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GAMETE BIOLOGY

Key factors enhancing sperm fertilizing ability are transferred from the epididymis to the spermatozoa via epididymosomes in the domestic cat model

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Abstract
Purpose Spermatozoa undergo critical changes in structure and function during the epididymal transit. Our previous studies in the domestic cat demonstrated that incidence of cenexin—a key protein involved in the centrosomal maturation—progressively increases in sperm cells from caput to cauda epididymidis. The objectives of the study were to (1) characterize mechanisms involved in transferring key factors—using the cenexin as a marker—between the epididymis and maturing sperm cells and (2) demonstrate the impact of such mechanisms on the acquisition of functional properties by spermatozoa.
Methods Epididymides were dissected from adult cat testes to assess the presence and localization of cenexin in testicular tissues and each epididymal segment (caput, corpus, and cauda) via immunofluorescence, Western blot, and mass spectrometry.
Results Results showed that tissues, luminal fluid, and isolated epididymosomes from each segment contained cenexin. Co-incubation of immature sperm cells for 3 h with luminal fluid or epididymosomes followed by immunostaining revealed that percentages of sperm cells containing cenexin significantly increased in samples co-incubated with epididymosome suspensions. Additionally, epididymosome co-incubation with immature spermatozoa resulted in sustained motility compared to untreated spermatozoa while there was no significant effect on acrosome integrity.
Conclusions Taken together, these results suggest that epididymosomes play a critical role in epididymal sperm maturation and could be ideal vehicles to assist in the enhancement or suppression of male fertility.

Keywords Epididymis · Domestic cat · Sperm maturation · Centrosome · Motility · Epididymosomes

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Introduction

Understanding the physiological processes leading to structurally and functionally mature spermatozoa is critical for overcoming common problems with infertility or developing efficient contraception strategies. Specifically, sperm maturation in the epididymis is fundamental for the cell to acquire the ability to fertilize an oocyte. Even though thorough reports exist on the sperm maturation in several species [1], many mechanisms remain to be deciphered regarding the epididymal sperm maturation in the domestic cat [2]—a critical model for biomedical studies and wild felid conservation. Besides important morphological changes, sperm maturation is associated with the integration of specific factors including peptides and microRNA [3, 4]. Classical methods of protein secretion by the epithelium of the epididymis involve the merocrine pathway in which proteins contain signal sequences and are secreted individually [3, 5]. These signal peptides then direct proteins to binding sites on the sperm cell to serve as coating proteins or are internalized [6–8]. Conversely, other secreted proteins without a signal sequence are secreted within small vesicles termed "epididymosomes." These small vesicles are secreted from the apical pole of the epididymal

epithelial cells in an apocrine manner [9]. This type of protein secretion and delivery to the maturing sperm cells via small vesicles has been demonstrated in other species, including the rat, hamster, cow, and human [10–13]. Interestingly, epididymosomes have been observed in domestic cat, but their role has not been demonstrated [14].

Although previous studies have assessed the content of epididymosomes (microRNA, peptides, proteins, and other factors [4]), much remains unclear about their impact on sperm acquisition of fertilizing ability and motility. Previous studies suggested that epididymal vesicles likely promote sperm motility as they contribute to significant incorporation of multiple factors that modulate motility including aldose reductase, sorbitol dehydrogenase, and macrophage migration inhibitory factor [7]. Using the domestic cat model, we already have determined that key proteins such as cenexin are supplied to the spermatozoa throughout the epididymal transit [15]. A critical aspect is the centrosomal maturation allowing the sperm cell to form a large sperm aster after penetration into the oocytes followed by successful embryo development as demonstrated in the cow [16] and in the domestic cat [17]. However, the influence of epididymosomes on the acquisition of sperm motility has not been demonstrated yet. The objectives of the study were to (1) characterize mechanisms involved in the transfer of key factors—using the cenexin as a marker—between the epididymis and the maturing sperm cells and (2) demonstrate the impact of such mechanisms on the acquisition of functional properties by the spermatozoa.

Materials and methods

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

Sample collection and preparation

Testes from adult (> 1 year) domestic cats were harvested during routine orchiectomy and donated from local veterinary clinics. Tracts were transported and stored in phosphate-buffered saline (PBS) at 4 °C until processing. Testicular and epididymal tissues were dissected and isolated according to the different regions (rete testis, caput, corpus, and cauda epididymidis) with a scalpel blade in PBS and kept separately. Part of the epididymal tissues were sliced with a scalpel blade to recover the luminal fluids and release sperm cells that were recovered after centrifugation at 300×*g* for 8 min in PBS medium. Cell debris was discarded from the supernatant by a series of centrifugations at 700×*g* for 10 min and 3000×*g* for 10 min at room temperature. The epididymosome fraction was isolated from the remaining luminal fluid by ultracentrifugation at 100,000×*g* for 2 h at 4 °C and re-suspended in fresh PBS. Aliquots of both luminal fluid and epididymosome samples were stored at − 20 °C until further processing.

Electron microscopy

Pellets obtained after ultracentrifugation were exposed to uranyl acetate for 1 min (negative staining) followed by multiple observations using a transmission electron microscope (Zeiss 10 CA transmission electron microscope) at the University of Maryland Laboratory for Biological Ultrastructure, USA.

Tissue processing and immunostaining

Tissue samples were fixed with 4% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned at a thickness of 5 μm. Samples then underwent 10-min antigen retrieval at 95 °C (10 mM citric acid, 3 mM ethylenediaminetetraacetic acid supplemented with 1% Triton-X), and permeabilized with 0.1% Triton X-100 in PBS (PBS-T) for 3 min. The non-specific antigenic sites were blocked in 5% bovine serum albumin in PBS (1 h, room temperature) and incubated with anti-cenexin (1:100, Millipore) antibodies overnight at 4 °C in a humidified chamber. After washings (5 min each) in PBS twice and PBS-T once, samples were incubated with secondary antibodies labeled with a fluorescent probe for 1 h at 37 °C (goat anti-mouse 1:100) before observation under a microscope fitted with epifluorescence (Olympus BX41). Negative control treatments were also included in which primary antibody omitted and samples were labeled with the secondary fluorescent antibody.

Western blot analysis and mass spectrometry confirmation

Tissue samples from each segment were also isolated and homogenized in Tween-20 lysis buffer (150 mM sodium chloride, 50 mM Trizma base, 1% Tween-20) and centrifuged at 14,000×*g* for 15 min at room temperature for further analyses via Western blot. Samples were diluted with SDS loading buffer (Boston BioProducts) and incubated at 95 °C for 10 min. Samples were then separated by one-dimensional electrophoresis (Bio-Rad 4–15% Mini-PROTEAN TGX Gel) with tissue, epididymosome, and luminal fluid samples separated based on equivalent quantity of proteins (30 μg total protein per lane as determined by Bio-Rad assay kit using bovine serum albumin as standard). Proteins were transferred to a nitrocellulose membrane (Bio-Rad) and blocked for 1 h at room temperature in 1× Tris-buffered saline (154 mM Trizma HCl, 1 M sodium chloride supplemented with 1% Tween-20 and 7% skim milk powder). Membranes were then incubated overnight at 4 °C with anti-cenexin (1:1000, Millipore) and for 1 h at room temperature with goat anti-mouse secondary antibody coupled with horseradish peroxidase (1:2000, Sigma) in 1× Tris-buffered saline supplemented with 1%

Tween-20 and 5% skim milk powder, followed with Clarity Western ECL substrate (Bio-Rad) and imaged with ChemiDoc XRS imaging system (Bio-Rad). Results were also confirmed by gel digest analysis via liquid chromatography-mass spectrometry (LC-MS/MS) with a Waters nanoACQUITY HPLC system interfaced to a Thermo Fisher Q Exactive at MS Bioworks, USA.

Co-incubation assays

Epididymosome and luminal fluid samples were diluted to a concentration of 4 μg total protein/μL as determined by Bio-Rad assay kit using bovine serum albumin as standard. Immature testicular spermatozoa (8×10^6 sperm/mL) were then co-incubated for 3 h at 38 °C with epididymosome and luminal fluid samples. Following incubation, 20 μL of each sperm suspension was smeared on a glass slide and fixed with 4% paraformaldehyde (1 h, room temperature) and further prepared following the same procedures detailed above for the immunofluorescent staining of epididymal tissue. Controls included testicular spermatozoa incubated in plain media or testicular and cauda spermatozoa fixed without any treatment. Negative control treatments were also included in which primary antibody omitted and samples were labeled with the secondary fluorescent antibody only.

A proportion of sperm cells that exhibited positive staining at the location of the centrosome were recorded for each treatment, analyzing 200 cells in total per treatment of each individual male. Images of positively labeled sperm (30 images/treatment of each individual) were captured using SPOT Basic 5.1 software (Diagnostics Instruments). The pixel intensity of immunofluorescence (mean gray value) was then analyzed using ImageJ version 1.47 software to indicate the relative amount of protein bound with the centrosome. To calibrate the intensities between different sperm cells, the area measured was adjusted according to the size of each cell's centrosome and the intensity expressed as the averaged mean gray value recorded per pixel. Following these measurements, variations between treatments of each individual were also calibrated by averaging the background intensity of the negative control sample and the value subtracted from each positively labeled cell. The change in relative intensity of each treatment was then analyzed in comparison to the testicular spermatozoa treatment incubated in plain media.

Motility and acrosome integrity assays

Epididymosome samples were diluted to a concentration of 4 μg total protein/μL with Hepes-Ham F10 medium (25 mM Hepes, 1.0 mM pyruvate, 2.0 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 5% fetal calf serum) as determined by Bio-Rad assay kit using bovine serum albumin as standard. Immature spermatozoa from the caput epididymidis (with minimal motility) were then co-incubated for 3 h at 38 °C with the epididymosome samples (sperm cell concentration = 8×10^6 sperms/mL). Controls included sperm cells from caput and cauda epididymis incubated in plain buffer media. Percentage of motile spermatozoa and forward progressive motility (scale 0 = non-motile, 5 = fast and straight motility) were assessed (using standard methods developed in our laboratory [18]) every 30 min and then normalized to 100% motility or FPM at time 0 for each sample. Acrosome integrity also was assessed before and after the 3-h co-incubation via fixation in paraformaldehyde and stained with Coomassie Brilliant Blue R-250 (0.1% Coomassie Brilliant Blue R-250, 50% methanol, and 10% glacial acetic acid) and mounted with Permount™ Mounting Medium (Fisher Scientific, Pittsburgh, PA, USA). A proportion of sperm cells with intact acrosomes were recorded for each treatment, analyzing 200 cells total per treatment of each individual male.

Experimental design and statistical analysis

For initial immunostaining of tissues and isolated spermatozoa, 4 males were used in 4 different days (4 replicates). For Western blot and mass spectrometry, 4 tissue samples from 2 males each, 4 epididymal sperm samples from 10 males each, and 4 samples of luminal fluid and epididymosomes from 5 males each were used in 4 replicates. For electron microscopy, 1 pooled sample of 5 males each was used. A total of 10 photographs were captured to ensure accurate assessment. For co-incubations, spermatozoa were collected from 6 different males and incubated with luminal fluid and epididymosomes isolated from 6 pools of 5 males. Motility and acrosomes were assessed on spermatozoa collected from 4 different males and incubated with epididymosomes isolated from 4 pools of 5 males.

Statistical analyses were conducted via SAS software version 9.3 and GraphPad Prism software version 6. The proportion of positively stained spermatozoa following each treatment and the corresponding intensity of immunofluorescent stain were analyzed via analysis of variance with results further compared via protected Tukey's test, blocking for individual variation. The change in percent motile spermatozoa, forward progressive movement, and caput sperm acrosome integrity was analyzed using analysis of variance with repeated measures, and results were further compared via protected Tukey's test, blocking for individual variation. Change in cauda sperm acrosome integrity was analyzed using a paired *t* test.

Results

Presence and localization of cenexin in testicular tissues, epididymal segments, luminal fluid, and sperm cells

Cenexin was detected in all seminiferous tubules and interstitial cells of testicular tissues (Fig. 1a); in epithelial cells from

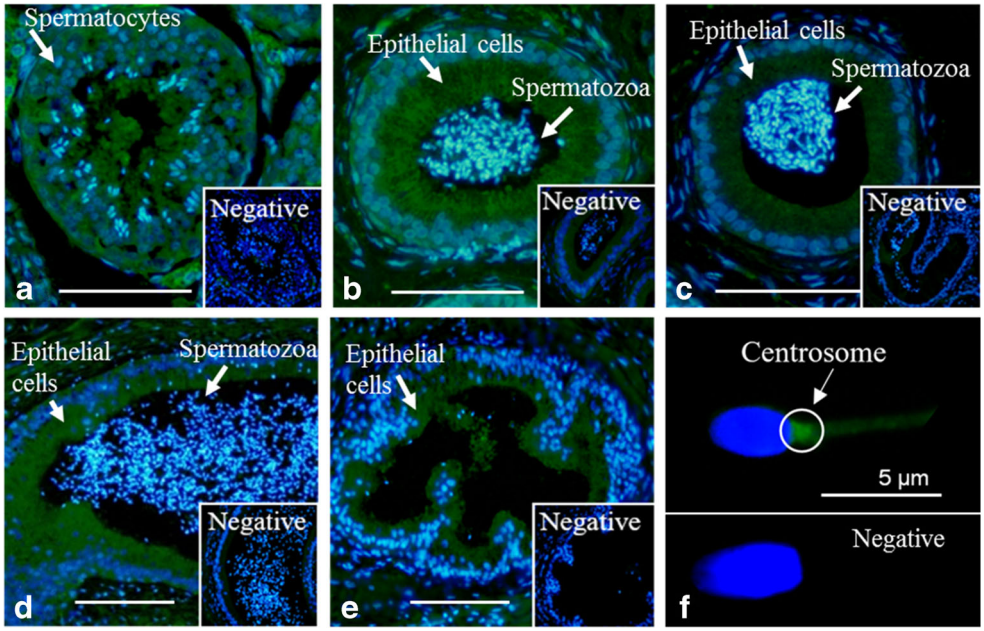

Fig. 1 Localization of cenexin by immunofluorescence (FITC; insets are negative controls) and nuclear chromatin counterstaining (Hoechst) in cross sections of **a** seminiferous tubules, **b** caput epididymidis, **c** corpus epididymidis, **d** cauda epididymidis, **e** vas deferens (scale bars = 100 μm), and **f** sperm centrosome from a cell collected in the cauda epididymidis

caput, corpus, and cauda epididymis (Fig. 1b–d), and in the vas deferens (Fig. 1e). When epididymal sperm cells were stained for cenexin, the labeling was located in the centrosomal area of the sperm neck/mid-piece (Fig. 1f). Western blot analysis (as well as mass spectrometry; data not shown) confirmed the presence of cenexin in tissues and in sperm cells isolated from the epididymis (Fig. 2a). Luminal fluids recovered from the different segments of the epididymis also contained cenexin (Fig. 2b). Epididymosomes isolated from luminal fluids in all segments of the epididymis contained cenexin (Fig. 2b). Electron microscopy revealed that epididymosome suspensions contained micro-vesicles of similar sizes and were free of contaminations (Fig. 3).

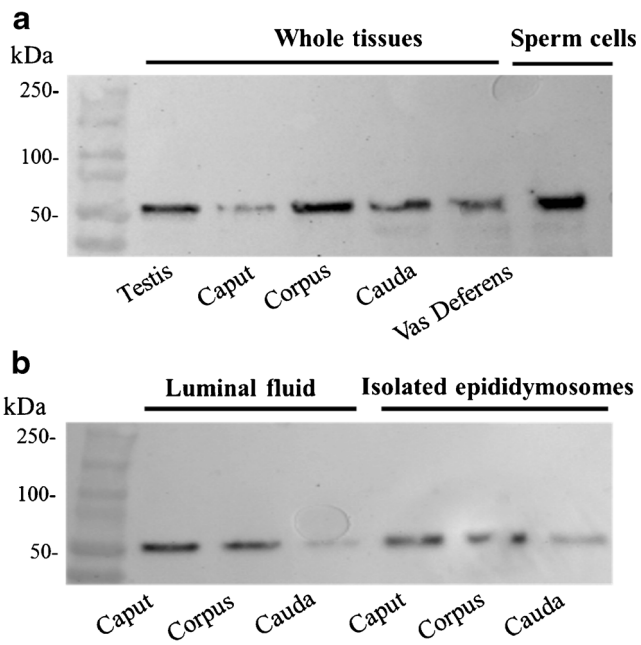

Fig. 2 Cenexin detection by Western blots in **a** whole testicular and epididymal tissues as well as pooled sperm samples from epididymal segments and **b** luminal fluid and isolated epididymosomes from epididymal segments

Role of epididymosomes in the transfer of cenexin between epididymal epithelium and sperm cells

Percentages of spermatozoa containing cenexin were higher ($P < 0.05$) in cells isolated from the cauda epididymidis (86.2 ± 7.0%) compared to testicular sperm cells (7.6 ± 1.6%; Fig. 4). Percentages of sperm cells with cenexin detected at the centrosome significantly increased ($P < 0.05$) in testicular spermatozoa co-incubated with epididymosome fractions (60.5 ± 4.3%) compared to controls (7.6 ± 1.6%) and in sperm samples co-incubated with plain buffer medium (12.6 ± 1.9%) or with luminal fluid (34.0 ± 8.6%; Fig. 4). Fluorescence intensity in positive cells did not differ between the treatment groups ($P > 0.05$); however, the intensity in immature sperm cells co-incubated with epididymosome fractions tended to be similar to the positive controls ($P = 0.06$; Fig. 5).

Influence of epididymosomes on the sperm motility and acrosome integrity

Exposure of immature sperm cells to epididymosomes resulted in relatively higher and sustained motility throughout incubation compared to immature sperm cells incubated in plain buffer medium or the control ($P < 0.05$, Fig. 6a). Forward progressive motility was affected by the epididymosome exposures at 1.5, 2.5, and 3 h of incubation ($P < 0.05$; Fig. 6b).

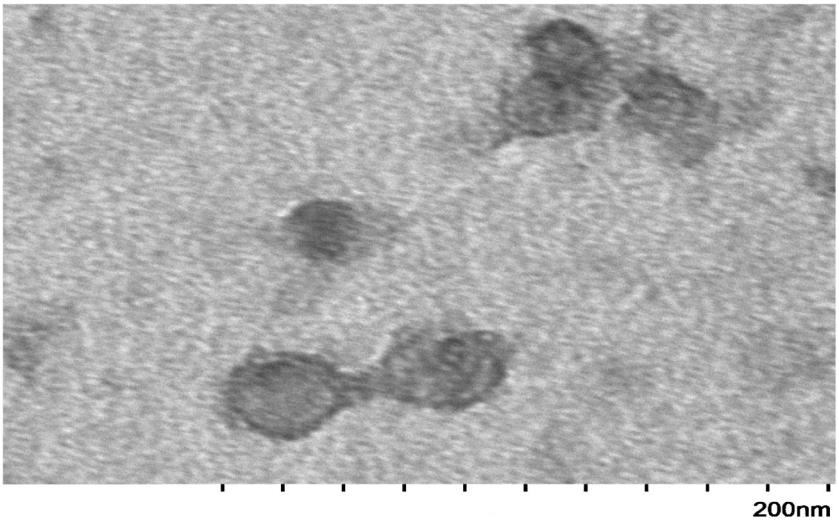

Fig. 3 Transmission electron micrograph of isolated epididymosome fractions

There were no significant effects of incubations on percentages of sperm cells displaying an intact acrosome regardless of the treatment ($P > 0.05$, range 80–93%).

Discussion

The main finding of this study is the evidence for the transfer of cenexin—a maturational marker—from the epididymal epithelium and sperm cells via epididymosomes. Our results also provide evidence that epididymosomes may have multiple roles, with increasing sperm cell protein content, and stimulate sperm motility without influence on acrosome integrity.

Analysis of tissues and sperm cells showed that cenexin was expressed in the epithelial cells of the epididymis, consistent with observations made in other somatic cells [19]. As such, this was the epididymal source of cenexin, which becomes critical in assessing the availability and physiological role of this protein. Detection of cenexin in sperm cells confirmed earlier studies from our laboratory showing that proportions of sperm cells with cenexin increase throughout epididymal transit [15] and could serve as a good marker of centrosomal maturation [19]. Principal cells of the epididymal epithelium have previously been reported to secrete numerous proteins via epididymosomes involved in the sperm maturation process in the rat [20–22]. Likewise, cenexin was detected in the luminal fluid and more specifically in epididymosomes isolated from the epididymal segments, suggesting that these cells are a principal source of cenexin from the epithelium. While cenexin has been previously reported to weigh approximately 95 kDa in the mouse [23], our analyses in the cat (confirmed by gel digest and LC-MS/MS) revealed a smaller protein weighing 50 kDa. This difference in molecular weight may be due to post-translational modification of the protein specific to the domestic cat, or possible proteolytic cleavage during sample processing.

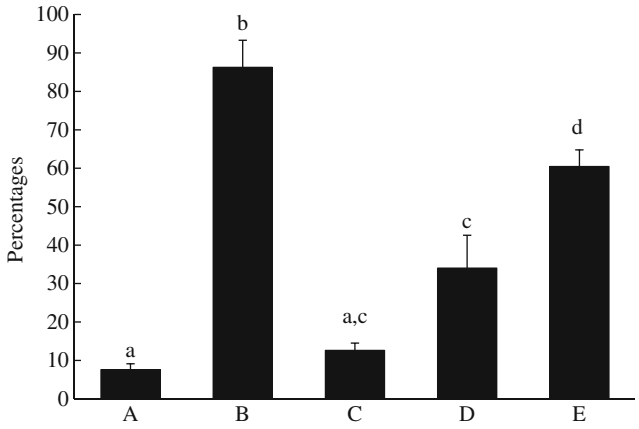

Fig. 4 Percentages (mean ± SEM) of spermatozoa positively stained for cenexin. Testicular spermatozoa non-exposed to epididymosomes (control, A) and mature spermatozoa (positive control, B). Testicular spermatozoa incubated for 3 h with plain buffer medium (C), luminal fluid (D), or epididymosome fraction (E). Bars with different letters significantly differ ($P < 0.01$)

Furthermore, our studies strongly suggested that cenexin is transported via epididymosomes to the sperm cells, based on the significant increase in centrosome-localized cenexin in the samples co-incubated with small vesicles. This was also supported by the fact that cenexin does not contain its own signal peptide sequence (as determined by sequence analysis via SignalP, version 4.0, D-score = 0.117) [24]. Indeed, proteins that do not contain a signal sequence are not secreted from epididymal epithelial cells via the merocrine pathway, and instead are packaged and transported to the sperm cells within the epididymosome vesicles [9]. Similarly, other proteins including aldolase reductase, p26h, macrophage migratory inhibition factor, and methylmalonate-semialdehyde dehydrogenase that lack a signal peptide were documented as delivered via epididymosomes [7, 11, 22]. Together, these results indicate that the epididymosomes supplied cenexin, and potentially other proteins important to centrosomal maturation. Further

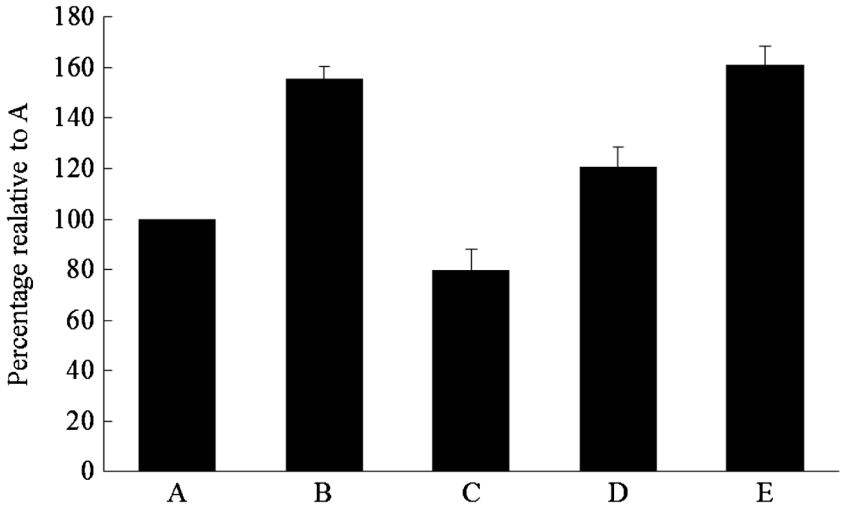

Fig. 5 Changes in the fluorescence intensity (mean ± SEM) of sperm cells positively stained for cenexin compared to the untreated control testis sample (A). Testicular spermatozoa non-exposed to epididymosomes (control, A) and mature spermatozoa (positive control, B). Testicular spermatozoa incubated for 3 h with plain buffer medium (C), luminal fluid (D), or epididymosome fraction (E)

proteomic analyses currently are underway in our laboratory to determine the exact composition of these epididymosomes. Protein content of small vesicles in the bovine model differs from the proteins in the surrounding luminal fluid compared to

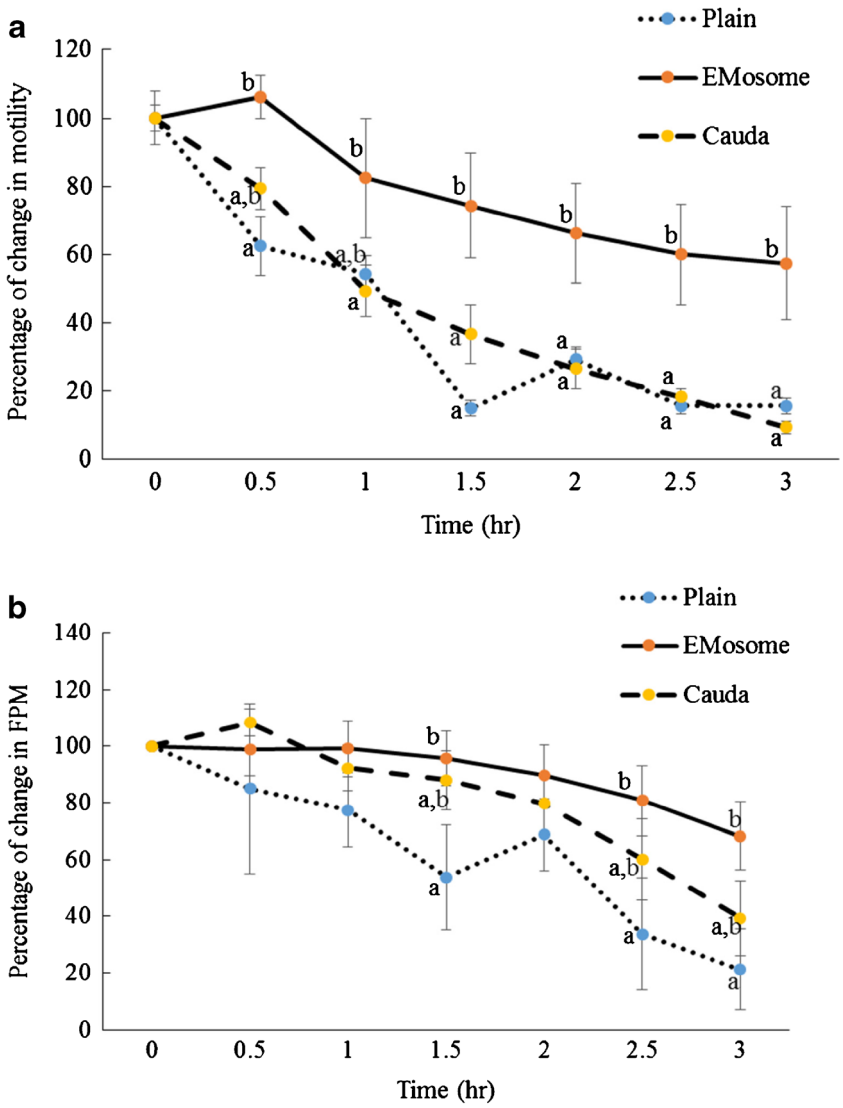

Fig. 6 Percentages (mean ± SEM) of sperm motility (**a**) and forward progressive motility (FPM; **b**) normalized to 100 at time 0 in samples of matured spermatozoa (Cauda; positive control), immature spermatozoa incubated with plain buffer medium (Plain; negative control), or incubated with epididymosomes (Emosomes). Within time points, different letters indicate significant difference ($P < 0.01$)

the higher content of the epididymosomes, which differs in the consecutive segments of the epididymis [25]. The exact role of each epididymal segment in production and secretory pattern still needs to be investigated in the cat model with the prediction of a sequential pattern.

Acquiring motility is one of the key properties gained by sperm cells as they transit through the epididymis. Influence of epididymosomes on this functional acquisition is not well understood; however, previous research indicated a supportive role [7, 26]. Exposure of immature sperm cells to epididymosomes resulted in sustained motility throughout the 3-h incubation, thus indicating that the epididymosomes may be supplying proteins that aid in the acquisition of motility. The same beneficial effect was observed on the forward progressive motility which suggests that epididymosomes also could supply nutrients or facilitate metabolism for greater motility [25, 26]. While previous studies have reported that a subpopulation of epididymosomes may provide proteins enhancing the sperm-egg interaction [25–27], we did not observe visible effects of co-incubation on the acrosome integrity. More analyses have to be conducted in our laboratory to assess the effects of co-incubation on sperm-egg binding in vitro. We also realize that our experiments involved epididymosome suspensions that were more concentrated than in the physiological luminal fluid. We hypothesize that the high concentration only accelerated the incorporation (in 3 h in vitro vs. several hours or days in vivo [2]) thereby providing insight into the physiological process, albeit at a faster rate.

In sum, our study demonstrates for the first time the mechanisms associated with secretion and incorporation of cenexin into immature spermatozoa during the cat sperm maturation. Furthering the understanding of these physiological processes will improve techniques of assisted reproductive techniques including in vitro sperm maturation. Impaired centrosomal maturation is a source of infertility in the cat, human, and cattle [16, 17, 28], but the ability to rescue normal functions of this organelle has yet to be achieved. Findings of this study are contributing to the development of in vitro culture strategies to overcome this type of infertility. Lastly, a better knowledge of that complex process is critical to potentially induce the suppression of male fertility.

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