HAMSTER OVIDUCTIN ENHANCES TYROSINE PHOSPHORYLATION OF SPERM PROTEINS DURING CAPACITATION

by

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ABSTRACT

Capacitation is essential for fertilization of ovulated oocytes. Capacitation is correlated with activation of a signal transduction pathway leading to protein tyrosine phosphorylation, an essential prerequisite for fertilization. Oviductin has been shown to bind to the acrosomal cap and the equatorial segment region of the sperm head. In light of findings reported in previous studies, we hypothesized that estrus stage-specific oviductin (EOV) enhances tyrosine phosphorylation. Immunofluorescent detection by light and confocal microscopy and immunogold labeling by electron microscopy and surface replica techniques were used to localize tyrosine phosphorylated proteins to the equatorial segment region and midpiece after incubation in medium in the presence or absence of EOV. In the presence of EOV, an increase in tyrosine phosphorylation in the equatorial segment region was observed as early as 5 minutes after incubation. On prolonging incubation in medium containing EOV immunostaining further increased, indicative of increased levels of tyrosine phosphorylation of sperm proteins as capacitation proceeds. Regardless of the presence or absence of EOV, phosphotyrosine expression was observed along the tail, specifically at the midpiece. However, this reactivity was enhanced in the presence of EOV. Western blot analysis of NP-40 extractable and non-extractable sperm proteins confirmed these observations. NP-40 extractable sperm proteins (25, 37, 44kDa) and non-extractable sperm proteins (70, 83, 90kDa) showed increased intensity when sperm were capacitated in the presence of EOV after 5-, 60-, 120- and 180-minutes of capacitation. Mass spectrophotometric analysis identified enolase, ATP-specific succinyl CoA, succinate CoA ligase, zona pellucida binding protein, heat shock protein 90, aconitase and hexokinase as proteins that undergo
enhancement in tyrosine phosphorylation in the presence of EOV. The proteins identified are known to be involved in specific functions including cellular metabolism, molecular chaperoning and normal sperm development. In summary, the present investigation has provided new evidence showing that sperm capacitated in vitro in the presence of EOV display an enhanced expression of tyrosine phosphorylation compared to sperm incubated in capacitating medium alone. These results indicate that inclusion of oviductin in media used for in vitro fertilization (IVF) may improve success rates of IVF by enhancing the signaling pathways involved in sperm capacitation.
~ Dedication ~

I would like to dedicate this thesis
to my Mom and Dad.
I can never thank you
enough for your faith in me!
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# TABLE OF CONTENTS

| ABSTRACT ................................................................................................................ | ii |
| DEDICATION .............................................................................................................. | iv |
| ACKNOWLEDGEMENTS ................................................................................................. | v |
| LIST OF TABLES ....................................................................................................... | viii |
| LIST OF FIGURES AND ILLUSTRATIONS ..................................................................... | ix |
| LIST OF ABBREVIATIONS .......................................................................................... | xi |

## CHAPTER 1: INTRODUCTION
1. LITERATURE REVIEW ................................................................................................. 1
   1.1 Female Reproductive Tract .................................................................................. 1
   1.1.1 Anatomical Structures of the Oviduct ............................................................ 2
   1.1.2 Histological Structures of the Oviduct ......................................................... 3
   1.1.3 Oviductal Fluid .............................................................................................. 6
   1.2 General Overview of Oviductin .......................................................................... 7
      1.2.1 Localization of Oviductins and Their Possible Roles .................................. 9
      1.2.1.1 Immunolocalization of Oviductin in the Oviduct ................................... 9
      1.2.1.2 Immunolocalization of Oviductin in the Uterus .................................... 10
      1.2.1.3 Immunolocalization of Oviductin to Sperm ......................................... 11
      1.2.2 Molecular and Biochemical Characterization of Oviductins ...................... 13
   1.3 Mammalian Spermatozoa ................................................................................... 16
      1.3.1 Structural Features of Mature Mammalian Sperm ...................................... 17
      1.3.1.1 Head .................................................................................................... 18
      1.3.1.2 Tail .................................................................................................... 21
      1.3.2 Capacitation .............................................................................................. 23
      1.3.3 Tyrosine Phosphorylation of Sperm Proteins ............................................. 28
      1.3.4 Functional Roles and Localization of Tyrosine Phosphorylation of Sperm Proteins ................................................................. 30

## CHAPTER 2: MATERIALS AND METHODS
2. HYPOTHESIS ........................................................................................................... 34
3. OBJECTIVES .......................................................................................................... 34

| 2.1 Animals ............................................................................................................... 34 |
| 2.2 Purification of Oviductin .................................................................................... 34 |
| 2.3 Collection and Capacitation of Sperm ............................................................. 36 |
| 2.4 Western Blot Analysis of Oviductin Binding to Sperm and Loading Control ... 37 |
| 2.5 Localization of Tyrosine Phosphorylated Sperm Proteins Using Immunofluorescence and Confocal Microscopy ......................................................... 38 |
| 2.6 Preparation of Colloidal Gold and Protein A-Colloidal Gold Complex .......... 39 |
| 2.7 Ultrastructural Localization of Tyrosine Phosphorylated Sperm Proteins by Electron Microscopy ................................................................. 40 |
### CHAPTER 3: RESULTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Lectin-Affinity Purification and Immunodetection of Estrus Stage-Specific Oviducin</td>
<td>47</td>
</tr>
<tr>
<td>3.2 Western Blot Analysis of Binding of Oviducin to Sperm</td>
<td>48</td>
</tr>
<tr>
<td>3.3 Immunolocalization of Tyrosine Phosphorylated Sperm Proteins using Indirect Immunofluorescence</td>
<td>49</td>
</tr>
<tr>
<td>3.4 Immunolocalization of Tyrosine Phosphorylated Sperm Proteins using Confocal Microscopy</td>
<td>50</td>
</tr>
<tr>
<td>3.5 Ultrastructural Localization of Tyrosine Phosphorylated Sperm Proteins using Electron Microscopy</td>
<td>52</td>
</tr>
<tr>
<td>3.6 Localization of Tyrosine Phosphorylated Sperm Proteins by Surface Replica Technique</td>
<td>53</td>
</tr>
<tr>
<td>3.7 Comparison of Sperm Protein Extraction Techniques (1% NP-40 vs. Sonication)</td>
<td>54</td>
</tr>
<tr>
<td>3.8 SDS-PAGE and Western Blot Analysis of Tyrosine Phosphorylated Hamster Sperm Proteins</td>
<td>55</td>
</tr>
<tr>
<td>3.9 Density Analysis of Tyrosine Phosphorylated Hamster Sperm Proteins Enhanced in the Presence of EOV</td>
<td>57</td>
</tr>
<tr>
<td>3.10 Immunoprecipitation of Tyrosine Phosphorylated Sperm Proteins for Mass Spectrophotometry</td>
<td>58</td>
</tr>
</tbody>
</table>

### CHAPTER 4: DISCUSSION                                                                                                           | 88   |

### CONCLUSIONS AND CLOSING REMARKS                                                                                                  | 100  |
### REFERENCES                                                                                                                        | 101  |
### APPENDICES                                                                                                                          | 126  |
<table>
<thead>
<tr>
<th>TABLE</th>
<th>DESCRIPTION</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Molecular Masses analyzed and identified by search of NCBI database with MASCOT program for each SDS-PAGE band of interest</td>
<td>59</td>
</tr>
<tr>
<td>Table 2</td>
<td>Hamster sperm proteins identified by Mass Spectrophotometry</td>
<td>60</td>
</tr>
</tbody>
</table>
# List of Figures and Illustrations

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Description</th>
<th>Page #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Schematic representation of the internal organs of the human female reproductive system</td>
<td>1</td>
</tr>
<tr>
<td>Figure 2</td>
<td>Illustration of the female hamster reproductive tract</td>
<td>2</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Electron micrograph showing the ciliated and non-ciliated secretory cells of the female oviduct</td>
<td>5</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Structure of rodent sperm head</td>
<td>20</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Fine structures of the rodent sperm tail</td>
<td>22</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Sperm capacitation pathway</td>
<td>24</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Western blot analysis of HPA-purified oviductin from the estrus stage (EOV) of the estrous cycle</td>
<td>61</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Western blot analysis of EOV-sperm binding and loading control</td>
<td>62</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Immunostaining of tyrosine phosphorylated sperm proteins during capacitation in the presence or absence of 60µg/mL EOV using indirect immunofluorescent microscopy</td>
<td>63</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Confocal microscopy of immunostaining of tyrosine phosphorylated sperm proteins during capacitation in the presence or absence of 60µg/mL EOV</td>
<td>64</td>
</tr>
<tr>
<td>Figure 11</td>
<td>Confocal microscopic imaging of immunostaining of tyrosine phosphorylated sperm proteins of capacitated sperm permeabilized with Triton-X-100</td>
<td>65</td>
</tr>
<tr>
<td>Figure 12</td>
<td>Confocal microscopic imaging of immunostaining of protein tyrosine phosphorylation of sperm cells permeabilized with NP-40</td>
<td>66</td>
</tr>
<tr>
<td>Figure 13</td>
<td>Localization of tyrosine phosphorylated sperm proteins in the sperm head by electron microscopy</td>
<td>67</td>
</tr>
<tr>
<td>Figure 14</td>
<td>Ultrastructural localization of tyrosine phosphorylated sperm proteins in the midpiece by electron microscopy</td>
<td>68</td>
</tr>
<tr>
<td>Figure 15</td>
<td>Control sections showing the absence of immunolabeling in equatorial segment of the sperm head and midpiece</td>
<td>69</td>
</tr>
<tr>
<td>Figure 16</td>
<td>Surface replicas showing the equatorial segment region of the sperm head after 5 minutes of capacitation in the presence and absence of EOV</td>
<td>70</td>
</tr>
<tr>
<td>Figure 17</td>
<td>Surface replicas of the equatorial segment region of the sperm head after 180 minutes of capacitation in the presence and absence of EOV</td>
<td>71</td>
</tr>
<tr>
<td>Figure 18</td>
<td>Surface replica of control sample showing the sperm head</td>
<td>72</td>
</tr>
<tr>
<td>Figure 19</td>
<td>Surface replicas showing the distribution of immunogold labeling of tyrosine phosphorylated sperm proteins in the midpiece of the sperm tail</td>
<td>73</td>
</tr>
<tr>
<td>Figure 20</td>
<td>Control surface replica showing the midpiece of the sperm</td>
<td>74</td>
</tr>
<tr>
<td>Figure 21</td>
<td>Western blot comparing NP-40 and sonication non-extractable</td>
<td>75</td>
</tr>
</tbody>
</table>
tyrosine phosphorylated hamster sperm protein

**Figure 22** Western blot comparing NP-40 and sonication extractable tyrosine phosphorylated hamster sperm proteins

**Figure 23** Western blot analysis of tyrosine phosphorylated sperm proteins from whole sperm capacitated in non-supportive capacitation medium (NCM)

**Figure 24** Western blot analysis of the NP-40 non-extractable tyrosine phosphorylated sperm proteins

**Figure 25** Western blot analysis of the NP-40 extractable tyrosine phosphorylated sperm proteins

**Figure 26** Histogram showing the mean density (± SEM) of tyrosine phosphorylated insoluble sperm protein of molecular weight of approximately 90 kDa

**Figure 27** Histogram showing the mean density (± SEM) of tyrosine phosphorylated insoluble sperm protein of molecular weight of approximately 83 kDa

**Figure 28** Histogram showing the mean density (± SEM) of tyrosine phosphorylated insoluble sperm protein of molecular weight of approximately 70 kDa

**Figure 29** Histogram showing the mean density (± SEM) of tyrosine phosphorylated soluble sperm protein of molecular weight of approximately 25 kDa

**Figure 30** Histogram showing the mean density (± SEM) of tyrosine phosphorylated soluble sperm protein of molecular weight of approximately 37 kDa

**Figure 31** Histogram showing the mean density (± SEM) of tyrosine phosphorylated soluble sperm protein of molecular weight of approximately 44 kDa

**Figure 32** Immunoprecipitation of monoclonal anti-phosphotyrosine antibody, clone 4G10-reactive NP-40 non-extractable sperm proteins

**Figure 33** Immunoprecipitation of monoclonal anti-phosphotyrosine antibody, clone 4G10-reactive NP-40 extractable sperm proteins
LIST OF ABBREVIATIONS

AC- adenyl cyclase
Ac- acrosomal cap
AKAP- A kinase anchoring protein
AKAP3- A kinase anchoring protein 3
AR- acrosome reaction
ATP- adenosine triphosphate
BME- beta-mercaptoethanol
BSA- bovine serum albumin
Ca^{2+} - calcium
CABYR - calcium binding tyrosine phosphorylation regulator protein
cAMP- cyclic adenosine monophosphate
cDNA- complimentary deoxyribonucleic acid
CES- caudal epididymal sperm
ddH₂O- double distilled water
DLD- dihydrolipoamide dehydrogenase
D_{1}OV- oviductin purified from the diestrus stage of the estrous cycle
DNA- deoxyribonucleic acid
DTT- dithiothreitol
EGF- epidermal growth factor
EGFR- epidermal growth factor receptor
EOV- oviductin purified from the estrus stage of the estrous cycle
Eq- equatorial segment
ERE- estrogen response element
ERK- extracellular signal regulated kinase
FITC-GAM- fluorescein isothiocyanate goat-anti-mouse antibody
FS- fibrous sheath
FSP95- fibrous sheath protein
GalNAc- N-acetyl-D-galactosamine
GPX4- phospholipid hydroperoxide glutathione peroxidase
H$_2$O$_2$- hydrogen peroxide
HCO$_3$- bicarbonate
HPA- *Helix pomatia* agglutinin
HSP90- heat shock protein 90
IAM- inner acrosomal membrane
IgG- immunoglobulin G
kDa- kilodalton
LFMS- low fat milk solution
mAb- monoclonal antibody
mAb 4.12 F6- monoclonal anti-oviductin antibody
MALDI- Matrix assisted laser desorption ionization
MEK- mitogen-activated protein kinase
mRNA- messenger ribonucleic acid
MP- midpiece
ms- mitochondrial sheath
MS- mass spectrophotometry
NaCl- sodium chloride
NaHCO₃- sodium bicarbonate
NCM- non-supportive capacitation medium
NCBI- National Center for Biotechnology Information
NE- nuclear envelope
NH₄HCO₃- ammonium bicarbonate
NP-40- non-ionic detergent Nonidet P40
OsO₄- osmium tetroxide
OAM- outer acrosomal membrane
ODF- outer dense fibers
OF- oviductal fluid
OPAL- outer peri-acrosomal layer
OV- oviductin
p- value of probability
P₄- progesterone
Pa- postacrosomal sheath
PA-CG- protein A-colloidal gold complex
PDHB- pyruvate dehydrogenase
PEG- polyethylene glycol
pI- isoelectric point
PBS- phosphate buffered saline
PKA- protein kinase A
PM- plasma membrane
PMSF- phenylmethanesulphonyl fluoride

PP-principle peice

ProOV- oviductin purified from the proestrus stage of the estrous cycle

PT- perinuclear theca

PTK- protein tyrosine kinase

PVA- polyvinyl alcohol

PVDF- polyvinylidene fluoride

QqTOF- quadrupole time-of-flight

RAM-HRP- rabbit-anti-mouse immunoglobulin G conjugated to horseradish peroxidase

RER- rough endoplasmic reticulum

rhaOvm- polyclonal antibody against recombinant hamster oviductin

ROS- reactive oxygen species

rpm- revolutions per minute

SDS- sodium dodecyl sulfate

SDS-PAGE- sodium dodecyl sulfate polyacrylamide gel electrophoresis

Ser- serine

SEM- standard error of the mean

TALP- modified Tyrode’s salt solution

TBST- tris buffered saline + tween 20

Thr- threonine

TK- tyrosine kinase

Tyr- tyrosine

UTR- untranslated region
V- volts

ZP- zona pellucida
CHAPTER 1.0 INTRODUCTION

1.1 Female Reproductive Tract

The mammalian female reproductive system consists of ovaries, oviducts, uterus, cervix and vagina (Figure 1). During coitus, sperm are deposited into the vagina and make their way through the female reproductive tract to meet ovulated ova in the ampulla of the oviduct where fertilization occurs. Studies performed for the present research pertain to the hamster oviduct, its secretions and the interaction between oviductal secretions and sperm during transit though the female reproductive tract.

Figure 1: Schematic representation of the internal organs or the human reproductive system. Illustrated here are the vagina, cervix, uterus, oviduct and ovary and fimbria.
1. 1.1 Anatomical Structures of the Oviduct

The oviduct is a dynamic organ that provides a suitable environment for gamete function, fertilization and early embryo development. Among mammalian species the structure of the oviduct varies from a highly coiled structure, seen in the Golden hamster, to a straight, uncoiled structure as seen in humans. Figure 2 shows the four main regions of the hamster oviduct including the 1) infundibulum with fimbria, 2) ampulla, an enlarged portion, which is the site of fertilization, 3) isthmus and 4) uterotubal junction leading to the uterus (Moore and Agur, 2007).

Figure 2: An illustration of the female hamster reproductive tract. The isthmus and ampulla of the oviduct are tightly coiled in the hamster. The fimbria are contained within the ovarian bursa with the ovary. A small segment of the uterine horn is also shown.
1.1.2 Histological Structures of the Oviduct

Once functional, at the time of sexual maturity, the oviduct is an active, dynamic, secretory organ. The oviductal epithelium is simple columnar and consists of two main types of cells: 1) ciliated cells and 2) non-ciliated, secretory cells (Young and Heath, 2000). Ciliated cells have long cilia that extend into the lumen of the oviduct and beat in the direction of the uterus. The non-ciliated, secretory cells synthesize secretory granules in the apical part of the cells and secrete glycoproteins into the lumen of the oviduct (Figure 3). The glycoproteins become major constituents of the oviductal fluid (Oliphant, 1986). Near the nucleus of the oviductal epithelial cells is a well-developed Golgi apparatus and within the cytoplasm are secretory granules that contain macromolecules destined for the lumen of the oviduct (El-Mestrah and Kan et al., 1999).

A marked difference in the type and number of secretory granules within the various regions of the oviductal epithelium has been reported (Abe and Oikawa, 1991). These variations may possibly play a role in the important reproductive events occurring in different regions of the oviduct. For example, secretions in the ampullary region may contribute to sperm-egg interactions as it is the site of fertilization, whereas the secretions of the isthmic region may be involved in events related to sperm capacitation or the maintenance of early embryos.

Examination of oviductal epithelium revealed a difference in the level of ciliation during the stages of the estrous cycle. During the follicular phase the ampulla and fimbria are heavily ciliated, while during the luteal phase large numbers of cilia are hidden. In comparison, the cilia of the isthmus and uterotubal junction show insignificant variation throughout the estrous cycle (Abe and Oikawa, 1992).
In order to confirm the functional differences between the regions of the oviduct, Grippo et al. (1995) showed that sperm incubated in fluid collected from the ampullary region of the oviduct have more acrosome reactions compared to sperm incubated in fluid from the isthmic region. There has been evidence indicating a sperm reservoir in the isthmic portion of the oviduct near the uterotubal junction (Smith et al., 1987). Attachment of sperm to the isthmic epithelium seems to be a major factor responsible for the storage of viable sperm (Smith and Yanagamachi, 1991). Sperm bind via the acrosomal region to the ciliated cells and after detachment sperm cells maintain their motility and fertilizing ability. The sperm reservoir seems to play a role in coordinating the fertilizing capability of sperm in relation to ovulation in order to ensure fertile sperm are available in the ampulla at the time of ovulation (Wagner et al., 2002).
Figure 3: An electron micrograph showing ciliated (CC) and non-ciliated, secretory (SC) cells of the hamster oviduct. Ciliated cells contain a large round nucleus (Nu), mitochondria (m) and cilia (Ci) projecting into the oviductal lumen (Lu). The non-ciliated, secretory cells contain an irregularly shaped nucleus and many secretory granules (sg) in the apical region of the cells. Small microvilli (Mi) projecting into the lumen are also shown on the non-

1.1.3 Oviductal Fluid

Oviductal fluid (OF), produced and secreted by the oviduct, facilitates gamete transport, maturation, fertilization and early embryo development. Chemical analysis of oviductal fluid indicates a mixture of constituents from the plasma and proteins secreted by the oviductal epithelium (Leese, 1988). Water is the major contributor to oviductal fluid and it diffuses freely in response to osmotic gradients. Sodium and calcium are the main ions within OF and they contribute to the regulation of pH and osmolarity (Oliphant, 1986). Glucose, pyruvate, lactate and amino acids are non-electrolytes found in OF and these are important for gamete and embryo metabolism (Leese, 1988 and Leese et al., 2001). Macromolecules derived from blood make up 5-10% of the plasma protein content of OF. Albumin and immunoglobulin G are the most abundant proteins in OF. The oviduct also synthesizes macromolecules that are secreted into the oviductal lumen. A family of high-molecular-weight glycoproteins have been detected and identified within OF. Also known as oviductins, these oviduct-specific glycoproteins are synthesized and secreted into the lumen of the oviduct by the non-ciliated, secretory cells of the oviductal epithelium (For a review see Buhi, 2002).

Ehrenwald et al. (1990) reported that the concentration of high-density lipoproteins from human OF is elevated during the follicular phase of the menstrual cycle and that they can serve as acceptors for cholesterol. Cholesterol efflux is a major event during capacitation of sperm and occurs within the female reproductive tract. It has also been determined that incubation of sperm in conditioned media from oviductal explants
enhanced sperm capacitation and increased the incidence of acrosome reaction (Anderson and Killian, 1994 and Grippo et al., 1995). Ehrenwald et al. (1990) reported that OF protein concentrations are elevated as serum levels of progesterone (P₄) decrease around the time of estrus in the bovine. Collectively, these findings implicate OF in having an important role in initiating the capacitation process, which is required for sperm to reach its fertilizing capacity.

1.2 General Overview of Oviductin

Fox and Shivers (1975) were the first to report the association of a glycoprotein of oviductal origin, now known as oviductin, with the zona pellucida (ZP) of post-ovulatory oocytes. This glycoprotein is detectable in oviductal cells, suggesting that the latter is the site of oviductin biosynthesis (Léveillé et al., 1987). Immunocytochemical analysis of hamster ovarian and oviductal tissues revealed an intense labeling of oviductin that is restricted to the epithelial cells of the oviduct. Specifically, labeling was found to be associated with secretory granules and the Golgi apparatus of non-ciliated secretory cells (Kan et al., 1989, Araki et al., 1987). Since the discovery of this oviduct specific-glycoprotein, investigators have undertaken studies to examine the synthesis, localization and molecular and biochemical characteristics of oviductins. Thus far, these studies have given insight into the structure, properties and roles of mammalian oviductins in the female reproductive tract.

Oviductins have been detected in many mammalian species including the human (Rapisarda et al., 1993), non-human primate (Verhage et al., 1990), hamster (Kan et al., 1989), rat (Abe and Abe, 1993), cow (Boice et al., 1990), rabbit (Oliphant et al., 1984), sheep (Gandolfi et al., 1991) and pig (Buhi et al., 1993). Hamster oviductin belongs to a
A group of high-molecular-weight glycoproteins (Araki et al., 1987; Leveille et al., 1987; Robitaille et al., 1988) that are synthesized and secreted into the oviductal lumen by the non-ciliated secretory cells of the oviductal epithelium (Buhi, 2002). These oviduct-specific glycoproteins have been suggested to have biological functions during reproduction. Araki and colleagues (2003) were able to produce oviductin gene null mice in order to study the physiological significance of this glycoprotein. This group reported no statistical difference in the reproductive properties of oviductin gene null mice and wild-type mice. However, evidence showing sub-fertility (i.e. smaller litter size and a lowered ability of sperm to fertilize oviductin gene-null oocytes) was reported. Therefore, although oviductin is not a necessity for successful reproduction in vivo, its absence results in lowered reproductive proficiency.

A monoclonal antibody produced against hamster oviductin was found to react with group A red blood cells which led to the suggestion that the antigenic determinant of hamster oviductin may consist of N-acetyl-D-galactosamine (GalNAc) residues (Araki et al, 1987). Kan et al. (1990) also reported that oviductin contains terminal GalNAc residues on its sugar chains as it reacts with *Helix pomatia* agglutinin lectin (HPA). *Helix pomatia* agglutinin was shown to be specific for GalNAc residues (Hammerstrom and Kabat, 1971) and binds hamster oviductin through GalNAc residues of the oligosaccharide side chains attached to the polypeptide backbone.

Northern blot analysis has shown that mRNA transcripts for oviductin were found exclusively in the oviduct and not in any other tissues (Sendai et al., 1995; Suzuki et al., 1995). Previous results obtained with a monoclonal antibody against oviductin used in immunofluorescence studies indicated that there is a gradual increase in the production of oviductin as the animal becomes sexually mature (Malette et al., 1995a). Oviductin was
first detected in 7-day old female hamsters and by day 14 all of the oviductal secretory cells contained oviductin. The production of oviductin increased until day 28 at which time sexual maturity is reached in the Golden hamster (Malette et al., 1995a). Abe and Oikawa (1989) reported that the Golgi apparatus and rough endoplasmic reticulum (RER) in hamster oviductal epithelial cells were not well developed until day 5.5. But, by day 10, the Golgi apparatus, RER and immature secretory granules were all present. The appearance of oviductin seems to parallel the hormonal changes that occur during sexual maturity. Therefore, it has been suggested that the biosynthesis of oviductin is estrogen-dependent. However, studies carried out in our laboratory (McBride et al., 2004) showed that there is no significant difference in oviductin protein expression in the oviduct secretory cells throughout the five stages of the estrous cycle of mature female hamsters. As a result, it has been proposed that protein expression of oviductin is not under hormonal control but that any hormonally dependent modifications to hamster oviductin occurs post-translationally (McBride et al., 2004).

1.2.1 Localization of Oviductin and Its Possible Roles

1.2.1.1 Immunolocalization of Oviductin in the Oviduct

Immunocytochemical analysis indicated that oviductin (OV) is localized to the non-ciliated secretory cells in the oviductal epithelium of many mammalian species including the human (Rapisarda et al., 1993), non-human primate (Verhage et al, 1990), hamster (Kan et al., 1989), rat (Abe and Abe, 1993), cow (Boice et al., 1990), rabbit (Oliphant et al., 1984), sheep (Gandolfi et al., 1991) and pig (Buhi et al., 1993). Quantitative immunocytochemical studies of OV labeling in various species showed maximal
production of OV at the time of ovulation (i.e. estrus stage/follicular phase) (O’Day-Bowman et al, 1995, Abe et al., 1993). Due to this increase in OV production at the time of ovulation it has been suggested that this oviduct-specific glycoprotein may play multiple roles during the fertilization process.

In the hamster, once secreted into the oviduct, oviductin became associated with the ZP of ovulated oocytes and with early embryos (Kan et al, 1988, 1989, 1990; Bleau and St.Jacques, 1988). Oviductin has been shown to be associated with the ZP of oocytes in all species studied including the human with the exception of the mouse (Kapur and Johnson, 1986) and rat (unpublished results obtained in our laboratory) where it is seen in the perivitelline space. By use of immunocytochemistry, oviductin was found to be distributed uniformly throughout the thickness of the zona pellucida of post-ovulatory hamster oviductal oocytes (Kan et al, 1988) but not in ovarian oocytes (Araki et al., 1987). This finding suggested that oviductin may play a role in the reproductive events occurring in the oviduct after ovulation (i.e. sperm-egg binding). A study carried out by Kan and Roux (1995) detected oviductin over the plasma membrane and in the perivitelline space of developing young blastocysts. Their results showed that the blastomeres internalize the oviductin shedding from the ZP. However, the significance of internalization of oviductin by blastomeres of early embryos is not known.

1.2.1.2 Immunolocalization of Oviductin in the Uterus

Due to the direction of flow of oviductal fluid (OF) oviductin can gain access to the uterus. Using the silver enhancement immunogold staining technique, hamster OV showed homogeneous distribution over the uterine epithelium at all stages of the estrous cycle. The apical regions of the surface epithelial cells and glandular epithelial cells showed a high
intensity of immunostaining (Martoglio and Kan, 1996). A study carried out with high resolution immunocytochemistry at the electron microscopic level showed that OV binds to the cell surface of a certain population of uterine epithelial cells during the estrous cycle in the hamster (Roux et al., 1997). Immunocytochemical and biochemical analysis showed that hamster oviductin binds to a subpopulation of uterine epithelial cells where it is internalized and degraded by these cells (Roux et al., 1999). During early gestation, immunolabeling of oviductin over the cell surface of the uterine epithelium was found to decrease in intensity from day 1 to day 6. At day 4 of gestation the decrease in immunoreactivity was evident and by day 6, around the time of embryo implantation, the signal was almost undetectable. The disappearance of OV from the uterine epithelium at the time of implantation suggests a possible role for oviductin in regulating uterine receptivity (Roux et al., 1997).

1.2.1.3 Immunolocalization of Oviductin in Sperm

Binding of OV to sperm in vitro has been demonstrated by several research groups. Boatman and Magnoni (1995) have reported binding of hamster oviductin to the head region of homologous sperm. This OV-sperm binding showed regional and temporal modifications during sperm capacitation. Studies showed that oviductin binds to the anterior acrosomal region and the apical aspect of the post-acrosomal region of hamster sperm and the labeling intensity in these regions of the sperm head was found to increase in capacitated sperm (Kimura et al., 1994). A more detailed quantitative immunocytochemical study showed the binding of OV to the equatorial segment region of hamster sperm and this signal is also increased in capacitated sperm (Kan and Esperanzate, 2006). During sperm transit through the oviduct, the head of hamster sperm has been shown to attach to the
isthmus region (sperm reservoir) of the oviduct and only these attached sperm had the ability to fertilize (Kimura et al., 1994). Therefore, binding of OV to the acrosomal region of sperm is thought to be important in the sperm-oviduct interaction.

Abe et al. (1995) reported the observation of labeling of OV in the posterior region of the head and along the midpiece and tail of bovine sperm. In humans, oviduct-specific glycoprotein has been detected over the surface of the head region (Lippes and Wagh, 1989) but immunofluorescence results showed that when human sperm were capacitated in the presence of human OV this same region was devoid of immunoreaction (O’Day-Bowman, 1996).

A study carried out by Boatman and Magnoni (1995) showed that OV binding to the acrosomal region of hamster sperm decreases as the capacitation process proceeds whereas labeling of oviductin in the post-acrosomal region increases during capacitation. The anterior rim of the acrosomal region is the site of sperm-oviductal epithelium binding (Suarez, 1987). The equatorial segment has been implicated in sustaining the binding of sperm to the zona pellucida (Yanagamachi et al., 1981). In the hamster, the increase in OV-sperm binding and relocation of OV binding from the anterior acrosomal region to the equatorial segment as the capacitation process proceeds indicates that OV is involved during the membrane changes that take place in the sperm head prior to fertilization.

Studies of bovine sperm carried out in vivo showed that OV increases the number of acrosome reacted sperm (Abe et al., 1995). Since acrosome reaction did not occur in vivo unless sperm were fully capacitated, it has been suggested that OV can facilitate capacitation (King et al., 1994). The same study also reported that sperm capacitated in the presence of OV increased the ability of sperm to penetrate ova and increased the fertilizing ability of sperm. Oviductin sustained sperm viability (Satoh, 1995; Abe et al., 1995) and
this action was dose-dependent (Abe et al., 1995). It has been suggested that sperm viability is sustained by OV due to its physiochemical properties related to negative charges of sialic acid moieties in the OV molecule (Satoh, 1995).

The biological significance of the binding of oviductin to sperm remains to be explored. However, a role played by oviductin in sperm-egg binding, sperm-oviduct interaction, egg-activation signaling, prevention of polyspermy and cortical granule release have been suggested (King et al., 1994, Kouba, 2000).

1.2.2 Molecular and Biochemical Characterization of Oviductin

Oviductin cDNA has been cloned in several mammalian species including the baboon (Donnelly et al., 1991; Jaffe et al., 1996), bovine (Sendai et al., 1994), human (Arias et al., 1994), sheep (DeSouza and Murray, 1995), mouse (Sendai et al., 1994), pig (Buhi et al., 1996), monkey (Verhage et al., 1997) and hamster (Suzuki et al., 1995; Paquette et al., 1995). There is prominent conservation within the N-terminal amino acid sequences of the mammalian oviductin cDNA (Jaffe et al., 1996; Sendai et al., 1994; Arias et al., 1994; DeSouza and Murray, 1995; Sendai et al., 1994; Buhi et al., 1996; Verhage et al., 1997; Suzuki et al., 1995; Paquette et al., 1995). Within the C-terminal region there is sequence divergence and low identity reported among species (Takahashi et al., 2000; Verhage et al., 1997). The coding region of the oviductin gene in the hamster is distributed across 11 exons and 10 introns (Merlen and Bleau, 2000). This is also true for the mouse and human (Takahashi et al., 2000; Agarwal et al., 2002). The coding of the oviductin gene also showed a very short 5’-untranslated region (UTR) and the identical imperfect estrogen response element (ERE) in these three species (Merlen and Bleau, 2000). The promoter region of the human oviductin gene has a putative TATA box (Agarwal et al., 2002).
whereas the hamster (Merlen and Bleau, 2000) and mouse (Takahashi et al., 2000) lack consensus TATA boxes, properly located CAAT boxes and GC-rich regions. A study carried out by Miyoshi et al. (2002) reported that the imperfect ERE of the mouse remains functional and since the hamster oviductin gene contains the identical imperfect ERE, it is assumed that the hamster oviductin ERE is also functional.

The amino acid and full length nucleotide sequences of hamster oviductin were reported independently by Suzuki et al. (U15048; 1995) and Paquette et al. (D32218; 1995). The amino acid sequence of human (GenBank U09550) and bovine (GenBank D16639) oviductin shares 71% and 68% identity, respectively, with the amino acid sequence of hamster oviductin (Jaffé et al., 1996). The baboon partial C-terminal sequence shares 58% identity with the hamster oviductin amino acid sequence (Jaffé et al., 1996). The hamster oviductin cDNA sequence codes for 635 amino acids and contains six identical repeats of 45 nucleotides, coding for six repeats of 15 amino acids containing Ser or Thr (potential acceptor sites for O-glycans) with one N-glycosylation consensus sequence in five of these six repeats (Paquette et al., 1995). Oviductin is an N- and O-glycosylated glycoprotein that appears to undergo cyclic variation of glycosylation during the estrous cycle with maximal glycosylation occurring at the time of estrus (McBride et al., 2004). The biosynthesis of O-glycans has been studied and O-glycan biosynthetic pathways have been proposed. The polypeptide α-GalNAc-transferase is the enzyme responsible for initiation of the O-glycosylation pathway and the addition of GalNAc to serine or threonine residues of the polypeptide backbone. This enzyme activity in cooperation with various other glycosyltransferases form several core structures. The collective enzyme activities have shown a distinct pattern throughout the stages of the
estrous cycle with the peak occurring immediately before and at the onset of ovulation, after which the levels decrease (McBride et al., 2004).

The region of tandem repeats of oviductin is very divergent among various mammalian species whereas the remainder of the molecule is highly conserved (Lapensee et al., 1997). In human (Arias et al., 1994), baboon (Jaffe et al., 1997) and rhesus monkey (Verhage et al., 1997) the C-terminal region contains four tandem repeats whereas the hamster contains six tandem repeats (Paquette et al., 1995). The bovine (Sendai et al., 1994), sheep (DeSouza and Murray, 1995) and pig (Buhi et al., 1996) contain incomplete or no tandem repeat sequences. In the mouse C-terminal region, there were 21 tandem repeats and each consisted of seven amino acid residues (Sendai et al., 1995).

Variability exists in the native molecular weight of mature oviductin (90-95 kDa in domestic animals; 120 kDa in primates; 160-350 kDa in rodents) but the core protein size of several species studied to date is approximately the same (~70kDa). The variability in molecular weights is attributed to differences in glycosylation patterns (Schmidt et al., 1997; Roux et al. (1997). The molecular mass of the core protein and isoelectric point of hamster oviductin have been determined to be 70.89kDa and 6.15, respectively (Roux et al., 1997). Oviductins purified from the Golden hamster consist of high molecular weight, heterogeneous glycoproteins that have a molecular mass of 160-350kDa (Paquette et al., 1995; St-Jacques and Bleau, 1988; Robitaille et al., 1988).

Studies performed using a polyclonal antibody against recombinant hamster oviductin (rhaOvm) have indicated that hamster oviductin mRNA and protein expression is not hormonally controlled, however the glycosylation of oviductin seems to be differentially regulated throughout the estrous cycle. Therefore, the post-translational
glycosylation of hamster oviductin appears to be under hormonal control (McBride et al., 2004).

As mentioned earlier, the amino acid sequence of hamster oviductin has been deduced. The heavily glycosylated protein presented characteristics, including serine or threonine rich tandem repeats, similar to those of typical mucins (Paquette et al., 1995). Because of the biochemical and genetic properties of oviductin it is considered to be a mucin-like glycoprotein.

1.3 Mammalian Sperm

During coitus millions of mature sperm cells are deposited into the female reproductive tract. However, only a fraction of these cells manage to find their way through the obstructions of the female reproductive tract to reach the ampulla of the oviduct for fertilization. Both physical and chemical barriers hinder the transit of the sperm cells allowing only the most viable sperm to reach the ovulated ova for fertilization (Eddy et al., 1994).

Sperm cells develop in the testes from germ cells by a process termed spermatogenesis. The newly formed cells mature in the cauda epididymis prior to deposition into the female reproductive tract (Lui et al., 2003). Morphological and physiological changes occur during sperm maturation and these changes result in the production of highly compartmentalized, polarized individual cells. The compartmentalization and polarization of cellular components, resulting in the formation of the acrosome and tail, allow the sperm cells to reach the site of fertilization, interact with ovulated ova and subsequently complete the fertilization process (Travis et al., 2001).
1.3.1 Structural Features of Mature Mammalian Sperm

Mammalian sperm are highly compartmentalized cells containing very little cytoplasm and only the necessary organelles required to perform specific functions. Surrounding the sperm cell is the dynamic plasma membrane (PM) which is a continuous structure covering the two main compartments of the cell: the head and the tail. During the development of sperm cells in the testes, the plasma membrane and other cell organelles are arranged and organized to prepare the sperm for subsequent interaction with the oocyte. During epididymal transit the plasma membrane of mammalian sperm undergoes a series of modifications by the adsorption and release of proteins and lipids (Eddy et al., 1994). The plasma membrane overlying the sperm head is separated from the plasma membrane overlying the midpiece by the neck and the midpiece plasma membrane is separated from the principal piece plasma membrane by the annulus (Flesch and Gadella, 2000). In the head region the plasma membrane overlying the acrosome is separated from the post-acrosomal membrane by the equatorial segment (Flesch and Gadella, 2000). The equatorial segment region is a membrane domain of the sperm head involved in adhesion and fusion of the male and female gametes (Gadella, 2008). Studies using freeze fracture techniques indicate that the plasma membrane domains of mammalian sperm contain different concentrations and distributions of intramembranous particles representing transmembrane proteins (Eddy et al., 1994). The biological significance of modifications of the sperm cell surface during sperm capacitation is not fully understood, however certain proteins adsorbed onto the sperm cell surface during capacitation are believed to be involved in sperm-egg binding (Keisuke and Kudo, 2004).
1.3.1.1 Head

The head of rodent sperm has a hooked appearance and is enclosed by the plasma membrane. The components of the sperm head are shown in Figures 4 A and B. The head of the sperm consists of the acrosome and highly condensed haploid nucleus. The nucleus is surrounded by a nuclear envelope in which the nuclear pore complexes become less in number during spermiogenesis (Sutovsky and Manandhar, 2006). Protecting the nucleus is the perinuclear theca. It forms a rigid shell around the nucleus and is composed of disulfide bond-stabilized structural proteins (Oko, 1995). Some of the perinuclear theca proteins function in cell signaling once released into the oocyte cytoplasm at the time of fertilization (Oko and Maravei, 1994). This cytoskeletal element is divided in two regions, the subacrosomal layer (also known as the perforatorium in murids) which underlies the acrosome, and the postacrosomal sheath, which extends towards the tail beyond the acrosomic system and directly underlies the plasma membrane. The subacrosomal layer is located between the inner acrosomal membrane (IAM) and the outer nuclear membrane. The postacrosomal sheath is located in the distal region of the head between the plasma membrane and the outer nuclear membrane. It is continuous with a layer of material referred to as the outer periacrosomal layer (OPAL), which is located between the plasma membrane and the outer acrosomal membrane and is compositionally similar with the perinuclear theca (Oko and Maravei, 1994).

The acrosome is derived from the Golgi apparatus during spermiogenesis and contains enzymes that digest proteins and complex sugars (Tusiani et al., 1994). The acrosome forms a cap in the anterior region of the head. The acrosome contains proteases and receptors required for sperm interaction with the zona pellucida of the oocyte (Tulsiani et al., 2004). The acrosome is divided into two regions, the anteriorly and posteriorly
located regions. The posteriorly located region, including the equatorial segment, is reportedly more stable and stays intact during the initial stages of sperm-egg binding (Sutovsky and Manandhar, 2006). Upon binding with the oocyte the plasma membrane and outer acrosomal membrane (OAM) become vesiculated allowing the enzymes stored in the acrosome to be released and lyse the outer coverings of the egg (Gerton, 2002). The inner acrosomal membrane (IAM) remains and fuses with the PT after primary sperm-egg binding. It is thought that the fusion of the PT and IAM stabilizes receptor molecules responsible for secondary binding of the sperm head to the egg zona pellucida after acrosomal exocytosis (For reviews see Gerton, 2002 and Yoshinaga and Toshimori, 2003).
Figure 4: Structural organization of the hamster sperm head. These illustrations depict the structures of the sperm head with a cross section (top image) including the overlying plasma membrane, nucleus (n), equatorial segment, acrosome, postacrosomal sheath and subacrosomal layer. Also shown here is a sagittal section (bottom image) of the sperm head displaying its structures. The plasma membrane is closely associated with the outer acrosomal membrane forming an area containing the acrosome at the apical region, or acrosomal region of the sperm head. The acrosome is situated between the outer acrosomal membrane and the inner acrosomal membrane. The space between the inner acrosomal membrane and nuclear envelope is the subacrosomal layer which spans the acrosomal region and equatorial segment. The post-acrosomal sheath is situated between the plasma membrane and the nuclear envelope. The space between the inner acrosomal membrane and nuclear envelope (ne), spanning the acrosome, equatorial segment and postacrosomal sheath, contains the perinuclear theca.
1.3.1.2 Tail

The sperm tail has the function of propelling the cell through the female reproductive tract to meet with the ovulated ova. The tail is separated into three main segments which all contain the common innermost structure of the microtubule-based axoneme and differ in external structures. The segments are referred to as 1) the midpiece, 2) principal piece and 3) end piece.

The midpiece of mammalian sperm contains mitochondria (Figures 5A and 5C) that are coiled helically around the axoneme but retain their individuality (Fawcett, 1975). The midpiece is protected by the mitochondrial sheath. In the midpiece, the axoneme is paralleled by 9 outer dense fibers (Fawcett, 1975). At the distal end of the midpiece is the annulus. This dense circular structure is firmly attached to the plasma membrane, separating the midpiece from the principal piece which consists of a series of circumferentially oriented ribs that extend half way around the tail and terminate in two longitudinal columns which run along the dorsal and ventral aspects of the sheath for its entire length (Fawcett, 1975). In the principal piece outer dense fibers 3 and 8, terminating near the end of the midpiece, are occupied by the longitudinal columns of the fibrous sheath (FS) that attach to appendages on doublets 3 and 8. The principal piece is protected by the FS. The FS provides support for the axoneme and contains proteins that seem to hold protein kinases required for sperm capacitation and hyperactivation (Eddy et al., 2003). The end piece contains axonemal doublets and the terminal ends of the FS and ODF.
Figure 5: Fine structures of the hamster sperm tail. A) Longitudinal section of the hamster sperm tail midpiece containing mitochondria (mi) and outer dense fibers (odf). Also shown is the sperm head containing the nucleus (n). X 9,000. B) Longitudinal section of the hamster sperm tail principal piece showing the plasma membrane (pm) and odf. X 12,000. C) Cross section of the hamster sperm tail midpiece displaying mi, odf, and the axoneme consisting of the central pair of microtubules (cp) surrounded by microtubule doublets (pd). X 12,000.
1.3.2 Capacitation

Upon deposition into the female reproductive tract mature sperm do not possess the ability to fertilize an oocyte. More than 50 years ago studies confirmed that exposure of mature sperm to the oviductal environment brought about the changes necessary for sperm to fertilize an ovulated oocyte. The process of sperm undergoing these cellular changes is termed capacitation (Austin, 1951; Chang, 1951). Capacitated sperm display a change in flagellar beat, which is thought to aid in sperm transport as well as penetration of the egg vestments. This pattern of motion is referred to as hyperactivated motility (Yanagimachi, 1981). This hyperactivity aids sperm in its progression through the viscous environment of the female reproductive tract (Luconi et al, 2006). The penetration of the thick oviductal mucus, cumulus oophorus and the zona pellucida also requires that sperm be hyperactivated so it can undergo the acrosome reaction and eventually fuse with the oocyte (Suarez and Pacey, 2005). Capacitation is a prerequisite for the acrosome reaction. Capacitation occurs over several hours in the Golden hamster and is reversible whereas the acrosome reaction is fast and irreversible (de Lamirande et al., 1997). The capacitation pathway is shown in Figure 6.
Figure 6: Sperm capacitation pathway. In vitro, bovine serum albumin (BSA) sequesters cholesterol (CHOL) resulting in a CHOL efflux from the plasma membrane (PM). This causes increased membrane fluidity and destabilization. The increase in plasma membrane fluidity allows for an influx of Ca\(^{2+}\) and HCO\(_3^-\) which act to activate adenylate cyclase (AC) within the PM. The activation of AC stimulates the production of cAMP which in turn initiates the activities of protein kinase A (PKA). It is uncertain whether PKA acts to stimulate or suppress the actions of proteins tyrosine kinase (PTK) and protein tyrosine phosphatase (Ptyr-Ptase) in order to phosphorylate or dephosphorylate tyrosine residues of sperm proteins, respectively. PKA may also play a direct role in the capacitated state and the hyperactivation of sperm.
In vitro capacitation is achieved by incubation of mature sperm cells in a medium mimicking the oviductal milieu. It is through in vitro conditions that the majority of the information we know about the capacitation process is derived. A consistently successful method for inducing sperm capacitation in vitro was reported by Bavister (1989). The report described the composition of the constituents required for a capacitating medium, a balanced salt solution, with the inclusion of bovine serum albumin (BSA), calcium (Ca\textsuperscript{2+}) and sodium bicarbonate (HCO\textsubscript{3}\textsuperscript{-}). Studies carried out in various mammalian species suggest that the presence of BSA, Ca\textsuperscript{2+} and HCO\textsubscript{3}\textsuperscript{-} is required for capacitation (see Visconti et al., 2002 for a review). Although the precise role of these constituents is still under investigation, it is now known that the absence of any one prevents tyrosine phosphorylation and capacitation from occurring (Visconti and Kopf, 1998). Motility factors including catecholamine ((-) epinephrine) and a β-amino acid (taurine or hypotaurine) are also required by mature hamster sperm in order to attain a fully capacitated status in vitro (Leibfried and Bavister, 1982). Capacitation is correlated with hyperpolarization of the sperm plasma membrane, which could be due to the opening of potassium channels that are sensitive to alkalinization of the cytosol (Visconti and Kopf, 1998). It has also been suggested that voltage-gated Ca\textsuperscript{2+} channels in the sperm plasma membrane will open and induce the Ca\textsuperscript{2+} influx as a consequence of membrane hyperpolarization (Flesch and Gadella, 2000).

Serum albumin is thought to sequester cholesterol from the plasma membrane (Cross, 1996) resulting in increased membrane fluidity and allowance of an efflux of cholesterol. This action is a known requirement of capacitation (Visconti et al., 1999).

Evidence for the role of Ca\textsuperscript{2+} in the biochemical events leading to capacitation has been accumulating over the years (for review see Naz and Rajesh, 2004; and Dubé et al.,
The capacitation process involves an intracellular increase in Ca\(^{2+}\). *In vitro*, Ca\(^{2+}\) has been shown to have both positive and negative effect on sperm capacitation. Visconti et al. (2002) and Naz and Rajesh (2004) have demonstrated that increasing amounts of extracellular Ca\(^{2+}\) can enhance capacitation-associated tyrosine phosphorylation in the mouse and human, respectively. However, other authors have described an opposite effect indicating that Ca\(^{2+}\) has a negative effect on tyrosine phosphorylation (Carrera et al., 1996). Although the effect of Ca\(^{2+}\) is controversial, the increase in intracellular Ca\(^{2+}\) concentration among several mammalian species indicates an important role of this divalent cation during sperm capacitation.

The requirement of HCO\(_3^-\) during capacitation has been documented, although the mechanism responsible for regulating capacitation is unclear (Naz and Rajesh, 2004). The intracellular rise in the pH of sperm is attributed to HCO\(_3^-\) but the role of pH is unclear as increasing pH does not induce capacitation (Naz and Rajesh, 2004). The presence of bicarbonate in the capacitating medium not only raised intracellular pH but also stimulated respiratory activity, regulated the opening of voltage-gated Ca\(^{2+}\) channels and also directly activated sperm AC (Jaiswal and Conti, 2001). Inhibitors of anion transporters inhibited the action of HCO\(_3^-\) on sperm functions such as capacitation and AR. These results imply the presence of functional anion transporters in sperm (Visconti et al., 1990).

The increase in Ca\(^{2+}\) and intracellular HCO\(_3^-\) has been suggested to be involved in activating adenyl cyclase (AC) (Okamura et al., 1985) and altering hydrogen peroxide (H\(_2\)O\(_2\)) concentrations. These cellular changes collectively activate AC and this activation results in increased production of cyclic AMP (cAMP) which activates protein kinase A (PKA) and controls its activity. Protein kinase A is responsible for the phosphorylation of certain sperm proteins (for a review see Breitbart and Naor, 1999).
Although phosphorylation of Ser/Thr residues is common in eukaryotic cells, researchers have found that phosphorylation of tyrosine residues, a rarer occurrence, on sperm proteins during capacitation is a time-dependent event and is a crucial step in order for sperm to achieve full capacitation (Carrera et al., 1996, Urner and Sakkas, 2003).

Capacitation is a complex process and the mechanisms controlling this process remain elusive. In a review by De Lamirande and O’Flaherty (2007), reactive oxygen species (ROS) have been implicated in playing a role in the capacitation process. During capacitation sperm generate low and controlled levels of ROS. These ROS aid in regulating many other complex events leading to final protein tyrosine phosphorylation. These events include an increase in cAMP, PKA activation, phosphorylation of PKA substrates and the phosphorylation of proteins on serine (Ser) and threonine (Thr) residues. In addition, ROS are involved in the phosphorylation of MEK (extracellular signal regulated kinase[ERK] kinase)-like proteins and the threonine-glutamate tyrosine motif and finally the tyrosine phosphorylation of sperm proteins (O’Flaherty et al., 2006). It is unclear whether a specific ligand is responsible for induction of the transduction cascade leading to tyrosine phosphorylation. However, one possible ligand is epidermal growth factor (EGF). EGF interacts with its tyrosine kinase receptor (EGFR) and has been identified on the head of bovine sperm (Breitbart, 2003). Although there is much information available on the pathways mediating the capacitation process, the present investigation focuses mainly on tyrosine phosphorylation of sperm proteins. Therefore, the present LITERATURE REVIEW will be focused on the general pathway leading to this event as shown in Figure 6.
1.3.3 Tyrosine Phosphorylation of Sperm Proteins

Sperm are highly compartmentalized cells, transcriptionally inactive and cannot synthesize new proteins. Therefore, mature sperm undergo modifications including protein phosphorylation in order to attain full function. Urner and Sakkas (2007) define protein phosphorylation as a post-translational modification of proteins that allows the cell to control various cellular processes and this modification is controlled by the activity of protein kinases and phosphatases. Phosphorylation of sperm proteins was first reported by Leyton and Saling (1989). Several phosphorylated proteins with molecular mass of 52, 75 and 95kDa have been identified by this group. A study by Komatsu et al. (1990) reported that as capacitation proceeds there is a change in the phosphorylated proteins with an increase in cAMP. Visconti et al. (1995b) used cAMP analogues to analyze the effect of cAMP on protein tyrosine phosphorylation. Capacitation and the increase in tyrosine phosphorylation was not observed when the latter components were absent from the incubation medium (Visconti et al, 1995A). However, this was restored by the addition of biologically active cAMP analogues. These findings showed that cAMP is required for protein tyrosine phosphorylation to occur in vitro. The main intracellular target for cAMP is protein kinase A (PKA). Caudal epididymal sperm cells incubated with inhibitors of PKA showed reduced ability to fertilize an egg. This indicated that PKA activation by cAMP may be a regulatory pathway responsible for controlling protein tyrosine phosphorylation and capacitation. It has been demonstrated that the capacitation-associated increase in tyrosine phosphorylation is downstream of a cAMP/PKA pathway in several mammalian species (Visconti et al, 1995b; Galantino-Homer et al., 1997; Kalab et al., 1998; Leclerc et al., 1996).
Post-translational protein modifications through serine/threonine and tyrosine phosphorylation play a role in many cellular processes, as mentioned above. PKA is a protein serine/threonine kinase that is unable to directly phosphorylate tyrosine residues but is often required for the activation of tyrosine kinases (TK). This means that tyrosine kinases must be involved in capacitation and suggests that a possible mechanism of interaction between PKA and protein tyrosine phosphorylation exists. The capacitation pathway seems to be tightly regulated by the constituents (calcium, bicarbonate and serum albumin) of the capacitating medium in vitro. In knockout mice lacking the testis-specific PKA catalytic subunit, sperm were not able to display active motility or display an increase in tyrosine phosphorylation. These results confirmed that the capacitation-associated increase in tyrosine phosphorylation is tightly controlled by the up-regulation of cAMP by components in the medium (Nolan et al., 2004).

There is a close association between protein tyrosine phosphorylation and capacitation in various mammalian species examined to date including the mouse (Visconti et al, 1995a, 1995b), boar (Bailey et al, 2005), bovine (Gualtieri et al, 2005), hamster (Kulanand and Shivaji, 2001), dog (Petrunkina et al, 2003), and human (Naz et al, 1991, Baldi et al, 1996). Immunofluorescence of tyrosine-phosphorylated proteins has shown that capacitation as well as exposure of sperm to zona pellucida proteins (i.e. ZP3) had an enhancing effect on the intensity of immunostaining observed on sperm cells and on the number of individual cells displaying immunostaining (Naz et al., 1991). As the capacitation process proceeds, there was a time-dependent increase in protein tyrosine phosphorylation in all the aforementioned species. Some tyrosine phosphorylated sperm proteins have been identified such as the fibrous sheath protein (AKAP3) of 95kDa, calcium binding tyrosine phosphorylation regulator protein (CABYR), E1β subunit of
pyruvate dehydrogenase (PDHB), A kinase anchoring proteins (AKAPs), dihydrolipoamide dehydrogenase (DLD), phospholipid hydroperoxide glutathione peroxidase (GPX4) and heat shock protein (HSP90) of 90kDa (Reviewed by Kumar et al., 2006). Ultimately, the regulation of events associated with the capacitation pathway such as membrane initiation and regulation of flagellar motility may be dependent on tyrosine phosphorylation.

1.3.4 Functional Roles and Localization of Tyrosine Phosphorylated Sperm Proteins

Phosphorylation of tyrosine residues results in conformational changes in the protein structure. Many receptors and enzymes are switched “on” and “off” by phosphorylation and dephosphorylation. Switching from a phosphorylated state to a dephosphorylated state (or vice versa) results in conformational changes in the structure of enzymes and receptors causing activation or deactivation. The addition of a phosphate group to a polar R group on an amino acid can turn a hydrophobic portion of a protein into a polar and highly hydrophilic portion of the molecule. In this way, it can introduce a conformational change in the structure of the protein via interaction with other hydrophobic and hydrophilic amino acid residues in the protein (Cozzen, 1988). It is well documented that phosphorylation of tyrosine residues is involved in a variety of cellular processes such as growth regulation, cell cycle control, cytoskeleton assembly, ionic current regulation, and receptor regulation (Clark et al., 1994; Hunter, 1996).

Evidence indicating that initiation and regulation of flagellar motility involved protein phosphorylation and the presence of kinases and phosphatases in the axoneme suggests a possible involvement of protein phosphorylation in the regulation of sperm motility (Porter and Sale, 2000). In humans, the motility of ejaculated sperm is improved by the addition of phosphatase inhibitors (Leclerc et al, 1996) and similar compounds initiate
motility in epididymal bovine sperm suggesting that protein kinase and protein phosphatase activities may control motility in these cells (Huang et al., 2004).

As mentioned earlier, sperm cells are transcriptionally inactive, highly compartmentalized and unable to synthesize new proteins. Because of these specializations, post-translational modifications such as protein phosphorylation are important in regulating the function of sperm cells. A better knowledge of the localization, distribution and identification of tyrosine phosphorylated sperm proteins is necessary for understanding the relationship between the regulation of phosphorylated protein regulation and sperm function. Immunohistochemical localization studies have been performed on several mammalian species including the hamster (Kulanand and Shivaji, 2002; NagDas et al., 2005), human (Ficarro et al., 2003; Moseley et al., 2005; Liu et al., 2006), pig (Tardif et al, 2001), bovine (Gualtieri et al., 2005), monkey (Mahony and Gwathmey, 1999), mouse (Urner et al., 2001), rat (Lewis and Atken, 2001) and dog (Petrunkina et al., 2003). In the above studies, using a monoclonal anti-phosphotyrosine antibody, these researchers have reported labeling of phosphorylated proteins along the sperm flagellum during capacitation.

In a review by Urner and Sakkas (2003), tyrosine phosphorylation of sperm proteins in the principal piece during capacitation has been reported in human, monkey, hamster, rat and mouse, but not the boar (Tardif et al., 2001). A time-dependent increase in tyrosine phosphorylation of flagellar proteins was found to occur during capacitation. Urner et al. (2001) reported that mouse sperm bound to the ZP displayed a tyrosine phosphorylated principal piece (PP) and midpiece (MP). Sperm bound to the oolemma of oocytes displayed tyrosine phosphorylation in the pp and mp until fusion between the sperm and the oocyte occurred. This pattern of phosphorylation suggests that phosphorylation of sperm proteins
is a critical event in sperm-egg binding and possibly in sperm penetration of the egg
vestments.

An enhancement of tyrosine phosphorylation in sperm after exposure to cAMP has
been observed in several mammalian species (For a review see Urner and Sakkas, 2003)
indicating that tyrosine phosphorylation is regulated by the cAMP/PKA signaling pathway
(Urner and Sakkas, 1996).

Hyperactivated sperm motility has been linked to protein tyrosine phosphorylation
and hyperactivation is required for transit of sperm through the reproductive tract, sperm
penetration of the cumulus and ZP of the oocyte (Si and Okuno, 1999). Some tyrosine
phosphorylated sperm proteins have been identified and these include the A-kinase
anchoring proteins (AKAPs) and fibrous sheath protein (FSP95). These proteins are
localized to the fibrous sheath and are the most prominent tyrosine phosphorylated proteins
(Carrera et al., 1996). A human insoluble 86kDa tyrosine phosphorylated protein has been
localized to the pp and has been identified as CABYR (Calcium-binding and tyrosine
phosphorylation-regulated protein). Since this is a calcium binding protein it may play a
role in sequestering the calcium required to maintain capacitation and hyperactivation.
Vijayaraghavan et al. (1997) localized a soluble tyrosine phosphorylated flagellar protein of
55kDa on bovine sperm cells. It is thought that this protein may also play a role in sperm
motility.

Tyrosine phosphorylation of sperm head proteins has also been explored. Several
authors reported the appearance of tyrosine phosphorylated proteins in the head region of
dog (Petrunkina et al., 2003), bovine (Gualtieri et al., 2005), human (Lui et al., 2003), pig
(Tardif et al., 2001) and hamster (Kulanand and Shivaji, 2003) sperm. Jones and colleagues
(2008) have reported that the monoclonal anti-phosphotyrosine antibody, clone 4G10 binds
strongly to the equatorial subsegment of mouse, rat, boar, bull and ram sperm. In addition, Jones et al. (2008) have also shown that a small area of the posterior acrosomal region in rat and mouse is enriched in tyrosine phosphorylated proteins. Using freeze fracture electron microscopy, Jones et al. (2008) reported the appearance of tyrosine phosphorylated proteins over the three overlying membranes of the equatorial subsegment: the plasma membrane, the outer acrosomal membrane and the inner acrosomal membrane. The tyrosine phosphorylated proteins in the equatorial subsegment were identified by mass spectrophotometry as orthologues of human sperm accessory 1 (SPACA 1) (Jones et al., 2008). The appearance of tyrosine phosphorylated proteins in the head region implies a role for these proteins in sperm-egg binding/fusion. Tyrosine phosphorylation of sperm head proteins may also be involved in the sperm-oviduct binding prior to fertilization. It is also interesting to note that hyperactivation has been thought to aid in breaking the sperm-oviduct (sperm reservoir) binding (Demott and Suarez, 1992). Enhancement and redistribution of tyrosine phosphorylated sperm proteins has indicated that capacitation of sperm initiates changes in the active phosphorylation sites of specific proteins on the sperm surface. Increased affinity of sperm to the ZP (Pukazhenthi et al., 1998), and an increase in acrosome reactions (Benoff, 1998) and sperm hyperactivation (Nassar, 1999) has been linked with tyrosine phosphorylation of sperm proteins.
2. HYPOTHESIS

Oviductin binds to specific structures of the mammalian egg which are involved in the fertilization process (Oliphant et al., 1984, Araki et al., 1987, Kan et al., 1988, Sendai et al., 1995). It has also been shown that sperm binds oviductin in vitro (Kimura et al., 1994, Boatman and Magnoni, 1995). This binding is localized to specific regions of the sperm head and is increased in capacitated sperm (Kan and Esperanzate, 2006). In light of the findings reported in previous studies, we hypothesize that the presence of oviductin enhances in vitro capacitation through tyrosine phosphorylation of sperm proteins.

3. OBJECTIVES

i) To determine whether lectin-affinity purified oviductin from the estrus stage of the estrous cycle (EOV) has the capacity to enhance the tyrosine phosphorylation of hamster sperm proteins during capacitation.

ii) To determine the location of tyrosine phosphorylated hamster sperm proteins during capacitation in the presence of EOV.

iii) To identify the hamster sperm proteins which undergo an enhancement in tyrosine phosphorylation when capacitated in the presence of EOV.
CHAPTER 2.0 MATERIALS & METHODS

2.1. Animals

The Golden hamsters (*Mesocricetus auratus*) used for this research were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, Indiana, USA). Female hamsters were between 6-8 weeks of age and housed in a temperature-controlled room with exposure to light 12 hours/day (6 am- 6 pm). The female hamsters were examined for vaginal discharge that is indicative of the metestrus stage of the estrous cycle. The discharge is viscous, yellow in color and has a pungent odor. The females were allowed to complete 4-5 cycles to establish regularity. Male Golden hamsters were 7-9 weeks of age and were housed under the same conditions with the females. All research was done in accordance to the guidelines of the Canadian Council on Animal Care, and the University Animal Care Committee at Queen’s University approved all procedures.

2.2 Lectin-Affinity Purification and Immunodetection of Estrus Stage-Specific Oviductin

Once cycling regularly, female hamsters were sacrificed by cervical dislocation according to the stage from which purified oviductin was desired (i.e. estrus). Oviducts of female Golden hamsters were excised and stored in liquid nitrogen until use. A minimum of 12 oviducts were homogenized at 4°C in homogenization buffer (1ml/6 oviducts) containing protease inhibitors (0.2mM PMSF, 0.2mM benzamidine, 1µM leupeptin and 35µg/mL aprotinin). The homogenate was then centrifuged (Beckman Optima XL-100K Ultracentrifuge, rotor # 50Ti) at 35,000 rpm for 1 hr at 4°C. The Bio-Rad Protein Assay (Bio-Rad, Mississauga, Ontario) was performed on the supernatant in order to determine
the protein concentration. Stage-specific oviductin was purified from the total protein homogenate using *Helix pomatia* agglutinin (HPA)-agarose, and N-acetyl-D-galactosamine (GalNAc) binding lectin by a procedure reported by Malette and Bleau (1993). The total protein concentration of the purified oviductin was measured once again using the Bio-Rad Protein Assay (Bio-Rad, Mississauga, Ontario) using bovine serum albumin (BSA) as the protein standard. Samples (~1.6 μg) of oviductin purified from the estrus stage of the estrous cycle (EOV) were prepared and analyzed by SDS-PAGE on an 8% acrylamide gel. The proteins were electrophoretically transferred to a PVDF membrane for Western blotting and the membrane was blocked overnight at 4°C in 5% low-fat milk solution in TBST (pH 7.4). Following blocking, the membrane was incubated for 1 hour at room temperature with anti-hamster oviductin monoclonal antibody (mAb 4.12F6) (1:100), washed thoroughly in TBST and then incubated for 1 hour at room temperature with horseradish peroxidase conjugated to rabbit anti-mouse IgG (RAM-HRP) (Sigma, Canada) diluted 1:10,000. The primary antibody recognizes a carbohydrate epitope of hamster oviductin and its tissue specificity has been previously established (St-Jacques et al., 1992). The membrane was thoroughly washed after incubation with the secondary antibody and immunoreaction was detected using Western Lightening Chemiluminescence Reagents (PerkinElmer, Boston, MA) according to the manufacturer’s instructions.

2.3 Collection and Capacitation of Sperm

For each experiment at least one male Golden hamster was used. The animal was sacrificed by cervical dislocation at 7-9 weeks of age and the epididymides excised. The
epididymides were placed in mineral oil extracted with TL-PVA, a modified Tyrode’s medium (Bavister, 1989), and the caudal region (indicated by the appearance of larger tubules) was punctured with a 23-gauge needle. The caudal epididymis contains mature sperm cells which were chosen for use over ejaculated sperm because of the absence of seminal fluid contents and the ease of retrieval from the male Golden hamster. The caudal epididymal sperm (CES) were allowed to seep into the oil. CES were considered viable if >80% of the cells displayed normal morphology and motility when examined under an inverted light microscope. The CES were diluted in capacitating media (See Bavister, 1989 for a complete list of reagents), a modified Tyrode’s medium. Cell counting was done using the Bright Line Counting Chamber (Hausser Scientific, Horsham, PA). For each experiment, 4-5 x 10^6 cells were used per sample and a minimum of three repetitions were performed.

CES were capacitated in the presence or absence of 60μg/mL of oviductin, previously determined in our laboratory to be the optimal concentration for successful sperm capacitation, for various time points (5-, 60-, 120-, 180-min). Prior to incubation, motility factors including penicillimine (8.0 mM), hypotaurine (40.0 mM) and epinephrine (1.0 mM) were added to the samples and then overlaid with extracted mineral oil. The samples were incubated at 37°C in 5% CO₂.

2.4 Western Blot Analysis of Oviductin Binding to Sperm and Loading Control

Once sperm were incubated in the presence or absence of EOV for 5-, 60-, 120- and 180-minutes the cells were removed from the incubator and centrifuged (3000 rpm for 5 minutes) after the addition of 0.2mM sodium orthovanadate, an inhibitor of protein tyrosine phosphatases. The cells were washed twice in 0.01M PBS and 50μL of 1X
loading buffer, containing 5% v/v beta-mercaptoethanol (BME), was added to each sample. The samples were boiled for 5 minutes then loaded on an 8% acrylamide gel for electrophoresis. Once the proteins were separated, the membrane was incubated with mAb 4.12 F6 (1:150) and anti-α-tubulin antibody (1:20,000) (Sigma, Canada) for 1 hour at room temperature. After incubation of the membrane in the primary antibodies the membrane was washed 3 times (10 minutes/wash) with TBST. The membrane was then incubated with RAM-HRP (1:10,000) (Sigma, Canada) for 1 hour at room temperature. The membrane was then washed 3 times (10 minutes/wash) with TBST and the affinity of oviductin binding to sperm as well as equal loading of the samples was detected. The band density was measured using Image J software. Statistical analysis was performed using the student’s two-tailed t-test in Microsoft Excel.

2.5 Localization of Tyrosine Phosphorylated Sperm Proteins Using Immunofluorescence and Confocal Microscopy

Sperm cells were capacitated as mentioned above in the presence or absence of 60μg/mL EOV. At 5- and 180-minutes, samples with and without oviductin were removed from the incubator, smeared on glass slides and allowed to air dry completely. To localize immunoreactions to specific regions, samples were “chemically dissected” with non-ionic detergents 0.2% Triton-X-100 (15 minutes) and 1% NP-40 (2 hours) and ionic detergent 2% SDS (2 hours) after capacitation in the absence of EOV at 5- and 180-minutes. After treatment with detergents the samples were centrifuged (3000 rpm for 1 minute) at 4°C, washed (0.01M PBS) and allowed to air dry completely on glass slides. The cells were fixed for 2 hours at room temperature in 0.1M cacodylate (CaCo) buffer with 3% paraformaldehyde and 0.1% glutaraldehyde containing 0.2M sucrose. The slides
were washed 2 times with 0.01M PBS and blocked overnight in 2% BSA at 4°C. The next day the slides were incubated with monoclonal anti-phosphotyrosine antibody clone 4G10 (1:1000) for 1 hour at room temperature. All successive steps were performed in the dark. The slides were washed with 0.01M PBS and incubated with goat-anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC-GAM) in a 1:300 dilution for 45 minutes at room temperature. After secondary incubation the slides were washed twice with 0.01M PBS and allowed to air dry completely. Coverslips were applied using non-fluorescent mounting medium and sealed with nail varnish. The slides were stored under dark conditions at 4°C until ready to be viewed using immunofluorescence and confocal microscopy. To detect background staining several slides were incubated with FITC-GAM only and used as a control.

2.6 Preparation of Colloidal Gold and Protein A-colloidal Gold Complex

Colloidal gold particles (~10nm) were prepared for immunolabeling by mixing two solutions (Slot and Geuze, 1985). In solution A, 1ml of 1% tetrachloroauric acid was added to 79mL of double distilled water (ddH₂O). In solution B, 4mL of 1% tri-sodium citrate was added to 30 μL of 1% tannic acid and 15.97mL of ddH₂O. Both solutions were heated separately to 60°C while agitating solution A. When both solutions reached the temperature of 60°C, solution B was added rapidly to solution A and the mixture was agitated constantly with a magnetic rod until the colloidal gold solution turned a red wine color (approx. 20 minutes). The colloidal gold solution was boiled further for 10 minutes and then allowed to cool to room temperature and stored at 4°C.

Protein A-colloidal gold complex preparation involved adjusting the pH of the colloidal gold solution to pH 5.6 then adding 1.0 mg of protein A per 10.0 mL of
colloidal gold solution and mixing. To test for the presence of free gold particles, a few drops of 10% NaCl was added to a small aliquot (1.0 mL) of the freshly prepared protein A-colloidal gold complex (PA-CG) and appearance of a rose color indicated successful conjugation of protein A to gold particles. A violet color resulting from this test is indicative of free colloidal gold that has not bound to protein A and more protein A is needed to complete the conjugation. To stabilize the final PA-CG prepared, 0.1mL of 1% polyethylene glycol (PEG 20000) for every 10.0mL of protein A-Gold was added to the mixture. This PA-CG was then centrifuged for 45 minutes at 26500 rpm at 4°C. A pellet formed at the base of the centrifuge tube after centrifugation. The supernatant was removed and discarded and the bottom pellet, which contained the purified protein A-Gold complex (PA-CG), was resuspended in 0.01M PBS, pH 7.2 containing 0.02% PEG 20000 (1.0mL per 10mL colloid gold stock). The protein A-Gold complex was then stored at 4°C until use.

2.7 Ultrastructural Localization of Tyrosine Phosphorylated Sperm Proteins by Immunoelectron Microscopy

Sperm cells were collected using the aforementioned technique and capacitated for 5- and 180-minutes as described above. When incubation was complete, the cells were transferred with a pipette into 1.5mL microcentrifuge tubes, centrifuged and washed 2 times in 0.01M PBS. The cells were blocked in 0.05M glycine then 1% ovalbumin for 10 minutes each at room temperature. The cells were then washed and resuspended in 0.01M PBS/ 0.1% BSA containing monoclonal anti-phosphotyrosine antibody clone 4G10 (1:500) for 2 hours at room temperature. The cells were washed 2 times in 0.01M PBS/ 0.1% BSA for 10 minutes at room temperature. Following blocking, the cells were
exposed to protein-A-gold complex for 1.5 hours at room temperature. After being washed, the cells were fixed as above and stored overnight at 4°C in 0.01M PBS. A solution of 30% BSA crossed-linked with 2.5% glutaraldehyde (in 0.01M PBS) was used as a gel matrix to keep the cells together. Each sample was postfixed in 1% osmium tetroxide (OsO₄) in double distilled water containing 1.5% potassium ferrocyanide for 1 hour at room temperature then processed through an acetone dehydration series and embedded in Epon according to the following procedure:

a. 30% Acetone → 10min at room temperature
b. 50% Acetone → 10min at room temperature
c. 70% Acetone → 10min at room temperature
d. 95% Acetone → 10min at room temperature
e. 100% Acetone → 10min at room temperature x 3
f. Epon/100% Acetone (1:1) → 1hr at room temperature
g. Epon/100% Acetone (2:1) → 1hr at room temperature
h. Epon/100% Acetone (3:1) → overnight at room temperature
i. Pure Epon → 5 hr at room temperature
j. Embedment in pure Epon → 48hr at 60°C

Semi-thin sections (1μm) of the Epon-embedded cells were cut and examined with a light microscope in order to locate the cells. Ultra-thin sections from the selected regions were then prepared and counterstained for 10 minutes with 4% aqueous uranyl acetate followed by 2 minutes with lead citrate and viewed on a Hitachi 7000 transmission electron microscope operated at 75kV.

For quantifying the immunoreactions of ultra-thin sections, the numbers of gold particles over cross-sectional profiles of sperm incubated in capacitation supportive medium in the presence or absence of EOV for 5- and 180-minutes were counted. The areas of the equatorial segment region and midpiece of the sperm cells were measured with a digitizer (MITABLET-II, Graphic Corp.) connected to a personal computer. Labeling densities are expressed as numbers of gold particles/μm². The averages of gold
particles from ten representative images were used for quantification of immunoreactions in both the midpiece and equatorial segment region. Statistical analysis was performed using the student’s two-tailed t-test in Microsoft Excel.

2.8 Preparation of Surface Replicas of Immunogold Labeled Sperm

Epididymal sperm cells were prepared and capacitated for 5- and 180-minutes in the presence or absence of EOV and at the designated time points the samples were collected and washed following the procedure mentioned above. The cells were fixed for 2 hours with 3% paraformaldehyde and 0.1% glutaraldehyde in 0.1M CaCo buffer containing 0.2M sucrose. The cells were washed twice and successively blocked for 10 minutes in 0.05M glycine and 1% ovalbumin. The cells were incubated with monoclonal anti-phosphotyrosine antibody clone 4G10 (1:300) for 1.5 hours and then washed twice in 0.01M PBS. The cells were blocked once again in 1% ovalbumin for 10 minutes and then exposed to protein A-gold (1:2) for 1 hour at room temperature. The cells were washed twice in 0.01M PBS and fixed overnight at 4°C with 3% paraformaldehyde and 0.1% glutaraldehyde in 0.1M CaCo buffer containing 0.2M sucrose. Control samples were exposed to protein A-gold only.

Droplets of the fixed, labeled cells and controls were placed on formvar-coated nickel grids (200 mesh) and allowed to air dry completely. The grids were mounted on a receptacle which was transferred into the chamber of a Balzers 400D freeze-fracture unit (Balzers AG, Balzers, Liechtenstein) for replication. Platinum-carbon replication of the air-dried, labeled sperm samples by electron beam gun evaporators was carried out as previously described by Kan and Esperanzate (2006).
After replication the samples were removed from the chamber. Each replicated sample was transferred to porcelain wells containing a solution of 70% ethanol and sodium hypochlorite (1:4) in order to digest the formvar and separate the replica from the nickel grid. The separated replicas were then transferred to another porcelain well containing fresh solution of sodium hypochlorite for complete digestion and removal of sperm debris. Each replica was then washed 3 times in double distilled water and mounted on copper grids (75x300 mesh). The grids were viewed using a Hitachi 7000 transmission electron microscope operated at 75kV.

For quantifying the immunoreactions of the surface replicas, the numbers of gold particles over surface profiles of sperm incubated in capacitation supportive medium in the presence or absence of EOV for 5- and 180-minutes were counted. The areas of the equatorial segment region and midpiece of the sperm cells were measured with a digitizer (MITABLET-II, Graphic Corp.) connected to a personal computer. Labeling densities are expressed as numbers of gold particles/µm². The average of gold particles from ten representative images was used for quantification of immunoreactions in both the midpiece and equatorial segment region. Statistical analysis was performed using the student’s two-tailed t-test in Microsoft Excel.

2.9 SDS-PAGE and Western Blot Analysis of Tyrosine Phosphorylated Sperm Proteins

At each designated time point sperm samples, prepared as mentioned above incubated with or without oviductin, were removed from the incubator and centrifuged (3000 rpm for 5 minutes) after the addition of 0.2mM sodium orthovanadate, an inhibitor of protein tyrosine phosphatases. The supernatant was discarded and the pellet washed twice in 1X PBS. The pellet was resuspended in 1% NP-40 and incubated overnight at
After incubation the samples were centrifuged (3000rpm for 1 minute) resulting in two fractions; 1) the non-extractable fraction (pellet) containing the cytoskeletal elements of the sperm, and 2) the extractable fraction (supernatant) containing the acrosomal contents, plasma membrane, inner and outer acrosomal membranes and loose cytoplasm of the tail. The pellet was resuspended in 1X loading buffer and boiled for 5 minutes. After boiling the pellet fraction was centrifuged (12,000 rpm for 4 minutes) and the resulting supernatant was kept and stored at -70°C.

A protein assay was performed on the supernatant fraction (S) in order to determine the protein concentration. Approximately 60μg of protein from each sample was diluted with 2X loading buffer and stored at -70°C. Prior to loading the samples on a 10% acrylamide gel, the samples were removed from the freezer and 5% v/v BME was added to each sample and boiled for 5 minutes. Sperm protein samples (~40 μL) from each fraction were subjected to SDS-PAGE. The proteins were then transferred to a PVDF membrane for 2 hours (0.23A) and blocked overnight at 4°C in a 5% low-fat milk solution (LFMS) in TBST. After blocking, the membrane was incubated with monoclonal anti-phosphotyrosine antibody clone 4G10 (Upstate, Lake Placid, NY) in a 1:3,000 dilution (in 5% LFMS) for 1 hour at room temperature. The membrane was then washed 3 times (10 minutes/wash) with TBST. After washing, the membrane was incubated with rabbit-anti-mouse IgG conjugated to horseradish peroxidase (RAM-HRP) in a 1:10,000 dilution for 1 hour at room temperature. The membrane was washed as described above and detection of tyrosine phosphorylated proteins was performed using Western Lightening Chemiluminescence Reagents (Roche, Canada). The density of the bands detected was measured using Image J software (NIH, USA). Statistical analysis was performed using the student’s two-tailed t-test in Microsoft Excel.
In order to confirm equal loading of protein per lane and to show the affinity of oviductin to sperm, after detection of tyrosine phosphorylated proteins the membrane was stripped by placing it in 1X phosphate buffered saline (PBS), 7 μL/mL BME, and 2% SDS for 45 minutes at 37°C. The stripped membrane was then washed in TBST for 30 minutes and blocked overnight at 4°C in 5% LFMS.

As a control, sperm were incubated in a non-supportive capacitation medium (NCM), processed as described above and Western blot analysis was performed using monoclonal anti-phosphotyrosine antibody, clone 4G10.

2.10 Immunoprecipitation of Tyrosine Phosphorylated Sperm Proteins and Protein Identification by Mass Spectrophotometry

Sperm cells were capacitated for up to 180 minutes using the procedure described above. Following capacitation the cells were collected, centrifuged for 1 minute (3000rpm) and washed twice in 1X PBS. Sperm cells were then separated into extractable (S) and non-extractable (I) fractions in 1% NP-40 overnight at 4°C. The total protein concentration of each fraction was measured using the Bio-Rad Protein Assay (Bio-Rad, Mississauga, Ontario) using BSA as the protein standard.

Immunoprecipitation was performed on each fraction using the Catch and Release Phosphotyrosine clone 4G10 Immunoprecipitation Kit (Upstate) according to the manufacturer’s protocol. Proteins from whole S and I fractions, immunoprecipitated S and I and flow through S and I fractions were separated on a 10% acrylamide gel and subjected to SDS-PAGE for protein analysis. The gel was then fixed in a solution of 40% methanol and 10% acetic acid for 30 minutes. The gel was stained using 0.025 % Coomassie blue standard staining solution for 1 hour at room temperature and destained
in double distilled water (changed frequently) for approximately 2 hours at room
temperature. Bands of interest were carefully removed and the samples were sent to the
Mass Spectrophotometry Laboratory (Dept. of Chemistry, Queen’s University) for
analysis. In-gel digestion of the Coomassie-stained protein bands was performed
following a standard protocol. The excised protein bands were crushed into small pieces
and destained with a mixture of 25mM ammonium bicarbonate (NH₄HCO₃) and
acetonitrile. The protein in the gels was reduced with 10mM dithiothreitol (DTT) solution
(in 100mM NH₄HCO₃) at 56°C for 1 hour, and then incubated with 55mM iodoacetamide
at room temperature for another 45 minutes. Following the complete dryness of the gel
particles by SpeedVac centrifuge, the protein was digested overnight with sequencing
grade 20ng trypsin (Calbiochem) in 25mM ammonium bicarbonate (pH 7.6) solution.
The proteolytic peptides were sequentially extracted using 0.1 % formic acid, 60%
acetonitrile/0.1% formic acid and pure acetonitrile, finally the collected fraction was dried
by SpeedVac centrifuge and C18 Ziptip clean for MALDI MS and MS/MS analyses.

Matrix assisted laser desorption ionization (MALDI) data were acquired by an
Applied Biosystems/MDS Sciex QStar XL quadrupole time-of-flight (QqTOF) mass
spectrometer (at the Department of Chemistry, Queen’s University) equipped with an
oMALDI II source and a nitrogen laser operating at 337nm. The sample was prepared at
the ratio of 1:1 (v/v) of the peptide digest to matrix (i.e. 2,5-dihydroxybenzoic), and
subsequently dried on a stainless steel MALDI plate. After MALDI MS mapping, the
peptide sequences were then identified by MS/MS measurements using argon as the
collision gas.

The peptide fingerprinting masses were searched by MS-Fit program against the
NCBI database using Protein Prospector at the UCSF web site
(http://prospector.ucsf.edu), whereas the MS/MS ions search on each tandem mass spectrum were performed through Mascot search engine (MatrixScience, http://www.matrixscience.com). These searches have been taken into account of up to two missed trypsin cleavage sites, and the common modifications of methionine oxidation, carbamidomethylation, asparagine and glutamine deamidation to asparate acid and glutamic acid, N-terminal pyroglutamation. The mass tolerance between calculated and observed masses used for database search was considered at the range of ± 100ppm for MS peaks and ± 0.2 Da for MS/MS fragment ions.

CHAPTER 3.0 RESULTS

3.1 Lectin-Affinity Purification and Immunodetection of Estrus Stage-Specific Oviductin

Immunostaining of the Western blot transferred 8% SDS-PAGE gel of HPA-purified hamster oviductin from the estrus stage (EOV) is consistent with results that have been previously obtained with purified hamster oviductin (Malette and Bleau, 1993). The immunoblot showed that the monoclonal antibody against hamster oviductin used in conjunction with the rabbit anti-mouse conjugated to horseradish peroxidise (RAM-HRP) secondary antibody reacted specifically with a broad, polydispersed band ranging from 160-350kDa (Figure 7). The large band does not appear uniform and is spread at the edges. This is due to the high level of glycosylation of oviductin at the estrus stage of the estrous cycle. The purpose of the present study is to examine the role of purified oviductin in sperm function.
3.2 Western Blot Analysis of Binding of Oviductin to Sperm

For oviductin-sperm binding analysis, caudal epididymal sperm were capacitated in the presence or absence of 60µg/mL EOV. The sperm proteins were separated on a 10% acrylamide gel and transferred to a PVDF membrane. The proteins were exposed to a monoclonal antibody against hamster oviductin in conjunction with rabbit anti-mouse IgG conjugated to horseradish peroxidase (RAM-HRP). The immunoreaction revealed a high molecular weight band ranging from 160- 360kDa in the 5-, 60-, 120-, and 180-minute samples incubated in the presence of EOV (Figure 8A). The experiment was performed in triplicate. Despite the increasing intensity of the bands over time shown in this representative blot, density analysis using ImageJ Software showed no significant binding pattern.

For each experiment, 3-4 x10^6 cells were capacitated. From these cells 40µg of sperm protein were added to each well. This concentration of sperm protein was chosen as it was sufficient to show immunoreactions and was consistently available after the capacitation of the cells. In order to confirm equal loading of protein per well anti-α-tubulin antibody was used for the detection of tubulin as a loading control. A protein band of ~52kDa of equal intensity of immunoreaction was observed in each lane confirming that each sample contained the same amount of protein (Figure 8B). The loading control indicated that any increase in band intensity is not due to unequal loading of protein in a particular sample.
3.3 Immunolocalization of Tyrosine Phosphorylated Sperm Proteins using Indirect Immunofluorescence

Caudal epididymal sperm capacitated for 5- and 180-minutes in the presence or absence of EOV were labeled with monoclonal anti-phosphotyrosine antibody clone 4G10 and FITC-GAM shown in Figure 9. Sperm from the 5-min/EOV- samples displayed very weak phosphotyrosine reaction in the head and tail regions. Sperm cells from the 5-min/EOV+ samples displayed a much more intense phosphotyrosine reaction in the head and tail regions. Sperm capacitated for 180-minutes showed an increase in protein tyrosine phosphorylation compared to the 5-minute samples which is consistent with previous results showing that a time-dependent increase in tyrosine phosphorylation occurs during capacitation (Carrera et al., 1996, Urner and Sakkas, 2003). Sperm from the 180-min/EOV- samples displayed a relatively weak phosphotyrosine reaction in the head region in comparison with the 180-min/EOV+ samples. Phosphotyrosine labeling of the 180-min/EOV- sperm also showed an uneven, yet intense reaction along the midpiece. After a 180-minute capacitation in the presence of EOV (180-min/EOV+) a very intense and evenly distributed labeling was seen along the midpiece and head. Control samples labeled with FITC-GAM alone were prepared and a very faint immunoreaction, considered as background immunostaining, was detected along the entire length of the tail.
3.4 Immunolocalization of Tyrosine Phosphorylated Sperm Proteins by Confocal Microscopy

Localization of tyrosine phosphorylated sperm surface proteins was performed by confocal microscopy of fixed caudal epididymal sperm capacitated for 5- and 180-minutes in the presence or absence of EOV (Figure 10). The sperm plasma membrane is fragile so manipulation of the sperm cells required delicate handling to ensure that they were not disrupted. Unless the cells were permeablized, we presumed that the plasma membrane was present.

Sperm capacitated for 5-minutes in the absence of EOV displayed distinct immunostaining of the plasma membrane overlying the equatorial segment region of the sperm head and midpiece. In the presence of EOV sperm capacitated for 5-minutes displayed the same labeling pattern but with a seemingly more intense and even distribution of immunoreaction along the midpiece. After 180-minutes of capacitation in the absence of EOV labeling was present but appeared weaker in the plasma membrane overlying the equatorial segment region and an increase in intensity was observed along the midpiece. This time-dependent increase in protein tyrosine phosphorylation is consistent with previous observations (Carrera et al., 1996, Urner and Sakkas, 2003). Sperm capacitated for 180-minutes in the presence of EOV displayed a more apparent intense immunoreaction in the plasma membrane overlying the midpiece and principal piece. Labeling of the equatorial segment region was maintained in the 180-min/EOV+ samples. A weak signal was present along the entire length of the tail in control samples labeled with FITC-GAM alone.
The mammalian sperm head consists of a complex membrane system made up of the plasma membrane, outer (OAM) and inner (IAM) acrosomal membranes and the associated perinuclear theca. Although indirect immunofluorescence and confocal microscopy localized tyrosine phosphorylated proteins to the equatorial segment region of the sperm head, there is a possibility that the procedure used for sperm preparation might have disrupted the plasma membrane and exposed the underlying membrane structures accessible for labeling. To verify if the immunoreaction observed in the equatorial segment region could be associated with the OAM, IAM, perinuclear theca or a combination of any one of the three, sperm were capacitated for 5- and 180-minutes then permeabilized using 0.2% Triton-X-100 (Figure 11) and 1% NP-40 (Figure 12A) in order to remove the plasma membrane.

At 5-minutes of capacitation, treatment with Triton-X-100 revealed localization of tyrosine phosphorylated proteins to the equatorial segment region of the sperm head and along the midpiece (Figure 11). After 180-minutes of capacitation localization of tyrosine phosphorylated proteins was detected in the equatorial segment region and along the midpiece as well as principal piece (Figure 11). Again, tyrosine phosphorylation of sperm proteins was found to increase over time.

Figure 12A displays NP-40 permeabilized sperm cells capacitated for 5-minutes with protein tyrosine phosphorylation localized to the equatorial segment region of the sperm head, however, labeling along the sperm tail was absent. After 180-minutes of capacitation, the sperm cells permeabilized with 1% NP-40 maintained the labeling in the equatorial segment region of the head (Figure 12A). After 180-minutes of capacitation the sperm cells displayed an increase in immunoreaction at the equatorial segment region, midpiece and principal piece (Figure 12A).
3.5 Ultrastructural Localization of Tyrosine Phosphorylated Sperm Proteins by Immuno-Electron Microscopy

For ultrastructural localization of sperm proteins undergoing tyrosine phosphorylation during capacitation, sperm were capacitated in the presence or absence of EOV for 5- and 180-minutes as described earlier using 3-4x10⁶ cells/experiment. The experiment was repeated three times. The cells were labeled with mAb anti-phosphotyrosine, clone 4G10 (1:600) and then exposed to protein-A-colloidal gold (10nm). At 5-minutes of capacitation immunogold labeling was found to be associated only with the OAM at the equatorial segment region of the sperm head (Figure 13A). A significant increase in labeling of this region was seen when the cells were capacitated in the presence of EOV (Figure 13B). The appearance of few gold particles along the midpiece of the sperm tail was also observed at 5-minutes of capacitation and a significant increase was observed between the samples capacitated in the presence or absence of EOV (Figures 14A and B).

Capacitation for 180-minutes in the presence or absence of EOV revealed an increased immunogold labeling of the OAM at the region of the equatorial segment compared to that observed at the 5-minute time point (Figures 13C and D). Cells capacitated for 180-minutes in the presence of EOV (Figure 13D) displayed a significant increase in labeling along the equatorial segment region compared to cells capacitated in capacitating medium alone. Labeling along the midpiece was also increased after 180-minutes of capacitation and, again, the cells capacitated in the presence of EOV showed an increased affinity for immunogold particles along the midpiece (Figures 14C and D). It is important to note that in both the 5- and 180-minute samples only sections of the
midpiece devoid of the plasma membrane showed immunogold labeling suggesting that proteins of the mitochondrial sheath are undergoing tyrosine phosphorylation.

Control samples were prepared by exposing the cells to protein-A-colloidal gold alone and showed a negative reaction (Figure 15). Histograms comparing the labeling densities of the equatorial segment region and the midpiece are shown in APPENDIX B.

### 3.6 Localization of Tyrosine Phosphorylated Sperm Proteins by the Surface Replica Technique

The surface replica technique was utilized to further define the localization and distribution of tyrosine phosphorylated proteins at the equatorial segment region and along the tail midpiece. Sperm capacitated for 5-minutes in the presence or absence of EOV showed immunogold labeling over the equatorial segment region (Figure 16). In the presence of EOV sperm capacitated for 5-minutes (Figures 16C and D) revealed a significant increase in affinity for gold particles compared to sperm capacitated in the absence of EOV (Figures 16A and B). After 180-minutes of capacitation a time-dependent increase in protein tyrosine phosphorylation was observed again confirming the previous observations reported by Carrera et al. (1996) and Urner and Sakkas (2003). Sperm capacitated for 180-minutes in the presence of EOV (Figures 17C and D) also showed an increased affinity for gold particles at the equatorial segment region in comparison to 180-minute incubation in capacitating medium alone (Figures 17A and B). Few gold particles were seen on the control surface replicas, exposed to protein-A-colloidal gold only, showing the presence of some non-specific binding (Figure 18).

Localization of tyrosine phosphorylated proteins along the sperm tail midpiece using the surface replica technique showed results corroborating findings obtained with
indirect immunofluorescence and confocal microscopy carried out in the present study. Immunogold labeling of tyrosine phosphorylated sperm proteins along the midpiece increased during capacitation and capacitation of sperm in the presence of EOV revealed an increase in immunoreaction at the 5- and 180-minute time point (Figure 19). Control samples were also prepared by exposing sperm capacitated for 5- and 180-minute to protein-A-colloidal gold alone (Figure 20). A few gold particles were detected indicating the presence of some background labeling. Again, histograms comparing the labeling densities of the equatorial segment region and the midpiece are shown in APPENDIX B.

3.7 Comparison of Sperm Protein Extraction Techniques (1% NP-40 versus Sonication)

Whole caudal epididymal sperm were exposed either to 1% NP-40 or a brief sonication in order to fractionate the sperm into NP-40 extractable and non-extractable sperm proteins. Approximately 40 µg of NP-40 non-extractable sperm protein prepared with both techniques were separated on a 10% acrylamide gel for comparison (Figure 21). Both techniques revealed an abundance of similar proteins with a molecular weight ranging from approximately 10-250kDa. Also, each method of extraction showed a time-dependent increase in tyrosine phosphorylation of NP-40 non-extractable sperm proteins. The NP-40 extraction technique revealed higher concentrations of NP-40 non-extractable sperm proteins as indicated by protein assay (results not shown).

Sonicated or 1% NP-40 treated extractable caudal epididymal sperm proteins were also separated on a 10% acrylamide gel for comparison (Figure 22). Both extraction techniques revealed proteins of similar molecular weights with the exception of a ~15
kDa protein which appeared only in the NP-40 treated samples. Again, NP-40 treated samples contained higher concentrations of proteins. Each lane was loaded with ~40µg of NP-40 extractable sperm proteins and the experiment was preformed in triplicate.

### 3.8 SDS-PAGE and Western Blot Analysis of Tyrosine Phosphorylated Hamster Sperm Proteins

Figure 23 shows that whole sperm incubated in non-supportive capacitation medium (NCM) did not display the characteristic time-dependent increase in tyrosine phosphorylation. Endogenous phosphorylation of whole sperm proteins has been reported (Tardiff et al., 1999) so the bands observed in the NCM incubated cells were considered as background.

Separation of extractable and non-extractable sperm proteins using 1% NP-40 was performed as described above. Each extract of proteins (extractable and non-extractable) was separated by one-dimensional SDS-PAGE on 10% polyacrylamide gels and transferred electrophoretically to polyvinylidene fluoride (PVDF) membranes.

The immunoblot of the NP-40 non-extractable proteins (Figure 24) revealed multiple bands indicating that the NP-40 non-extractable fraction is rich in tyrosine phosphorylated proteins. A time-dependent increase in protein tyrosine phosphorylation of sperm incubated in the presence or absence of EOV was also observed and this is consistent with previous results reported (Carrera et al., 1996, Urner and Sakkas, 2003). This experiment was performed in triplicate.
Three NP-40 non-extractable protein fraction bands of molecular weight 70, 83 and 90kDa from sperm samples incubated in the presence of EOV showed an increase in protein tyrosine phosphorylation compared to samples incubated in the absence of EOV (Figure 24). This indicates an enhancement in tyrosine phosphorylation of certain sperm proteins when sperm are exposed to homologous purified EOV. It is also interesting to note that several NP-40 non-extractable proteins from sperm incubated in the presence of EOV showed a decrease in immunoreaction (Figure 24). This indicates that capacitation is a complex process involving not only post-translational protein tyrosine phosphorylation but also protein tyrosine dephosphorylation.

NP-40 extractable hamster sperm proteins from sperm capacitated for 5-, 60-, 120- and 180-minutes in the presence and absence of EOV were extracted with 1% NP-40 for Western blot analysis using monoclonal anti-phosphotyrosine antibody clone 4G10. Figure 25 shows the appearance of numerous protein bands with a molecular weight ranging from approximately 15-100kDa. Tyrosine phosphorylation of the NP-40 extractable sperm proteins showed a time-dependent increase with a peak at 120-minutes in the absence of EOV and at 60-minutes in the presence of EOV (Figure 25). Tyrosine phosphorylated proteins of molecular weight 25, 37 and 44kDa displayed an increase in intensity when incubated in the presence of EOV (Figure 25). This suggests that in the presence of homologous EOV, some sperm proteins of the NP-40 extractable fraction underwent an enhancement in tyrosine phosphorylation. This experiment was performed in triplicate.
Density Analysis of Tyrosine Phosphorylated Hamster Sperm Proteins

Enhanced in the Presence of EOV

Density analysis of the bands undergoing enhancement in the presence of EOV was performed using ImageJ software. Three representative Western blots of the NP-40 non-extractable fraction were analysed to determine the mean density (±SEM). Statistical significance was determined using a two-tailed student’s t-test (Microsoft Office Excel, 2008) and a p<0.05 confidence level. A significant increase in tyrosine phosphorylation of the 90kDa was found in the NP-40 non-extractable protein fraction recovered from sperm after 5-, 60- and 120- minutes of capacitation in the presence of EOV compared to the corresponding NP-40 non-extractable protein from sperm after 5-, 60-, 120-minutes of capacitation in the absence of EOV (Figure 26). An NP-40 non-extractable sperm protein of molecular weight of 83kDa showed a significant increase in tyrosine phosphorylation when capacitated in the presence of EOV at each time point (Figure 27). Capacitation in the presence of EOV did not result in a significant increase in tyrosine phosphorylation for the NP-40 non-extractable 70kDa sperm protein (Figure 28).

Density analysis of tyrosine phosphorylated sperm proteins enhanced in the presence of EOV from the NP-40 extractable fraction was performed to determine the mean density (± SEM and p<0.05) of three representative Western blots. A NP-40 extractable tyrosine phosphorylated protein of molecular weight of 25kDa showed a significant increase in density of immunolabeling when sperm were capacitated in the presence of EOV at 5-, 60- and 180-minutes (Figure 29). No significant difference in labeling density was found in the 25kDa NP-40 extractable protein from sperm after capacitation for 120-minutes either in the presence or absence of EOV (Figure 29).
Density analysis of the tyrosine phosphorylated NP-40 non-extractable proteins showed a significant increase in density of immunolabeling of a 37kDa protein after 5- and 60-minutes of capacitation in the presence of EOV (Figure 30). Interestingly, there was no significant increase in density of the 37kDa protein capacitated in the presence of EOV after 120- and 180-minutes of incubation. A NP-40 extractable tyrosine phosphorylated 44kDa sperm protein also showed an increase in density after 5-, 60- and 180-minutes of capacitation in the presence of EOV (Figure 31). However, after 120-minutes of capacitation in the absence of EOV the NP-40 extractable tyrosine phosphorylated 44kDa protein showed a significantly higher density than the corresponding 44kDa NP-40 extractable protein from sperm incubated in the presence of EOV.

3.10 Immunoprecipitation of Tyrosine Phosphorylated Sperm Proteins for Mass Spectrophotometry

Western blots of NP-40 non-extractable (Figure 32) and NP-40 extractable (Figure 33) fraction revealed the presence of six major bands that appear to have undergone tyrosine phosphorylation. An array of tyrosine phosphorylated sperm proteins was observed after Western blot analysis but only those which showed an enhancement after incubation in the presence of EOV were chosen for mass spectrophotometry (MS) analysis. The bands selected for identification were obtained by immunoprecipitation of the tyrosine phosphorylated proteins from each fraction. As shown in Table 1, peptide sequences from a total of three major phosphorylated protein bands from the NP-40 non-extractable fraction designated I70, I83 and I90 (Figure 32) and the NP-40 extractable fraction designated S25, S37 and S44 (Figure 33) were used to search the NCBI database and matches to the following proteins were obtained: enolase 1 variant (accession no.
BAD96237.1), ATP-specific succinyl-CoA synthetase beta subunit (AAC64398), Succinate-CoA ligase, GDP-forming, alpha subunit (XP_532985), zona pellucida binding protein (NP_056600), surface sperm protein (p26h, AAD03695), heat shock protein 90 (P11499), hexokinase (AAA37804), aconitase 2 (NP_542364) and serum albumin precursor (P02769). A total of nine proteins from the excised bands were identified by MS, digested and sequenced from the 10% SDS-PAGE gel containing NP-40 extractable and NP-40 non-extractable sperm proteins. These proteins were identified by MALDI QqTOF MS. The results of the mass spectrophotometry data (Appendix A) are summarized in Table 1 and Table 2.

Table 1: Molecular Masses analyzed and identified by search of NCBI database with MASCOT program for each SDS-PAGE band of interest

<table>
<thead>
<tr>
<th>Band</th>
<th>Expected Mol. Mass (kDa)</th>
<th>Accession No.</th>
<th>Protein MW (kDa)</th>
<th>% Sequence Covered</th>
<th>No. Significant Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>S44a</td>
<td>44</td>
<td>BAD96237.1</td>
<td>Enolase 1 variant</td>
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<td>51.6</td>
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<tr>
<td>S44b</td>
<td>44</td>
<td>AAC64398</td>
<td>ATP-specific succinyl-CoA synthetase beta subunit</td>
<td>46</td>
<td>6.1</td>
</tr>
<tr>
<td>S37a</td>
<td>37</td>
<td>XP_532985</td>
<td>Succinate-CoA ligase, GDP-forming, alpha subunit</td>
<td>34.9</td>
<td>9</td>
</tr>
<tr>
<td>S37b</td>
<td>37</td>
<td>NP_056600</td>
<td>Zona pellucida binding protein</td>
<td>44.9</td>
<td>3.9</td>
</tr>
<tr>
<td>S25</td>
<td>25</td>
<td>AAD03695</td>
<td>Surface sperm protein p26h</td>
<td>25.7</td>
<td>36</td>
</tr>
<tr>
<td>I90a</td>
<td>90</td>
<td>P11499</td>
<td>Heat Shock Protein 90-beta Hexokinase</td>
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<td>25.4</td>
</tr>
<tr>
<td>I90b</td>
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<td>AAA37804</td>
<td>Aconitase 2, mitochondrial</td>
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<td>17.9</td>
</tr>
<tr>
<td>I83</td>
<td>83</td>
<td>NP_542364</td>
<td>Aconitase 2, mitochondrial</td>
<td>85.5</td>
<td>24.2</td>
</tr>
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</table>
Table 2: Hamster sperm proteins identified by Mass Spectrophotometry

<table>
<thead>
<tr>
<th>Band ID</th>
<th>Protein Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>S25</td>
<td>Surface sperm protein, p26h</td>
<td>Epididymal sperm maturation</td>
</tr>
<tr>
<td>S37a</td>
<td>Succinate-CoA ligase, GDP-forming, alpha subunit</td>
<td>Kreb cycle, cell metabolism</td>
</tr>
<tr>
<td>S37b</td>
<td>Zona pellucida binding protein</td>
<td>Normal morphology and acrosome compaction</td>
</tr>
<tr>
<td>S44a</td>
<td>Enolase 1 variant</td>
<td>Kreb cycle, cell metabolism</td>
</tr>
<tr>
<td>S44b</td>
<td>ATP-specific succinyl-CoA synthetase beta subunit</td>
<td>Kreb cycle, cell metabolism</td>
</tr>
<tr>
<td>I70</td>
<td>Serum albumin precursor</td>
<td>Blood contamination</td>
</tr>
<tr>
<td>I83</td>
<td>Aconitase 2, mitochondrial</td>
<td>Kreb cycle, cell metabolism</td>
</tr>
<tr>
<td>I90a</td>
<td>Heat Shock Protein 90-beta</td>
<td>Molecular chaperone protein</td>
</tr>
<tr>
<td>I90b</td>
<td>Hexokinase</td>
<td>Glycolytic pathway, cell metabolism</td>
</tr>
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Figure 7: Western blot analysis of HPA-purified oviductin from the estrus stage (EOV) of the estrous cycle. Anti-hamster oviductin monoclonal antibody 4.12 F6 was used for immunolabeling followed by incubation with RAM-HRP. A high-molecular-weight band ranging from ~160-350kDa was revealed.
Figure 8: Western blot analysis of EOV-sperm binding and loading control with monoclonal anti-α-tubulin antibody. A) Western blot of whole sperm protein extracts prepared from sperm after capacitation for 5-, 60-, 120- and 180-minutes in the presence (+EOV) or absence (-EOV) of EOV. Immunolabeling using monoclonal anti-hamster oviductin antibody 4.12 F6 followed by incubation with RAM-HRP. B) Western blot of whole sperm protein extracts prepared from sperm after capacitation for 5-, 60-, 120- and 180-minutes in the presence or absence of EOV. Immunolabeling using monoclonal anti-α-tubulin antibody followed by incubation with RAM-HRP reveals a 52kDa band (arrow) of consistent immunostaining intensity under different capacitation conditions, confirming equal loading of whole sperm protein extracts shown in Figure 16A.
Figure 9: Immunostaining of tyrosine phosphorylated sperm proteins during capacitation in the presence or absence of 60µg/mL EOV using indirect immunofluorescent microscopy. Caudal epididymal sperm cells were incubated for 5- and 180-minutes in the presence or absence of EOV then exposed to monoclonal anti-phosphotyrosine antibody followed by incubation with and FITC-GAM (X 200).
Figure 10: Confocal microscopy of immunostaining of tyrosine phosphorylated sperm proteins during capacitation in the presence or absence of 60µg/mL EOV. Confocal microscopic imaging of non-permeablized caudal epididymal hamster sperm shows the appearance of tyrosine phosphorylated sperm proteins over the plasma membrane of the equatorial segment of the sperm and the midpeice of the sperm tail. The intensity of the immunoreaction increased overtime and also increased in the presence of EOV in both the 5- and 180-minute samples (X 1000). Column 1: phase contrast confocal images; Column 2: epifluorescence confocal images; Column 3: merge of phase contrast and epifluorescence (FITC) confocal images. Scale bar = 30µm.
Figure 11: Confocal microscopic imaging of immunostaining of tyrosine phosphorylated sperm proteins after capacitation for 5- and 180-minutes. Permeabilization of the caudal epididymal sperm was performed using 0.2% Triton-X-100 (X 1000). Column 1: phase contrast confocal images; Column 2: epifluorescence confocal images; Column 3: merge of phase contrast and epifluorescence (FITC) confocal images. Scale bar = 30µm.
Figure 12: Confocal microscopic imaging of immunostaining of tyrosine phosphorylated sperm proteins after capacitation for 5- and 180-minutes. A) Permeabilization of the caudal epididymal sperm was performed using 1% NP-40 (X 1000). Column 1: phase contrast confocal images; Column 2: epifluorescence confocal images; Column 3: merge of phase contrast and epifluorescence (FITC) confocal images. Scale bar = 30µm. B) Confocal image of high magnification of the sperm heads showing tyrosine phosphorylated proteins localized to the equatorial segment of the sperm head after 5-minutes of incubation in capacitation media (X 3000). Scale bar = 9.29µm.
A) FITC Overlay

5 min

180 min

B) Overlay BF FITC

*
Figure 13: Localization of tyrosine phosphorylated sperm proteins to the sperm head by electron microscopy. Sperm cells were capacitated for 5- and 180-minutes in the presence or absence of 60µg/mL of EOV. After capacitation the cells were incubated in monoclonal anti-phosphotyrosine antibody clone 4G10 (1: 500) then exposed to protein-A-gold (10nm). Longitudinal sections showing protein-A-gold labeling of the tyrosine phosphorylated proteins of the sperm head equatorial segment (arrows); A) 5min/EOV- (X10,000); B) 5min/EOV+ (X15,000); C) 180min/EOV- (X10,000); D) 180min/EOV+ (X15,000).
Figure 14: Ultrastructural localization of tyrosine phosphorylated sperm proteins in the midpeice by electron microscopy. Caudal epididymal hamster sperm were capacitated in the presence or absence of 60µg/mL of EOV for 5- and 180-minutes. After capacitation the cells were incubated with monoclonal anti-phosphotyrosine antibody (1:500) then exposed to protein-A-gold (10nm). Ultrastructural localization of tyrosine phosphorylated proteins with protein-A-gold (open arrows) A) Cross-sections of the sperm midpeice after 5-minutes of capacitation; 5min/EOV- (X15,000); B) Cross-sections of the sperm midpeice; 5min/EOV+ (X15,000); C) Longitudinal section of the sperm midpeice; 180min/EOV- (X10,000; insert: increased magnification- X15,000); D) Longitudinal section of the sperm midpeice; 180min/EOV+ (X12,000; insert: cross section of midpeice- X 17,000).
Figures 15: Control samples, using protein A-gold without monoclonal antiphosphotyrosine antibody, showing the absence of immunolabeling in the midpiece of the sperm tails (A, X8,000) or at the equatorial segment region of the sperm head (B, X10,000) after 5- and 180-minutes (A and B respectively) incubation in capacitation medium.
Figure 16: Surface replicas showing the en-face view of the distribution of immunogold labeling of tyrosine phosphorylated sperm proteins at 5-minutes of capacitation in the absence or presence of EOV. A) Surface replica of protein A-colloidal gold labeling of tyrosine phosphorylated proteins in the equatorial segment at 5-minutes of capacitation in the absence of 60µg/mL EOV (X12,000). B) High magnification of the boxed region shown in (A) (X18,200). C) Capacitation for 5-minutes in the presence of 60µg/mL EOV (X 9,000). D) High magnification of the boxed region shown in (C) (X19,800). Ac= acrosomal cap; Eq= equatorial segment; Pa= post-acrosomal sheath.
Figure 17: Surface replicas showing the *en-face* view of the distribution of immunogold labeling of tyrosine phosphorylated sperm proteins after 180-minutes of capacitation in the absence or presence of EOV. A) Surface replica of protein-A-colloidal gold labeling of tyrosine phosphorylated proteins in the equatorial segment after 180-minutes of capacitation in the absence of 60µg/mL EOV (X15,000). B) High magnification of the boxed region shown in (A). (X37,500). C) Capacitation for 180-minutes in the presence of 60µg/mL EOV (X8,000). D) High magnification of the boxed region shown in (C) (X18,000). Ac- acrosomal cap; Eq- equatorial segment; Pa- postacrosomal sheath.
Figure 18: Control surface replica sample showing only a few gold particles at the background level in the equatorial segment region (Eq) of the sperm head after 180-minutes of capacitation in the presence of 60µg/mL EOV. Sperm were capacitated for 5- and 180-minutes in the presence or absence of EOV and exposed to proteinA-colloidal gold only. This representative surface replica (180min/EOV+) displays the sperm head including the acrosomal cap (Ac), equatorial segment region (Eq) and postacrosomal sheath (Pa) (X12,000).
Figure 19: Surface replicas showing the en-face view of the distribution of immunogold labeling of tyrosine phosphorylated sperm proteins in the midpeice of the sperm tail. Sperm cells were capacitated for 5- and 180-minutes in the presence or absence of 60µg/mL EOV and incubated with monoclonal anti-phosphotyrosine antibody clone 4G10 then exposed to protein-A-colloidal gold. (A) 5min/EOV- (X12,000), B) 5min/EOV+ (X15,000); C) 180min/EOV- (X 15,000); D) 180min/EOV+ (X20,000).
Figure 20: Control surface replica showing the absence of immunogold particles over the midpiece when sperm capacitated for 180-minutes in the presence of EOV were labeled with protein-A-colloidal gold alone (X15,000). pp: principal piece; mp: midpiece.
Figure 21: Western blot of non-extractable tyrosine phosphorylated hamster sperm proteins. Comparison of the 1% NP-40 and sonication extraction technique using monoclonal anti-phosphotyrosine antibody clone 4G10. Immunoblot of caudal epididymal hamster sperm after 5-, 60-, 120- and 180-minutes of capacitation. Lanes 1-4 show the non-extractable tyrosine phosphorylated proteins prepared with the 1% NP-40 technique. Lanes 5-8 show the non-extractable tyrosine phosphorylated proteins prepared with the sonication technique.
Figure 22: Western blot of extractable tyrosine phosphorylated hamster sperm proteins. Comparison of the 1% NP-40 and sonication extraction technique using anti-phosphotyrosine mAb, clone 4G10. Immunoblot of caudal epididymal hamster sperm after 5-, 60-, 120- and 180-minutes of capacitation. Lanes 1–4 show the extractable tyrosine phosphorylated proteins prepared with the 1% NP-40 technique. Lanes 5–8 show extractable tyrosine phosphorylated proteins prepared with the sonication technique.
Figure 23: Western blot analysis of tyrosine phosphorylated proteins from whole sperm incubated in non-supportive capacitation medium. Sperm cells were incubated in non-supportive capacitation medium (NCM) for 5-, 60-, 120- and 180-minutes. Immunolabeling with monoclonal anti-phosphotyrosine antibody, clone 4G10 followed by incubation with RAM-HRP revealed that the time-dependent increase in tyrosine phosphorylation does not occur in NCM. Supplementing NCM with EOV did not have any effect (results not shown).
Figure 24: Western blot analysis examining the effect of estrus stage affinity-purified oviductin (60µg/mL) on protein tyrosine phosphorylation of 1% NP-40 non-extractable hamster caudal epididymal sperm proteins. Sperm cells were previously capacitated in the presence (+EOV) or absence (-EOV) of EOV for 5-, 60-, 120- and 180-minutes. Immunolabeling with monoclonal anti-phosphotyrosine antibody clone 4G10 followed by incubation with RAM-HRP revealed numerous bands. Three bands (arrows) of molecular weight of approximately 70, 83 and 90kDa showed an increase in intensity when incubated in the presence of EOV. NP-40 non-extractable proteins displaying dephosphorylation during capacitation in the presence of EOV are within the box outlined in red.
Figure 25: Western blot analysis examining the effect of estrus stage affinity-purified oviductin (60µg/mL) on protein tyrosine phosphorylation of 1% NP-40 extractable hamster caudal epididymal sperm proteins. Sperm cells were previously capacitated in the presence (+EOV) or absence (-EOV) of EOV for 5-, 60-, 120- and 180-minutes. Immunolabeling with monoclonal anti-phosphotyrosine antibody clone 4G10 followed by incubation with RAM-HRP revealed numerous bands. Three bands (arrows) of molecular weight of approximately 25, 37 and 44kDa showed an increase in intensity when incubated in the presence of EOV.
Figure 26: Histogram showing the mean density (± SEM) of tyrosine phosphorylated NP-40 non-extractable sperm proteins of molecular weight of approximately 90kDa after 5-, 60-, 120- and 180-minutes of capacitation in the presence or absence of 60µg/mL EOV. This density analysis corresponds to Figure 24. ( *= p<0.05)
Figure 27: Histogram showing the mean density (± SEM) of tyrosine phosphorylated NP-40 non-extractable sperm proteins of molecular weight of approximately 83kDa after 5-, 60-, 120- and 180-minutes of capacitation in the presence or absence of 60µg/mL EOV. This density analysis corresponds to Figure 24. (*= p<0.05)
Figure 28: Histogram showing the mean density (± SEM) of tyrosine phosphorylated NP-40 non-extractable sperm proteins of molecular weight of approximately 70kDa after 5-, 60-, 120- and 180-minutes of capacitation in the presence or absence of 60µg/mL EOV. This density analysis corresponds to Figure 24. (*= p<0.05)
Figure 29: Histogram showing the mean density (± SEM) of tyrosine phosphorylated NP-40 extractable sperm proteins of molecular weight of approximately 25kDa after 5-, 60-, 120- and 180-minutes of capacitation in the presence or absence of 60µg/mL EOV. This density analysis corresponds to Figure 25. (*= p<0.05)
Figure 30: Histogram showing the mean density (± SEM) of tyrosine phosphorylated NP-extractable sperm proteins of molecular weight of approximately 37kDa after 5-, 60-, 120- and 180-minutes of capacitation in the presence or absence of 60µg/mL EOV. This density analysis corresponds to Figure 25. (*= p<0.05)
Figure 31: Histogram showing the mean density (± SEM) of tyrosine phosphorylated NP-40 extractable sperm proteins of molecular weight of approximately 44kDa after 5-, 60-, 120- and 180-minutes of capacitation in the presence or absence of 60µg/mL EOV. This density analysis corresponds to Figure 25. (* = p<0.05)
Figure 32: Immunopurification of monoclonal anti-phosphotyrosine antibody, clone 4G10-reactive NP-40 non-extractable proteins from caudal epididymal hamster sperm after 180-minutes capacitation in the absence of EOV. Coomassie Brilliant Blue staining showing the NP-40 non-extractable protein profile. Lane 1: Molecular weight standard; Lane 2: Whole 1% NP-40 non-extractable sperm protein extract (whole); Lane 3: Tyrosine phosphorylated immunoprecipitated (IP) NP-40 non-extractable proteins; Lane 4: Flow through (FT) containing the remaining proteins of the whole NP-40 non-extractable fraction that were not precipitated out using monoclonal anti-phosphotyrosine antibody, clone 4G10. The bands chosen for mass spectrophotometry analysis are indicated by arrows.
Figure 33: Immunopurification of monoclonal anti-phosphotyrosine antibody, clone 4G10-reactive NP-40 extractable proteins from caudal epididymal hamster sperm after 180-minutes capacitation in the absence of EOV. Coomassie Brilliant Blue staining showing the NP-40 extractable protein profile. Lane 1: Molecular weight standard; Lane 2: Whole 1% NP-40 extractable sperm protein extract (whole); Lane 3: Flow through (FT) containing the remaining proteins of the whole NP-40 extractable fraction that were not precipitated out using monoclonal anti-phosphotyrosine antibody, clone 4G10; Lane 4: Tyrosine phosphorylated immunoprecipitated (IP) NP-40 extractable proteins. The bands chosen for mass spectrophotometry analysis are indicated by arrows.
CHAPTER 4.0 DISCUSSION

This work provided information on the localization of tyrosine phosphorylated sperm proteins as hamster sperm undergo the biochemical and biophysical changes associated with capacitation. In the present study we observed that proteins in the equatorial segment region of the sperm head and the midpiece of the sperm tail undergo post-translational modifications through phosphorylation of tyrosine residues. We are the first to provide evidence that the time-dependent increase in tyrosine phosphorylation is enhanced when sperm are capacitated in the presence of homologous oviductin, an oviduct-specific glycoprotein which was shown to bind to the sperm head (Kan and Esperanzate, 2006) and increase sperm viability and motility (Abe et al., 1995) as well as sperm-egg binding (King et al., 1994). We also provided preliminary information on the identification of several tyrosine phosphorylated proteins that undergo enhancement when sperm are capacitated in the presence of oviductin.

In the present investigation we supplemented capacitation supportive medium with affinity-purified hamster oviductin from the estrus stage of the estrous cycle for several reasons. First, surface mapping of oviductin binding to sperm during in vitro capacitation revealed binding of oviductin to the acrosomal cap and the area of the postacrosomal sheath and this labeling appeared to be more intense in capacitated sperm (Kan and Esperanzate, 2006). Secondly, ovulation occurs during the estrus stage and previous studies performed in our laboratory found that estrus stage-specific oviductin (EOV) is fully glycosylated (McBride et al., 2004). The basis of the experiment was to create an in vitro environment that closely mimicked the in vivo environment of the female reproductive tract at the time of ovulation in order to elucidate the impact of
oviductin on sperm capacitation by measuring changes in protein tyrosine phosphorylation (an indicator of capacitation).

In the present study and in earlier studies carried out in our laboratory it was shown that sperm incubated in non-supportive capacitation medium do not display the characteristic time-dependent increase in tyrosine phosphorylation. The addition of oviductin to the non-supportive capacitation medium did not initiate or amplify the level of sperm capacitation. In earlier studies and in the present investigation, a medium that was supportive of capacitation was used and sperm cells displayed the characteristic time-dependent increase in tyrosine phosphorylation. Using immunofluorescence and the surface replica labeling technique, we showed that protein tyrosine phosphorylation distribution in the sperm head is restricted to the equatorial segment region throughout the capacitation process. The equatorial segment region of the sperm head is a specialized area as it retains its integrity after the acrosome reaction which is necessary for subsequent fusion with the oolemma of the egg (Yanagamachi and Noda, 1970). The plasma membrane overlying the equatorial segment is distinguishable from the plasma membrane overlying other regions of the acrosome. After the acrosome reaction, the integrity of the plasma membrane over the equatorial segment is retained to allow initial fusion with the oolemma of the egg to occur. In the present investigation immunostaining of phosphotyrosine in sperm cells after treatment with non-ionic detergents (Triton X-100 and NP-40) to remove the plasma membrane revealed that tyrosine phosphorylation of proteins in the equatorial segment region was associated with structural features underlying the plasma membrane such as the outer acrosomal membrane. This was confirmed by immunogold labeling with monoclonal anti-phosphotyrosine antibody which localized tyrosine phosphorylated proteins to the region of the outer acrosomal
membrane. These observations indicate that tyrosine phosphorylation is present in the plasma membrane overlying the equatorial segment region and possibly associated with the outer acrosomal membrane. In support of this finding, Jones et al. (2008) have recently localized tyrosine phosphorylated proteins to the outer acrosomal membrane in the equatorial segment region of the bull, boar and ram. They also provided preliminary evidence of a similar labeling pattern in the rat and mouse. However, we cannot exclude the possibility that tyrosine phosphorylated proteins detected in the equatorial segment region underlying the plasma membrane could be associated with the outer periacrosomal layer which is compositionally continuous with the subacrosomal layer of the perinuclear theca (for reviews see Oko et al. 1991; Oko and Maravei, 1994). Post-embedding immunogold labeling could be used in the future to explore the possibility whether tyrosine phosphorylation also occurs in the subacrosomal region. Having established the locality of tyrosine phosphorylation in the sperm head we were able to enhance tyrosine phosphorylation of sperm proteins by the addition of estrus stage-purified oviductin in chemically defined medium supportive of capacitation.

In our study, the samples from the initial time point involved exposure of sperm in a capacitation medium with or without the presence of oviductin for as short as 5-minutes. Whole sperm capacitated in non-supportive capacitation medium showed the presence of endogenous phosphorylation which was also observed by Tardiff et al. (1999). These samples were used as a non-capacitated control. Incubation of sperm in a capacitation supportive medium lacking oviductin showed tyrosine phosphorylation of sperm proteins. Interestingly, an increase in tyrosine phosphorylation of sperm incubated in medium containing oviductin was observed instantly indicating that addition of this glycoprotein had an immediate enhancing effect on tyrosine phosphorylation of the
proteins sequestered in the equatorial segment region. Chang (1984) defined sperm capacitation as the time interval of in vitro sperm incubation that is required to bring about the final functional maturation of sperm. Our findings suggest that the maturational changes associated with the capacitation process begin immediately upon exposure to in vitro capacitating conditions. In other words, tyrosine phosphorylation of sperm proteins occurs more so in capacitation medium supplemented with oviductin than in the standard capacitation supporting medium that is currently utilized.

Using immunofluorescence we showed that phosphotyrosine labeling is present in the sperm tail during the later stages of sperm capacitation. In vitro capacitation for 180-minutes revealed tyrosine phosphorylation along the midpiece and principal piece which was not observed at the earlier time points. Immunofluorescence was found to be most intense along the midpiece and the labeling intensity was further increased when sperm were capacitated in the presence of oviductin. In the present study, immunogold labeling of capacitated sperm showed that gold particles were not present on the plasma membrane overlying the midpiece but on the mitochondria where the overlying plasma membrane had been disrupted or removed. The midpiece of the sperm tail is heavily packed with mitochondria which provide energy to the cell in the form of ATP. The Kreb cycle, which generates ATP, involves tyrosine phosphorylation of structural proteins of the mitochondrial capsule, such as phospholipid hydroperoxide glutathione peroxidase (NagDas et al., 2005). This premise is supported by observations made in the current investigation of a time-dependent increase in phosphotyrosine labeling of mitochondria in the tail midpiece. Sperm capacitated in the presence of EOV significantly increased the phosphotyrosine labeling. Recent studies have provided evidence indicating that mitochondrial proteins undergo post-translation phosphorylation on tyrosine residues
Members of the Src kinase family (Salvi et al., 2002) and the tyrosine phosphatase Shp-2 (Salvi et al., 2004) were the first tyrosine kinases and phosphatases shown to be located in this organelle indicating that tyrosine phosphorylation is involved in mitochondrial ATP production. Taken together, these findings and results obtained in the present investigation indicate that tyrosine phosphorylation may be involved in ATP production in sperm and that oviductin could play a role in mediating the efficiency of the Kreb’s cycle through tyrosine phosphorylation of mitochondrial proteins during sperm capacitation.

Because sperm are such highly compartmentalized cells, it was decided to treat these cells with non-ionic detergent NP-40 or with sonication at various intervals during capacitation in vitro. After localization of the tyrosine phosphorylated sperm proteins that appear during capacitation, we next focused on determining the quantity and identity of these proteins. These extraction techniques were applied in order to analyze tyrosine phosphorylation of extractable and non-extractable sperm proteins separately. Western blot analysis of the extractable and non-extractable tyrosine phosphorylated sperm proteins after treatment of the cells with NP-40 or sonication (after capacitation for 5-, 60-, 120- and 180-minutes) revealed that NP-40 is capable of extracting a wider variety of proteins compared to the sonication method. This was confirmed by protein assay of the extractable fractions (results not shown). Exposure of sperm to the non-ionic detergent serves as a chemical dissection capable of solubilizing membranes and hence releasing proteins from these membranes. In the case of sperm, NP-40 solubilizes the plasma membrane and outer and inner acrosomal membranes releasing the acrosomal contents. It also partially solubilizes the nuclear envelope and the mitochondrial sheath presumably releasing some proteins from these membrane structures. It should be emphasized that the
majority of sperm proteins are cytoskeletal in nature and thus resistant to NP-40 extraction (Oko and Maravei, 1994; Oko, 1995). Sonication on the other hand is normally used to break the junction between the sperm head and tail but in doing so removes the plasma membrane and the outer acrosomal membrane from the head releasing the contents of the acrosome (Mountjoy et al., 2008).

Immunoblotting of the NP-40 and sonication non-extractable sperm proteins revealed the characteristic time-dependent increase in tyrosine phosphorylation. However, the NP-40 and sonication extractable fractions showed some variability between the two extraction techniques which was expected as NP-40 extracts more membranous components than sonication. Proteins with a molecular weight ranging from 60kDa to 83kDa were seen in both the NP-40 and sonication extractable fractions. Western Blot analysis of the NP-40 extractable fraction revealed the presence of one other protein with an approximate molecular weight of 15kDa. It is possible that the 15kDa protein originates from the mitochondrial sheath as it appeared to be the only protein absent in the sonication extractable fraction. The proteins with a higher molecular weight from the NP-40 extractable fraction displayed a less intense phosphotyrosine signal than their counterparts in the sonication extractable fraction. This diminution in intensity could have been caused by a protein dilution effect as the NP-40 extractable fraction most likely contained a more diverse protein profile compared to the sonication fraction. For the present investigation we decided to use the NP-40 extraction method due the consistence of its reproducibility.

The proteins of the NP-40 non-extractable fraction displayed the characteristic time-dependent increases in tyrosine phosphorylation when sperm were incubated with or without EOV. The 70, 83 and 90kDa bands underwent an enhancement in tyrosine
phosphorylation when sperm were incubated in the presence of EOV. Immunoblotting of NP-40 extractable proteins also revealed a time-dependent increase in protein tyrosine phosphorylation, however, samples incubated in supportive capacitating medium alone showed a peak in tyrosine phosphorylation only after two hours of capacitation. In contrast, when these extractable sperm samples were incubated in supportive capacitating medium with the addition of EOV, a peak in tyrosine phosphorylation appeared after only one hour of capacitation. This implies that the addition of oviductin to the capacitating medium stimulates an earlier onset of the signal transduction events associated with capacitation. Currently the mechanism by which oviductin enhances tyrosine phosphorylation is unknown and further investigation is necessary to elucidate the mechanisms underlying the relationship between oviductin and the events associated with capacitation. Density analysis of the bands displaying an increase in phosphotyrosine reactivity revealed that a significant increase in tyrosine phosphorylation occurs even after only 5-minutes of incubation in capacitation medium supplemented with EOV. This is an important finding as it shows the immediate enhancing effect that oviductin has on sperm upon initial exposure to the glycoprotein.

Interestingly, there were also some NP-40 non-extractable proteins that displayed a decrease in tyrosine phosphorylation when the cells were capacitated in the presence of EOV. Affected polypeptides of ~29 and 37kDa suggest that dephosphorylation of proteins may be a common occurrence. This decrease in immunoreaction is markedly less in sperm capacitated in the presence of EOV. Knowing that capacitation does occur in vitro without the need for biological fluids from the female reproductive tract leads us to believe that capacitation and the events associated with this process (i.e. tyrosine phosphorylation) are controlled intrinsically by sperm itself as long as specific requirements are met. This
intrinsic control may involve a series of phosphorylation and dephosphorylation which would mediate the preparedness and precise timing required for successful sperm-egg interaction, acrosome reaction and subsequent fertilization. There are few studies that address the incidence of dephosphorylation of sperm proteins during capacitation, however, it is also known that tyrosine phosphorylation is reversible so long as induction of the acrosome reaction has not occurred (Bedu-Addo et al., 2005). As mentioned earlier, we are not aware of the mechanisms underling the enhancing effect that oviductin has on capacitation-associated tyrosine phosphorylation or dephosphorylation but there have been studies performed on human sperm establishing that motility of ejaculated sperm is improved by the addition of phosphatase inhibitors (Leclerc et al, 1996). Huang et al. (2004) have shown that compounds similar to phosphatase inhibitors initiated motility in epididymal bovine sperm which suggests low protein kinase and high protein phosphatase activities may control motility in these cells (Huang et al., 2004). It may also be considered that oviductin may somehow activate components of the capacitation-related signaling pathway such as adenylyl cyclase, cAMP, PKA or inhibit phosphoprotein phosphatase. Therefore, the effect of oviductin on those molecules that regulate capacitation-associated tyrosine phosphorylation should be the focus of future studies addressing the role of oviductin in signal transduction events.

In order to further elucidate the role of oviductin (OV) during sperm capacitation, immunoprecipitation of the proteins showing an increased density when capacitated in the presence of OV was performed. We excised six immunoprecipitated proteins from the 10% SDS-PAGE gel of the NP-40 non-extractable (I70, I83 and I90) and extractable (S25, S37 and S44) fraction and identification by MALDI-TOF MS analysis was performed. The functional distribution of the six bands is shown in Table 2. In the present
investigation we used one-dimensional SDS-PAGE and immunoprecipitation to identify
the proteins showing enhancement in tyrosine phosphorylation when capacitated in the
presence of oviductin. Using this method we found that more than one protein can be
identified from one single band. In future studies in our lab, two-dimensional SDS-PAGE
will be used to circumvent this problem. Because of the limitations involved in
identification of phosphorylated proteins using electrophoresis and immunoblotting, new
emerging technologies are being developed. The use of immobilized metal affinity
chromatography (IMAC) prior to mass spectrophotometry analysis to increase the
selectivity for phosphopeptides is one technique currently employed (Ficarro et al., 2003).
The proteomic analysis presented here is preliminary and future studies will be required
to analyze these proteins further. Because of the limitations in proteomic analysis of
tyrosine phosphorylated sperm proteins, the proteins identified here are only to be
considered for future study of capacitation-associated tyrosine phosphorylation.

In the present study the majority of the group of identified proteins corresponds to
proteins involved in energy production. Previous in vitro studies have strongly suggested
that the main contribution of ATP required for sperm motility is through the citric acid
cycle (Storey and Kayne, 1975). A total of nine identifications were made and four of
these identified proteins are known to be involved in the Kreb’s cycle; 1) enolase 1
variant, 2) ATP-specific succinyl CoA synthetase, beta subunit, 3) succinate-CoA ligase,
GDP-forming alpha-subunit and 4) aconitase. Because of the aforementioned lack of
sensitivity and difficulties in identifying proteins undergoing phosphorylation we cannot
be certain that the four latter proteins are all phosphorylated on tyrosine residues.
However, potential tyrosine phosphorylation sites were determined using PROSCAN
(http://npsa-pbil.ibcp.fr/cgi_bin/npsa_automat.pl?page=/NPSA/npsa_proscan.html) with a
similarity level set at 100%. ATP-specific succinyl CoA synthetase beta subunit was the only peptide of the four identifications involved in the Kreb’s cycle that showed the presence of a potential tyrosine phosphorylation site (RDNDKTRY and KSPDDPSRY).

Recent in vivo studies showed that glycolysis at the level of the fibrous sheath is essential for mouse sperm function (Miki at al., 2004). Hexokinase is the first enzyme in the glycolytic pathway and a key regulator of glycolysis (Wilson, 1995) meaning that increased hexokinase activity may enhance the rate of glycolysis and ATP production. Analysis of the mass spectrophotometry generated sequence for hexokinase using PROSCAN revealed one tyrosine phosphorylation site (KKIDKYLY). However, Naz et al. (1996) reported contradictory evidence claiming that sperm hexokinase is a glycoprotein that is not phosphorylated at tyrosine residues.

One out of the nine identifications was found to be serum albumin precursor and it did not show a significant increase in density although visual analysis suggested there was an increase in tyrosine phosphorylation when exposed to EOV during capacitation. This is most likely due to the fact that this protein often appears as a result of blood contamination of the sample (personal communication with Dr. Y. She). In the present study, using MALDI-TOF MS analysis, the molecular chaperone protein, heat shock protein (HSP)-90 was identified. Cross-immunoprecipitation experiments confirm that HSP-90 is tyrosine phosphorylated (Ecroyd et al., 2003) and results from PROSCAN identify the tyrosine phosphorylation sites at RGFEVY, KHNDEFQY, RLSLEEY, KHNDEFQY, KRGFEVY and RRLSELLRY. HSP-90 has been shown to be a crucial component of a variety of signaling pathways where it acts together with other components to ensure the correct folding of proteins (Ecroyd et al., 2003). Since the capacitation signaling pathway is mediated through cAMP and PKA (Visconti et al.,
1995a and Visconti et al., 1999) the involvement of protein tyrosine phosphorylation is suggested. Therefore HSP-90 has the potential to control tyrosine kinase signaling in the pathway leading to the overall augmentation in protein tyrosine phosphorylation in the capacitation process. In boar sperm, HSP-90 has also been implicated in playing a role in sperm motility (Huang et al., 2000) and this notion is supported by MALDI-TOF MS analysis which identified HSP-90 in the NP-40 non-extractable protein fraction containing the cytoskeletal elements of the tail. Although evidence suggesting the involvement of HSP-90 in sperm motility is accumulating, its involvement in the signaling events associated with capacitation remains elusive.

Sperm surface protein, p26h is known to be abundant in the luminal fluid of the proximal region of the hamster epididymis (Robitaille et al., 1991). P26h accumulates on sperm during epididymal maturation (Sullivan and Robitaille, 1989) and is exclusively localized to the covering of the acrosomal cap of mature sperm cells, the domain involved in ZP binding and fertilization related events (Bérubé and Sullivan, 1994). Another protein identified in the present investigation was zona pellucida binding protein (Zpbp). This protein has been localized to the acrosomal cap and is named for its binding to the oocyte zona pellucida. Lin et al. (2007) studied the in vivo functions of this protein using male mice lacking Zpbp1 and Zpbp2 (Zpbp1’s novel paralog). Zpbp1-null males were sterile with abnormal sperm head morphology and no forward sperm motility (Lin et al., 2007). Also, ultrastructural studies showed that Zpbp1 is required for proper acrosome compaction (Lin et al., 2007). Zpbp2-null mice were subfertile and produced dysmorphic sperm with reduced ability to penetrate the ZP. Since this protein is localized exclusively to the sperm head and this is also the site of OV-sperm binding, it is possible that tyrosine
phosphorylation of this protein is enhanced in the presence of EOV in capacitation medium.

Although the requirements for *in vitro* sperm capacitation have been well described for some time (Visconti et al., 1995a and b; Bavister, 1989), we report here for the first time that the addition of EOV to capacitating supportive medium has an enhancing effect on capacitation associated tyrosine phosphorylation and dephosphorylation. It appears that the inclusion of EOV in medium supporting capacitation initiates the events occurring during capacitation at a more efficient rate compared to *in vitro* capacitation in capacitating supportive medium alone. The addition of EOV to the capacitating supportive medium more accurately represents the milieu of the female reproductive tract *in vivo*. At this point the mechanism(s) by which oviductin enhances capacitation-associated tyrosine phosphorylation is not known. Oviductin may act to facilitate the action of BSA on the removal of cholesterol from the sperm plasma membrane, increasing membrane fluidity and permeability to Ca$^{2+}$ and HCO$_3^−$. Because activation of tyrosine kinases or phosphoprotein phosphatases are requirements of the capacitation signaling pathway, oviductin may play a role in mediating these events. Further studies to determine whether oviductin has a positive regulatory effect on capacitation-associated tyrosine phosphorylation are required and it would be interesting to see if affinity-purified oviductin from other stages of the estrous cycle, where oviductin is not fully glycosylated, has a similar enhancing effect on tyrosine phosphorylation during capacitation. In the future, studies focusing on changes in tail velocity (i.e. sperm hyperactivation) may provide further information on the ability of EOV to potentiate capacitation through protein tyrosine phosphorylation.
CONCLUSIONS AND CLOSING REMARKS

Throughout this investigation we observed an enhancing effect of oviductin on tyrosine phosphorylation of hamster sperm proteins during capacitation. The increase in tyrosine phosphorylation was especially strong in two of the sperm domains; 1) the equatorial segment region and 2) the midpiece of the tail. The equatorial segment region and the midpiece have long been thought to be involved in sperm-egg binding and sperm motility, respectively. The ability of oviductin to enhance the capacitation by potentiating tyrosine phosphorylation is a novel finding, however, additional work in this area determining the mechanism(s) mediating the oviductin-sperm interaction is required.
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APPENDIX A

Mass spectrum and sequences from analysis of the NP-40 extractable and non-extractable sperm proteins showing an enhancement in capacitation-associated tyrosine phosphorylation when incubated in the presence of oviductin (matching peptides are shown in red).

NP-40 extractable 25kDa protein

1 MKLNFTGLRA LVTGAGR GIG RGTAKALHAS GAVVAVSLI NEDLVSLAKE CPGIEPV CVD
61 LGDWATEKA LRIGPV DLLL VNNAAVALVQ PFILSTKEVF DRSFNVNVRS VLOVSQ MVAK
121 GMINR GVGAS IVNISMVAY VTFPGLATYS STKGAITMLT KAMAMELPY KIRVNSVNPT
181 VVLTDKGKV SADPEAKKL KERHRLRKFA EVEDVNSIL FLLSDSSAST SGSGILVDAG
241 YLAS

[Mass spectrum graph with peaks labeled and sequences shown in red highlights]
NP-40 extractable 37kDa protein

A

1 MASGSSGLAA ARLLRSFLLL QONGIRHCSY IASRKHLYVD KNTKVICQGF TGKQGTFHSQ
61 QALDYGTQLV GGTTPGKKGGK THLGLPVFNT VKEAKEETGA TASVIYVPPP FAAAAINEAV
121 EAEVPLVCI TEGIQOQDVMV RKKHKLLOIQG KTRLIGPNCGP GINPGECXI GIMPQIHIIK
181 GRIGIVRSTG TLTEYAVHTQ TVQVGLQGSLC VGIGGDPFNG TDFIDCLEIF LNDPATEGII
241 LIGEIGNAGAE ENAAEFLKOH NSGPKAKPVV SFIAGLTAPP GRRMGHAGAI IAGGKGGAKE
301 KISALQSAGV VVSMSPAQLG TTIYKEFEKR KLL

B

1 MCTQAGSQAS TPPAHPSGS GASVGPSPFRP AVCQLPAAGG RAPGNDLRR GSAVGGVWME
61 ALAPGRAPRG RRAGASGSV LSPLSLAAVL LCALLRAPPV GHLARLPRS IHLTQDSLKI
121 VGSTHFPVSV YMVLHMKSPH VLCVTQRLRN TELVPSFQW HSGPKGLVSE NTTAQVTSTG
181 SIFSQFESMS MGVYTFCLNE YKPTVEESIK NLQKYIYVYA YREPRFYQM TARYWAAPCN
241 SIYNSFEKK LQOILSLKLVL DLSCEISLKV SECHRVMQG AGLQENLFFFT FSVASIDTEK
301 GSIPCTDHC EASKRLSNAK NLIERFFIQQ VEVLGKRAEP LPEIYYEIGT LQMWWVNRCF
361 PGYGINVLK HPKCEPECCVVC SPGSFNRDG THLQCNNSL VYGAFTCM
NP-40 extractable 44kDa protein

A)

1 FNKHGLQVQO QQQRTLSLHE YLSMELOQEA GVSPKGFA KSSDEAYAIA KKLGSKDVVI
61 KAQLAGGREG KGTFTSQLKG GVIVFSPEE AKAQSSQMIG QKLTKQCTGE KGRICNQVLI
121 CERKYPRREY YFAITMERSF QGPVLIGSAQ GGVDNEWVAA ENPEAIVKEP IDIVEGKK
181 QAVTLAQKMG FSNIVDSDAA ENMIKLYNFL KKYDATMVEI NPMVEDSDBG VKLMDAKINF
241 DNSAYRQKK IFDLDWQWSQ DERDKEAANA DINYIGLDSG ICLVNGAGL AMATMIIKL
301 HGGTPANFLD VGGGATVQQV TEAFKLTSKD KKVQAILVNI FGGIMCDV AQGIVMAVKD
361 LEIRIPVVR LGTRVDDAK ALIADSGLKI LACDDLDEAA KMVVLSEIV TLAKEAHVDV
421 KFQLP
1 MSILKIHARE IFDSRGNPTV EVDLFTSKGL FRAAVPSGAS TGIYEALEL R
51 DNDKTRYMGK GVS KAVEHIN KTIAPALASK KLNVT EQEKI DKLIMEMGT
101 ENKSK FGANA ILGVLASVV AGAVEKGVPL YRHIA DLGN SEVILPVPAF
151 NVINGSHAG NKLAMQEFMI LPVGAANFRE AMRIGAEVYH NLKNVI KEKY
201 GKDATNVGDE GGFAPNILEN KEGLELLKTA IGKAGYTDKV VIGMDVAASE
251 FFRSGKYLDL FKSPDDPSRY ISPQLADLY KSFIKDVYPV SIEDPFDQDD
301 WGA WQKFTAS AGI GVVGDDL TVTNPKRIAK AVNEKSCNCL LLKVNQIGSV
351 TESLQACKLA QANGWGMVS HRSGETDF T IADLVGLCT GQIKTGAPCR
401 SERLAKYNQL LRIEELGSK AKFAGRNFRN PLAK

m/z, amu

Intensit y, count s

0 100 200 300 400 500 600
1000 1500 2000 2500 3000 3500 4000 4500

1779.977
1691.897
1588.862
1540.778
1519.825
1398.734
1341.748
2033.052
2154.063
2017.941
1907.991
1804.941
1519.789
3535.764
2277.135
1907.991
2750.390
3011.567
2510.116
233.052
3519.789
2655.328
3011.567
3616.734
3616.734
3519.789
3011.567
2750.390
3501.939

NP-40 non-extractable 70kDa protein

1 MKWVTFIGLL LFSSAYRSA G VFRDTHKSE IAHRFKDLGE EHFKGLVLJA FSOYLQQCPF
21 DEHVKLNVV TEFAKTCVAD ESHAGEKLSE HTLFGEDELCK VASLRETYGD MADCEKEQEP
121 ERNECFLSHK DDSDPLPKL PDPNLCDEF KADEFKFWGGK YLYEIARRHP YFYAPELLEYY
181 ANKYNVFQIE CCQADAEGKAC LLPIETMRE KVLAASFQQR LRCASIQKFG ERALKAWLSVA
241 RLSQKFFFKE FVEVTKLVID LTKVHKECCH GDLEACDR ADLAKYICDN QDITSSKLKE
301 CDDKPLEKS HCLAEEKDA IPEPNLPLTA DPAEDKLVCK NYQEAKDAFL GSFLYEYSRR
361 HPEAYASVALL RLAKYEATL EECACADPH ACYSTVFADKL KHLVDEPQNL IKQNCDFQFEK
421 LGEYGFQNAL IVRYTRKVFPQ VSTPTLVEVS RSLGKVGTRC CTKPESERMP CTEDYLSIL
481 NRLCMLHEKT PVSEKVTCC TESLVNRPCC FSAETPETY VPKFDEKLFL TFHADICLTP
541 DEKQIKKQQT ALVEFLKHKP KATEEQLKTV MENFVAFVDK CCAADDKEAC FAVEGPVLV
601 STQTALAA
NP-40 non-extractable 83kDa protein

1  MIAAQLLAYY FTELKDDQVK KIDKYLYAMR LSDEILIDIL TRFKKEMKNG LSROYNPTAS
61  VKMLPTFVRS IPDGSEKGDF IAALDGGSF S RILRQVHNE KSNQVSMESVE VYDTPEIVHV
121  GSGSQLFDHV AECLGDFMEK RKIKDDKLPY GFTFSFPCRQ SKIDEAVLIT WTKRFKASGV
181  EAADVVPKLLN KAIKKGKDYG ANIVAVNDT VGTMMTCGYD DQCEVGLLI GTGTVNYME
241  ELRHIDLVEG DEGRMCINTE WGAFFGDGSL EDIRTEFDRE LDRGSLNPQK QLFEKVMQSM
301  YMGELVRLIL VMKMAESLLF EGRITPELLT RGKFTTSDVA AIETDKEGQ VNAKELTRLG
361  VIPSIVDCCVS VQHVCITVSF RSLKVAATL GAILNRDRDN KGTPRLRTTV GVGDGSYLMHM
421  PQYSSRHFHT LRRLPVPSDV RFLSESGSG KGAAMVATA YRLAEGHRQI EETLSHFRLS
481  KQALMEVKKK LRESEMEGRK KETRNRATVK MLPSYVRSP DGTEHGOFLA LDLGGTFRFRY
541  LLYKIRSGKKRTVENHNVK SIPEIMOQGT GDELFDHVSV CSVILFDYMMG KGPSMPLGF
601  TFSPPCPKOTS LDCGILITWT KGFKATDCVG HDVATLLLDRD VAEREDFDL VVAVVNDTVG
661  TMTCAYEERP SCIEGLIVGT GSNACMEEM KNVEMEGNQ GQMNINMERS AFGDNCLLDD
721  IRTDFDKVVD EYSLNFGQKR FEKMNISMGL GEIVRNLID FTKK GFLFRG QISEPLKTRG
781  IFETKFLSQI ESDRLALLQV RAIQLQGLN STCSDSILVG TVCGVVSQRA AQLCAGAGMA
841  VVEKIRENRC LDHNLNVTVG DGTLYKLHPH FSRIMHQTQV ELSPKCTVSF LLSEDGSGKRG
901  AALITAVGVR LRGDPTNA

![Graph showing m/z and amu values](image-url)
NP-40 non-extractable 90kDa protein

A)

1 MPEEVHGGEE EVETFAFQAE IQLMSLIIN TFYSNK
2 RELISNASDA
51 LDIKRYESLT DPSKLDGKE LKIDILPNPQ ERTLTVDTG
IGMTKADLIN
101 NLGTIAKSGT KAFMEALQAG ADISMIGQFG VGFYSAVLVA
EKVVVITKHN
151 DDEQYAWESS AGGSFTVRAD HGEPIGRGTV VILHLKEDQT
EYLEERRVKE
201 VVKHHSQFIG YPITLYEKE REKEISDEAA EEEKGEKEEE
DKEDEEKPKI
251 EDVGSDEEED SGDKKKKTK KIKEKYIDQE ELNKTKPIWT
RNPDITQEE
301 YGEFYKSLTN DWEDHLAVKH FSVEGQLEFR AFLFIPRRAP
FDLFENKKKK
351 NNIKLVVRV FIMDSCDELI PEYNFIRGV VDSEDLPLNI
CPRMLQGSKL
B)

1 MIAAQLLAYY FTELDDQVK KIDKYLYAMR LSDEILDIL TRFKEMKNG LSRDYNPTAS
2 VKMLPTFVRS IPDGSEK GDF IALDLGGSSF RILRVQNHE KSQNVMESE VYDTPENIVH
3 GSGSDLHAE ACLGDFMEK RKIKDKKLPSK GFTSFPCQ SKIDEAVLIT WTFRFKAGV
4 EGADVKLKN KAIKRGYD ANIVAVNNDT VGTMCTCYGD DQCEVGLII GTGTNACYM,
5 ELRHIDLVEG DEGRMCINTE WGAFCDDQG EDLRGSNPGK QLFKEMVSGM
6 YMGELVRLIL VKMAKSFLL EQRTPELLELT RGFKFTSDVA AIETDKEGVQ NAKEILTRGL
7 VEPHDDCVS VQHVCITVSV RSAMLVATL GAILNRLDN KGTPRLRTIV GVTOGSYKHM
8 PQYSSRFKHT LRLVLPDDSV RFLLESGSG KGAAMVATA YRLAEQHRQI EETLSHFRLS
9 KQALEDVKKK NRSEMELGR KETNSRATVK MLPSYVRSIP DGTEHDFLA LDLGGTNRV
10 LLVKIRSGKK RTVEMHKKVI SIPEIMEQGT GDELFHHS GCSDFLDYM NKGRPMPLQF
11 TFSFPCKDTS LDCGILITW TGFKAATDCVG HDVCATTLLRDVA VKKRRQFDLD VVAAVNDTVG
12 TMMTCAEPEEP SCEIGLIVGT GSACAYMEEM KNVEMVGPQ GQMPNMEWQ
13 AFGDNGCDD
14 IRDFDKVVD EYSLNSGOQ FEKMSGMYF GEIVRNILD FTKKGFHFRG QISEPLKTRG
15 EFSKFLSOE EDSDRLAQV RAILQLQLGLN STCSDSILVK TVCGVSKRA AQELAGAAM
16 VVEKIRENRG LDHNLNTVP GDLYKLPHQ FSRIHQTQV ELSPKCTSVF LLSEDSGKCG
17 AALITAVGVR LRGDPNATNA

133
APPENDIX B

Quantification of gold particles after immuno-electron microscopy and surface replica with colloidal gold showing a significant increase in the number of gold particles/pm2 in the equatorial segment region and midpiece after 5- and 180-minutes of incubation in capacitation supportive medium in the presence or absence of 60µg/mL of EOV.

**Quantification of Gold Particles in the Equatorial Segment Region (Electron Microscopy)**

**Quantification of Gold Particles in the Midpiece (Electron Microscopy)**

**Quantification of Gold Particles in the Equatorial Segment Region (Surface Replica)**
Quantification of Gold Particles in the Midpiece (Surface Replica)

![Graph showing quantification of gold particles (gold particles/pm²) over time (5-min and 180-min) with and without EOV. The graph indicates a significant increase in gold particles with EOV compared to without EOV.](image)

- **Without EOV**: The bars for 5-min and 180-min show a low number of gold particles, with 5-min being the lowest.
- **With EOV**: The bars for 5-min show a significant increase compared to without EOV, while the 180-min bar shows an even greater increase with an asterisk indicating significance.