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# Uroplakin expression in the male reproductive tract of rat

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ABSTRACT

Uroplakins (UPKs) play an important role in the normal and pathophysiology of the urothelium. They protect the urothelium and play a crucial role during urothelial infections by Uropathogenic *E. coli*. However, their functions beyond this organ system remain unexplored. A wide variety of proteins secreted in the male reproductive tract tissues contribute to spermatogenesis, sperm maturation, fertilization and innate immunity. However, the presence of UPKs and their possible contribution to the male reproductive tract physiology is not yet reported. Hence, in this study, we characterized UPKs in the male reproductive tract of rats. To the best of our knowledge, for the first time, we report the expression of UPKs in the male reproductive system. *Upk1a, Upk1b, Upk2* and *Upk3b* mRNA and their corresponding proteins were abundantly expressed in the caput, cauda, testis, seminal vesicles and the prostate. Their expression was not developmentally regulated. UPK protein expression was also localized on the spermatozoa, suggesting a role for these proteins in sperm function. To study the role of UPKs in innate immunity, *Upk* mRNA expression in response to endotoxin challenge was evaluated *in vitro* and *in vivo*. In the rat testicular and epididymal cell lines, *Upk* mRNA levels increased in response to lipopolysaccharide challenge. However, in the caput, cauda, testes, seminal vesicle and prostate obtained from LPS treated rats, *Upk* mRNA expression was significantly reduced. Results of this study indicate a role for UPKs in male reproductive physiology and innate immune responses.

## 1. Introduction

Uroplakins (UPKs) are a group of proteins that form the major constituents of urothelial plaques. In the mammals, five UPKs, namely, UPK1a, UPK1b, UPK2, UPK3a and UPK3b (a minor isoform of UPK3a) are characterized. Besides these, UPK2b and UPK3c and UPK3d were identified in other species (Desalle et al., 2014, Garcia-Espana et al., 2006, Wu et al., 1994). Mammalian UPKs are divided into two groups. UPK1a and UPK1b belong to the tetraspanin family, whereas UPK2 and UPK3 are grouped under the monospanin family (Yu et al., 1994, Lin et al., 1994, Wu and Sun 1993). The former family of proteins span the plasma membrane four times whereas the later span the plasma membrane only once. The plaques formed by UPKs generates an asymmetric unit membrane (AUM), which in turn functions to provide permeability barrier and structural stability to the urothelium. The formation of plaques involves specific interaction between the UPKs. UPK1a interacts with UPK2 whereas UPK1b interacts with UPK3a (Wu et al., 1994, Hu et al., 2005, Tu et al., 2006, Tu et al., 2002).

The functional significance of UPKs vary across the species. In mammals, though their major function is important for development, differentiation and homeostasis of the urothelium (Carpenter et al., 2016), in the *Xenopus* oocytes UPK3a and UPK1b form a complex to mediate sperm-egg interaction and fertilization (Sakakibara et al., 2005, Hasan et al., 2011, Mahbub Hasan et al., 2005). In the zebrafish, UPK3a related protein contributes to epithelial cell polarization and morphogenesis of pronephric tubules (Mahbub Hasan et al., 2005). A recent study indicates that the UPK2/3 proteins are related to phosphotyrosine phosphatases and thus may have a role in cellular signaling (Chicote et al., 2017). UPK knock out mice display renal failure and high rates of mortality (Kong et al., 2004, Hu et al., 2000). Mutations in the UPK genes resulted in renal hypo dysplasia, adysplasia and renal failure in humans (Jenkins et al., 2005, Schonfelder et al., 2006). Further, UPKs serve as anchors for Uropathogenic *E. coli* (UPEC) during infections (Wu et al., 2009). They allow binding of the type-1 fimbriae expressing UPEC strains and facilitate their binding to the urothelial surface and triggering a cascade of events that favor bacterial infection and migration not only in the bladder, but also in the upper urinary tract organs such as urethra and renal pelvic urothelia (Wu et al., 1996, Mulvey et al., 1998, Martinez et al., 2000). Altered levels of UPKs in urothelial carcinomas are considered as useful markers for the diagnosis, detection and prediction of urothelial carcinomas (Wu et al., 2009, Huang et al., 2007, Zupancic et al., 2011).

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Because of the tetraspanin nature of UPKs, they are predicted to play a key role in a variety of functions such as cell migration, immune signaling, membrane architecture and infection (Hemler 2003, Levy and Shoham 2005). The presence of UPKs in respiratory, ocular and other epithelial cell types indicate a wider role for these proteins beyond the urothelial functions (Adachi et al., 2000, Olsburgh et al., 2003). The spatiotemporal expression of UPK3b in the epididymal and testicular sperm and ovarian follicles implicates them in gametogenesis and the development of gamete delivery organs (Kuriyama et al., 2017). Recently it was demonstrated that mouse UPKs are expressed in non-urothelial lineages (Liao et al., 2018). All the known uroplakins were found to be expressed on the oocytes and associated with multivesicular bodies. Further, they were localized on the hook of the sperm. *In vitro* assays demonstrated reduced fertilization rates in mice eggs under conditions of knock down or antibody protection. Further, UPII/IIIa double knock out mice had smaller litter size suggesting are important in reproduction (Liao et al., 2018). Though UPKs have been well characterized in the urinary tract, their role in other organ systems are not well studied. The organs of male reproductive system has many anatomical similarities with the urinary system and both these are subjected to infections by UPEC, the bacterial species that expresses the type 1 fimbriae. In light of these similarities and the scarce of studies on the role of UPKs in other organ systems, the aim of this study was to analyze the expression pattern of UPk gene and their protein products in the male reproductive system of rat and to study their possible functional role during endotoxin challenge was analyzed. We observed that UPKs are abundantly expressed in the male reproductive tract of rat and their expression altered during endotoxin challenge both *in vitro* and *in vivo*.

## 2. Materials and methods

### 2.1. In silico analyses

The rat genome available at UCSC Genome Browser rat assembly RGSC6/rn6 was used to download all the known *Upk* gene sequences. Various properties of the genes and their proteins were predicted using *in silico* tools (Supplementary Table 1). Based on the genome assemblies deposited in Ensembl, gene neighborhood analyses was carried out. Self-Optimized Prediction Method with Alignment (SOPMA) was used to predict the percentage of all the secondary structures (α-helix, β-turn, extended strand and random coil) (Geourjon and Deléage 1995, Frishman and Argos 1995).

### 2.2. Molecular modeling

Amino acid sequences of UPK1a, UPK1b, UPK2, UPK3a and UPK3b were obtained from NCBI database and submitted to the GPCR-I-TASSER server (https://zhanglab.ccmb.med.umich.edu/GPCR-I-TASSER/) in the FASTA format. The server usually employs a hybrid approach that integrates experimental mutagenesis data with *ab initio* transmembrane helix assembly simulations to construct the final 3D-model of target proteins (Zhang et al., 2015). Models with high confidence score (C-score) were selected, which is typically in the range of (-5 to 2), where a C-score of a higher value signifies a model with a higher confidence and vice-versa. Further, the structural validation was performed using PROCHECK (Laskowski et al., 1993) and Ramachandran plot analysis (Ramachandran et al., 1963). Models with 90% and above residues in the most favored or allowed or additionally allowed regions were considered for further analysis. PyMOL was used for visualization of the selected models, structural comparison and picture representation.

### 2.3. Animals and tissue collection

Wistar rats were purchased from National Center for Laboratory Animals, National Institute of Nutrition, Hyderabad, India. The rats were housed at animal facility maintained at ambient temperature (25 °C) with free access to food and water and were daily monitored for their well-being. For collection of tissues, the animals were terminated in a carbondioxide containing jar. All procedures involving animals were conducted using the guidelines for the care and use of laboratory animals to minimize suffering and this study was specifically approved by the Institutional Animal Ethics Committee of University of Hyderabad (IAEC/UH/151/2017/01/SY/P7).

To determine the overall tissue distribution patter of *Upk*s, brain, heart, lungs, liver, kidney, spleen, ovary, uterus, cervix, bladder, caput (without the initial segment and corpus), cauda, testis, seminal vesicles and prostate were collected from adult Wistar rats (aged 90 days; n = 5 each of male and female). Epididymides and testes obtained from 1- to 60-day old Wistar rats (n = 5 for each age group) were used to study the developmental regulation. The tissues were placed in PBS, removed any excess fat and snap frozen in liquid nitrogen and stored at −80 °C until use. For collection of spermatozoa, cauda epididymides from adult Wistar rats were placed in PBS and squeezed gently with forceps. The spermatozoa were resuspended in PBS for further processing.

### 2.4. Polymerase chain reaction (PCR)

Total RNA isolated from different tissues using commercially available kits (Qiagen) was subjected to DNAse digestion to remove any contaminated DNA. 2 μg of total RNA was reverse transcribed and the expression of *Upk*s in these tissues was carried out using exon spanning gene specific primers (Supplementary Table 2) in a thermal cycler (cycling conditions: 94 °C for 1 min followed by 30 cycles at 94 °C for 30 sec, 58 °C for 30 sec and 72 °C for 30 sec, and with a final round of extension at 72 °C for 10 min). No reverse transcriptase and no template controls were included to confirm that the amplification is specific. The PCR amplicons were electrophoresed on 2% agarose gels and the images captured using a gel documentation system. The identity of the PCR amplicons was confirmed by sequencing though a commercial source (Bioserve Technologies Hyderabad, India). For quantification of gene expression, real time PCR was carried out using SYBR master mix kit (Applied Biosystems, Warrington, UK) in a thermal cycler (Applied Biosystems) using standard conditions.

### 2.5. Immunoblotting

Urinary bladder, caput, cauda, testes seminal vesicle and prostate tissues of 90 day old Wistar rats were homogenated in RIPA buffer (25 mM Tris-HCL, pH 7.6; 150 mM NaCl; 1% each of NP-40, sodium deoxycholate and sodium dodecyl sulfate;) and centrifuged at 12,857g for 10 min. The protein in the supernatant was quantified by Lowry's method. Total protein (100 μg) was electrophoresed on 15% SDS PAGE and transferred to nitrocellulose membrane (Hybond ECLTM; GE Healthcare, Little Chalfront UK) in a transfer buffer (25 mM Tris; 192 mM Glycine, 20% methanol (v/v); pH 8.3) with a constant voltage of 25 V for 16 h. 5% skim milk was used to block the membrane for 2 h at room temperature followed by probing (for 1 h) with primary antibody against the respective UPK protein. (UPK1a (Cat # ab183503) or UPK1b (Cat# ab185970) raised in rabbit (Abcam Biotechnology, Cambridge, UK) or UPK2 (Cat# sc-15178) raised in goat (Santa Cruz Biotechnology, Dallas, USA) or UPK3b (Cat# sc-165867) raised in goat (Santa Cruz). The blots were then washed thrice with TBS (Tris-buffered saline) and TBS-T (Tris-buffered saline, 0.1% Tween-20) followed by incubation with anti-rabbit secondary antibody (Cat# 65120) raised in goat or anti-goat secondary antibody (Cat# 611620) raised in rabbit (Invitrogen, Carlsbard, USA). After the incubation the membranes were washed thrice with TBS and TBS-T each for 10 min. At the end of washing, the membrane was developed using enhanced chemiluminescence kit (GE Healthcare, Buckinghamshire, UK).

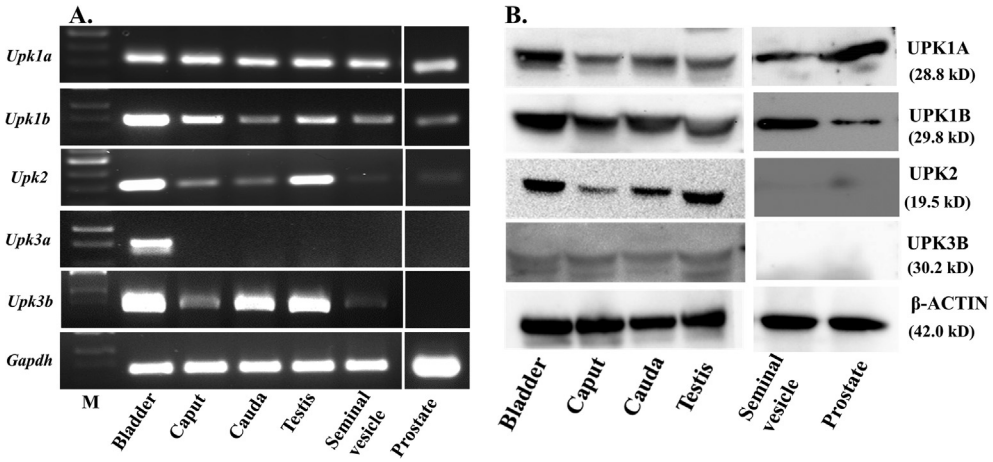


**Fig. 1.** Expression of *Upk* mRNA and protein in the rat male reproductive tissues. (A) *Upk* mRNA expression. Total RNA isolated from bladder, caput, cauda, testis, seminal vesicle and prostate was reverse transcribed and the cDNA obtained was used as template for amplifying the *Upk1a, Upk1b, Upk2, Upk3a* and *Upk3b* by gene specific PCR. *Gapdh* expression was analyzed to serve as internal control. (B) UPK protein expression. 100 µg of the protein obtained from bladder, caput, cauda and testis homogenates were electrophoresed on 15% SDS-PAG and transferred to nitrocellulose membrane. The immunoblots were probed with UPK1a or UPK1b antibody (raised in rabbit) or UPK2 antibody (raised in goat) or UPK3b antibody (raised in goat); followed by incubation with labelled anti-rabbit secondary antibody raised in goat or anti-goat secondary antibody raised in rabbit.

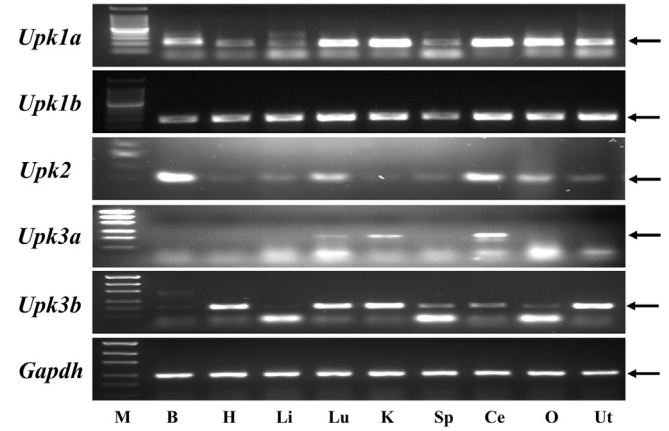


**Fig. 2.** Tissue distribution of *Upk*s. Total RNA isolated from Brain (B), Heart (H), Lungs (Lu), Liver (Li), Kidney (K), Spleen (Sp), Cervix (Ce), Ovary (O) and Uterus (Ut) was reverse transcribed and the cDNA obtained was used as template for amplifying the *Upk1a, Upk1b, Upk2, Upk3a* and *Upk3b* by gene specific PCR. *Gapdh* expression was analyzed to serve as internal control. M indicates DNA ladder. Arrow indicates the amplicon of interest.

### 2.6. Immunofluorescence

Epididymides (caput, cauda) and testes obtained from adult Wistar rats were fixed in Bouin's solution and 4% paraformaldehyde solution respectively and embedded in paraffin. Five micron sections (obtained by serial sectioning) placed on a glass slide were kept on a platform preheated to 60 °C for 5 min followed by washings with xylene, gradient alcohol (70–100%) and PBS for 10 min each. Slides were placed in a container filled with 10 mM citrate buffer, pH 6.5 maintained at 60C for 12 min (for antigen retrieval) and subjected to permeabilization with PBS containing 1% triton X-100 (PBST) for 15 min. 10% goat serum was then added and incubated for 45 min to allow blocking. Sections were incubated for one hour with the respective primary antibody as indicated in the immunoblotting section above. The primary antibody was washed off thoroughly and the sections incubated with anti-rabbit (Cat# 656111; Invitrogen) or anti-goat (Cat# sc-2777; Santa Cruz) -FITC-labelled secondary antibody. Nucleus was stained with 4′, 6-diamidino-2-phenylindole (DAPI; Sigma Aldrich, St. Louis, USA) followed by addition of mounting medium and covered with cover slip. Multiple images (around 10) for each sample were recorded by taken in a trinocular fluorescence microscope with excitation and emission at 495 nm and 519 nm respectively. In the case of spermatozoa, smears on glass slides were prepared, air dried and fixed with 4% paraformaldehyde. They were then permeabilized with PBST, blocked with 10% goat serum and processed in a similar way as that of tissue sections.

To determine the specificity of antibodies, appropriate controls were included. Since UPKs are difficult to express using recombinant technology, pcDNA vector that encodes either one of the UPK proteins was transfected into HEK cells and incubated with primary and FITC-labelled secondary antibody (positive control). Untransfected HEK cells incubated with primary and FITC-labelled secondary antibody served as negative control. Further, another negative control to test the non-specific binding of secondary antibody was also included by incubating the tissue sections with only FITC-labelled antibody.

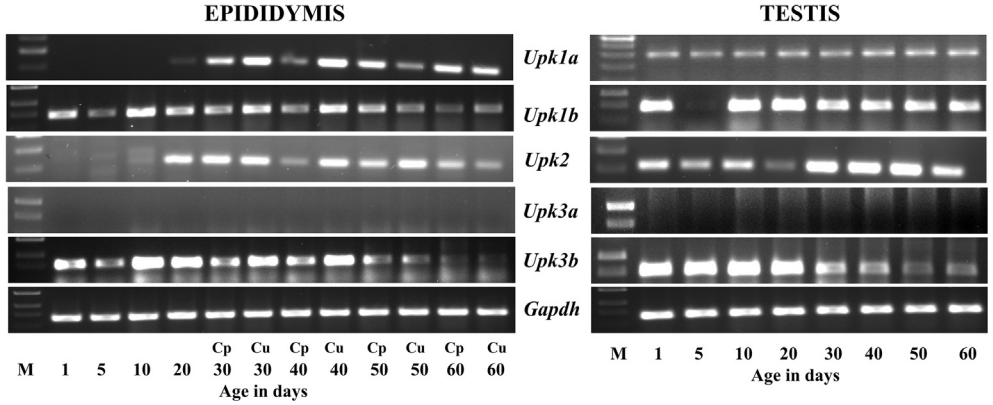


**Fig. 3.** Developmental regulation of *Upk* genes in epididymis and testes. RNA from epididymis and testes collected from 1, 5, 10, 20, 30, 40, 50 and 60 day old rats was reverse transcribed and the cDNA obtained was used as template for amplifying the *Upk1a, Upk1b, Upk2, Upk3a* and *Upk3b* by gene specific PCR. *Gapdh* expression was analyzed to serve as internal control. Cp-caput, Cu-cauda.

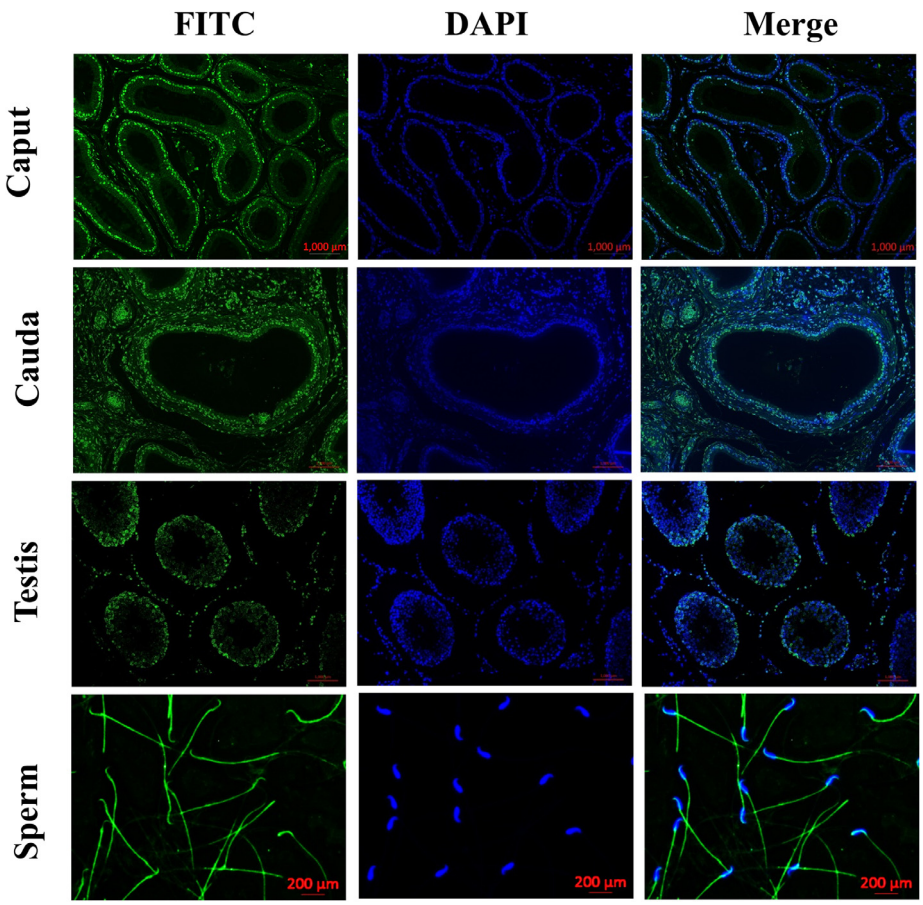


**Fig. 4.** Immunolocalization of UPK1a. Serial sections (5 µm thickness) of the rat testes and epididymides and smears of spermatozoa were fixed and incubated for one hour with UPK1a antibody (raised in rabbit) followed by incubation with FITC labelled anti-rabbit secondary antibody raised in goat. Nucleus was stained with 4′, 6-diamidino-2-phenylindole and images taken using fluorescence microscope with excitation and emission at 495 nm and 519 nm respectively. Magnification: 10X.

### 2.7. Treatment with LPS in vitro and in vivo

Rat testicular carcinoma cells (LC540) were obtained from National Centre for Cell Science, Pune, India and were maintained in EMEM medium (Sigma Aldrich, St. Louis, USA) containing 5% FCS at 37 °C. The rat caput immortalized cell lines (RCE) were a kind gift from Dr. Daniel Cyr, University of Quebec, Canada. They were maintained in DMEM medium containing 5% FBS at 32 °C. $1 \times 10^6$ cells were plated in each well of a 24 well plate (Sigma Aldrich, St. Louis, USA) and allowed to adhere. Following replacement of culture medium, they were treated with 100 ng/ml *E. coli* lipopolysaccharide (LPS; Sigma Aldrich, St. Louis, USA) and incubated up to 24 h. The treatment dose and time points were chosen basing on our previous studies (Biswas and Yenugu 2013). Cells were collected at different time points (0, 3, 6 12 and 24 h after treatment) for RNA isolation. Since LPS was dissolved in phosphate buffered saline (PBS), pH 7.4, control cells were treated with PBS. The experiments were conducted three times in triplicates. To determine the effect of LPS on *Upk* gene expression, adult male Wistar rats (n = 5 for each group) were treated intra peritoneally with 1 mg/kg body weight of *E. coli* LPS and sacrificed at different time points (0, 6, 12 and 24 h after LPS injection). The dosage of LPS was based on our previous studies (Biswas and Yenugu 2011). Control animals (n = 5) received PBS equivalent to the volume that was used for LPS treatment. Caput, cauda, testis, seminal vesicle and prostate were collected at each of these time points. They were then snap frozen in liquid nitrogen and stored at −80 °C until further use.

### 2.8. Statistical analyses

Statistical analyses were performed using one way ANOVA (multiple comparison; Holm-Sidak test) and Student's *t*-test available in Sigma Plot software 12.5, Build 12.5.0.38 (SPSS Inc., Chicago, IL, USA). Values shown are Mean ± S.D. *p < 0.05 denotes statistical significance.

## 3. Results

### 3.1. In silico analyses

To gain insight into the role of UPKs in the male reproductive system, we initiated *in silico* studies to gather first-hand information on the characteristic features of their genes and proteins. A thorough search in the rat genome indicated that the gene sequence of *Upk1a*, *Upk1b*, *Upk2* and *Upk3a* (NM_001108911.1, NM_001024253.1, NM_001109523.1 and NM_001130507.1 respectively) are already available in the GenBank. Besides these, alternate transcripts for *Upk1a* (XM_017589474.1; XM_008759176.2; XM_017589475.1) and *Upk1b* (XM_006248367.2; XM_006248368.3) were found to be predicted. Besides the reported *Upk* gene sequences, identification and characterization of the alternate transcripts is an important aspect in studying the functional characterization. Hence, using cDNA from rat urinary bladder, the amplicons corresponding to *Upk1a* alternate transcript 2 (XM_008759176.2), *Upk1b* alternate transcript 1 (XM_006248367.2) were amplified and sequenced. Rat *Upk3b* is not yet reported. Two alternate transcripts (XM_341056.8 and XM_006249208.3) are predicted. To characterize rat *Upk3b* further, we amplified XM_341056.8. The sequence obtained was submitted to GenBank and were assigned the accession numbers KU310911 (*Upk1a* alternate transcript 2), KU310910 (*Upk1b* alternate transcript 1) and KU310912.1 (*Upk3b* alternate transcript 1). For practical purposes, in this manuscript, we labelled the *Upk1a* alternate transcript 2, *Upk1b* alternate transcript 1 and *Upk3b* alternate transcript 1 as *Upk1a*, *Upk1b* and *Upk3b* respectively. We observed that *Upk3b* gene was located at chromosome 12q.12 (Supplementary Fig. 1), whereas *Upk1a, Upk1b,*

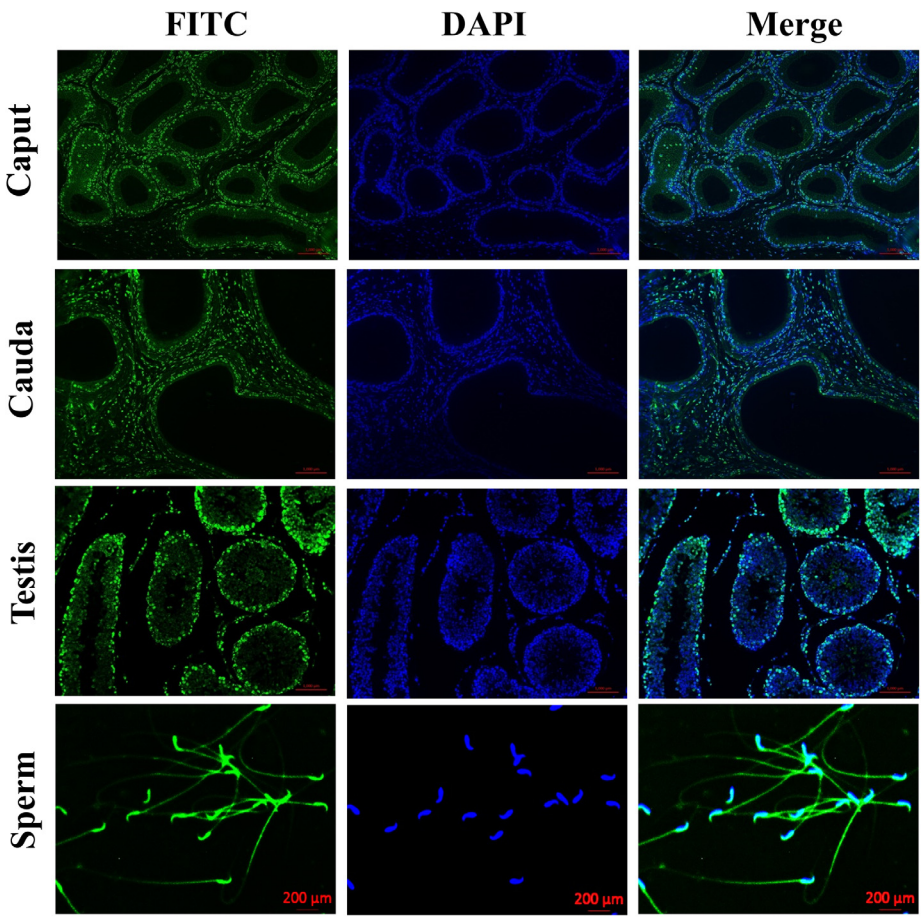


**Fig. 5.** Immunolocalization of UPK1b. Serial sections (5 µm thickness) of the rat testes and epididymides and smears of spermatozoa were fixed and incubated for one hour with UPK1b antibody (raised in rabbit) followed by incubation with FITC labelled anti-rabbit secondary antibody raised in goat. Nucleus was stained with 4′, 6-diamidino-2-phenylindole and images taken using fluorescence microscope with excitation and emission at 495 nm and 519 nm respectively. Magnification: 10X.

*Upk2* and *Upk3a* genes were localized to the chromosomal positions 1q21, 11q21, 8q22 and 7q34 respectively (as per the information available at NCBI). The coding region of the mRNA and the corresponding amino acid sequence of *Upk1a, Upk1b* and *Upk3b* characterized in this study are shown in Supplementary Figs. 2 and 3. The general characteristic features of all the rat *Upk* genes and their protein products were compiled based on the information available in the rat genome at NCBI and are given in Supplementary Table 3.

Depending on their organization in the plasma membrane, UPK1a and UPK1b belong to tetraspanin family, whereas the other UPKs are included in the monospanin family. Analyzing sequence similarity among a group of proteins provides information on the possible functional roles. Such an analyses is lacking for the rat UPK proteins. To determine the similarity among the rat UPKs, their sequences were aligned using T-coffee program (Supplementary Figs. 4 and 5). Among UPK1a and UPK1b, the sequence similarity was about 53% (Supplementary Table 4). The similarity score of UPK2 with UPK3a and UPK3b was 23 and 24 respectively. UPK3a and UPK3b had a similarity score of 39 (Supplementary Table 4). The rat, mouse and human UPK sequences were aligned to determine the homology of each of the UPK among these species (Supplementary Fig. 6). Rat UPK1a displayed 98 and 70% similarity with the mouse and human counterparts respectively (Supplementary Table 4). Similarly, UPK1b, UPK2 and UPK3a and UPK3b displayed a very high similarity with the mouse and human counterparts. (Supplementary Table 4).

Gene expression is influenced by the nature of genes present in its vicinity and this can be determined by neighborhood analyses. *Upk* genes are located on different chromosomes and such an analyses to determine the nature of genes that flank them in the rat and the comparison with the mouse and human genomes is not reported. We show that *Psenen, U2af1l4, Igflr1, Zptb32, Cox6b1* and *Rbm42* genes were found to be commonly present in the rat, mouse and human chromosomal regions where *Upk1a* was located (Supplementary Fig. 7). In the case of *Upk1b*, the genes *Igs11, B4galt4, Tmem39a* and *Poglut1* were present in the neighborhood region in all the three species (Supplementary Fig. 7). Genes that were commonly present in the neighborhood of *Upk2* were *S1c37a4, Trappc4, Bcl9l* and *Cxcr5* (Supplementary Fig. 8). *Smc1b, Ribc2, Fam118a* and *Fbln1* were the genes commonly present in the *Upk3a* neighborhood in all the three species (Supplementary Fig. 8). Genes that were commonly present in the neighborhood of *Upk3b* were *Dtx2, Zp3, Ssc4d, Ywhag* and *Hspb1* (Supplementary Fig. 8).

Though the three dimensional structures for UPKs in some species are reported, such an attempt was not made for rat UPKs. Because of the differences in the amino acid sequence homology of these proteins among the species, the three dimensional structure of rat UPKs may vary. Hence, to gain further insight into the structural aspects of UPKs, their three dimensional structure was obtained (Supplementary Fig. 9). The templates used for generating the three dimensional structures of UPKs varied significantly (Supplementary Table 5). Ramachandran plots generated for each of the UPKs indicated that majority of the amino acids are in the allowed regions and the structures predicted are valid (Supplementary Table 5). UPK1a and UPK1b that belong to tetraspanin family display more of alpha-helix, whereas UPK2, UPK3a and UPK3b that belong to monospanin family tend to have more random coil (Supplementary Fig. 9). To correlate the observations made in the three dimensional structure, the percentage of different structural features were determined by Self-Optimized Prediction Method with Alignment (SOPMA). UPK1a was found to have 40.07, 23.34, 8.17 and 28.40% of alpha-helix, extended strand, beta-turn and random coil respectively (Supplementary Table 6). UPK2 also showed such a similar distribution wherein the percentage of alpha-helix, extended strand,

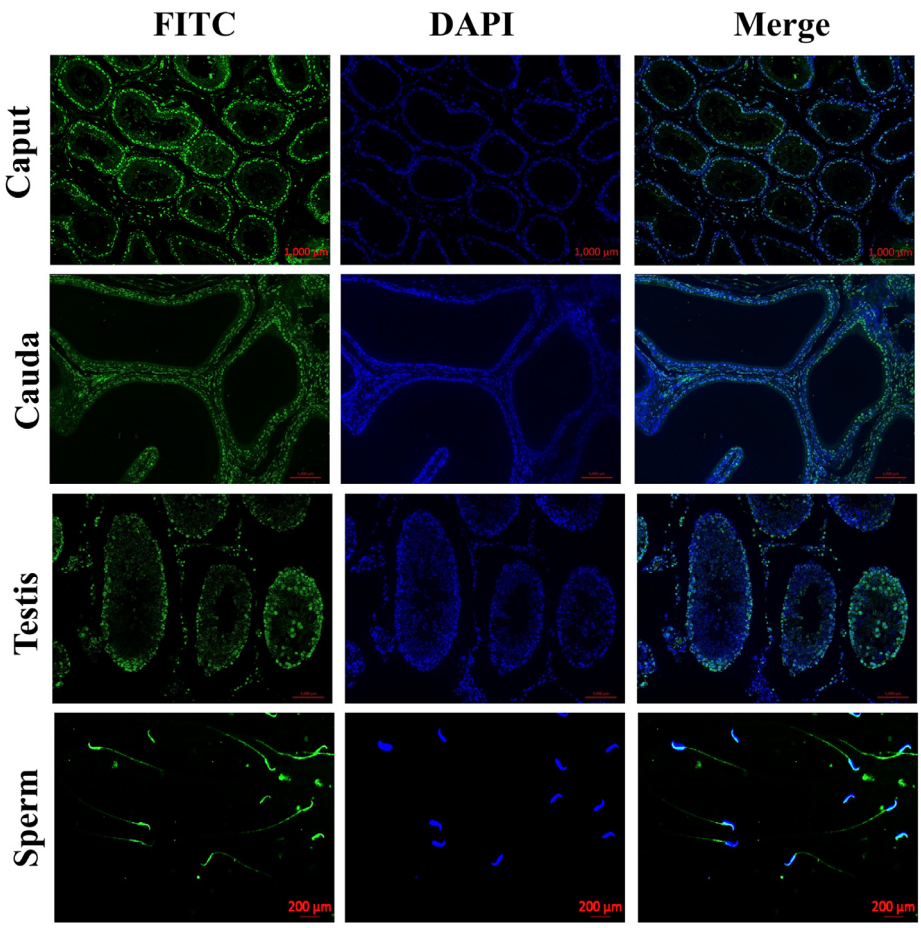


**Fig. 6.** Immunolocalization of UPK2. Serial sections (5 µm thickness) of the rat testes and epididymides and smears of spermatozoa were fixed and incubated for one hour with UPK2 antibody (raised in goat) followed by incubation with FITC labelled anti-goat secondary antibody raised in rabbit. Nucleus was stained with 4′, 6-diamidino-2-phenylindole and images taken using fluorescence microscope with excitation and emission at 495 nm and 519 nm respectively. Magnification: 10X.

beta-turn and random coil were 42.85, 18.11, 7.31 and 31.70 respectively (Supplementary Table 6). It appears that UPK1a and UPK1b have higher content of alpha-helix followed by random coil, extended strand and beta-turn. In UPK2, the percentage of alpha-helix, extended strand, beta-helix and random coil content was 32.06, 22.28, 10.32 and 35.32 (Supplementary Table 6). UPK3a is predicted to have 19.51, 26.82, 10.45 and 43.20 percent of alpha-helix, extended strand, beta-helix and random coil (Supplementary Table 6). UPK3b contains 12.72, 22.18, 5.45 and 59.63 percentage of alpha-helix, extended strand, beta-helix and random coil respectively (Supplementary Table 6). From these analyses it appears that the percentage of random coil was higher followed by extended strand, alpha helix and beta-turn in the case of members belonging to monospanin family (UPK2, UPK3a and UPK3b).

### 3.2. Uroplakin expression in the rat

Analyzing *Upk* gene and protein expression pattern in the male reproductive tract and their possible contribution to this organ system has gained importance in the recent past. In this study, to gain an in depth understanding of *Upk* expression in the male reproductive system of rats, their mRNA expression pattern was analyzed using PCR. *Upk1a* and *Upk1b* were expressed in the caput, cauda, testis, seminal vesicle and prostate (Fig. 1A). *Upk2* was predominantly expressed in the testis with lower level of expression in the caput, cauda, seminal vesicle and prostate. *Upk3a* mRNA was not detected in all the male reproductive tract tissues. *Upk3b* expression was evident in the caput, cauda, testis and seminal vesicle with no expression in the prostate (Fig. 1A). All *Upks* were expressed abundantly in the bladder, which was used as a positive control. Western blotting indicated the expression of UPK1a, UPK1b and UPK2 and UPK3b proteins in the caput, cauda, testis, seminal vesicle and prostate (Fig. 1B). To further confirm whether *Upks* are male reproductive specific, their expression was analyzed in other tissues of male rats and in the reproductive tissues of female rats. *Upk1a*, *Upk1b*, *Upk2* and *Upk3b* were found to be expressed in all the tissues analyzed, whereas *Upk3a* was restricted to lung, kidney and cervix (Fig. 2). Since gene regulation in the testis and epididymis is highly regulated by androgens during development, *Upk* mRNA expression pattern in the epididymis and testis during different stages of development was analyzed (Fig. 3). In the epididymis, *Upk1a* and *Upk2* expression was evident from 20 days of postnatal development, where as *Upk1b* and *Upk3b* were detected in all the stages. *Upk3a* was not detected at all the stages analyzed (Fig. 3). In the testis, except for *Upk3a*, other *Upks* were found to be expressed at all the developmental stages analyzed (Fig. 3).

To determine if UPKs may have a role in male reproductive physiology, sperm function and fertilization, we examined their localization in the caput, cauda, testis and male gametes by immunofluorescence microscopy using a trinocular fluorescent microscope. Prior to the localization analyses in the tissues, the specificity of the antibodies were tested by including positive and negative controls. We put our best efforts for recombinant expression of UPK proteins to be used in peptide controls. However, we met with little success. Alternatively, HEK cells were transfected with PCDNA vector encoding either one of the UPK proteins and incubated with the respective primary and secondary antibodies (positive control). Further, untransfected HEK cells were incubated with primary and secondary antibody served as negative controls. HEK cells transfected with the vector encoding the UPK protein showed abundant fluorescence (Supplementary Fig. 10), whereas such a fluorescence was not observed in untransfected cells (Supplementary Fig. 11). Further, we also checked for the non-specific binding of the secondary antibody by incubating the tissue sections only with FITC-labelled secondary

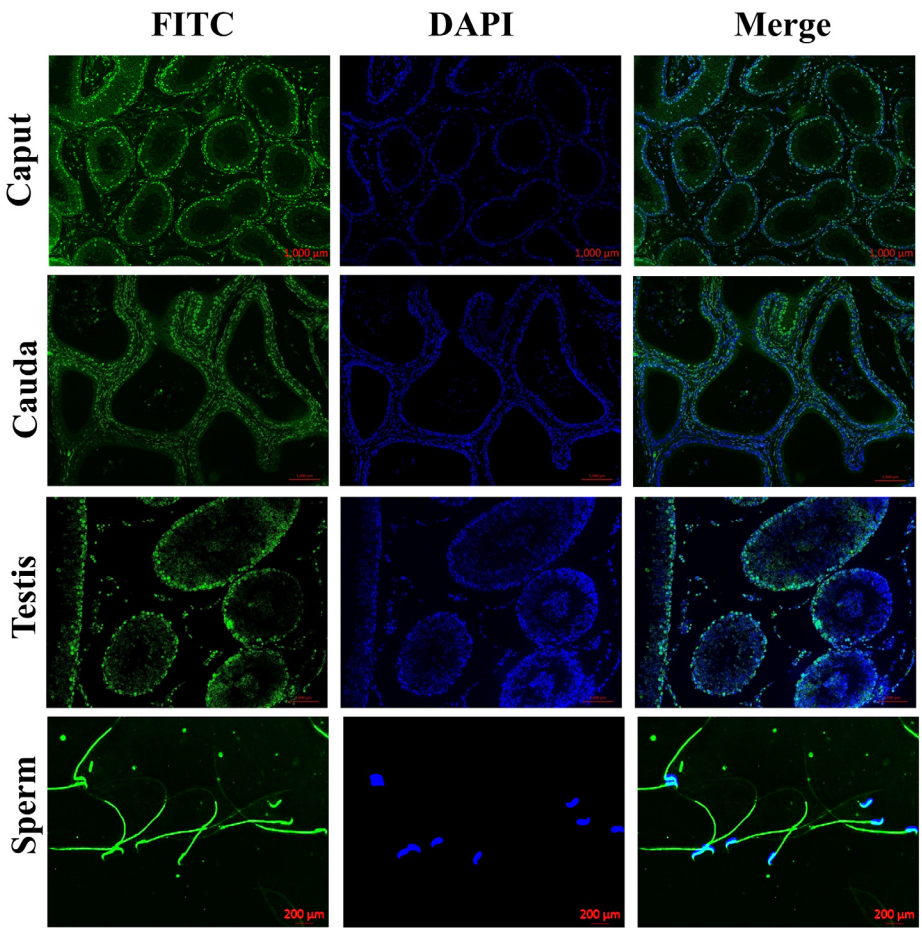


**Fig. 7.** Immunolocalization of UPK3b. Serial sections (5 µm thickness) of the rat testes and epididymides and smears of spermatozoa were fixed and incubated for one hour with UPK3b antibody (raised in goat) followed by incubation with FITC labelled anti-goat secondary antibody raised in rabbit. Nucleus was stained with 4′, 6-diamidino-2-phenylindole and images taken using fluorescence microscope with excitation and emission at 495 nm and 519 nm respectively. Magnification: 10×.

antibody. The negative controls (incubated only with the secondary antibody) did not shown any fluorescence (Supplementary Fig. 12). These observations suggest that the antibodies used were specific to the respective UPK protein. UPK1a was localized in majority of the cells that are closer to the basement in the caput and cauda. In the testis, the localization was observed throughout the seminiferous tubules and also in the interstitial cells. Further, it was localized in all the regions of the sperm (Fig. 4). Similar staining pattern was observed for UPK1b (Fig. 5), UPK2 (Fig. 6) and UPK3b (Fig. 7). In the sperm, all the UPKs were localized in the head, mid piece and tail regions (Figs. 4–7).

### 3.3. Modulation of Upk gene expression by LPS

Male reproductive tract proteins are known to have roles in innate immunity besides sperm function. Since we observed that UPKs are abundantly expressed in the male reproductive tract tissues, we anticipate that they may have additional role in innate immune responses and this could be a novel function of UPKs that has not been reported till now. In order to understand the possible functional role of UPKs in the male reproductive system immune responses, we analyzed the dynamics of their mRNA levels during endotoxin challenge using *in vitro* and *in vivo* model systems. Since *Upk1a*, *Upk1b* and *Upk3b* were expressed in LC540 and RCE cells, further analyses were carried out on only these genes. In LC540 cells, *Upk1a* expression increased significantly up to 12 h after treatment, whereas such an increased expression of *Upk1b* was observed only up to 3 h. However, *Upk3b* expression was decreased 24 h after LPS challenge (Fig. 8). In the RCE cells, *Upk1a*, *Upk1b* and *Upk3b* expression increased significantly up to 6 h after LPS treatment followed by a decline below the control levels at the later time points (Fig. 8). The effect of endotoxin challenge was also evaluated *in vivo* using rat as the model system. In the caput obtained from rats challenged with LPS, the mRNA levels of *Upk1a*, *Upk2* were not altered; whereas, a time dependent significant decline in *Upk1b* and *Upk3b* was observed (Fig. 9). In the cauda, the mRNA levels of all the *Upks* were found to be significantly decreased following LPS administration (Fig. 9). Testicular *Upk1a* and *Upk1b* expression was decreased at all the time points after LPS treatment. *Upk2* though increased at the 3 h time point was found to be decreased at the later time points. *Upk3b* expression remained unchanged. (Fig. 9). *Upk1a*, *Upk1b*, *Upk2* mRNA levels were significantly reduced in the seminal vesicles. *Upk3b* mRNA levels were down regulated at 6 and 24 h after LPS challenge, (Fig. 9). In the prostate, though *Upk1a* and *Upk2* mRNA levels increased in a time dependent manner after LPS challenge up to12 h followed by a decline, the mRNA levels of *Upk1b* was significantly reduced at all the time points analyzed (Fig. 9).

## 4. Discussion

UPKs are implicated to play a crucial role in urothelial physiology and pathogenesis (Carpenter et al., 2016, Wu et al., 2009, Liao et al., 2018, Zupancic and Romih 2013). They have been projected as key factors to indicate urothelial infections and cancers of the urothelial system (Lee 2011). However, their expression beyond the urothelial systems was proposed (Lee 2011). *Upk* expression was reported in the human female genital tract (Shapiro et al., 2000) and may have a role in the developmental process (Cunha et al., 2017) and pathophysiology (Ogawa et al., 1999). Reduced *Upk* gene expression is associated with poor prognosis in colorectal and adenocarcinoma patients (He et al., 2014, Zheng et al., 2014). UPKs were implicated to be crucial in the fertilization process (Liao et al., 2018). Analyzing the role of UPKs in other organ system has been an emerging area of research in the recent past. This study aims at investigating the possible functions of UPKs in

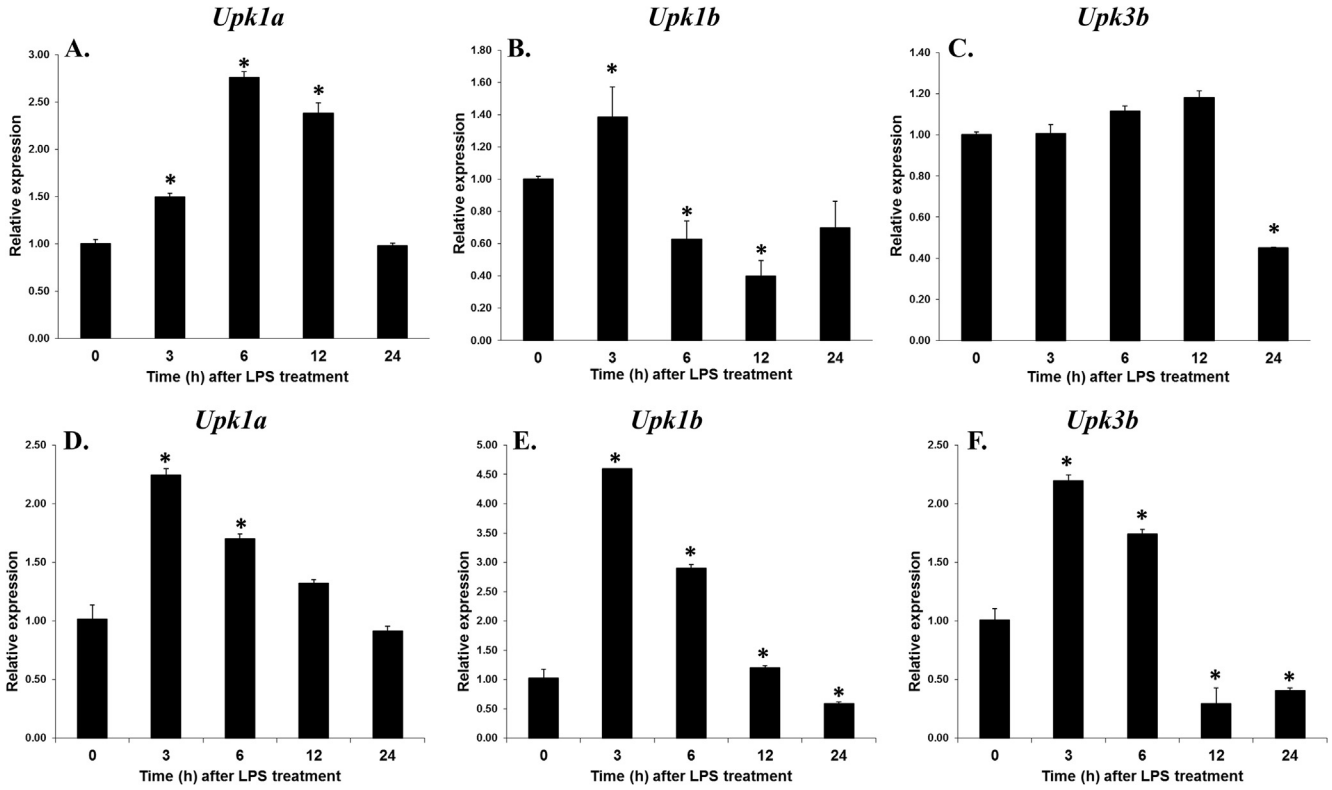


**Fig. 8.** Effect of endotoxin challenge on *Upk* gene expression *in vitro*. $1 \times 10^6$ cells were plated in each well of a 24 well plate and treated with 100 ng/ml LPS. RNA isolated from cells were collected at 3, 6, 12 and 24 h after LPS treatment was reverse transcribed and the resulting cDNA was used to analyze *Upk1a, Upk1b* and *Upk3a* gene expression by real time PCR. A-C, LC540 cells; D-F, RCE cells Values shown are Mean ± S.D. * denotes $p < 0.05$ compared to 0 h control.

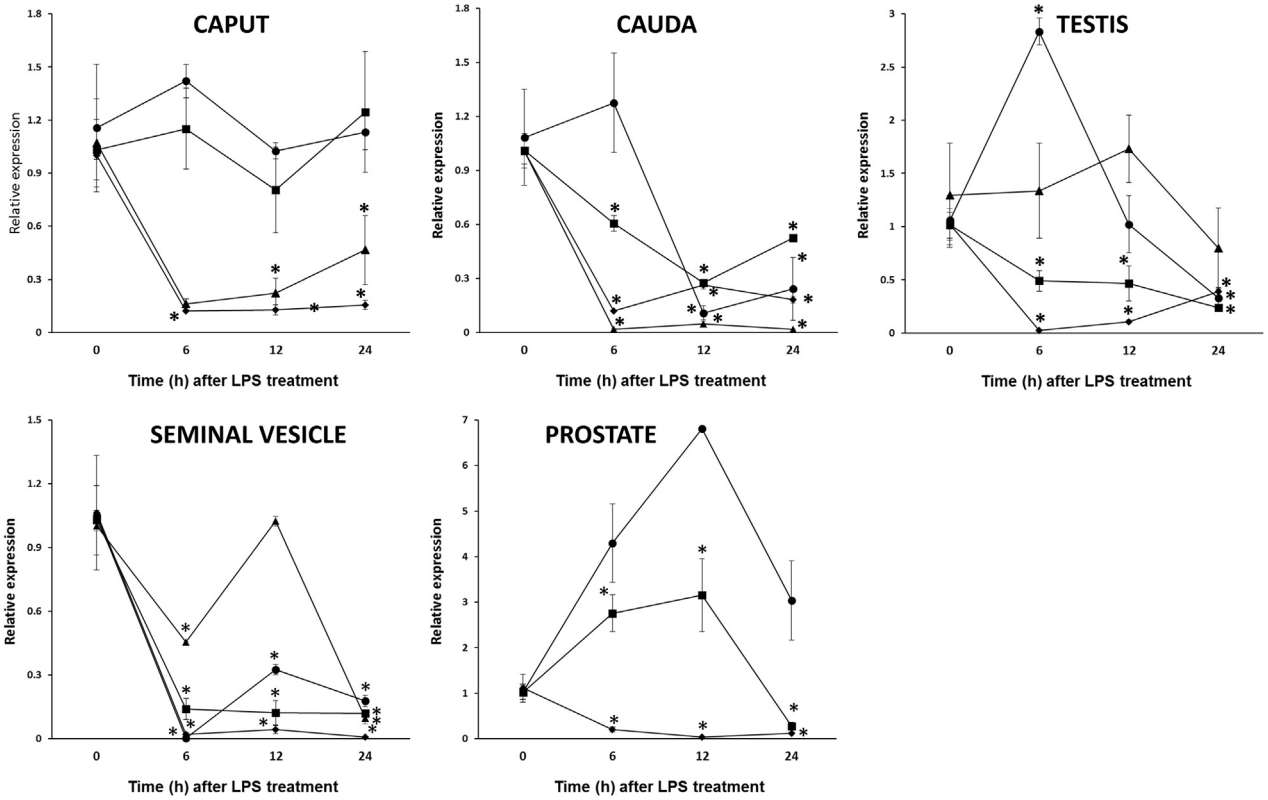


**Fig. 9.** *Upk* gene expression in the male reproductive tract upon endotoxin challenge. Rats were challenged with a single intraperitoneal dose (1 mg / kg body weight) of LPS. Caput, cauda, testis, seminal vesicle and prostate were collected at 0, 6, 12 and 24 h after LPS treatment. RNA isolated from these tissues was reverse transcribed and the resulting cDNA was used for analyzing *Upk* gene expression by real time PCR. ■ – *Upk1a*; ♦ – *Upk1b*; ● – *Upk2*; ▲ – *Upk3b*. Values shown are Mean ± S.D. * denotes $p < 0.05$ compared to 0 h control.

the male reproductive system using rat as the model system. The rationale is based on the fact that male reproductive system and urothelium are of same embryonic origin and thus UPKs may play a crucial role in many cellular and tissue processes.

UPKs first emerged in cartilaginous fish, which could be a common ancestor of vertebrates (Garcia-Espana et al., 2006). Over a period of time UPKs were identified in many species and are found to be highly conserved and have common evolutionary ancestors (Desalle et al., 2014, Wu et al., 1994). Recently three new UPKs were identified and this demonstrates the continued interest to study the role of UPKs in mammalian physiology (Desalle et al., 2014). Rat genome wide search indicated the presence of five *Upk* genes, namely, *Upk1a*, *Upk1b*, *Upk2*, *Upk3a* and *Upk3b*, of which *Upk3b* sequence is predicted. Sequence analyses revealed that the rat *Upk3b* is located on chromosome 12. Similar to the rat, the mouse and human *Upk*s identified till date are located on different chromosomes, suggesting that they are not clustered though they contribute to the common function. We identified and reported the rat *Upk3b* gene and this is a novel aspect of this study. *Upk3b* gene contains seven exons and is transcribed to an 1158 nucleotide mRNA that encodes a protein with 275 amino acids. Rat UPK3b contains a single transmembrane domain similar to that of mouse and human monospanins. However, its sequence similarity with UPK2 and UPK3a was found to be only 14 and 28 percent respectively. Rat UPK3b shows 92 and 82 percent homology with its mouse and human counterparts respectively. Though the rat UPK3b is classified as a monospanin and displays similarities to that of human in terms of localization of the gene on a separate chromosome and the predicted function in urothelium, sequence homology reveals that it appears to be closely related only to the mouse UPK3b. Gene neighborhood analyses for rat *Upk* genes indicated similarities in the nature of genes present in the vicinity of *Upk* genes of mouse and human. A number of genes were commonly present in the vicinity of *Upk*s in all the three species. It is possible that the location of *Upk* genes in relation to the other genes is conserved through evolutionary process across the species. Three dimensional structure analyses of rat UPKs indicate that UPK1a and UPK1b (tetraspanin family members) and UPK2, UPK3a and UPK3b (monospanin family members) are similar in appearance. The high content of alpha-helix in the tetraspanin family members and that of random coil in the monospanin family members appears to correlate with the number of spans they make in the plasma membrane. The 3D structure of UPKs proposed in this study are indicative and whether the proposed structures can fit into the existing models of asymmetric unit membrane particles needs to be validated using advanced methods such as nuclear magnetic resonance spectroscopy. Information presented on the sequence similarity among rat UPKs and between UPKs of different species, genome neighborhood and three dimensional structure are relatively less reported for UPKs of rat and other species; and thus contributes to the further understanding of the functional role of these proteins.

Though, UPKs were predicted to be expressed in other tissues (Lee 2011), their presence and importance in the male reproductive tract is gaining importance in the recent years. The importance of UPKs in fertilization was recently demonstrated in the mouse (Liao et al., 2018). In the rat model system, characterization of UPKs still remains to be explored. Determination of expression pattern and functional role of UPKs may vary from species to species and hence this study aimed to understand the contribution of UPKs in the rat male reproductive tract. *Upk1a*, *Upk1b*, *Upk2* and *Upk3b* mRNA was detected in the caput, cauda, testis, seminal vesicle and prostate, whereas *Upk3a* was not detected in any of the tissues. Presence of UPK1a, UPK1b, UPK2 and UPK3b in the caput, cauda and testis as detected by immunoblotting further confirmed the expression of UPKs in male reproductive tract. The expression of *Upk* mRNAs in other tissues of the rat suggest that these genes are ubiquitously expressed and may have functions beyond the urinary system. In the rat, UPK expression in the urethra and vaginal introitus was associated with the developmental process (Cunha et al., 2017). Such a correlation between *Upk* gene expression and postnatal development was not studied in the male reproductive tract. Developmental regulation of the male reproductive tract in many species is governed by dynamic changes in gene expression pattern, which in turn is determined by the fluctuating levels of androgens (Rodriguez et al., 2001, Harris and Bartke 1974). Our results indicate that, in the epididymis, *Upk1b* and *Upk3b* are constitutively expressed starting from early stages of development, whereas *Upk1a* and *Upk2* are detected at later stages. In the testis, except for *Upk3a*, all other *Upk*s seem to be expressed in all the stages of development. It appears that some *Upk*s are essential at all stages of development whereas some are governed by androgen levels. Testosterone levels vary greatly during the developmental process. A steady increase in testosterone levels occurs in the rete testis of 30–130 day old rats (Harris and Bartke 1974, Harris and Bartke 1981). Androgen levels in the epididymis of rat decline from birth until 20 days and a normal level of 10 ng/g tissue (35 nM) is maintained until approximately 40 days after which, the levels begin to increase to that of the adult, between 15 and 20 ng/g (Charest et al., 1989). Serum testosterone levels in the young rat remain low and do not begin to increase to adult levels until 35–40 days of age (Nayfeh et al., 1966). It can be hypothesized that the androgens may influence the expression of *Upk* genes directly by binding to their promotor elements or through modulation of other transcription factors. Immunofluorescence microscopy revealed the localization of UPKs in the rat male reproductive tract tissues and on the spermatozoa. We previously demonstrated the localization of proteins that play an important role in spermatogenesis and sperm maturation. They were found to be abundant in the epithelial lining of caput, cauda and testis and on spermatozoa (Rajesh and Yenugu 2012, Rajesh and Yenugu 2015, Narmadha et al., 2011, Narmadha and Yenugu 2016). In this study, we show that UPKs are predominantly localized in the epithelial lining of caput, cauda and testis, suggesting that they may a play a crucial role in spermatogenesis and sperm maturation. A recent study reported the localization of UPK1b in the apical region of the cauda epididymis (Liao et al., 2018). Since the tetraspanin UPKs are expected to be localized in the vesicles, they should be detected in the apical regions. However, the localization pattern we demonstrate is different from that is reported by Liao et al. This could be due to variation in the species being studied. Further, imaging using high resolution microscopy are warranted to demonstrate the subcellular localization of UPKs in the tissues analyzed in this study. All the UPKs analyzed were localized throughout the sperm. Liao et al reported that all UPK proteins were localized only to the head region of the mouse sperm (Liao et al., 2018). The pattern of localization of UPKs i.e. throughout the rat sperm could be due to species variation. The presence of these proteins on the sperm indicates their possible role in many functions such as capacitation, acrosome reaction, sperm egg recognition and fertilization. Since the urothelial and reproductive tract tissues originate from the same embryonic origin, this could be an important reason for the presence of UPKs in both these organ systems. The exact role of UPKs in the male reproductive tract and their contribution to sperm function needs further investigation.

Proteins in the male reproductive tract have been demonstrated to have functions beyond spermatogenesis and sperm maturation. Ample evidence exists that some of them play a crucial role in innate immunity. For example, members belonging to defensin, Sperm Associated Antigen 11 (SPAG11), Prostate and Testis expressed (PATE), Lysozyme like (LYZL) families were found to be altered in response to endotoxin challenge or in model systems that mimic an infection (Biswas and Yenugu 2011, Rajesh and Yenugu 2012, Narmadha et al., 2011). Their expression was epigenetically regulated (Biswas and Yenugu 2014). Since UPKs were found to be expressed abundantly in the male reproductive tract, it is possible that may also contribute to innate immunity. Implicating UPKs for their multiple roles in the same organ system has not been demonstrated earlier and our approach in this direction is novel. We observed that *Upk* expression was in

generally down regulated when challenged with LPS both *in vitro* and *in vivo*. It is well established that Uropathogenic bacteria use UPKs as an advantage to infect the urothelial tissues (Mulvey et al., 1998, Martinez et al., 2000). UPK3a signaling is very crucial for bladder response during bacterial infection (Thumbikat et al., 2009). Presence of surfactant D protein acted as a competitor to FimH (fimbrial tip-positioned adhesive protein) of UPEC because of its ability to bind to UPK1a and thereby reducing the available levels of UPK1a and thus the bacteria induced inflammation (Kurimura et al., 2012). In view of the above evidences, it is possible that UPKs may have a significant role during endotoxin or bacterial induced responses in the male reproductive system. Since LPS is a component of the capsule of Uropathogenic bacteria, the down regulation of *Upk* mRNAs in the male reproductive tract tissues during LPS challenge could be a protective mechanism. It is possible that down regulation of UPKs may result in non-availability of UPKs for Uropathogenic bacterial binding and thereby lowering the infection severity. We previously demonstrated that LPS induced changes in the defensin expression involved TLR (toll-like receptor) mediated NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) activation and epigenetic changes such as DNA methylation and histone acetylation / methylation (Biswas and Yenugu 2014, Biswas et al., 2015). *UP1a* promoter methylation is implicated in bladder carcinoma (Cunha et al., 2017), suggesting that the promoters of *Upk*s could be modulated by epigenetic changes under altered physiological conditions. It would be very interesting to study the molecular mechanisms (signaling pathways and epigenetic changes) by which LPS modulates *Upk* gene expression and also the interaction of Uropathogenic bacteria with UPKs of male reproductive system.

We conclude that UPKs are abundantly expressed in the male reproductive tract of rat and may contribute to the general physiology of this organ system, spermatogenesis, sperm function and immune responses.

## 5. Authors' contributions

SBM carried out the experimental work. SY conceptualized the ideas, wrote the manuscript and provided the reagents and other facilities.

## Declaration of Competing Interest

None.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.06.003.

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