Combination Therapy Using Human Adipose-derived Stem Cells on the Cavernous Nerve and Low-energy Shockwaves on the Corpus Cavernosum in a Rat Model of Post-prostatectomy Erectile Dysfunction

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OBJECTIVE
To investigate combined therapeutic efficacy of human adipose-derived stem cells (h-ADSCs) application on injured cavernous nerve and low-energy shockwave therapy (SWT) on the corpus cavernosum in a rat model of post-prostatectomy erectile dysfunction.

MATERIALS AND METHODS
Rats were randomly divided into 5 groups: control, bilateral cavernous nerve injury (BCNI), adipose-derived stem cell (ADSC) (BCNI group with h-ADSCs on the cavernous nerve), SWT (BCNI group with low-energy SWT on the corpus cavernosum), and ADSC/SWT (BCNI group with a combination of h-ADSCs and low-energy SWT). After 4 weeks, erectile function was assessed using intracavernosal pressure. The cavernous nerves and penile tissue were evaluated through immunostaining, Western blotting, and a cyclic guanosine monophosphate assay.

RESULTS
ADSC/SWT significantly improved intracavernosal pressure compared to the other experimental group. ADSC had significantly increased β-III tubulin expression of the cavernous nerve, and SWT had a markedly enhanced vascular endothelial growth factor expression in corpus cavernosum. The ADSC/SWT group had a significantly increased in alpha smooth muscle actin content (P < .05), neural nitric oxide synthase (nNOS) of the dorsal penile nerve (P < .05), endothelial nitric oxide synthase (eNOS) protein expression (P < .05), and cyclic guanosine monophosphate level (P < .05) compared to the ADSC or SWT alone group. In addition, ADSC/SWT reduces the apoptotic index in the corpus cavernosum.

CONCLUSION
In this study, h-ADSCs showed an effect on the recovery of injured cavernous nerve and low-energy SWT improved angiogenesis in the corpus cavernosum. The h-ADSCs combined with low-energy SWT showed beneficial effect on the recovery of erectile function in a rat model of postprostatectomy erectile dysfunction.

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Prostate cancer (PCa) is common in North America and Europe, but in recent years, its incidence has started to increase rapidly across Asia.1 Around 94% of the PCa cases are identified as clinically localized and are generally treated with radical prostatectomy (RP). RP, however, has a number of complications that have been affecting an increasing number of PCa patients. One of these complications, post-prostatectomy erectile dysfunction (ED), is observed in more than 50% of patients treated with RP.2 Additionally, the patients affected with post-prostatectomy ED demonstrate injury to the cavernous nerve. Therefore, the ideal method to treat this complication should
target and restore the injured cavernous nerve, prevent apoptosis in the corpus cavernosum, and boost levels of the necessary vasodilatory factors.3

Two methods have been extensively studied for their potential in treating post-prostatectomy ED: injection of stem cells and shockwave treatment. Albersen et al4 demonstrated that intracavernous injection of adipose-derived stem cells (ADSCs) significantly improved erectile function and prevented apoptosis of the cells in the corpus cavernosum. Several other studies have demonstrated the therapeutic effects of shockwave therapy (SWT), a method that has become more widely recognized in recent years for its ability to improve penile hemodynamics.5

Because stem cells have the potential to induce recovery of the cavernous nerve6,7 and to prevent apoptosis of the cavernosal smooth muscle and endothelial cells,8 and low-energy SWT has the potential to induce neovascularization and angiogenesis,8,9 we hypothesized that combining these 2 treatments would yield better erectile function recovery in a rat ED model with bilateral cavernous nerve injury (BCNI) than applying the 2 treatments separately. Unlike previous stem cell approaches to treating ED that involved injecting ADSCs intracavernously,4 we chose to directly inject human adipose-derived stem cells (h-ADSCs) around the cavernous nerve. We believe that damage to this nerve is the ultimate cause of ED. Further, h-ADSCs were deliberately chosen for their substantial adipose tissue mass, simple collection procedure, and multilineage differentiation ability.10

Ultimately, the purpose of the present study was to measure the combined therapeutic effects of h-ADSCs injected around the cavernous nerve and low-energy SWT on the corpus cavernosum in a rat model of postprostatectomy ED.

MATERIALS AND METHODS

Preparation of h-ADSCs

The h-ADSCs used in the present study were obtained from the surplus stock of frozen cells at a cell bank (K-STEMCELL, Seoul, Korea). Human abdominal subcutaneous fat tissues were acquired by simple liposuction from a donor who provided informed consent, according to the guidelines of the Institutional Review Board of K-STEMCELL. h-ADSCs were then isolated from the fat stromal vascular fraction by culturing them on growth medium (mesenchymal stem cell proliferation media; K-STEMCELL) at subconfluent levels to prevent spontaneous differentiation. All detailed studies were performed according to the methods outlined in the study by Ra et al.11

Experimental Animal and Study Design

Eight-week-old male Sprague-Dawley rats weighing 270-300 g (Orient Bio Co., Seongnam, Korea) were used in this study. All animal experiments in this study were approved by the Institutional Animal Care and Use Committee (CUMC-2014-0141-01) of the Catholic University of Korea. The rats were randomly divided into 5 groups (N = 10 per group): a control group with age-matched rats (control), a group with BCNI, a group with BCNI treated with h-ADSCs (ADSC), a group with BCNI treated with low-energy SWT (SWT), and a group with BCNI treated with h-ADSCs in conjunction with SWT (ADSC/SWT).

Cavernous Nerve Injury and h-ADSCs Application

After exposing the urinary bladder and prostate by a lower abdominal incision, we identified and located the cavernous nerve stretching toward the rat’s penis from the major pelvic ganglion, as shown in Supplementary Figure S1.

The rats’ abdomens in the control group were closed without any direct cavernous nerve manipulation (sham operation). BCNI was performed in all groups except for the control group. The cavernous nerves on both right and left sides below the major pelvic ganglion were first identified and were then compressed with a hemostat clamp for 2 minutes. In the ADSC and ADSC/SWT groups, 1 × 106 h-ADSCs (diluted in phosphate-buffered saline) were injected around the injured cavernous nerve as seen in Supplementary Figure S1. To track the location of the administered h-ADSCs, they were labeled with a fluorescent dye (Cell Tracker™ CM-Dil; Molecular Probes, Eugene, OR) according to the manufacturer’s protocol.

Shockwave Treatment

Three days after the BCNI surgery, rats in the SWT and ADSC/SWT groups were treated with shockwaves. After anesthesia, the penis was drawn out of the prepuce in a supine position. Ultrasonic gel was applied to the penis, and then the Piezo Wave2 shockwave applicator (Richard Wolf GmbH, Knittlingen, Germany) was placed on the penis. A total of 300 shocks were delivered at an energy level of 0.1 ml/mm² and a frequency of 2 shocks/second during each SWT session. SWT was repeated 3 times per week with a day’s break in between each session, for a total duration of 3 weeks.

Measurement of Erectile Function

Erectile function was assessed 4 weeks after BCNI surgery. The carotid artery and cavernous nerve were exposed to measure the mean arterial pressure (MAP) and the intracavernosal pressure (ICP), respectively.6,7 A 23-gauge butterfly needle containing 250 U/mL heparin solution was inserted into the proximal corpus cavernosum and then connected to a pressure transducer (Grass model S48 K; Astro-Med Inc., West Warwick, RI), to measure the ICP. A bipolar stainless-steel electrical stimulator was used to stimulate the major pelvic ganglion at 10 V for 50 seconds and 2.4 mA with a pulse width of 2.5 milliseconds. The maximal ICP during nerve electrostimulation was calculated from an isometric force transducer and recorded on a computer with a PowerLab commercial data acquisition system (AD Instruments, Dunedin, New Zealand). BD Intramedic PE-50 tubing (BD, Franklin Lakes, NJ) was inserted into the carotid artery for the measurement of MAP. The ratio of ICP to MAP was used to determine the level of erectile function.

After erectile function was assessed, the cavernous nerve and penis were harvested. The cavernous nerve and penis samples were fixed in 4% paraformaldehyde for 24 hours at 4°C before creating a paraffin block. For Western blot analysis and ELISA, the corpus cavernosum samples were frozen in liquid nitrogen.

Immunohistochemistry

The cavernous nerve paraffin sections were immunostained with the primary antibody neuron-specific β-III tubulin (diluted 1:200; Abcam, Cambridge, UK) and mounted with 4,6-diamidino-2-phenylindole (DAP; Vector Laboratories, Inc., Burlingame, CA) to stain the nuclei. The penis paraffin sections were immunostained with rabbit anti-β-III tubulin (1:2000; Abcam, Cambridge, UK) and chicken anti-β-III tubulin (1:2000, Abcam, Cambridge, UK) to stain the nuclei.
with the following primary antibodies: neuron-specific β-III tubulin, neuronal nitric oxide synthase (nNOS), smooth muscle actin (α-SMA), and vascular endothelial growth factor (VEGF; diluted 1:200; Santa Cruz Biotechnologies, Santa Cruz, CA), alpha smooth muscle actin (α-SMA, diluted 1:500; Abcam), and vascular endothelial growth factor (VEGF; diluted 1:200; Santa Cruz Biotechnologies), and were mounted with DAPI to stain the nuclei. Digital images were obtained using a Zeiss LSM 510 Meta confocal microscope (Zeiss, Oberkochen, Germany), and the mean intensity was calculated using ZEN 2009 (Zeiss).

**Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay**

Penis paraffin sections were stained by TUNEL using the In Situ Cell Death Detection Kit, TMR red (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's protocols. The apoptotic index was expressed as the number of TUNEL and DAPI-positive cells in 3 randomly chosen sections of the corpus cavernosum per animal, which were digital images obtained using a Zeiss LSM 510 Meta confocal microscope (Zeiss), and colocalization was calculated using ZEN 2009 (Zeiss).

**Western Blot Analysis**

Corpus cavernosum tissue was homogenized using ice-cold RIPA buffer (Cell Signaling Technology, Danvers, MA) containing ethylene diamine tetra acetic acid-free protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche Diagnostics GmbH). The homogenized sample was then centrifuged at 12,000 × g for 10 minutes at 4°C and its supernatant extracted. This supernatant was electrophoresed on NuPAGE 4%-12% bis-Tris gel (Invitrogen, Carlsbad, CA) and then transferred onto a nitrocellulose membrane. After the transfer, the membrane was blocked with 5% skim milk at room temperature for 1 hour and then incubated with the primary antibodies for rabbit endothelial NOS (eNOS), diluted 1:500 (Abcam), and VEGF, diluted 1:200 (Santa Cruz Biotechnologies). After this, the membrane was incubated with a secondary antibody conjugated to horseradish peroxidase for 1 hour at room temperature. The enhanced chemiluminescence method (Amersham, Arlington Heights, IL) was used for protein detection.

**Assay of Cyclic Guanosine Monophosphate (cGMP)**

Equal amounts of penile tissue from all experimental groups were weighed and treated with 350 mL of 0.1 M HCL and silica beads were added (BioSecEnviro, Inc., Guelph, Canada) for each 60 mg of penile tissue. The resulting sample was processed through a homogenizer (Precellys 24; Bertin Technologies, Montigny-le-Bretonneux, France), centrifuged at 12,000 × g for 10 minutes at 4°C, and then its supernatant was extracted. The cGMP direct immunoassay kit (K372-100; BioVision, Mountain View, CA) was used for detection of cavernous cGMP levels.

**Statistical Analysis**

Statistical analyses were performed using the SPSS version 22.0 software (IBM, Armonk, NY). Data are expressed as the mean ± standard error. The multiple groups were compared using analysis of variance with Tukey’s multiple comparison test for post-test analysis. $P < .05$ was considered statistically significant.

**RESULTS**

**Analysis of Erectile Function**

Representative ICP tracings are shown in Figure 1A. The ICP/MAP ratios in the control, BCNI, ADSC, SWT, and ADSC/SWT groups were $0.69 \pm 0.08$, $0.23 \pm 0.06$, $0.42 \pm 0.12$, $0.40 \pm 0.08$, and $0.55 \pm 0.09$, respectively (Fig. 1B). The ICP/MAP ratio was significantly higher in the ADSC/SWT group than in the ADSC and SWT groups ($P < .05$).

**Histological Analysis of the Cavernous Nerve**

β-III tubulin staining was performed to visualize the cavernous nerve present at the treatment site. The representative images of the control, BCNI, ADSC, SWT, and ADSC/SWT groups are shown in Figure 2A.

The mean intensities of β-III tubulin-positive areas in the control, BCNI, ADSC, SWT, and ADSC/SWT groups were $702.7 \pm 91.11$, $190.9 \pm 45.81$, $365.85 \pm 122.78$, $187 \pm 52.25$, and $377 \pm 153.35$, respectively. β-III tubulin expression increased significantly in the ADSC and ADSC/SWT group compared to that in the BCNI and SWT group (Fig. 2B). CM-DiI-labeled h-ADSCs were localized around the cavernous nerve injury site, indicated by a red color (Fig. 2A).

**Analysis of Smooth Muscle and Apoptosis Index in the Corpus Cavernosum**

In the corpus cavernosum, smooth muscle-positive areas were analyzed by immunohistochemical staining (Fig. 3A). The mean intensities of α-SMA-positive areas in the control, BCNI, ADSC, SWT, and ADSC/SWT groups were $259.8 \pm 22.95$, $58.89 \pm 23.34$, $116.6 \pm 49.26$, $109.5 \pm 26.92$, and $196.89 \pm 59.83$, respectively (Fig. 3B). α-SMA expression was significantly higher in the ADSC/SWT group than in the ADSC and SWT groups ($P < .05$).

TUNEL staining showed nuclear colocalization with DAPI, with cell nuclei stained blue and dead cells stained red (Fig. 3C). The apoptosis indices in the control, BCNI, ADSC, SWT, and ADSC/SWT groups were $9.54 \pm 5.43\%$, $40.5 \pm 4.62\%$, $33.52 \pm 4.42\%$, $30.46 \pm 3.72\%$, and $21.2 \pm 7.74\%$, respectively (Fig. 3D). The apoptotic index was significantly lower in the ADSC/SWT group than in the ADSC and SWT groups ($P < .05$).

**Analysis of VEGF Expression in the Corpus Cavernosum**

Analysis of the immunohistochemical staining showed that the mean intensities of VEGF-positive areas in the control, BCNI, ADSC, SWT, and ADSC/SWT groups were $39.58 \pm 7.69$, $4.11 \pm 8.27$, $27.49 \pm 8.06$, $35.79 \pm 8.27$, and $41.81 \pm 7.69$, respectively (Fig. 4B). In addition, in the Western blot analysis, VEGF expression levels for the control, BCNI, ADSC, SWT, and ADSC/SWT groups were $0.528 \pm 0.14$, $0.303 \pm 0.09$, $0.485 \pm 0.11$, $0.679 \pm 0.12$, and $0.717 \pm 0.14$, respectively (Supplementary Fig. S2C). VEGF expression was significantly higher in both the ADSC/SWT and SWT groups than in the ADSC group ($P < .05$).

**Analysis of NO/cGMP Signaling Pathway**

In the dorsal penile nerve, nerve fibers and nNOS were analyzed by immunohistochemical staining (Fig. 4C). The mean intensities of nNOS-positive areas for the control, BCNI, ADSC, SWT, and ADSC/SWT groups were 70.2, 187, 187, and 153.35, respectively. nNOS expression increased significantly in the ADSC and ADSC/SWT group compared to that in the BCNI and SWT group (Fig. 4B). CM-DiI-labeled h-ADSCs were localized around the cavernous nerve injury site, indicated by a red color (Fig. 2A).
BCNI, ADSC, SWT, and ADSC/SWT were 435.8 ± 90.05, 191.6 ± 41.38, 289.1 ± 39.26, 281.2 ± 54.31, and 382.4 ± 81.29, respectively (Fig. 4D). The nNOS content was significantly higher in the ADSC/SWT group than in the ADSC and SWT groups (P < .05).

The expression of eNOS in the corpus cavernosum, as measured by Western blotting, is shown in Supplementary Figure S2A. The relative band intensities indicating eNOS expression for the control, BCNI, ADSC, SWT, and ADSC/SWT groups were 1.414 ± 0.41, 0.534 ± 0.15, 0.793 ± 0.12, 0.754 ± 0.14, and 1.071 ± 0.23, respectively (Supplementary Fig. S2B). eNOS expression was significantly higher in the ADSC/SWT group than in the ADSC and SWT groups (P < .05).

Finally, cGMP levels measured by ELISA for the control, BCNI, ADSC, SWT, and ADSC/SWT groups were 83.1, 48.2, 59.2, 58.9, 70.5, and 100.2 pmol/g, respectively (Supplementary Fig. S2D). The cGMP level was significantly higher in the ADSC/SWT group than in the ADSC and SWT groups (P < .05).

**COMMENT**

Although robot-assisted laparoscopic RP and nerve-sparing techniques are performed worldwide, there are complications that inevitably occur, including laceration, mechanical stretching, and heat damage to the cavernous nerve. Compromised function of the cavernous nerve after RP is the main cause of atrophy of the corpus cavernosum, loss of neurotransmitters, and fibrosis, all of which contribute to post-prostatectomy ED. Cavernous nerve injury in a rat model induces the apoptosis of penile tissue, which leads to the damage of the subcutaneous smooth muscle cells, eventually giving rise to veno-occlusive dysfunction and thus ED. As a first-line treatment for post-prostatectomy ED, phosphodiesterase type-5 inhibitors are commonly used. However, due to limitations in nerve regeneration, these drugs remain largely ineffective in this population.

Stem cells hold great promise in the future of regenerative medicine due to their multilineage differentiation capacity and neuroregenerative effects, and they have already been utilized in several ED models. Fandel et al reported that intracavernously injected ADSCs initiated neural regeneration in the major pelvic ganglia, most likely due to migration of ADSCs to the injured region. However, conventional studies have only assessed direct injection of cells into the corpus cavernosum. Thus, to enhance the current strategy of ED treatment, we applied stem cells...
directly around the injured cavernous nerve, which we believe is the ultimate cause of ED.

Low-energy SWT, on the other hand, has been known to stimulate vasodilation and angiogenesis, but it has only recently been examined for its potential use in treating ED. Qiu et al observed that ED could be associated with diabetes mellitus induced by streptozotocin. Diabetes mellitus-induced ED involves dysfunction of the nerves, endothelium, and smooth muscle, so Qiu et al utilized low-energy SWT and found that the treatment was able to restore the function of the erectile tissues. However, while low-energy SWT was examined for treating diabetes-associated ED, it had not yet been applied specifically for post-prostatectomy ED. Thus, our study hypothesized that combining the use of h-ADSCs and low-energy SWT would significantly improve the treatment of postprostatectomy ED.

h-ADSCs injected around the injured cavernous nerve demonstrated no significant differences in β-III tubulin expression between the BCNI and SWT groups or between the ADSC and ADSC/SWT groups. This result suggests that low-energy SWT applied to the penis has no role in cavernous nerve recovery, as this addition did not change β-III tubulin expression.

Our results suggest that the combination of h-ADSCs and low-energy SWT could play a key role in preventing smooth muscle atrophy of the corpus cavernosum. However, the exact mechanism of erectile function recovery due to dual therapeutics utilizing h-ADSCs and low-energy SWT still remains unclear.

It is speculated that h-ADSCs injection might contribute to the recovery of the cavernous nerve. ADSCs have been shown to improve conditions of ischemia by accelerating the recovery of the murine nervous system. Furthermore, ADSCs are important precursors to a range of supporting structures of the mature peripheral nervous system, such as Schwann-like cells. Moreover, ADSCs release a range of neurotrophic factors such as epidermal growth factor, transforming growth factor beta-1, VEGF, basic fibroblast growth factor, hepatocyte growth factor,
Figure 3. Alpha smooth muscle actin expression and apoptotic index in the corpus cavernosum. (A) Representative image of alpha-smooth muscle actin (α-SMA) staining in the penile sections. α-SMA is stained green and nuclei are stained blue. Original magnification: ×200. (B) Relative quantification of α-SMA mean intensity in the corpus cavernosum. Each bar shows the mean values (standard deviation). *P < .05 compared with the bilateral cavernous nerve injury (BCNI) group, **P < .01 compared with the BCNI group, ***P < .05. (C) Representative images of apoptosis in the corpus cavernosum. Apoptotic cells are stained red via in situ terminal deoxynucleotidyl transferase dUTP nick end labeling detection, while nuclei are stained blue with 4,6-diamidino-2-phenylindole. Original magnification: ×200. (D) Apoptotic index. *P < .05 compared with the BCNI group, **P < .05.
Figure 4. Vascular endothelial growth factor (VEGF) expression in the corpus cavernosum and neuronal nitric oxide synthase (nNOS) expression in the dorsal penile nerve. (A) Representative images of vascular endothelial growth factor (VEGF) staining (green) in the corpus cavernosum. Original magnification: ×400. (B) Mean intensity of VEGF expression. Each bar shows the mean values (standard deviation). *P < .05 compared with the bilateral cavernous nerve injury (BCNI) group, **P < .05. (C) Immunostaining for nNOS (red) and β-III tubulin (green) in the dorsal penile nerve. Magnification: ×400. (D) Mean intensity of nNOS expression for the dorsal penile nerve cross section. Each bar shows the mean values (standard deviation). *P < .05 compared with the bilateral cavernous nerve injury (BCNI) group, **P < .05.
insulin-like growth factor-I, and brain-derived neurotrophic factor, secreted at different phases of tissue regeneration during the recovery of a damaged peripheral nerve.\textsuperscript{14,15} Although the recovery of the cavernous nerve after h-ADSCs injection is thought to be the result of several neurotrophic factors working in synergy, further study is required to address this hypothesis.

VEGF expression increased as well in the ADSC/SWT group compared to its expression in the BCNI, ADSC, and SWT groups. Meanwhile, ICP/MAP also improved for SWT and ADSC/SWT groups, compared to BCNI, demonstrating that SWT can be used to improve penile blood flow.

SWT has long been noted to trigger high levels of VEGF,\textsuperscript{27,28} which is known to protect endothelial cells from apoptosis.\textsuperscript{29} We demonstrated that the SWT and ADSC/SWT groups had a significantly decreased apoptotic index compared to the BCNI group.

Researchers have shown that VEGF stimulated the phosphorylation of eNOS and elevated the cGMP level, which stimulated penile erection.\textsuperscript{30} Our results also showed that markedly elevated levels of eNOS and cGMP were noted in the ADSC/SWT group compared to that in the ADSC or SWT alone group.

This study has shown that h-ADSCs are capable of enhancing nerve regeneration, as demonstrated by the increased β-III tubulin and nNOS expression. In addition, this study has shown that SWT could upregulate the expression of VEGF and induce neovascularization without causing any adverse effects in the penile tissue or enhancing neurological damage on the cavernous nerve. While the precise molecular mechanism underlying the increased efficacy of the dual treatment is not entirely clear, our results clearly demonstrate that a combination therapy of injecting h-ADSCs and applying low-energy SWT was more effective than separate treatments at enhancing erectile function.

Although our study demonstrated improved erectile function with a combination therapy of h-ADSCs injection and low-energy SWT, further study and research are required. Moreover, our study used 4 weeks after initial h-ADSCs injection and SWT as the end time point, so it is yet unknown if the reported effects last as long as 6 months or 1 year. Still, as the first study to report the beneficial effects of a combination therapy of h-ADSCs injection and SWT, this study can still serve as the basis for future clinical application.

In summary, combination therapy using h-ADSCs and low-energy SWT improves erectile function more than ADSC or SWT alone in our model. The enhanced recovery in the ADSC/SWT group may be related to increased VEGF expression, nNOS, eNOS, higher cGMP levels, and reduced apoptosis of the corpus cavernosum. While h-ADSCs injection plays a role in the recovery of the injured cavernous nerve, the low-energy SWT results in significantly increased expressions of VEGF and vascular supply to the penis and decreased apoptosis of the corpus cavernosum.

### CONCLUSION

The application of h-ADSCs on injured cavernous nerves had an effect on nerve recovery and low-energy SWT on corpus cavernosum improved angiogenesis in the corpus cavernosum. The combination therapy of h-ADSC and low-energy SWT significantly increased VEGF, nNOS, and eNOS expression. In addition, the combination therapy reduced apoptosis in the corpus cavernosum and decreased smooth muscle atrophy.

### References


APPENDIX

SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.urology.2015.10.021.
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