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Effects of L-Arginine on Bladder Function in Bladder Outlet-Obstructed Rats

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Nitric oxide (NO) is thought to play an important role in the lower urinary tract. We therefore studied the effects of NO on bladder function in bladder outlet-obstructed rats. Twelve-week-old female Wistar rats had their bladder outlets partially obstructed by ligating the urethra over which a catheter was placed. Micturition parameters and urodynamic parameters were measured in 6 groups of rats: Group I, 1 week after sham operation; Group II, 1 week after surgical induction of bladder outlet obstruction; Group III, 150-mg/kg/day L-arginine injected intraperitoneally for 1 week after surgical induction of bladder outlet obstruction; Group IV, 6 weeks after sham operation; Group V, 6 weeks after surgical induction of bladder outlet obstruction and Group VI, 150-mg/kg/day L-arginine injected intraperitoneally for 6 weeks after surgical induction of bladder outlet obstruction. Moreover, the area density of smooth muscle versus connective tissue was determined in bladder specimens using color assisted computer image analysis in these groups. The number of micturitions per day of the 1- and 6-week obstructed rats significantly increased over that of the sham-operated rats. The number of micturitions per day of the obstructed rats treated with L-arginine for 1 and 6 weeks significantly decreased in comparison to that of the obstructed rats. In the urodynamic study, the maximum detrusor pressure of the obstructed rats for 1 and 6 weeks significantly increased over that of the sham-operated rats. Residual urine volume of the obstructed rats for 1 and 6 weeks significantly increased over that of the sham-operated rats. The maximum detrusor pressure and residual urine volume of the obstructed rats treated with L-arginine for 1 and 6 weeks significantly decreased over those of the obstructed rats. In the histological study, the area density of smooth muscle versus connective tissue of the 1- and 6-week obstructed rats significantly increased over that of the sham-operated rats. Histological changes in the 1- and 6-week obstructed rats were prevented by treatment with L-arginine. These results indicate that L-arginine has a beneficial effect on bladder dysfunction in bladder outlet-obstructed rats. This might be due to L-arginine's influence in increasing NO levels and the rate of blood flow in the obstructed bladder.

Key words: bladder dysfunction; bladder outlet obstruction; L-arginine; rat

Bladder outlet obstruction is one of the most common urological problems in elderly male patients (McGuire, 1984; Berggren and Uvelius, 1998). Bladder outlet obstruction secondary to benign prostatic hyperplasia (BPH) induces various bladder dysfunctions. Increases in urinary frequency and urgency are typical signs of the early stages of BPH (McGuire, 1984). During the later stages of BPH, patients suffer from increasing amounts of residual urine and are subject to overflow incontinence due to retention (McGuire, 1984). These symptoms may be related to the lack of ability of the detrusor to compensate for bladder outlet obstruction and result in impaired detrusor contractility (Saito et al., 1993). But the association between

Abbreviations: BPH, benign prostatic hyperplasia; NIH, National Institutes of Health; NO, nitric oxide; NOS, nitric oxide synthase

bladder outlet obstruction and the resulting changes in bladder function is poorly understood (Greenland and Brading, 2001). In recent years, some authors have reported that increased intravesical pressure and bladder outlet obstruction are associated with decreased bladder blood flow (Nielsen, 1995; Greenland and Brading, 1996; Lin et al., 1998). Nielsen (1995) showed that a median decrease of 47% in blood flow rate was observed after distension in the 2-month obstructed mini-pig bladder. After 2 weeks of partial bladder outlet obstruction in rabbits, Lin et al. (1998) reported an 80% decrease in blood flow rate. Also, Greenland and Brading (2001) showed that significant detrusor ischemia occurs during prolonged voiding contractions in obstructed pigs.

Nitric oxide (NO) mediates relaxation of smooth muscle. Until recently, there have been few reports of this type of relaxation in the detrusor smooth muscle. Andersson and Persson (1995) have described that the detrusor had a lower sensitivity to NO, which makes it less likely that it can play the role of a relaxant neurotransmitter. Moreover, NO is a major blood flow regulator that is synthesized and released by NO synthase (NOS). Although there have been no reports on measurement of NO in the obstructed bladder, there has been speculation regarding the role of NO in bladder ischemia of the obstructed bladder (Sutherland et al., 1998).

The goal of this study was to determine whether L-arginine, a precursor of NO synthesis, prevents bladder dysfunction induced by bladder outlet obstruction. This is the first report to study the effects of L-arginine in experimental bladder dysfunction of bladder outlet-obstructed rats.

Table 1. Surgical and medical treatments in the various groups of rats

Group	Duration (week)	Surgical induction of bladder outlet obstruction	Medical treatment
I	1	Not done	None
II	1	Done	None
III	1	Done	L-arginine
IV	6	Not done	None
V	6	Done	None
VI	6	Done	L-arginine

Materials and Methods

Twelve-week-old female Wistar rats weighing 200 to 250 g (SLC, Shizuoka, Japan) were used. All animal experiments were performed in accordance with the guidelines of the Tottori University Committee for Animal Experimentation. All rats were housed in a room with controlled lighting and were allowed access to food and water ad libitum. Rats were divided into 6 groups, each containing 7 rats as follows: Group I, 1 week after sham operation; Group II, 1 week after surgical induction of bladder outlet obstruction; Group III, L-arginine (150 mg/kg/day) was injected intraperitoneally for 1 week after surgical induction of bladder outlet obstruction; Group IV, 6 weeks after sham operation; Group V, 6 weeks after surgical induction of bladder outlet obstruction and Group VI, L-arginine (150 mg/kg/day) was injected intraperitoneally for 6 weeks after surgical induction of bladder outlet obstruction (Table 1).

Surgical bladder outlet obstruction was created in Groups II, III, V and VI according to Mattiasson and Uvelius (1982). After anesthesia with intraperitoneal sodium pentobarbital (50 mg/kg), the abdomen was opened through a midline suprapubic incision and the bladder and the proximal urethra were exposed without causing damage to the bladder. A constant degree of urethral obstruction was produced by tying a ligature of 2-0 silk around the urethra and a catheter with an outer diameter of 1.70 mm. The catheter was then removed. The presence of the catheter ensured that the ligature did not significantly compress the urethra. The bladder was returned to normal position within the abdomen and the skin incision was closed. Sham-operated animals (Groups I and IV) underwent a similar procedure with dissection around the urethra, but with no placement of the silk ligature.

At end of the experiment, micturition behavior in the metabolic cage was monitored. Three days after the measurement of micturi-

tion behavior, a cystometrogram under urethane anesthesia was performed. Immediately after the cystometrogram, all rats were sacrificed and their bladders were removed for weighing. After the weights were recorded, each bladder was used for histological examination.

Voiding behavior studies

The rats were placed in a metabolic cage containing a urine collection funnel and placed over an electronic balance (HF200, A.N.D., Tokyo, Japan) to measure micturition behavior. Plastic 250-mL beakers were placed on the balance pans. The balances were connected to a personal computer (Macintosh Powerbook G3, Apple Computer, Cupertino, CA) via a multiport controller (Maclab/400, AD Instruments, Castle Hill, Australia) to monitor the cumulative weight of the collected urine. All rats received water ad libitum when initially placed in the cages. Micturition frequency and volumes were sampled by the computer continually every 150 s for 24 h and the data was stored on harddisk. Each monitoring started at 18:00. The following parameters of the micturition reflex were obtained: urine volume per micturition, maximal micturition volume, micturition frequency and total urine output.

Cystometrogram

Cystometric studies were performed according to Watanabe and Miyagawa (1999). A cystometrogram was performed under urethane anesthesia (1.0 g/kg subcutaneously). The cystometry was done using a 24 G catheter inserted into the apex of the bladder dome for the purpose of recording pressure and filling the bladder with physiological saline. External bladder filling was carried out using an infusion pump (5200, TOP, Tokyo) at a constant rate of 0.4 mL/min until micturition was detected. A cystostomy catheter was connected to an external pressure transducer (P2310, Gould, Eastlake, OH) for the measurement of intravesical pressure. Intravesical pressure was recorded on the personal computer Macintosh Powerbook G3 via a bridge amplifier (ML112, AD Instruments) and the multiport controller Maclab/400. During voiding, filter paper was carefully placed at the meatus to absorb the voided urine without spillage and weighed to measure the voided volume.

To establish a reliable baseline, a period of at least 20 min was allowed to elapse after surgery before measurements were taken, and cystometrograms were repeated 3 times in each group. The following parameters were evaluated: bladder capacity, bladder compliance, maximum detrusor pressure, voided volume and residual urine volume. The maximum detrusor pressure was defined as instantaneous pressure minus post-contraction resting pressure.

Histological examination of the rat bladder

The bladders were cut free of surrounding tissue and weighed on a Mettler Basbal scale (Delta Range, Tokyo). After each bladder was transected at the level of the ureteric orifices, it was immediately fixed with 20% formalin. After fixation, the tissues were embedded in paraffin. Five micron-thick tissue sections were cut from these paraffin blocks. The tissue sections were deparaffinized with xylene and rehydrated with a graded series of ethanol. All of the bladder specimens were stained using the Elastica-van Gieson method according to Schlote and Gaus (1994). Smooth muscle and collagen are stained yellow and red, respectively. Each section was viewed under a light microscope at a magnification of 400.

Color-assisted quantitative image analysis

The stained tissue sections were viewed under a microscope (BX50, Olympus, Tokyo) using a digital camera (HC-3000, Fujix, Tokyo). The image of the histologic section which was displayed on a color monitor (PVM-20M4V, Sony, Tokyo) was printed using a digital color printer (CP700DSA, Mitsubishi, Tokyo). The image was digitized using a scanner (GT-8000, EPSON, Tokyo) operated by the Macintosh Powerbook G3. The automated analysis was performed using the National Institutes of

Table 2. Comparison of bladder weight among control, obstructed and L-arginine-treated obstructed rats

Group	Bladder weight (g)	
I	0.146 ± 0.006	**
II	0.184 ± 0.010	**
III	0.142 ± 0.003	
IV	0.131 ± 0.006	**
V	0.251 ± 0.025	**
VI	0.134 ± 0.005	

Shown are mean ± SEM.
** $P < 0.01$.

Health (NIH) Image 1.55 (Research Service Branch, NIH). The NIH Image 1.55 is an image analysis system which discriminates the color differences of stained tissue sections. The NIH Image 1.55 was utilized to discriminate the differentially stained smooth muscle and connective tissue elements. The area densities corresponding to the smooth muscle and connective tissue components were calculated per full screen. At least 10 different fields were examined from each tissue section. The ratio of percent area density of smooth muscle versus connective tissue was determined for each subject.

Statistical analysis

All results are expressed as the mean value ± SEM. Statistical comparison of differences between groups was performed using analysis of variance and Fisher's test for the multiple comparison. A probability of $P < 0.05$ was considered to be statistically significant.

Drugs and chemicals

L-arginine, pentobarbital and urethane were purchased from Sigma (St. Louis, MO). All other chemicals were of a commercial base.

Results

Bladder weight

The results of bladder weight are recorded in Table 2. After 1 and 6 weeks of bladder outlet obstruction, the bladder weight of obstructed rats was significantly increased compared to that of sham-operated rats. Six weeks of obstruction induced an increase in bladder mass of over twice that of the sham bladder weight. This increase was significantly prevented by treatment with L-arginine.

Voiding behavior studies

The results of the voiding behavior study are recorded in Table 3. The frequency of micturition was greater during the dark cycle than during the light cycle. After 1 and 6 weeks, bladder outlet obstruction induced an increase in the number of micturitions per day and a decrease in the average micturition volume. Also, there was a significant difference in the number of micturitions per day and the average micturition

Table 3. Comparison of micturition behavior among control, obstructed and L-arginine-treated obstructed rats

Group	Volume excreted per day (mL)	Number of micturitions per day		Average micturition volume (mL)		Maximal micturition volume (mL)	
I	20.4 ± 1.4	22.3 ± 1.3	**	0.91 ± 0.05	**	1.79 ± 0.06	*
II	20.9 ± 1.7	53.8 ± 8.5	**	0.42 ± 0.05	**	1.30 ± 0.14	
III	19.8 ± 1.0	27.5 ± 3.3		0.77 ± 0.08		1.61 ± 0.23	
IV	17.9 ± 0.6	16.7 ± 0.7	**	1.09 ± 0.06	**	2.12 ± 0.07	
V	20.6 ± 1.5	38.7 ± 3.6	**	0.56 ± 0.06	**	2.12 ± 0.18	
VI	18.0 ± 3.3	15.2 ± 2.4		1.20 ± 0.13		2.50 ± 0.23	

Shown are mean ± SEM.
*$P < 0.05$; **$P < 0.01$.

Table 4. Comparison of urodynamic parameters among control, obstructed and L-arginine-treated obstructed rats

Group	Bladder capacity (mL)	Maximum detrusor pressure (cm H_2O)	Voided volume (mL)	Residual urine volume (mL)	Bladder compliance (mL/cm H_2O)
I	0.28 ± 0.03	27.4 ± 1.3 **	0.27 ± 0.04	0.01 ± 0.01 **	0.06 ± 0.01
II	0.31 ± 0.06	41.3 ± 2.3 **	0.25 ± 0.05	0.07 ± 0.02 **	0.07 ± 0.02
III	0.25 ± 0.08	23.7 ± 1.7	0.23 ± 0.06	0.02 ± 0.01	0.08 ± 0.03
IV	0.30 ± 0.06 **	28.6 ± 0.7 **	0.28 ± 0.06	0.02 ± 0.01 **	0.08 ± 0.02
V	0.83 ± 0.17 *	58.3 ± 4.5 **	0.27 ± 0.06	0.56 ± 0.17 **	0.12 ± 0.02
VI	0.40 ± 0.08	33.8 ± 2.6	0.36 ± 0.07	0.04 ± 0.03	0.09 ± 0.01

Shown are mean ± SEM.
*P < 0.05; **P < 0.01.

volume between the obstructed rats and the obstructed rats treated with L-arginine, although no differences were seen in the volume excreted per day and the maximal micturition volume between them.

Cystometrogram

The results from cystometric experiments are recorded in Table 4. After 1 and 6 weeks of bladder outlet obstruction, the maximum detrusor pressure and the residual urine volume significantly increased in the obstructed rats compared to that of the sham-operated rats. The maximum detrusor pressure and the residual urine volume were significantly improved in the obstructed rats treated with L-arginine compared to the obstructed rats, although there were no differences in the voided volume and the bladder compliance among them. After 6 weeks of bladder outlet obstruction, the bladder capacity significantly increased in the obstucted rats compared to the sham-operated rats and this change was significantly prevented by treatment with L-arginine.

Table 5. Area densities of smooth muscle versus connective tissue

Group	Smooth muscle area density/ connective tissue area density
I	1.80 ± 0.06 **
II	9.71 ± 0.41 ** **
III	2.79 ± 0.06 **
IV	2.11 ± 0.08 **
V	11.09 ± 0.26 ** **
VI	3.46 ± 0.05 **

Shown are mean ± SEM.
**P < 0.01.

Histological examination of the rat bladder: the area densities of smooth muscle versus connective tissue

The results of histological examination are recorded in Table 5. The area densities of smooth muscle versus connective tissue were calculated from tissue sections by means of the Elastica-van Gieson method using color-assisted computer image analysis. Examples of bladder specimens are shown in Figs. 1 and 2. After 1 and 6 weeks of bladder outlet obstruction, the area densities of smooth muscle versus connective tissue significantly increased in the obstructed rats compared to the sham-operated rats and these changes were significantly prevented by treatment with L-arginine.

Discussion

In this study, Wistar rats whose urethral diameter was reduced by placing a suture around the urethra were used as a model for bladder outlet obstruction. In order to experimentally reproduce and study the change of bladder function in regard to the bladder outlet obstruction, ani-

mal models of bladder outlet obstruction have been developed in several species including the rabbit (Kato et al., 1988), guinea pig (Mostwin et al., 1991) and rat (Malmgren et al., 1987; Steers and De Groat, 1988). Obstruction has been variously created by surgical placement of a ligature (Malmgren et al., 1987; Steers and De Groat, 1988), cuff (Kato et al., 1988) or ring (Mostwin et al., 1991) around the urethra. In the rat, a variety of methods have been used to create a model of bladder outlet obstruction (Constantinou, 1996) but the most common methodology to create a model of bladder outlet obstruction involves reduction of the urethral diameter by placing a suture around the urethra. This model was developed by Malmgren et al. (1987), and they showed that strips dissected from most rat bladders 6 weeks after urethral obstruction demonstrated increased spontaneous activity. Further studies using this model demonstrated a significant increase in micturition frequency, micturition pressure and residual urine volume. Although differences between the rat model and humans concerning bladder outlet obstruction exist, similarities in their bladder dysfunction argue that this animal model remains valid for investigating the effects of obstruction (Steers, 1994).

The present study found that L-arginine prevents bladder dysfunction induced by bladder outlet obstruction. Evidence shows that an increase in the number of micturitions per day and a decrease in average micturition volume in bladder outlet-obstructed rats are prevented by treatment with L-arginine. Also, cystometric investigation indicates that bladder outlet obstruction causes increases in maximum detrusor pressure and residual urine volume, both of which are prevented by treatment with L-arginine. Although there was a significant difference in the average micturition volume between the sham-operated rats and the obstructed rats in the voiding behavior study, there were no differences in the voided volume between the sham-operated rats and the obstructed rats in the cystometric study. In the voiding behavior study, rats were placed individually in metabolic cages to measure urine output for 24 h. In contrast, the cystometric study was performed using a catheter inserted into the apex of the bladder dome under urethane anesthesia. In rats, anesthesia suppresses both spontaneous

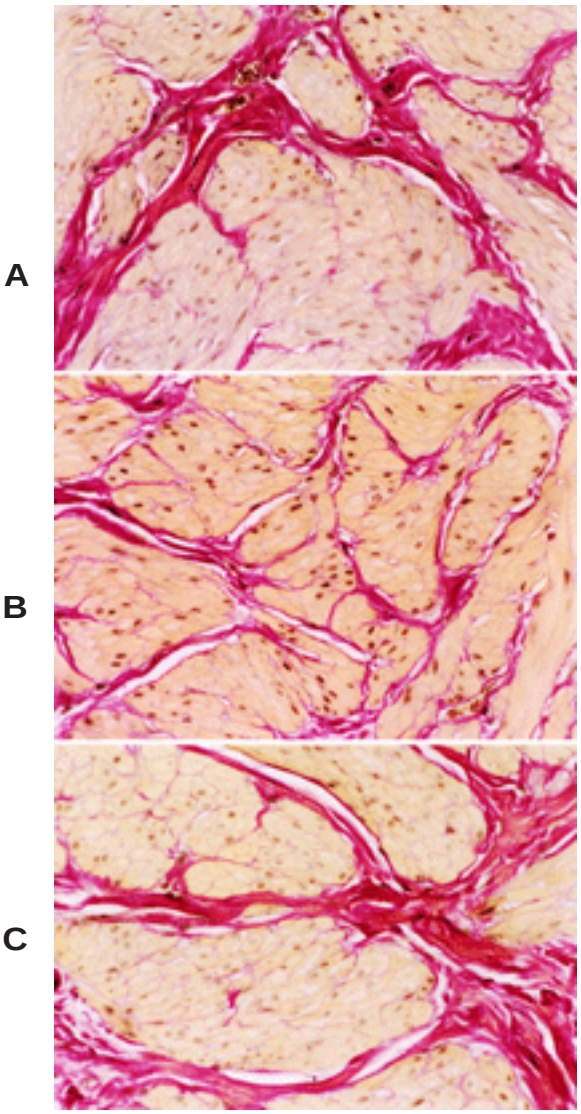

Fig. 1. Sections of rat bladder stained using the Elastica-van Gieson method (original magnification, × 400). **A:** Group I, 1 week after sham operation. **B:** Group II, 1 week after surgical induction of bladder outlet obstruction. **C:** Group III, L-arginine was injected intraperitoneally for 1 week after surgical induction of bladder outlet obstruction.

activity and the micturition reflex (Malmgren et al., 1987). Thus, the difference between the voided volume of the obstructed rats in the voiding behavior study and that of the obstructed rats in the cystometric study may be due to a difference in the response of the detrusor related to anesthesia and methodology.

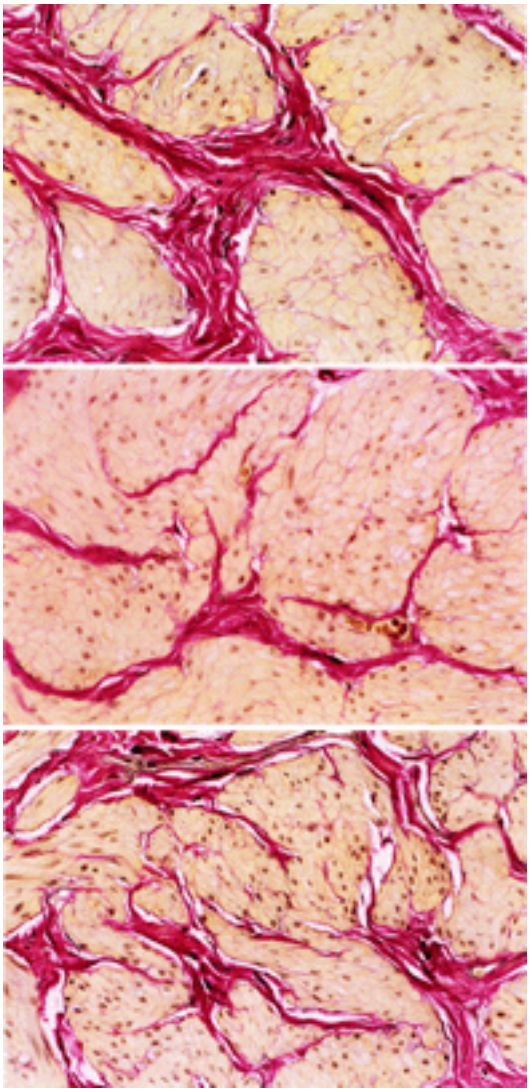

Fig. 2. Sections of rat bladder stained using the Elastica-van Gieson method (original magnification, × 400). **A:** Group IV, 6 weeks after sham operation. **B:** Group V, 6 weeks after surgical induction of bladder outlet obstruction. **C:** Group VI, L-arginine was injected intraperitoneally for 6 weeks after surgical induction of bladder outlet obstruction.

NO is synthesized from L-arginine in a reaction catalyzed by NOS (Burnett, 1995). There is no published evidence on whether or not treatment with L-arginine increases tissue levels of NO in the bladder. However, the chronic administration of oral L-arginine has been examined for use in modification of other NO-related processes, such as prevention of atheroma formation in rabbits and erectile response in rats (Cooke et al., 1992; Moody et al., 1997). Moody et al. (1997) showed that long-term oral L-arginine administration increased penile levels of L-arginine, and that L-arginine would up-regulate penile NOS activity. Also, Yang et al. (1998) reported that L-arginine supplementation increased urine NO production. Therefore, in the present study it is presumed that treatment with L-arginine may increase levels of NO in the obstructed bladder.

Consequent to the discovery by Furchgott and Zawadzki (1980) of an endothelial factor responsible for relaxation of vascular smooth muscle, Palmer et al. (1987) proposed that NO was the responsible agent, a hypothesis that has been confirmed by others (Malmgren et al., 1987). NO plays a major role in the regulation of blood flow within the bladder (Shabsigh et al., 2000). The magnitude of the decrease in bladder mucosal blood flow was seen after systemic NOS inhibitors in vivo (Kelm and Schrader, 1990). Functionally, NO mediates the relaxation of smooth muscle both directly and indirectly (Gaw and Bevan, 1993). These effects have been studied extensively in many organ systems, including the gastrointestinal system (Keef et al., 1994), the genitalia (Burnett et al., 1992) and both the upper (Bachmann and Mundel, 1994) and lower urinary tracts (Dokita et al., 1991; Persson et al., 1992). Additionally, NO has been found throughout the lower urinary tract, especially in the bladder neck and urethra (Sutherland et al., 1997). Thus, L-arginine's prevention of bladder dysfunction in bladder-obstructed rats could be

accounted for in 2 ways: i) L-arginine causes an increase of blood flow in the bladder via NO; ii) The resistance of the urethra in bladder outlet-obstructed rats may be decreased by L-arginine treatment and this reduction of urethral resistance may cause a decrease in intravesical pressure. Moreover, the decrease of intravesical pressure may decrease the degree of detrusor ischemia in bladder outlet obstruction. Therefore, the L-arginine/NO pathway may increase the blood flow of the obstructed bladder both directly and indirectly. On the other hand, Saito et al. (1998) reported that NO works as a cell/tissue damaging agent in ischemia-reperfusion injury in the rat bladder. Thus, NO works as both a cell/tissue damaging agent and a cell/tissue protecting agent in the rat bladder. The present study suggests that NO works as a cell/tissue protecting agent in bladder dysfunction in bladder outlet-obstructed rats.

The present study utilized color assisted computer image analysis to determine the effect of L-arginine on bladder morphometry in bladder outlet-obstructed rats. The bladder is comprised primarily of smooth muscle and connective tissue elements. These bladder components were stained different colors using the Elastica-van Gieson method. The bladder smooth muscle and connective tissue were stained yellow and red, respectively, using the Elastica-van Gieson method. Computer-assisted color image analysis discriminated the staining properties of smooth muscle and connective tissue. In the present study, bladder outlet obstruction caused a significant increase in bladder weight, and this result is the same as that reported by several authors (Uvelius and Mattiasson, 1984; Malkowicz et al., 1986; Kato et al., 1988; Steers and De Groat, 1988; Mostwin et al., 1991). The histologic basis of this increased mass varys depending on the model, and can include smooth muscle hypertrophy, smooth muscle hyperplasia, fibroblast hyperplasia, and increases in intrinsic and extrinsic connective tissue (Uvelius and Mattiasson, 1984). Lemack et al. (1999) used Masson-trichrome and computer assisted color image analysis to quantify the ratio of smooth muscle to the extracellular matrix of obstructed mouse bladders. They did not determine the changes in collagen and smooth muscle after the bladder was obstructed. However, in the present study the area densities of smooth muscle versus connective tissue increased after 1 and 6 weeks of bladder outlet obstruction. These data and histological findings may suggest that the increase of bladder weight in bladder outlet-obstructed rats is related to smooth muscle hypertrophy. Moreover, increases in the area densities of smooth muscle versus connective tissue in bladder outlet-obstructed rats is prevented by treatment with L-arginine. These data suggest that L-arginine may protect morphological changes induced by bladder outlet obstruction. This is probably because L-arginine functionally causes a decrease in detrusor pressure on bladder outlet obstruction. However, further studies are needed to clarify the effects of L-arginine on morphological changes induced by bladder outlet obstruction.

In conclusion, L-arginine improved bladder dysfunction and the morphological changes of the bladder induced by bladder outlet obstruction. This may be due to the effect of L-arginine to increase levels of NO and the rate of blood flow in the obstructed bladder. In addition, NO may play an important role in the damage induced by bladder outlet obstruction.

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