EVIDENCE SUPPORTING THE PRESENCE OF INTRAEPITHELIAL MAST \CELLS IN RAT UROTHELIUM: A LIGHT AND ELECTRON MICROSCOPY STUDY.

Alexandru Cristian Popescu[1,2], Catalin Gabriel Manole[1,2], Andrei Marian Niculae[1,2], Mihail Eugen Hinescu[1,2], Mihaela Gherghiceanu[1,2]

[1] Carol Davila University of Medicine and Pharmacy, Bucharest, Romania
[2] Victor Babes National Institute of Pathology, Bucharest, Romania

ABSTRACT

Introduction and Objectives. C-Kit-positive cells involved in the ureteral peristalsis have been described throughout the human urinary tract. The nature and localization of C-kit-positive cells are still under debate, considering that many interstitial cells, mast cells (MCs) included, could express this marker. Intraepithelial mast cells (MCs) were identified as a population of mast cells (MCs) within the gastric, intestinal, upper airways, alveolar, and kidney tubule epithelium but have not been investigated in the urothelium.

Materials and Methods. We used electron microscopy to identify cells in rat urothelium and a rat experimental model to investigate c-kit-positive urothelial cells' potential role in the progression of obstructive ureteral pathology.

Results. Using electron microscopy, we found MCs with different shapes with round or oval membrane-bound dense vesicles in lamina propria, muscularis, and adventitia as it is known. We also found MCs in the urothelium of normal ureters and those with obstruction. There was no difference in MC distribution in the normal ureters from the control, unobstructed ureters, and ureters below the obstruction. A significant reduction of IEMCs was observed in the ureters above the obstruction.

Conclusions. For the first time, we have identified MCs located inside the urothelium of the rat ureter. The IEMCs identified in the ureter's urothelium may explain the presence of intraepithelial c-Kit-positive cells previously described. The presence of IEMCs is significant as MCs are involved in urothelial cancer, opening up new prospects for research and potential treatments.

Keywords: urothelium, mast cell, interstitial cell, c-Kit positive cells

Correspondence to:

e-mail: alexandru.popescu@umfcd.ro

Introduction

The interstitial cells (ICs) with various names found in the upper urinary tract (UUT) are studied because they are involved in the ureteral peristalsis. In the urinary tract, the urine generated in the kidneys is propelled through the ureters to the bladder. The peristaltic contractions are spontaneous, of myogenic nature, continuing in-vivo after denervation, which implies the existence of pacemaker cells. In uni-calyceal kidneys, the spontaneous electrical impulse originates in the most proximal regions of the pelvic-papilla border [1,2] and the mid and distal renal pelvis RP [3]. In multi-calyceal kidneys, the spontaneous electrical impulse originates in one minor calyx at a time, spontaneously moving between calyces [4,5]. The pacemaker cells fire 'pacemaker' action potentials with a higher frequency than the 'driven' action potentials of the smooth muscle contracting cells, and 'intermediate' action potentials are fired by the interstitial cells of Cajal-like cells [6].

The different amounts of connective tissue composed of fibers, ground substance, and different resident cells (fibroblasts, myofibroblasts, macrophages, mast cells, adipocytes, and adult stem cells) and transient cells (lymphocytes, plasma cells, neutrophils, eosinophils, basophils, and monocytes) are forming the interstitial tissue found in between different components of various organs including the urinary tract. All those connective cells can be called interstitial or stromal cells. Only a few non-connective cells with special functions in the interstitium of various organs are called interstitial cells (ICs). In the UUT there are some ICs that over time received different names: interstitial cells of Cajal (ICCs) [7]; interstitial cells of Cajal-like cells (ICC-LCs) [6,8]; Cajal cells [9]; c-Kit positive ICs [7]; kit positive cells [10]; fibroblast-like cells [11,12]; renal interstitial cells [13]; ICs [10]; telocytes [14]; PDGFRα(+) ICs [15]; PDGFRα(+) fibroblast-like ICs [16]; Kit+ interstitial cell (KIC) [17]; PDGFRα(+) interstitial cell type 1 [17]; PDGFRα(+) interstitial cell type 2 previously referred to as atypical smooth muscle cells [17]; sub-urothelial ICs [18] later renamed basal epithelial cells [19]. The types, names, and functions of these ICs present in UUT are still being investigated using different techniques and searching for specific markers because they are considered to have an important role in the peristaltic contraction of the ureter. Their dysfunction has been reported in some congenital anomalies of the kidney and urinary tract, such as the congenital ureteropelvic junction obstruction [20].

C-Kit type III tyrosine kinase receptor (CD117) is considered to be the specific marker for ICCs in the digestive tract [10,21] and also to be a reliable marker for identifying ICC-LCs [7 22]. Researchers soon realized that some ICs were positive or negative for c-Kit, but their identification highly varies by species, clones, and fixation methods [7]. Another problem was correctly differentiating ICs from mast cells (MCs), which are also c-Kit positive. There are some specific markers to MCs, so co-labeling is the solution, but it is rarely used. Investigators prefer to differentiate the c-Kit positive cells by their aspect: spindle-shaped with two or more cytoplasmic processes for the ICs and larger, rounded for MCs [23]; the method is not always reliable because some MCs are smaller or elongated as they are wandering cells and the ICs in cross-section are rounded, and the cytoplasmic processes are not always obvious.

Van der AA and colleagues first noticed that c-Kit-positive urothelial cells were sporadically in the whole human urinary tract [7]. In a series of articles, Metzger and colleagues [23,24,25] studied in different species (humans, pigs, rodents, carnivores and cows), sections from pyelon and proximal, middle and distal ureter and only porcine bladder regions using immunohistochemistry, multiple monoclonal and polyclonal antibodies recognizing c-Kit receptor (CD117) applied on serial frozen sections, observed the distribution of three types of c-Kit positive cells.

Two types of cells are usually described as c-Kit positive cells: the MCs and the ICC-LCs [6,8]. Their morphological and antigenic profile identified MCs as c-Kit-positive and CD34-positive round cells found in all three segments of the ureters of all studied species. They occur in various amounts in all layers of the ureteral wall (mucosa, muscular layer, and adventitia) except the urothelium, and the number of MCs seems to be highest in humans and porcine and lowest in rats and mice [24]. A second c-Kit positive but CD34 negative spindle-shaped cell type, identified as ICC-LCs, was found in the lamina

propria and among the inner and outer smooth muscle layers in all the three segments of the ureter of all the studied species with the highest number in the pyelon and slightly decreased distally [24].
Interepithelial cells have been found to express the c-Kit receptor (CD117) and have been described for the first time within the urothelium only in the human and porcine ureter, as basal and vertically oriented spindle cells with an oval nucleus, between the urothelial cells, originating from the basal lamina but never found in the superficial layer of umbrella (dome-shaped) cells [25]. C-Kit mRNA was also detected by real-time PCR within the urothelium [25]. The authors noted that the interepithelial cells could have been a previously unnoticed cell population with speculative receptor function, mechanosensory to stretch, or a type of urothelial stem cell.
Intraepithelial mast cells (MCs) were identified as a population of mast cells (MCs) within the gastric, intestinal [26], upper airways, alveolar, and kidney tubules epithelium [27] but not in the urothelium.
Until now, cells that could be positive for c-kit in urothelium have not been investigated or identified at the ultrastructural level. We used electron microscopy to identify cells in rat urothelium and an experimental model to explore the potential role of c-kit-positive urothelial cells in the progression of this pathology.

Materials and Methods

Animal tissue samples

An experimental model for unilateral ureteral obstruction was performed on rats. We use adult, 16 female Wistar rats weighing 170-230 g, provided by the INCD "Victor Babeş" animal facility. The study was approved by the Bioethics Committee of the "Victor Babes" Institute of Pathology, Bucharest, according to international standards. The animals, held and fed ad libitum according to local ethical guidelines, were divided into control (n=3) and ureteral obstruction (n=10). The left ureter was surgically discovered after a substernal incision of the abdomen. Unilateral partial upper ureteral obstruction was created using a polypropylene 5-0 suture ligature of the ureter, and a metallic guide was removed after ligation, resulting in a partial reduction of the ureter's diameter. The abdomen was closed, and the animals returned to the nursery and received analgesic and antibiotic treatment. From animals in all groups, both kidneys with the pelvis and ureters and control and obstructed ureters from study rats were harvested after 30 days and processed for electron microscopy.

Transmission Electron Microscopy

Tissue samples were fragmented into small pieces (about one mm3) and fixed in 4% glutaraldehyde (in 0.1M cacodylate buffer) at pH 7.3 for four h, at 4°C. After a brief wash in 0.1 M cacodylate buffer, tissue samples were post-fixed with 1% osmium tetroxide in 0.1M cacodylate buffer at 4°C, followed by dehydration in a graded series of ethanol and embedded in epoxy resin (Agar, UK), polymerized for 24h at 60°C. For light microscopy, the tissue blocks were cut into 1 µm thick sections (semithin sections) using a Leica EM UC7 ultramicrotome (Leica Microsystems, Germany), stained with 1% toluidine blue. Semithin sections were used for histologic examination in light microscopy. Representative areas have been further processed for electron microscopy. Ultrathin sections were cut at 60-80 nm thickness using a Leica UM UC7 ultramicrotome (Leica Microsystems, Germany), collected on 3 mm formvar-coated copper grids, and stained with uranyl acetate and Reynolds solution (Agar, UK). The images were acquired using a FEI Morgagni 268 Transmission Electron Microscope (FEI Company, USA) at 80 kV with a MegaView III CCD camera and iTEM software (Olympus, Hamburg, Germany).

Results

Light microscopy examination has been performed for the ureters of control rats (Image 1A), unobstructed ureter (Image 1B), obstructed ureters above ligature (Images 1 C, 2A), and obstructed ureters beneath ligature (Images 1D, 2B).
Examining semithin blue sections from the normal ureter from the control as well as the semithin sections through the ligated ureter from the area below the ligature, we observed in both control and below ligature of the upper ureter, the presence of intraepithelial granular cells (Image 1A, B, D). We found signs of inflammatory reaction in the adventitia of ureters above the obstruction (Images 1C, 2A). Few MCs have been found in all layers of the

ureter (Image 3A, 3B). The MCs were observed in the basal part of the urothelium, interspersed among the basal cells, and in the middle part among the intermediate cells (Image 2) but never in the superficial layer of umbrella cells. We have identified cells with granular content in the urothelium of control and obstructed ureters. The ureter above the ligature showed a wide lumen and fewer MCs than the segment below the ligature (Image 2). The ureteral IEMCs showed various shapes (round, oval, spindle, pear-shaped, or irregular) and locations. The MC granules stain intensely bluish-purple in rats (Image 2). In large numbers, the granules appear as a solid mass that might obscure the pale staining nucleus (Image 1, Image 2). surrounded by urothelial cells in the middle part of the urothelium, we identified elongated, spindle IEMCs, oval-round IEMCs, or pear-shaped IEMCs (Images 1, 2).

After glutaraldehyde fixation, mast cell granules can be displayed with the primary dye toluidine blue, which stains the granules intensely and metachromatically bluish-purple (Image 2B). When in large numbers, the granules tend to appear as a solid mass and might obscure the pale staining nucleus. The non-segmented nucleus, granules amount and distribution, and ultrastructural aspect of granules (Images 3, 4) exclude other granulated wandering cells. Mast cell vesicles of the rat are very electron-dense and homogenous (Images 3, $), different from lattice or scroll-like vesicles found in human MCs [28, 29]. We saw single membrane-bound dense vesicles with a granular homogeneous appearance. We have not found degranulated MCs and free granules in our EM sections. Light microscopy shows IEMCs in the upper part of the urothelium but with umbrella-shaped cells separating them from the lumen (Image 4).

Electron microscopy showed MCs with different shapes (Image 3) having round or oval membrane-bound dense vesicles either in the adventitia (Image 3A), muscularis (Image 3B), lamina propria (Image 3C), or urothelium (Image 3D-3F). The nucleus is large, often eccentric, with a regular or irregular outline, and few mitochondria are present. Small cellular extensions of MCs interdigitate with urothelial cells (Images 3D, 4B). IEMCs have been located close to the basal part of the urothelium with urothelial cells closing the connection of Mcs to LP where we can see small capillary, tiny unmyelinated nerve fibers, parts of ICs and processes and bundles of collagen (Image 3E, 3F). The membrane-bounded patchy vesicles inside neighboring urothelial cells might be secondary lysosomes with endocytic MCs granules (Image 4B).

Discussion and Conclusions

MCs are intensely granulated wandering cells of connective tissue described until now inside lamina propria, muscular layer, and adventitia of UUT, in higher numbers in humans and pigs and the lowest number in rats and mice. Using light and electron microscopy, we describe the intraepithelial MCs in the urothelium of the rat ureter. IEMCs would explain the c-Kit positive interepithelial cells described by Metzger and his colleagues [24] inside the ureter's urothelium (only in humans and pigs); cells remained unidentified until now.

We used glutaraldehyde fixation, plastic-embedded tissues, and semi-thin sections (1 µm) stained with toluidine blue. In contrast, Metzger and colleagues used formalin-fixed and paraffin-embedded tissues sectioned at 2 to 4 µm, stained with toluidine blue and mast cell markers [23]. We have to mention that Metzger and colleagues wrote that "MCs were found in a varying amount throughout the entire wall of the urinary tract, except the urothelium" [24, 25]. They did not mention the possibility that the c-kit-positive interepithelial cells they discovered might be mast cells.

Intraepithelial MCs in mice were found in gastric, intestinal, upper airways, and kidneys but not in skin or other organs [29]. IEMCs were first observed in 1919, residing within the intestinal epithelium between enterocytes [30, 31]. IEMCs were identified as a specialized MC population within the intestinal epithelium [31, 32]. Their functions have been linked with a phase of type 2 immunity involving crosstalk between mast cells and epithelial cells [31]. The feedback loop in which cytokine signals from damaged epithelial cells activate MCs that regulate tissue environments, essential for epithelial barrier function restoration and maintenance of tissue homeostasis, has been described [33]. Investigating the urothelial IEMCs will show if that's also the case in the urinary tract.

The degranulation of MCs and the release of histamines, heparin, and proteases like tryptase may be induced by many factors. For example, tryptase is a potent growth factor for epithelial cells and could be involved in urothelial carcinoma [34]. This study showed the presence of IEMCs in rat urothelium and reduced the number of MCs above the ureteral obstruction. Even if we do not find an explanation for the differences in IEMCs distribution in an obstructive experimental rat model, further investigation might determine the role and interactions of urothelial IEMCs with epithelial cells with impact on therapy.

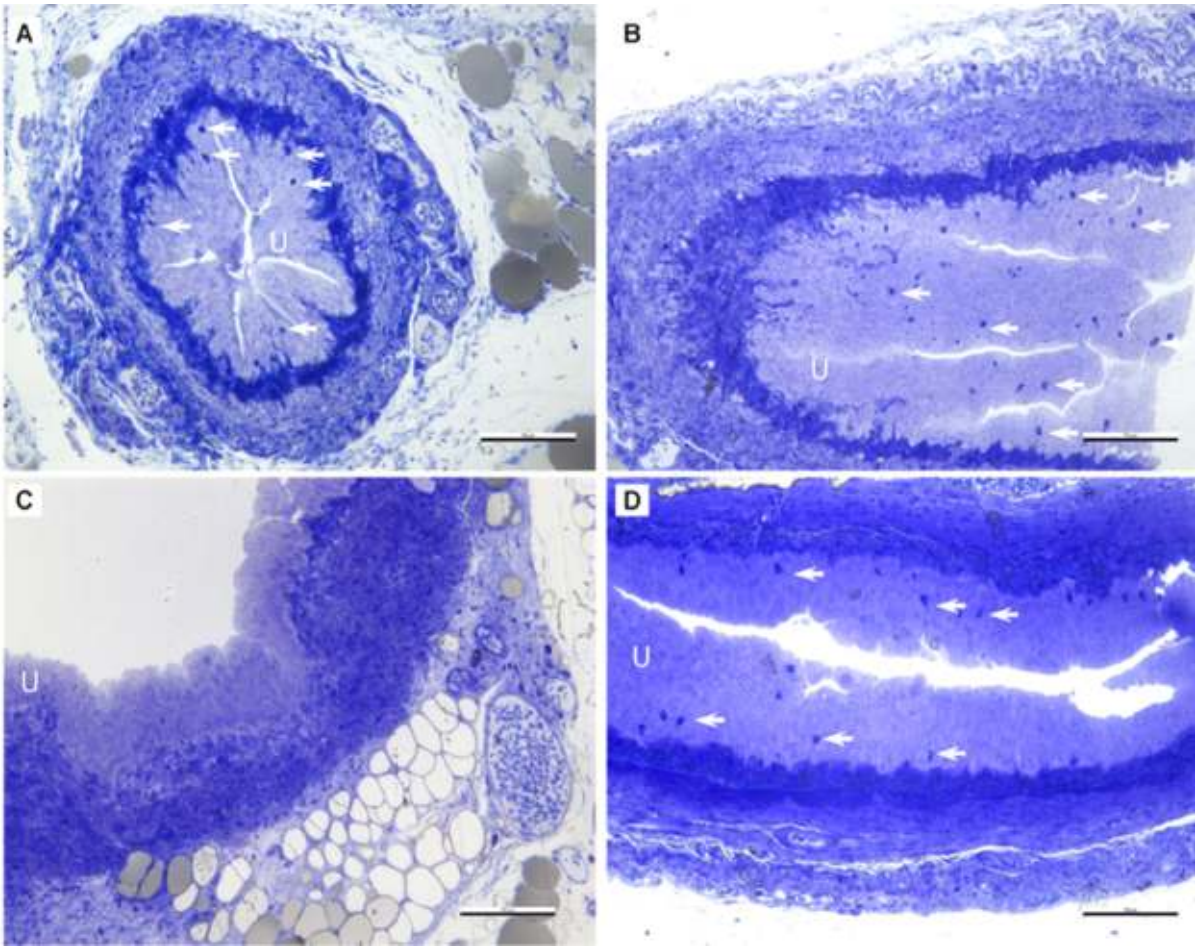


Image 1. Light microscopy on semithin sections from control ureters (A, B) and obstructed ureters (C) – above the ligature, (D) – below the ligature. U – urothelium. Arrows indicate granulated cells with various shapes in the urothelium (A, B, D). (C) The urothelium above the ligature shows no clear MCs, but inflammation is present in the adventitia

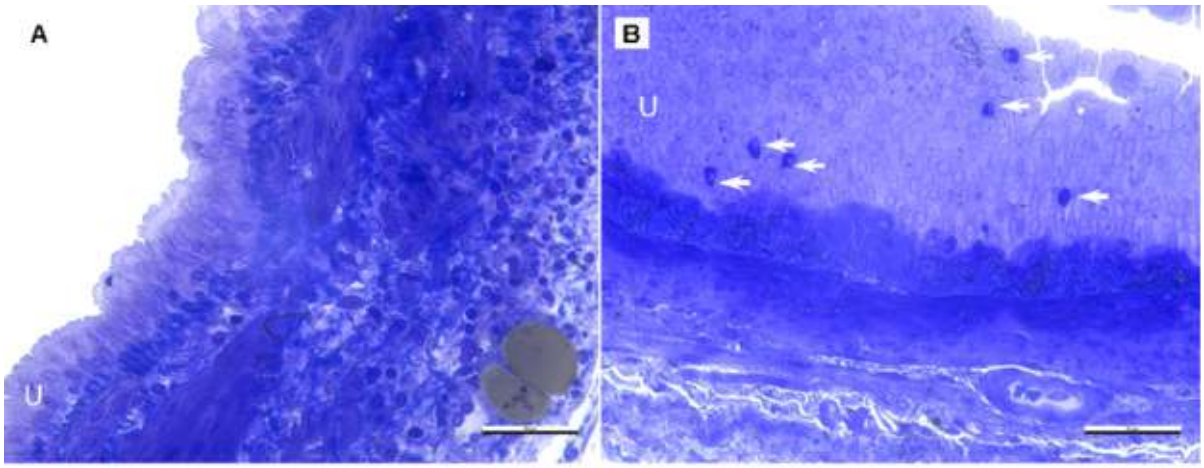
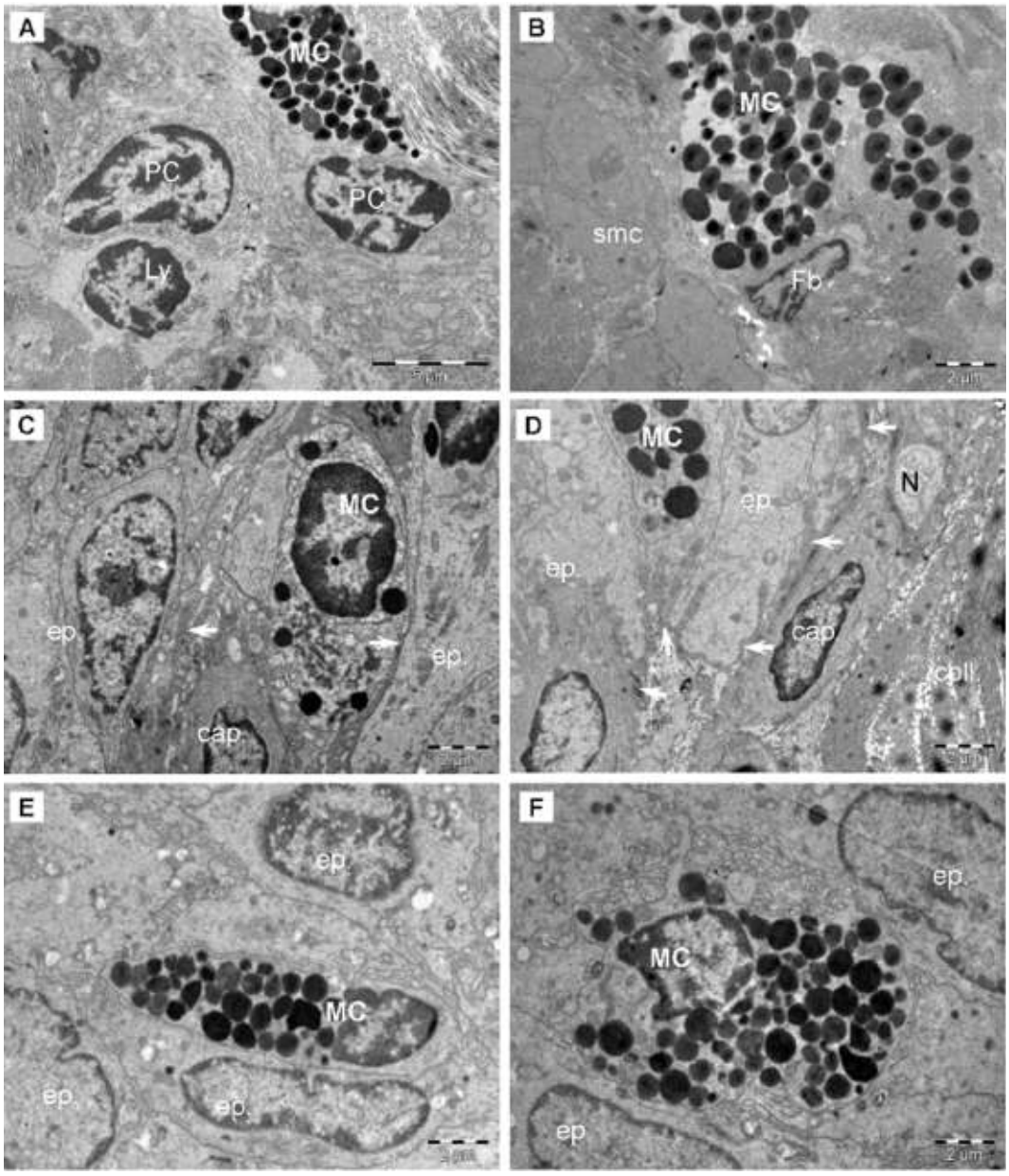


Image 2. Light microscopy on semithin sections from obstructed ureters: (A) – above the ligature, (B) – below the ligature. (A) Inflammatory infiltrate is visible around the ureter. (B) Arrows indicate cells with granular cytoplasm characteristic of mast cells. U – urothelium



IImage 3. Transmission electron microscopy images show mast cells (MC) present in lamina propria (A), muscularis (B), and urothelium (D, E, F). MCs contain single membrane-bound dense vesicles with a homogeneous appearance and are in close contact with surrounding cells. PC – plasma cells, Ly – lymphocyte, Fb – fibroblast, SMC - smooth muscle cells, ep. epithelial – urothelium, cap – capillaries, N – nerve fiber. Arrows in (C) and (D) indicate basal lamina of the urothelium.

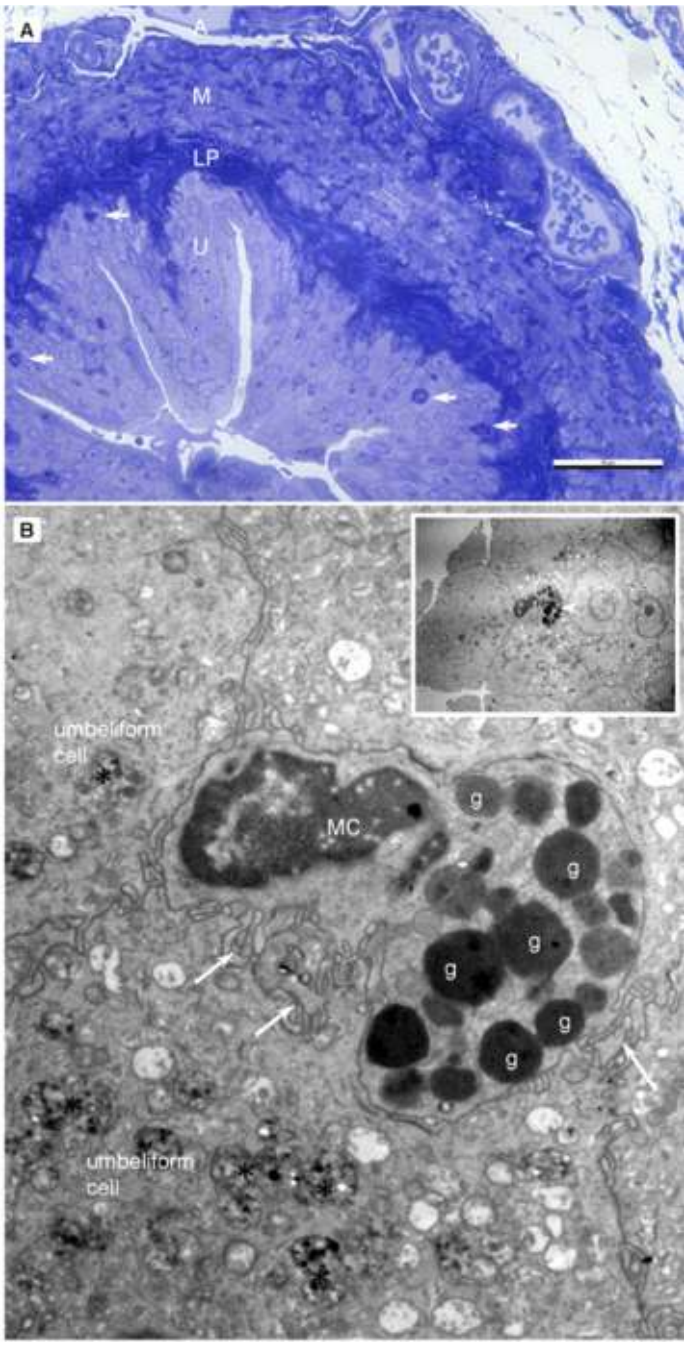


Image 4. (A) Light microscopy of a normal rat ureter showing all layers: U- urothelium, LP- lamina propria, M - muscular layer, and A- adventitia. Arrows indicate intraepithelial mast cells. (B) Electron microscopy of urothelium showing a mast cell (MC) between urothelial cells. The inset locates the MC beneath the umbrella cell's layer. Arrows indicate interdigitations of urothelial cells and mast cells. The membrane-bounded heterogeneous vesicles (*) inside umbrella cells.

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