Effects of Intravenous Injection of Adipose-Derived Stem Cells in a Rat Model of Radiation Therapy-Induced Erectile Dysfunction

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Abstract

Introduction—Radiation therapy (RT) for prostate cancer is frequently associated with post-treatment erectile dysfunction (ED).

Aim—To investigate whether injection of adipose-derived stem cells (ADSCs) can ameliorate RT-associated ED.

Methods—Thirty male rats were divided into 3 groups. The Control+PBS group received tail-vein injection of phosphate-buffered saline (PBS). The Radiation+PBS group received radiation over the prostate and tail-vein injection of PBS. The Radiation+ADSC group received radiation over the prostate and tail-vein injection of ADSCs, which were labeled with 5-ethynyl-2-deoxyuridine (EdU). Seventeen weeks later erectile function was evaluated by intracavernous pressure (ICP) in response to electrostimulation of cavernous nerves (CN). Penile tissue and major pelvic ganglia (MPG) were examined by immunofluorescence (IF) and EdU staining.

Main Outcome Measures—Erectile function was measured by ICP. Protein expression was examined by IF, followed by image analysis and quantification.

Results—Radiation over the prostate caused a significant decrease in erectile function and in the expression of neuronal nitric oxide synthase (nNOS) in penis and MPG. Cavernous smooth muscle (CSM) but not endothelial content was also reduced. Injection of ADSCs significantly restored erectile function, nNOS expression, and CSM content in the irradiated rats. EdU-positive cells were visible in MPG.

Conclusions—Radiation appears to cause ED via CN injury. ADSC injection can restore erectile function via CN regeneration.

Introduction

Radiotherapy (RT) is an effective and common treatment for prostate cancer; however, it is frequently associated with post-treatment erectile dysfunction (ED) [1–3]. While the

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etiology of post-RT ED remains unclear, clinical studies have suggested that radiation might damage blood vessels and nerves that supply the penile tissues [1–3]. In addition, studies using RT animal models have shown that radiation caused a reduction of nNOS-containing erectile nerves [4,5] and fibrosis of penile arteries [5,6]. Thus, similar to radical prostatectomy (RP), RT may cause ED via the destruction of cavernous nerves (CN) [1–3].

RT-induced ED is most commonly treated with phosphodiesterase-5 inhibitors (PDE5Is) [1–3]. However, PDE5Is are ineffective in approximately one-third of patients and can produce adverse side effects such as headache and flushing [1–3]. As such, there is a need to identify alternative treatments for patients who cannot be effectively treated with PDE5Is. Previously we and others have demonstrated in animal models that RP-associated ED could be effectively treated with various types of stem cells (SCs) [7–11]. Specifically, after the induction of ED via CN transection or crush injury, intracavernous (IC) injection of SCs significantly improved erectile function when compared to saline-treated controls. Furthermore, the improved erectile function was accompanied by regeneration of nNOS-containing nerves in the corpus cavernosum. Thus, SC therapy has the potential to restore the natural erectile capability, as opposed to symptomatic relief afforded by agents such as PDE5Is.

In the present study we tested two hypotheses: (1) RT can cause ED via destruction of nNOS-containing nerves, and (2) intravenous (IV) injection of adipose-derived stem cells (ADSCs) can restore erectile function via regeneration of nNOS-containing nerves.

Materials and methods

Animal groups

Thirty-five 12-weeks-old Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA, USA). Five of them were used for the purpose of ADSC isolation while the remaining ones were used for the actual experimentation. The Control +PBS group (n=6) received tail-vein injection of phosphate-buffered saline (PBS). The Radiation+PBS group (n=12) received radiation over the prostate and tail-vein injection of PBS. The Radiation+ADSC group (n=12) received radiation over the prostate and tail-vein injection of ADSCs. All procedures were approved by Institutional Animal Care and Use Committee. Note that in our previous study [12] injection of ADSCs into normal rats (equivalent of a Control+ADSC group) did not have an effect on the erectile function.

ADSC isolation and labeling

ADSCs were isolated from paratesticular fat and cultured as described previously [7,12]. They were labeled with thymidine analog 5-ethynyl-2-deoxyuridine (EdU) (Invitrogen, Carlsbad, CA, USA) for 48 h before injection.

Radiation and ADSC injection

Initially, rats in the Radiation+PBS and Radiation+ADSC groups were irradiated according to van der Wielen et al [6]. However, half of the rats died within two weeks. Thus, we reduced both the radiation dosage and window size, as described in the following procedure. The rats were anesthetized with ketamine (100mg/kg) and midazolam (5mg/kg). A 3-mm-thick lead shield with a 25mm×30mm window was used to limit the radiation to the prostate. Radiation was delivered with a J.L. Shepherd & Associates Mark I radiator (Model: 68-A, San Fernando, CA, USA) operating at 662 keV with a dose rate of 1.65 Gy/min. For the first radiation session, 4 Gy/day was delivered for 5 consecutive days. One week later, the rats were anesthetized with isoflurane and injected through the tail vein with one million ADSCs in 400μl PBS or with 400μl PBS alone. Eleven weeks later, the rats were irradiated with 2

Gy/day for 5 consecutive days. Another 6 weeks later, the rats were evaluated for erectile function.

Erectile function evaluation

Intracavernous pressure (ICP) in response to electrostimulation of the CN was used to evaluate erectile function. Briefly, under ketamine (100mg/kg) and midazolam (5mg/kg) anesthesia, the major pelvic ganglion (MPG) and CN were exposed via midline laparotomy. The corpus cavernosum was cannulated with a heparinized (200U/ml) 25G needle connected to a pressure transducer (Utah Medical Products, Midvale, UT, USA). The stimulus parameters were 20Hz, pulse width of 0.2 ms, and duration of 50s with three different current settings: 0.5 mA, 1.0 mA and 1.5 mA. The maximum increase of ICP of three stimuli per side was selected for statistical analysis in each animal. ICP was normalized to mean arterial pressure (MAP), which was recorded using a 25G needle inserted into the aortic bifurcation.

Histology

At the conclusion of erectile function evaluation, the rats were sacrificed and their penis and MPG harvested for histology. The mid-shaft of the penis and MPG were fixed with 2% formaldehyde and 0.002% picric acid in 0.1M PBS for 4 hours, followed by immersion in 30% sucrose in PBS overnight at 4°C. The fixed tissues were then embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA), cut into 5 μm-thick sections, mounted on glass slides (3 sections per slide), and subjected to immunofluorescent (IF) and EdU staining. For IF staining, the slides were placed in 0.3% H_2O_2/methanol for 10 min, washed twice in PBS for 5 min and incubated with 3% horse serum in PBS/0.3% Triton X-100 for 30 min at room temperature. After draining this solution from the tissue section, the slides were incubated at room temperature with rabbit anti-nNOS (1:100, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), mouse anti-α smooth muscle actin (α-SMA) (1:1000, Sigma-Aldrich, St. Louis, MO, USA), mouse anti-S100 (1:500, Chemicon, Temecula, CA, USA), or mouse anti-von Willebrand (vWF) (1:400, Abcam Inc., Cambridge, MA, USA) overnight. Control tissue sections were similarly prepared except no primary antibody was added. After rinses with PBS, the sections were incubated with Alexa-488- or Alexa-594-conjugated secondary antibody (Invitrogen). After rinses with PBS, the slides were incubated with freshly made Click-iT reaction cocktail containing Alexa-594 (Invitrogen) for 30 min at room temperature without light followed by staining with 4',6-diamidino-2-phenylindole (DAPI, for nuclear staining, 1 μg/ml, Sigma-Aldrich).

Image analysis and quantification

The stained tissues were examined with Nikon Eclipse E600 fluorescence microscope and photographed with Retiga 1300 Q-imaging camera using the ACT-1 software (Nikon Instruments Inc., Melville, NY). For evaluation of cavernous smooth muscle and endothelium contents, two fields (both sides of the corpus cavernosum at 100X magnification) on each tissue section were photographed. For evaluation of arterial endothelium content, two fields (both dorsal arteries at 400X magnification) on each tissue section were photographed. For evaluation of dorsal nerve nNOS expression, two fields (the two largest dorsal nerve branches at 400X magnification) on each tissue section were photographed. For evaluation of MPG nNOS expression, two random fields at 400X magnification on each tissue section were photographed. In each of these photographic recordings the images were generated in green, red, and blue channels, and these single-color images were then superimposed to generate the multi-color figures. For quantification, the single-color images were analyzed with Image-Plus 5.1 software (Media Cybernetics, Bethesda, MD, USA). To quantify cavernous smooth muscle, α-SMA-stained area (in green) was measured and expressed as a percentage of the entire corpus cavernosa. To

quantify cavernous endothelium, vWF-stained area (in red) was measured and expressed as a percentage of the entire corpus cavernosa. To quantify arterial endothelium, vWF-stained area (in red) was measured and expressed as a ratio (in percentage) to the α-SMA-stained area. To quantify nNOS in dorsal nerves, nNOS-stained area (in red) was measured and expressed as a percentage of the entire dorsal nerve. To quantify nNOS in MPG, nNOS-positive cells (in red) per 400× field were counted.

Statistical analysis

Data were analyzed using Prism 4 (GraphPad Software, San Diego, CA, USA) and expressed as mean ± standard error of mean (SEM). Multiple groups were compared using one-way analysis of variance followed by the Tukey-Kramer test for post-hoc comparisons. Statistical significance was set at $p < 0.05$.

Results

ADSCs restore radiation-impaired erectile function

Radiation over the prostate significantly impaired erectile function as seen in the sharp decline of the ICP/MAP value in radiated rats versus normal control (Fig. 1). Injection of ADSCs significantly restored erectile function to levels similar to normal control.

ADSCs promote nerve regeneration

Radiation over the prostate caused significant decreases of nNOS-containing nerves in the penis and nNOS-expressing neurons in the MPG (Fig. 2). Injection of ADSCs partially but significantly restored these nNOS-positive nerves and neurons.

ADSCs improve cavernous smooth muscle content

Radiation over the prostate caused a significant decrease of CSM (Fig. 3). Injection of ADSCs partially but significantly restored the CSM content.

Radiation does not affect endothelial content

Radiation over the prostate had no effect on the endothelial content in either the dorsal arteries or cavernous sinusoids (Fig. 4). Injection of ADSCs did not affect these endothelial contents either.

IV-injected ADSCs migrate to MPG

Seventeen weeks after their IV injection, ADSCs were undetectable in the penis but were scantly visible in the MPG (Fig. 5).

Discussion

Although highly effective in treating prostate cancer, RT and RP are both frequently associated with post-treatment ED. However, while RP-associated ED has been studied extensively, RT-associated ED has received little attention in terms of animal model-based studies. Likewise, while there is a large body of literature in the experimental treatment of RP-associated ED, no such studies for RT-associated ED exist. As such, the present study was undertaken to address these deficiencies in ED research.

Despite using different radiation protocols, all animal studies, including the present study, have confirmed clinical findings that radiation over the prostate can cause ED [4,5,13,14]. However, among those studies that have conducted histological examination of the penis, their focuses differ and outcomes vary. First, Carrier et al found decreased nNOS-containing

nerves in the proximal penises of rats 5 months after a single irradiation of 10 or 20 Gy [4]. Second, the study by van der Wielen et al, which did not measure erectile function, described reduced smooth muscle and increased collagen in dorsal arteries and cavernosal arteries in the proximal penises of rats 2 to 9 weeks after fractionated irradiation of 7.4 Gy/day for 5 consecutive days [6]. Whether there were changes in the nerves, cavernous smooth muscle (CSM), or endothelium was not mentioned. Third, in a recent study by Kimura et al, a single fraction of 20 Gy was delivered to rat prostate [5]. Four weeks later reduced nNOS expression was noted in CN, and 9 weeks later reduced expression of smooth muscle actin and eNOS was noted in the corpus cavernosum while CD31 expression was unaffected. In the present study, we also noted reduced expression of nNOS and smooth muscle actin as well as unchanged endothelial vWF expression. Thus, it appears that radiation on the prostate causes CN injury, and which leads to CSM atrophy. This hypothesis, while requiring further investigation, suggests that both RP- and RT-associated ED are mediated by similar mechanisms, namely, initial CN injury and subsequent CSM atrophy.

RP-associated ED has been the subject of experimental SC therapy in recent years, and the overall outcome appears promising [15]. However, the mechanism responsible for such therapeutic efficacy remains poorly understood. In particular we have raised the question on how the commonly employed intracavernous (IC) injection of SC can lead to regeneration of CN when the nerve cell bodies are located some distance away in the major pelvic ganglia (MPG) [16]. By tracking fluorescently labeled SCs we discovered that IC injected SCs traveled to bone marrow and MPG [12,16]. We thus suggested that, due to similarities between cavernous sinusoids and blood vessels, IC injection is similar to IV injection, and IC injected SCs therefore can travel through bloodstream to various organs and tissues [16]. We also discovered that the MPG of CN injury rats expressed SC homing factor SDF-1, suggesting its role in attracting IC injected SCs to the MPG [12]. In another study we showed that ADSCs exerted neurotrophic effects on CN by secreting CXCL5 cytokine at a high level [17]. Together, these studies establish that CN injury induces SDF-1 expression in MPG, thereby attracting SC migration, and SCs secrete neurotrophic factors, thereby promoting CN regeneration.

In the present study we first confirmed previous findings [4,5] that RT can cause CN injury. We then demonstrated for the first time that IV-injected ADSCs could travel to MPG, repair CN, rebuild CSM, and restore erectile function. Because ADSCs can be easily and safely isolated in large quantity, their transplantation through IC or IV injection should represent a promising therapy for RT-associated ED. However, it should be cautioned that the present study is still preliminary and requires further validation. For example, to better simulate clinical situations, we need to test whether SC injection can prevent the progression of CN degeneration by conducting a single-course radiation (i.e., without the post-ADSC radiation, which was designed to test ADSC's "protective" effects). We also need to test various cell dosages and time points in order to validate the present study and identify the optimal therapeutic regimen.

Conclusions

Radiation over the prostate can cause ED via CN injury. IV injection of ADSCs can restore erectile function via CN regeneration.

Acknowledgments

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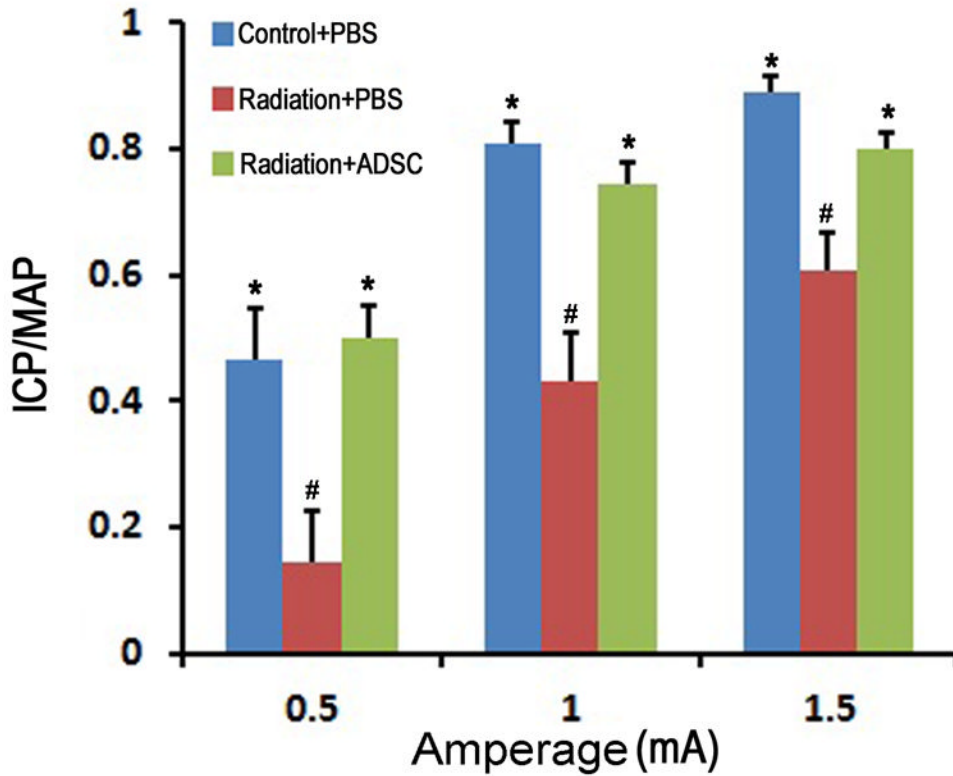
Figure 1. Evaluation of erectile function
Rats in Control+PBS group (n=6) received tail-vein injection of PBS. Rats in Radiation +PBS group (n=12) received radiation over the prostate and tail-vein injection of PBS. Rats in Radiation+ADSC group (n=12) received radiation over the prostate and tail-vein injection of ADSCs. Their erectile function was evaluated as response in ICP to electrostimulation of CN at three different amperages (0.5, 1.0, and 1.5). ICP was normalized with MAP. # and * denote $p < 0.05$ compared to the Control+PBS and Radiation+PBS groups, respectively.
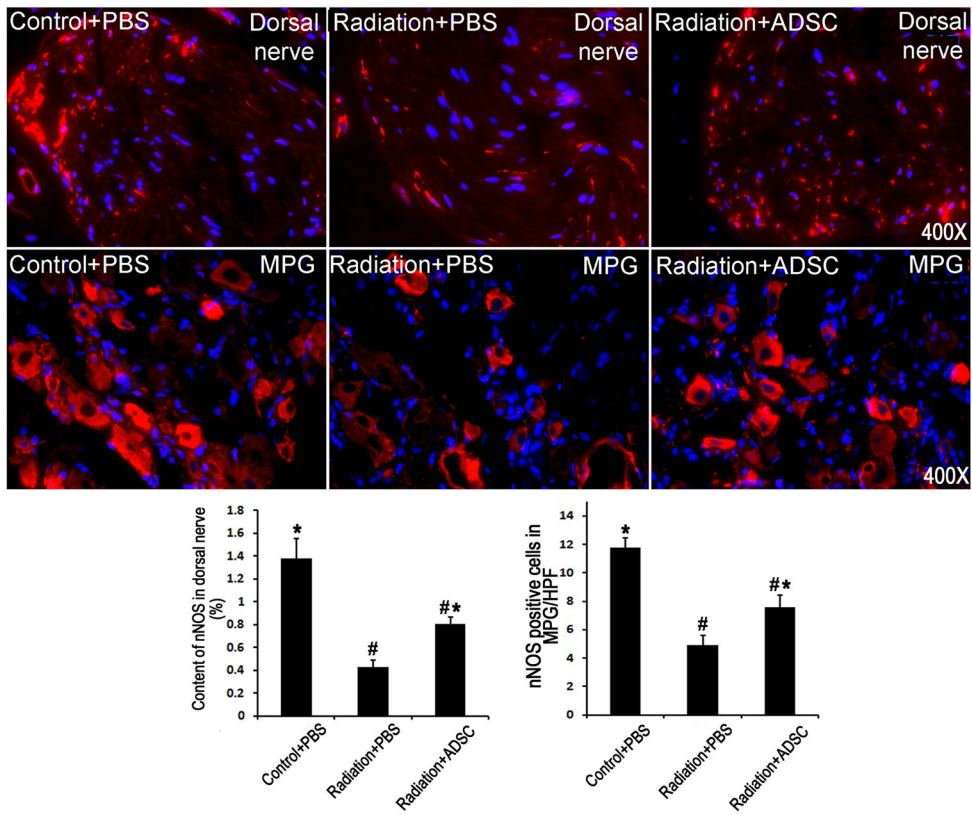
Figure 2. Evaluation of nNOS expression in penile dorsal nerves and MPG
Rats in Control+PBS group (n=6) received tail-vein injection of PBS. Rats in Radiation +PBS group (n=12) received radiation over the prostate and tail-vein injection of PBS. Rats in Radiation+ADSC group (n=12) received radiation over the prostate and tail-vein injection of ADSCs. Their penile tissues and MPG were examined by immunofluorescence staining for nNOS expression. The results are shown in the representative histological images with red and blue stains indicating nNOS expression and cell nuclei, respectively. Quantification of nNOS expression in penile dorsal nerves is shown in the left bar chart. Numbers of nNOS-positive cells in MPG are shown in the right bar chart. # and * denote $p<0.05$ compared to the Control+PBS and Radiation+PBS groups, respectively.
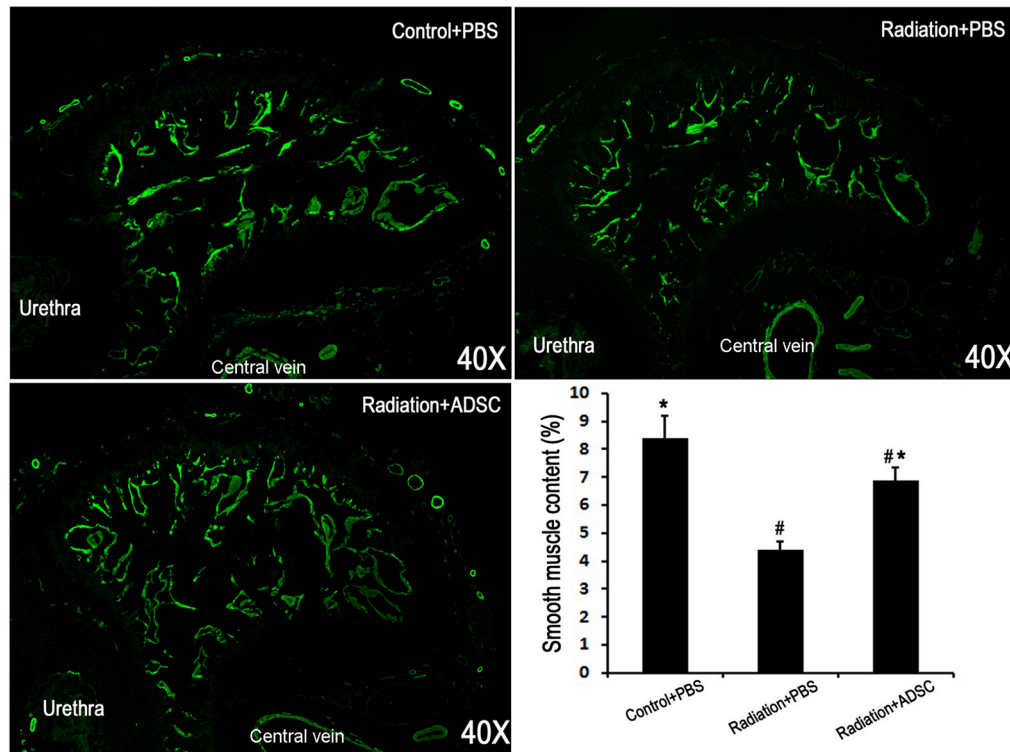
Figure 3. Evaluation of smooth muscle content in corpus cavernosum
Rats in Control+PBS group (n=6) received tail-vein injection of PBS. Rats in Radiation
+PBS group (n=12) received radiation over the prostate and tail-vein injection of PBS. Rats
in Radiation+ADSC group (n=12) received radiation over the prostate and tail-vein injection
of ADSCs. Their penile tissues were examined by immunofluorescence staining for α-SMA
expression. The results are shown in the representative histological images with green stain
indicating α-SMA expression. Quantification of α-SMA expression in corpus cavernosa is
shown in the bar chart. # and * denote $p < 0.05$ compared to the Control+PBS and Radiation
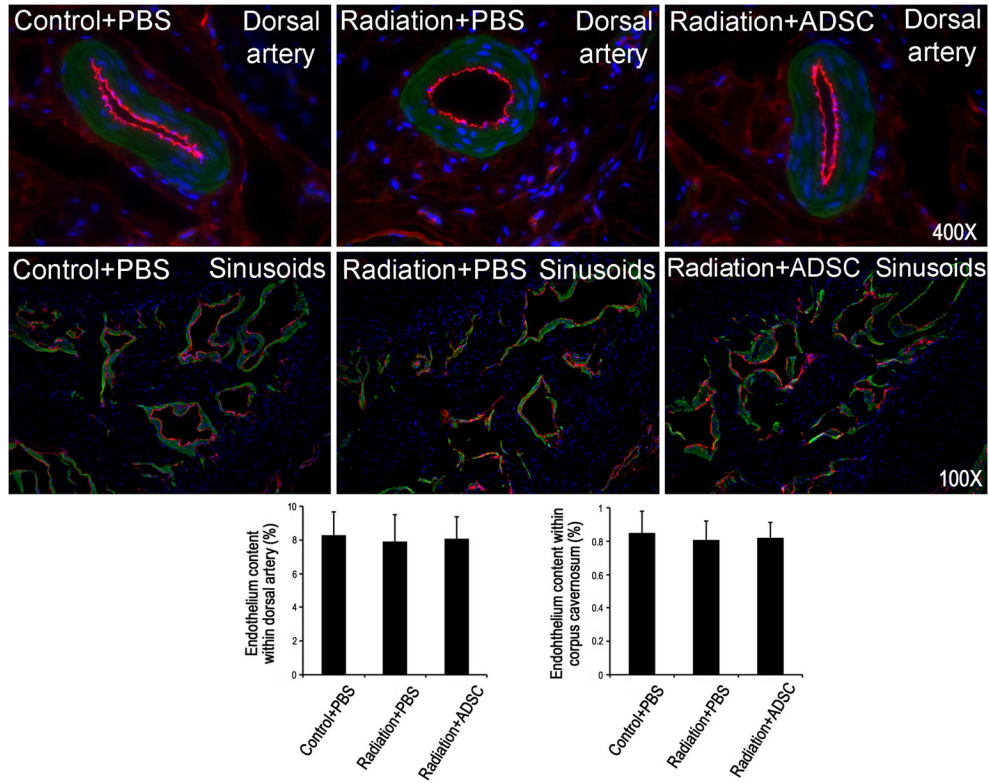+PBS groups, respectively.

Figure 4. Evaluation of endothelial content in dorsal arteries and corpus cavernosa
Rats in Control+PBS group (n=6) received tail-vein injection of PBS. Rats in Radiation
+PBS group (n=12) received radiation over the prostate and tail-vein injection of PBS. Rats
in Radiation+ADSC group (n=12) received radiation over the prostate and tail-vein injection
of ADSCs. Their penile tissues were examined by immunofluorescence staining for vWF
and α-SMA expression. The results are shown in the representative histological images with
red, green, and blue stains indicating vWF, α-SMA, and cell nuclei, respectively.
Quantification of vWF expression in dorsal arteries and corpus cavernosa is shown in the
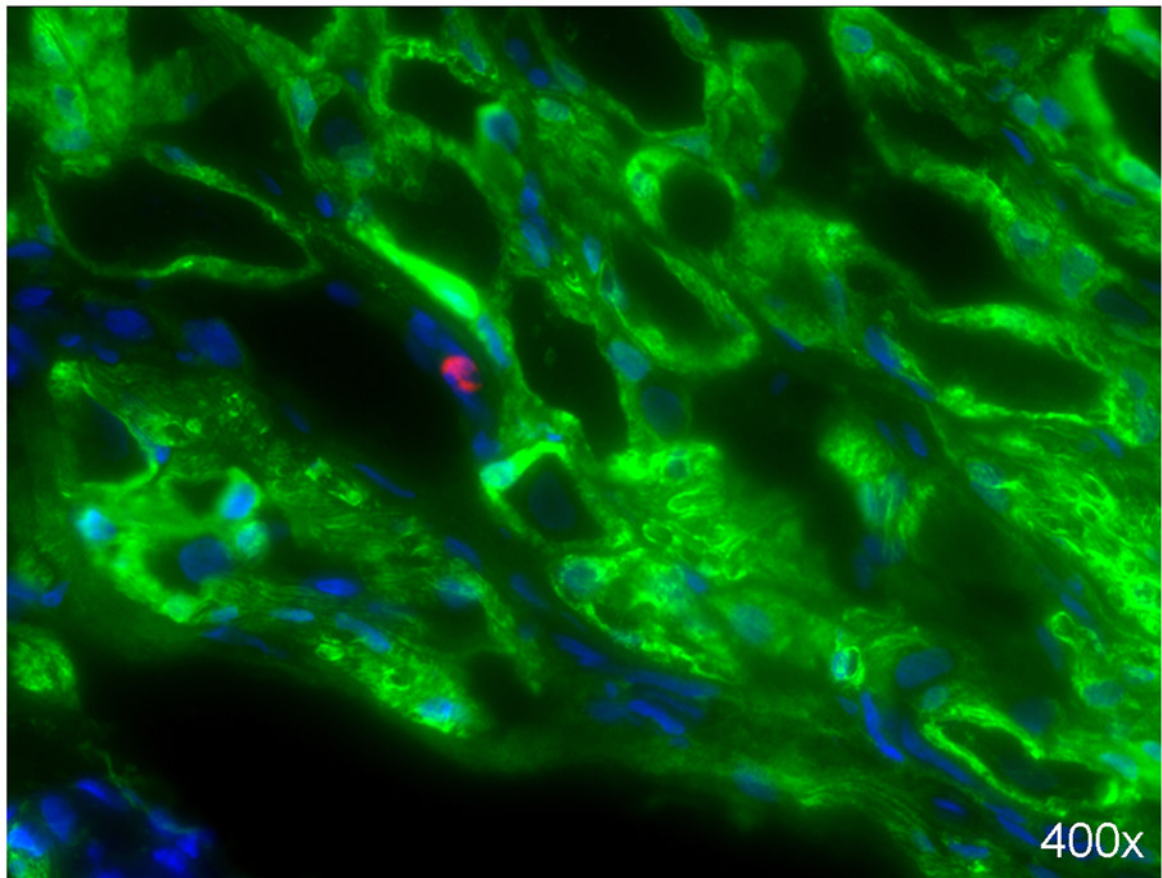left and right bar charts, respectively.

Figure 5. Identification of transplanted ADSCs in MPG
ADSCs were labeled with EdU prior to their injection into rats in the Radiation+ADSC group. Seventeen weeks later, a few ADSCs (red stain) were visible in the MPG, in juxtaposition to S100-stained (green) nerves. Cell nuclei are stained blue.