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Age-associated bladder and urethral coordination impairment and changes in urethral oxidative stress in rats

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ABSTRACT

Aims: We examined age-associated changes in bladder and urethral coordination involving the nitric oxide (NO)/soluble guanylyl cyclase (sGC) system, which induces urethral smooth muscle relaxation, and urethral ischemic/oxidative stress changes in rats.

Main methods: Sixteen female Sprague-Dawley rats were divided into young (3 months old) and middle-aged (12–15 months old) groups. Urethral activity was evaluated by simultaneously recording intravesical pressure under isovolumetric conditions and urethral perfusion pressure (UPP) under urethane anesthesia. Sodium nitroprusside (SNP, 0.1 mg/kg), an NO donor, and BAY 41-2272, a novel NO-independent stimulator of sGC (0.1 mg/kg), were administered intravenously to both groups. N-nitro-L-arginine methyl ester hydrochloride (L-NAME, 100 mg/kg) was also injected intravenously, to inhibit NO synthase activity in both groups. Staining for the ischemic marker, hypoxia-inducible factor-1α (HIF-1α), and the oxidative stress markers, 8-hydroxy-2′-deoxyguanosine (8-OHdG) and malondialdehyde (MDA), was performed on tissue sections of the urethra, in both groups.

Key findings: Baseline UPP and UPP changes were significantly lower in middle-aged rats than in young rats. After administration of SNP and BAY 41-2272, baseline UPP and UPP nadir were significantly decreased in both groups. After administration of L-NAME, UPP change/bladder contraction amplitude in young rats was still lower than at baseline but was completely restored to control levels in middle-aged rats. Immunoreactivity of HIF-1α, 8-OHdG, and MDA was higher in middle-aged rats than in young rats.

Significance: Age-associated ischemic and oxidative stress in the urethra might be correlated with impairment of the NO/sGC system and with coordination of the bladder and urethra.

1. Introduction

Voiding is performed by coordinated action between the bladder and urethra, which is voluntarily or involuntarily controlled by the spino-bulbospinal micturition reflex pathway via the pontine micturition center [1,2]. Due to the intricacy of these systems, coordination of the bladder and urethra can be damaged by numerous pathological conditions, such as spinal cord injury [3] or diabetes [4]. Previous reports [5–7] have suggested that aging also impairs coordination, which can cause an underactive bladder with residual urine, ultimately reducing the quality of life [8]. Moreover, incontinence is more prevalent in women than men, especially after menopause. In Japan, it has been reported that the prevalence rate of urge incontinence and stress incontinence for 1 or more times per week was 15.8% and 19.4%, respectively, among women ages 40 years or older [9]. In our recent studies [6,7], urethral smooth muscle-mediated relaxation during bladder contractions was diminished in 12–24-month-old rats, as shown by simultaneously recording intravesical pressure and urethral perfusion pressure (UPP). However, the precise mechanisms underlying the coordinated action between the bladder and urethra, as a function of age, are not well understood.

Acetylcholine from the parasympathetic nerves contracts the

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bladder, simultaneously, the release of nitric oxide (NO) relaxes the urethral smooth muscle [9]. The external urethral sphincter (EUS) also relaxes during voiding, and rhythmic contractions and relaxation of the EUS during voiding occur to expel urine, which is speculated to generate high-frequency oscillations (HFOs) in UPP, which is only identified in rats and dogs [10,11]. In both animal and human experiments, NO synthesis in the bladder, urethra, and prostate and acetylcholine release from pelvic nerve have been reported to decrease with age, whereas non-neuronal acetylcholine release from the urothelium increased with age, suggesting reduced bladder contractility and increased urinary frequency [12,13]. In our recent study, aging was also assumed to induce dysfunction in NO-mediated urethral smooth muscle relaxation during voiding; however, L-arginine, a NO substrate, could not restore impaired urethral smooth muscle relaxation except urethral contraction during UPP change [6]. NO activates soluble guanylyl cyclase (sGC), resulting in an increase in intracellular cyclic guanosine monophosphate (cGMP), which relaxes urethral smooth muscle [13]. Thus, we hypothesized that the NO/sGC system, which induces urethral smooth muscle relaxation, is impaired by aging.

Therefore, in this study, we used young and middle-aged rats to investigate urethral function by simultaneously recording intravesical pressure and UPP, before and after intravenous injections of sodium nitroprusside (*SNP*), a NO donor, BAY 41-2272 [14], a novel NO-independent stimulator of sGC, and N-nitro-L-arginine methyl ester hydrochloride (L-NAME), a NO inhibitor. To explore the impairment in the NO/sGC mechanism, we also examined the ischemic and oxidative status of the urethra by immunostaining for the oxidative stress markers, hypoxia-inducible factor-1α (HIF-1α), 8-hydroxy-2-deoxyguanosine (8-OHdG), and malondialdehyde (MDA).

2. Material and methods

2.1. Animal model

A total of 16 female Sprague–Dawley rats (young: 3 months old, n = 8; middle-aged: 12–15 months, n = 8) were used in this study. We used rats aged 12–15 months because they are equivalent to middle-aged humans who are mostly affected by lower urinary tract dysfunction. Rats were housed at a constant room temperature and maintained on a standard diet (Nihonkurea, Tokyo, Japan) with free access to water. The Institutional Animal Care and Use Committee of the University of Ryukyus approved this study protocol.

2.2. Surgical procedure

Under isoflurane anesthesia, a polyethylene catheter (PE-10; Clay Adams, Parsippany, NJ, USA) was inserted into the jugular vein for drug injection. The bladder and ureter were exposed using a lower midline abdominal incision. The ureter was cut from both sides and the distal end was tied. The bladder neck was tied just below the ureterovesical junction to separate the functions of the bladder and urethra. A PE-90 catheter (Clay Adams) was inserted into the bladder from above the bladder wall to record intravesical pressure, and a PE-50 catheter (Clay Adams) was inserted from the urethra to record the UPP. These catheters were connected to a pressure transducer (Transbridge 4 M; World Precision Instruments, Sarasota, FL, USA) and an infusion pump via a three-way stopcock. Following surgery, isoflurane anesthesia was terminated and replaced with urethane anesthesia (1.2 g/kg, intraperitoneal and subcutaneous; Sigma Chemical Company, St. Louis, MO). The abdomen was then closed using sutures.

2.3. Simultaneous recordings of intravesical pressure under isovolumetric conditions and UPP in young and middle-aged rats

Saline was infused at a rate of 0.05 ml/min into the bladder to exceed the threshold volume (0.4–1.2 ml) and induce rhythmic contractions of equal volume. Saline was continuously infused into the urethral catheter at a rate of 0.05 ml/min. PowerLab (AD Instruments Pty, Ltd., Castle Hill, New South Wales, Australia) was used for data acquisition and manipulation. The maximum amplitude of isovolumic contractions and the intravesical pressure threshold to induce urethral relaxation were measured when rhythmic bladder contractions were stable for at least 30 min. Urethral pressure was changed by contraction of the bladder. Therefore, the following specific parameters of UPP were measured during bladder contractions: UPP nadir during reflex urethral relaxation and the baseline UPP between reflex bladder contractions. The change in UPP was calculated as the value just below the UPP nadir, minus the baseline UPP. Negative changes in UPP indicate a bladder-urethral synergistic pattern. Because urethral activity varies depending on bladder activity, UPP changes and bladder contraction amplitude were also evaluated. The mean velocity (Hz) and the amplitude of the urethral striated muscle HFOs during reflex bladder contractions, were also measured (Fig. 1). These parameters were averaged over 30 min and used as control (baseline) levels.

After a one-hour control period, SNP (Sigma-Aldrich Co., Tokyo, Japan) was injected intravenously (0.1 mg/kg) to increase the level of NO. After bladder contraction stabilized, SNP (0.1 mg/kg) and BAY41-2272 (0.1 mg/kg) (NAMIKI SHOJI Co., Tokyo, Japan) were simultaneously injected intravenously.

After SNP and BAY41-2272 treatment, rats were injected intravenously with L-NAME (100 mg/kg; Sigma-Aldrich Co.) to inhibit NO synthase activity. The amount of dosage was decided according to previous reports [14] and our preliminary experiments. Bladder and urethral pressure parameters were measured after each intravenous drug injection, were averaged, and then compared to the control values.

2.4. Immunohistochemistry

The bladder and urethra were removed from euthanized rats, fixed in phosphate-buffered formalin (10%), embedded in paraffin, and cut into 4 μm-thick sections.

Immunohistochemical analysis was performed using formalin-fixed, paraffin-embedded sections. Primary antibodies for 8-OHdG (Santa Cruz Biotechnology, TX, USA), MDA (Abcam, UK), and HIF-1α (Novus Biologicals, USA) were applied to the samples. All the sections were deparaffinized in xylene and rehydrated using graded ethanol solutions. Antigen retrieval was performed using 0.01 M sodium citrate buffer (pH 6.0) at 95 °C for 40 min. All sections were then immersed in 3% hydrogen peroxide for 15 min to block endogenous peroxidase activity. The sections were incubated overnight with the primary antibody (8-OHdG 1:500; MDA 1:100; HIF-1α 1:40) at 4 °C, and then washed with 0.05% Tween-20 in phosphate-buffered saline (PBS). The sections were incubated for 60 min in peroxidase using the labeled polymer method, with Dako EnVision+™ Peroxidase (Dako, Carpinteria, CA). The peroxidase reaction was visualized using a liquid 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate kit (Muto Pure Chemicals Co., Tokyo, Japan). Sections were counterstained using hematoxylin, dehydrated through graded alcohol solutions, cleared using xylene, and mounted with Poly-Mount mounting medium (Polysciences, Inc., Warrington, PA, USA). Masson's trichrome staining was also performed to compare the morphological differences in the bladder between young and middle-aged rats.

2.5. Statistical analysis

Data are presented as the mean ± standard error of the mean, except for in the evaluation of HIF-1α. Statistical comparisons were performed using paired or unpaired *t*-tests, where appropriate. The Mann-Whitney *U* test was used to evaluate the immunohistochemically stained cells. Statistical significance was set at $p < 0.05$. All statistical analyses were two-sided and performed using StatView for Windows (version 5.0; Abacus Concepts, Inc., Berkeley, CA, USA).

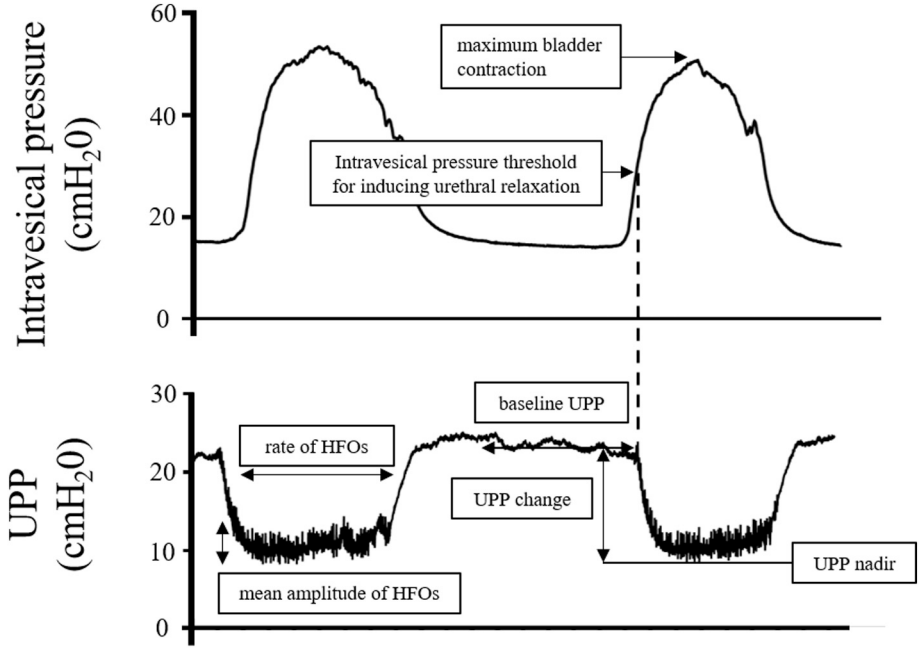

Fig. 1. Simultaneous recording of intravesical pressure and UPP under isovolumetric conditions. UPP, urethral perfusion pressure; HFOs, high-frequency oscillations.

3. Results

3.1. Body weight differences between young and middle-aged rats

The body weight of middle-aged rats was significantly increased (553.5 ± 18.2 g, $p < 0.01$) compared to young rats (265.6 ± 14.4 g).

3.2. Differences between the control young and middle-aged rats

Under isovolumetric conditions, UPP measurements reflected urethral relaxation accompanied by HFOs when bladder contractions occurred in young and middle-aged rats. Baseline UPP was significantly lower in the middle-aged rats, compared to young rats ($p = 0.022$; Table 1, Fig. 2). The mean UPP nadir during amplitude bladder contractions was not significantly different between young and middle-aged rats (9.7 ± 0.7 and 9.1 ± 0.9 cmH_2O, respectively; $p > 0.05$). However, UPP changes during urethral relaxation were significantly smaller in middle-aged rats compared with young rats (−9.5 ± 0.9 vs −14.8 ± 1.8 cmH_2O, respectively; $p = 0.021$). The maximum amplitude of isovolumetric contractions and the intravesical pressure threshold for inducing urethral relaxation, and UPP change/bladder contraction amplitudes were not significantly different between the two groups ($p > 0.05$). Additionally, the mean amplitude of HFOs was not significantly different between young and middle-aged rats (4.3 ± 0.8 vs 4.5 ± 0.2 cmH_2O, respectively; $p > 0.05$); however, the mean rate of HFOs was smaller in middle-aged rats compared with young rats (4.4 ± 0.2 and 5.3 ± 0.2, respectively; $p = 0.003$).

3.3. SNP treatment-induced changes in young and middle-aged rats

After SNP (0.1 mg/kg) administration, baseline UPP was significantly decreased in both young and middle-aged rats ($p = 0.029$ and $p = 0.016$, respectively; Figs. 3 and 4) compared with the UPP measured before the SNP injection (control). The UPP nadir did not change in either young or middle-aged rats. However, UPP changes were significantly decreased in both young and middle-aged rats ($p = 0.035$ and $p = 0.013$, respectively). The maximum bladder contraction amplitude was significantly decreased in both young and middle-aged rats ($p = 0.017$ and $p = 0.011$, respectively). The intravesical pressure threshold for inducing urethral relaxation was not significantly changed in young rats, however, was significantly decreased in middle-aged rats ($p = 0.008$, from 20.4 ± 1.8 cmH_2O to 16.8 ± 2.4 cmH_2O). UPP change/bladder contraction amplitude was significantly decreased in both young and middle-aged rats ($p = 0.035$ and $p = 0.048$, respectively).

In middle-aged rats, HFO amplitude was significantly decreased ($p = 0.008$), but the mean rate of HFOs did not change in either young or middle-aged rats.

3.4. SNP and BAY 41-2272 treatment-induced changes in young and middle-aged rats

After simultaneous intravenous injection of SNP (0.1 mg/kg) and BAY41-2272 (0.1 mg/kg), baseline UPP was significantly decreased in both young and middle-aged rats ($p = 0.003$ and $p < 0.001$, respectively; Figs. 3 and 4) compared with the UPP levels before injection (control). UPP nadirs were significantly decreased in both young and middle-aged rats ($p = 0.002$ and $p < 0.001$, respectively). In young rats, UPP nadir after administration of SNP and BAY41-2272 was further decreased compared to the UPP nadir after administration of SNP alone ($p = 0.044$). UPP changes were significantly decreased in both young and middle-aged rats ($p = 0.0.35$, $p = 0.013$, respectively) compared to control levels. The maximum amplitude of isovolumetric contractions and the intravesical pressure threshold for inducing urethral relaxation did not change in either young or middle-aged rats. After administration of combined SNP and BAY41-2272, UPP change/bladder contraction amplitude was significantly decreased in both young and middle-aged rats ($p = 0.009$, $p = 0.004$) compared to control levels; however, these changes were not different from those induced by administration of SNP alone, in both young and middle-aged rats.

The mean amplitude of HFOs was not significantly different before and after the administration of combined SNP and BAY41-2272, in both young and middle-aged rats. However, the mean rate of HFOs was decreased in both groups ($p = 0.029$ and $p = 0.014$, respectively), compared to the control measurement.

3.5. L-NAME treatment-induced in young and middle-aged rats

When L-NAME (100 mg/kg) was intravenously administered after

Table 1
UPP and intravesical parameters before (control) or after SNP, SNP plus BAY 41-2272, and L-NAME.

	Control (mean ± SE)	SNP (mean ± SE)	SNP + BAY41-2272 (mean ± SE)	L-NAME (mean ± SE)
Young rats (n = 8)				
Max reflex bladder contraction amplitude (cmH_2O)	53.8 ± 3.0	48.3 ± 1.9*	49.4 ± 2.7	48.9 ± 3.8
Urethral relaxation intravesical pressure threshhold (cmH_2O)	27.9 ± 4.1	25.5 ± 3.6	26.8 ± 3.2	26.4 ± 3.8
Baseline UPP (cmH_2O)	24.4 ± 1.8	18.5 ± 1.8*	16.6 ± 1.5*	18.5 ± 2.2*
UPP nadir (cmH_2O)	9.7 ± 0.7	8.5 ± 0.5	7.2 ± 0.4*, ‡	11.5 ± 0.9*, §
UPP change (cmH_2O)	−14.8 ± 1.8	−10.1 ± 1.8*	−9.4 ± 1.7*	−7.0 ± 1.9*
UPP change/bladder contraction amplitude	0.29 ± 0.04	0.21 ± 0.04	0.19 ± 0.04*	0.14 ± 0.03*
HFO amplitude (cmH_2O)	4.3 ± 0.8	3.2 ± 0.4	3.8 ± 1.0	3.1 ± 0.4
HFO rate (Hz)	5.3 ± 0.2	5.2 ± 0.1	5.0 ± 0.1*	5.2 ± 0.1
Middle-aged rats (n = 8)				
Max reflex bladder contraction amplitude (cmH_2O)	50.4 ± 4.6	44.6 ± 4.7*	45.0 ± 3.7	45.3 ± 3.4
Urethral relaxation intravesical pressure threshhold (cmH_2O)	20.4 ± 1.8	16.8 ± 2.4*	19.9 ± 2.3	20.9 ± 1.9
Baseline UPP (cmH_2O)	18.6 ± 1.4¶	13.2 ± 1.7*	10.6 ± 0.7*	15.8 ± 1.3§
UPP nadir (cmH_2O)	9.1 ± 0.9	7.4 ± 0.9	6.2 ± 0.5*	9.8 ± 0.9§
UPP change (cmH_2O)	−9.5 ± 0.9¶	−5.8 ± 1.2*	−4.4 ± 0.6*	−6.0 ± 0.8*
UPP change/bladder contraction amplitude	0.20 ± 0.03	0.14 ± 0.03*	0.10 ± 0.01*,¶	0.13 ± 0.02
HFO amplitude (cmH_2O)	2.5 ± 0.2	1.7 ± 0.3*	1.9 ± 0.4	2.4 ± 0.4
HFO rate (Hz)	4.4 ± 0.2¶	4.2 ± 0.2	3.9 ± 0.2*	4.4 ± 0.1§

SNP: Sodium nitroprusside, L-NAME: N-nitro-L-arginine methyl ester hydrochloride, UPP: urethral perfusion pressure, HFO: high frequency oscillations, SE: standard error.
SNP: 0.1 mg/kg, BAY41-2272: 0.1 mg/kg, L-NAME: 100 mg/kg.
* $P < 0.05$ vs Control.
‡ $P < 0.05$ vs SNP.
§ $P < 0.05$ vs SNP + BAY41-2272.
¶ $P < 0.05$ vs young.

combined SNP and BAY41-2272 treatment, baseline UPP was not altered in young rats and was still lower (18.5 ± 2.2 cmH_2O, $p = 0.252$; (Figs. 3 and 4)) compared to the levels before injection (control). However, in middle-aged rats, baseline UPP was significantly increased after L-NAME (15.8 ± 1.3 cmH_2O, $p = 0.004$) administration, and recovered to control levels. The UPP nadirs during reflex bladder contractions were significantly increased in both groups (young:11.5 ± 0.9, middle-aged:9.8 ± 0.9 cmH_2O). Changes in UPP were not significantly altered by L-NAME in young and middle-aged rats (−7.0 ± 1.9 cmH_2O and −6.0 ± 0.8 cmH_2O, respectively). The maximum amplitude of isovolumetric contractions was not changed in young and middle-aged rats (48.9 ± 3.8 and 45.3 ± 3.4 cmH_2O, respectively). The UPP change/bladder contraction amplitude was still smaller after L-NAME (0.14 ± 0.03, $p < 0.001$) compared with the control levels in young rats and recovered to the control level in middle-aged rats (0.13 ± 0.02).

L-NAME treatment did not affect the mean amplitude of HFOs in either group. However, the mean rate of HFOs was increased in middle-aged rats (4.4 ± 0.1 Hz, $p = 0.019$) compared with SNP and BAY41-2272 treatment.

3.6. Immunohistochemistry

As shown in Fig. 5A–D, immunoreactivity levels of 8-OHdG and MDA in the urethra epithelium of middle-aged rats were significantly higher than those of young rats ($p < 0.001$). Supplementary figure also showed higher immunoreactivity levels of 8-OHdG and MDA in the bladder epithelium of middle-aged rats than those of young rats. On the other hand, the ratio of HIF-1α-staining in urothelial cells (5.66 ± 0.17%, median = 5.65, interquartile range = 5.28–6.08) was significantly higher ($p < 0.001$) compared to that of young rats (2.25 ± 0.20%, median = 2.4, interquartile range = 2.00–2.6; Fig. 5E and F). In Masson's trichrome staining, atrophy of the smooth muscles and fibrosis between and around smooth muscles in middle-aged rats were stronger compared to those in young rats (Fig. 5G and H).

4. Discussion

The coordination between the bladder and the urethra maintains storage and facilitates the elimination of urine [1]. The results of this study indicate that: (1) middle-aged rats have impaired urethral relaxation during voiding, as evidenced by reduced UPP changes during urethral relaxation (similar to our previously published results) [6,7]; (2) middle-aged rats show a decrease in the efficacy of their NO/sGC system, as evidenced by a decrease in baseline UPP induced by combined SNP and BAY41-2272, and blockade of these reactions by administration of L-NAME; (3) there are age-associated changes in oxidative stress and ischemia in the urethra which may be involved in the impairment of coordination between the bladder and urethra, as evidenced by immunostaining of oxidative stress markers (8-OHdG, MDA) and ischemic markers (HIF-1α) in the urethra.

Urethral relaxation is important for efficient voiding. There are fewer reports exploring the effects of aging on urethral function compared to those on bladder function. Lluel et al. reported that anesthetized rats with increased residual urine volume showed a decrease in resting urethral pressure at the micturition threshold and a significant delay in urethral relaxation during micturition itself [5]. The present study confirmed our previous work showing that UPP change was significantly lower in middle-aged rats than in young rats [6,7]. Similarly, other work done in rabbits has provided evidence for an age-related decrease in NO-mediated relaxation and nitrinergic innervation of the prostate [15]. Additionally, NO release in human prostate tissue has also been shown to decrease with age [16]. These results suggest that age-associated changes affect NO synthesis in the lower urinary tract, resulting in dysfunctional urethral smooth muscle relaxation. In our recent study, decreased UPP change during reflex bladder contractions was not restored in middle-aged rats after treatment with L-arginine, a NO substrate, except for in alterations of urethral contractions during UPP change [6]. Studies using streptozotocin-induced diabetic rats have reported that impaired urethral relaxation was partially recovered by L-arginine treatment [4]. However, in the present study, both baseline UPP and nadir were significantly decreased by SNP alone or in combination with BAY41-2272, and these changes were completely restored by L-NAME, in aged rats. These results suggest that the NO/sGC system, which induces smooth muscle relaxation in the urethra, might be impaired by age, and that SNP and BAY41-2272 could restore reduced NO/sGC system more effectively than L-arginine. Because baseline UPP reacted more than UPP nadir by SNP or combined SNP and BAY41-2272 in middle-aged rats, UPP change (as defined by baseline UPP minus UPP nadir) might appear to be decreased. However, baseline UPP and UPP nadir were also decreased in young rats, suggesting that additional NO activation (induced by combined SNP and BAY41-2272) results in increased intracellular cGMP, thus relaxing the urethral smooth muscle in young rats. The change in UPP seems more prominent than bladder activity with age because the amplitude of bladder contractions did not

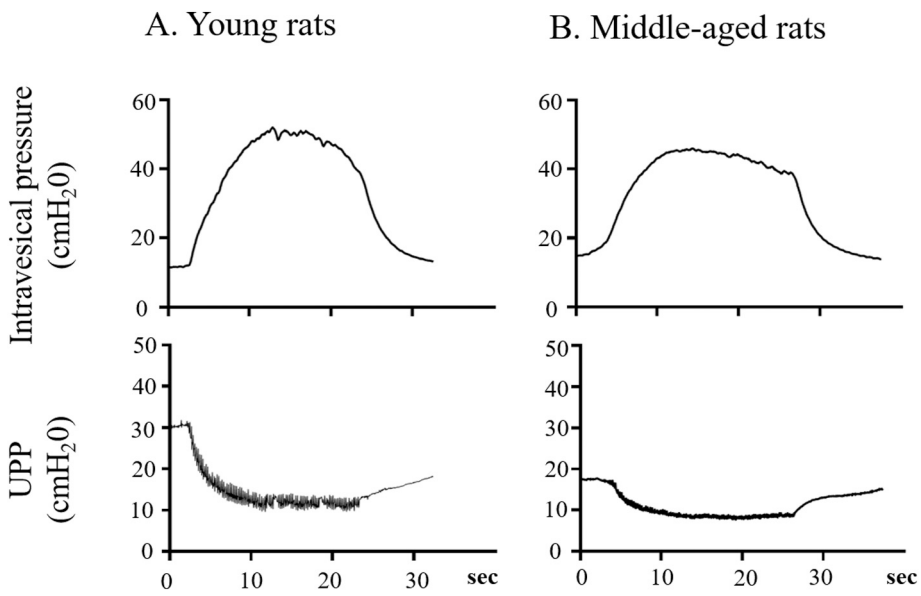

Fig. 2. Representative traces of simultaneous recordings of intravesical pressure under isovolumetric conditions and in UPP of (A) young and (B) middle-aged rats. In middle-aged rats, the UPP change was smaller compared with young rats. However, maximum bladder contraction amplitude did not differ between both groups. UPP, urethral perfusion pressure.

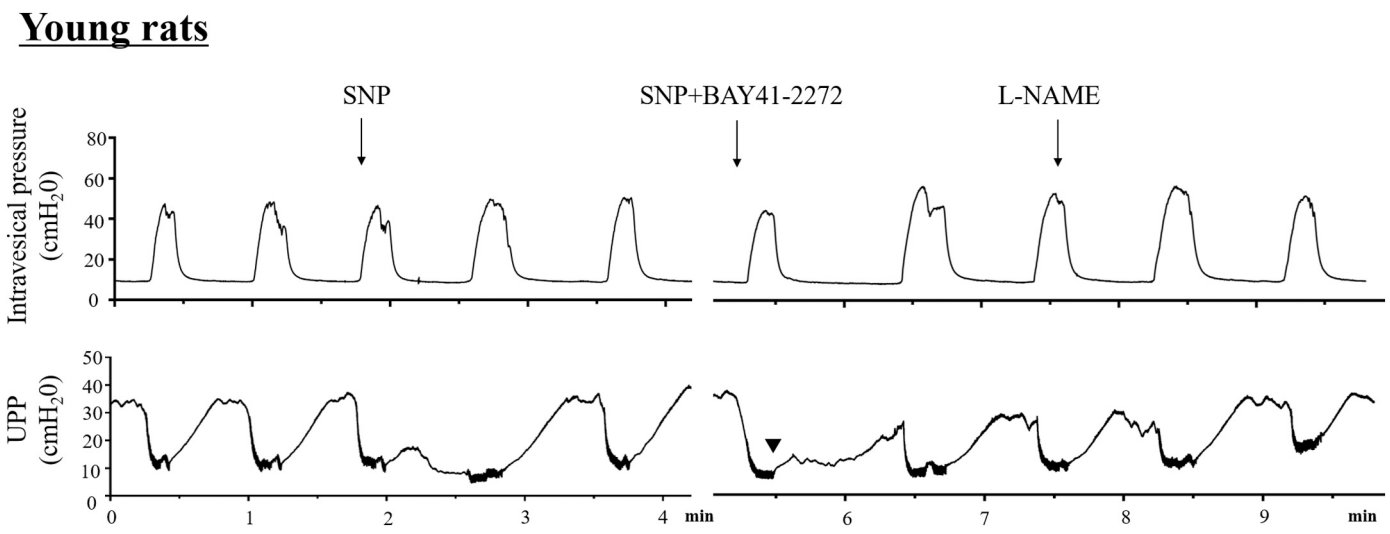

Fig. 3. Representative traces of simultaneous recordings of intravesical pressure under isovolumetric conditions and UPP, in young rats. During control periods, large negative UPP changes during bladder contractions were observed. After SNP treatment, baseline UPP and UPP negative change was decreased and were recovered after about 1 min. After SNP plus BAY 41-2272 treatment, UPP nadir was further decreased (▼). After L-NAME, baseline UPP and nadir were increased, and UPP change was partially recovered to the control level. UPP: urethral perfusion pressure; SNP: Sodium nitroprusside; L-NAME: N-nitro-L-arginine methyl ester hydrochloride.

differ between young and middle-aged rats in the present study, as well as in our recent studies [6,7]. Overall, the decreased activity of the NO/sGC system might be a contributor to impairment of bladder and urethral smooth muscle coordination in aged rats (prominent in urethral relaxation impairment).

Aging is associated with impaired vascular function which is primarily characterized by endothelial dysfunction [17]. The mechanisms for age-dependent endothelial dysfunction include a reduction in NO bioavailability due to decreased NO synthesis (NOS) or increased NO scavenging and oxidative stress, leading to peroxynitrite formation [18,19]. In the present study, levels of HIF-1α (ischemic marker), 8-OHdG, and MDA (lipid oxidative stress marker) in the urethral epithelium, which could affect NO synthesis, were also higher in middle-aged rats than in young rats. In a rat model of chronic bladder ischemia, impaired vascular occlusion has been reported to cause fibrosis and reduce bladder contractility [20]. Furthermore, Gotoh et al. [21] reported that within the urothelial layer of the bladder in diabetic rats, expression of HIF-1α and 8-OHdG was stronger than in non-diabetic rats. These results suggest that local ischemia and accumulation of oxidative stress in the urethra may impair urethral and bladder function. In the urethra of sheep, neural NOS immunoreactivity was present in the intramural nerve and in the sarcolemma of some striated muscle fibers and was denser at the neuromuscular junction [22]. NOS is expressed in endothelium lining the cavernous spaces and urethral epithelium of rats, and NOS protein is also decreased in diabetic rats [23]. The other possibility is that low cyclical estrogen levels might have affected urethral ischemia, especially in middle-aged female rats. Levin and co-workers reported that ovariectomy resulted in lower bladder contractility and

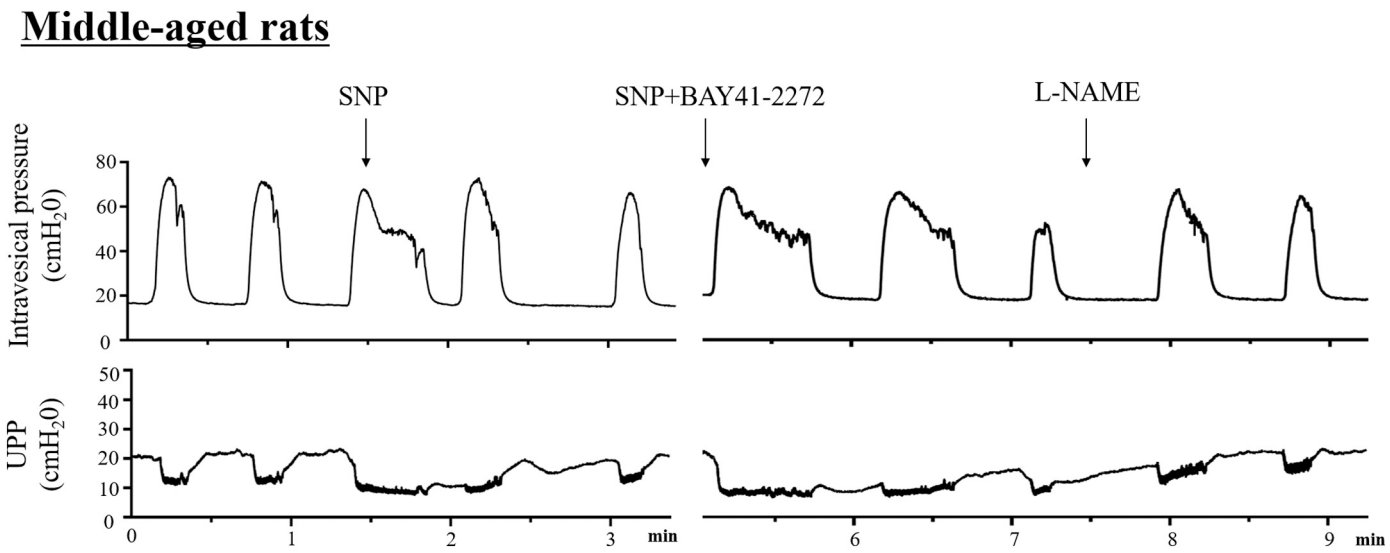

Fig. 4. Representative traces of simultaneous recordings of intravesical pressure under isovolumetric conditions and UPP in middle-aged rats. During the control periods, a low UPP negative change during bladder contractions was observed. After SNP injection, the baseline UPP and UPP negative changes decreased, and they recovered after about 2 min. After administration of combined SNP and BAY 41-2272, baseline UPP and UPP negative changes were decreased, similar to SNP alone. After administration of L-NAME, baseline UPP and nadir were increased, and the UPP change completely recovered to control levels. UPP, urethral perfusion pressure; SNP, sodium nitroprusside; L-NAME, N-nitro-L-arginine methyl ester hydrochloride.

higher lipid peroxidation in vitro ischemia/perfusion experiments, whereas estrogen supplementation protected the bladder against oxidative stress [24,25]. Chronic stress related to urethral ischemia may also impair urothelial mitochondria structure and function [26]. In the present study, atrophy of the smooth muscles and fibrosis in middle-aged rats were significantly stronger compared to those in young rats. Age-related ischemia and accumulation of oxidative stress in the bladder might have caused atrophy of the smooth muscles and fibrosis. Based on previous studies [20,21,23–26] and our present results, the specific cellular mechanisms involved with the aging process in the female bladder/urethra could be hypothesized as follows: (i) blood flow to the bladder and urethral muscle and mucosa, which are under estrogenic control, decreased as circulating estrogen decreased or when there is atherosclerosis of the pelvic arteries; (ii) decreased blood flow to the bladder and urethral smooth muscles and mucosa resulted in localized tissue hypoxia and reactive oxygen species (ROS) and reactive nitrogen species (RNS) free radical generation (i.e., accumulation of oxidative stress); and (iii) tissue hypoxia and oxidative stress resulted in neuronal and mitochondrial damage, impairment of NO/sGC system, as well as increased connective tissue formation, smooth muscle atrophy, and bladder/urethra dysfunction. Our future study will aim to clarify detailed mechanisms of chronic urethral ischemic influence on the NO/sGC system. This study is the first to report the accumulation of ischemic and oxidative stress in the urethra of aged rats fed a standard diet.

HFOs are the pumping mechanism of the EUS, which increases the efficiency of voiding. In our previous work [6,7], the amplitude of HFOs was lower in middle-aged rats than in young rats. In the present study, the amplitude of HFOs in middle-aged rats tended to be lower than that in young rats ($p = 0.06$). Additionally, using EUS-electromyography, we have previously shown EUS bursting activity during voiding, accompanied by clear active and silent phases in young rats but unclear active and silent phases in aged rats (i.e., detrusor-sphincter dyssynergia pattern) [7]. In the present study, the amplitude of HFOs was decreased after SNP administration, in accordance with the reaction to the urethral smooth muscles in middle-aged rats, and was restored after L-NAME administration. These results indicate that the urethral smooth muscle and the EUS work together, and that aging induces both types of urethral dysfunctions, which may lead to a persistent cycle of discoordination between the bladder and urethra.

In the chronic phase of spinal cord injury, bladder-sphincter coordination is impaired, leading to detrusor-sphincter dyssynergia [3]. Torimoto et al. [4] also reported that streptozotocin-induced diabetic rats showed partial detrusor-sphincter dyssynergia changes. These lower urinary tract dysfunctions then cause various problems, such as urinary incontinence, recurrent urinary tract infection, and vesicoureteral reflux, with or without upper urinary tract deterioration. The present study also demonstrated bladder and urethral discoordination as a function of age. Thus, the urethra might be a promising target to improve lower urinary tract symptoms in the aging bladder.

This study has several limitations. First, we used 12–15-month old rats as middle-aged rats, which might not be the best representative age range for bladder dysfunction. Second, we only used female rats because sex hormones influence nitrergic- or adrenergic-dependent urethral function [27]; in addition, middle-aged females are mostly affected by lower urinary tract dysfunction such as incontinence because of low cyclical estrogen levels. Third, all experiments were performed under urethane anesthesia, which might have affected the results of this study, given that urethane affects the NO pathway and urethral function [28,29]. Fourth, evidenced by an SNP-induced decrease in the maximum amplitude of isovolumetric contractions and intravesical pressure threshold for inducing urethral relaxation in middle-aged rats, we show that the NO/sGC system also affects bladder function. Despite these limitations, the outcomes of the present study may be of significance for future studies on aging-related changes in the urethra.

5. Conclusion

This study provides evidence that aged rats have impaired bladder and urethral coordination. After combined administration of SNP, an NO donor, and BAY41-2272, an sGC activator, the baseline UPP and nadir were decreased, and the blockade of these reactions by L-NAME administration, an NO inhibitor, restored all parameters to baseline levels, only in middle-aged rats. This supports our initial hypothesis that the efficacy of the NO/sGC system is diminished by aging. Additionally, the urethra of aged rats showed higher staining for HIF-1α, 8-OHdG, and MDA levels. Therefore, age-associated ischemic and oxidative stress in the urethra might be correlated with the occurrence of bladder and urethral coordination.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2021.119690.

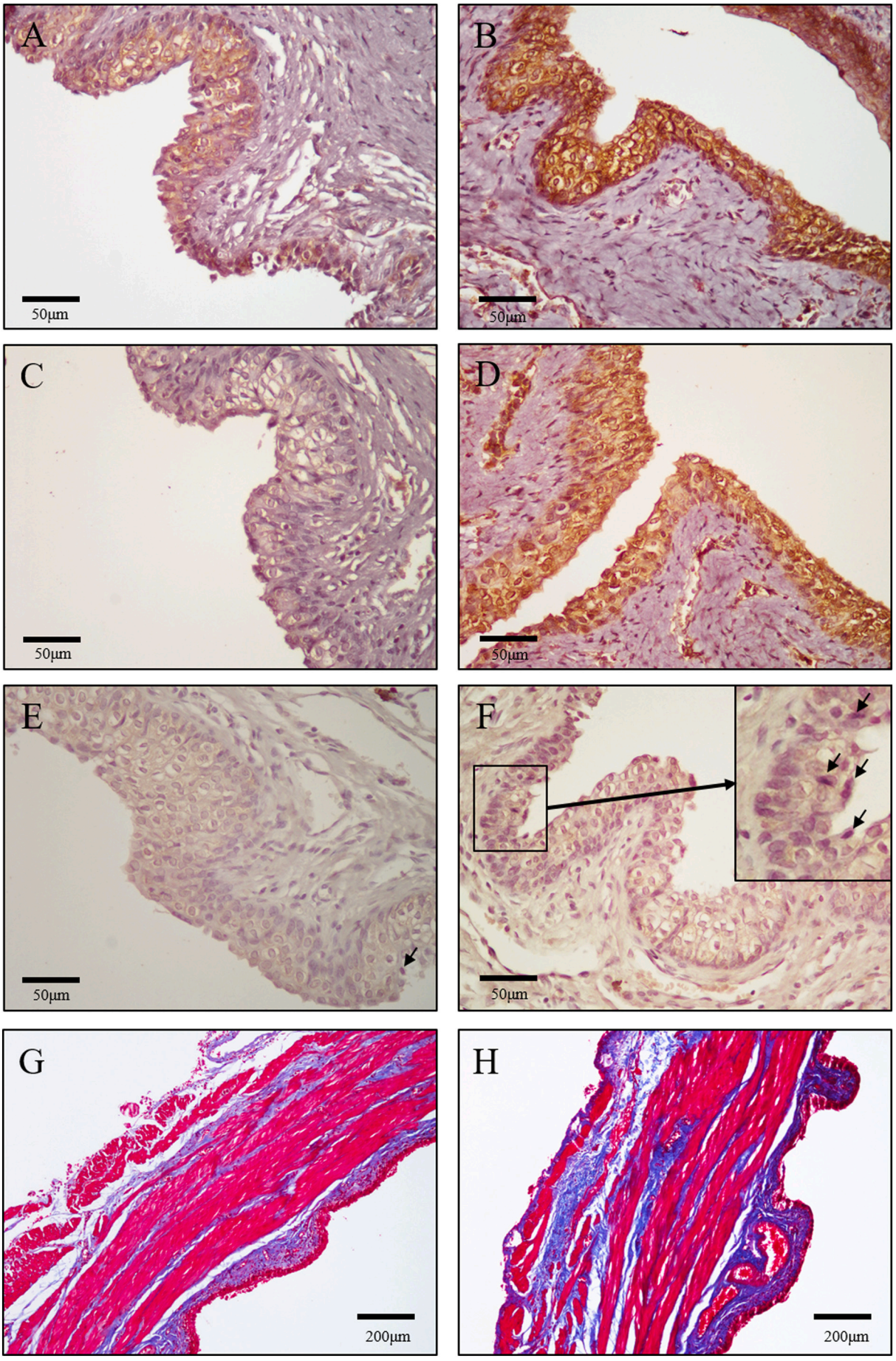

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Fig. 5. 8-hydroxy-2-deoxyguanosine (8-OHdG) immunohistochemical staining in the urethra of young rats (A) and middle-aged rats (B). Malondialdehyde (MDA) immunohistochemical staining in the urethra of young rats (C) and middle-aged rats (D). Hypoxia-inducible factor-1α (HIF-1α) immunohistochemical staining in the urethra of young rats (E) and middle-aged rats (F). Masson's trichrome staining in the bladder of young rats (G) and middle-aged rats (H). Expression levels of 8-OHdG and MDA in the urethral epithelium in middle-aged rats were remarkably higher than those in young rats. Likewise, the frequency of HIF-1α-positive cells (arrows: nucleus in the urethral epithelium) in middle-aged rats was significantly higher compared to that in young rats. In Masson's trichrome staining, atrophy of smooth muscle and fibrosis in middle-aged rats were significantly stronger compared to those in young rats.

CRediT authorship contribution statement

The contributions of each author are as follows: (1) Substantial contributions to conception and designed experiments: AO, MM, TO. (2) Performing the experiments and analyzing data: AO, MM, TO, TM, YM. (3) Drafting and revising the article critically for important intellectual content: AO, MM, TO, RK, TM, and YM. (3) Final approval of the version to be published: AO, MM, TO, RK, TM, YM, and HS.

Declaration of competing interest

The authors declare no conflict of interest.

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