Exploration of the Mechanism of Action of Human Oviduct-Specific Glycoprotein (OVGP1) in Regulating Sperm Capacitation

By

Sydney Vanderkooi

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Queen’s University

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Abstract

The present research is of significance to the field of fertility treatment as better understanding the mechanism that regulates the function of oviduct-specific glycoprotein (OVGP1) could lead to its supplementation to culture medium used in assisted reproductive technologies, potentially improving the fertilizing competence of sperm. The mammalian oviductal non-ciliated secretory cells synthesize and secrete a major glycoprotein known as oviductin or OVGP1. Various mammalian in vitro studies have implicated native OVGP1 in enhancing sperm capacitation, sperm motility, sperm penetration and sperm-egg binding. Our laboratory has successfully produced recombinant human OVGP1 (rHuOVGP1) which has been shown to enhance tyrosine phosphorylation of sperm proteins, a biochemical hallmark of capacitation that takes place in the sperm tail, enhance the potential of sperm undergoing acrosome reaction, and increase sperm-egg binding. In the present study, we set out to explore the mechanism of human OVGP1 in enhancing key events of sperm capacitation. A major mechanism of inducing sperm capacitation is through the increase of intracellular calcium concentration ([Ca^{2+}]_i). The cation channels of sperm (CatSper) have been found to control the entry of Ca^{2+} ions into the sperm tail during capacitation and to be required for male fertility. Progesterone (P4) is known to bind to the plasma membrane P4 receptor of sperm and activate the CatSper channels, thus inducing the influx of Ca^{2+}. We hypothesized that human OVGP1 enhances tyrosine phosphorylation of sperm proteins through increasing [Ca^{2+}]_i, similar to P4, via the CatSper channels during human sperm capacitation. Results showed that the presence of rHuOVGP1 in the capacitating medium was able to further increase the level of [Ca^{2+}]_i in human sperm. Additionally, treatment of sperm with rHuOVGP1 further increases and sustains the level of [Ca^{2+}]_i in sperm following treatment with P4. The inhibition of CatSper channels with the selective inhibitor HC-056456 impedes the effects
of rHuOVGP1 on [Ca^{2+}]_i. Furthermore, the present study showed that P4 alone and in combination with rHuOVGP1 can further enhance the level of sperm protein tyrosine-phosphorylation. In summary, OVGP1 and P4 function most effectively in combination to enhance sperm capacitation through upregulating [Ca^{2+}]_i and protein tyrosine-phosphorylation.
Co-authorship

Sydney Vanderkooi, Yuewen Zhao and Frederick W. K. Kan all designed the experiments. Sydney Vanderkooi and Yuewen Zhao performed all experiments. Patricia Lima provided assistance with the design of and in carrying out flow cytometry and live cell imaging experiments. The present thesis was written by Sydney Vanderkooi and revised by Frederick W. K. Kan.
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AKAP3</td>
<td>A-Kinase Associated Protein 3</td>
</tr>
<tr>
<td>ART</td>
<td>Assisted reproductive technology</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BWW</td>
<td>Briggers-Whitten-Wittingham medium</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CatSper</td>
<td>Cation channels of sperm</td>
</tr>
<tr>
<td>[Ca$^{2+}$]_i</td>
<td>Intracellular calcium concentration</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>HBS</td>
<td>HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-buffered saline</td>
</tr>
<tr>
<td>HC-056456</td>
<td>3,4-Bis(2-thienyl)-1,2,5-oxadiazole-N-oxide</td>
</tr>
<tr>
<td>HEK 293</td>
<td>Human embryonic kidney cells 293</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>ICSI</td>
<td>Intracytoplasmic sperm injection</td>
</tr>
<tr>
<td>ODF</td>
<td>Outer dense fibers</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IVF</td>
<td><em>In vitro</em> fertilization</td>
</tr>
<tr>
<td>OVGP1</td>
<td>Oviductin, or oviduct-specific glycoprotein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>pY</td>
<td>Tyrosine phosphorylation</td>
</tr>
<tr>
<td>P4</td>
<td>Progesterone</td>
</tr>
<tr>
<td>rHuOVGP1</td>
<td>Recombinant human oviductin</td>
</tr>
<tr>
<td>sAC</td>
<td>Soluble adenylyl cyclase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-buffered saline containing 0.05% Tween 20</td>
</tr>
<tr>
<td>2AG</td>
<td>2-arachidonoylglycerol</td>
</tr>
<tr>
<td>7AAD</td>
<td>7-Aminoactinomycin D</td>
</tr>
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</table>
Chapter 1

Introduction and Literature Review
Introduction

Worldwide, approximately 15% of couples who seek to conceive suffer from infertility, a condition defined as the failure to achieve a successful pregnancy after at least 12 months of regular unprotected intercourse (Sharlip et al., 2002). Male infertility is the sole or contributing cause of infertility in about 50% of all infertile couples (Agarwal et al., 2015). Many infertile couples seek fertility treatment with assisted reproductive technologies (ART) which are medical procedures used to achieve pregnancy. Intracytoplasmic sperm injection (ICSI) is a common ART procedure that is accomplished by injecting a single sperm into the cytoplasm of an oocyte (Zhao et al., 2011; Repping, 2019). Once fertilization is achieved in vitro, the early embryo is delivered into the female reproductive tract for further development and subsequent implantation of the blastocyst in the uterus. Another common ART is conventional in vitro fertilization (IVF) which involves co-incubating sperm and the oocyte in a culture dish where the environment mimics that of the oviductal fluid in the oviduct where fertilization normally occurs in vivo (Zhao et al., 2011).

Total fertilization failure, which is the failure of fertilization of oocytes by sperm, occurs in 5-15% of IVF cycles despite apparently normal sperm quality (Krog et al., 2015). Defective sperm-egg binding and penetration are the major causes for zero or low fertilization rates in conventional IVF procedure (Oehninger et al., 2014). Today, ICSI has become the widely used technique for couples with previously failed fertilization with conventional IVF (Oehninger et al., 2014). However, ICSI is a relatively more expensive and invasive treatment compared to conventional IVF that relies on the subjective selection of sperm (Bouwmans et al., 2008; Dang et al., 2019; Repping, 2019). Consequently, there are accumulating reports on the association of increased genetic anomalies and birth defects in ICSI concepti (Simpson, 2014; Krog et al., 2015).
Considering the potential adverse effects of ICSI, investigations to improve the fertilization success rates of conventional IVF are merited to encourage its use in patients with mild male factor infertility.

Mammalian fertilization occurs via a comprehensive progression of events in an orderly manner. After ejaculation, sperm undergo a series of biochemical and physiological changes, collectively known as capacitation, during their transit through the female reproductive tract (Jin and Yang, 2017). During capacitation, changes in membrane properties, enzyme activity, and motility allow the sperm to become responsive to stimuli that induce the subsequent acrosome reaction which is required for sperm to penetrate and fertilize the oocyte (Salicioni et al., 2007; Jin and Yang, 2017). Chang (1951) first discovered that sperm that have reached and resided in the oviduct acquire the maximum level of fertilizing capacity. The mammalian oviductal non-ciliated secretory cells synthesize and secrete a major glycoprotein known as oviductin, or oviduct-specific glycoprotein (OVGP1) (Aviles et al., 2010; Saint-Dizier et al., 2014). Previous research has shown that OVGP1 is able to bind to sperm and the zona pellucida of oocytes, as well as increase sperm motility, sperm capacitation, sperm-egg binding and penetration rates, decrease polyspermy and enhance embryo quality and development (Kouba et al., 2000; Killian, 2004, 2011; Coy et al., 2008; Saccary et al., 2013). Similar to the importance of the oviductal fluid in fertilization in vivo, the culture medium used for ART in fertility centres plays a key role in the advancement of fertility treatments (Aviles et al., 2010). Even though mimicking the physiological condition of the female oviductal fluid has made conventional IVF possible, the culture medium currently used in fertility clinics and centres lacks OVGP1 which is normally present in the lumen of the oviduct in vivo (Gruber and Klein, 2011). The implicated biological functions of OVGP1 suggest that its
supplementation to the culture medium could increase the fertilization rate of IVF, however, the mechanisms of the physiological functions of this glycoprotein are not fully understood. This is mainly due to the difficulty in isolating and purifying adequate amounts of OVGP1 for functional studies which are required to justify the rationale for using this glycoprotein in clinical settings. To circumvent this limitation, our laboratory has successfully used recombinant DNA technology to produce and purify a secretory form of recombinant human OVGP1 (rHuOVGP1) from stably transfected human embryonic kidney (HEK 293) cells (Zhao et al., 2016). These cells have been shown to successfully express rHuOVGP1, which could be used to enhance the capacitation and fertilizing capabilities of human sperm in vitro (Zhao et al., 2016). The capacitation of sperm is accompanied by a time-dependent increase in the level of tyrosine phosphorylation of sperm proteins which is a biochemical hallmark of capacitation (Naz and Rajesh, 2004). Previous research in our laboratory has shown that rHuOVGP1, used at an optimal concentration of 50 µg/mL, enhances tyrosine phosphorylation levels of sperm proteins predominantly in the sperm tail (Zhao et al., 2016; Zhao and Kan, 2019). Moreover, rHuOVGP1 can bind to human sperm and to the zona pellucida of human oocytes, and its presence in the culture medium can also enhance the potential of sperm to undergo acrosome reaction, and increase sperm-egg binding (Zhao and Kan, 2019). However, the functional mechanism of how rHuOVGP1 enhances tyrosine phosphorylation of sperm proteins is not fully understood and needs to be further investigated.

As mentioned above, the changes associated with capacitation are modulated by components of the oviductal fluid. After ovulation, there is a release of the sex hormone progesterone (P4) from cumulus cells surrounding the oocyte (Gatica et al., 2013; López-Torres and Chirinos, 2017). One of the initial changes described in capacitating sperm is the influx of
Ca\textsuperscript{2+} ions (Baldi \textit{et al.}, 1991). The cation channels of sperm (CatSper), located in the principal piece of the sperm tail, have been found to control the entry of Ca\textsuperscript{2+} ions into sperm and to be required for male fertility (Lishko \textit{et al.}, 2011). Evidence has shown that P4 binds to the P4 receptor on the plasma membrane of sperm and activates the CatSper channels, thus inducing the influx of Ca\textsuperscript{2+} that initiates a cascade of signal transduction events that lead to sperm capacitation (Lishko \textit{et al.}, 2011; Jin and Yang, 2017). The link between CatSper mediated Ca\textsuperscript{2+} signaling and tyrosine phosphorylation of sperm proteins in mice was investigated by Chung \textit{et al.} (2014) where they demonstrated that CatSper organizes Ca\textsuperscript{2+} signaling domains that orchestrate the timing and extent of tyrosine phosphorylation cascade. The latter research suggests that disruption of the CatSper channel complex deregulates the capacitation-initiated tyrosine phosphorylation pathway (Chung \textit{et al.}, 2014). Previous research has shed light on the mechanism by which P4 induces Ca\textsuperscript{2+} influx in sperm capacitation and the link between Ca\textsuperscript{2+} signaling and tyrosine phosphorylation, however, the effects of P4 on tyrosine phosphorylation of human sperm proteins during capacitation has not been well studied. As the principal piece of the tail in human sperm is the predominate site where CatSper channels are located and where tyrosine phosphorylation of sperm proteins occurs, we sought to gain an understanding of the mechanism of rHuOVGP1 and to determine if this glycoprotein enhances intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) and tyrosine phosphorylation of sperm proteins by working synergistically with P4.
Literature Review

1.1 Oviduct

1.1.1 Anatomical Structure of the Human Oviduct

The female reproductive tract is composed of the paired ovaries and oviducts, the uterus, the cervix, the vagina, and the external genitalia (Figure 1). The present thesis focuses on studies related to the oviducts, commonly known as the fallopian or uterine tubes in humans. The mammalian oviducts provide the environment necessary for gamete transport, fertilization, embryo transport and nutritional support for early embryonic development (Aviles et al., 2010). The paired oviducts, supported by ligaments and mesenteries, are tube-like structures that extend from the uterus and have openings into the peritoneal cavity near the ovaries (Mescher, 2018). The oviduct is divided anatomically into four regions; 1) the infundibulum is the funnel-shaped portion at the distal end closest to the ovary with finger-like extensions called fimbriae; 2) the ampulla is the longest region where fertilization normally occurs; 3) the isthmus is the narrow proximal portion; and 4) the intramural or uterine region that passes through the uterine wall and opens into the interior of the uterus (Mescher, 2018).

1.1.2 Histological Features of the Oviduct

The wall of the oviduct is composed of three layers; 1) an inner mucosa with longitudinal folds, 2) a thick muscularis with inner circular and outer longitudinal layers of smooth muscle; and 3) a thin outer serosa covered by visceral peritoneum (Mescher, 2018). The mucosa with the lamina propria is lined by simple columnar epithelium that contains interspersed ciliated and non-ciliated secretory cells (Mescher, 2018). The ciliated cells are characterized by the presence of long cilia that extend into the lumen of the oviduct that aid in transporting oocytes into the oviduct and
sweeping fluid toward the uterus. (Lyons et al., 2006; Mescher, 2018). On the other hand, the non-ciliated secretory cells are elongated with microvilli located on their apical surface (Kan et al., 1989). These secretory cells are responsible for the synthesis and release of secretory material via secretory granules into the lumen of the oviduct which provides a suitable environment for the events that take place in this region (Abe, 1996). Regional variations in the oviductal epithelium have been found where the difference in the number of secretory granules and type of secretions are thought to be associated with the role of each oviduct region in fertilization events (Abe, 1996).

1.1.3 The Oviductal Fluid

The oviductal fluid within the oviductal lumen is a dynamic fluid that influences gamete transport, oocyte maturation, sperm selection and capacitation, acrosome reaction, fertilization, prevention of polyspermy, early embryonic development, and transport of the embryo to the uterus (Pérez-Cerezales et al., 2018). The oviductal fluid is a complex mixture formed by secreted components from oviductal non-ciliated epithelial cells and plasma transudate (Buhi et al., 2000; Aviles et al., 2010). This fluid contains metabolic components, including glucose, lactate, pyruvate and amino acids, the concentrations of which differ from those of the uterine fluid and plasma (Aviles et al., 2010). A large number of proteins have been identified in oviductal fluid, including growth factors, cytokines and receptors, hormones and receptors, proteases and inhibitors, antioxidant protective agents, defense agents, glycosidases and glycosyltransferases, chaperones and heat shock proteins, glycosaminoglycans and proteoglycans (Buhi et al., 2000; Aviles et al., 2010; Soleilhavoup et al., 2016). These components influence and may contribute to the optimal development of the different processes that occur in the oviduct, including but not limited to immunity against viral infection, gamete transport, fertilization, and embryo transport (Aviles et
Albumin, an abundant protein found in the oviductal fluid, plays a key role in sperm capacitation via the removal of cholesterol from the plasma membrane (Pérez-Cerezales et al., 2018). Protein secretions of the oviductal fluid differ in expression according to specific stages of the estrous cycle (Pérez-Cerezales et al., 2018). The differential regulation of oviductal proteins has been shown in studies carried out in cows (Bauersachs et al., 2004), sheep (Soleilhavoup et al., 2016) and humans (Tone et al., 2008; George et al., 2011). Proteomic analysis of oviductal epithelial cells in sheep demonstrated a 79% increase in the relative abundance of proteins at the estrus stage compared to a 21% increase at the luteal stage (Soleilhavoup et al., 2016). Furthermore, the composition of the oviductal fluid influences sperm transport in the oviduct where the mucus in the oviductal lumen during the pre-ovulatory stage is extremely viscous and may suppress sperm motility, while after ovulation it becomes less viscous and facilitates the progression of sperm migration towards the ampulla region of the oviduct where fertilization normally occurs (Coy et al., 2012).

1.2 Oviductin/Oviduct-Specific Glycoprotein (OVGP1)

1.2.1 General Review of OVGP1

Among the proteins in the oviductal fluid, a major glycoprotein named oviductin, or oviduct-specific glycoprotein (OVGP1) is synthesized and released specifically by the non-ciliated secretory cells in the oviductal epithelium. Since its discovery in hamsters (Fox and Shivers, 1975), OVGP1 has been further characterized in hamsters (Léveillé et al., 1987) and also identified in many other mammalian species including mice (Kapur and Johnson, 1985), rabbits (Oliphant and Ross, 1982; Oliphant et al., 1984), baboons (Verhage and Fazleabas, 1988), rhesus monkeys (Verhage et al., 1997b), sheep (Gandolfi et al., 1989), pigs (Buhi et al., 1990), cows (Malayer et
al., 1988), goats (Pradeep et al., 2011), cats (Hachen et al., 2012), dogs (Saint-Dizier et al., 2014), and humans (Verhage et al., 1988). The regulation of protein and gene expression of OVGP1 appears to be dependent on the stage of the estrous cycle with an increase in estrus stage compared to diestrus (Buhi, 2002; Soleilhavoup et al., 2016).

OVGP1 DNA sequences have been identified in baboons (Jaffe et al., 1996), cows (Sendai et al., 1994), hamsters (Suzuki et al., 1995), humans (Arias et al., 1994), mice (Sendai et al., 1995), pigs (Buhi et al., 1996), rhesus monkeys (Verhage et al., 1997a, 1997b) and sheep (Desouza and Murray, 1995). These OVGP1s share high sequence identity (70-78%) and similarity (76-87%) in the N-terminal region compared to low sequence identity (37-63%) and similarity (50-75%) in the C-terminal region (Buhi, 2002). The core protein size of OVGP1 of several different species have been found to be approximately the same (~70 kDa), however, the glycosylated OVGP1 varies in molecular weights (domestic animals 90-95 kDa; primates 120 kDa; rodents 160-350 kDa) (Schmidt et al., 1997). In humans, the size of the core protein is 75 kDa while the secreted form of OVGP1 is around 110-130 kDa (O’Day-Bowman et al., 1995). The variability in molecular weights are likely due to the differences in the extent of glycosylation (Schmidt et al., 1997). In addition, the glycosylation of OVGP1 appears to be essential for maintaining its extracellular solubility as the complete deglycosylation of OVGP1 resulted in an insoluble protein (Satoh et al., 1995). Furthermore, the ability of bovine OVGP1 to maintain sperm viability was found to be greatly reduced after the removal of sialic acid and N-linked glycans (Satoh et al., 1995). Therefore, glycosylation of OVGP1 appears to play a major role in the biological activity of the glycoprotein.
As mentioned earlier, OVGP1 is produced and secreted exclusively by non-ciliated oviductal epithelial secretory cells (Buhi, 2002). In the hamster, OVGP1 was found to be localized in the secretory granules and the Golgi apparatus of non-ciliated secretory cells but not in ciliated cells of the oviductal epithelium (Kan et al., 1989). OVGP1 has been shown to bind to sperm and appears to regulate sperm functions. Boatman and Magnoni (1995) reported binding of hamster OVGP1 to the head region of sperm where the glycoprotein binds to the acrosomal region in non-capacitated sperm, but it binds to the equatorial segment and post-acrosomal region following capacitation. The anterior region of the acrosome is the site where non-capacitated sperm attach to the oviductal epithelium, while the equatorial segment is involved in maintaining the binding of penetrating sperm to the zona pellucida during fertilization (Boatman and Magnoni, 1995). Therefore, the binding of OVGP1 to the head region of sperm is thought to be involved in sperm-oviduct interactions. In bovine sperm, OVGP1 binds to the posterior region of the head, the mid-piece, and the principal piece of the sperm tail (Abe et al., 1995). As described later below, research in our laboratory using recombinant human OVGP1 revealed the localization of this glycoprotein to specific membrane domains of human sperm.

The binding of OVGP1 to sperm appears to have positive effects on sperm function, where incubation of bovine sperm with purified OVGP1 has been shown to increase sperm motility and viability (Abe et al., 1995), increase the potential of sperm to undergo acrosome reaction, and enhance the fertilizing capability of sperm (King et al., 1994). Hamster OVGP1 purified from the estrus stage can enhance the level of tyrosine phosphorylation of sperm proteins, a biochemical hallmark of sperm capacitation, in a time-dependent manner in vitro (Saccary et al., 2013). A characteristic feature of OVGP1 is its localization to the zona pellucida of oviductal oocytes (Araki
et al., 1987; O’Day-Bowman et al., 1996, 2002; Coy et al., 2008). The ovarian follicles in the ovary lack OVGP1 but the glycoprotein is added to the zona pellucida of post-ovulated oocytes during their passage through the oviduct (Araki et al., 1987; Kan et al., 1989; Abe and Oikawa, 1990). However, in contrast to all other mammalian species studied to date where OVGP1 is localized to the zona pellucida, mouse OVGP1 is found in the perivitelline space of post-ovulatory oocytes (Kapur and Johnson, 1986). Association between OVGP1 and the zona pellucida appears to influence the functions of the zona pellucida.

Incubation of hamster sperm and oocytes with hamster OVGP1 as well as incubation of human sperm and hemizonae with human OVGP1 resulted in an increase in sperm-egg binding and penetration (Boatman and Magroni, 1995; O’Day-Bowman et al., 1996). OVGP1 has been found to increase zona pellucida resistance to digestion with pronase and consequently decrease polyspermy (Coy et al., 2008; Pradeep et al., 2011). The finding of endocytosis of OVGP1 by oocytes soon after fertilization suggests that OVGP1 may also influence early embryo development (Kan and Roux, 1995). In particular, studies have shown the presence of OVGP1 in the perivitelline space and endocytic vesicles of hamster embryos and early blastocysts indicating that OVGP1 is internalized by fertilized oocytes and by blastomeres of early embryos upon its shedding from the zona pellucida (Kan et al., 1993; Kan and Roux, 1995). In sheep, incubation of sperm and oocytes with OVGP1 during in vitro fertilization showed a significant increase in cleavage rates (Hill et al., 1996) as well as a higher blastocyst yield and a shorter time for blastocyst formation (Hill et al., 1997). Similarly, in pigs, the presence of OVGP1 in the capacitating medium during in vitro fertilization resulted in an increase in the number of embryos that developed to blastocysts despite no detected differences in cleavage rates (Kouba et al., 2000). Altogether, the
results obtained in the above studies demonstrate the beneficial biological effects of OVGP1 on sperm, oocytes, and early embryo development (Figure 2).

1.2.2 Recombinant Human Oviductin (rHuOVGP1)

While the findings from animal studies support the role of OVGP1 in sperm capacitation, fertilization, and early embryo development, information concerning the mechanisms that regulate human OVGP1 in these processes had been lacking. This is mainly due to the ethical issues and technical difficulties in collecting adequate quantities of human oviductal fluid for purification of OVGP1 for functional and mechanistic studies, and for use clinically in the future. To circumvent this limitation, our laboratory has successfully used recombinant DNA technology to produce recombinant human OVGP1 (rHuOVGP1) from stably transfected human embryonic kidney (HEK 293) cells (Zhao et al., 2016). In membrane-intact human sperm, rHuOVGP1 has been shown to bind to the head, neck and mid-piece and, to a lesser extent, to the principal piece of the sperm tail (Zhao et al., 2016). Interestingly, when the sperm plasma membrane was removed, rHuOVGP1 was found to bind to the equatorial and post-acrosomal regions in the sperm head and throughout the mid-piece and principal piece of the sperm tail. These findings suggest that rHuOVGP1 may exert its function through binding not only to the surface of the sperm head and tail regions but also to certain structural elements underlying the plasma membrane of human sperm (Zhao et al., 2016).

The biological functions of rHuOVGP1 that have been revealed in *in vitro* studies are illustrated in Figure 3. The addition of rHuOVGP1 in the capacitating medium can increase the level of tyrosine phosphorylation of sperm proteins, a characteristic feature of sperm capacitation,
detected predominantly in the fibrous sheath in the principal piece of the sperm tail (Zhao et al., 2016; Zhao and Kan, 2019). The fibrous sheath influences the degree of flexibility, motion and shape of the flagellar beat while functioning as a protein scaffold for signaling pathways involved in sperm motility and capacitation (Eddy et al., 2003). A major tyrosine phosphorylated protein enhanced by the presence of rHuOVGP1 migrates at 105 kDa (p105) (Zhao et al., 2016) and has been shown to be associated with A-kinase anchoring protein 3 (AKAP3) in the fibrous sheath (Zhao and Kan, 2019). Phosphorylated AKAP3 binds to and confines protein kinase A (PKA), a key enzyme involved in sperm capacitation, to subcellular compartments of sperm and plays an important role in sperm motility (Taskén and Aandahl, 2004; Luconi et al., 2005). Incubating sperm with rHuOVGP1 can further potentiate them to undergo acrosome reaction, which is linked to increased fertilization rates (Zhao et al., 2016). Similar to OVGP1 in the animal models discussed above, rHuOVGP1 binds to the zona pellucida of human oocytes (Zhao and Kan, 2019). Pre-treatment of sperm and hemi-zonae with rHuOVGP1 prior to co-incubation can increase the rate of sperm-egg binding as compared to similar conditions without rHuOVGP1 (Zhao and Kan, 2019). The success of producing rHuOVGP1 via cell cultures using HEK 293 cells has enabled our laboratory to investigate the functions of human OVGP1 and has opened up new avenues for future investigations, such as the present study, into the role of human OVGP1 in reproductive functions and the mechanisms that regulate these functions.

1.3 Mammalian Sperm

1.3.1 Structure of Sperm

Mammalian spermatozoa are produced in the seminiferous tubules of the testis through a complex process known as spermatogenesis (Neto et al., 2016). Upon their release from the
seminiferous epithelium, the spermatozoa travel to the cauda epididymis where they mature prior to being deposited into the female reproductive tract after ejaculation (Dacheux and Dacheux, 2014). The mammalian sperm can be divided into two parts: the head and the flagellum (tail). The head region of spermatozoa contains the extensively compact haploid nucleus with very little cytoplasm, contributing to its small size (Mescher, 2018). The equatorial segment of the head is the boundary between the anterior and the posterior head and marks the initial site of sperm-egg membrane fusion (Mortimer, 2018). The acrosome is a membrane-enclosed vesicle that covers the anterior two-thirds of the head region and contains hydrolytic enzymes and molecules required for digesting the zona pellucida that surround the oocyte during fertilization. The post-acrosomal sheath is a structure that wraps around the posterior of the sperm head and is the region of the fertilizing sperm that first binds to the plasma membrane of the oocyte (Mortimer, 2018). The flagellum or sperm tail is structurally divided into four major parts: the connecting piece, the mid-piece, the principal piece, and the end piece (Mescher, 2018). The connecting piece connects the head and flagellum together. The core of the flagellum contains an array of microtubules forming the axoneme, composed of 9 outer doublets and 2 central singlet of microtubules (Lehti and Sironen, 2017). Peripheral to the axoneme are the outer dense fibers (ODF) that run longitudinally from the connecting piece to the principal piece. The mid-piece contains 9 ODFs that are surrounded by the mitochondrial sheath, a tightly packed helix of mitochondria responsible for ATP production (Lehti and Sironen, 2017). The principal piece of the tail is defined by the presence of a fibrous sheath, where 2 of the ODFs are replaced by the longitudinal columns of the fibrous sheath that are connected by circumferential ribs (Lehti and Sironen, 2017). The fibrous sheath is believed to play a role in the degree of flexibility, plane of flagellar motion and the shape of the
flagellar beat (Eddy et al., 2003). The end piece is the final segment of the tail and contains only the axoneme. The sperm cell is covered from head to tail by a plasma membrane.

1.3.2 Sperm Capacitation

Mammalian fertilization occurs via a comprehensive progression of events in an orderly manner. After ejaculation, sperm undergo a series of biochemical and physiological changes as they travel through the female reproductive tract, collectively known as capacitation, that render sperm capable of fertilizing the oocyte. Chang (1951) first discovered that freshly ejaculated sperm in vivo are not able to fertilize oocytes unless sperm have resided for a period of time in the female reproductive tract. The process of sperm capacitation is summarized in Figure 4. One of the initial changes of capacitation of mammalian spermatozoa involves the transfer of cholesterol from the sperm plasma membrane to albumin present in the oviductal fluid in vivo or in the capacitating medium under in vitro conditions (Naz and Rajesh, 2004; Jin and Yang, 2017). Cholesterol is a decapacitation factor that stabilizes the plasma membrane of sperm during transit through the epididymis. In doing so, cholesterol prevents the intermolecular interactions described below that lead to a capacitated state (Aitken and Nixon, 2013). An increase in membrane fluidity after cholesterol efflux increases the permeability of the sperm plasma membrane to various ions such as calcium (Ca$^{2+}$) and bicarbonate (HCO$_3^-$), leading to a cascade of signal transduction events that result in sperm capacitation (Jin and Yang, 2017). Simultaneously, progesterone (P4) binds to the sperm plasma membrane and induces Ca$^{2+}$ influx through the Ca$^{2+}$ channels (Naz and Rajesh, 2004; Jin and Yang, 2017).
Activation of the cyclic adenosine monophosphate (cAMP) dependent protein kinase A (PKA) pathways is a key aspect of inducing sperm capacitation. The influx of Ca\(^{2+}\) and HCO\(_3^-\) activates soluble adenylyl cyclase (sAC), converting ATP to cAMP (Signorelli et al., 2012). PKA is a cAMP-dependent ubiquitous tetrameric enzyme that contains two regulatory subunits (R2) and two catalytic subunits (C2) (Signorelli et al., 2012). The binding of cAMP to the regulatory subunits allows for the regulatory subunits to dissociate from the R2C2 complex, thus exposing the active sites of the catalytic subunits (Signorelli et al., 2012). The free catalytic subunits interact with proteins to phosphorylate the serine-threonine residues of sperm (Signorelli et al., 2012; Jin and Yang, 2017). The phosphorylation of these PKA substrates eventually leads to phosphorylation of tyrosine residues of sperm proteins (Aitken and Nixon, 2013). This extensive increase in tyrosine phosphorylation is a characteristic feature of sperm capacitation in many mammalian species including the human (Leclerc et al., 1996), bovine (Galantino-Homer et al., 1997), porcine (Kaláb et al., 1998), hamster (Visconti et al., 1999), mouse (Visconti et al., 1995) and rat (Lewis and Aitken, 2001). Nevertheless, the exact mechanism of PKA-mediated tyrosine phosphorylation remains to be determined.

During the process of capacitation, sperm undergo a change in the pattern of their motility known as hyperactivation (Suarez, 2008). When sperm become hyperactivated, their motility is characterized by a vigorous and asymmetrical swimming pattern. Hyperactivation is critical to fertilization as it enhances the ability of sperm to travel through the female reproductive tract and to penetrate the oviductal mucus in vivo and, finally, to penetrate the zona pellucida surrounding the oocyte. Moreover, capacitated sperm acquire the potential to undergo acrosome reaction, an important prerequisite for fertilization. The acrosome reaction, triggered by sperm binding to the
Zona pellucida of the oocyte, results in the release of the acrosomal contents and the exposure of the molecules present on the inner acrosomal membrane responsible for the ability of sperm to penetrate through the zona pellucida and subsequently fuse with the plasma membrane of the oocyte (Bailey, 2010). In short, capacitation encompasses a series of changes and preparatory steps required for sperm to gain fertilizing competence.

1.4 Progesterone

1.4.1 Functions of Progesterone in Mammalian Sperm

After ovulation, progesterone (P4), a sex steroid hormone, is produced and secreted by the cumulus cells that surround the oocyte (Gatica et al., 2013). Consequently, the participation of P4 in sperm function has been well studied. P4 elevates the level of intracellular Ca\(^{2+}\) to alter flagellar bending and to trigger hyperactivated motility, a key aspect of sperm capacitation (Uhler et al., 1992; Suarez, 2008; Sagare-Patil et al., 2012; Servin-Vences et al., 2012). Additionally, P4 has been implicated in increasing chemotaxis, the mechanism that guides sperm towards the oocyte (Teves et al., 2006). The secretion of P4 from the cumulus cells creates a P4 gradient towards the oviduct (López-Torres and Chirinos, 2017). Sperm are highly sensitive to P4 as they alter their orientation towards the oocyte in the presence of picomolar concentrations of P4 (Teves et al., 2006, 2009). The mechanism of human sperm chemotaxis mediated by P4 appears to involve the cAMP-PKA pathway and Ca\(^{2+}\) mobilization (Teves et al., 2009). Lastly, P4 has been implicated in stimulating acrosome reaction, which is believed to be attributed to the P4-induced increase in intracellular Ca\(^{2+}\) (Harper et al., 2006; López-Torres and Chirinos, 2017). Chen and colleagues (2019) reported a positive correlation between P4-induced acrosome reaction and in vitro
fertilization rate. Altogether, the evidence suggests that P4 is involved in regulating many important sperm functions that are critical for sperm to fertilize the oocyte.

1.4.2 Progesterone and the Cation Channels of Sperm (CatSper)

It has previously been shown that P4 induces Ca^{2+} entry into human spermatozoa, one of the initial changes that occur during sperm capacitation (Baldi et al., 1991; Naz and Rajesh, 2004). The cation channels of sperm (CatSper) have been localized in the principal piece of the sperm tail and have been found to control Ca^{2+} influx into sperm (Lishko et al., 2011). It has been reported that CatSper is required for sperm motility and male fertility, as targeted disruption of the CatSper gene resulted in male sterility in otherwise normal mice (Ren et al., 2001). Evidence has shown that P4 binds to the human sperm P4 receptor, alpha/beta hydrolase domain containing protein 2 (ABHD2) (Miller et al., 2016). This receptor acts as a P4-dependent lipid hydrolase by depleting endocannabinoid 2-arachidonoylglycerol (2AG), sperm CatSper channel inhibitors, from the plasma membrane (Miller et al., 2016). The subsequent removal of 2AG activates CatSper channels, thus inducing the influx of Ca^{2+} (Lishko et al., 2011; Miller et al., 2016). A rise in Ca^{2+} initiates a cascade of signal transduction events essential for sperm capacitation and hyperactivated motility which are required for sperm to ascend the female reproductive tract and fertilize the oocyte (Jin and Yang, 2017).

1.5 Hypothesis and Objectives

Our laboratory has previously shown that rHuOVGP1 can enhance the level of tyrosine phosphorylation of sperm proteins in human sperm (Zhao et al., 2016). However, the mechanism that regulates the increased level of tyrosine phosphorylation of sperm proteins by rHuOVGP1 is
unknown. With the information above regarding the interplay between P4, intracellular Ca\(^{2+}\) and CatSper channels during sperm capacitation, we hypothesized that one of the mechanisms could be that human OVGP1 can enhance tyrosine phosphorylation of sperm proteins through increasing intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), similar to P4, via the CatSper channels during human sperm capacitation.

**Study objectives**

The present investigation has four study objectives:

1. To determine whether rHuOVGP1 can increase [Ca\(^{2+}\)]\(_i\) levels.

2. To examine the effect of rHuOVGP1 alone and in combination with P4 on [Ca\(^{2+}\)]\(_i\) specifically in human sperm tail.

3. To investigate whether rHuOVGP1, similar to P4, increases [Ca\(^{2+}\)]\(_i\) through CatSper channels during sperm capacitation.

4. To determine if P4 alone can enhance tyrosine phosphorylation of sperm proteins and, if so, to determine if the effect can be further enhanced by use of a combination of P4 and rHuOVGP1.
Figures
Figure 1. Schematic diagram of the human female reproductive tract.

The human female reproductive tract composed of the ovaries, oviducts, uterus, and vagina. The oviduct is divided anatomically into four regions; 1) the infundibulum; 2) the ampulla; 3) the isthmus; and 4) the uterine or intramural region. Created with BioRender.com.
**Figure 2.** Schematic diagram showing the biological functions of OVGP1 derived mainly from *in vitro* functional studies using native OVGP1.

OVGP1 is synthesized and secreted by mammalian oviductal cells under the influence of estrogen (E2). *In vitro* functional studies from various mammalian species have shown that OVGP1 is able to bind to sperm and zona pellucida and enhances sperm capacitation, sperm motility and viability, sperm-egg binding, penetration and fertilization rates, decreases polyspermy and enhances embryo cleavage rate and blastocyst yield (Modified from Zhao, 2019).
**Figure 3.** Schematic diagram showing the biological functions of OVGP1.

Using rHuOVGP1 as a model, *in vitro* experiments revealed that rHuOVGP1 can bind to human sperm and enhance capacitation presumably through increasing the level of tyrosine phosphorylation of sperm proteins, and consequently increasing acrosome reaction. Additionally, OVGP1 has been shown to bind to the zona pellucida of oocytes and enhance sperm-egg binding (Modified from Zhao, 2019).
Figure 4. Schematic diagram of sperm capacitation.

In brief, sperm capacitation is a series of physiological and biochemical changes that facilitate sperm-egg binding and penetration of sperm into the oocyte. The initial stage of capacitation involves the transfer of cholesterol from the sperm plasma membrane to albumin present in the oviductal fluid in vivo or in capacitating medium in vitro. This destabilizes the plasma membrane, increasing its fluidity and permeability, thus enabling the influx of calcium (Ca\(^{2+}\)) and bicarbonate (HCO\(_3^-\)). Simultaneously, progesterone binds to the sperm plasma membrane and induces Ca\(^{2+}\) influx through the cation channels of sperm (CatSper). Calcium and bicarbonate bind to soluble adenylyl cyclase (sAC), allowing for the production of cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). The binding of cAMP to the regulatory subunits of protein kinase A (PKA) triggers the dissociation and activation of the two catalytic subunits (C). Activated PKA binds to and activates tyrosine kinases which, in turn, increase the tyrosine phosphorylation levels of sperm proteins during capacitation. The acquisition of capacitation enables sperm to adopt a hyperactivated motility. Furthermore, capacitated sperm can undergo acrosome reaction which is a requirement for fertilization. The mechanism of OVGP1 that regulates the enhancement of intracellular Ca\(^{2+}\) concentration and the level of tyrosine phosphorylation of sperm proteins remains in question. Created with BioRender.com.
Chapter 2
Materials and Methods
2.1 Materials

The following chemical reagents and materials were obtained from the sources indicated: Percoll, Fluo3, 7-Aminoactinomycin D (7AAD), and goat anti-mouse IgG-HRP antibody (Thermo Fisher Scientific, Waltham, MA, USA); HC-056456 (3,4-Bis(2-thienoyl)-1,2,5-oxadiazole-N-oxide) (Cedarlane, Burlington, ON, Canada); monoclonal mouse anti-phosphotyrosine antibody, monoclonal mouse anti-α-tubulin antibody, Concanavalin A (Con-A) (Sigma, St. Louis, MO, USA); progesterone (Life Technologies, Carlsbad, CA, USA); protein ladder (FroggaBio, Concord, ON, Canada); clarity Western enhanced chemiluminescence (ECL) substrate (Bio-Rad Laboratories Inc., Hercules, CA, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

The rHuOVGP1 used in the present study was previously prepared in our laboratory. The secretory form of rHuOVGP1 was produced from HEK 293 cells using recombinant DNA technology as described in detail by Zhao et al. (2016). rHuOVGP1 was purified using ammonium sulfate precipitation and gel filtration chromatography. The resulting rHuOVGP1 showed a single band corresponding to the 120-150 kDa size range of native human OVGP1, and its identity as human OVGP1 was further confirmed by mass spectrometry (Zhao et al., 2016).

2.2 Preparation of sperm from fresh human semen samples

The present study (Research Project #: DBMS-030-14; see attached in Appendix B) was approved by the Queen’s University Health Sciences & Affiliated Teaching Hospitals Research Ethics Board (HSREB). Human semen samples were obtained from Flow Labs Kingston (see Appendix C for Consent Form), located in Kingston General Hospital (Kingston, Ontario, Canada), via donors by masturbation after 2 to 3 days of abstinence. Upon collecting the samples,
they were liquefied in a 35°C water bath. The volume and viscosity of the semen as well as the sperm motility were assessed according to guidelines of the World Health Organization (WHO, 2010). To prepare the sperm samples for subsequent experiments, liquefied semen was layered on top of a Percoll gradient made up of 2 mL fractions each of 40% and 65% and 1 mL of 95% Percoll, followed by centrifugation at 1000×g for 30 minutes at 25°C to isolate sperm from seminal fluid. Percoll was prepared in 1X HEPES-buffered saline (HBS; 25mM HEPES, 130mM NaCl, 4mM KCl, 0.5mM MgCl₂, 14mM D-fructose, pH 7.6). After centrifugation, sperm from the 65%-95% interface and within the layer of 95% Percoll were collected and used for subsequent experiments, as they represent a highly motile population. In general, sperm which are most motile are indicative of better quality and viability. Sperm were then washed with 10 mL of HBS and centrifuged at 500×g for 10 minutes at 25°C. The supernatant was removed, and sperm concentration was then determined using a hematocytometer counter chamber device.

2.3 Preparation of sperm for assessment of [Ca²⁺]ᵢ

The Fluo3 is a fluorescence indicator of intracellular calcium. The Fluo3 stock solution was purchased as 1 mg in powder form and prepared at a concentration of 1 mM in DMSO. Once sperm cells were counted, they were added at a concentration of 1 x 10⁷ cells/mL into the non-capacitating medium (120 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 0.25 mM Na pyruvate, 21.6 mM Na lactate, 10 mg/L phenol red, 5.6 mM D-glucose, 10 mM HEPES, pH 7.4), in order to prevent the onset of capacitation during preparation. Subsequently, the sperm cell solution was supplemented with Fluo3 (2 µM) and incubated at 37°C with 5% CO₂ for 30 minutes. The rest of the cells were added to the non-capacitating medium at a concentration of 1x 10⁷ cells/mL without Fluo3 for subsequent use as controls. At the end of incubation, cells were
removed from the incubator (Sanyo MCO-17AIC CO₂ incubator, Marshall Scientific, Hampton, NH, USA) and centrifuged at 1000×g for 5 minutes at room temperature. Sperm were then washed with 1 mL of HBS and centrifuged again. The supernatant was removed, and the remaining volume was measured.

2.4 Flow cytometry

Following preparation for assessment of [Ca²⁺]ᵢ, sperm cells at a concentration of 4 x 10⁶ cells/mL were added to 500 µL of modified Biggers-Whitten-Wittingham medium (BWW; 94.6 mM NaCl, 4.8 mM KCl, 1.7 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 0.25 mM Na pyruvate, 21.6 mM Na lactate, 10 mg/L phenol red, 25.1 mM NaHCO₃, 5.6 mM D-glucose, 10 mM HEPES, pH 7.4) supplemented with 0.3% BSA. The indicator 7-Aminoactinomycin D (7AAD) (2 µL per 2 x 10⁶ sperm cells) was added to all experimental reactions to assess sperm viability prior to data acquisition. Intracellular Ca²⁺ was then assessed using flow cytometry (FL2 525/50; SH800S, Sony, SanJose, USA). Following 3 min of baseline fluorescence reading, rHuOVGP1 (25, 50, 75, and 100 µg/mL) was added, respectively, to the cell suspension (4 x 10⁶ cells/mL), and the data were acquired for a further 10 minutes. As controls, the fluorescent intensity of unstained non-capacitated sperm cells (4 x 10⁶ cells/mL) as well as cells in the presence of Fluo3 alone, 7AAD alone, and Fluo3 with 7AAD was measured. Data were analyzed using FlowJo (Ashland, USA), and the median of Fluo3 fluorescence was recorded as a measurement of intracellular Ca²⁺.

2.5 Calcium imaging

Sperm cells were prepared for assessment of [Ca²⁺]ᵢ as described above. Sperm cells (2 x 10⁶ cells/mL) in non-capacitating medium were plated in 35 mm dishes (no. 1.5 Coverslip; 10 mm
Glass Diameter; MatTeK Life Sciences) coated with the Concanavalin A (a carbohydrate-binding protein; 1 mg/mL in PBS) and left in the incubator for 5 minutes at 37°C with 5% CO₂ to allow adherence of the sperm cells (Sánchez-Cárdenas et al., 2014). Sperm cells were imaged using the OkoLab stage-top microscope incubator (OkoLab Bold Line, Pozzuoli, Italy) located in Queen’s CardioPulmonary Unit (QCPU, Queen’s University, Kingston, ON, Canada). Live cell imaging was performed using the Leica TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany) located in QCPU. Sperm cells were imaged for 1 minute in capacitating (BWW) medium to obtain baseline measurements, followed by subsequent imaging of the same cells treated, respectively, with rHuOVGP1 (100 μg/mL), the CatSper channel blocker, HC-056456 (3 or 10 μM), and progesterone (P4; 1 μM), as below:

1) Baseline (1 min) → rHuOVGP1 (10 mins) → P4 (5 mins)
2) Baseline (1 min) → control medium (10 mins) → P4 (5 mins)
3) Baseline (1 min) → HC-056456 (3 μM; 5 mins) → rHuOVGP1 (10 mins) → P4 (5 mins)
4) Baseline (1 min) → HC-056456 (3 μM; 5 mins) → control medium (10 mins) → P4 (5 mins)
5) Baseline (1 min) → HC-056456 (10 μM; 5 mins) → rHuOVGP1 (10 mins) → P4 (5 mins)
6) Baseline (1 min) → HC-056456 (10 μM; 5 mins) → control medium (10 mins) → P4 (5 mins)

HC-056456, an inhibitor of CatSper channels, was used at both concentrations of 3 and 10 μM because previous research demonstrated that the use of HC-056456 at these concentrations was found to be an effective blocker of CatSper channels (Carlson et al., 2009; Orta et al., 2019). While our current findings using flow cytometry demonstrated that rHuOVGP1 can enhance the level of [Ca²⁺]i when used at a concentration of 50, 75, and 100 μg/mL, we chose to examine the
effects of 100 μg/mL rHuOVGP1 on [Ca\(^{2+}\)]; by use of live cell imaging. During the calcium imaging experiments, we were unable to mix in the added reagents in order to image the same sperm cells throughout the entire experiment. Therefore, we decided to add a higher concentration of rHuOVGP1 (100 μg/mL) assuming that, at a higher concentration, the glycoprotein would be well distributed among the sperm cells.

2.6 Western blot analysis

Our laboratory has previously reported that the tyrosine phosphorylation level of p105 can be further enhanced in the presence of rHuOVGP1 at an optimal concentration of 50 μg/mL in capacitating medium (Zhao et al., 2016), therefore, rHuOVGP1 at a concentration of 50 μg/mL was used in the present study to assess the effect of rHuOVGP1 on tyrosine phosphorylation of p105 by Western blot. Aliquots of the sperm (2 x 10^7 cells/mL) were prepared in the absence or presence of 50 μg/mL of rHuOVGP1 with different concentration of P4 (0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} M). These samples were capacitated via incubation at 37°C with 5% CO\(_2\) in modified Biggers-Whitten-Wittingham medium supplemented with 0.3% BSA for different time-points (0-4 hours). The capacitating medium was pre-equilibrated overnight at 37°C with 5% CO\(_2\). During the incubation period, samples were mixed gently every 15 minutes in order to resuspend the cells. At the end of the incubation period, the samples were washed with 1mL of 1X HBS and centrifuged at 1000×g for 5 minutes at room temperature. For Western blot analysis, the sperm samples were vortexed for 30 seconds in sample buffer (6% SDS, 30% glycerol, 12% β-mercaptoethanol, 0.02% bromophenol blue, 6mM Na\(_3\)VO\(_4\), 187.5mM Tris-HCl, pH 6.8). Following preparation, the samples were boiled for 5 minutes to denature the proteins and centrifuged at 16000×g for 5 minutes at room temperature. Samples were loaded into wells in the gel apparatus. The first lane
was reserved for a protein ladder, which consisted of a mixture of proteins of known molecular weights that were later visualized as coloured bands. Sperm proteins were separated by SDS-PAGE (SDS polyacrylamide gel electrophoresis) and transferred onto polyvinylidene difluoride (PVDF).

### 2.7 Evaluation of tyrosine phosphorylation of sperm proteins

As tyrosine phosphorylation of sperm proteins is a biochemical hallmark of sperm capacitation, changes in the level of tyrosine phosphorylation of sperm proteins were determined by Western blot analysis. To begin, non-specific binding on the PVDF membrane was blocked with 5% milk in 1X Tris-buffered saline (150 mM NaCl, 50 mM Tris, pH 7.5) containing 0.05% Tween 20 (TBST) for 45 minutes. Next, the PVDF membrane was incubated in the cold room for 1-2 hours with a monoclonal mouse anti-phosphotyrosine antibody (diluted in 1X TBST at a final concentration of 0.1 μg/mL). After four washes with 1X TBST, the PVDF membrane was incubated with goat anti-mouse IgG-HRP antibody (diluted in blocking solution at 0.02 μg/mL) for 1 hour at room temperature. Following another four washes, the PVDF membrane was placed in ECL (enhanced chemiluminescence) which detects horseradish peroxidase (HRP) and allows for visualization of the protein bands of interest.

To assure that an equal amount of protein was loaded into each well of the gel apparatus, the same PVDF membrane was stripped of previous antibodies and re-probed for 1 hour with a monoclonal mouse anti-α-tubulin antibody diluted in 1X TBST at a final concentration of 0.1 μg/mL. Following four washes with 1X TBST, the membrane was incubated for 1 hour with goat
anti-mouse IgG-HRP antibody (diluted in blocking solution at 0.02 µg/mL). Bands were visualized as previously outlined.

### 2.8 Statistical analysis

Statistical tests were performed using Prism 9 (GraphPad Software, San Diego, CA, USA). Changes of \([\text{Ca}^{2+}]_i\) were determined by measuring the level of intensity of fluorescent staining of Fluo3. The assessment of \([\text{Ca}^{2+}]_i\) with flow cytometry for each experimental sample was compared to its initial baseline (-200 seconds) prior to the addition of rHuOVGP1 and expressed as a fold increase using two-way ANOVA with Dunnett’s *post hoc* test. The comparison of the intensity of fluorescent staining between rHuOVGP1-treated cells at different concentrations of rHuOVGP1 and the untreated cells at a specific time-point was analyzed using Student’s *t*-tests.

The assessment of \([\text{Ca}^{2+}]_i\) with live cell imaging over the 16- and 21-minute incubation periods were recorded and averaged, respectively, to 15 and 20 time-points and expressed as a fold increase from the initial fluorescent intensity. The comparison of the intensity of fluorescent staining between the cells treated with rHuOVGP1 and the untreated cells at a specific time-point was analyzed using Student’s *t*-tests.

For assessment of protein tyrosine-phosphorylation via Western blot analysis, the band intensities were quantified by densitometry using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). The intensities of the bands were normalized to those of α-tubulin. The level of tyrosine phosphorylation of sperm proteins at each time-point was compared to the 0-hour time-point and expressed as a fold increase using two-way ANOVA with Dunnett’s *post hoc*
test. The comparison between treated cells and untreated cells at a specific time-point was analyzed using Student’s $t$-tests. $P$-values of <0.05 were considered significant.
Chapter 3
Results
3.1 Effects of rHuOVGP1 on \([\text{Ca}^{2+}]_i\) of human sperm

Due to the shared localization of calcium channels and tyrosine phosphorylation of sperm proteins in the principal piece of the sperm tail (Lishko et al., 2011; Zhao and Kan, 2019), we sought out to investigate the effects of OVGP1 on \([\text{Ca}^{2+}]_i\). Flow cytometry experiments were carried out where sperm samples were incubated in capacitating medium followed by the addition of different concentrations of rHuOVGP1 (0, 25, 50, 75 or 100 \(\mu\text{g/mL}\)) at \(t = 0\) for 10 minutes. Additionally, a sample incubated in non-capacitating medium was analyzed to act as a baseline for fluorescent intensity. The level of \([\text{Ca}^{2+}]_i\), indicated by the intracellular calcium indicator Fluo3, in sperm appeared to gradually increase when sperm cells were exposed to capacitating (BWW) medium (Figure 4A). However, the level of \([\text{Ca}^{2+}]_i\) appeared to be further increased when sperm were subjected to treatment with increasing concentrations of rHuOVGP1 (Figure 4A). The level of \([\text{Ca}^{2+}]_i\) over the period of 470-600 seconds after the addition of rHuOVGP1 is significantly higher in sperm treated, respectively, with 50, 75 and 100 \(\mu\text{g/mL}\) of rHuOVGP1 compared to untreated sperm (Figure 4B). Table S1 displays the results from a two-way ANOVA statistical analysis comparing the level of Fluo3 median intensity over time to that of the time-point at 45 seconds prior to the addition of rHuOVGP1 indicated as -45 seconds. The results showed that the time-points that show a significant increase in \([\text{Ca}^{2+}]_i\) occur earlier when sperm were treated with higher doses of rHuOVGP1 during the 10-minute incubation period. Treating sperm with rHuOVGP1 significantly increased the level of intracellular calcium in a time-dependent manner. Overall, these results indicated that the presence of OVGP1 increases \([\text{Ca}^{2+}]_i\) at the beginning of sperm capacitation and maintains this increase in \([\text{Ca}^{2+}]_i\) over the 10-minute time course.
3. 2 Influence of treating sperm with rHuOVGP1 prior to P4 on sperm [Ca\textsuperscript{2+}].

The effects of OVGP1 on [Ca\textsuperscript{2+}] were further studied using fluorometric confocal microscopy with live cell imaging. Sperm cells were imaged in capacitating (BWW) medium to obtain baseline measurements, followed by subsequent imaging of the same cells treated with or without rHuOVGP1 (100 µg/mL) for 10 minutes and then treated with P4 (1 µM) for 5 minutes. Representative images of the live cell imaging over time are shown in Figure 5A. Sperm cells treated with rHuOVGP1 showed an increasing trend in the level of intracellular calcium compared to untreated cells (Figure 5B and 5C: time-points A-C). As expected, based on evidence that P4 induces calcium influx in sperm (Lishko et al., 2011; Strünker et al., 2011; Miller et al., 2016), the addition of P4 alone resulted in an increase in [Ca\textsuperscript{2+}] followed by a gradual decrease (Figure 5A and 5B). However, this increase in [Ca\textsuperscript{2+}] was further enhanced when sperm cells were treated with rHuOVGP1 followed by the addition of P4 (Figure 5A-C: time-point D). Moreover, the increase in [Ca\textsuperscript{2+}] observed in sperm treated with rHuOVGP1 was sustained over the 5-minute period following treatment with P4 (Figure 5A-C: time-points E-G). These results demonstrated that treating sperm with OVGP1 amplifies the level of intracellular Ca\textsuperscript{2+} in human sperm tail following treatment with P4.

3.3 Inhibition of CatSper channels impedes the effects of rHuOVGP1 on [Ca\textsuperscript{2+}] in human sperm

CatSper channels play a key role in male fertility by controlling Ca\textsuperscript{2+} entry into sperm (Carlson et al., 2009; Lishko et al., 2011; Miller et al., 2016; Sun et al., 2017). To investigate whether rHuOVGP1 increases [Ca\textsuperscript{2+}] through CatSper channels in a manner similar to P4 in increasing [Ca\textsuperscript{2+}], further live cell imaging was performed using the selective CatSper channel
inhibitor HC-056456. The compound HC-056456 has previously been shown to lower the CatSper current in whole cell patch-clamp recordings and reversibly prevent the development of hyperactivated motility during sperm capacitation (Carlson et al., 2009). In the present study, sperm cells were imaged for 1 minute in capacitating (BWW) medium to obtain baseline measurements, followed by imaging the same cells treated with HC-056456 (at 3 or 10 μM), and treatment with or without rHuOVGP1 (100 μg/mL) for 10 minutes and subsequently treatment with P4 (1 μM) for 5 minutes. As anticipated, inhibiting CatSper channels resulted in a decrease in the level of Fluo3 labeling intensity of [Ca\(^{2+}\)]\(_i\) (Figure 6). As P4 is known to induce calcium influx through CatSper channels (Lishko et al., 2011; Strünker et al., 2011; Miller et al., 2016), no increase in [Ca\(^{2+}\)]\(_i\) following the addition of P4 was observed in sperm cells pre-treated with HC-056456 alone (Figure 6). Likewise, no increase in [Ca\(^{2+}\)]\(_i\) following the addition of rHuOVGP1 or subsequent addition of P4 was observed in sperm cells pre-treated with HC-056456 (Figure 6). These results indicated that inhibition of the CatSper channels with HC-056456 impedes the effects of rHuOVGP1 on [Ca\(^{2+}\)]\(_i\) in the human sperm tail, suggesting that the CatSper channel may be involved in the mechanism by which OVGP1 increases intracellular calcium.

3.4 Effects of P4 on tyrosine phosphorylation of sperm protein

As time-dependent increase in the level of sperm protein tyrosine phosphorylation is a characteristic feature of sperm capacitation and P4 is known to increase intracellular calcium during sperm capacitation (Lishko et al., 2011; Miller et al., 2016), the effects of P4 on enhancing protein tyrosine phosphorylation were assessed. Sperm cells were incubated for 0 to 4 hours in capacitating (BWW) medium alone or supplemented with different concentrations of P4 (10\(^{-9}\), 10\(^{-8}\), 10\(^{-7}\), 10\(^{-6}\), or 10\(^{-5}\) M). Western blot analysis was used to detect the expression level of protein
tyrosine phosphorylation by probing the membrane with an anti-phosphotyrosine antibody. The intensity of the bands was further analyzed by ImageJ software to obtain a graphical analysis of these results summarized in Figure 7. Our results showed that the most abundantly tyrosine-phosphorylated human sperm protein migrates at 105 kDa (p105), comparable to previous studies in our laboratory (Zhao et al., 2016), and was further enhanced by the presence of P4 in the capacitating medium (Figure 7A and 7B). As shown in Figure 7C, the level of tyrosine phosphorylation is further increased with higher concentrations of progesterone. Specifically, the level of tyrosine phosphorylation is significantly increased at 3 hours when treated with 10^{-7} M of P4 but is significantly further increased at 4 hours when treated with 10^{-6} M of P4 (Figure 7C). Table S2 displays the results from a two-way ANOVA statistical analysis of tyrosine phosphorylation of each treatment of P4 over time compared to non-capacitated sperm cells. The results showed that the time-points that yielded a significant increase in the level of tyrosine phosphorylation of p105 occurred earlier as the concentration of P4 increased to 10^{-6} M of P4, followed by a later increase in the level of tyrosine phosphorylation at a concentration of 10^{-5} M of P4. Overall, these results showed that P4 can enhance the tyrosine phosphorylation of sperm p105 in a time- and dose-dependent manner and suggest that 10^{-6} M may be the optimal P4 concentration.

3.5 Combined effects of P4 and rHuOVGP1 on tyrosine phosphorylation of sperm protein

Our laboratory has previously reported that the tyrosine phosphorylation level of p105 can be further enhanced in the presence of rHuOVGP1 at an optimal concentration of 50 µg/mL in the capacitating medium (Zhao et al., 2016). Based on these previous results and the current findings that P4 can increase the level of tyrosine phosphorylation of p105, we hypothesized that OVGP1
and P4 may work in synergy to enhance tyrosine phosphorylation of sperm p105. Western blot analysis was carried out to examine whether the increase in tyrosine phosphorylation observed in sperm treated with rHuOVGP1 and P4 each alone can be further enhanced when sperm are treated with rHuOVGP1 and P4 in combination. As shown in Figure 8, the intensity of protein tyrosine phosphorylation was faintly detected at 0 hour in non-capacitated sperm. The tyrosine phosphorylation level of p105 exhibited an increase in response to the presence of rHuOVGP1, similar to results previously reported (Zhao et al., 2016), and in response to P4 compared to the control. Interestingly and importantly, the strongest intensity of p105 was detected when P4 was used in combination with rHuOVGP1. Statistical analysis was also performed confirming that the level of tyrosine phosphorylation of p105 was significantly increased with rHuOVGP1 alone after 2 and 3 hours as well as with P4 alone after 2 and 4 hours (Figure 8C). However, the level of tyrosine phosphorylation of p105 was substantially increased at 2, 3 and 4 hours after treatment with P4 and rHuOVGP1 in combination (Figure 8C). The present results suggest that P4 and rHuOVGP1 may work in concert to increase tyrosine phosphorylation of p105 which could, in part, lead to enhancement of sperm capacitation.
Figures
**Figure 5.** rHuOVGP1 increases [Ca$^{2+}$]$_i$ in human sperm at the beginning of capacitation.

(A) Line graph of the median fluorescent intensity of Fluo3 from the flow cytometry analysis of [Ca$^{2+}$]$_i$ in sperm. Following 3 minutes (shown as -200 to -20 seconds) of baseline fluorescence reading, rHuOVGP1 (0, 25, 50, 75, and 100 µg/mL) was added, respectively, to the cell suspension (time = 0 seconds), and the data were acquired for a further 10 minutes. (B) Histogram of the mean fluorescent intensity over the period of 470-600 seconds after the addition of rHuOVGP1 (0, 25, 50, 75, and 100 µg/mL). Data are represented as fold of change in fluorescent labeling +/- SEM; n = 7; * p<0.05.
A)
B) Intracellular Calcium Concentration ([Ca^{2+}]_i) in Sperm Tail

- **BWW only + P4**
- **BWW + rHuOVGP1 + P4**

**Graph:**
- **Y-axis:** Fold of Change in Fluo3 Mean Intensity
- **X-axis:** Time (s)

Legend:
- **Black Circle:** BWW only + P4
- **Red Square:** BWW + rHuOVGP1 + P4

Additional Notes:
- A B C
- D E F G
- */+ OVGP1
- +P4

Significance Levels:
- ***:** p < 0.05
- ****: p < 0.01
- ****: p < 0.001
Figure 6. Treatment of sperm with rHuOVGP1 further increases and sustains the level of $[\text{Ca}^{2+}]_i$ in human sperm tail following treatment with P4.

(A) Representative images of calcium live cell imaging experiments with or without rHuOVGP1 (100 µg/mL) treatment. High magnifications of sperm cells indicated by blue (pre-P4) and red (post-P4) dashed boxes are shown on the right side of the figure with regions of interest (ROIs) of sperm tails outlined in yellow. Sperm cells were imaged for 1 minute in BWW to obtain baseline measurements, followed by subsequent imaging the same cells treated with or without rHuOVGP1 (100 µg/mL) for 10 minutes and progesterone (P4; 1 µM) for 5 minutes. (B) Line graph of the relative mean fluorescent intensity of Fluo3 from the live cell imaging analysis of $[\text{Ca}^{2+}]_i$ in sperm tails treated with or without rHuOVGP1 (100 µg/mL) followed by the treatment with P4 (1 µM). (C) Histograms of the relative mean fluorescent intensity from $[\text{Ca}^{2+}]_i$ analysis for sperm incubated with or without rHuOVGP1 at various time-points (500 (A), 585 (B), 645 (C), 715 (D), 800 (E), 885 (F), and 945 (G) seconds). Data are represented as fold of change in fluorescent labeling +/- SEM; n = 6 patients (36 sperm cells in total) for each treatment; * p<0.05, ** p<0.01.
A)
Figure 7. Inhibition of CatSper channels impedes the effects of rHuOVGP1 on $[\text{Ca}^{2+}]_i$ in human sperm tail.

(A) Representative images of calcium live cell imaging experiments with or without rHuOVGP1 (100 µg/mL) treatment in the presence of CatSper channel inhibitor HC-056456. Sperm cells were imaged for 1 minute in BWW to obtain baseline measurements, followed by the subsequent imaging of the same cells treated with HC-056456 (3 or 10 µM) for 5 minutes, with or without rHuOVGP1 (100 µg/mL) for 10 minutes and with progesterone (P4; 1 µM) for 5 minutes. (B) Line graph of the relative mean fluorescent intensity of Fluo3 from the live cell imaging analysis of $[\text{Ca}^{2+}]_i$ in sperm tails treated with HC-056456 (3 or 10 µM), with or without rHuOVGP1 (100 µg/mL) followed by treatment with P4 (1 µM). Data are represented as fold of change in fluorescent labeling +/- SEM; n = 3 patients (18 sperm in cells total) for each treatment.
A) p105

- 0 M P4
- 10^{-9} M P4
- 10^{-6} M P4
- 10^{-7} M P4
- 10^{-8} M P4
- 10^{-9} M P4

Relative Intensity of pY vs. Time (hr)

B) Western Blot Analysis

- P105
  - 0 hr
  - 1 hr
  - 2 hr
  - 3 hr
  - 4 hr

IB: pY
IB: α-tubulin

M of P4 Concentration: 10^{-9} to 10^{-5} M
Figure 8. P4 enhances the level of tyrosine phosphorylation of human sperm protein.

(A) Line graph showing the effect of different P4 concentrations (0, 10⁻⁹, 10⁻⁸, 10⁻⁷, 10⁻⁶, or 10⁻⁵ M) on the level of tyrosine phosphorylation (pY) of p105 following 0 to 4 hours of capacitation. (B) Representative Western blot analysis showing the effect of different P4 concentrations on the level of tyrosine phosphorylation of p105 following 0 to 4 hours of capacitation. (C) Histograms showing the relative intensity of tyrosine phosphorylation (pY) levels of p105 in sperm treated with different P4 concentrations after 2, 3, and 4 hours of capacitation. Data are represented as relative intensity of pY +/- SEM; n = 6; * p<0.05; ns: statistically not significant.
**Figure 9.** rHuOVGP1 further enhances the positive effects of P4 on the level of tyrosine phosphorylation of protein in human sperm.

(A) Line graph showing the effect of P4 (0 or 1 μM) with (+) or without (-) rHuOVGP1 (50 μg/mL) on the level of tyrosine phosphorylation (pY) of p105 following 0 to 4 hours of capacitation. (B) Histograms showing the relative intensity of tyrosine phosphorylation (pY) levels of p105 in sperm treated with P4 (0 or 1 μM) in the presence (+) or absence (-) of rHuOVGP1 (50 μg/mL) after 2, 3, and 4 hours of capacitation. Data are represented as relative intensity of pY +/- SEM; n = 7; * p<0.05; ns: statistically not significant.
Chapter 4
Discussion and Future Directions
4.1 Discussion

Mammalian oviductal fluid is a complex mixture formed by secreted components from oviduct epithelial cells and plasma transudate (Aviles et al., 2010). Accumulated evidence has shown that OVGP1, a major glycoprotein secreted by the oviductal epithelium, is able to bind to sperm and zona pellucida in several mammalian species, and enhance early fertilization events and embryo quality and development (Abe et al., 1995; Boatman and Magnoni, 1995; Kouba et al., 2000; Saccary et al., 2013). Further studies of the mechanism of action of OVGP1 have been limited by the difficulty in collecting adequate amounts of oviductal fluid for purification and functional studies. Our laboratory has circumvented this limitation by producing the secretory form of rHuOVGP1 in HEK 293 cells. Previous findings have demonstrated that rHuOVGP1 can bind to sperm to enhance tyrosine phosphorylation, a biochemical hallmark of capacitation, of a sperm protein of 105 kDa (p105) in the principal piece of the sperm tail (Naz and Rajesh, 2004; Zhao et al., 2016). However, the exact mode of action of how rHuOVGP1 enhances tyrosine phosphorylation of sperm proteins is not clearly understood and needs to be further investigated.

Compiled evidence has shown that P4, another key component of the oviductal fluid that is secreted by cumulus cells surrounding the oocyte, induces Ca^{2+} influx through the CatSper channel which is also located in the principal piece of the sperm tail and is an important aspect of capacitation (Lishko et al., 2011; Strünker et al., 2011; Miller et al., 2016). Chung et al. (2014) demonstrated that CatSper organizes Ca^{2+} signaling domains that orchestrate the timing and extent of tyrosine phosphorylation cascade. Interestingly, protein tyrosine phosphorylation was found to delocalize with disruption of CatSper channels in mouse sperm, suggesting a functional connection between these two major events of sperm capacitation (Chung et al., 2014). While previous
research has illustrated the effect of P4 on Ca\(^{2+}\) influx and a link between Ca\(^{2+}\) signaling and tyrosine phosphorylation, the effects of P4 on tyrosine phosphorylation have not been extensively studied. A study conducted by Sagare-Patil et al. (2012) found that the level of protein tyrosine phosphorylation increased in a dose-dependent manner in response to P4 over the first hour of human sperm capacitation. Pre-incubation of sperm with Mibefradil, a non-selective calcium channel blocker, reduced the P4-mediated tyrosine phosphorylation after 10 and 30 minutes of sperm capacitation (Sagare-Patil et al., 2012). These results further imply a connection between Ca\(^{2+}\) signaling and tyrosine phosphorylation during sperm capacitation. With the information above outlining the interplay between P4, Ca\(^{2+}\) influx, and CatSper channels, we hypothesized that a possible mechanism regulating rHuOVGP1-enhanced tyrosine phosphorylation of sperm proteins could be mediated by an increase in intracellular Ca\(^{2+}\) via CatSper channels in a manner similar to that of P4 in inducing Ca\(^{2+}\) influx through CatSper channels during sperm capacitation.

In the present study, \([\text{Ca}^{2+}]_i\) was detected in human sperm using the intracellular Ca\(^{2+}\) indicator Fluo3. Our results obtained with flow cytometry demonstrated that the level of intracellular Ca\(^{2+}\) gradually increases in human sperm at the onset of capacitation, yet the concentration further increased when sperm were treated with rHuOVGP1 (Figure 4A). Particularly, the presence of rHuOVGP1 in the capacitating medium was found to increase the level of \([\text{Ca}^{2+}]_i\) in a time- and dose-dependent manner at the beginning of human sperm capacitation (Figure 4 and Table S1). This is the first report to show that rHuOVGP1 regulates \([\text{Ca}^{2+}]_i\) at the onset of sperm capacitation. We went further to investigate the influence of rHuOVGP1 on \([\text{Ca}^{2+}]_i\) specifically in the tail of sperm. Live cell imaging was carried out where sperm were capacitated in the absence or presence of rHuOVGP1 followed by subsequent
treatment with P4. We found that treating sperm with P4 alone increased \([\text{Ca}^{2+}]_i\) (Figure 5A and 5B), which is supported by finding of previous studies showing that P4 induces Ca\(^{2+}\) influx through activating CatSper channels in sperm (Lishko et al., 2011; Strünker et al., 2011; Miller et al., 2016). Moreover, there was an increasing trend in the level of \([\text{Ca}^{2+}]_i\) when sperm were treated with rHuOVGP1 (Figure 5A-C: time-points A-C). Interestingly, there was a further increase in \([\text{Ca}^{2+}]_i\) following the addition of P4 in the capacitating medium where sperm cells were treated with rHuOVGP1 and the positive effects persisted over time (Figure 5A-C: time-points D-G). Incubation of sperm with rHuOVGP1 appears to amplify the positive effects of P4 on \([\text{Ca}^{2+}]_i\) in the sperm tail, presumable this glycoprotein works synergistically with P4 to upregulate \([\text{Ca}^{2+}]_i\).

Accumulating findings reported in literature have linked P4-induced Ca\(^{2+}\) influx in human sperm to CatSper channels (Lishko et al., 2011; Strünker et al., 2011; Miller et al., 2016). Results obtained in the present study supported and extended the previous findings by demonstrating that treatment of human sperm with rHuOVGP1 can further enhance and sustain the positive effects of P4 on \([\text{Ca}^{2+}]_i\) \textit{in vitro}. In order to decipher the mechanism of OVGP1 in regulating this event, we used a specific inhibitor for CatSper channels, HC-056456, and performed live cell imaging to examine if rHuOVGP1 increases \([\text{Ca}^{2+}]_i\) by way of the CatSper channels. As anticipated and based on reports in the literature, P4 was unable to induce an influx of Ca\(^{2+}\) when CatSper channels were blocked by the inhibitor (Figure 6). Inhibition of CatSper channels by HC-056456 also hindered the effects of rHuOVGP1 in enhancing \([\text{Ca}^{2+}]_i\) that we observed earlier. Taken together, the present results indicate that OVGP1 increases \([\text{Ca}^{2+}]_i\) during capacitation at least, in part, through CatSper channels located in the sperm tail.
A time-dependent increase in protein tyrosine phosphorylation of sperm proteins is associated with capacitation (Naz and Rajesh, 2004). Hence, we investigated whether P4 could enhance sperm capacitation by increasing tyrosine phosphorylation of sperm protein p105, similar to our previous findings showing that rHuOVGP1 can enhance tyrosine phosphorylation of p105 (Zhao et al., 2016; Zhao and Kan, 2019). We conducted a dose response of P4 (0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} M) on tyrosine phosphorylation of sperm p105 over a time course of 4 hours. Western blot analysis revealed that the level of tyrosine phosphorylation p105 was increased in sperm incubated in capacitating medium in the presence of higher concentrations (10^{-7}, 10^{-6}, and 10^{-5} M) of P4 (Figure 7A and 7B). These results are consistent with previous findings by Sagare-Patil et al. (2012) where 10^{-6}-10^{-5} M of P4 were found to increase the level of tyrosine phosphorylation of sperm proteins over the first hour of capacitation. Our results also showed that the level of tyrosine phosphorylation of p105 was significantly increased in sperm capacitated in the medium containing 100 nM of P4 after 3 hours (Figure 7C). However, a significantly greater increase in the tyrosine phosphorylation level of p105 was observed in sperm capacitated in the medium containing 1 μM of P4 after 4 hours (Figure 7C). Altogether, the present results demonstrated that P4 alone can increase the tyrosine phosphorylation level of p105 in a dose- and time-dependent manner (Figure 7 and Table S2). As the tyrosine phosphorylation level of p105 has been found previously to be under the influence of rHuOVGP1 (Zhao et al., 2016) and now shown in the present study to be under the influence of P4, we were curious of their influence on tyrosine phosphorylation when used in combination. Western blot analysis showed an increase in tyrosine phosphorylation of p105 when sperm were capacitated in the presence of rHuOVGP1 alone or P4 alone as compared to the untreated cells (Figure 8). However, the level of tyrosine phosphorylation was found to be further enhanced when sperm were capacitated in the medium containing both
rHuOVGP1 and P4, with a peak intensity of tyrosine phosphorylation at 3 hours of capacitation (Figure 8). Thus, the present results clearly demonstrated that tyrosine phosphorylation of sperm protein p105 can be further enhanced when the capacitation medium is supplemented with both P4 and rHuOVGP1. A recent study carried out in our laboratory revealed that the sperm protein, p105, could be A-Kinase Anchoring Protein 3 (AKAP3) (Zhao and Kan, 2019), a prominent tyrosine phosphorylated protein associated with the fibrous sheath of the sperm tail (Ficarro et al., 2003; Baker et al., 2010). AKAP3 binds to the regulatory subunits of protein kinase A (PKA) and confines PKA to sub-cellular compartments of the sperm cell to regulate the cAMP-PKA pathway, a key signaling pathway for sperm capacitation (Michel and Scott, 2002; Taskén and Aandahl, 2004; Luconi et al., 2005). Tyrosine phosphorylation of AKAP3 is associated with an increased binding of the protein to PKA, resulting in the recruitment and activation of PKA to the fibrous sheath, ultimately, leading to enhanced sperm motility (Luconi et al., 2005).

Collectively, our experimental results support our hypothesis that rHuOVGP1 and P4 function most effectively in combination to enhance human sperm capacitation through increasing $[\text{Ca}^{2+}]_i$ and protein tyrosine-phosphorylation. With regard to the mode of action of OVGP1, based on the results obtained in the present study, we propose that this glycoprotein may work synergistically with P4 to upregulate $[\text{Ca}^{2+}]_i$ by way of CatSper channels in the sperm tail at the onset of capacitation which, in turn, leads to the downstream event of tyrosine phosphorylation of sperm proteins and an enhancement of sperm capacitation and fertilizing competence of sperm.
4.2 Limitations and Future Directions

This study had some limitations, including the small sample size, the variability among human sperm, and the limits of resources. The number of patients was limited due to availability of samples from FlowLabs Kingston as well as closure of FlowLabs Kingston during the COVID-19 pandemic. Particularly, our live cell imaging results showing that the use of rHuOVGP1 alone yielded a non-significant increase in the level of intracellular Ca\(^{2+}\) in the sperm tail (Figure 5) could be due to the small sample size. Future studies using a larger sample size will determine whether the use of rHuOVGP1 alone can significantly increase the level of intracellular Ca\(^{2+}\) in the sperm tail, especially taking into account the intra- and inter-variability that exist in concentrations, motility and vitality of human sperm (Álvarez et al., 2003).

In terms of the limit of resources, in order to further investigate whether OVGP1 induces its positive effects on intracellular Ca\(^{2+}\) in part through CatSper channels, we had initially planned to perform electrophysiological recording of CatSper currents. We attempted to record CatSper currents in the whole sperm configuration using the patch-clamp technique as previously described (Strünker et al., 2011; Lishko et al., 2013). Unfortunately, we were unable to replicate the technique as outlined by Strünker et al. (2011) and Lishko et al. (2013) due to several technical issues. 1) We were not able to visualize the cytoplasmic droplet (site of attachment) of the sperm cells due to lack of a proper objective lens; 2) Our attempts resulted in low success in formation of gigaohm seal, a seal between the cell and the micropipette that has an electrical resistance in the order of a gigaohm which makes it possible to isolate the currents measured across the cell; and 3) Despite successful formation of gigaohm seal, we were not able to successfully establish whole-cell by rupturing the sperm membrane, thus providing access to the intracellular space of the cell.
Specifically, we were unable to replicate the high voltage as outlined by Strünker et al. (2011) and Lishko et al. (2013) to rupture the human sperm plasma membrane. We did attempt to zap individual human sperm cells as outlined for rupturing the plasma membrane of mouse sperm, however, we were unable to transition to whole-cell without killing the sperm cell in the process (Lishko et al., 2013). In order to further examine if OVGP1 increases $[Ca^{2+}]_i$ in part through CatSper channels using electrophysiology, we may need to collaborate with an outside facility with the proper technical resources to perform the patch-clamp experiments in sperm cells.

It is well known that hyperactivated motility is an important feature of sperm capacitation whereby protein tyrosine phosphorylation regulates capacitation as well as hyperactivation (Urner and Sakkas, 2003). In the present study, we have shown the beneficial effect of rHuOVGP1 and P4 on sperm capacitation through their positive synergistic effect on tyrosine phosphorylation of a major sperm protein. Higher concentrations of P4 have previously been shown to induce hyperactivation in human sperm (Sagare-Patil et al., 2012). In the future, it will be of interest to examine if rHuOVGP1 alone and in combination with P4 can further stimulate sperm hyperactivation using a computer-aided sperm analyzer system that records different functional parameters of sperm motility.

According to the WHO laboratory manual for the examination and processing of human semen, the lower motility limit for normozoospermic samples is 40% (WHO, 2010). Due to the limited availability of patient samples in the present study, a small portion of experiments that we conducted involved the use of samples with motility lower than normal. We separated our live cell imaging results previously presented in Figure 5 into low motility ($\leq 40\%$ motility) and normal
motility (> 40% motility) groups. It appears that the influence of rHuOVGP1 on amplifying the positive effects of P4 on \( [\text{Ca}^{2+}]_i \) in human sperm tail is more pronounced in sperm with low motility compared to sperm with normal motility (Figure S1). It will be of great interest to examine the effects of rHuOVGP1 on \( [\text{Ca}^{2+}]_i \); specifically in sperm with low motility using a larger sample size as this could further validate the benefits of rHuOVGP1 for patients with mild male factor infertility. Since poor sperm motility can be a cause of male factor infertility, addition of rHuOVGP1 in the capacitating medium, along with P4 secreted by cumulus cells surrounding the oocyte, could enhance the sperm with low motility to move efficiently. This could lead to improved sperm-egg binding and higher fertilization rate in the conventional IVF procedure.

4.3 Conclusion

The present investigation aimed at unraveling the mechanism of human OVGP1 in enhancing sperm capacitation. The present research revealed that rHuOVGP1 increases \( [\text{Ca}^{2+}]_i \) in human sperm at the onset of capacitation. Furthermore, treatment of sperm with rHuOVGP1 further increases and sustains the level of \( [\text{Ca}^{2+}]_i \) in the sperm tail following the addition of P4. Inhibition of CatSper channels impedes the effects of rHuOVGP1 on \( [\text{Ca}^{2+}]_i \) in human sperm. This suggests that OVGP1 may in part influence \( \text{Ca}^{2+} \) influx through the CatSper channels, similar to the manner in which P4 regulates \( [\text{Ca}^{2+}]_i \). Lastly, we have demonstrated that P4 alone can increase tyrosine phosphorylation of a major human sperm protein, yet yield further increase when P4 and rHuOVGP1 are used in combination. The current findings are of significance to the field of infertility as a better understanding of the mechanism that regulates the function of OVGP1 could improve the success rates of conventional IVF procedures by supplementing the capacitating media currently used in fertility clinics with rHuOVGP1. Addition of rHuOVGP1 to the
capacitating media, accompanied by the secretion of P4 by cumulus cells encircling the oocyte, may be particularly valuable for treatment of male factor infertility. Improving fertility treatments for infertile couples remains a high priority in reproductive medicine.
Chapter 5
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Appendix A Supplemental Data
**Table S1.** rhOVGP1 increases [Ca\(^{2+}\)]\(_i\) in human sperm at the beginning of capacitation.

Table 1 shows the level of Fluo3 median intensity from the flow cytometry analysis of [Ca\(^{2+}\)]\(_i\) in sperm. Following 3 minutes (shown as -200 to -20 seconds) of baseline fluorescence reading, rhOVGP1 (0, 25, 50, 75, and 100 µg/mL) was added, respectively, to the cell suspension (time = 0 seconds), and the data were acquired for a further 10 minutes. The level of Fluo3 median intensity of each concentration of rhOVGP1 (0, 25, 50, 75, and 100 µg/mL) over time was compared to that prior to the addition of rhOVGP1 at -45 seconds. n = 7; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001.

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Table S2. P4 enhances the level of tyrosine phosphorylation of human sperm protein.

Table showing the effect of different P4 concentrations (0, $10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$, or $10^{-5}$ M) on the level of tyrosine phosphorylation of p105 from Western blot analysis following 0 to 4 hours of capacitation. The relative intensity of tyrosine phosphorylation of p105 for treatment with each concentration of P4 over time (1-, 2-, 3-, and 4-hour) compared to that of 0-hour capacitation. n = 6; * p<0.05, ** p<0.01, *** p<0.001.
**Figure S1.** Segregation of results for normal and low motility sperm treated with rHuOVGP1 followed by subsequent treatment with P4.

Line graph of the relative mean fluorescent intensity of Fluo3 from the live cell imaging analysis of $[Ca^{2+}]_i$ in sperm tail treated with or without rHuOVGP1 (100 μg/mL) followed by the treatment with P4 (1 μM). Data are represented as fold of change in fluorescent labeling +/- SEM; control group n = 6 (36 sperm cells in total); sperm with > 40% motility n = 4 (24 sperm cells in total); sperm with ≤ 40% motility n = 2 (12 sperm cells in total).
Appendix B Human Ethics Approval
QUEEN'S UNIVERSITY HEALTH SCIENCES & AFFILIATED TEACHING HOSPITALS RESEARCH ETHICS BOARD (HSREB)

HSREB Amendment Acknowledgement/Ethics Clearance

June 12, 2020

Dr. Frederick W. K. Kan
Department of Biomedical and Molecular Sciences
Queen's University

TRAQ #: 6012967
Department Code: DBMS-036-14
Study Title: "DBMS-036-14 The Role of Recombinant Human Oviductal Glycoprotein (rHuOVGP1) in Human Sperm Function" Review Type: Delegated
Date Ethics Clearance Issued: June 12, 2020

Dear Dr. Kan:

The Queen's University Health Sciences & Affiliated Teaching Hospitals Research Ethics Board (HSREB) has reviewed the amendment application and granted ethics approval/acknowledgement for the documents listed below:

<table>
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<th>Document Name</th>
<th>Comments</th>
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<tr>
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NOTE: In-person contact should be eliminated due to COVID-19. If you wish to continue data collection for your research that currently involves in-person contact, submit an amendment event form to outline methods for remote data collection, including all privacy and security considerations. There are human participant research policies, in relation to hospital and non-hospital based research, that are being continually updated. Many restrictions are now in place with respect to in-person research. For the most
current information on the COVID-19 impact on research, please visit https://www.queensu.ca/vpr/covid-19. For information directly related to GREB/HSREB please visit the Research Ethics FAQs.

Regards,

Albert F Clark, PhD
Chair, Queen’s University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board

The HSREB operates in compliance with, and is constituted in accordance with, the requirements of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the international Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Product Regulations; Part 3 of the Medical Devices Regulations, and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The HSREB is qualified through the CTO REB Qualification Program and is registered with the U.S. Department of Health and Human Services (DHHS) Office for Human Research Protection (OHRP). Federalwide Assurance Number: FWA#: 00004184, IRB#: 00001173. HSREB members involved in the research project do not participate in the review, discussion or decision.
Appendix C Consent Form
CONSENT FORM

DATE: Feb 1st, 2021

TITLE OF PROJECT: The Role of Recombinant Human Oviductal Glycoprotein (rHuOVGP1) in Human Sperm Function

BACKGROUND INFORMATION:

You are being invited to participate in a research study directed by Dr. Frederick W.K. Kan from Queen’s University to evaluate the usefulness of recombinant human oviductal glycoprotein (rHuOVGP1) to improve the sperm function in assisted fertilization procedures when compared to the standard procedure. Gwyneth Fairfield, the laboratory technologist at FlowLabs, will read through this consent form with you and describe procedures in detail and answer any questions you may have. This study is being sponsored by a research grant from the Canadian Institutes of Health Research (CIHR) awarded to Dr. Frederick W.K. Kan. This study has been reviewed for ethical compliance by the Queen’s University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board.

DETAILS OF THE STUDY:

Aim of the study:

Oviduct-specific glycoprotein (OVGP1), also named oviductin, is a major glycoprotein secreted by the secretory cells of the Fallopian tube (oviduct). Secretion of OVGP1 occurs in abundance during late follicular and peri-ovulatory phases. OVGP1 has been shown to be associated with ovulated oocytes, blastomeres, and acrosomal regions of mammalian sperm. Previous studies have shown that the presence of OVGP1 in the culture medium can benefit sperm motility and viability, enhance sperm capacitation, acrosome reaction, increase sperm-egg binding and cleavage rate of early embryos, and prevent polyspermy. The objective of the present investigation is to elucidate the mechanisms of recombinant human OVGP1 (rHuOVGP1) in enhancing human sperm capacitation.

Procedure:

Individuals who are sending their semen samples for semen analysis at FlowLabs will be approached for this research. After semen analysis steps are completed at FlowLabs, semen samples will be anonymized before sending to Dr. Frederick Kan’s research lab at Botterell Hall, Queen’s University. Semen samples with 3 - 5 days sexual abstinence will be collected for this study. No treatment will be performed on the volunteering patients. Semen samples will be obtained by masturbation, and samples will be collected into a sterile test tube with a wide opening.

Research Techniques:

The sperm samples will be washed and incubated with a capacitating medium in the presence or absence of rHuOVGP1 protein. Thereafter, sperm samples will be subjected to intracellular Calcium concentration analysis and proteomic analysis. The unused portion of sperm sample from
the donor will be discarded according to standard biological sample disposal procedure. None of the sperm donated for this research project will be used to produce an embryo, a baby or a pregnancy. The sperm sample that is used in this study will not be subjected to medical screening test for diseases or genetic disorders.

**Risks/Side-Effects:**

There are no foreseeable physical risks or side-effects in donating sperm for this research project. Should any unusual symptoms occur, please report immediately to Gwyneth Fairfield.

**Benefits:**

This research project is not intended to provide any direct medical benefit to you or anyone else. You would be donating your sperm solely for the advancement of this research project in general. While you may not benefit directly from this study, results from this study may improve the understanding of rHuOVGP1 in sperm function and may benefit patients undergoing assisted fertilization procedures in the future.

**Exclusions:**

If you have less than 3 days of sexual abstinence, you will not be considered for this study. Transportation of the semen sample should not exceed 1 hour from the time of ejaculation to the clinics. Patients should not consider participating in this study if transportation takes more than 1 hour.

**Confidentiality:**

All information obtained during the course of this study is strictly confidential and your anonymity will be protected at all times. Identification codes will be used instead of donors’ names, and all records will be kept in a private database. Data will be stored in locked files and will be available only to principal investigator and the Health Protection Branch in Canada. Local and other regulatory agencies, and project sponsors and funding agencies may review the research project records to ensure that your rights as a sperm donor were adequately protected. However, your identity will not be readily discoverable to these individuals. To protect your genetic and biological privacy, only your identification code, not your name, will be discoverable to the researches that perform sperm function analysis. Any report that the researchers publish will not include any information that will make it possible for readers to identify you as a sperm donor.

**Voluntary nature of study/Freedom to withdraw or participate:**

Your participation in this study is voluntary. You have alternative to choose not to participate in this study. You may withdraw from this study at any time before your sperm sample is used for this research project. You sample will be discarded according to standard biological sample disposal procedure. Your decision not to participate or withdraw will not affect your future medicalcare with your physician or at this hospital.
Liability:

In the event that you are injured as a result of the study procedures, medical care will be provided to you until resolution of the medical problem. By signing this consent form, you do not waive your legal rights nor release the investigator(s) and sponsors from their legal and professional responsibilities.

Payment:

We appreciate and value your participation in this research. However, no payment will be given for donating sperm sample.

PARTICIPANT STATEMENT AND SIGNATURE SECTION:

I have read and understand the consent form for this study. I have had the purposes, procedures and technical language of this study explained to me. I have been given sufficient time to consider the above information and to seek advice if I chose to do so. I have had the opportunity to ask questions which have been answered to my satisfaction. I am voluntarily signing this form. I will receive a copy of this consent form for my information.

If at any time I have further questions, problems or adverse events, I can contact Principal Investigator, Dr. Frederick W.K. Kan at (613)533-2863

or

Collaborators of this Study, Dr. Keith Jarvis at (613)417-0444

or

Department Head, Dr. Lynne Postovit at (613)533-2452

If I have questions regarding my rights as a research participant, I can contact Dr. Albert Clark, Chair, Queen’s University Health Sciences and Affiliated Teaching Hospitals Research Ethics Board at (613)533-6081.

By signing this consent form, I am indicating that I agree to participate in this study.

_____________________________ ________________________
Signature of Participant, Date

_____________________________ ________________________
Signature of Gwyneth Fairfield, Date
STATEMENT OF INVESTIGATOR:

I, or one of my colleagues, have carefully explained to the subject the nature of the above research study. I certify that, to the best of my knowledge, the subject understands clearly the nature of the study and demands, benefits, and risks involved to participants in this study.

_________________________  __________________
Signature of Principal Investigator Date