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Original Article

Voided volumes predict degree of partial bladder outlet obstruction in a murine model

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Abstract: The partial bladder outlet obstruction animal model (pBOO) is commonly used as a model for obstructive uropathy. Unfortunately, pBOO demonstrates variable degrees of obstruction requiring bladder weight (BW) or urodynamic studies to determine true obstruction. Our objective is to identify extent of obstruction by correlating early post-operative Void Stains on Paper (VSOP) assays with ultimate BW in mice. pBOO was performed on 32 mice 1- and 4-week VSOPs were quantified for mean voided volume (mVV). At 4 weeks, bladders were harvested and weighed. Correlation was evaluated through bivariate kernel density estimation and a Pearson correlation coefficient (SAS). Single variable histogram of the data established groups based on BWs and mVV. mVV's and bladder weights within group pairings were averaged and plotted to render a non-linear regression model. A significant correlation was found between 1-week mVVs and 4-week BWs upon bivariate analysis with a correlation coefficient of -0.758 ($p = 0.0294$). A non-linear regression of plotted data defined a statistically significant fit equation correlating 1-week mVV to 4-week BW. We demonstrate a novel method for forecasting degree of obstruction in pBOO based on 1-week post-operative VSOP mVV.

Keywords: Bladder, bladder obstruction, partial bladder outlet obstruction, obstructive uropathy, posterior urethral valves, VSOP, surgical and invasive medical procedures

Introduction

The murine partial bladder outlet obstruction model (pBOO) is commonly used [1-3] to simulate human diseases such as benign prostatic hyperplasia (BPH) [4, 5] and posterior urethral valves (PUV) [6-8]. However, this model contains inherent challenges in achieving a similar degree of obstruction between surgeries, leading to variable outcomes between biological replicates. Oftentimes, appropriate degree of obstruction cannot be determined until the time of bladder harvest at the end of the experiment.

Two objective measures useful for evaluating pBOO are bladder weight at the time of animal sacrifice and Void Stains on Paper (VSOP). The most quantifiably objective means of determining obstruction is bladder mass [9, 10]. While valuable, this does not allow the researcher to make assessments on the degree of obstruction until completion of the study, at which point valuable resources and time may have been wasted. Void Stains on Paper (VSOP) is another objective measure that can be useful in determining bladder function longitudinally after pBOO; however, its relationship with bladder outlet obstruction has not been established. Despite its potential utility, no statistically significant correlation has been put forth in the literature. Such correlation would allow researchers to use early VSOP data to predict the bladder weight, reflecting the extent of obstruction at the end of the study. This would prevent any undue pain and suffering in the animals deemed unobstructed and therefore not useful for the study. Additionally, time and labor-inten-

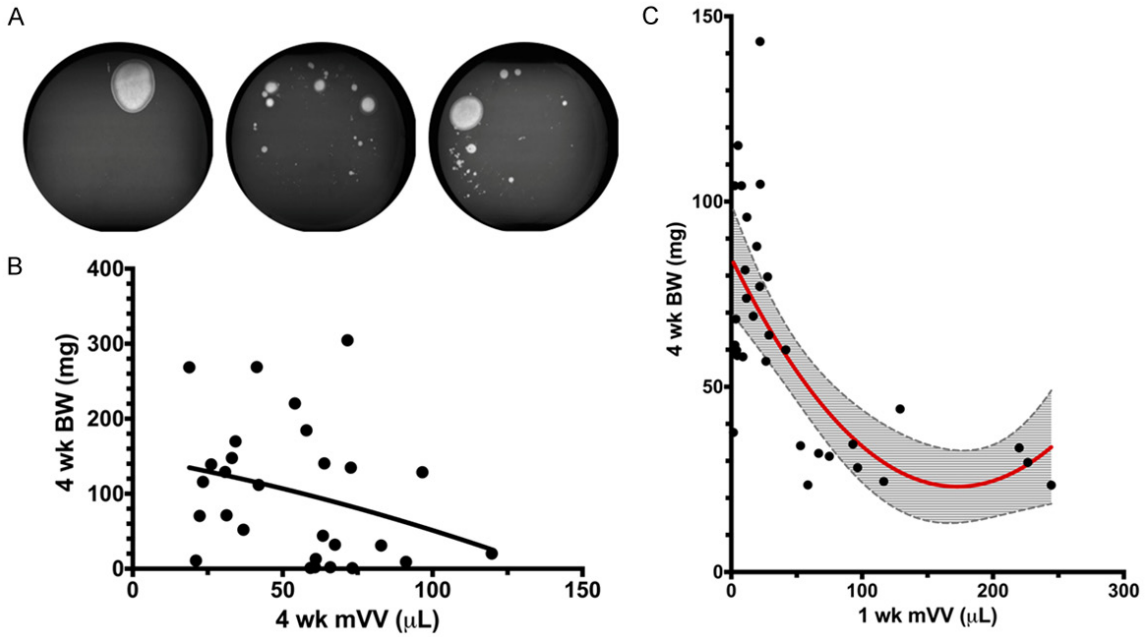

Figure 1. Evaluation and graphical representation of VSOPs at 1- and 4-week time points. A. Images of VSOPs obtained from metabolic cages for sham (left) and pBOO (middle, right) surgeries with a representative image of a VSOP with both obstructed and unobstructed characteristics (right). B. Scatter plot of 4-week mVVs, obtained from VSOP quantification, and their corresponding 4-week BW (n = 28) with a nonlinear fit of $y = -0.00433x^2 - 0.49x + 143$ (Weighted $R^2 = 0.2543$). C. Scatter plot of 1-week mVVs, obtained from VSOP quantification, and their corresponding 4-week BW (n = 32) with a nonlinear fit (red line) of $y = 0.00207x^2 - 0.72x + 84.71$ (Weighted $R^2 = 0.5789$) with a weighting factor of $1/Y^2$. The 95% asymptotic confidence interval is indicated by the dark gray dotted line. Data points in the light gray shaded region fall within the 95% confidence interval.

sive data collection on mice would be reduced. With this motivation in mind, we set out to identify a correlation between average void volumes obtained from VSOPs at 1-week post-op and bladder weights obtained at 4-weeks post-op.

Materials and methods

Experimental animals

The Institutional Animal Care and Use Committee of Northwestern University approved all animal studies. Animals included in this study were male, non-genetically or hormonally altered, wild-type mice with a C57 BL/6 (Charles River) background.

Partial bladder outlet obstruction mode

Mice, 6 to 8 weeks of age, were used in the partial outlet obstruction surgeries. A Matrix Medical Inc. Spartan VMC anesthesia unit was used to induce anesthesia with 3% vaporized isoflurane (Baxter) at a flow rate of 1.5 L/min. Mice were transferred to the operating table and anesthesia was maintained using a rodent anesthesia mask. The abdomen was opened and the bladder was dissected free. To create the partial bladder outlet obstruction, the urethra was ligated at the bladder neck over a 23 g angiocath with 6-0 Prolene. The muscle and skin were then closed with running 4-0 Vicryl. Sham surgeries were performed by dissecting the bladder free followed by closing the abdomen in a similar fashion. Upon completion of surgery, mice were placed into a post-operative recovery cage on a heating pad and monitored until they were alert and moving without limitations. Pain was treated by injecting 0.1 mg/kg of buprenorphine into the scruff every 12 hours for 72 hours with intermittent post-operative checks.

Void stains on paper (VSOP)

Following pBOO and sham (control) surgeries, mice were evaluated by VSOP on postoperative days 7, 14, and 28. Mice were placed into individual metabolic cages for 2 hours and voids were captured with Whatman paper at the base

Table 1. Nonlinear fit equations

$y = ax^2 + bx + c$	$a \times 10^{-3}$	b	c	Weighted R^2
Eq. 1	-4.33	-0.49	143.00	0.2543
Eq. 2	2.07	-0.72	84.71	0.5789
Eq. 3	1.89	-0.66	78.40	0.6349
Eq. 4	2.36	-0.78	82.35	0.9012

of the cage. The Whatman paper was then imaged with UV light and voids were quantified using ImageJ. Void areas were calculated and converted to void volumes by multiplying the area by a conversion constant of 12.688. This constant was determined by pipetting variable known volumes of human urine onto the Whatman paper and establishing a correlation curve. Finally, an average void volume was calculated from the 10 largest voids.

Bladder harvesting

Mice were euthanized 4-weeks post-surgery; and total body weight and bladder weights were determined.

Statistical analysis

Multivariate statistical analysis was performed using SAS. Non-linear regression analysis and formation of residual plots were constructed using Prism 7.

Results

Initial correlation assessment

VSOPs were used to elucidate a correlation between 1-week mean voided volumes (mVV) and 4-week bladder weight (BW). Following pBOO, many animals demonstrated voiding patterns that were not definitively associated with obstruction yet were not clearly normal (**Figure 1A**). A scatter plot of the raw BW and mVV was constructed and a non-linear fit equation was calculated for the 1-week and 4-week time points. A statistically significant correlation could not be concluded when comparing 4-week mVV and 4-week BW (**Figure 1B**) as indicated by the poor r^2 value of 0.2543 for the non-linear regression curve (**Table 1**, *Eq. 1*). However, the 1-week mVV followed an expected trend (**Figure 1C**)-as mice with larger BWs had smaller mVVs. At 1-week post-surgery, a correlation became more apparent and the non-linear regression began taking shape (**Table 1**, *Eq. 2*) given the increased r^2 value of 0.5789.

From this, the 1-week mVV time point presented itself as a suitable variable for our correlation study. Using Pearson correlation coefficient (r), the 1-week mVV showed a significant correlation with BW (r = -0.5789, p = 0.0006 at a 95% confidence interval).

Construction of correlation curve

Since bladder weights may increase with total body weight, bladder weights ($m_{bladder}$) were normalized to reduce the effect of larger body weights (m_{body}) as a confounding variable. The average body weight of the C57 BL/6 mice used in this study was 25.5 g. This value was used to normalize bladder weight as outlined in the equation below,

$$aBW = \left(\frac{25.5g}{m_{body}}\right) m_{bladder}$$

This equation gave an adjusted bladder weight (aBW), leading to a marginal improvement in the non-linear regression fit (**Table 1**, *Eq. 3*) (**Figure 2A**) and an enhanced correlation (r = -0.6349, $p < 0.0001$).

A kernel density estimation was used to initially visualize the distribution of a hypothetically continuous variable. This estimation is useful since it is non-biased towards any underlying distribution for an individual variable (mVV or aBW). For this estimation, the bandwidth was approximated using SAS. The heatmap overlying the scatter distribution (**Figure 2B**) provides evidence that there is no efficient univariate method for grouping or binning data. To confirm this observation, data was binned based on frequency analysis, with bin width determined using Sturge's Rule:

$$K = 1 + \log_2 N$$

where K is the bin width and N is the number of observations in the set [11]. This was applied to both the aBW (**Figure 2C**) and mVV (**Figure 2D**) variables. In doing so, large deviation is observed when averaging data within the bins rendering any statistical correlation curve obsolete. For example, averaging data within each aBW bin leads to significant mVV error at low bladder weights, with minute x- and y-error at larger bladder weights. The opposite also held true when attempting to do the same process based solely on mVV bins.

To overcome this hurdle, bins rendered from each frequency distribution were assigned a

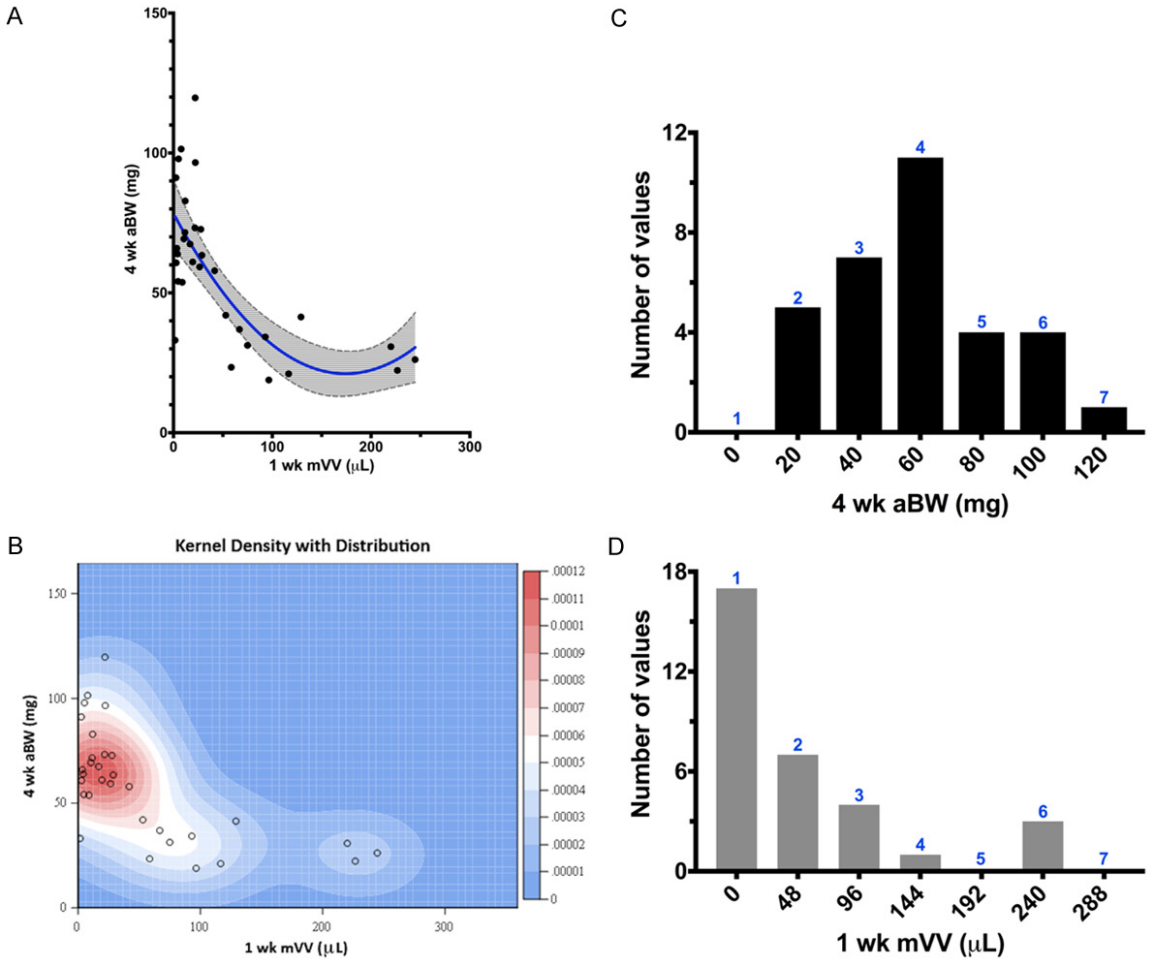

Figure 2. Bladder weight normalization and correlation analysis. A. Scatter plot of 1-week mVVs and their corresponding 4-week aBW (n = 32) with a nonlinear fit (blue line) of $y = 0.00189x^2 - 0.66x + 78.40$ (Weighted R^2 = 0.6349) with a weighting factor of $1/Y^2$. The 95% asymptotic confidence interval is indicated by the dark gray dotted line. Data points in the light gray shaded region fall within the 95% confidence interval. B. 1-week mVVs and 4-week aBWs distribution overlaying the Kernel Density. Bivariate statistics rendered from Kernel Density include covariance = -831, corelation -0.60. C. Frequency distribution histogram of 4-week aBWs (n = 32) with a bin width of 20 mg and a mean of 58 mg (SEM = ± 5 mg). D. Frequency distribution histogram of 1-week mVVs (n = 32) with a bin width of 48 µL and a mean of 53 µL (SEM = ± 10 µL). C, D. Bins are assinged a numerical label indicated by the number in blue above each bar. Numerical labels were used to establish grouping of data in the final correlation curve.

numerical value and assigned to the data point within each bin. Subsequently, data were categorized into groups that shared the same bin number pairings. For example, data points that fell into aBW bin 5 and mVV bin 2 were placed into a new group with a new alphanumeric label. Of note, one datum was left unpaired in its bin. However, since this was a mouse from a sham surgery with aBW and mVV values similar to that of bin h, it was included in that average. This is an acceptable change since sham mice are the unobstructed control, and our goal is to measure the degree of obstruction, not unobstruction.

Correlation curve

A second order quadratic standard curve was interpolated from these data with a weighting factor of $1/Y^2$. This resulted in a correlation curve with a suitable R square of 0.9012 (**Table 1**, *Eq. 4*) (**Figure 3**). To assess whether our weighting introduced bias on our non-linear fit, a test for appropriate weighting was performed (Prism). This test determines if there is a correlation between y-values and the absolute value of the weighted residual. Low *p*-values in this case suggest a correlation between the two and thus an inappropriate biased weighting.

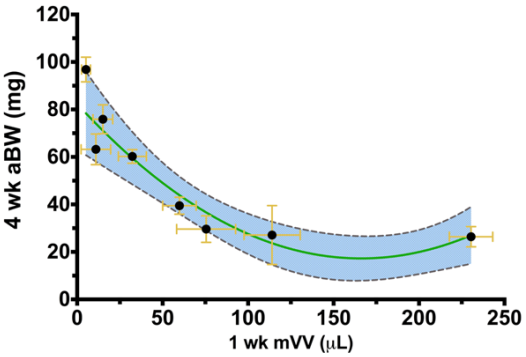

Figure 3. Correlation of 1-week mVV and 4-week aBW. Scatter plot of binned (n = 8) 1-week mVVs and their corresponding 4-week aBW with a nonlinear fit (green line) of $y = 0.00236x^2 - 0.78x + 82.35$ (Weighted $R^2 = 0.9012$, dof = 5) with a weighting factor of $1/Y^2$. The 95% asymptotic confidence interval is indicated by the dark gray dotted line. Data points in the light blue shadded region fall within the 95% confidence interval. Error bars represent the standard deviation of the mVVs (x-error) and aBW (y-error) of the data contained in each bin.

Table 2. Comparison summary of predicted and observed 4-week aBW

Surgery/ Animal	Observed 4 wk aBW (mg)	Predicted 4 wk aBW (mg)	Percent Error
Sham.v1	20.27	24.42	20%
Sham.v2	20.29	17.44	14%
pBOO.v1	43.41	56.90	31%
pBOO.v2	47.74	52.54	10%
pBOO.v3	52.04	53.37	3%
pBOO.v4	69.69	63.72	9%
pBOO.v5	70.00	81.60	17%
pBOO.v6	52.16	53.52	3%
pBOO.v7	82.56	77.42	6%
pBOO.v8	89.54	79.51	11%
pBOO.v9	67.45	72.85	8%
pBOO.v10	73.47	81.09	10%
pBOO.v11	76.23	73.92	3%
pBOO.v12	96.23	79.49	17%
Average Percent Error			12%

This test resulted in a *p*-value (one tailed) > 0.05, of 0.2309, therefore passing the test for appropriate weighting.

The Runs test was also performed to evaluate if our correlation curve deviates systematically from our data. Here, a large deviation will be indicated by a small *p*-value while minimal deviation will be noted by a large *p*-value [12]. This test reported a large *p*-value of 0.8857, concluding that there is non-significant deviation from our model. Finally, the Pearson correlation coefficient was calculated once more yielding a coefficient of -0.758 ($p = 0.0294$). A summary of the fit equations found in this study are outlined in **Table 1**.

Validation

Further studies were put forth to test the ability of the correlation curve to predict the 4-week aBW from 1-week mVV. An additional 14 biological replicates received either sham or pBOO surgeries. VSOPs at 1-week post-op were quantified, and their predicted aBW was calculated using *Eq. 4* (**Table 1**). Mice were then euthanized at 4 weeks post-operation and their bladder weight was measured. The results from this validation study are summarized in **Table 2**. Our correlation curve was able to predict the 4-week aBW from 1-week mVV within a margin of error of 14% on average. This can be broken down into 17% error for animals with sham surgeries (n = 2) and 11% error for animals with pBOO surgeries (n = 12).

Bladders on the polar ends of the pBOO model (mildly (aBW < 50 mg) and severely (aBW > 90 mg) obstructed) contain larger percent errors with the largest coming from the mildly obstructed and sham surgeries. The wide range of 1-week mVVs (100-250 µL) for sham animals caused increased percent error when estimating the 4-week aBW. This can be attributed to the natural differences in bladder function, volume, and pressure between biological replicates [5].

Of note, there is a slight underestimation in bladder weights when approaching the upper boundaries of the pBOO surgical model, with the exception of pBOO.v5 (**Table 2**). We argue that the underestimations are not concerning as they will provide a more conservative estimation of the 4-week aBW from the 1-week mVV. Furthermore, the conservative estimate is appropriate since this model is attempting to provide statistically significant evidence that a mouse is obstructed. For example, although the model for pBOO.v12 predicts a 4-week aBW of 79.49 mg, the observed weight is 96.27 mg. Regardless of the underestimation, both values arrive at the same conclusion that the animal is obstructed.

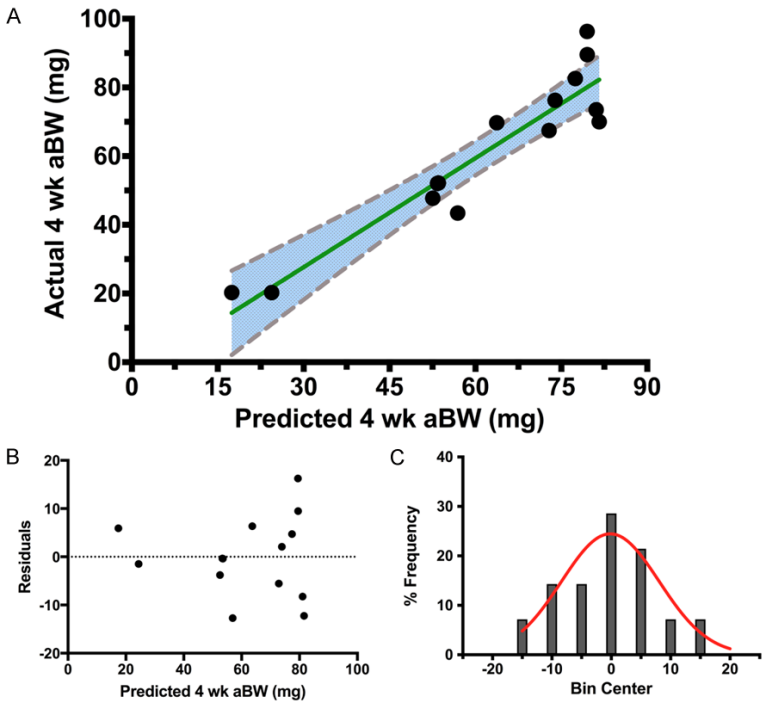

Figure 4. Graphical validation of proposed correlation curve. A. Linear regression (green) of predicted and observed 4-week aBW (n = 14) fit by the equation (R^2 = 0.8749). The 95% asymptotic confidence interval is indicated by the dark gray dotted line. Data points in the light blue shadded region fall within the 95% confidence interval. B. Residual plot rendered from the linear regression. C. A Gaussian curve (red) was calculated from the frequency distribution (bin width = 5) of residuals and centered at -0.16 (R^2 = 0.8366).

The predicted and observed 4-week aBW were plotted for further evaluation (**Figure 4A**). The linear regression fitting these data contained a slope of 1.1 ± 0.1 with an R^2 of 0.8749 and a *p*-value < 0.0001, further strengthening the relationship between our predicted and observed 4-week aBW.

A number of methods exist that effectively evaluate the relationship between two variables. A residual plot is one such method. Residuals are calculated from the difference between a dependent variable and a predicted variable, and the residual plot is generated on a scatter plot of the independent variable against the residuals. A residual plot that shows a strong relationship between two variables will have points plotted at random above and below the zero residual line and equally dispersed across the x-axis [13]. Although our residual plot (**Figure 4B**) begins to meet these criteria, it does exhibit marginal qualities of heteroscedasticity meaning residuals get larger as the predicted aBW increases. This can be overcome in future correlation studies by increasing the number of data points on the linear regression curve.

Frequency analysis was then performed on the residuals and a Gaussian curve was subsequently computed (**Figure 4C**). The Gaussian curve fit these data at a mean value of -0.16 and R^2 of 0.8366 (dof = 5). This provides evidence of minimal bias towards any positive or negative residual as indicated by the mean value closely centered at 0.

Discussion

Study of bladder outlet obstruction in animal models is necessary to determine therapies to prevent or reverse maladaptive remodeling that occurs in human bladders subject to injury. Any researcher that has utilized the pBOO model is familiar with the inherent variability that exists in the study. Despite our best efforts at standardizing surgical technique between animals, a mortality rate of greater than 15% has been described [14, 15]. In addition, while a urethral ligature may be placed, some animals do not demonstrate any evidence of bladder outlet obstruction histologically at the time of animal sacrifice. This raises the question of reliability for the pBOO model. We set out to determine an early method of accurately identifying animals with an appropriate degree of bladder remodeling at the time of sacrifice.

We hypothesized that early changes identified on VSOP following pBOO were reflective of the bladder's direct response to the degree of obstruction rather than bladder remodeling. This was supported by data that demonstrated that minimal changes were identified in regard to bladder to body weight ratio and TGFB levels early after pBOO [16, 17]. We have also shown that mice that undergo sham surgery without a bladder outlet ligature recover normal pattern on VSOP by 1 week, whereas mice that undergo

pBOO will continue to demonstrate an obstructive pattern [1]. As the outlet is constricted by the ligature, bladder pressure rises, leading to the initiation of molecular changes that eventually govern bladder remodeling. However, we believe the early VSOP findings of decreased voided volumes and increased urinary frequency with a spraying pattern are a direct effect of obstruction. By the 4-week time point, remodeling has occurred in a variable fashion. Thus, VSOP at that time reflects these changes, making 4-week VSOP less reliable in predicting bladder weight. At this time point, it was not uncommon to have obstructed bladders (aBW > 100 mg) with seemingly unobstructed mVV > 50 μL as demonstrated by an insignificant R^2 from the nonlinear regression (**Figure 1B**).

We primarily utilized bladder weight as evidence of true established bladder outlet obstruction. This is an established quantifiable response of bladder remodeling [2]. Though functional testing such as murine cystometry is commonly utilized, multiple potential pitfalls with cystometry have been previously demonstrated [18]. In particular, evaluating bladder pressures following surgically induced bladder outlet obstruction can be extremely challenging given the degree of scarring that occurs around the bladder. Furthermore, murine cystometry is typically not a procedure than can be used to track mice longitudinally, and therefore in an obstructive model, it cannot be utilized to identify sufficiently obstructed animals early in post-obstructive course. We have used cystometry following pBOO and found similar evidence of elevated voiding and baseline pressures with significant differences in remodeling [1]. Therefore, we chose not to utilized cystometry in this study.

Void Stain on Paper was initially described by Sugino et al. [19]. They demonstrated a linear correlation between the area of the void stain and voided volume. Furthermore, they were able to utilize VSOP to identify differences in voided volume and voiding interval between mice with cyclophosphamide induced cystitis and sham mice. This allowed for non-invasive, longitudinal monitoring of bladder function, a significant advantage compared to murine cystometry which is typically a terminal process. In our study, we utilize static paper in modified metabolic cages to track void stains. However, we believe these results will equally apply to alternate methods of measuring mouse voiding such as continuous transported paper or continuous urine weight recording [18, 20, 21].

Using this formula, 1-week VSOP can be utilized to predict 4-week bladder weight, allowing researchers to terminate experiments or exclude mice who do not demonstrate a sufficient degree of BOO. This can be a significant benefit by reducing cost and effort. Exclusion of samples that do not meet criteria for true BOO can allow researchers to better understand obstructive physiology and prevent cluttering data with non-obstructed animals. This can increase reproducibility of experiments as we become more accurate in utilizing only samples that demonstrate true obstruction.

Perhaps the most important benefit in having a reliable early test to identify adequate obstruction with pBOO is the ability to evaluate the efficacy of genetic or pharmaceutical interventions that prevent bladder hypertrophy and fibrosis. A common criticism of the pBOO model lies in its inherent variability. When interventions limit the maladaptive effects of pBOO, the first question that comes to mind is whether that group of mice was sufficiently obstructed. The ability to pre-determine the degree of obstruction inflicted with pBOO becomes particularly important in evaluating the effect of treatment to prevent deleterious change with BOO. Concern is regularly raised when remodeling following pBOO doesn't follow expected patterns: is this merely because the obstruction was insufficient, or is it due to the genetic or pharmaceutical intervention? If 1-week VSOP is an indication of appropriate bladder outlet obstruction, deviation from the predicted bladder weight can be evidence of therapeutic effectiveness.

Conclusion

We have demonstrated that an inexpensive and easy to perform test can reliably predict the degree of bladder hypertrophy that will occur following partial bladder outlet obstruction. This formula can be utilized to screen mice for appropriate obstruction following pBOO. Furthermore, if deviations from the predicted bladder weight occur, it may be evidence of alterations in bladder remodeling that have occurred due to genetic or pharmaceutical interventions.

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Disclosure of conflict of interest

None.

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