Novel proteomic profiling of epididymal extracellular vesicles in the domestic cat reveals proteins related to sequential sperm maturation with differences observed between normospermic and teratospermic individuals.

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Abstract

Extracellular vesicles (EVs) secreted by the epididymal epithelium transfer to spermatozoa key proteins that are essential in promoting motility and subsequent fertilization success. Using the domestic cat model, the objectives were to (1) characterize and compare protein content of EVs between segments of the epididymis, and (2) compare EV protein compositions between normo- and teratospermic individuals (producing >60% of abnormal spermatozoa). Epididymal EVs from adult cats were isolated and assessed via liquid chromatography tandem mass spectrometry. Both male types shared 3,008 proteins in total, with 98 and 20 EV proteins unique to normospermic and teratospermic males, respectively. Expression levels of several proteins changed between epididymal segments in both male types. Several proteins in both groups were related to sperm motility (hexokinase 1, adenylate kinase isoenzyme) and zona pellucida or oolemma binding (disintegrin and metalloproteinase domain proteins, zona binding proteins 1 and 2). Interestingly, seven cauda-derived EV proteins trended downward in teratospermic compared to normospermic males, which may relate to poor sperm quality. Collective results revealed, for the first time, EV proteins related to sequential sperm maturation with differences observed between normospermic and teratospermic individuals.

Key words: proteomics / spermatozoa / domestic cat / epididymis / extracellular vesicles

Introduction

Mammalian sperm cells undergo structural modifications throughout epididymal transit. This includes sperm membrane changes as well as addition of key surface proteins which are critical in sperm progression through the cumulus cells, binding and crossing of the zona
pellucida, and finally binding and fusing with the oolemma (1-5). Sperm composition also progressively evolves in the epididymis with the sequential integration of specific key peptides and microRNA (1, 3, 7). The presence of miRNA influences post-transcriptional regulation of gene expression and the mRNA content of sperm cells, which subsequently alter intercellular communication during the embryonic development (7-9, 10, 11). However, compared to small RNAs, proteins have more immediate effects on sperm motility, fertilization, and early embryonic development (12, 1, 4, 5, 12).

Where do these proteins originate and how are they integrated by the maturing sperm cell? Classical protein secretion by the epididymal epithelium is achieved through the merocrine pathway in which proteins contain signal sequences and are secreted individually from the epithelium (1, 13). Once secreted into the luminal fluid, proteins bind with the sperm surface before being incorporated into the cell. Another secretion pathway has been identified in which proteins without a signal sequence are secreted within membranous vesicles termed, extracellular vesicles (EV), ranging in size from approximately 30-300 nm (3, 6, 14-17). Epididymal EVs will fuse with the outer plasma membrane before cellular uptake (6, 15, 16). This type of secretory process has been identified in several species including the mouse (18), rat (19), hamster (20), bovine (21), human (22) and cat (23). Additionally, previous bovine and mouse studies have reported epididymal EV proteins incorporated by the sperm cells in a sequential manner (16, 24). Using the domestic cat model, our laboratory has been investigating this complex process. We demonstrated that exposing immature sperm cells in vitro to these EVs improved motility and allowed the acquisition of key peptides; thereby contributing to the maturation of the centrosome (25, 26). However, the protein profile of epididymal EVs in the domestic cat is not well
characterized; elucidating the function of these key factors will contribute to our understanding of sperm maturation processes in mammals.

Discerning key proteins contained within EVs is critical to developing interventions for fertilization failure and early embryo loss. This is especially vital for individuals that exhibit teratospermia; a condition in which more than 60% of sperm cells exhibit morphological abnormalities (27, 28). This condition has been observed in several species, including humans and in wild species (29). Research suggests this condition may result from detrimental membrane modifications during the sperm maturation process, which subsequently hinders spermatozoa structural integrity (30, 31). Supporting evidence for this hypothesis are in examples of these modifications observed in humans including acrosome and nuclear abnormalities (32), resulting in diminished ability of the sperm cell to penetrate the zona pellucida (33). This reduced sperm functionality has also been observed in the teratospermic domestic cat (29, 34, 35). The vast majority of research in cats focused on abnormalities which arise during spermatogenesis, resulting in primary abnormalities, adversely impacting sperm functionality (e.g. micro-, macro-, or polycephalic heads, acrosomal defects, mitochondrial sheath defects, biflagellate or tightly coiled flagellum; 29, 36-38). Studies on secondary abnormalities are scarcer, rendering it difficult to understand the underlying etiology of these defects (e.g. detached head or flagellum, retained cytoplasmic droplets, bent midpiece or flagellum; 29, 38). While not as severe, these secondary abnormalities still greatly diminish sperm quality and overall fertility. Comparing EV protein content of teratospermic males to the normospermic baseline will further elucidate the underlying basis of teratospermia, which is rampant in the 38 species of felids that are listed as threatened or endangered as reported by the International Union for Conservation of Nature (39). The domestic cat serves as an ideal model because it is a close relative of wild felids.
Furthermore, studies in the domestic cat have improved our basic understanding of human reproductive physiology, including potential physiological sources of teratospermia (40).

The objectives of the study were to (1) characterize and compare protein content of EVs between segments of the cat epididymis, and (2) compare EV protein compositions between normo- and teratospermic adults.

**Materials and Methods**

All chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA), unless otherwise stated.

**Tissue collection**

The study did not require the approval of the Animal Care and Use Committee of the Smithsonian Conservation Biology Institute because cat testes were collected at local veterinary clinics as byproducts from owner-requested routine orchiectomies. Adult (>1 year) domestic cat testis samples were supplied by local veterinary clinics following routine orchiectomy (n = 20 male tracts total). Tracts were transported and stored in phosphate buffered saline (PBS) at 4°C until processing within a 24-hour time period. Epididymal tissues were then removed from the rest of the testis in PBS using a scalpel blade until further processing.

**Classifying normospermic vs teratospermic samples**

A small sample of spermatozoa was isolated by making one 3 mm incision into the distal end of the cauda segment (42). The sample was then fixed by diluting 1:1 with 4% paraformaldehyde (PFA) and stained with Coomassie Brilliant Blue R-250 (0.1% Coomassie Brilliant (Blue R-250, 50% methanol, and 10% glacial acetic acid), then mounted with Permount™ Mounting Medium (Fisher Scientific, Pittsburgh, PA, USA). A proportion of sperm
cells were recorded, analyzing 200 cells total per each individual male. Individuals were considered teratospermic if \( \geq 60\% \) of cauda sperm cells had a morphological abnormality (34).

To first assess overall abnormalities in this sample type, sperm cells were isolated from the cauda segment of individuals until a sample size of \( n = 5 \) normospermic males, and \( n = 7 \) teratospermic males were attained, and the different morphological abnormalities recorded. It should be noted that the prevalence of teratospermia in the samples used in this study does not represent the overall prevalence of the general domestic cat population. A separate set of samples were then assessed for the collection of EVs (\( n = 4 \) normospermic males, and \( n = 4 \) teratospermic males).

EV samples were subsequently collected within hour to reduce sample loss and protein degradation.

**EV collections**

Following identification of whether a sample was normospermic or teratospermic, entire epididymides were separated into the different, consecutive, segments (caput, corpus, cauda; 42) and further minced using a scalpel blade in PBS (\( n = 4 \) normospermic males, and \( n = 4 \) teratospermic males). Luminal fluid was allowed to seep for 5-10 minutes before collected into microcentrifuge tubes.

Cell debris was discarded from the supernatant by a series of centrifugations at 700 x g for 10 min and 3,000 x g for 10 min at room temperature, with the supernatant transferred to a new microcentrifuge tube following each centrifugation. The EV fraction was isolated from the remaining luminal fluid by ultracentrifugation at 100,000 x g for 2 h at 4°C and re-suspended in fresh PBS (Beckman Coulter Optima L-90K, SW 55 Ti rotor, 3.5 mL polycarbonate tubes catalogue number: 349622, filled to 3 mL each, with full dynamic braking to 0 rpm, \( K_{adj} = 88 \)).
Aliquots of EV samples were then stored at -20°C. Samples were processed, and analyzed via mass spectrometry (described below) within one month’s time. Successful isolation of EVs was previously confirmed via observations performed using a transmission electron microscope (Zeiss 10 CA Transmission Electron Microscope) at the University of Maryland Laboratory for Biological Ultrastructure, USA (Rowlison et al., 2018). All relevant data of collection may be found in the EV-TRACK knowledgebase (EV-TRACK ID: EV200074, https://evtrack.org/). It should be noted that this study is using the operative term, “Extracellular Vesicle” (EV), in accordance to the guidelines set forth by the International Society for Extracellular Vesicles (17). ISEV endorses the use of this term when defining an isolated sample that has not been further analyzed for EV subtype (e.g. exosome, microvesicle, apoptotic vesicle, etc.).

**Mass spectrometry analysis**

EV samples were isolated from each consecutive segment of the epididymis (caput, corpus, and cauda), as described above, and the mass spectrometry analyses carried out by MS Bioworks (Ann Arbor, Michigan, USA) using nano liquid chromatography-tandem mass spectrometry.

Sample processing by MS Bioworks: The volume of each submitted sample was adjusted to 50μL with 50mM Tris.HCl, pH 8.0 and transferred to a 1.5mL microcentrifuge tube. 50μL of 2X modified RIPA buffer was added to each sample and sonicated briefly. Samples were incubated at 60°C for 15 minutes prior to clarification by centrifugation. The protein concentration of each sample was determined by Qubit fluorometry. 10μg of protein from each sample was processed by SDS-PAGE using a 10% Bis-Tris NuPAGE gel (Invitrogen) with the MES buffer system, the gel was run approximately 2cm and the mobility region was excised into
10 equally sized bands. In-gel digestion with trypsin was performed on each band using a ProGest robot (DigiLab) protocol: gels were first washed with 25mM ammonium bicarbonate followed by acetonitrile, reduced with 10mM dithiothreitol at 60°C, followed by alkylation with 50mM iodoacetamide at room temperature. Reduced gels were digested with trypsin (Promega) at 37°C for 4h, and quenched with formic acid. The supernatant was analyzed directly without further processing.

Analyses were performed with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher QExactive HF. Peptides were loaded on a trapping column and eluted over a 75μm analytical column at 350nL/min; both columns were packed with Luna C18 resin (Phenomenex). The mass spectrometer was operated in data-dependent mode, with the Orbitrap operating at 60,000 FWHM and 17,500 FWHM for MS and MS/MS respectively. The fifteen most abundant ions were selected for MS/MS.

Proteomic searching

All RAW cat epididymosome files were converted to mzML files using Proteowizard msconvert (version 3.0.18172-e4a23b1bb). These mzML files were then searched using MetaMorpheus 0.0.305 (41) against the Felis catus proteome (19,651 entries, downloaded from Uniprot April 16, 2020) on the Smithsonian High Performance Computing Cluster (Smithsonian Institution High Performance Computing Cluster, Smithsonian Institution). MetaMorpheus was configured as follows: 1) Calibration settings: default settings, 2) G-PTM-D settings were used: protease = trypsin; maximum missed cleavages = 2; minimum peptide length = 7; maximum peptide length = unspecified; initiator methionine behavior = Variable; max modification isoforms = 1024; fixed modifications = Carbamidomethyl on C, Carbamidomethyl on U; variable modifications = Oxidation on M; G-PTM-D modifications count = 150; precursor mass
tolerance(s) = ±5 ppm; G-PTM-D modifications: Acetylation on K; Acetylation on X; ADP-ribosylation on S; Butyrylation on K; Carboxylation on D; Carboxylation on E; Carboxylation on K; Citrullination on R; Crotonylation on K; Dimethylation on K; Dimethylation on R; Formylation on K; Glu to PyroGlu on Q; Glutarylation on K; HexNAc on Nxs; HexNAc on Nxt; HexNAc on S; HexNAc on T; Hydroxybutyrylation on K; Hydroxylation on K; Hydroxylation on N; Hydroxylation on P; Malonylation on K; Methylation on K; Methylation on R; Nitrosylation on C; Nitrosylation on Y; Phosphorylation on S; Phosphorylation on T; Phosphorylation on Y; Pyridoxal phosphate on K; Succinylation on K; Sulfonation on Y; Trimethylation on K; Acetylation on S; Acetylation on T; Amidation on X; Biotinylation on K; Carbamidomethyl on D; Carbamidomethyl on E; Carbamidomethyl on H; Carbamidomethyl on K; Carbamidomethyl on S; Carbamidomethyl on T; Carbamidomethyl on X; Carbamidomethyl on Y; Carboxymethylation on C; Carboxymethylation on K; Carboxymethylation on W; Carboxymethylation on X; Decarboxylation on D; Decarboxylation on E; Dehydroalanine on C; Dehydroalanine on S; Dehydrobutyryne on T; Didehydro on Y; Dimethylation on N; Dioxidation on C; Dioxidation on F; Dioxidation on K; Dioxidation on M; Dioxidation on P; Dioxidation on R; Dioxidation on W; Dioxidation on Y; Ethylation on D; Ethylation on E; Ethylation on K; Ethylation on X; Farnesylation on C; Formylation on S; Formylation on T; Formylation on X; Methylation on C; Methylation on D; Methylation on E; Methylation on H; Methylation on I; Methylation on L; Methylation on N; Methylation on Q; Methylation on S; Methylation on T; Methylation on X; Myristoylation on C; Myristoylation on G; Myristoylation on K; Oxidation and then loss on oxidized M side chain; Oxidation on C; Oxidation on D; Oxidation on E; Oxidation on F; Oxidation on H; Oxidation on I; Oxidation on L; Oxidation on Q; Oxidation on R; Oxidation on S; Oxidation on T; Oxidation on U; Oxidation on V; Oxidation on W; Oxidation
on Y; Oxidation to Kynurenine on W; Palmitoylation on C; Palmitoylation on K; Palmitoylation on S; Palmitoylation on T; Proline pyrrole to pyrrolidine six member ring on P; Propionamidation on C; Propionamidation on K; Propionamidation on X; Propionylation on K; Pyrroolidinone on P; Quinone on W; Quinone on Y; Reduction on D; Reduction on S; Reduction on T; Succinylation on X; Sulfonation on S; Sulfonation on T; Trioxidation on C; Water loss on D; Ammonia loss on C; Ammonia loss on N; Carbamyl on C; Carbamyl on K; Carbamyl on M; Carbamyl on R; Carbamyl on X; Deamidation on N; Deamidation on Q; Water Loss on E; Calcium on D; Calcium on E; Cu[I] on D; Cu[I] on E; Fe[II] on D; Fe[II] on E; Fe[III] on D; Fe[III] on E; Magnesium on D; Magnesium on E; Potassium on D; Potassium on E; Sodium on D; Sodium on E; Zinc on D; Zinc on E; product mass tolerance = ±20 ppm. 3) Search setting: protease = trypsin; maximum missed cleavages = 2; minimum peptide length = 7; maximum peptide length = unspecified; initiator methionine behavior = Variable; fixed modifications = Carbamidomethyl on C, Carbamidomethyl on U; variable modifications = Oxidation on M; max mods per peptide = 5; max modification isoforms = 1024; precursor mass tolerance = ±5 ppm; product mass tolerance = ±20 ppm; report PSM ambiguity = True. PSMs, peptides, and proteins were filtered at 1% false discovery rate (FDR) based on a slided decoys.

To confirm the successful isolation of EVs and assess the quality of isolates, proteins known to be associated with exosomes, as well as commonly found contaminants, were assessed as according to the International Society for Extracellular Vesicles (17).

**Experimental design and statistical analysis**

Spermatozoa were isolated from the cauda epididymal segment and assessed for morphological abnormalities (n = 5 normospermic males, and n = 7 teratospermic males).
Statistical analyses were conducted using Graphpad Prism (version 6). Percent of sperm cells displaying each abnormality was then compared between treatment groups via Student’s T-test. EVs were isolated between segments (caput, corpus, and cauda) and kept separately for mass spectrometry analyses (n = 4 normospermic males, and n = 4 teratospermic males). Subsequently, the resultant MetaMorpheus data was analyzed by the FlashLFQ Bayesian fold-change tool (43). The following parameters were used: 10 ppm tolerance, normalize intensities, match between runs, Log$_2$ Fold-change cutoff 0.5. For searches that required two runs (i.e., Normal/Teratospermic caput vs corpus vs cauda), the same random seed was utilized to allow similarity in MCMC outcomes. Significant differences were restricted to 1% FDR or <10% posterior probability, whichever was smaller. Fold changes were evaluated by groups in Table 1.

Results

Comparative sperm morphological abnormalities between normospermic and teratospermic males

Morphological analyses revealed both primary and secondary abnormalities in mature, cauda-derived sperm samples. As expected, samples isolated from teratospermic individuals (as evidenced by ≥ 60% of spermatozoa with one or more morphological abnormalities) had significantly (p < 0.05) more abnormal cells compared to samples isolated from normospermic individuals (p = 0.0034; Table 2). Of the abnormalities observed, three were classified as primary (i.e. micro- or macrocephalic heads, polycephalic, as well as biflagellate) and five were secondary (i.e. missing acrosome, abnormal head-tail junction, bent midpiece, bent flagellum, and retained cytoplasmic droplet). Teratospermic samples had a significantly higher percentage of sperm cells with bent flagellum as compared to normospermic samples (p = 0.0085). While
not significant, teratospermic samples also trended toward having higher percentages of missing acrosomes as compared to normospermic (p = 0.0522) as well as abnormal head-tail junctions (p = 0.0773).

**Qualitative analysis of EV content**

Proteomic analyses of normospermic males identified a total of 3,029 proteins including 115, 10, and 28 proteins unique to caput, corpus, and cauda-derived EVs, respectively, and 2,687 proteins present in all three segments (Figure 1A). Analyses of teratospermic males identified a total of 3,028 proteins including 101, 15, and 48 proteins unique to caput, corpus, and cauda-derived EVs, respectively, and 2,685 proteins present in all three segments (Figure 1B). Several known proteins associated with exosomes were detected including 30 transmembrane or GPI-anchored proteins, and 29 cytosolic proteins (Table 3) as according to the International Society for Extracellular Vesicles (17). Additionally, three proteins known to be common contaminants of exosome isolates were also detected (Table 3; 17).

Normospermic and teratospermic males shared a total of 3,008 proteins, with 98 and 20 proteins unique to normospermic and teratospermic males, respectively (Figure 1C). Further analysis of each epididymal segment identified differences in protein composition when comparing samples isolated from normospermic versus teratospermic males (Table 4). Several identified EV proteins in both sample groups had functions related to sperm motility (e.g. cysteine-rich secretory protein 1- CRISP1, hexokinase 1, adenylate kinase isoenzyme 1, sorbitol dehydrogenase), as well as zona and oolemma binding (e.g. acrosin, zona binding protein 1,2-ZPBP1,2, sperm acrosome associated proteins 1,3, 4, a disintegrin and metalloproteinase domain 2,7,28, 32- ADAM-2,7,28,32, sperm equatorial segment protein 1; Supplementary File 1).
Gene Ontology analysis (DOI: 10.5281/zenodo.3727280; Gene Ontology annotations: 2020-03-23) was then performed on the PANTHER 15.0 platform (http://pantherdb.org/) to classify identified EV proteins based on known or potential molecular function. Overall, only limited differences were noted when comparing the distribution of molecular functions of the whole epididymis between normospermic and teratospermic males, as well as when comparing between the consecutive segments of each (Table 5). Proteins unique to normospermic or teratospermic males were also classified based on their known or potential biological processes (Figure 2 A-B, Table 6, Supplementary File 2), with teratospermic males lacking numerous proteins with key biological functions including cellular structure, stimulus response, signaling, multicellular organismal processes, developmental processes, adhesion, locomotion, and immune system support.

**Quantitative analysis of EV content**

Significant changes in protein expression (FDR ≤ 0.01) was observed between EVs isolated from each of the consecutive epididymal segments in both normospermic and teratospermic males (Figures 3 A-C, and 4 A-C, respectively, FDR ≤ 0.01). When comparing EVs isolated from each epididymal segment between normospermic versus teratospermic males, only 7 proteins were observed to trend toward a significant change in expression in the cauda segment (i.e. calnexin, dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit, calreticulin, uncharacterized protein- accession: ENSFCAG00000050424, zona pellucida binding protein 1, dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit, peptidyl-prolyl cis-trans isomerase; Figures 5 A-D). A complete list of identified proteins with corresponding expression quantification is included in Supplementary File 1.
Discussion

This study is the first to characterize the proteomic profile of epididymal EVs in the cat model, showing conserved molecular functions similar to other organisms including men, bull and mouse (16, 22, 24). Morphological analyses of teratospermic sperm samples showed a significant increase in the percentage displaying bent flagellum, a secondary abnormality that occurs from suboptimal maturation during epididymal transit (29, 38). Main findings from this study suggest that proteins brought by EVs can be specific to caput, corpus, or cauda segments. Several proteins were associated with reproductive processes (i.e. sperm motility, zona and oolemma binding) in specific segments. Furthermore, this was the first study to compare epididymal EV protein content between normospermic and teratospermic individuals of any species. Normospermic and teratospermic males shared many similar proteins (3,008 in total); however, 98, and 20 proteins were unique to normospermic and teratospermic males, respectively, with additional variances observed when comparing the consecutive segments between each group.

In total, 3,029 and 3,028 proteins were identified in EVs isolated from normospermic and teratospermic individuals, respectively. Comparative studies in the mouse and human resulted in much fewer identified proteins (1,640, and 1,022, respectively); however, this may be a result of intrinsic interspecies variation, or the use of different methods for collecting and processing of tissue samples, as well as protein identification, especially as the study in humans was published years prior when methods were not as advanced (16, 22). The reference proteomes used may also have included fewer annotated proteins at the time of analysis. Regardless, there were many similar GO-classified molecular functions observed in all species including catalytic activity, binding, and transportation.
Numerous proteins known to be associated with exosomes were detected including several transmembrane or GPI-anchored proteins as according to the International Society for Extracellular Vesicles (17), confirming the successful isolation of these epididymal exosomes. Previous analysis of the EVs using transmission electron microscopy also confirmed successful isolation (26). Additionally, three proteins known to be common contaminants of exosome isolates were also detected likely as a result of the collection method (17). Mincing of the epididymal tissue proved to be the most feasible option, as compared to profusion of the luminal fluid; however, this also resulted in the release and collection of extraneous lipoproteins and albumin. While effort was made to reduce the amount of these contaminants, it should be noted that these were still detected in the overall collection.

Multiple candidate proteins brought by EVs during sperm maturation have a direct effect on the fertilization and subsequent quality of the preimplantation embryo in the domestic cat. There were also several key proteins which aid in reproductive processes identified in this study that were also observed within the EVs of the other species. These included sorbitol dehydrogenase which was previously reported in the bovine and mouse (16, 24), interleukin enhancer binding factor (also reported in the mouse; 16), cysteine rich secretory protein and zona pellucida binding proteins (mouse and human; 16, 22), as well as disintegrin and metalloproteinase domain-containing proteins which were identified in all three models (16, 22, 24). Comparisons between these species illustrate the evolutionary conservation of certain proteins previously seen in other studies which contribute to the structure and function of the sperm cell (44-46). In addition to the conservation of certain proteins, it is also of note that the mechanism itself in which these vesicles are secreted and acquired by the maturing sperm population appear to be very similar between the studied animal models (3, 6, 14-17, 23, 47).
Proteomic analyses of EVs identified several candidate proteins contributing to the zona binding process including CRISP1, ZPB1, ZPB2, ADAM2, ADAM7, ADAM28, ADAM32 and acrosin. Specifically, ZPB1, and ZPB2 function in the binding of the sperm cell to the zona pellucida, while acrosin acts as a protease to aid in the penetration (1, 4, 5). Proteins CRISP1, and ADAM2, ADAM 7, ADAM 28, ADAM 32 then subsequently aid in the sperm penetration of the oolemma. Other key proteins identified from these analyses have also been previously reported to aid in various sperm functions in the human including motility (sorbitol dehydrogenase, interleukin enhancer binding factors 2 and 3, and hexokinase 1; 48-50) as well as maintenance of DNA integrity (DNA repair protein- RAD50; 51). Interestingly, the epididymal sperm binding protein (ELSPBP1) was also identified like in bovine EVs, which specifically targets dead sperm cells to presumably initiate degradation and removal from the maturing population (53). Together, acquiring these proteins can aid in the achievement of key functions while simultaneously eliminating poor-quality sperm cells to improve the condition of the overall population. As an example, Figure 6 illustrates predicted interactions between a subset of identified EV proteins in this study, and domestic cat sperm cell proteins that have been previously identified to aid in the fertilization process (STRING CONSORTIUM 2020, version 11.0). These interactions highlight the potential benefits that the content of EVs may provide to the developing sperm cell. Additionally, this figure highlights the potential interactions that EV proteins may have with each other, possibly altering their downstream effects and adding another layer of complexity to the study of these vesicles that to date has not been assessed in any species.

Comparing the EV content of normospermic individuals to those exhibiting teratospermia also provides novel information which will aid in better understanding the underlying physiology
of this condition. Previous research has implicated certain gene alterations in teratospermic individuals which may contribute to diminished sperm quality, but the downstream effects on protein expression have yet to be determined (29). Investigating the potential influence of epididymal EVs is also of great value as the etiology of secondary abnormalities, which occur during epididymal transit, are poorly understood. For example, sperm samples isolated from teratospermic individuals in this study were observed to have a significantly increased percentage of cells with bent flagellum, a secondary abnormality. As this deformity arises during the sperm epididymal transit, it is possible that the spermatozoa were unable to acquire the appropriate milieu of proteins in the proper sequential order as a result of altered EV content. Abnormalities in the head-tail junction region of the sperm cell also tended to increase in teratospermic males. Contained within this region is the sperm centrosome which is essential in coordinating the first mitotic division of the zygote, diminished functionality of this cellular structure may inhibit subsequent embryonic development (53, 54). Previous studies in our laboratory have observed significant improvement in the sperm centrosome maturation when exposing immature spermatozoa to EVs (25, 26). Therefore, it is also possible that modifying the content of these EVs in teratospermic individuals may contribute to the development of malformed centrosomes.

Certain factors, such as hormone imbalance, altered luminal composition, and pH have been found as contributing factors to secondary abnormalities, but how these interplay with the miRNAs and proteins supplied via EVs remains unknown (38). Additionally, variance of any of these factors may also influence protein expression at the cellular level, potentially altering which proteins are even secreted from the epididymal epithelium. For example, many of the identified proteins unique to normospermic males, and not present in teratospermic EVs, were
GO-classified in a number of vital biological functions including cellular processes (29.7%), intracellular transportation (13.5%), metabolism (13.5%), cellular structure (10.8%), stimulus response (8.1%), signaling (4.1%), multicellular organismal processes (4.1%), developmental processes (2.7%), adhesion (1.4%), locomotion (1.4%), and immune system support (1.4%). While it is possible that these proteins may be provided via another mechanism, their absence in these EVs may still hinder sperm cell maturation. This is further evident as many proteins supplied by EVs have been identified as lacking any signal peptide sequence (3, 6, 14-17). While these proteins may somehow be secreted into the luminal fluid, possibly through the classical merocrine pathway, successful binding with the sperm cell is still unlikely as there is no signal sequence available to achieve this. One protein of interest for example, zona pellucida binding protein1 (ZPB1), was observed to be trending toward a significant decrease in expression in the cauda-derived EVs of teratospermic males as compared to normospermic males. As this protein enables the sperm cell to penetrate the oocyte zona pellucida, a decrease in its acquisition may greatly hinder the success of fertilization.

Our collective results elucidate the type and quantity of proteins involved in the sperm cell maturation during epididymal transit. Proteomic analyses of the epididymal EV revealed vital information regarding mechanisms of protein transport and identified key paternal factors which may contribute to improved sperm quality and early embryonic development in the domestic cat model. Future studies will investigate roles of candidate proteins to assess the downstream effects on sperm maturation and developmental potential. Additional analyses will also be performed to further assess the subtype of extracellular vesicles secreted by the epididymal epithelium. Information gained from this study and future analyses will contribute to
progress in reproductive medicine, development of assisted reproductive techniques, as well as contraceptive strategies in human and many other species.

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Data Availability

All files from the MetaMorpheus search are deposited on MassIVE (ftp://massive.ucsd.edu/MSV000085833/) allowing for direct viewing of the annotated spectra with MetaMorpheus’s MetaDraw viewer.

Author’s Roles

T. R. designed and performed experiments as well as analyzed and interpreted data and wrote the article. T. C. analyzed the resultant MetaMorpheus data, and contributed to the article revision. M.A.O. and P.C. contributed to the study design, and data interpretation and article revision.

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Conflict of Interest

The authors declare no conflict of interest.

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Figure 1. Number and distribution of proteins identified in (A) normospermic males, 3,029 total, and (B) teratospermic males, 3,028 total. (C) Number of proteins identified in the whole epididymis of normospermic vs teratospermic males, 3,126 total.
Figure 2. Proportions of proteins based on biological processes which are only present in the whole epididymis of (A) normospermic males, and (B) teratospermic males.
Figure 3. Change in protein expression of normospermic males when comparing (A) corpus-derived EV content to the caput, (B) cauda-derived EV content to the caput, and (C) cauda-derived EV content to the corpus segment. Dotted line indicates 90% posterior probability.
Figure 4. Change in protein expression of teratospermic males when comparing (A) corpus-derived EV content to the caput, (B) cauda-derived EV content to the caput, and (C) cauda-derived EV content to the corpus segment. Dotted line indicates 90% posterior probability.
Figure 5. Change in protein expression when comparing teratospermic to normospermic male EVs derived from (A) whole epididymis, (B) caput, (C) corpus, and (D) cauda segments. Dotted line indicates 90% posterior probability.
Figure 6. Example of interactions observed between a subset of proteins identified in exosomes of normospermic males, and sperm cell proteins that are involved in the fertilization process (STRING 11.0).

| Protein     | Description                                             |
|-------------|---------------------------------------------------------|
| AADAT       | Aminotran_1_2 domain-containing protein                 |
| ACRBP       | Acrosin binding protein                                 |
| ARSA        | Sulfatase domain-containing protein                     |
| CLU         | Clusterin                                               |
| CRISP1      | Cysteine rich secretory protein 1                       |
| CSNK2A2     | Casein kinase II subunit alpha                           |
| HSPA4L      | Heat shock protein family A, member 4                   |
| LIPI        | Lipase domain-containing protein                         |
| PDHB        | Pyruvate dehydrogenase E1 component subunit beta        |
| ROPN1       | Rhophilin associated tail protein 1                     |
| SEC23IP     | SEC23-interacting protein                               |
| SPACA (1,3,4)| Sperm acrosome associated protein (1,3,4)               |
| SPESP1      | Sperm equatorial segment protein 1                      |
| SUCLA2      | Succinate-CoA ligase subunit beta                        |
| TEX101      | Testis expressed 101                                    |
| TPI1        | Triosephosphate isomerase                               |
| TSPAN6      | Tetraspanin 6                                           |
| ZP2         | Zona pellucida sperm-binding protein 2                   |
| ZPBP2       | Zona pellucida binding protein 2                         |
| Segment          | Normospermic | Teratospermic |
|------------------|--------------|---------------|
| Whole epididymides | 29741-29752 (averaged) | 29753-29764 (averaged) |
| Caput segment    | 297-41, 44, 47, 50 (averaged) | 297-53, 56, 59, 62 (averaged) |
| Corpus segment   | 297-42, 45, 48, 51 (averaged) | 297-54, 57, 60, 63 (averaged) |
| Cauda segment    | 297-43, 46, 49, 52 (averaged) | 297-55, 58, 61, 64 (averaged) |

Table 1. Groups used in fold-change analysis.
Table 2. Proportions of sperm morphological abnormalities assessed in normospermic, and teratospermic domestic cat males (values are expressed as mean ± SEM).

| Overall % of Abnormal Morphology | % of Primary Abnormalities | % of Secondary Abnormalities |
|----------------------------------|-----------------------------|-----------------------------|
|                                  | Micro or Macrocephalic      | Polyccephalic               | Biflagellate               | Missing Acrosome | Abnormal Head-tail Junction | Bent Midpiece | Retained Cytoplasmic Droplet | Bent Flagellum |
| Normospermic                     | 40.2 ± 11.0\(^a\)           | 0.6 ± 0.4                  | 0.4 ± 0.2                  | 0 ± 0              | 3.4 ± 1.5               | 3.0 ± 1.4      | 3.6 ± 1.4                  | 3.0 ± 1.4      | 33.2 ± 9.8\(^a\) |
| Teratospermic                    | 77.6 ± 3.0\(^b\)            | 1.1 ± 0.6                  | 0.1 ± 0.1                  | 0.4 ± 0.3          | 9.6 ± 2.1              | 10.3 ± 2.9     | 2.7 ± 0.8                  | 1.3 ± 0.5      | 65.0 ± 4.5\(^b\) |

Within columns, values with different superscripts (a, b) are statistically different (p < 0.05).
Table 3. EV characterization based on known associated proteins, as well as contaminants commonly found in EV isolates (17).

| Transmembrane/GPI-anchored EV proteins | Cytosolic EV proteins | Non-EV components |
|----------------------------------------|-----------------------|-------------------|
| CD47,63,81,82                          | VPS4A                 | APOA1             |
| GNA12,13,14                            | ARRDC1                | APOB              |
| ITGA1,2,3,4,7                          | FLOT2                 | ALB               |
| ITGB1,2,3,5                            | CAV1                  |                   |
| LAMP1,2                                | EHD1,2,3,4            |                   |
| SDC2                                   | PDCD6IP               |                   |
| BSG                                    | TSG101                |                   |
| NT5E                                   | FLOT1                 |                   |
| CD59                                   | RHOA                  |                   |
| TSPAN8                                 | ANXA1,2,3,4,5,6,7,9,11|                   |
| CD9                                    | HSPA8                 |                   |
| PECAM1                                 | HSP90AB1              |                   |
| EPCAM                                  | SDCBP                 |                   |
| THY1                                   | HSPA1A                |                   |
| PTPRC                                  | ACTB                  |                   |
| GYPA                                   | ACTN4                 |                   |
| ABCC1                                  | ACTG1                 |                   |
|                                        | TUBA1A                |                   |
Table 4. Number of identified proteins via LC-MS/MS in the different segments of the epididymis in normospermic versus teratospermic males.

|       | Both | Normospermic Only | Teratospermic Only |
|-------|------|-------------------|--------------------|
| Caput | 2839 | 88                | 56                 |
| Corpus| 2731 | 105               | 74                 |
| Cauda | 2616 | 213               | 261                |
Table 5. Proportions of EV proteins based on GO molecular function isolated from the whole epididymis as well as in each consecutive segment from normospermic, and teratospermic males.

|                      | Segment | Binding | Catalytic Activity | Molecular Function Regulation | Molecular Transduction | Structural Molecular Activity | Transcription Regulation | Translation Regulation | Transportation |
|----------------------|---------|---------|--------------------|-------------------------------|------------------------|-------------------------------|--------------------------|------------------------|---------------|
| **Normospermic Males** | Whole   | 37.80%  | 44.30%             | 4.70%                         | 1.20%                  | 4.30%                         | 1.40%                    | 1.60%                  | 4.70%         |
|                      | Caput   | 37.90%  | 44.70%             | 4.60%                         | 1.10%                  | 4.10%                         | 1.50%                    | 1.70%                  | 4.40%         |
|                      | Corpus  | 38.20%  | 44.30%             | 4.80%                         | 1.10%                  | 4.30%                         | 1.40%                    | 1.50%                  | 4.40%         |
|                      | Cauda   | 37.80%  | 44.30%             | 4.70%                         | 1.10%                  | 4.50%                         | 1.30%                    | 1.70%                  | 4.50%         |
| **Teratospermic Males** | Whole   | 37.70%  | 44.50%             | 4.80%                         | 1.10%                  | 4.10%                         | 1.40%                    | 1.60%                  | 4.70%         |
|                      | Caput   | 37.90%  | 44.60%             | 4.70%                         | 1.00%                  | 4.20%                         | 1.50%                    | 1.70%                  | 4.30%         |
|                      | Corpus  | 37.90%  | 44.90%             | 4.50%                         | 0.90%                  | 4.50%                         | 1.40%                    | 1.70%                  | 4.30%         |
|                      | Cauda   | 37.90%  | 44.70%             | 4.60%                         | 1.00%                  | 4.10%                         | 1.30%                    | 1.70%                  | 4.60%         |
Table 6. Identified proteins in EVs isolated from normospermic and teratospermic males.

| Protein Name                                           | Protein Accession | Protein Full Name                                           | Protein Accession |
|--------------------------------------------------------|-------------------|-------------------------------------------------------------|-------------------|
| Glyceraldehyde-3-phosphate dehydrogenase               | Q9N2D5            | Amine oxidase                                               | M3WIA6            |
| Actin alpha 1, skeletal muscle                         | A0A5F5XWN0        | Sodium/potassium-transporting ATPase subunit beta           | A0A5F5XVM0        |
| Tubulin alpha chain                                    | M3WDV2            | Sodium/potassium-transporting ATPase subunit alpha          | M3W6P5            |
| NSF attachment protein beta                            | A0A337S7C8        | Prostate and testis expressed 2                            | A0A2I2UR6         |
| Aminopeptidase N                                       | A0A337S27         | GP-PDE domain-containing protein                            | A0XBBX3           |
| Sodium/potassium-transporting ATPase subunit alpha     | M3WST3            | Solute carrier family 25 member 4                          | A0A2I2URL1        |
| Uncharacterized protein                                | A0A337SS6         | Chromosome E3 C16orf58 homolog N(alpha)-acetyltransferase 16, NatA auxiliary subunit | A0A2I2UA1         |
| COMM domain-containing protein                         | M3WQF8            | 4-hydroxyphenylpyruvate dioxygenase                         | M3WGC4            |
| ANK_REP_REGION domain-containing protein               | M3X4L9            | Zinc ribbon domain containing 2                            | A0A2I2UQX5        |
| Ig-like domain-containing protein                      | M3X1A8            | ATPase H+ transporting V1 subunit E2                       | M3WFD4            |
| Cytoplasmic FMR1-interacting protein                   | A0A5F5XD78        | ATP binding cassette subfamily B member 10                  | M3W846            |
| Uncharacterized protein                                | A0A2I2UZ67        | Protein tyrosine phosphatase receptor type F               | A0A5F5Y3R2        |
| Histone H2A                                            | A0A5F5XNL4        | Aldehyde dehydrogenase                                     | M3W3M4            |
| Deacetylase sirtuin-type domain-containing protein     | A0A5F5XQJ4        | B12-binding domain-containing protein                       | A0A2I2U7V7        |
| ATRX chromatin remodeler                               | M3WTQ4            | MYC binding protein 2                                       | M3W3D6            |
| MARVEL domain-containing protein                       | M3W9B8            | AP-1 complex subunit gamma                                 | A0A2I2U977        |
| ANK_REP_REGION domain-containing protein               | A0A5F5Y225        | Euchromatic histone lysine methyltransferase 2              | A0A5F5Y5V5        |
| WD_REPEATS_REGION domain-containing protein            | A0A5F5XL49        | 4-hydroxy-2-oxoglutarate aldolase 1                        | M3XDF5            |
| Strawberry notch homolog 1[Strawberry notch homolog 2 | A0A2I2UN15        | Additional Normospermic Proteins                           |                   |
| Uncharacterized protein                               | A0A337S3K8        | YTH domain containing 2                                    | M3WVZ7            |
| JmC domain-containing protein                          | A0A5F5YQ9         | WD_REPEATS_REGION domain-containing protein                 | M3VYE0            |
| ER lumen protein-retaining receptor                    | A0A5F5XG06        | Non-specific serine/threonine protein kinase               | A0A5F5Y0J8        |
| Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase      | A0A337S9W6        | PRKCSH domain-containing protein                            | M3WLS7            |
| Guanine nucleotide-binding protein subunit gamma       | A0A337SD1         | Uncharacterized protein                                     | A0A5F5X8P7        |
| Complement C2                                          | A0A5F5UX26        | Peptidyl-prolyl cis-trans isomerase                         | A0A2I2UVE4        |
| Epiplatin 1                                            | A0A5F5X6L2        | Solute carrier family 41 member 3                          | A0A337SN7G7       |
| RNA binding motif protein 39                           | M3WUX8            | Transket_pyr domain-containing protein                      | M3XER1            |
| alpha-1,2-Mannosidase                                  | A0A2I2VB17        | FXYD domain-containing ion transport regulator              | A0A5F5Y0E2        |
| S'-nucleotidase                                        | M3XB03            | IF rod domain-containing protein                            | M3VXQ9            |
| WD_REPEATS_REGION domain-containing protein            | A0A337SNM3        | TPR_MLP1_2 domain-containing protein                       | A0A337RXX4        |
| Uncharacterized protein                               | A0A2I2UCN7        | Myeloid leukemia factor 1                                  | A0A337SDK2        |
| Elongation of very long chain fatty acids protein 1    | A0A337SB82        | Heme binding protein 2                                      | A0A5F5XWR9        |
| Olfactory receptor                                     | A0A2I2UQ24        | Cytochrome b5 heme-binding domain-containing protein        | A0A337SAE3        |
| E3 ubiquitin-protein ligase RNF168                      | M3WSK4            |                                                           |                   |
| SET and MYND domain containing 3                      | A0A2I2UGW5        |                                                           |                   |
|                                                           |                   |                                                           |                   |
| Protein Description                                      | UniProt ID  |
|----------------------------------------------------------|-------------|
| Prostaglandin E synthase 2                               | M3WHE5      |
| Kinesin-like protein                                     | M3X169      |
| Uncharacterized protein                                  | A0A2I2UNB8  |
| Arm_2 domain-containing protein                          | M3X964      |
| Ubiquitin-like domain-containing protein                 | M3X178      |
| Hydrolase_4 domain-containing protein                    | M3VZ44      |
| Ig-like domain-containing protein| M3VUF6[M3XG03               |
| Centromere protein B                                     | A0A2I2UTF7  |
| IF rod domain-containing protein                         | M3WV73      |
| Dystrophin                                               | A0A5F5XPE7  |
| Transmembrane protein 64                                 | M3WX12      |
| Small integral membrane protein 7                        | M3WNE0      |
| IF rod domain-containing protein                         | M3WGY3      |
| MPN domain-containing protein                            | A0A337SUW7  |
| 4HBT domain-containing protein                           | A0A337ST35  |
| Amidase domain-containing protein                         | M3W0D6      |
| MAGE domain-containing protein                           | M3XET9      |
| Myotubularin 1                                           | M3X251      |
| Beta-defensin                                            | A0A2I2V201  |
| Phenylalanyl-tRNA synthetase 2, mitochondrial            | A0A5F5XFQ5  |
| ATP binding cassette subfamily A member 9                | A0A2I2U9T3  |
| Protein S100                                             | A0A2I2U4Z5  |
| Integrator complex subunit 4                             | M3XDA8      |
| Doublecortin domain containing 2C                       | A0A337SJL5  |
| Synaptotagmin like 1                                     | A0A337S460  |
| Gp_dh_N domain-containing protein                        | A0A5F5Y264  |
| Intraflagellar transport 57                              | M3WTC0      |
| Jacalin-type lectin domain-containing protein            | A0A5F5XGU8  |
| MARVEL domain-containing protein                         | M3WM87      |
| FA_desaturase domain-containing protein                   | M3X8E3      |
| DnaJ heat shock protein family (Hsp40) member B4         | M3X3F4      |
| Matrin-type domain-containing protein                    | A0A337SIR1  |
| WD_REPEATS_REGION domain-containing protein              | M3WFN9      |
| Schlafen family member 5                                 | M3VUD6      |
| Endo/exonuclease/phosphatase domain-containing protein   | A0A5F5Y4E8  |
| PH domain-containing protein                             | A0A2I2UZ86  |
| J domain-containing protein                              | A0A5F5Y4M2  |
| Sema domain-containing protein                           | M3VXW2      |
| Peroxisomal biogenesis factor 1                          | M3WL25      |
| ENTH domain-containing protein                            | M3X6K7      |
| Matrin-type domain-containing protein                    | M3X4F3      |
| RING-type domain-containing protein                      | M3WPS3      |
| BAF chromatin remodeling complex subunit BCL7B           | M3X7M8      |
| V-SNARE coiled-coil homology domain-containing protein   | A0A2I2UFB6  |
| Crystallin beta-gamma domain containing 3                | A0A2I2UFY1  |
| Calpastatin                                              | A0A2I2UML4  |
| START domain-containing protein                           | A0A337S826  |
