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Original Article

Autochthonous male urothelial carcinoma immune competent model: from induction to BCG transurethral treatment

Ana Clara C Salustiano[1], Eduardo Riccetto[2], Isis GA Kiehl[1], Marina V Ossick[1], Karen L Ferrari[1], Fernandes Denardi[2], Leonardo O Reis[1,2]

[1]*Medical Pathophysiology Program, School of Medical Sciences, University of Campinas, UNICAMP, Brazil;* [2]*UroScience, School of Medical Sciences, University of Campinas, UNICAMP and Pontifical Catholic University of Campinas, PUC-Campinas, Brazil*

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Abstract: Objective: To describe a new animal model of autochthonous urothelial cancer (UC) accessible by transurethral catheter in males, from induction to treatment. Seven-week-old male Fischer 344 rats were used. The first 10 animals were used to overcome and standardize the technical challenges of safe transurethral catheterization of male rats. The remaining 14 animals underwent intravesical N-Methyl-Nitrosourea (MNU) instillation for UC induction, of which six were randomized to undergo intravesical BCG treatment. The stretched male rat urethra travels 35 mm in a tortuous "S" shaped trajectory with a 180° angle behind the pubic bone, safely traversed by a 20G 36" 0.8 mm epidural catheter in a stretched, straightened urethra inserted after anterior dilation of the penile urethra with a 24G IV catheter. Histopathologic analysis of the urinary bladder demonstrated Stage pT1, pTa, and pTis lesions in the 8 controls, all with increased cell proliferation by Ki-67 expression and no pT1 or pTis in the animals 6 treated with BCG. This pioneering study describes an autochthonous, effective, and accessible transurethral animal model of immune-competent UC in males, and may help with understanding of the biology, immunology, and treatment of UC, which predominates in males.

Keywords: Male, urothelial bladder cancer, animal model, intravesical treatment, BCG, autochthonous

Introduction

Urinary bladder carcinoma (BC), the second most common malignant disease of the urinary tract and the 9th most common cancer worldwide, has great relevance in public health, with incidence rates 2 to 4 times higher in men than in women [1].

In vitro experiments suggest that androgen receptor (AR) mediated signals play a critical role in urothelial tumorigenesis and cancer progression [2]. There is also pre-clinical evidence that AR signaling is related to the resistance of some conventional non-surgical therapies currently available. Advances in cell culture offer new approaches to define specific urothelial tissue responses, but findings from *in vitro* models for BC study need validation in living models [3, 4].

Animal models are a fundamental tool for studies of bladder carcinogenesis. The animal model shows similar characteristics to human BC and may be central to the development of clinical treatment. The induction model using intravesical MNU was optimized and reproduced bladder tumors that are clinically observed in humans, and native to the urothelium [5].

Considering the growing evidence of an established relationship between androgenic environment and urothelial carcinogenesis, progression, and response to treatment [6, 7], we standardized an autochthonous animal model of immune-competent urothelial cancer (UC) in male rats from induction to transurethral treatment.

Methods

Animals and experimental procedure

Twenty-four isogenic male Fischer 344 rats, 7 weeks old, from the Bioterrorism Center of the State University of Campinas (CEMIB/UNICAMP)

were used and anesthetized with 2% Xylazine hydrochloride (5 mg/kg i.m.; König, São Paulo, Brazil) and 10% Ketamine hydrochloride (60 mg/kg, i.m.; Fort Dodge, Iowa, USA) in all procedures. The entire experimental protocol followed the ethical principles according to authorization no. 4541-1/2017 from the university's Animal Research Ethics Committee (CEUA/UNICAMP).

Preliminarily 10 rats were used to discover male urethral catheterization, of which 5 were used for feasibility and 5 for reproducibility and learning curve. Once the access route and appropriate catheters were elucidated, the step-by-step process was defined. We proceeded to intravesical instillation of 1.5 mg/kg dose of MNU (Sigma, St. Louis, MO, USA) dissolved in 0.3 ml of 0.9% saline solution in alternate weeks (0, 2, 4 and 6) [4]. The animals were positioned in dorsal decubitus and had their bladders emptied. The catheters were lubricated and the penis exposed. First, the 24G intravenous catheter, similarly used in female model catheterization [8], was inserted for urethral dilation, removed, and then the 20G 36" 0.8 mm epidural catheter was inserted, allowing access to the bladder. After instillation, the animals were kept in this state for 45 minutes to avoid spontaneous urination [9]. The instillations were confirmed with ultrasound images (US) in real time.

At the end of induction, the animals were randomized into 2 groups. In the Control Group, the animals received the intravesical dose of 0.3 mL of saline solution 0.9%, and in the BCG (Bacillus Calmete-Guerin) Group the animals received an intravesical dose of 10^6 UFC-1 mg of BCG (Ataulpho de Paiva Foundation, Rio de Janeiro, Brazil) diluted in 0.3 mL of 0.9% saline solution, both in weeks 8, 9, 10, 11, 12 and 13, totaling 6 doses [10].

The animals were sacrificed at the 15th week using deep anesthetic by a combination of 2% lidocaine hydrochloride (1 mg/kg) without vasoconstrictor 10 minutes before thiopental sodium (90 mg/kg) in a single intraperitoneal dose. All urinary bladders were collected, opened by incision from the urethra insertion t o the cranial apex, dividing the bladder trigone. The urothelium was inspected for macroscopic lesions, and fixed for histopathology and immunohistochemistry.

Ultrasonography

Both during the elucidation of the urethra catheterization, and soon after the intravesical instillations in the period of induction with MNU, and also at the time of sacrifice, the animals were submitted to genitourinary sonographic evaluation.

Two models of ultrasound apparatus were used: Mindray® Z5 with 10 mHz linear transducer and General Eletric®, Logiq E model with 12 mHz linear transducer.

The interpretation of the images was graduated by the veterinary surgeon in "Normal" (no changes, wall thickness, crystals, cellularity, or urolites); "+" (small single lesion up to 0.30 cm in diameter, without loss of stratification of the bladder wall), "++" (moderate size lesion, more than 0.30 cm in diameter, or multiple lesions, with loss of stratification of the bladder wall) and "+++" (various lesions, diffuse wall thickening).

Histopathologic analysis

Urinary bladders were fixed in buffered paraformaldehyde 4% for 24 hours at room temperature and transversely sectioned to increase the urothelium analysis area, accommodated in plastic cassettes and dehydrated through 1 h baths, in series, in alcohols of increasing concentrations: 70%, 80%, 95%, and 100%, cleared in xylol for 30 min and finally immersed in paraffin at 65°C.

The processed tissue fragments were included in blocks and sectioned in the rotating microtome Biocut 1130 (Reichert-Jung, Munich, Germany) with a thickness of 5 µm and fixed on silanized microscope slides and left overnight in the oven at 65°C. For staining, the slides were immersed in Erlich hematoxylin and 0.5% alcoholic eosin.

The pathologist followed the classification according to the staging proposed by the World Health Organization consensus [11] and read blindly.

Immunohistochemistry-cell proliferation analysis (Ki-67)

The same process to obtain the histopathology slides was used for the immunohistochemical analysis slides which were then rehy-

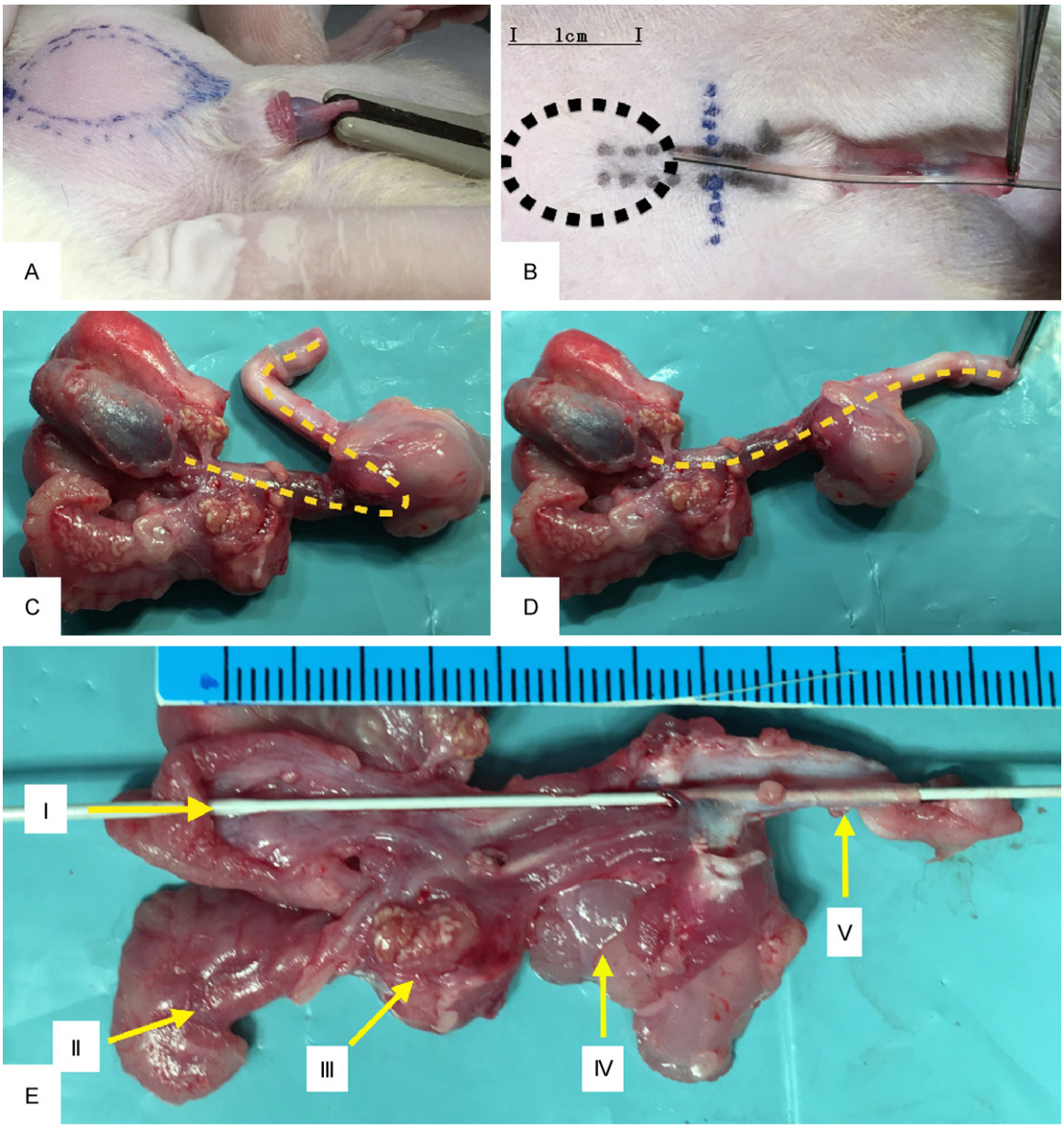

Figure 1. Anatomic and topographical male urethral visualization. A. Static topographic view. B. Static anatomic view. C. Stretched topographic view. D. Stretched anatomical view. E. Dissection of the urinary tract shows the path of the catheter through the urethra and urinary bladder. Anatomical structures: (I) Urinary bladder-internal view, (II) Seminal vesicle, (III) Prostate (altered by the action of carcinogenic agent), (IV) *Corpora cavernosa* crura, (V) Penile body with the exposed urethra.

drated. They were washed in TBS-T and in TBS and microwaved in a 0.01 mol/L citrate buffer bath with pH equal to 6, for 10 min to activate the epitopes of the proteins and then immersed in a solution of methyl alcohol and 5% hydrogen peroxide to block the endogenous peroxidase.

The sections were blocked with Bovine Serum Albumin (BSA) 3% and incubated overnight at 4°C in primary Anti-Ki-67 Rabbit Polyclonal Antibody solution (AB9260, Millipore, USA) and BSA 1%, concentration 1:50. The following day, they were incubated in secondary Anti Rabbit antibody solution and BSA 1%, concentration 1:200, followed by DAB ScyTek Laboratories (Logan, Utah, USA).

Ten random fields of each slide were photographed in 40× magnification under the same

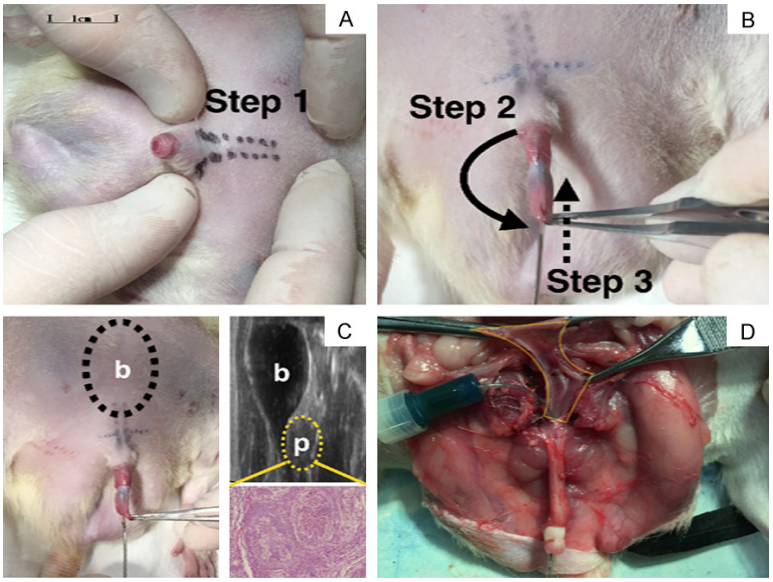

Figure 2. A, B. Step-by-step male urethra catheterization. C. Ultrasonography and histological (b) bladder and (p) prostate views. D. Dissected urethral path with open bladder (yellow outline).

lighting conditions using the Carl Zeiss AxioImager® A1 microscope (Carl Zeiss, Oberkochen, Germany) and the AxioVision® program (Carl Zeiss, Oberkochen, Germany). The area of interest (urothelium), was subjected to computerized analysis for investigation of the field marked nuclei using the ImmunoRatio plug-in [12].

Results

The learning curve occurs between the 3rd and 5th animals, and the average time per cauterization is 30 seconds. In relaxation, the distance from the navicular fossa to the bladder lumen appears to be a straight, short course of no more than 10 mm. However, anatomic dissection revealed that the male urethra travels an average of 35 mm in a tortuous, "S" shaped trajectory with an angle of more than 180° behind the pubic bone (**Figure 1A-D**). The anatomical topographic relationships are described in **Figure 1E**.

Three steps systematize the progress of the 35 mm epidural catheter into the stretched urethra of the male rat to the bladder. The penis is positioned at a 90° angle to the animal's body, and the 24G IV catheter is inserted into the animal's navicular fossa, maintaining traction on the penis and inserting the catheter until it meets resistance (Step 1). The rat's penis is aligned with the tail pointing caudally with traction maintained outward (Step 2), and the IV catheter is removed and the 20G 36" 0.8 mm epidural catheter is then inserted all the way in (Step 3) (**Figure 2A-D**). Often the bulbocavernosus reflex occurs when the catheter conquers the bladder neck.

At this stage, with the epidural catheter fully inserted up to the 35 mm mark, under real-time ultrasound visualization of the bladder, using a 12 Hz linear transducer (General Electric® Logiq E GE), it is possible to confirm, first, that the bladder was correctly emptied, and then, its repletion with the solution of interest. At the end of the 15-week protocol, the prostate gland was visibly inflamed on macroscopic and microscopic analysis but it did not prevent bladder catheterization (**Figure 2C**).

All 14 animals completed the four MNU instillation procedures confirmed by ultrasound imaging. Histopathologic analysis of the urinary bladder demonstrated 3 stage pT1, 3 pTa, and 1 pTis lesions among the 8 control animals (**Figure 3A, 3C** and **3E**), all with increased cell proliferation by Ki-67 expression (**Figure 3B, 3D** and **3F**), and no pT1 or pTis lesions in the 6 BCG-treated animals.

Discussion

Male rat catheterization methods have never been systematized in detail before in the literature [3, 5]. This work is a stepping stone to further male environment discovery in the immune-competent UC scenario. The success of the mouse model was proven by the histopathologicconfirmation of bladder urothelial cancer. It is comparable to the current standard, well-established five-decade-old female model, which has been regarded as human-like and easily reproducible [13-15].

The intravesical autochthonous MNU method is one of the most robust and most utilized methods, compared to transgenic and implantable

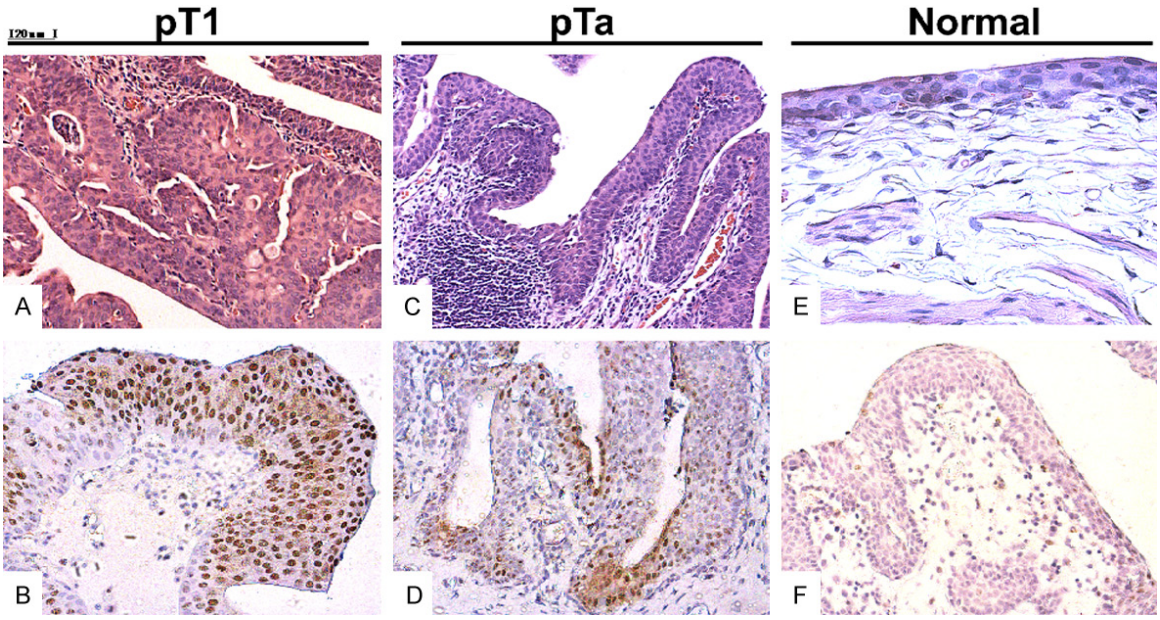

Figure 3. A, C, and E. Bladder histopathology (Hematoxylin/Eosin). B, D, and F. Ki-67 immunohistochemistry characterization.

tumor models [5]. However, even though over two-thirds of UC occurs in the male counterpart, the female model has been accepted as a translational reference so far [16-18].

The successful establishment of the male clinically significant urothelial carcinoma model was proved by its description in immunocompetent rats, and by its successful transurethral treatment with the standard of care intravesical BCG instillations, mimicking the human counterpart.

This catheterization method can be further explored for diverse immune-competent hypothetical models of prostatic diseases, upper tract UC [4], and benign bladder processes. Currently, we are studying the male castration effect on carcinogenesis and BCG treatment.

Beyond BCG intravesical immunotherapy, the proposed immune competent male model will allow further evaluation of intravesical instillment in the androgenic microenvironment with anti-PD-1 inhibitor, previously restricted to female mice description due to urethral access [19].

BC models might evolve to include large animals, though more expensive and laborious, with organ size and lifespan similar to humans, allowing the same tools and techniques applied in the clinic such as bladder tumor transurethral resection [20]. However, no model is perfect, and different models complement each other and serve different purposes.

Studies suggesting a role for androgens in the biology of bladder cancer and its response to treatment are largely hypothesis-generating, and the relative importance of direct effects on bladder tumors versus indirect effects on the immune microenvironment remains unknown [21]. The described immunocompetent male animal model provides a robust platform for further understanding of androgens impact on bladder tumors and on the immune microenvironment.

Conclusion

Access to the male mouse bladder by urethral catheterization is possible, simple, and easily reproducible, making autochthonous UC induction and BCG treatment feasible. This is a pioneering immunocompetent transurethral autochthonous UC animal model. It has the potential to reshape studies of translational urothelial carcinogenesis and treatment, significantly increasing the similarity of the pathophysiology and treatment of human UC, which is predominantly male.

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Disclosure of conflict of interest

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Address correspondence to: Dr. Leonardo O Reis, UroScience, University of Campinas, UNICAMP and Pontifical Catholic University of Campinas, PUC-Campinas, Campinas, SP, Av. John Boyd Dunlop - Jardim Ipaussurama, Campinas-SP, CEP: 13034-685, Brazil. Tel: (19) 3343-600; E-mail: reisleo.l@gmail.com

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