Diabetic Complications Consortium

Application Title: Intermittent Hypoxia and Urologic Complications of Diabetes

Principal Investigator: Chad Vezina

1. Project Accomplishments:

The independent and combined influence of sleep apnea, obesity, and type 2 diabetes on urinary function is unclear. To model these complications in mice, we used BTBR male mice carrying the *Lep*^{ob} mutation, which are subject to severe and progressive obesity and diabetes beginning at 6 wk of age. We focused on one specific manifestation of sleep apnea, intermittent hypoxia. A custom device was used to deliver continuous normoxia (Nx, control) or intermittent hypoxia (IH) to wild type and *Lep*$^{ob/ob}$ (mutant) mice for 2 wk. Intermittent hypoxia was delivered during the 12 hr inactive (lighted) period in the form of 90 sec of 6% O_2 followed by 90 sec of room air. Continuous room air was delivered during the 12 hr active (dark) period. We evaluated aspects of mouse anatomy, physiology, and urodynamics at the end of the 2 wk exposure period. The following endpoints were significantly higher in mutant mice compared to wild type controls: food and water consumption, body weight, blood and urine glucose concentrations, bladder volume, bladder wet weight

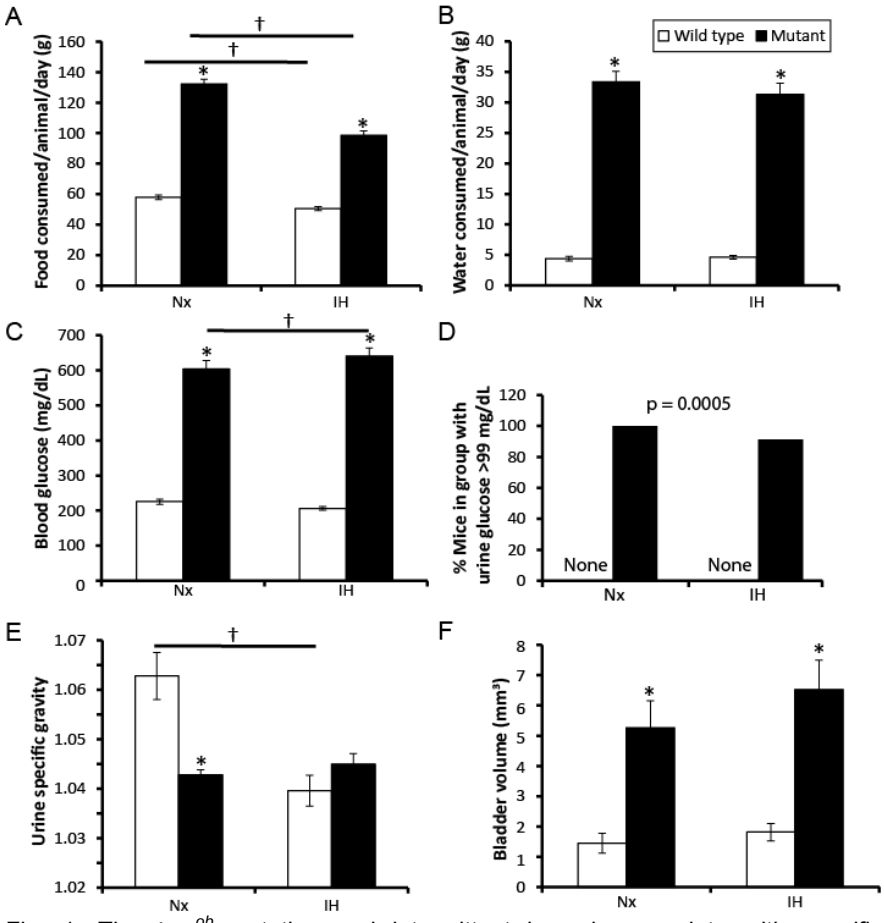

Fig. 1. The *Lep*^{ob} mutation and intermittent hypoxia associate with specific physiological changes in mice. Six week old *Lep*$^{ob/ob}$ (mutant) and wild type male mice were subjected to normoxia (Nx) or intermittent hypoxia (IH) for two weeks. Various physiological outcomes including (A) food and (B) water consumption were measured across the two week period, and other outcomes including (C) blood glucose, (D) urine glucose, (E) urine specific gravity, and (F) bladder volume were assessed at the end of the exposure period. For discrete variables assessed in A-C and E-F, results are mean ± SEM, n=16-25 mice per group, and differences between groups were determined by ANOVA followed by the Tukey HSD post-hoc test. "*" indicates significant differences between genotypes and within the same exposure group; "†" indicates differences within the same genotype and between exposure groups. For the discrete variable of urine glucose (measured by test strip) in D, results represent 5-23 mice per group and differences were assessed by Fisher's exact test.

and incidence of bacteriuria. The following endpoints were significantly lower in mutant mice compared to wild type controls: urine specific gravity, absolute and relative wet weights of anterior prostate, dorsal prostate, lateral prostate, ventral prostate, seminal vesicle, and urethra. In both wild type and mutant mice, IH decreased food consumption and body weight, increased relative bladder weight and changed the *in vitro* bladder strip contraction response to carbachol. IH uniquely decreased urine specific gravity in wild type mice, uniquely increased blood glucose concentration and appeared to increase the frequency of bacteriuria in mutant mice. Together, these results reveal that IH exposure and the *Lep*^{ob} mutation are capable of acting independently and together to modify mouse urinary function.

2. **Specific Aim:** Test the hypothesis that intermittent hypoxia causes urinary dysfunction in control mice and worsens urinary dysfunction in mice with T2DM.

Observations of mouse anatomy and physiology

Method: Feed and water consumption was determined by weighing water bottles and food at the beginning and end of the 2 wk exposure period. Blood glucose concentrations were determined by analyzing tail vein blood collected after a 4 hr fasting period with the AlphaTRAK 2 veterinary monitoring system (canine setting). Glucose concentrations of urine obtained by cystocentesis were determined with test strips. Specific gravity of freely caught urine was measured with a refractometer. Bladder volume measurements and wet weights of prostate lobes, seminal vesicle and urethra were determined within 15 min of euthanasia. Urethral measurements were conducted on the portion of the pelvic urethra spanning from the bladder neck to the bulbourethral gland.

Progress: Though these studies were not specifically listed in our initial proposal, they were needed because each endpoint has the capacity to influence urinary function directly or indirectly.

Results: Several of the physiological endpoints we assessed were consistent with reported observations made in obese, diabetic mouse models and results are shown in Figs. 1 and 2. Mutant mice consumed more food and water than wild type littermates over the 2 wk exposure period (Fig. 1). Blood glucose and urine glucose concentrations were higher and bladder volumes were larger in mutant mice. Intermittent hypoxia decreased food intake

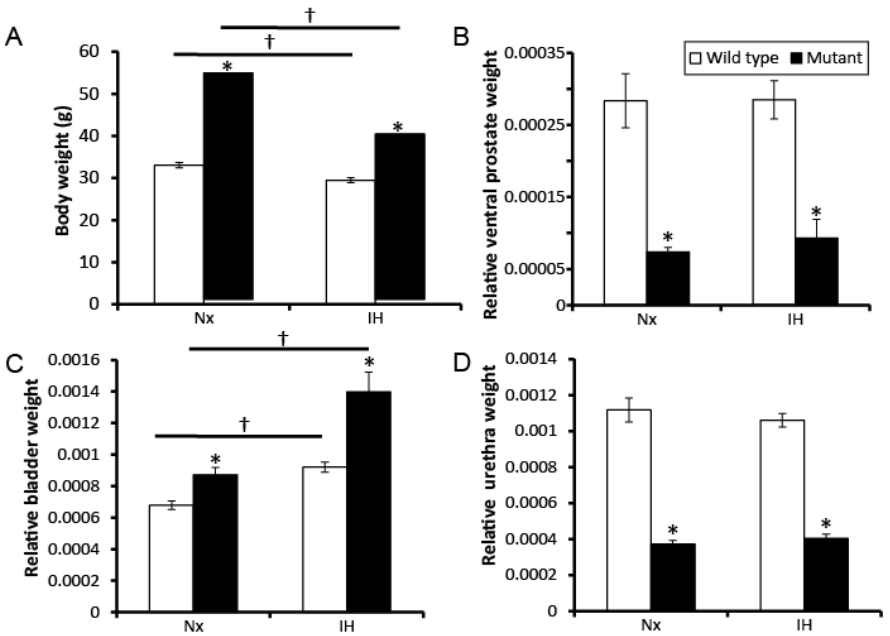

Fig. 2. The *Lep*$^{ob/ob}$ genotype and intermittent hypoxia associate with specific physiological changes in mice. Six week old *Lep*$^{ob/ob}$ (mutant) and wild type male mice were subjected to normoxia (Nx) or intermittent hypoxia (IH) for two weeks. (A) Body weight as well as wet weights normalized to body weight for (B) ventral prostate, (C) bladder, and (D) urethra were assessed at the end of the exposure period. Results are mean ± SEM, n=10-25 mice per group. Differences between groups were determined by ANOVA followed by the Tukey HSD post-hoc test. "*" indicates significant differences between genotypes and within the same exposure group; "†" indicates differences within the same genotype and between exposure groups.

among wild type and mutant mice and increased blood glucose concentrations in mutant mice. We also examined urine specific gravity, which was lower in mutant mice than in wild type littermates exposed to normoxia, consistent with dilute urine observed as a result of diabetic diuresis. Intermittent hypoxia decreased urine specific gravity among wild type mice. While body weight increased in mutant mice over wild type littermates (Fig. 2), we found that absolute and relative wet weights (normalized to body weight) of mutant prostate lobes, seminal vesicle and urethra decreased (representative results shown). In contrast, relative bladder wet weight increased in mutant mice, consistent with the larger bladder volumes observed in these mice. Intermittent hypoxia exposure caused loss of body weight while increasing relative bladder wet weight in both wild type and mutant mice compared to normoxia controls.

Assessment of urinary tract infection

Methods: Urine was obtained by cystocentesis of euthanized mice. An incision was made in the ventral midline and, for mice with full bladders, urine was extracted with a sterile 26.5ga syringe needle. For mice with empty bladders, a 26.5ga needle was used to lavage the bladder with sterile saline. One hundred microliters of urine or saline lavage was inoculated on blood agar plates and incubated overnight at 37°C. Plates were inspected the following morning and those with detectable growth were submitted to the UW-Madison Veterinary Pathology Laboratory for identification of bacterial strains.

Progress: These studies were not part of our initial proposal, but the endpoint was revealed as a confounding variable of urinary function in the mouse model used in our studies and therefore needed to be examined.

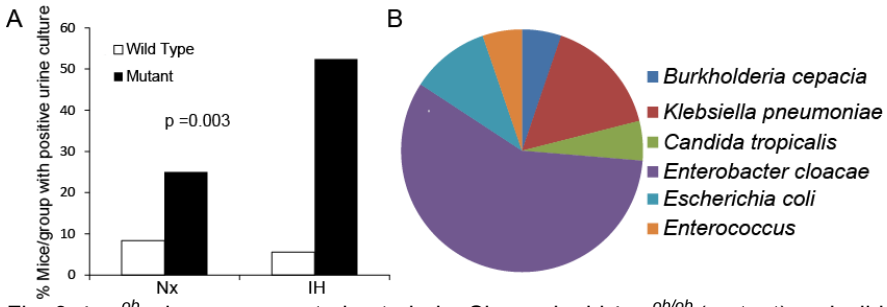

Fig. 3. *Lep*^{ob} mice are prone to bacteriuria. Six week old *Lep*$^{ob/ob}$ (mutant) and wild type male mice were subjected to normoxia (Nx) or intermittent hypoxia (IH) for two weeks. Urine was obtained by cystocentesis and cultured overnight on blood agar plates. (A) The percent of mice in each group with positive urine culture results is shown. Results represent 12-24 mice per group. Differences were assessed by Fisher's exact test. (B) Positive culture isolates were identified by the UW-Madison Veterinary Pathology laboratory. Several pathogens were identified and the proportion of each is shown.

Results: We found that about 40% of mutant male mice develop bacteriuria, and most of the isolated pathogens (Fig. 3) were enteric gram negative bacteria, consistent with ascending infection. Bacterial cystitis can be a clinically significant cause of urinary dysfunction in rodents and humans and may be a contributing factor to urinary dysfunction in mutant mice. Consistent with this, mutant mice with bacteriuria tended to exhibit histological evidence of bladder inflammation (inflammatory infiltrate with a neutrophilic pattern, urothelial thickening, edema, hemorrhage, results not shown). Examining inflammation was not part of our proposed aim, but future quantification of 16S bacterial DNA in mutant bladder, urethra and prostate, as well scoring of inflammation in these tissues, will provide an opportunity to address whether the prostate and urethra are influenced by bladder infection in mutant mice, and whether these aspects change urinary function in mutant mice.

Assessment of spontaneous voiding function (spontaneous void assay)

Method: Void assays were conducted and analyzed essentially as described previously (Keil et al., In Press). Mice were removed from their cages and singly housed in a clean, empty cage lined with 3MM Whatman filter paper (Fisher Scientific #057163W). Assays were conducted over 4 hr with access to food but not water. Assays were conducted in the same quiet location and at the same time of day. Filter papers were imaged using UV light on a transilluminator and analyzed using Image J Software (Version 1.46r). The following endpoints were analyzed: total urine spot number, total urine area, mean spot area, percent of filter paper area occupied by urine, primary (largest) void area, percent primary void area as percent of total urine area, and percent of total urine area in filter paper corners and center.

Progress: Assessments were completed in 5 wild type normoxia mice, 6 wild type intermittent hypoxia mice, 6 mutant normoxia mice, and 5 mutant intermittent hypoxia mice. We have met our proposed target for this subaim.

Results: Representative filter papers from each experimental group are shown in Fig. 4A. All parameters examined differed between wild type and mutant mice in at least one of the exposure groups (representative examples shown in Fig. 4B-C), with the exception of urine area in the center of the filter paper, which was not different between genotypic groups. In contrast, the only parameter that differed by treatment was the percent urine area in the center of the filter paper, which was significantly lower in the IH versus Nx groups for wild type mice.

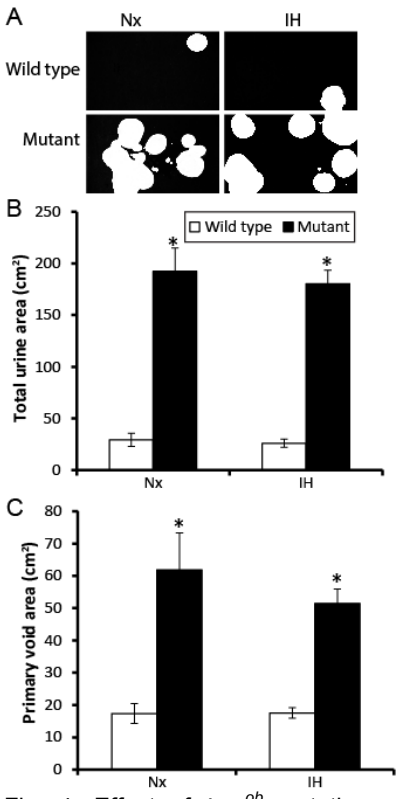

Fig. 4. Effect of *Lep*^{ob} mutation and intermittent hypoxia on spontaneous voiding in male adult mice mice. Six week old *Lep*$^{ob/ob}$ (mutant) and wild type male mice were subjected to normoxia (Nx) or intermittent hypoxia (IH) for two weeks. Void spot assays were conducted the day after the last Nx or IH exposure. (A) Representative images of filter papers after the 4 hr monitoring period. (B) Total urine area and (C) primary (largest) void area differed between genotypes. Results are mean ± SEM, n=5-6 mice per group. Differences between groups were determined by ANOVA followed by the Tukey HSD post-hoc test. "*" indicates significant differences between genotypes and within the same exposure group.

*Assessment of bladder function (*in vitro *tissue bath assay)*
Method: *In vitro* bladder bath studies were conducted essentially as described previously (Tengowski et al., 1997). Excised bladders were divided longitudinally from apex to bladder neck and weighed. 4-0 sutures were used to secure the bladder neck of each hemisection to the specimen arm and the bladder apex to a force displacement transducer (FT-03, Grass Instruments, Quincy MA). Bladder hemisections were suspended in a 37°C water-jacketed tissue chamber filled with Krebs solution (133mM NaCl, 16mM $NaHCO_3$, 5mM KCl, 1mM $MgCl_2$, 1.4mM NaH_2PO_4, 2.5mM $CaCl_2 \bullet 2H_2O$, 7.8mM d-glucose, pH 7.2) and aerated with 95% O_2/5% CO_2. Tissues were maintained at a tension of 1g for 75 min prior to experimentation. Tension was recorded using the AxoScope Application of pCLAMP™ Software (Molecular Devices, Sunnyvale, CA). Bladder hemisections were equilibrated for approximately 1 hr in Krebs solution and increasing concentrations (0-10μM) of the cholinergic agonist carbachol were added stepwise thereafter until a maximal response was achieved. Tissues were then washed with carbachol-free Krebs solution to return the tension to baseline and a maximal response

was generated by adding 60mM KCl to the baths. The average bladder hemisection response was determined for each animal and 5-6 animals per treatment group were evaluated.

Progress: Assessments were completed in 6 wild type normoxia mice, 5 wild type intermittent hypoxia mice, 5 mutant normoxia mice, and 6 mutant intermittent hypoxia mice. We have met our proposed target for this subaim.

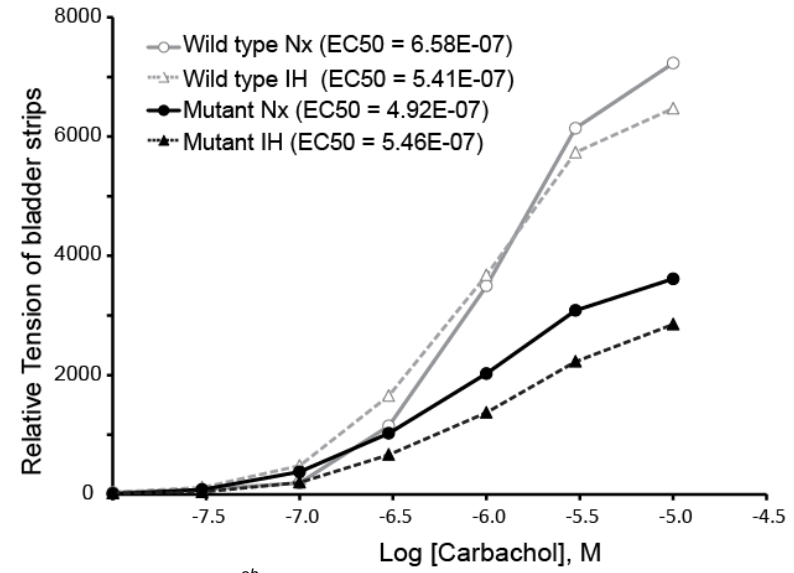

Fig. 5. Effect of *Lep*^{ob} mutation and intermittent hypoxia on carbachol-induced contraction of mouse bladder strips. Six week old *Lep*$^{ob/ob}$ (mutant) and wild type male mice were subjected to normoxia (Nx) or intermittent hypoxia (IH) for two weeks. Isolated bladders were hemisected longitudinally, the strips affixed to the arms of a force displacement transducer, submersed in bath medium and exposed to graded concentrations of carbachol. (A) Carbachol concentration-bladder contraction response curve. The relative tension is the percent maximal contraction (induced by KCl) normalized to bladder strip wet weight. Each data point is the mean from two bladder strips per mouse, averaged across 5-6 mice per group.

Results: Bladder muscle (detrusor) contraction is stimulated by cholinergic pathways. To test whether intermittent hypoxia and the *Lep*$^{ob/ob}$ genotype influence bladder contractile responses, *in vitro* organ bath studies were conducted with bladder hemisections. Carbachol was added stepwise to bath medium and a maximal contractile response was generated with KCl. A cumulative carbachol concentration – bladder contractile response curve was generated and is shown in Fig. 5. The curve was used to calculate the EC50 responses, which are also shown in Fig. 5. IH decreased the EC50 response in wild type mice but increased the EC50 response in mutant mice. Mutant mouse bladder strips exhibited reduced maximal carbachol responsiveness, but this effect was not further changed by IH. While there appears to be a difference in the carbachol-induced maximal response, there was no significant difference in the KCl induced maximal response (results not shown).

Assessment of bladder response to filling and emptying (cystometry)

Method: Mice were anesthetized with urethane (1.125mg/kg). A body wall incision was made in the abdominal wall to expose the bladder. Purse string sutures were inserted near the apex of the bladder using 6-0 thread. Catheters were created from PE50 tubing, cut 2mm shorter than the length of a 1.5 in, 26ga syringe needle, and one end of the tubing was melted to create a cuff. The needle was run through the catheter, then both needle and catheter were inserted through the apex and across the bladder wall. The needle was then removed and the purse string sutures tightened, followed by two circumferential sutures, to fix the catheter in place. The body wall and skin were sutured and the animal was allowed to recover for 45 min prior to saline infusion. During experimentation saline was perfused at a rate of 1.6ml/hr and voiding was recorded for at least one hour for each animal until a stable pattern was reached. Measurements were analyzed from at least six voiding events per animal with 3-6 animals per treatment group.

Progress: Assessments were completed in 6 wild type normoxia mice, 6 wild type intermittent hypoxia mice, 5 mutant normoxia mice, and 3 mutant intermittent hypoxia mice. We have not yet met the proposed goal of 5 mice per group, largely because of anesthesia complications. The mutant mice are extremely sensitive to urethrane dosing based on body weight, which resulted in overdose. We therefore modified the urethrane dosing scale such that it was equivalent to the amount given to an age-matched wild type littermate.

Results: Cystometry was performed on urethane anesthetized mice and the results are shown in Fig. 6. Voiding pressure versus time curves for representative mice are shown in Fig. 6A. Several parameters were analyzed from five consecutive representative voids per mouse and averaged across at least 3 mice per group. These include: threshold pressure (defined as the pressure needed to elicit a void), voiding frequency, peak voiding pressure, time to return to baseline following peak voiding pressure, intervoid interval, leak point pressure, non-voiding contraction frequency and peak non-voiding contraction pressure. The only parameter that differed significantly between groups was the time needed to return to baseline after achieving peak voiding pressure. Results for this parameter are shown in Fig. 6B.

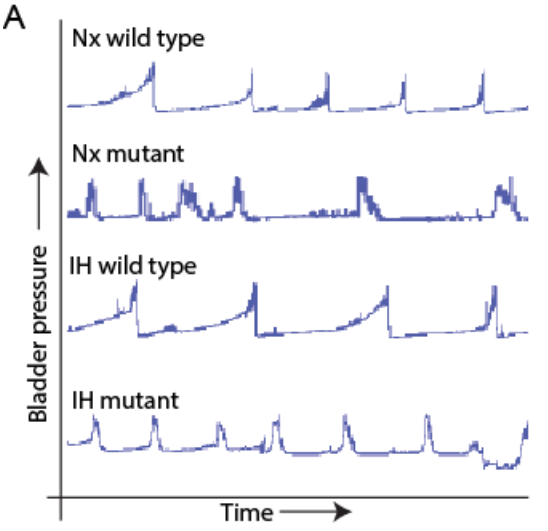

Fig. 6. Effect of *Lep*^{ob} mutation and intermittent hypoxia on bladder response to filling and emptying. Six week old *Lep*$^{ob/ob}$ (mutant) and wild type male mice were subjected to normoxia (Nx) or intermittent hypoxia (IH) for two weeks. Cystometry was performed on urethrane anesthetized mice. (A) Representative bladder pressure-time curves (cystometrograms) from each experimental group. Each peak represents a voiding event. (B) Elapsed time between peak voiding pressure and return to baseline. Results are mean ± SEM, n = 3-6 mice per group. Mutant Nx mice, compared to wild type Nx mice, took longer to return to baseline pressure after peak voiding pressure. Differences between groups were determined by ANOVA followed the Tukey HSD post-hoc test. "*" indicates significant differences between genotypes and within the same exposure group.

3. **<u>Publications:</u>** Abstracts were or will be presented at Society for Basic Urologic Research Meetings (2013 and 2014 listed below). We are completing analysis so that results can be submitted for publication within a year.

    1. Abler LL, Keil KP, Mehta V, Crader-Smith S, Wang Z-Y, Bjorling DE, Watters JJ, Vezina CM. Examining the Effects of Intermittent Hypoxia on Urinary Function in Mice. 10th World Congress on Urologic Research, Nashville, TN, November 2013. #89.
    2. Abler LL, Keil KP, Ouellet JN, Wang Z-Y, Wang P, Ricke WA, Bjorling DE, Watters JJ, Vezina CM. Investigating the Effects of Intermittent Hypoxia on Urinary Function in Diabetic Mice. Society for Basic Urologic Research Fall Symposium, Dallas, TX, November 2014.

4. References:

Keil, K.P., Abler, L.L., Altmann, H.M., Bushman, W., Marker, P.C., Ricke, W.A., Bjorling, D.E., Vezina, C.M., In Press. Influence of animal husbandry practices on void spot assay outcomes in C57BL/6J male mice. Neurourol Urodyn.

Tengowski, M.W., Bjorling, D.E., Albrecht, R.M., Saban, R., 1997. Use of gold-labeled ovalbumin to correlate antigen deposition and localization with tissue response. Journal of pharmacological and toxicological methods 37, 15-21.