

4. RESULTS

4.1 Citrate supports human sperm capacitation

We first determined whether increasing concentrations of citrate can support human sperm capacitation when incubated with FCSu, a well-known capacitation inducer. Levels of tyrosine phosphorylation were significantly lower in samples incubated in BWW-NES without FCSu, with or without citrate, compared to the FCSu-treated samples, indicating that the spermatozoa in these samples did not capacitate even in the presence of citrate (Figure 8). However, when spermatozoa were incubated with FCSu, levels of tyrosine phosphorylation were further increased when supplemented with 5 and 10 mM of citrate, indicating that citrate supports the process of capacitation. Surprisingly, levels of tyrosine phosphorylation in samples treated with FCSu and 5 or 10 mM of citrate were comparable to that of spermatozoa incubated with FCSu in regular BWW medium (BWW-REG; containing pyruvate, lactate, and glucose). It should be noted, however, that spermatozoa exposed to higher concentrations of citrate (15 and 20 mM) in BWW-NES exhibited decreased tyrosine phosphorylation levels comparable to spermatozoa incubated in FCSu alone.

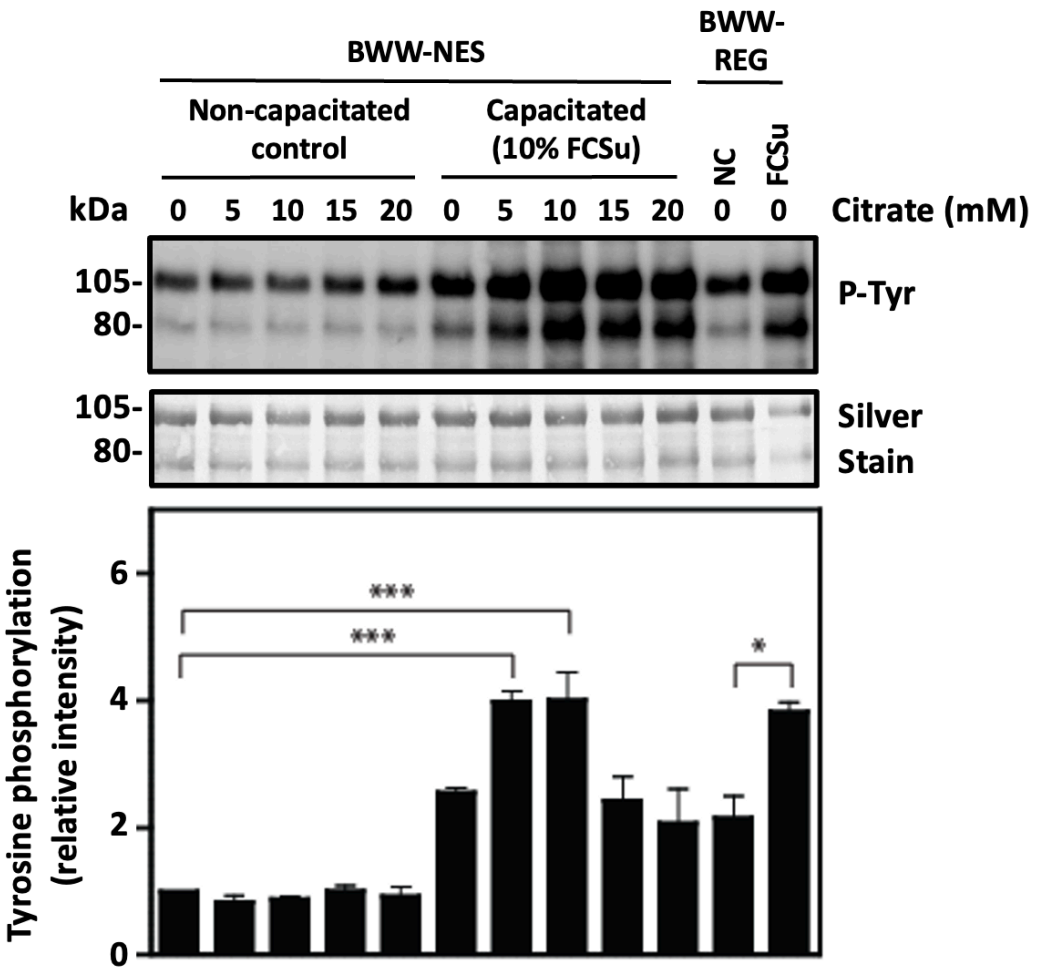
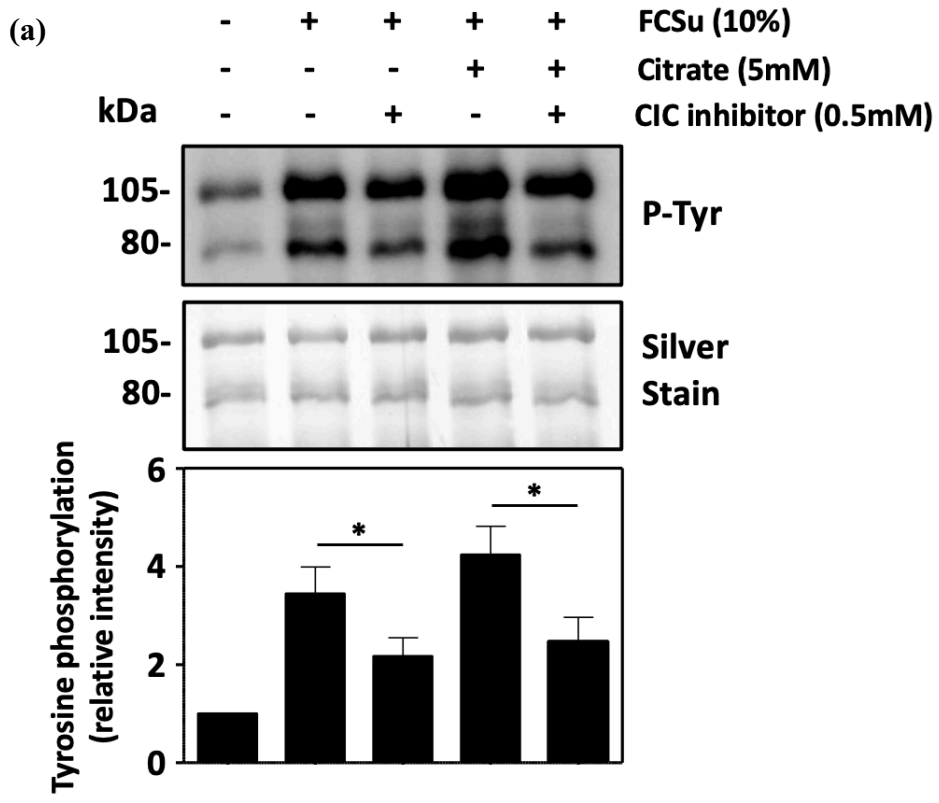


Figure 8. Citrate supports human sperm capacitation. Human spermatozoa were incubated in capacitating conditions in the absence and presence of FCSu, with different concentrations (5, 10, 15, and 20 mM) of citrate, in BWW medium containing no other energy substrates (BWW-NES). Immunoblotting using an anti-phosphotyrosine (P-Tyr) antibody reveals increased capacitation when spermatozoa were incubated with FCSu, along with 5 or 10 mM of citrate, at levels comparable to FCSu-treated samples in regular BWW (BWW-REG containing pyruvate, lactate, and glucose). However, samples incubated with FCSu and 15 or 20 mM of citrate exhibited decreased capacitation levels. This experiment was done a total of four times with different healthy donors, and a representative blot is shown.
*$p<0.05$, ***$p \leq 0.005$, ANOVA and Tukey's test, n=4.

4.2 Mitochondrial citrate transport supports human sperm capacitation

The role of the mitochondrial citrate transporter (CIC) was then determined, as spermatozoa were incubated with a competitive inhibitor of CIC under capacitating conditions in BWW-REG in the absence and presence of 5 mM citrate. Tyrosine phosphorylation levels following the incubation with 0.5 mM of the CIC inhibitor reveal that capacitation levels were significantly decreased, regardless of whether citrate was present in the capacitation media (Figure 9a). In addition, total sperm motility and progressive motility (measured via the CASA system) and sperm viability (assessed via the hypo-osmotic swelling test) were shown to be unaffected by the presence of 5 mM citrate and 0.5 mM CIC inhibitor under the same conditions, indicating no effect of the inhibitor on overall sperm health (Figure 9b-d).



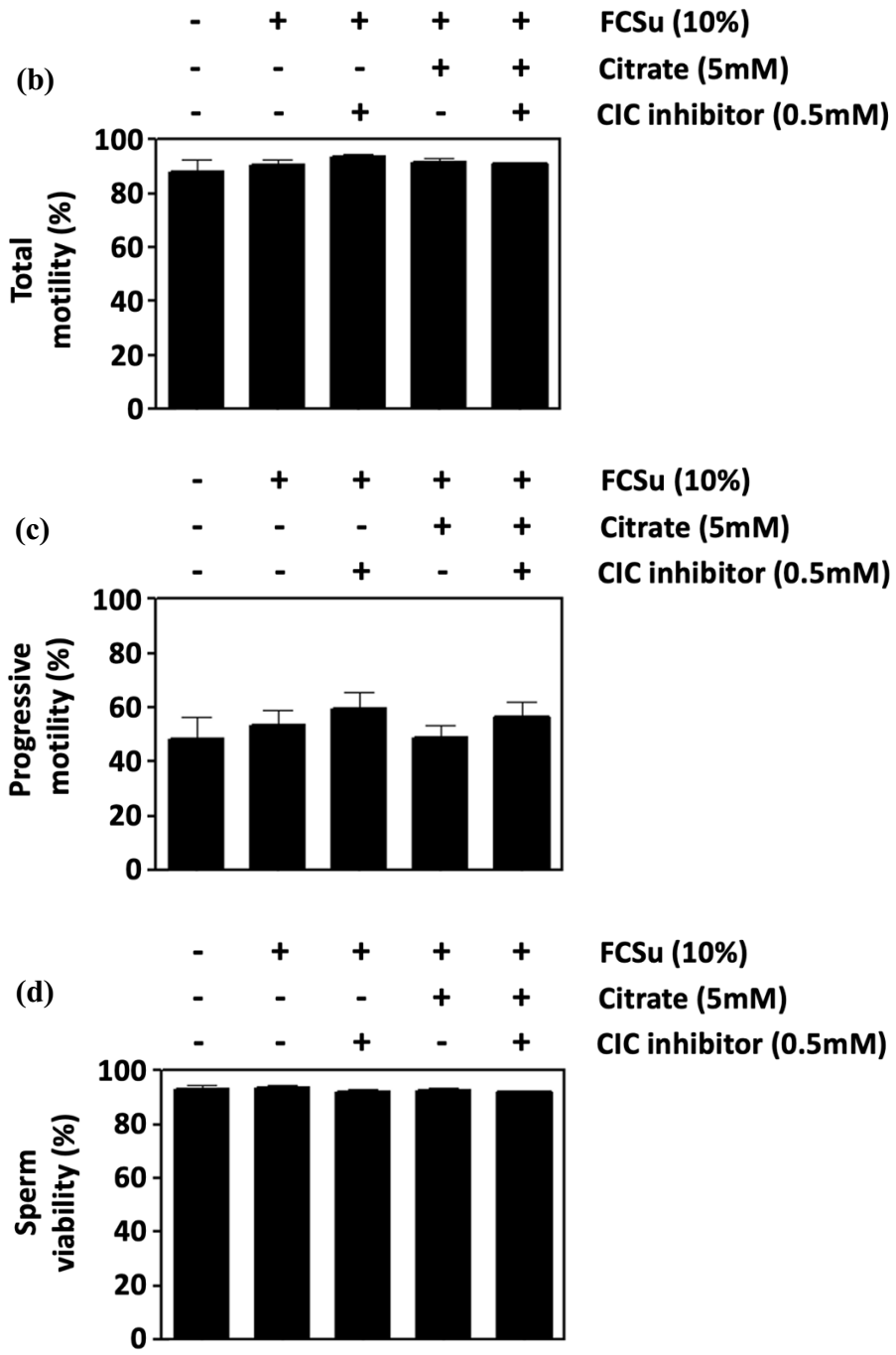
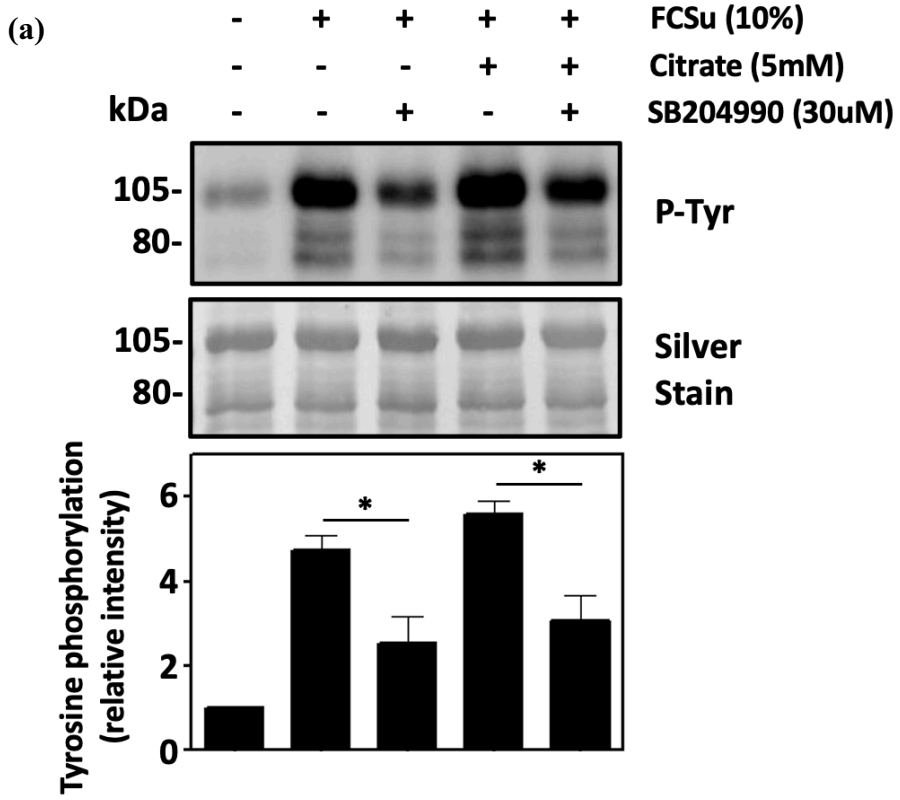


Figure 9. Mitochondrial citrate transport supports human sperm capacitation. Human spermatozoa were incubated in capacitating conditions with 10% FCSu and 5 mM citrate, with 0.5 mM of mitochondrial citrate transport (CIC) inhibitor. Immunoblotting using an anti-phosphotyrosine (P-Tyr) antibody reveals decreased capacitation levels in samples containing the CIC inhibitor in the presence and absence of citrate (a). Total motility, progressive motility, and sperm viability were all not affected by the incubation in CIC inhibitor under the same conditions (b-d). This experiment was done a total of four times with different healthy donors, and a representative blot is shown.
*P≤0.05, ANOVA and Tukey's test, n=4.

To explore the role of ATP-citrate lyase (ACLY) on human sperm capacitation, capacitating spermatozoa were incubated in BWW-REG with an inhibitor of the ACLY known as SB204990 (30 μM) in the absence and presence of 5 mM citrate. In each condition, immunoblotting using an anti-phosphotyrosine antibody revealed that the incubation of samples with SB204990 significantly decreases tyrosine phosphorylation, indicating decreased sperm capacitation (Figure 10a). Further studies on sperm motility and viability under the same conditions demonstrate that 30 μM of SB204990 had no effect on total sperm motility, progressive motility, or sperm viability, suggesting that any effect of decreased tyrosine phosphorylation was not due to a toxic effect of the inhibitor on spermatozoa (Figure 10b-d). These results reveal that the conversion of cytosolic citrate (which is supplied into the cytosol from the extracellular space or from the mitochondrion via CIC) into acetyl-CoA and oxaloacetate plays a major role in the process of human sperm capacitation.



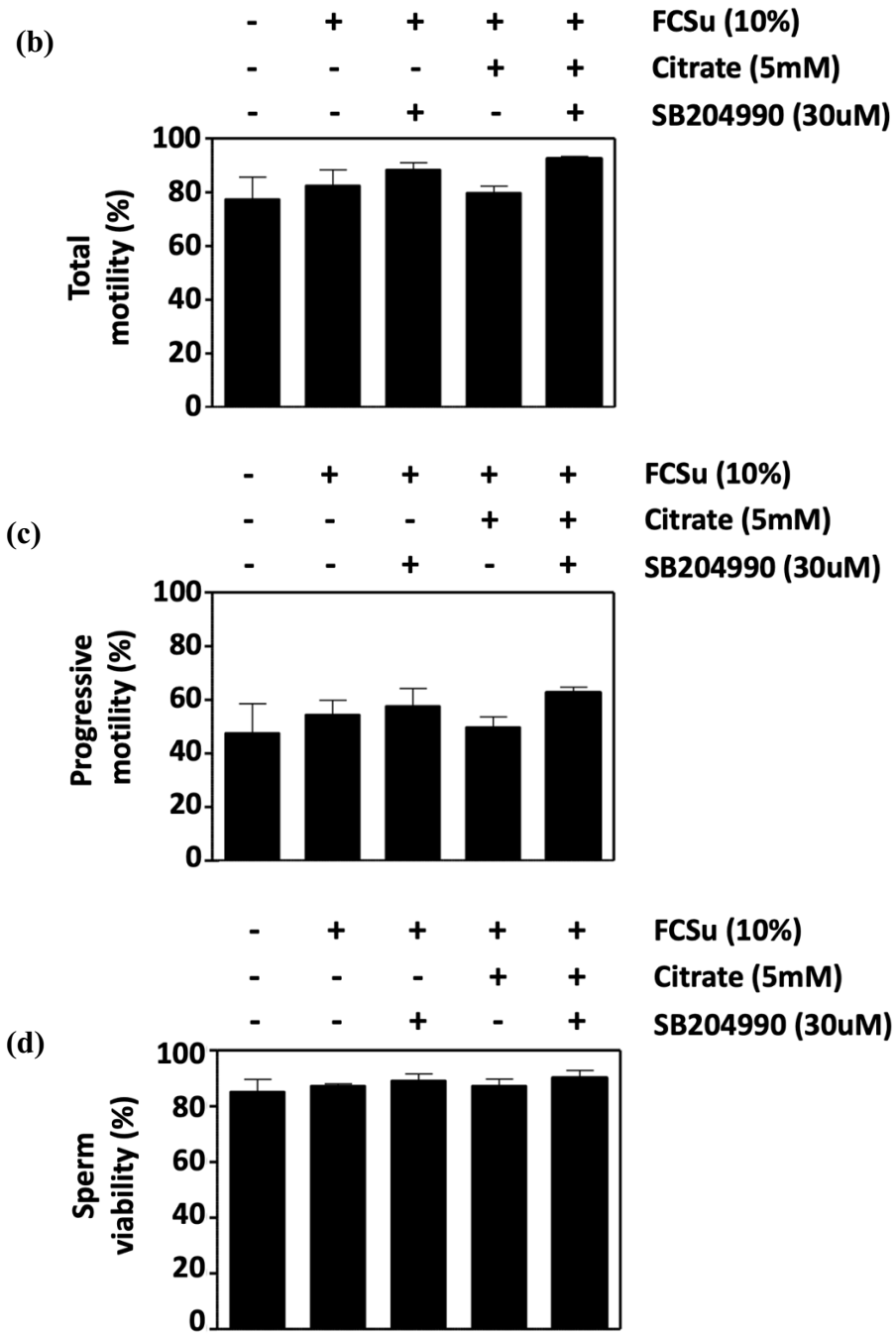


Figure 10. ATP-citrate lyase supports human sperm capacitation. Human spermatozoa were incubated in capacitating conditions with 10% FCSu and 5 mM citrate, with 30 μM of ATP-citrate lyase (ACLY) inhibitor SB204990. Immunoblotting using an anti-phosphotyrosine (P-Tyr) antibody reveals decreased capacitation levels in spermatozoa treated with SB204990 (a). Total motility, progressive motility, and sperm viability were all not affected by the incubation in the inhibitor under the same conditions (b-d). This experiment was done a total of four times with different healthy donors, and a representative blot is shown. *P≤0.05 ANOVA and Tukey's test, n=4.

4.4 ATP-citrate lyase is activated during sperm capacitation

Knowing that citrate lyase inhibition reduced tyrosine phosphorylation in capacitating spermatozoa, the next step was to determine the effect of citrate on levels of citrate lyase activation (through its phosphorylation). In spermatozoa incubated in BWW-REG, we found that levels of phospho-ATP-citrate lyase (P-ACLY) were increased when FCSu was present, and were further increased in the presence of 10 mM citrate (Figure 11). These findings are consistent with the increased tyrosine phosphorylation and capacitation that were observed in the same samples in the presence of citrate. Thus, it was demonstrated through these experiments that ATP-citrate lyase activation is increased in capacitating spermatozoa, and the presence of additional citrate increases the need of activated ACLY in the process of human sperm capacitation.

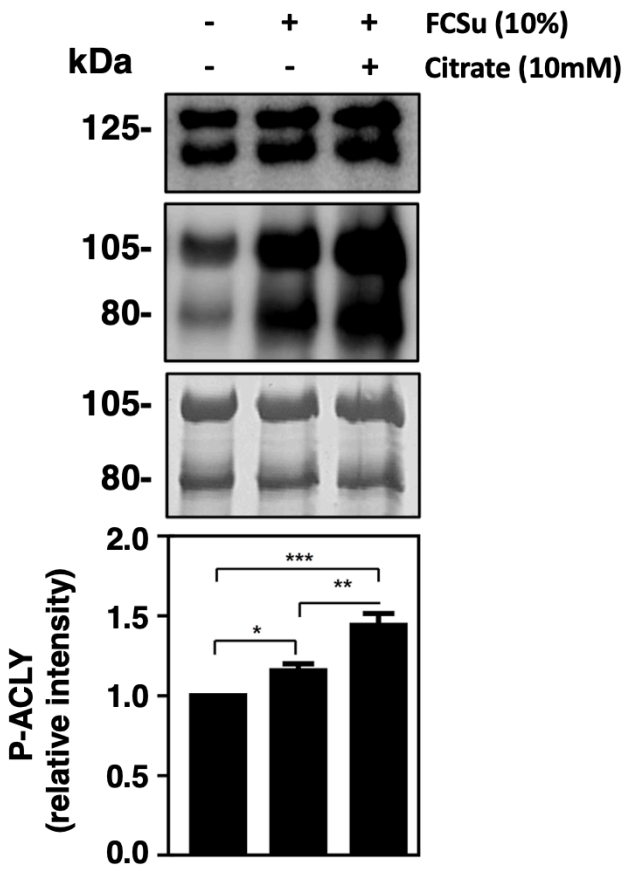
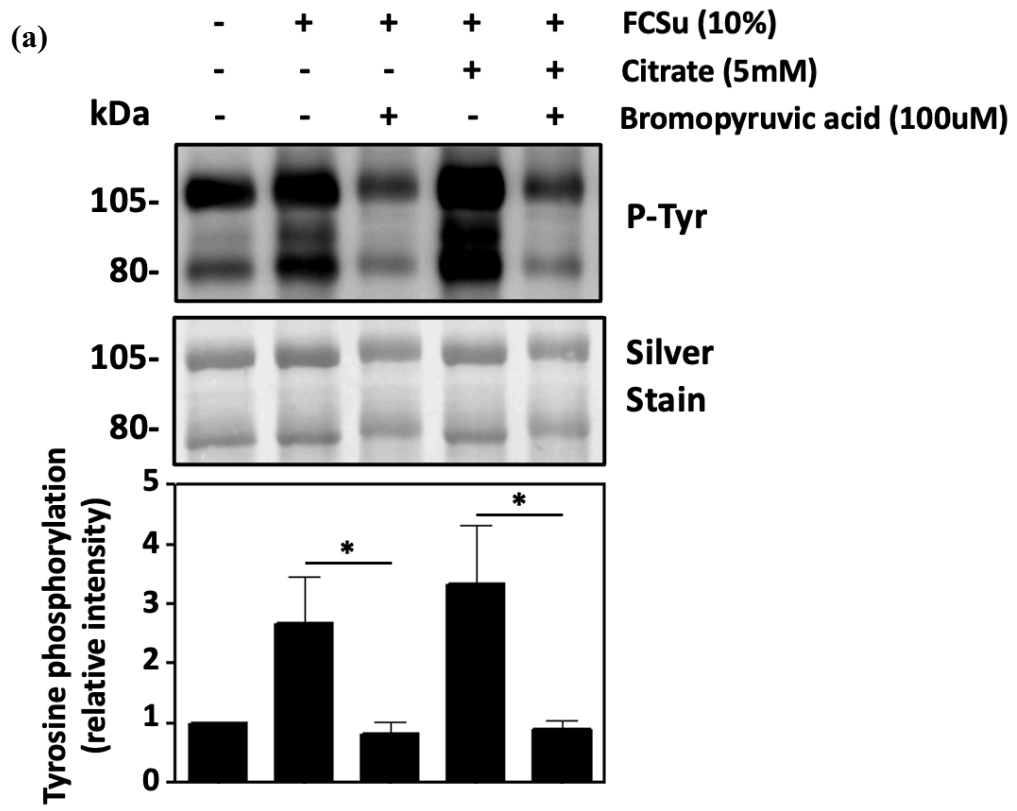


Figure 11. ATP-citrate lyase is activated during human sperm capacitation. Human spermatozoa were incubated in capacitating conditions with 10% FCSu and 10 mM citrate. Immunoblotting using anti-phospho-ATP-citrate lyase (P-ACLY) and anti-phosphotyrosine (P-Tyr) antibodies reveals increased ATP-citrate lyase activation in the presence of citrate, consistent with the increase in tyrosine phosphorylation observed in the same samples. This experiment was done a total of six times with different healthy donors, and a representative blot is shown.
*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.005$ ANOVA and Tukey's test, n=6

4.5 Malic enzyme supports human sperm capacitation

Following CIC and ACLY inhibition, the importance of the malic enzyme (ME) was determined in the proposed pathway of citrate-mediated capacitation. Capacitating spermatozoa in BWW-REG were incubated in 100 μM of bromopyruvic acid (BPA), a non-competitive inhibitor of ME, in the presence and absence of 5 mM of citrate. It was determined that the inhibition of ME led to a significant decrease in tyrosine phosphorylation in the absence and presence of citrate, indicating decreased levels of capacitation (Figure 12a). In addition, the inhibition of the ME using the same concentration of BPA did not impair total motility, progressive motility, and sperm viability, demonstrating that the decrease in tyrosine phosphorylation was not due to an effect on the overall health of spermatozoa (Figure 12b-d). Together, these results highlight the importance of ME activity in human sperm capacitation.



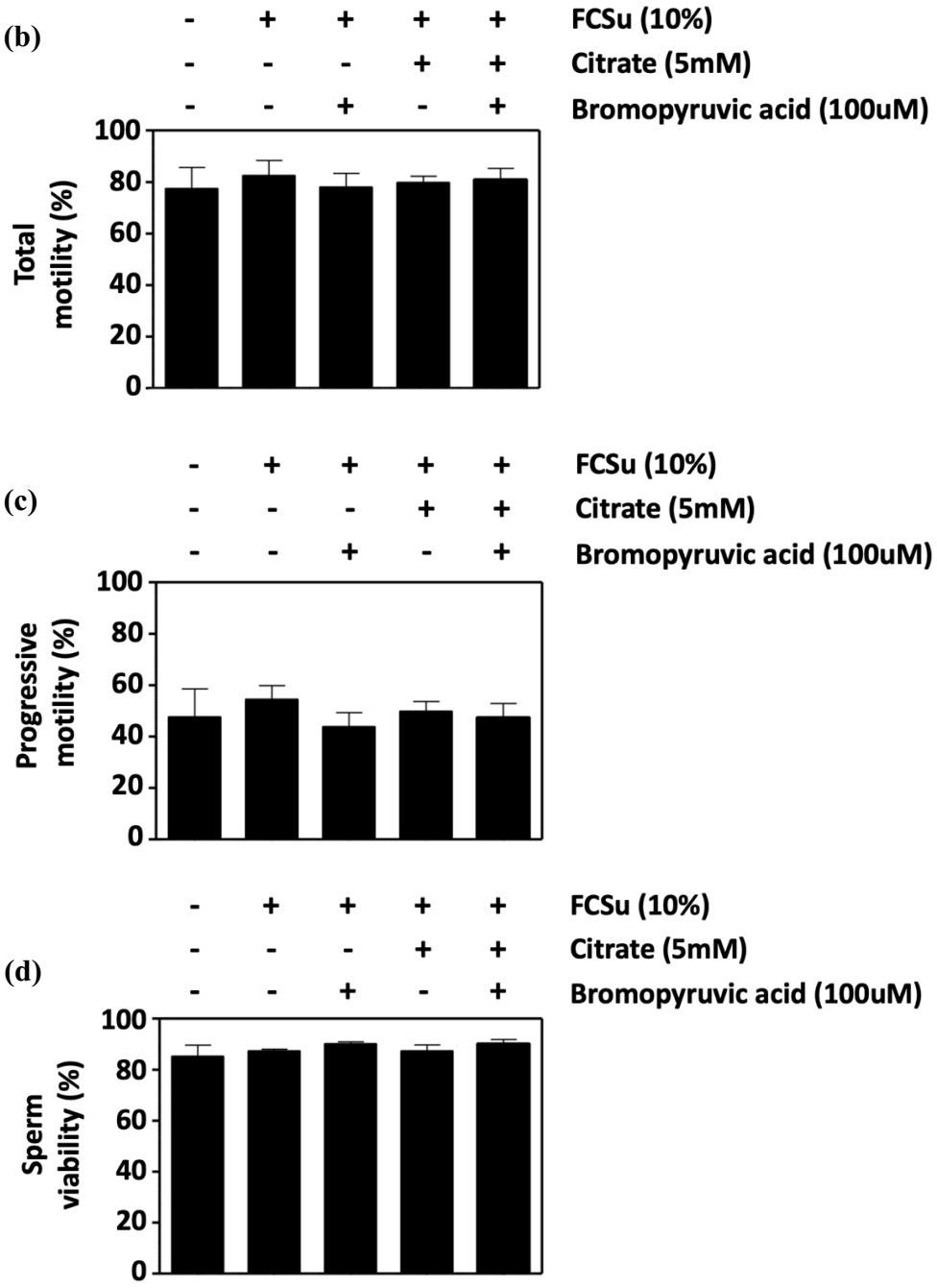


Figure 12. Malic enzyme supports human sperm capacitation. Human spermatozoa were incubated in capacitating conditions with 10% FCSu and 5 mM citrate, with 100 μM of bromopyruvic acid (an inhibitor of the malic enzyme). Immunoblotting using an anti-phosphotyrosine (P-Tyr) antibody reveals decreased capacitation levels in samples incubated with bromopyruvic acid in the absence and presence of citrate (a). Total motility, progressive motility, and sperm viability were not affected by the incubation in the inhibitor under the same conditions (b-d). This experiment was done a total of four times with different healthy donors, and a representative blot is shown.
*P≤0.05, ANOVA and Tukey's test, n=4.

4.6 Nitric oxide synthase is involved in citrate-mediated capacitation

Knowing that the ME plays a role in the process of capacitation, the next step was to determine whether citrate may be implicated in $NO^{\bullet}$ production in capacitating spermatozoa. In this experiment, human spermatozoa were incubated in BWW-REG under capacitating conditions with 1 mM of L-N^{G}-Nitro arginine methyl ester (L-NAME). L-NAME is a competitive inhibitor of L-arginine for NOS, which was previously reported to decrease capacitation in mammalian spermatozoa (Herrero et al., 1999; O'Flaherty et al., 2004; Thundathil et al., 2003). It was found that the incubation of spermatozoa in L-NAME resulted in a significant decrease in sperm capacitation, either in the absence or presence of 10 mM of citrate (Figure 13). The results of this experiment highlight the importance of $NO^{\bullet}$ production by nitric oxide synthase in sperm capacitation, a process which may be supported by intracellular citrate metabolism.

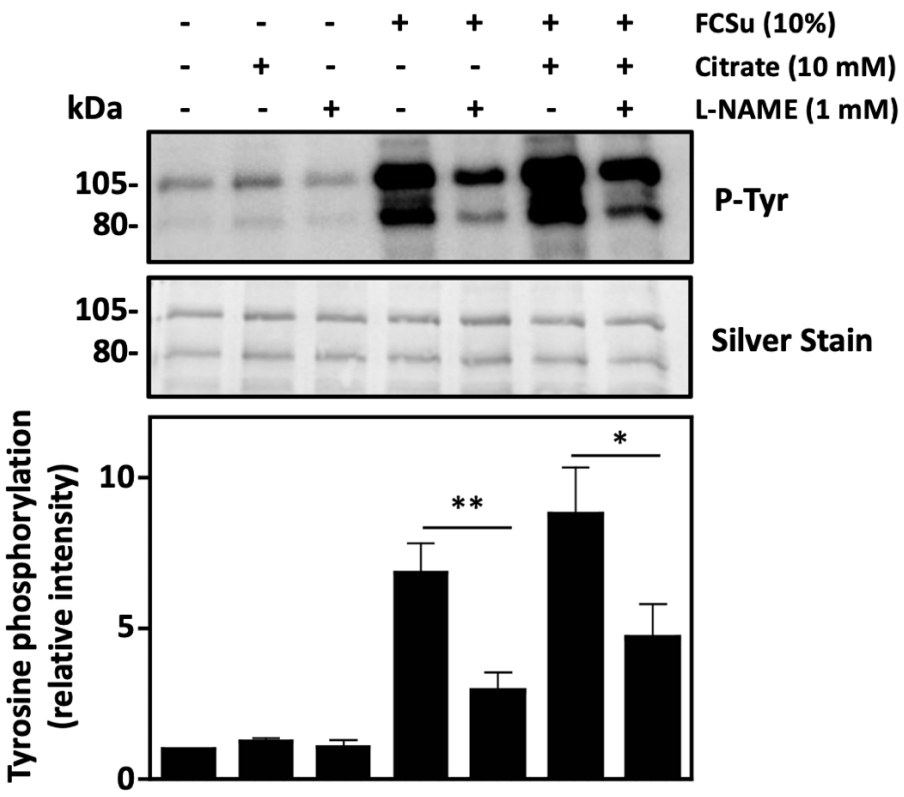
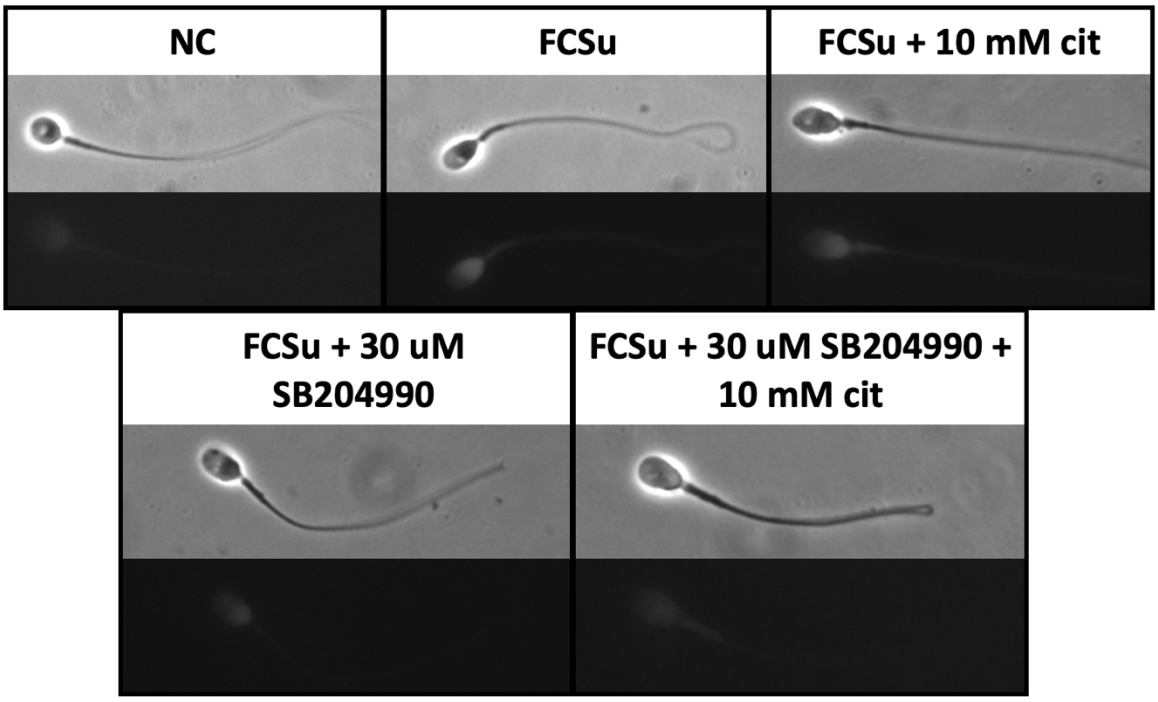


Figure 13. Nitric oxide synthase activity supports citrate-mediated human sperm capacitation. Human spermatozoa were incubated in capacitating conditions with 10% FCSu and 10 mM citrate, with 1 mM of L-N^G-Nitro arginine methyl ester (L-NAME, an inhibitor of nitric oxide synthase). Immunoblotting using an anti-phosphotyrosine (P-Tyr) antibody reveals decreased capacitation levels in the presence of the L-NAME in each condition. This experiment was done a total of four times with different healthy donors, and a representative blot is shown.
*P≤0.05; ** P≤0.01, ANOVA and Tukey's test, n=4

4.7 Citrate mediates NO• production in capacitating spermatozoa

To confirm whether citrate has an effect on intracellular nitric oxide production during sperm capacitation, levels of nitric oxide production following the incubation of spermatozoa with citrate were evaluated. Levels of NO• were found to be significantly increased in capacitating spermatozoa incubated in BWW-REG with FCSu or FCSu with 10 mM citrate, when compared to the non-capacitated control (Figure 14). Interestingly, NO• levels were significantly lower following the incubation of capacitating spermatozoa with 30 μM SB204990 compared to spermatozoa incubated with FCSu alone. Moreover, levels of NO• production in samples incubated with SB204990 were not increased when FCSu and citrate were both present in the capacitation media, highlighting the importance of ACLY activity in the process of NO• generation.

(a)



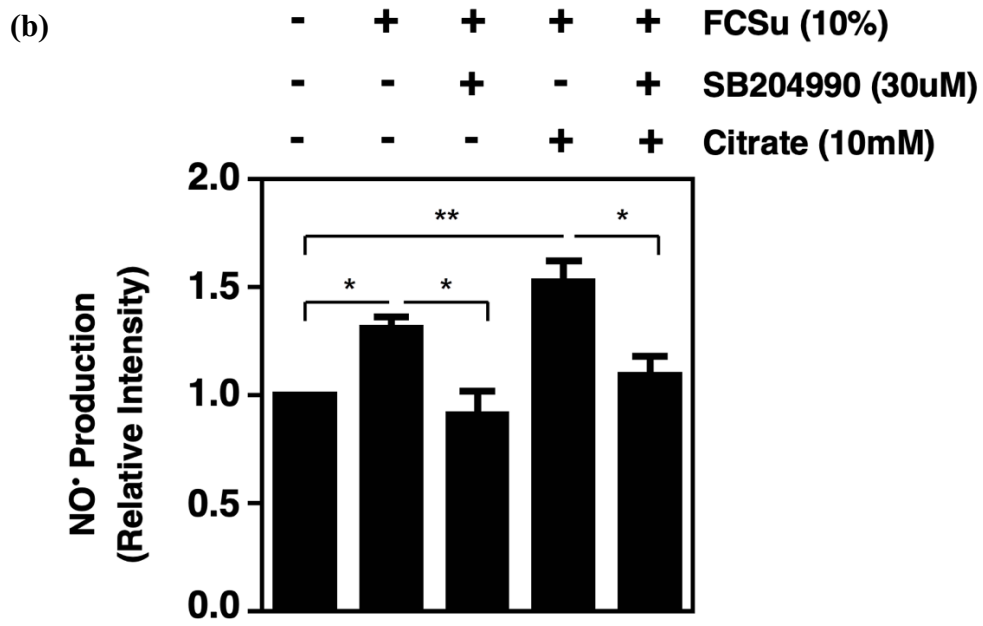


Figure 14. Citrate and ATP-citrate lyase promote nitric oxide production in capacitated spermatozoa. Human spermatozoa were incubated in capacitating conditions with 10% FCSu in the absence or presence of 10 mM citrate and 30 μM of SB204990. Fluorescence microscopy using DAF-2 DA as a fluorescent probe for intracellular NO• reveals that NO• levels are altered in spermatozoa incubated with citrate or SB204990 compared to the controls (a). Quantification of fluorescence intensity in each sample validates that NO• levels are decreased in capacitated spermatozoa incubated with SB204990 when compared to the non-capacitated (NC) control, but increased when incubated with citrate (b). This experiment was done a total of four times with different healthy donors, and a representative image from each sample is shown.
*$p \leq 0.05$, **$p \leq 0.01$ ANOVA and Tukey's test, n=4.

5. DISCUSSION

In this study, we aimed to uncover the role of cytosolic citrate in the process of human sperm capacitation. Although seminal plasma contains a very high concentration of citrate, its role in the context of sperm capacitation has remained very elusive. Through this research, we demonstrated that citrate supports the process of human sperm capacitation in humans through the activity of CIC, ACLY and ME in a process involving $NO^{\bullet}$ production. Thus, this work not only uncovers the importance of citrate in capacitation, but also an additional mechanism by which intracellular ROS can be produced during sperm capacitation.

Following incubation of spermatozoa with FCSu alone in BWW-NES (with no other energy metabolites present), levels of tyrosine phosphorylation in highly motile human spermatozoa were increased. This result demonstrates that spermatozoa may still undergo capacitation using only endogenous energy substrates or substrates loaded into the cell through contact with epididymal fluid and seminal plasma prior to capacitation. Additionally, the incubation of these samples with 5 and 10 mM of citrate led to a further increase in tyrosine phosphorylation, at levels comparable to spermatozoa incubated with FCSu in traditional BWW medium (BWW-REG; containing pyruvate, lactate, and glucose). This finding highlights that citrate can support tyrosine phosphorylation in capacitating spermatozoa. Interestingly, when spermatozoa were incubated with higher concentrations of citrate (15 and 20 mM), there was a notable decrease in tyrosine phosphorylation, at levels comparable to samples incubated with FCSu alone. A potential explanation for this may be the excessive production of $NO^{\bullet}$, yielding oxidative stress which may impair the spermatozoon's ability to capacitate (Luo et al., 2021). Thus, an optimal level of intracellular citrate exists, which yields the greatest amount of tyrosine phosphorylation and capacitation in spermatozoa.

The next step in our research was to determine the role of mitochondrial citrate transport, ATP-citrate lyase, and the malic enzyme on human sperm capacitation, by inhibiting the respective transporter and enzymatic activities (Table 2). Through these studies, we revealed that the inhibition of the mitochondrial citrate transporter (CIC) decreases tyrosine phosphorylation in capacitating spermatozoa incubated in regular BWW medium (BWW-REG). Surprisingly, the supplementation of these CIC-inhibited samples with 5 mM of citrate did not rescue tyrosine phosphorylation, indicating that the inhibitor may be causing dysregulation of other metabolites which rely on the CIC. For instance, CIC inhibition would also restrict the import of cytosolic malate into the mitochondrion, which is used in the citric acid cycle to produce energy. In addition, the accumulation of cytosolic malate arising from the inhibition of the CIC may result in excessive $NO^{\bullet}$ production (through ME and NOS activity), thus promoting oxidative stress.

Our next series of results aimed to elucidate the role of ATP-citrate lyase (ACLY) in human sperm capacitation. In these experiments, ACLY was inhibited via SB204990, and a notable decrease in tyrosine phosphorylation was observed in the absence and presence of citrate. This result highlights the importance of citrate conversion into acetyl-CoA and oxaloacetate in the process of human sperm capacitation. In addition, levels of ACLY phosphorylation were shown to be increased in samples incubated with the capacitation inducer (FCSu), and increased further in the presence of citrate. This corresponded to an increase in tyrosine phosphorylation, indicating that active ACLY is necessary for human sperm capacitation to occur.

Following inhibition of the malic enzyme (ME) with bromopyruvic acid, a similar decrease in tyrosine phosphorylation was observed in the absence and presence of citrate, demonstrating the importance of NADPH and pyruvate generated by the malic enzyme in sperm capacitation. It is worth noting that other sources of NADPH may be implicated in the process of capacitation as

well. For instance, the activity of glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD) from the pentose phosphate pathway, as well as cytosolic $NADP^+$-dependent isocitrate dehydrogenase (IDPc), can yield cytosolic NADPH which may be implicated in sperm capacitation (Koh et al., 2004; O'Flaherty, 2015; Stanton, 2012). In further studies, we show that citrate metabolism supports intracellular $NO^{\bullet}$ generation in capacitation, which we propose is mediated by the NADPH generated from the malic enzyme to drive nitric oxide synthase (NOS) activity. However, it must be noted that NADPH can serve other functions within the spermatozoon. For instance, NADPH is used in the biosynthesis of lipids, which may be useful to the spermatozoon for long-term energy storage (Jump, 2009). In addition, NADPH is essential in the PRDX antioxidant activity, as it is involved in the thioredoxin reductase/thioredoxin/NADPH system that protects the spermatozoon against excess PRDX oxidation (Fernandez & O'Flaherty, 2018; O'Flaherty, 2015).

We also investigated whether the decrease in tyrosine phosphorylation mediated by CIC, ACLY, and ME inhibition was due to sperm cell death. Interestingly, the inhibition of all these proteins using the same concentrations of inhibitors that were used prior did not have any significant effect on total motility, progressive motility, or sperm viability. These results highlight that the decreases in tyrosine phosphorylation observed in each experiment were not due to the inhibitors killing the spermatozoa, but rather due to a direct effect on the machinery needed for sperm capacitation. In addition, the incubation of spermatozoa with 5 mM citrate did not affect sperm viability or motility, validating that it does not affect the health of the spermatozoa.

Spermatozoa were then incubated in capacitating conditions with L-NAME, a competitive inhibitor of NOS, resulting in a significant decrease in tyrosine phosphorylation in the absence and presence of citrate. This result confirms the importance of NOS activity in the process of human

sperm capacitation, but also reveals an important mechanism by which citrate may support the process of capacitation. With the information that malic enzyme inhibition decreases sperm capacitation, we postulate that the NADPH being produced by the malic enzyme, downstream of cytosolic citrate metabolism, is used as a substrate for NOS to produce low levels of $NO^{\bullet}$ in the capacitation process.

Knowing that NOS activity may be mediating citrate-supported capacitation, we then quantified levels of $NO^{\bullet}$ in capacitating spermatozoa incubated with citrate and the ACLY inhibitor SB204990. In this experiment, it was shown that the inhibition of ACLY decreases $NO^{\bullet}$ production in capacitating spermatozoa, whereas the incubation with citrate supports this $NO^{\bullet}$ production. Yet, citrate could not increase sperm $NO^{\bullet}$ production in the presence of the ACLY inhibitor. This result confirms that citrate-mediated capacitation involves $NO^{\bullet}$ production, and uncovers a novel source of cytosolic ROS in capacitating human spermatozoa.

	CIC Inhibitor	SB204990	Bromopyruvic acid	L-NAME
Synonyms	4-chloro-3-{[(3-nitrophenyl)amino]sulfonyl}benzoic acid	3-furanacetic acid	3-bromo-2-oxopropanoic acid	L-N^G-nitro arginine methyl ester
Description	A competitive inhibitor of mitochondrial citrate transport protein (CIC).	A potent and specific inhibitor of ATP citrate lyase (ACLY) enzyme.	An alkylating agent, and a non-competitive inhibitor of the malic enzyme (ME)	A competitive inhibitor of nitric oxide synthase (NOS) for L-arginine
Effect on sperm capacitation	Decreased effect with 0.5 mM	Decreased effect with 30 µM	Decreased effect with 100 µM	Decreased effect with 1 mM
Effect on total sperm motility and viability	No effect with 0.5 mM	No effect with 30 µM	No effect with 100 µM	No effect with 1 mM

Table 2. Summary of inhibitors used, and their effects on human sperm capacitation. Spermatozoa incubated with different concentrations of CIC, ACLY, ME, and NOS inhibitors under capacitating conditions resulted in a decrease in sperm capacitation, as assessed through quantification tyrosine phosphorylation. However, each inhibitor had no effect on total sperm motility or viability at the concentrations used.

Altogether, the work presented demonstrates that cytosolic citrate can mediate $NO^{\bullet}$ production through oxaloacetate generation by ACLY. However, the question remains as to whether the acetyl-CoA generated by ACLY plays a role in capacitation. Acetyl-CoA is a molecule that is involved numerous biochemical reactions, including the initiation of fatty acid synthesis (Figure 15). Fatty acids are important components of lipids and are implicated in energy storage, cell signaling, and maintenance of cell structure. It is important to consider how fatty acids play a role

in sperm capacitation, as it has been shown that roughly 24% of the human sperm proteome is associated with lipid metabolism (Amaral et al., 2013; Furse et al., 2022). In addition, it has been suggested that the energy requirement for spermatozoa to reach the ovum is greater than can be supplied by glucose stores alone, demonstrating the potential role of fatty acid consumption in fertilization (Cosson, 2006; Furse et al., 2022). The energy stored through fatty acid synthesis can be released through β-oxidation within the mitochondrion, replenishing mitochondrial acetyl-CoA which can then enter the citric acid cycle to output ATP. Human spermatozoa may require this ATP store for hours during capacitation, an energy-intensive process which involves many ATP-dependent phosphorylation events. As mentioned previously, NADPH supplied by ME, G6PD, 6PGD, IDPc is also involved in fatty acid elongation (Jump, 2009). Thus, it is possible that the citrate, through acetyl-CoA production by ACLY and NADPH production by ME (along with NADPH produced from G6PD, 6PGD, and/or IDPc), can be used in fatty acid synthesis in spermatozoa in order to provide the ATP necessary for capacitation.

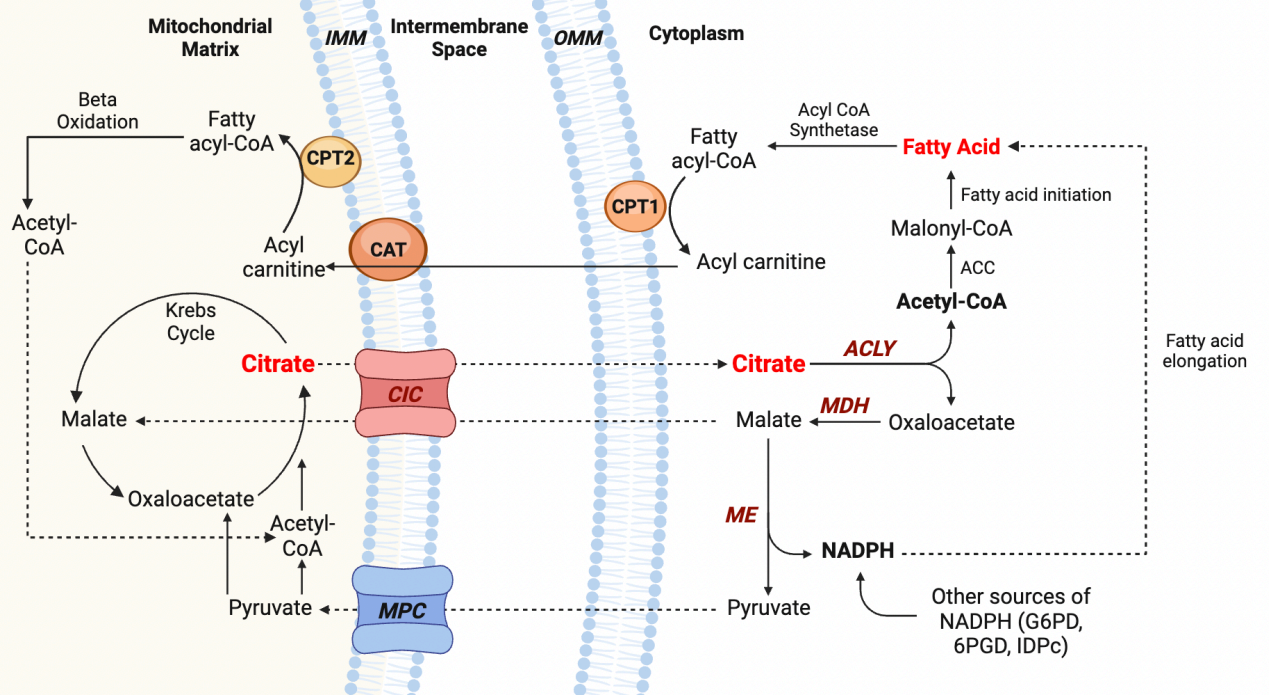


Figure 15. Cytosolic citrate metabolism, fatty acid synthesis, and mitochondrial beta oxidation. Mitochondrial citrate can be transported into the cytosol via the mitochondrial citrate transporter (CIC). Cytosolic citrate can then be used by ATP-citrate lyase (ACLY) to produce Acetyl-CoA and oxaloacetate. Acetyl-CoA can be used in the initiation of fatty acid synthesis through its conversion by the rate-limiting acetyl-CoA carboxylase (ACC). Oxaloacetate can lead to the downstream production of NADPH involved in fatty acid elongation, which is also supplied through glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD), and cytosolic $NADP^+$-dependent isocitrate dehydrogenase (IDPc). Fatty acids are converted into fatty acyl-CoA, which is then used by carnitine palmitoyltransferase I (CPT1) on the outer mitochondrial membrane (OMM) to produce acyl carnitine. Acyl carnitine enters the mitochondrion via the carnitine/acylcarnitine translocase (CAT), to then be converted by carnitine palmitoyltransferase II (CPT2) on the inner mitochondrial membrane (IMM) to produce fatty acyl-CoA. Beta oxidation leads to the conversion of fatty acyl-CoA into acetyl-CoA, which may then be used in the citric acid cycle to yield ATP.
This figure was generated using BioRender.

In addition to mediating fatty acid synthesis, the acetyl-CoA produced from ACLY may also mediate protein acetylation events. It is known that acetylation of specific proteins on lysine residues plays a role in sperm motility and capacitation, as the inhibition of sperm deacetylases leads to the onset of capacitation-associated events such as phosphorylation of PKA substrates, calcium influx, and hyperactivation (Ritagliati et al., 2018; Sun et al., 2014; Yu et al., 2015). Thus, it stands to reason that citrate metabolism may also play a role in sperm capacitation by maintaining protein acetylation events, but further studies are needed to support this statement.

Although the roles of acetyl-CoA in fatty acid synthesis and protein acetylation have been previously studied in spermatozoa, the modulation of these events in the context of citrate-mediated sperm capacitation remains to be fully elucidated. This work demonstrates how citrate supports capacitation through $NO^{\bullet}$ generation, yet there may be many other implications of citrate on sperm capacitation due to these branching metabolic pathways. Therefore, future experiments would be required to fully understand how citrate supports human sperm capacitation.

It should be emphasized that, although this work demonstrates the involvement of citrate in supporting capacitation, citrate has other effects on the spermatozoon which must be carefully balanced to maintain capacitation. For instance, citrate is known for its properties as an anticoagulant, which arises from its ability to chelate ionic calcium (Davenport & Tolwani, 2009). This decrease in intracellular calcium concentration may lead to an overall decrease in sperm capacitation, considering that calcium is a significant factor in the regulation of capacitation (Finkelstein et al., 2020). However, this potential issue was largely addressed by adding $CaCl_2$ to BWW media used in each experiment presented here. Moreover, sodium citrate is known to work as an alkalinizing agent, as its metabolism leads to the production of bicarbonate ions and the buffering of excess hydrogen ions, resulting in a change in intracellular pH that would, in turn,

affect capacitation levels in spermatozoa (Johnson & Hillier, 2007). However, it is important to note that this citrate-mediated change in pH was circumvented in each experiment through the use of HEPES-buffered BWW medium in each experiment, and no significant change in pH was observed upon the addition of citrate to the capacitation medium.

The importance of understanding citrate regulation in human sperm capacitation cannot be understated, as this work can be used to improve current treatment options for infertile couples. For instance, the inclusion of citrate in IVF media at may lead to improved sperm capacitation, leading to overall increased success rates for this ART, which currently stands at only 30-40% (Smith et al., 2015; Wade et al., 2015). Improving the outcomes of traditional IVF would not only be more cost-effective for couples, saving them thousands of dollars per cycle, but it would also improve the emotional distress that comes with infertility.

In addition to ameliorating success rates for IVF, the work presented here may be applied in the preparation of sperm before IUI. Since the spermatozoa of some infertile men may be exposed to insufficient citrate due to low citrate levels in the seminal plasma, the incubation of these spermatozoa in citrate before the IUI process may simulate the loading of citrate from the seminal plasma that occurs naturally in fertile men. This may lead to an increase in sperm capacitation and, consequently, an increase in success rates in IUI treatments. Since IUI is generally considered to be one of the most cost-effective and least invasive ARTs, citrate supplementation to spermatozoa may be a very promising avenue to explore to aid infertile couples.

We hope that this work will increase awareness and appreciation for the process of capacitation, which is generally not evaluated in fertility clinics. In addition, we hope that this work will shed light on the importance of citrate on male infertility, and that this research will be continued in order to understand the full scope of citrate metabolism on human sperm capacitation.

6. CONCLUSION

As the prevalence of infertility rises worldwide, more couples than ever are facing the financial and emotional distress that is associated with the disease. With this increased pressure, we must face the challenge of understanding how the spermatozoon recognizes and fertilizes the oocyte in order to improve current assisted reproductive technologies such as IUI, IVF or ICSI.

The experiments conducted in this study reveal a novel role of citrate in the process of human sperm capacitation in a process involving $NO^{\bullet}$ generation (Figure 16). This was demonstrated as the incubation of capacitating spermatozoa in citrate alone supported the capacitation process, yet the inhibition of CIC, ACLY, ME, and NOS decreased capacitation levels. In addition, the inhibition of ACLY reduced $NO^{\bullet}$ production in capacitating spermatozoa, whereas citrate was shown to support $NO^{\bullet}$ production.

This work explains a potential novel role of the oxaloacetate that is generated through cytosolic citrate metabolism, yet further studies are required to understand how other metabolic intermediates, such as acetyl-CoA, may be implicated in the process of human sperm capacitation. These studies will help understand how citrate regulates human sperm capacitation in an effort to ameliorate current alternatives for infertile couples and to improve the current state of the infertility crisis.

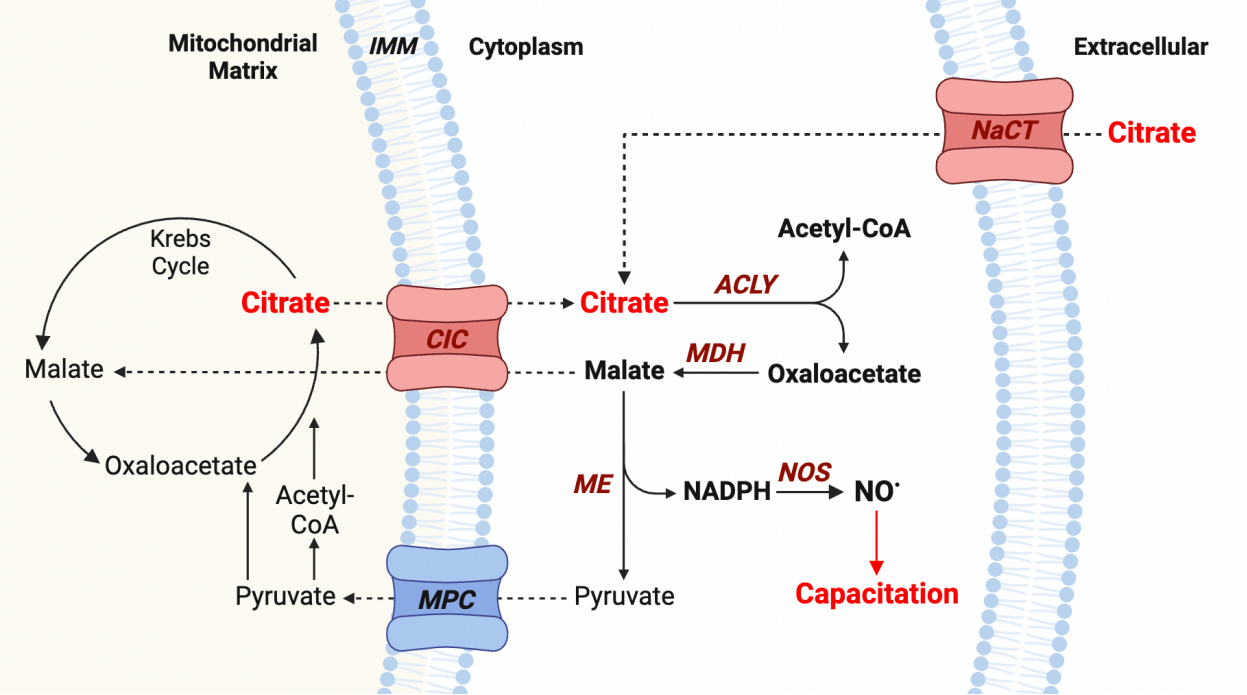


Figure 16. Cytosolic citrate metabolism and potential ROS generation in sperm capacitation. Extracellular citrate can enter the cytosol via the sodium-citrate cotransporter (NaCT) and the mitochondrial citrate transporter (CIC). Cytosolic citrate is then converted by ATP-citrate lyase (ACLY), malate dehydrogenase (MDH), and malic enzyme (ME) in sequence, leading to the production of NADPH. NADPH may then be used by nitric oxide synthase (NOS) to yield nitric oxide (NO•), which is required in the process of human sperm capacitation. The resulting pyruvate can re-enter the mitochondrion via the mitochondrial pyruvate carrier (MPC) to continue energy production.
This figure was adapted from Zara et al., 2022, and generated using BioRender.

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