

## Combination Histamine and Serotonin Treatment After Simulated Childbirth Injury Improves Stress Urinary Incontinence

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**Aims:** Histamine and serotonin-related pharmaceuticals have the potential to modulate micturition and continence. The aim of this study was to determine if treatment with histamine and/or serotonin improves stress urinary incontinence (SUI) in female rats. **Methods:** Twenty-six age-matched female rats underwent pudendal nerve crush and vaginal distension (PNC + VD), to produce SUI. One week after injury, rats were treated subcutaneously with saline, histamine (1.1 µg), serotonin (2 µg), or the combination of both twice daily for another week. A sham injured group received sham PNC + VD and were treated with saline (n = 7). Leak point pressure (LPP) testing with simultaneous external urethral sphincter (EUS) electromyography (EMG) was conducted 2 weeks after injury. The urethra was harvested for qualitative and quantitative histology. Data were analyzed with a one-way ANOVA and Student-Newman-Keuls posthoc test with  $P < 0.05$  indicating statistically significant differences between groups. **Results:** Combination treatment significantly increased LPP after PNC + VD compared to injured sham treatment and treatment with either histamine or serotonin alone. Compared to injured sham treated rats, all three treatments significantly increased EUS EMG amplitude at both baseline and peak pressure and EUS EMG firing rate at peak pressure during LPP testing. There were more consistent urethral striated muscle fibers and thicker smooth and striated muscle with combination and histamine treatment. There was a statistically significant shift to a greater proportion of thicker collagen fibers in the urethra in serotonin and combination treated rats compared with injured sham treated rats. **Conclusions:** Combination treatment was the most effective and may provide an effective therapy for SUI. *NeuroUrol. Urodynam.* 35:703–710, 2016. © 2015 Wiley Periodicals, Inc.

**Key words:** electromyography; external urethral sphincter; female; histology; leak point pressure; rat

### INTRODUCTION

Stress urinary incontinence (SUI), the involuntary loss of urine from increased abdominal pressure, is a common condition, impacting 1/3 of women between the ages of 40–59.<sup>1</sup> The greatest risk factor is vaginal delivery, which can damage maternal pelvic floor muscles.<sup>2</sup> Current mainstay therapy for SUI is surgery, and pharmacological options are limited.

Histamine can modulate bladder and urethral function, possibly via activation of the H<sub>1</sub> receptor and modulation of excitatory glutamate neurotransmission in the spinal cord.<sup>3,4</sup> Serotonin receptors and serotonin reuptake inhibitors, a class of medications for clinical depression and anxiety, have also been shown to modulate micturition and the latter have potential for therapeutic efficacy for SUI.<sup>5</sup>

We have developed a dual injury childbirth simulation model, consisting of pudendal nerve crush (PNC) and vaginal distension (VD) which causes more severe and longer lasting damage than either PNC or VD alone.<sup>6,7</sup> Dual injury induces a significant decrease in urethral function as measured by reduced leak point pressure (LPP) and external urethral sphincter (EUS) electromyography (EMG), lasting approximately 4 weeks after injury.<sup>6</sup> In addition, the morphology of the EUS is disrupted and urethral smooth muscle is edematous after injury.<sup>6,7</sup>

The goal of this study was to determine if subcutaneous administration of low dose histamine and/or serotonin improves SUI symptoms in the dual injury model of SUI.

### MATERIALS AND METHODS

All experiments were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic.

Karl-Erik Andersson led the peer-review process as the Associate Editor responsible for the paper.

Potential conflicts of interest: Dr. Damaser reports grants from Beech Tree Labs during the conduct of the study. She also reports personal fees from Astellas and research grants from Fate Therapeutics, Eli Lilly and Acorda Therapeutics outside the submitted work. Dr. Gurel, Dr. McMichael, Dr. Spaulding and Dr. Tobacyk report multiple patents issued and pending. Dr. Gurel and Dr. McMichael are employees of Beech Tree Labs which supported the research in this study.

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Thirty-three female, virgin, age-matched Sprague-Dawley rats (200–250 g; Harlan, Indianapolis, IN) were randomized into 5 groups. Nineteen rats underwent PNC + VD and were treated with either *histamine* (1.1  $\mu$ g; n = 6), *serotonin* (2  $\mu$ g; n = 6), or *histamine and serotonin (combination group)* (1.1  $\mu$ g and 2  $\mu$ g; n = 7). Seven rats in the *sham injured group* underwent sham PNC + VD and were treated with saline. Rats that underwent PNC + VD and were treated with saline (n = 7) served as an *injured sham treated group*. Histamine (catalog number 7099ED, Hollister Stier, Spokane, WA) and serotonin (catalog number H9523, Sigma-Aldrich, St. Louis, MO) were mixed to working concentrations and stored at 4°C until used. One week after surgery, each rat began treatment via subcutaneous injection (0.2 ml/rat) twice daily in a blinded fashion to simulate the sublingual delivery route used in a prior clinical trial.<sup>8</sup> After a week of treatment, 2 weeks after injury, the animals were anesthetized for LPP testing with simultaneous EUS EMG recordings. The rats were then euthanized and the urethra was harvested for histological assessment.

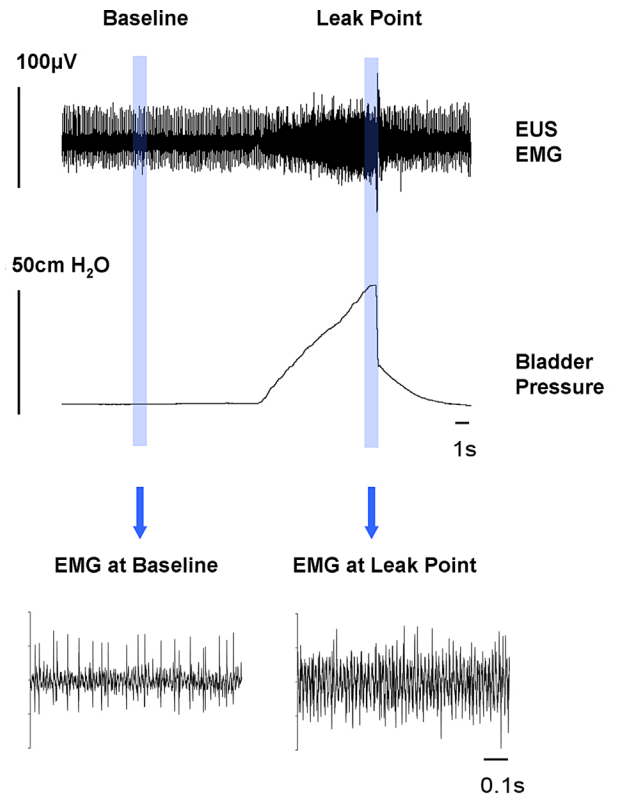
PNC + VD was performed as previously described.<sup>6,7</sup> In brief, after anesthesia with isoflurane, the pudendal nerve was isolated in the ischioanal fossa and crushed twice bilaterally with a Castro-Viejo needle holder for 30 sec. VD was performed immediately after PNC by accommodating the vagina with increasing sizes of Otis Bougie à Boule urethral dilators (24F–32F) to avoid rupture and bleeding. A modified 10F Foley balloon catheter was then inserted into the vagina and the balloon was inflated with 3 ml water for 4 hr. Sham injury was produced by a dorsal skin and muscle incision, which was closed and followed by vaginal accommodation with urethral dilators and placement of the Foley catheter for 4 hr without inflation of the balloon.

Rats were anesthetized with urethane (1.2 g/kg, intraperitoneal) for LPP with simultaneous EUS EMG recordings.<sup>5</sup> Wire electrodes were inserted into the mid-urethra bilaterally using a 30-gauge needle for EMG recordings as previously described.<sup>9</sup> A transurethral catheter was used to fill the bladder (5 ml/hr) and measure bladder pressure. To determine LPP, with the bladder approximately half full, intravesical pressure was gradually increased by slowly and gently pressing the abdominal wall with a cotton-tipped applicator. At the first indication of leakage at the urethral meatus, the externally applied abdominal pressure was rapidly removed (Fig. 1). The test was repeated 3–4 times in each animal. Bladder pressure and simultaneous EUS EMG data were amplified (Model P122, Grass technologies, Warwick, RI), filtered (60 Hz and 120 Hz), and recorded with an analog-to-digital recording system (Dash 8X, Astro-Med, Inc.; 10 k samples/sec).

LPP was calculated by subtracting baseline pressure from peak bladder pressure at the moment of leakage during LPP testing.<sup>7</sup> One second segments of EUS EMG were selected at baseline pressure just prior to LPP testing and at the point of peak pressure during LPP testing (Fig. 1).<sup>9</sup> Using a 15  $\mu$ V threshold, mean EMG amplitude and firing rate were calculated at both baseline and peak pressure as previously described.<sup>9</sup> The mean value of each outcome for each rat was calculated from 3–4 trials and was used to create a mean and standard error for each experimental group.

Rats were euthanized immediately after LPP and EMG recording. A 5 mm segment of the urethra, attached to the anterior portion of the vagina (~5 mm proximal to the urethral meatus), was dissected, immersion fixed in 10% formalin and sectioned transversely (5  $\mu$ m). Masson's trichrome stained slides were analyzed qualitatively to assess muscle and connective tissue.

Additional mid-urethral sections were stained with picosirius red and were analyzed quantitatively to determine changes to



**Fig. 1.** Example of leak point pressure (LPP) and external urethral sphincter (EUS) electromyography (EMG) data from a sham injured rat. The slow rise to peak in bladder pressure is from external pressure applied to the bladder. When leakage occurs, the external pressure is rapidly removed and the pressure quickly drops back to baseline. The vertical lines indicate the 1 sec segments used for quantitative data analysis.

collagen composition with injury and treatment. Circular polarizing microscopy was used to determine collagen thickness taking advantage of the birefringent properties of collagen, resulting in a color scale (green to yellow to orange) representing increasing thickness of collagen fibrils.<sup>10</sup> Color separation was performed on microscopy images (SigmaScan Pro, San Jose, CA) and the content in pixels of each of the three thickness ranges of collagen (represented by green, yellow, and orange) were quantified and expressed as percentage total collagen content calculated by adding the pixels of all 3 thickness ranges.

Additional mid-urethral sections underwent immunohistochemical staining to assess urethral innervation. Slides were dewaxed by heating them to 60°C for 20 min and rehydrating. Antigen retrieval was performed for 20 min using citrate buffer (Dako, Golstrup, Denmark, pH 6.0). Before blocking, slides were treated with a 1% TritonX-100 (Sigma-Aldrich), 3% H<sub>2</sub>O<sub>2</sub> (Sigma-Aldrich) solution for 20 min. Slides were blocked for 1 hr at room temperature with 1% BSA (Sigma-Aldrich) and 10% normal goat serum (Jackson Immune Research, West Grove, PA) in PBS. Slides were then incubated with mouse anti-PGP9.5 (Abcam, Cambridge, MA) at a concentration of 1:2000 overnight at 4°C. The following day the slides were incubated for 1 hr with a biotinylated goat anti-mouse secondary antibody (Vector laboratories, Burlingame, CA) at 1:1000 concentration at room temperature. The slides were then incubated for one hour with ABC peroxidase kit (Vector Laboratories Burlingame, CA) at room temperature. Diaminobenzidine (Sigma-Aldrich, St Louis, MO) was used as chromogen. Sections were mounted in

Permount (Fisher Scientific). Primary antibody was omitted in the negative control. The slides were qualitatively evaluated in a blinded manner.

Physiological and anatomical data were analyzed by an investigator blinded to the experimental groups. Quantitative data is presented as mean  $\pm$  standard error of the mean and was statistically compared using a one-way ANOVA followed by a Student-Newman-Keuls test since it demonstrated normality and equal variance, with  $P < 0.05$  indicating a statistically significant difference between groups.

## RESULTS

No rats died during this experiment. LPP in injured sham treated rats ( $31.6 \pm 3.3$  cmH<sub>2</sub>O) was significantly decreased compared to that of sham injured rats ( $46.8 \pm 2.2$  cmH<sub>2</sub>O; Fig. 2), consistent with previous results with this model.<sup>6</sup> LPP was significantly increased after injury with both combination ( $57.0 \pm 2.5$  cmH<sub>2</sub>O) and histamine ( $46.9 \pm 2.1$  cmH<sub>2</sub>O) treatments compared to injured sham treated rats, suggesting a therapeutic effect. Serotonin treatment did not significantly increase LPP ( $37.4 \pm 2.7$  cmH<sub>2</sub>O) compared to injured sham treated rats.

EUS EMG amplitude and firing rate at both baseline and peak pressure during LPP testing were significantly decreased in injured sham treated rats compared to sham injured rats (Fig. 2), as we have observed previously.<sup>6</sup> EUS EMG amplitude at both baseline and peak pressure and EUS EMG firing rate at peak pressure were significantly increased after PNC + VD with all treatments compared to injured sham treated rats and were not significantly different from sham injured rats. Although all treatment groups had greater EUS EMG firing rate at baseline than injured sham treated rats, only with combination treatment was this a significant increase. These results parallel but do not exactly mirror the LPP results, suggesting that LPP better discriminates between the treatment groups than EUS EMG.

Sham injured animals demonstrated thick and continuous EUS fibers and normal urethral smooth muscle with dense collagen and a rich venous plexus (Fig. 3). In contrast, signs of injury and collagen infiltration after PNC + VD were prominent in the EUS in injured sham treated animals, as we have observed previously.<sup>7</sup> Histology also revealed urethral smooth muscle edema and widening of the vascular plexus in injured sham treated animals (Fig. 3). Treatment with serotonin demonstrated poorly healed EUS and urethral smooth muscle compared to combination and histamine treatments, both of which resulted in increased thickness and relatively continuous EUS muscle and healthy appearing smooth muscle (Fig. 3), which may account for the significantly increased LPP in these two groups.

Circular polarization microscopy demonstrated a distribution of the three thickness ranges of collagen fibrils in urethras of sham injured rats with the thickest fibers dominating (thinnest:  $30.9 \pm 0.8\%$ ; intermediate:  $23.4 \pm 1.1\%$ ; thickest:  $44.8 \pm 1.3\%$ ). After injury with sham treatment, the distribution shifted such that intermediate fibers dominated (thinnest:  $33.0 \pm 2.2\%$ ; intermediate:  $40.7 \pm 2.1\%$ ; thickest:  $26.4 \pm 3.0\%$ ) with significantly more intermediate fibers and significantly fewer thick fibers after injury compared to sham injured rats (Fig. 4).

Treatment with serotonin (thinnest:  $14.1 \pm 3.4\%$ ; intermediate:  $29.2 \pm 2.8\%$ ; thickest:  $56.8 \pm 0.8\%$ ) or combination (thinnest:  $16.6 \pm 2.2\%$ ; intermediate:  $24.6 \pm 3.9\%$ ; thickest:  $58.8 \pm 2.3\%$ ) redistributed collagen fiber thickness such that thick fibers dominated. There were significantly fewer of the

thinnest fibers and significantly more of the thickest fibers in the serotonin and combination treated groups compared to sham injured, injured sham treated, and histamine treated rats (Fig. 4). Treatment with histamine resulted in a collagen fiber distribution with significantly fewer intermediate fibers and significantly more of the thickest fibers compared to injured sham treated rats (thinnest:  $27.3 \pm 2.6\%$ ; intermediate:  $30.7 \pm 3.4\%$ ; thickest:  $42.3 \pm 5.0\%$ ).

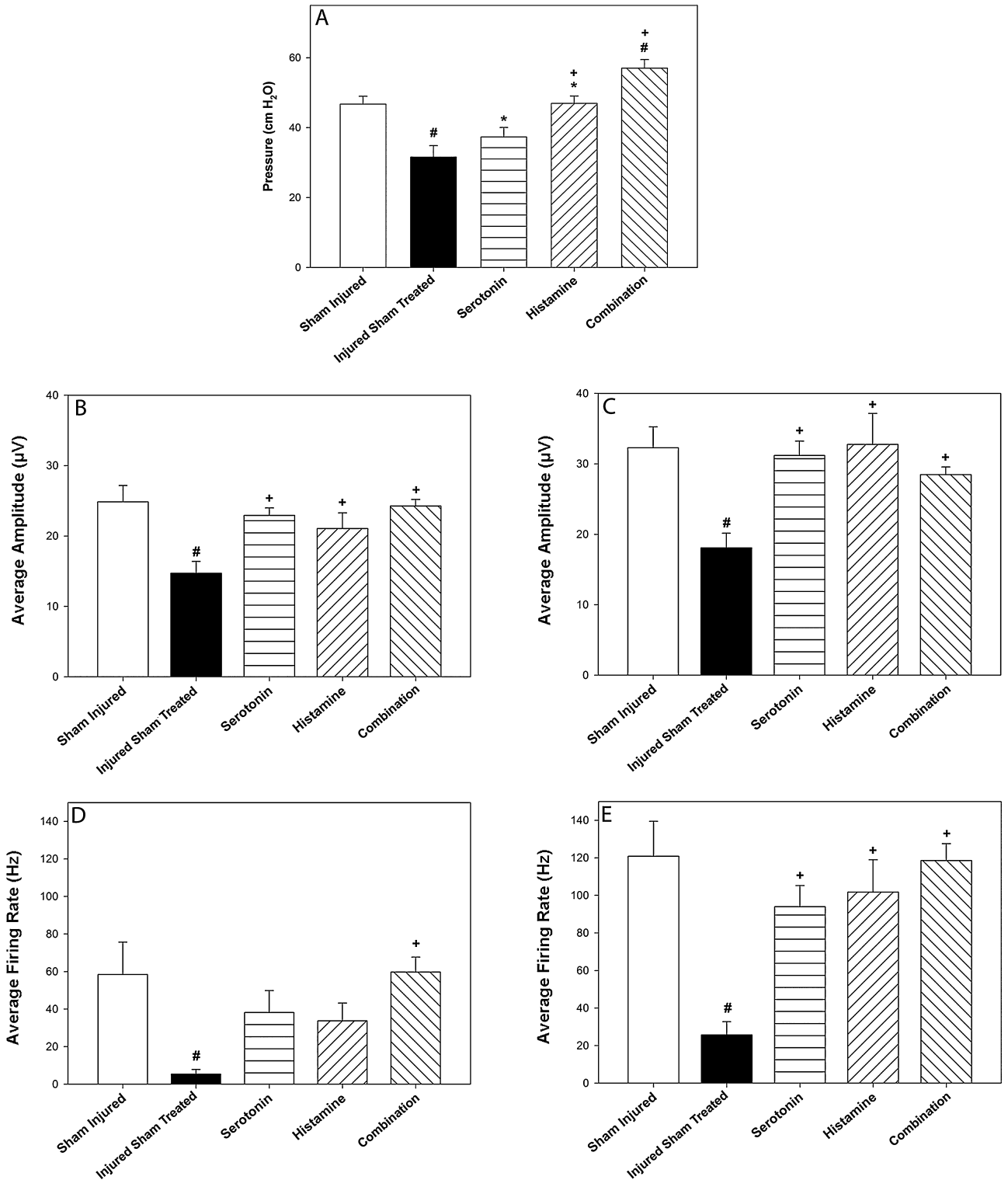
Sham injured rats demonstrated abundant nerves in the urethra with PGP9.5 staining, with nerves present both between the EUS muscle fibers, and outside of the muscle fibers (Fig. 5). In contrast, injured sham treated rats had fewer nerves and these nerves were located outside of urethral striated muscle fibers. Serotonin and combination treatments resulted in urethral neuroanatomy similar to that of sham injured rats, with abundant nerves located both between striated muscle fibers and outside of the muscle fibers. Histamine treatment resulted in neuroanatomy similar to injured sham treated rats with few nerve fibers, most of which were located outside of urethral striated muscle fibers (Fig. 5).

## DISCUSSION

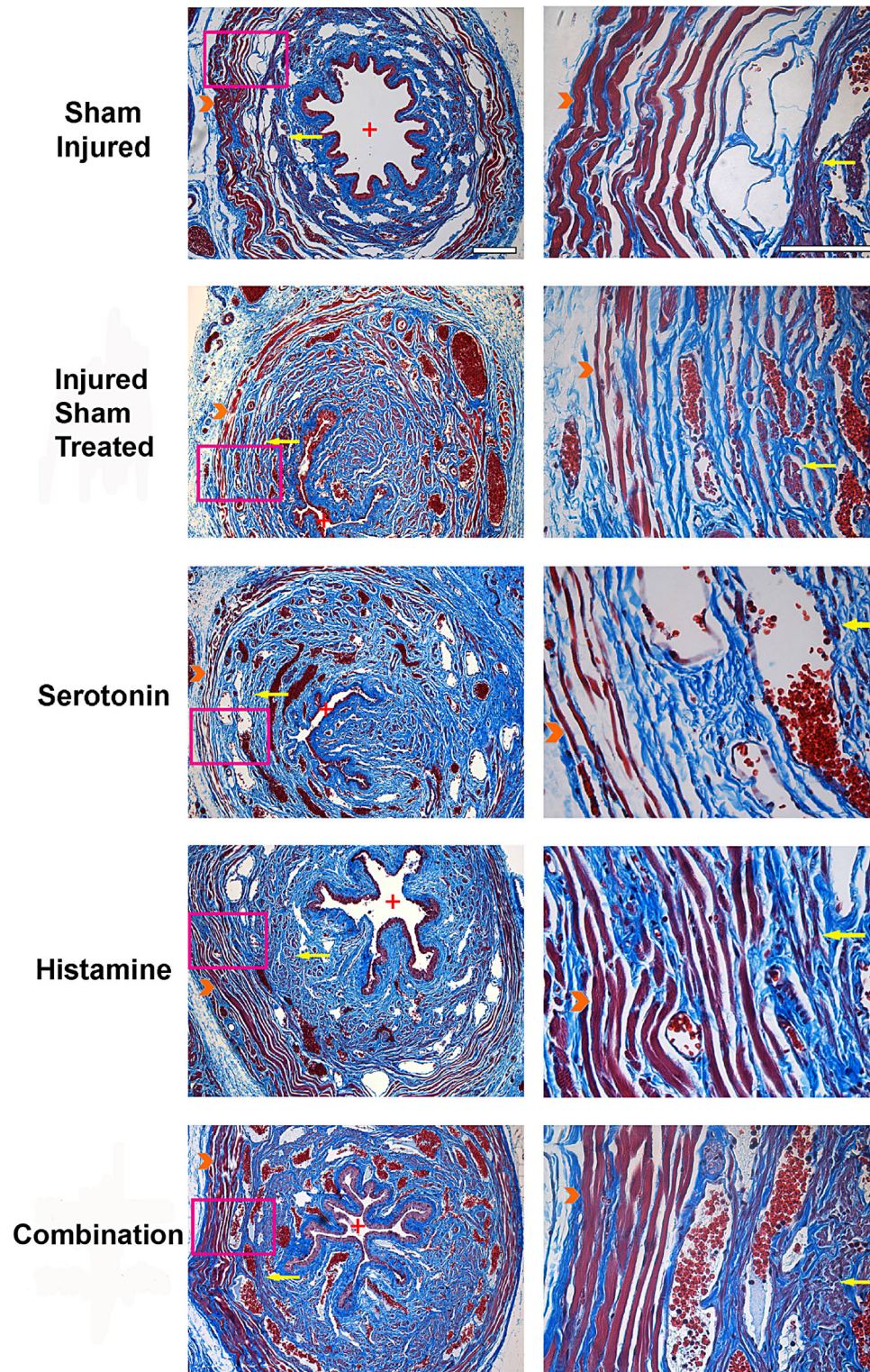
Depending on age, SUI prevalence ranges from 18.8 to 33.1%,<sup>1</sup> which can result in reduced quality of life, psychological morbidity, and increased financial burden.<sup>11</sup> Surgery is the primary treatment for SUI, but has high risk of complications.<sup>12</sup> Although pharmacological treatments have been introduced for management of SUI, none are currently in use in the U.S.<sup>13</sup>

Urethral smooth muscle is an important contributor to LPP recovery and leakage prevention in rats.<sup>14</sup> Since the H<sub>1</sub> receptor is located near smooth muscle and can trigger muscle contraction with histamine stimulation, histamine can have a contractile effect on urethrovesical smooth muscle,<sup>15</sup> which can be eliminated by an H<sub>1</sub> receptor blocker.<sup>4</sup> In cultured detrusor smooth muscle cells, H<sub>1</sub> and H<sub>3</sub> receptors have been shown to induce calcium release mainly through the 1,4,5-inositol triphosphate pathway or modulation of N-type calcium channels.<sup>16</sup> Acting as a neurotransmitter, histamine also modulates sympathetic and parasympathetic nerve activity through the H<sub>3</sub> receptor.<sup>17,18</sup> In addition, histamine promotes excitatory neurotransmission by facilitating N-methyl-D-aspartate (NMDA) receptors, which play an essential role in controlling EUS activity and bladder contractility.<sup>3</sup> Histamine can also stimulate fibroblasts to promote collagen production.<sup>19</sup> These mechanisms could partly explain the outcomes observed in our study, although it is not clear if histamine acts centrally and/or peripherally and the mechanisms of action remain to be explored.

Several serotonin receptors have been shown to control micturition and maintain continence.<sup>20</sup> However, different animal models demonstrate different functional outcomes.<sup>20</sup> Duloxetine, a selective serotonin/norepinephrine reuptake inhibitor has been shown to be effective for SUI,<sup>5</sup> as it can block preganglionic parasympathetic outflow to the bladder, inhibiting bladder contraction and facilitating urine storage during the filling phase.<sup>21</sup> Additionally, serotonin and norepinephrine enhance pudendal nerve activity through glutamate release in Onuf's nucleus leading to an increase in EUS tone.<sup>5,22</sup> Blockade of the  $\alpha$ -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) glutamatergic receptors abolished the positive effects of noradrenaline and serotonin on urethral resistance,<sup>23</sup> confirming this mechanism of action. Serotonin also has a regulatory effect on collagen synthesis by activating transforming growth factor- $\beta$ 1, a key cytokine for extracellular matrix production, cell proliferation and fibrosis.<sup>24</sup> Which



**Fig. 2.** Leak point pressure data (A) as well as amplitude (B and C) and firing rate (D and E) of external urethral sphincter electromyography recorded at both baseline during bladder filling (B and D) and at the peak of leak point pressure testing (C and E). Data are presented as mean ± standard error of the mean of 6–7 animals in each group. #Indicates a statistically significant difference compared to sham injured rats; + indicates a statistically significant difference compared to injured sham treated rats. † indicates a statistically significant difference compared to rats that received injury and combination treatment.

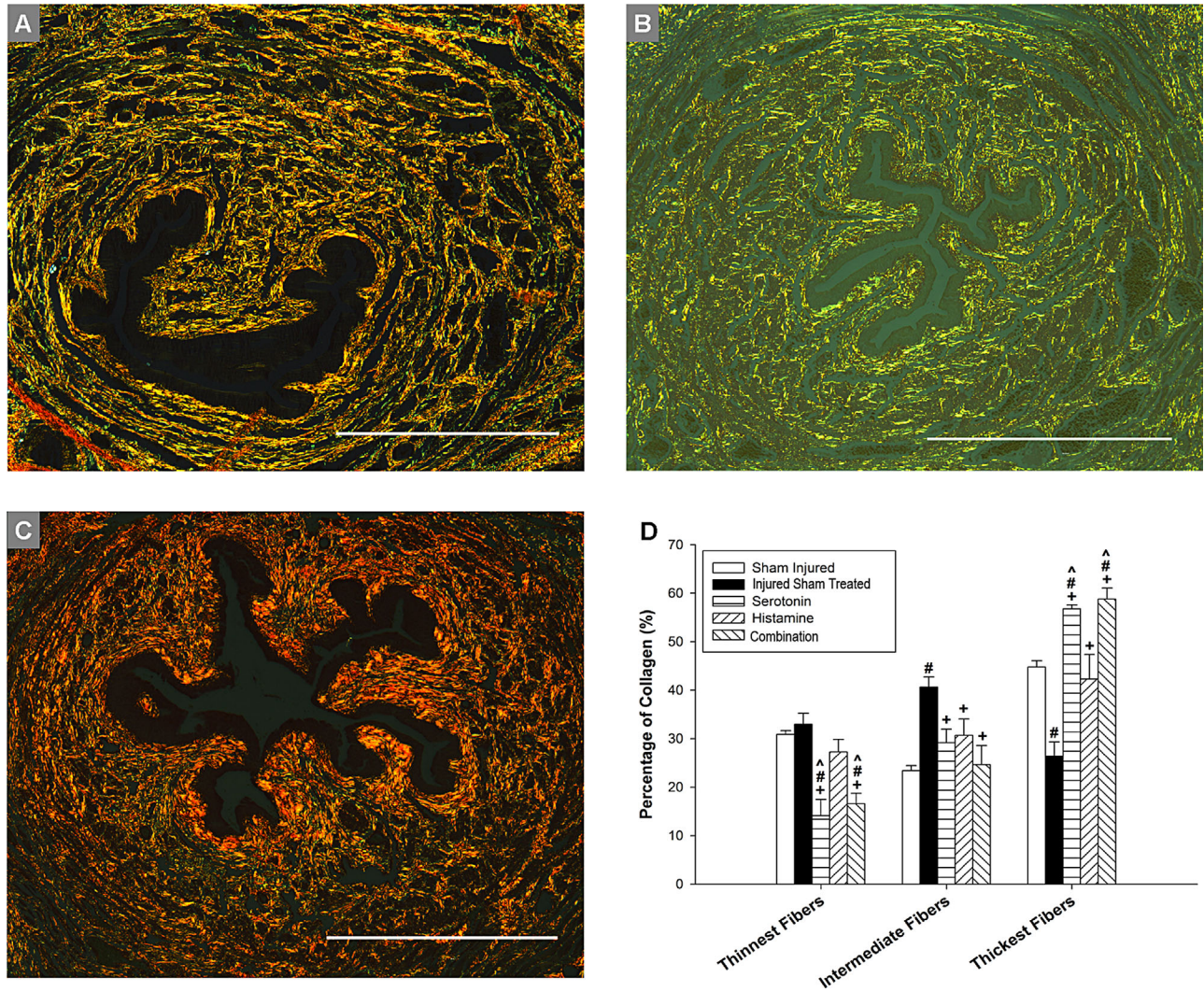


**Fig. 3.** Example Masson's trichrome-stained transverse sections of urethras from animals in all experimental groups. The red box in the left panel indicates the location of the corresponding higher magnification picture in the right panel. Bars = 500  $\mu\text{m}$  in both magnifications; red cross indicates urethral lumen; orange arrow head indicates external urethral sphincter striated muscle; yellow arrow indicates urethral smooth muscle.

mechanisms of action serotonin is acting upon in this study remain to be determined.

We have previously demonstrated that LPP and EUS EMG are significantly decreased and EUS morphology is disrupted after

dual injury, indicating a denervated and defunctionalized urethra.<sup>6,7</sup> LPP recovers 4–6 weeks after injury, while EUS EMG remains abnormal 6 weeks after injury.<sup>6</sup> In this study, we demonstrated that after only 1 week of treatment, both



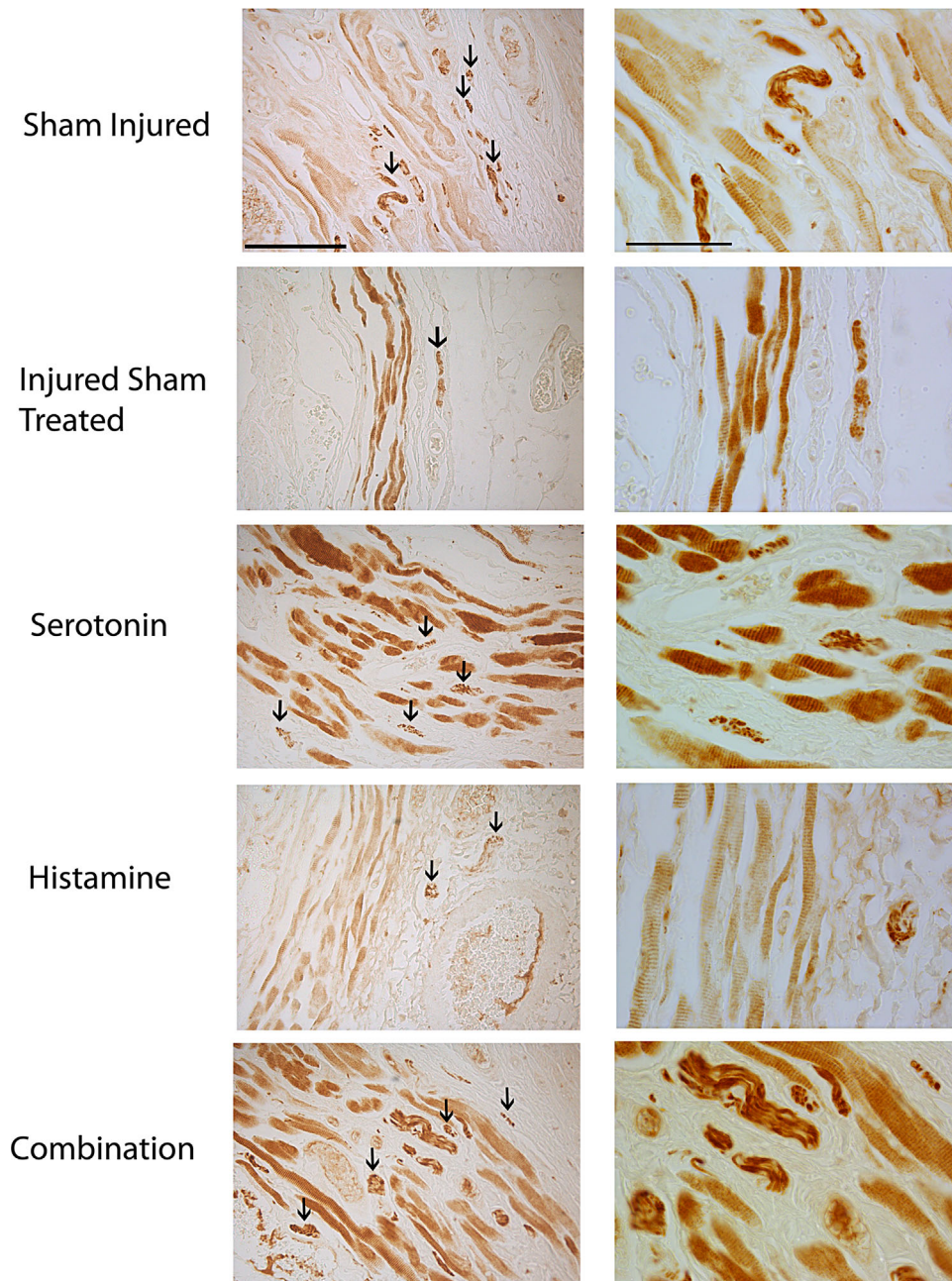
**Fig. 4.** Example Picosirius stained transverse sections of urethras from a sham injured rat (A), an injured sham treated rat (B) and an injured rat treated with the combination histamine and serotonin treatment (C). Green = thinnest collagen fibers, yellow = intermediate collagen fibers and orange = thickest collagen fibers. The scale bar in each picture represents 500 μm. The percentage of each of the collagen color groups in the control and experimental groups (D) is presented as mean ± standard error of the mean of 3–4 animals in each group. #Indicates a statistically significant difference compared to sham injured rats; + indicates a statistically significant difference compared to injured sham treated rats. ^Indicates a statistically significant difference compared to rats that received injury and histamine treatment.

histamine and combination treatment returned LPP to normal 2 weeks after injury, while both serotonin and combination treatment maintained urethral nerves near the EUS. Only combination treatment significantly increased EUS EMG firing rate at baseline, indicating that this treatment could restore normal EUS function. The increase in nerves, LPP, EUS EMG at baseline, and thick collagen fibers with combination treatment demonstrates maintenance of urethral striated muscle, its innervation, and extracellular matrix, resulting in improved urethral function over histamine or serotonin treatments.

LPP was significantly higher with combination treatment than in sham injured rats, suggesting potential overtreatment, which could possibly result in partial bladder outlet obstruction. While histamine treatment alone may improve LPP by enhancing urethral smooth muscle contraction,<sup>15</sup> combination treatment may affect excitability of both urethral smooth muscle and the EUS, which could explain the higher LPP.<sup>5,15</sup> The consistently thick EUS fibers and relatively healthy smooth

muscle in the histamine and combination treated groups demonstrated that the treatment could also maintain continence by facilitating striated and smooth muscle regeneration and proliferation accompanied by a redistribution of collagen fiber thickness.

Early in the healing process, collagen fibers are thinner and more loosely packed. With time and improved healing they become thicker and more tightly packed,<sup>10,25</sup> a progression that can be demonstrated with picosirius staining. In this study, all treatment groups demonstrated more rapid collagen fiber repair after injury than injured sham treated rats, with the serotonin and combination treatments having the most profound effect. Since collagen is slow to regenerate after injury,<sup>26</sup> longer duration treatment may result in more mature collagen fibers and greater differences between the treatment groups. Determination of an exact relationship between color under circular polarizing microscopy and exact collagen thickness cannot be made because birefringence color is also



**Fig. 5.** Examples of PGP 9.5 immunohistochemical stained transverse sections of urethras from rats in all experimental groups. The scale bar equals 100  $\mu\text{m}$  in the low magnification pictures (left column) and 50  $\mu\text{m}$  in the higher magnification pictures (right column). Black arrows point to nerve fascicles, which are magnified in the higher magnification images.

affected by experimental factors, such as section thickness and the surrounding medium, both of which were held constant in this experiment.<sup>27</sup> Therefore, we expressed our results as relative thickness of collagen fibers.

Using fragment-based lead discovery, a method to screen, synthesize and generate chemicals for drug development, significant overlap was found between  $H_4$  receptors and  $5\text{-HT}_{3A}$  receptors.<sup>28</sup> As a result, the similarity between the ligand-gated ion channels of histamine and serotonin receptors could serve as a shared therapeutic target, resulting in a synergistic effect. For example, both  $H_4$  and  $5\text{-HT}_{3A}$  receptors have been shown to play a role in irritable bowel syndrome.<sup>28</sup> Thus, the combination

treatment may have the greatest effect because of a combinatorial effect dominated by the influence of histamine on muscle and serotonin on nerves and collagen regeneration.

Although systematic histamine and/or serotonin treatment is related to potentially life-threatening reactions, such as dose-dependent serotonin syndrome,<sup>29</sup> we did not observe such lethal side effects in the treated animals at the low doses used in this experiment, suggesting its potential for the future clinical translation. Intraperitoneal LD50 of histamine is 1,550 mg/kg in rats.<sup>30</sup> Moreover, acute and sub-chronic subcutaneous administration of histamine in rats previously demonstrated that the no-observed-effect-level (NOEL) and

no-observable-adverse-effect-level (NOAEL) were 1 mg/kg/day and 10 mg/kg/day, respectively.<sup>31</sup> This NOAEL dose is 1,126 times higher than the dose administered to the animals in this study (8.88 µg/kg/day). A clinical safety trial has recently been conducted using the same concentrations administered in the animal study. Results of this study suggested that these doses are well tolerated and the combination did not affect hemodynamic changes in mean arterial blood pressure, heart rate, respiration, and temperature in participants as compared to the placebo treatment group (unpublished data).<sup>8</sup>

We used a simulated childbirth injury model in quadruped rodents whose pelvic orientation is not the same as bipedal humans. Nonetheless, using functional and histological assessments, repeated studies have suggested that animal models are feasible to produce SUI symptoms.<sup>6,7</sup> This study provides valuable insight into the potential benefit of the combination of histamine and serotonin in relieving SUI symptoms and suggests a mechanism of action of these agents.

### CONCLUSIONS

Subcutaneous administration of the combination of histamine and serotonin facilitates restoration of continence in an animal model of SUI, as evidenced by improved LPP and EUS EMG, maintenance of urethral striated muscle and its innervation, and thicker collagen fibers. The outcomes from this initial study set the stage for future mechanistic studies and clinical trials.

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