Original Article

A flavonoid derivative of icariside II (YS-10) improves erectile dysfunction in radiation-injured rats via oxidative stress pathway

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Background: We explored the preventive effect and mechanism of YS-10, a novel synthesized flavonoid derivative based on the structure of icariside II (ICA II), on a rat model of radiation-induced erectile-dysfunction (Ri-ED).

Methods: Eighteen 10-week-old male Sprague-Dawley (SD) rats were randomly divided into 3 groups. Six rats were used as the control group (Control), and the remaining 12 were given a single X-ray irradiation of 20 Gy in the prostate and then randomly divided into the radiation injury group (Ri-ED group) and YS-10 treatment group (Ri-ED+YS-10, 2.5 mg/kg/day). After 4 weeks of drug administration and a 2-week drug washout period in the YS-10 treatment group, the erectile function of the animals was evaluated, and the tissues were collected for histopathological analysis and detection of oxidative stress indicators.

Results: After radiation injury, the ratio of maximum intracavernosal pressure (ICP) to mean arterial pressure (MAP), the number of neuronal nitric oxide synthase (n-NOS) positive nerve fibers in the penis cavernosa, endothelial cell content, and n-NOS and endothelial nitric oxide synthase (e-NOS) proteins in the Ri-ED group were significantly lower than those in control group. Compared with the control group, the Ri-ED group had lower superoxide dismutase (SOD) levels and higher malondialdehyde (MDA) levels. Compared with the Ri-ED group, the YS-10 group had a significant increase in the ratio of ICP/MAP in the corpus cavernosum (0.59±0.06 *vs.* 0.43±0.06, P<0.01), the number of n-NOS positive nerve fibers, and the content of endothelial cells. The protein content of n-NOS and e-NOS in the corpus cavernosum increased and could significantly reduce the level of MDA (2.67±0.27 *vs.* 3.25±0.21, P<0.05).

Conclusions: As a novel ICA II derivative, YS-10 could significantly improve the erectile dysfunction and pathological damage in rats caused by radiation injury, and its mechanism may be related to the improvement of radiation-induced oxidative stress.

Keywords: Flavonoid derivative YS-10; radiation injury; erectile dysfunction; oxidative stress

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Introduction

Worldwide, nearly 200,000 men are diagnosed with prostate cancer each year (1). Radiotherapy is a common method for the treatment of prostate cancer, but the incidence of erectile dysfunction caused by radiation [radiation-induced erectile-dysfunction (Ri-ED)] can be as high as 50% (2). Sexual health problems severely impact the quality of life of patients and their partners (3,4). Phosphodiesterase 5 inhibitors (PDE5-Is) are recommended as first-line treatment for ED but are less effective in the treatment of Ri-ED. The results of one prospective clinical trial showed that the efficacy of PDE5-Is in Ri-ED patients decreased from 68% in the first year to 38% in the third year (5).

The reason for this difference in efficacy may be that PDE5-Is can only transiently improve the hemodynamics of cavernous tissue and have no therapeutic effect on other pathological lesions of Ri-ED, such as apoptosis of smooth muscle cells and endothelial cells and reduction of neuronal nitric oxide synthase (n-NOS)-positive fibers. Therefore, a new and more effective treatment method is needed. Currently, there are few studies on the pathogenesis of Ri-ED. Previous studies have shown that the level of reactive oxygen species (ROS) in tissues increases after radiotherapy, and oxidative stress injury may play an important role in the development of Ri-ED (6). After radiation damage, the pathological damage can be repaired through antioxidative drug treatment (3,7).

Previous studies have demonstrated that the traditional Chinese medicine *Epimedium* has the effect of improving erectile dysfunction in animal models (8). Both the monomeric icariin (ICA) extracted from *Epimedium*, and its metabolite icariside II (ICA II) (9) could improve penile erectile function in various animal models (10). The mechanism may be related to the expression level of nitric oxide synthase (NOS) genes and protein in corpus cavernosum (11,12). However, considering the limited availability of Herba Epimedii, the exorbitant cost of extraction, and low bioavailability of ICA and ICA II, it is infeasible for use in clinical settings (13). A new synthetic ICA derivative (YS-10) embodies the common structure of flavonoids, 8-prenylkaempferol. YS-10 can be obtained by manual synthesis. Therefore, this is readily seen at a much lower cost than ICAII derived from plant isolates. Recently, YS-10 has been reported to treat erectile dysfunction. Through animal experiments, it was shown that YS-10 had the effect of repairing the pathological damage of the corpus cavernosum and improving erectile function, and the efficacy was not lower than ICA II (14). However, the pharmacological mechanism of YS-10 is still unclear.

In the current study, YS-10 was analyzed for its possible mechanism in treating Ri-ED in a rat model. We present the following article in accordance with the ARRIVE reporting checklist (available at https://tau.amegroups.com/article/view/10.21037/tau-22-376/rc).

Methods

Animals and establishment of ED model

A total of 18 Sprague-Dawley (SD) male rats (10 weeks old, weighing 280±20 g) were purchased from Keyu Biotechnology Co. [Beijing, China, License No. SCXK (Beijing) 2018-0010] and raised in the Animal Breeding Center of the Affiliated Hospital of Changchun University of Chinese Medicine. Animal experiments were performed under a project license (No. 2020113) granted by Animal Ethics Committee of the Affiliated Hospital of Changchun University of Chinese Medicine, in compliance with Changchun University of Chinese Medicine (Jilin, China) guidelines for the care and use of animals. A protocol was prepared before the study without registration. Six rats were marked as control group (Control). The remaining 12 rats were given a single X-ray irradiation of 20 Gy to the prostate. Briefly, the rats were anesthetized by intraperitoneal injection of 3% sodium pentobarbital (30 mg/kg), and a single dose of 20 Gy (2 Gy/min) was delivered by a medical linear accelerator. After irradiation, the rats were randomly divided into 2 equal groups: Ri-ED group and YS-10 treatment group (Ri-ED+YS-10, 2.5 mg/kg/day) (14). Twenty-four hours after irradiation, the 3 groups were treated with a vehicle (Control and Ri-ED groups) and YS-10 (Ri-ED+YS-10 group, 2.5 mg/kg) dissolved in polyethylene glycol 400 (PEG400) via intragastric administration daily for 4 weeks, followed by a washout period of 2 weeks. All the rats were examined for erectile function, and the penises and major pelvic ganglia (MPG) were harvested for further investigations.

Evaluation of hemodynamic changes in corpus cavernous

After anesthetization with 5% pentobarbital sodium (30 mg/kg), two 24-gauge puncture needles were filled with 200 IU/mL heparin, and one end was connected to a MP150 multi-conductivity physiology instrument (Biopac Systems Inc., Goleta, CA, USA) through a PE-50 tube.

The left common carotid artery was dissociated, a puncture needle was inserted, and the mean arterial pressure (MAP) of the rats was monitored. The skin of the penis was incised to expose the rat corpus cavernosum, and another puncture needle was inserted into the corpus cavernosum sinus. Penile erection was induced by electrical stimulation of the cavernous nerve. The stimulation parameters were 5 V, 1.5 mA, 20 Hz, pulse width 1 ms, duration 60 seconds, and the maximum value of intracavernosal pressure (ICP) and MAP were recorded at the same time. Penile erection in rats was evaluated by comparing ICP/MAP in each group.

Masson staining and immunohistochemical (IHC)

The rat penis midsection tissue was taken, fixed with 4% paraformaldehyde for 24 hours, dehydrated with graded alcohol, embedded in paraffin, and sliced into 5-μm-thick sections for Masson staining and IHC. Staining was performed according to the instructions of the Masson's staining kit (Solarbio, Beijing, China) and the IHC and concentrated 3,3'-diaminobenzidine (DAB) kits (ZSGB Biotech, Beijing, China), and the slides were mounted with neutral gum. Images were observed and collected using a BX41 optical microscope (Olympus, Tokyo, Japan). Image J software was used to analyze the images. Collagen deposition in the corpus cavernosum was assessed as the ratio of smooth muscle (red) to collagen (green) areas in the image. Penile smooth muscle distribution was assessed using the proportion of smooth muscle positive area (brown).

Immunofluorescence

After dehydration with 30% sucrose for 2 days, penises were immersed in optimal cutting temperature (OCT) compound and cut into 5-μm-thick sections at –20 ℃. The sections were baked for 30 minutes, rinsed with phosphate-buffered saline (PBS) for 5 minutes, permeabilized with 0.3% Triton for 15 minutes, rinsed with PBS for 5 minutes again, then blocked with 5% bovine serum albumin (BSA; Amresco, Solon, OH, USA) for 1 hour. Primary antibodies, including alpha-smooth muscle actin (α-SMA, 1:200; Abcam, Cambridge, MA, USA), neurofilament (NF, 1:500; Abcam), n-NOS (1:200, Abcam), and von Willebrand factor (vWF, 1:100, Abcam), were incubated at 4 ℃ overnight. The sections were then incubated with secondary antibody (goat anti-rabbit IgG, 1:200; Abcam) for 1 hour in the dark for fluorescent labeling. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA, USA). All sections were detected and recorded using the DMI 6000B inverted microscope camera system (Leica, Wetzlar, Germany).

Western blot

The penile tissue proteins were extracted and quantified by bicinchoninic acid (BCA) assay. The proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% nonfat dry milk for 1 hour at room temperature and then incubated overnight at 4 ℃ with the corresponding primary antibodies at the following concentrations: anti-α-SMA (1:2,000, Abcam), anti-β-actin (1:10,000, Abcam), anti-n-NOS (1:250, Abcam), and anti-e-NOS (1:250, Abcam). After washing the membrane at room temperature, the membrane was incubated with secondary antibody (1:1,000) for 90 minutes. An enhanced chemiluminescence (ECL) kit was used to develop images, and the ChemiImager 4000 and gel imaging system (Alpha Innotech, Santa Clara, CA, USA) was used to photograph and measure the absorbance of each protein band.

Oxidative stress indicators

Tissue was homogenized in an ice bath at a ratio of 100 μL of sample preparation solution per 10 mg of tissue. The homogenate was centrifuged at 12,000 g for 5 minutes at 4 ℃, and the supernatant was taken as the sample to be tested. The operation was carried out in strict accordance with the instructions of the superoxide dismutase (SOD) and malondialdehyde (MDA) detection kits (Beyotime Biotech, Shanghai, China). The absorbance of the sample was recorded using an ELx808 absorbance light microplate reader (BioTeK, Winooski, VT, USA), and MDA content and SOD activity in the tissue were determined, respectively.

Statistical analysis

All experimental data are presented as mean ± standard deviation. Image J software was used for semiquantitative analysis of the density and proportion of positive areas. SPSS 26.0 statistical software was used to perform one-way analysis of variance (ANOVA) on the data. Statistical

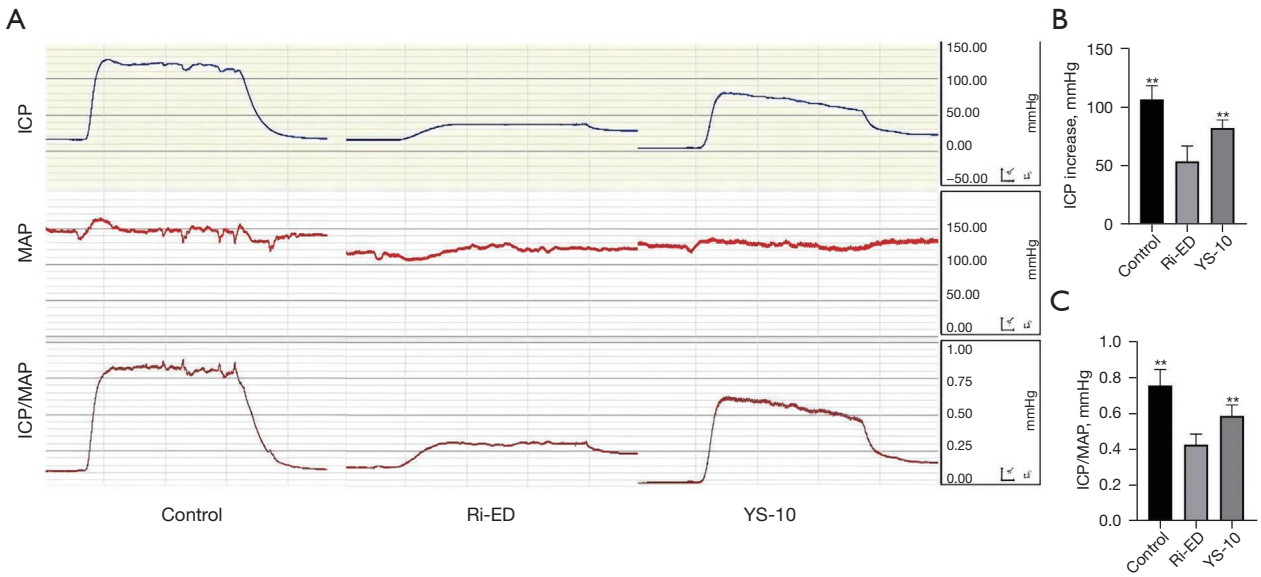

Figure 1 Ratio of ICP/MAP under 5 V electrical stimulation (n=6). (A) Representative raw recordings of ICP, MAP, and ICP/MAP. (B) The increase of ICP in the Control group, Ri-ED group, and YS-10 treatment group under 5 V electrical stimulation. (C) ICP /MAP ratio after treatment. The columns indicate the mean standard deviation ($\bar{x}$±s); **, P<0.01 versus the Ri-ED group. ICP, maximum intracavernosal pressure; MAP, mean arterial pressure; Ri-ED, radiation-induced erectile-dysfunction.

significance was set at P<0.05.

Results

YS-10 improves erectile function effectively

Erectile function was measured after drug clearance for 2 weeks in all groups. The ICP/MAP of the control group (0.76±0.09) was significantly higher than that of the Ri-ED group (0.43±0.06, P<0.01). After 4 weeks of intragastric administration, the growth value of ICP (0.59±0.06) and the ratio of ICP/MAP in the YS-10 group were significantly higher than those in the Ri-ED group (P<0.01) but were still slightly lower than those in the control group (P>0.05) (*Figure 1*).

Histological changes of corpus cavernosum

Masson staining showed that compared with the control group (0.09±0.02), the smooth muscle/collagen ratio of the Ri-ED group (0.05±0.02) was significantly decreased (P<0.01). After treatment, the smooth muscle/collagen ratio of the YS-10 group (0.08±0.01) was increased (P<0.05) (*Figure 2A,2B*).

IHC staining showed that compared with the control group (0.11±0.02), the positive area ratio of smooth muscle in the Ri-ED group (0.06±0.02) was significantly lower (P<0.05). After treatment, compared with the Ri-ED group, the positive area ratio of smooth muscle in the YS-10 group (0.09±0.01) was significantly improved (P<0.05). (*Figure 2C,2D*).

Western blot showed that the content of α-SMA in corpus cavernosum in the Ri-ED group (0.51±0.20) was significantly lower than that in the control group (1.52±0.34) (P<0.05), and the YS-10 group (0.83±0.16) was significantly lower than the Ri-ED group (0.83±0.16). Compared with the Ri-ED group, the content of α-SMA in the corpus cavernosum increased (P<0.05) (*Figure 2E,2F*).

YS-10 promotes endothelial cell and nerve function recovery and regeneration

Immunofluorescence staining (*Figure 3*) showed that compared with the control group (1.33±0.31), the content of vWF in the Ri-ED group (0.38±0.24) was significantly lower (P<0.01). After 4 weeks of treatment, compared with the Ri-ED group, the vWF content of the YS10 group (1.08±0.21) was significantly improved (P<0.01).

Fluorescence immunostaining of the MPG (*Figure 4*) showed that YS-10 could increase the mean density of NF

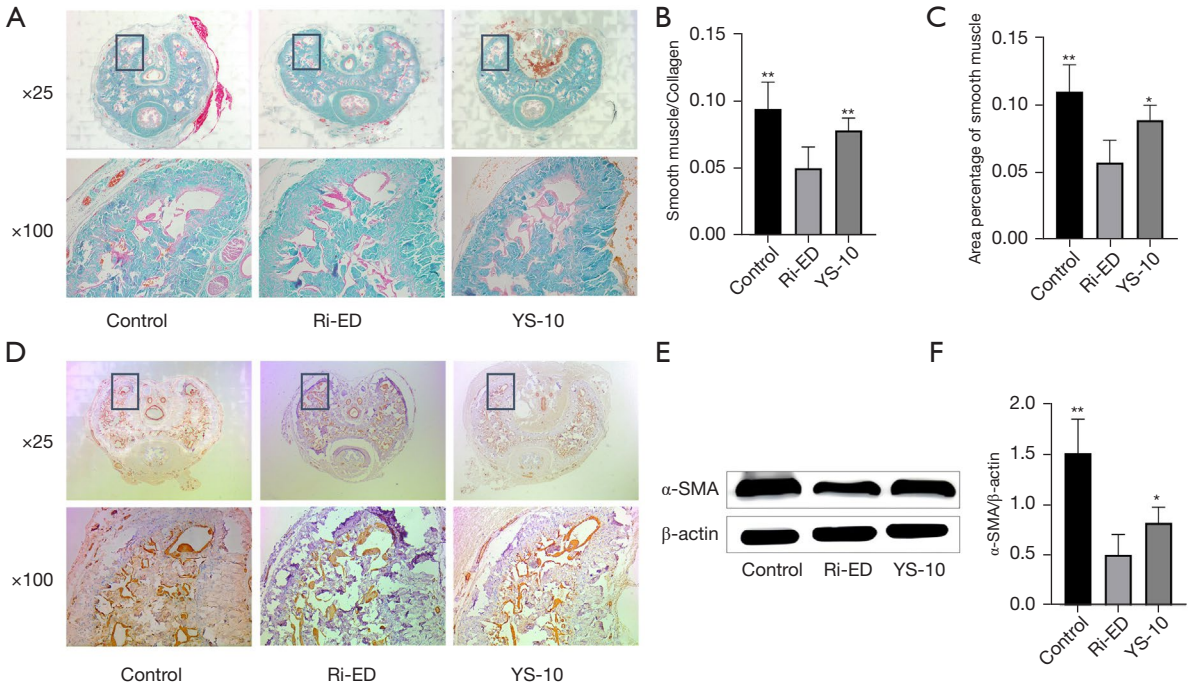

Figure 2 Expression of smooth muscle content in penile tissue of rats in each group (n=6). (A) Microscopic images of penile smooth muscle (red-indicated) and collagen (green-indicated); the lower 3 images are enlarged versions (×100) corresponding to the 3 boxes in the upper images (×25, Masson staining). (B) Ratio of penile smooth muscle to collagen; the columns indicate the mean standard deviation ($\bar{x}$±s). (C) The proportion of smooth muscle positive area in cavernosal tissue; the columns indicate the mean standard deviation ($\bar{x}$±s). (D) Immunohistochemical images of penile smooth muscle and cavernosal tissue; the lower 3 images are enlarged versions (×100) corresponding to the 3 boxes in the upper images (×25). (E) Western blot assessment of α-SMA expression in various groups of rats. (F) α-SMA in penile tissue of rats in each group; the columns indicate the mean standard deviation ($\bar{x}$±s). *, P<0.05 versus the Ri-ED group; **, P<0.01 versus the Ri-ED group. Ri-ED, radiation-induced erectile-dysfunction; α-SMA, alpha-smooth muscle actin.

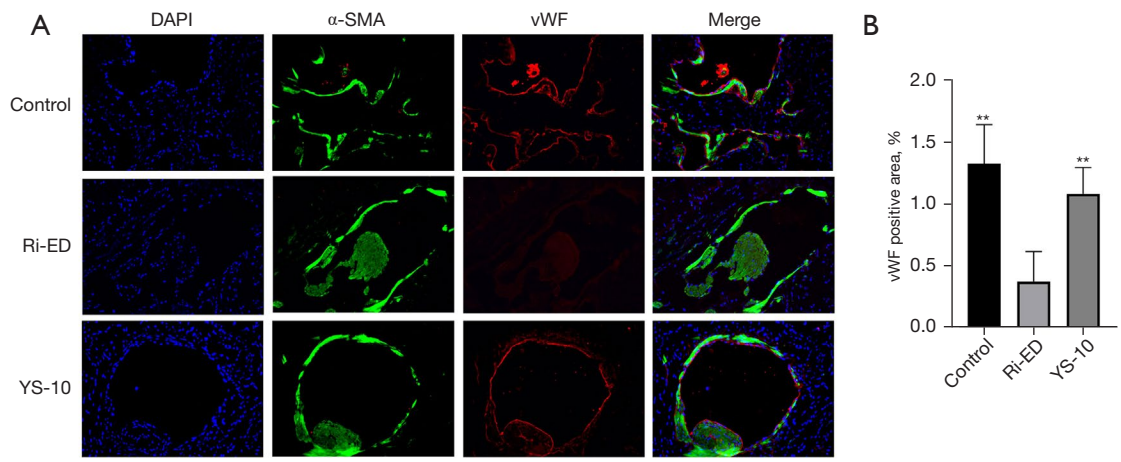

Figure 3 Immunofluorescence staining to assess the expression of endothelial cell content in the penile corpus cavernosum of rats in each group (n=6). (A) Penile tissues were examined for vWF (red) expression, a marker of endothelial cells (×400). (B) vWF expression in the penile tissue of rats in each group; the columns indicate the mean standard deviation ($\bar{x}$±s). **, P<0.01 versus the Ri-ED group. DAPI, 4,6-diamidino-2-phenylindole; α-SMA, alpha-smooth muscle actin; vWF, von Willebrand factor; Ri-ED, radiation-induced erectile-dysfunction.

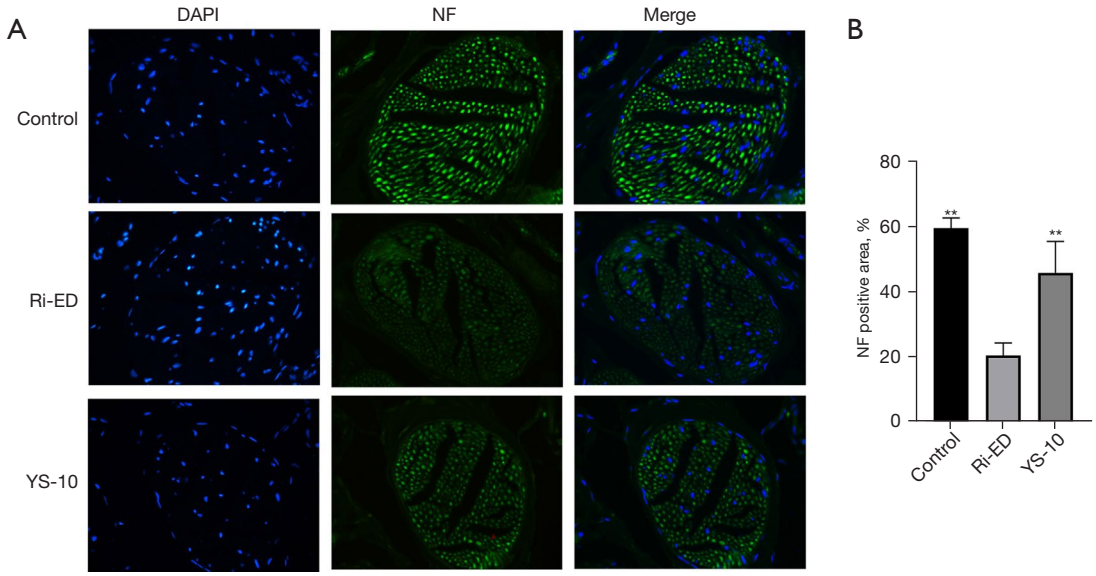

Figure 4 Evaluation of neural functions in MPG. (A) Representative immunofluorescence staining images of NF (green) and MPG (×400). (B) Semiquantitative analysis of the mean density of NF. **, P<0.01 compared to Ri-ED. DAPI, 4,6-diamidino-2-phenylindole; NF, neurofilament; Ri-ED, radiation-induced erectile-dysfunction; MPG, major pelvic ganglion.

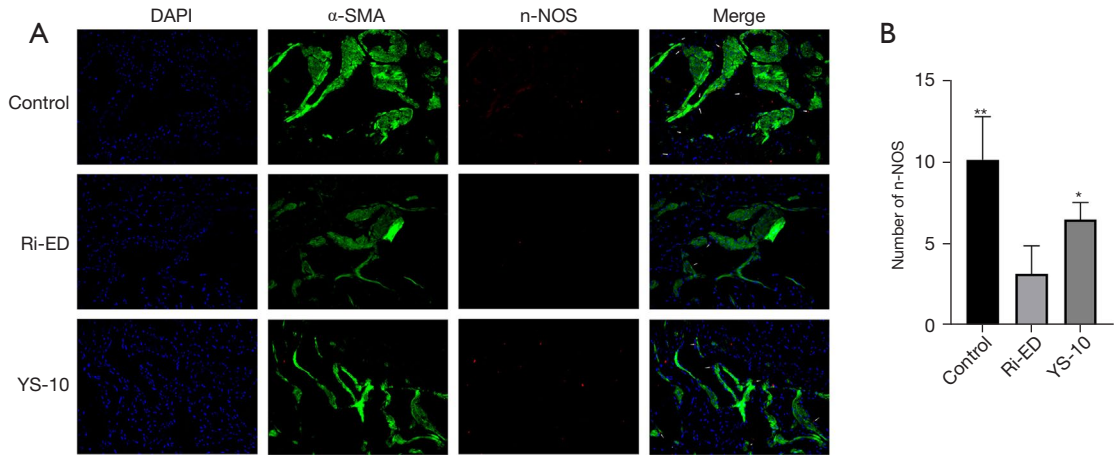

Figure 5 Changes in the number of n-NOS-positive nerves in the corpus cavernosal penis of each group (n=6). (A) Immunofluorescence images of n-NOS-positive nerves (green) in cross sections of cryoembedded penile midshaft (×400). (B) n-NOS-positive nerves expression in the penile tissue of rats in each group; the columns indicate the mean standard deviation ($\bar{x}$±s). *, P<0.05 versus the Ri-ED group; **, P<0.01 versus the Ri-ED group. DAPI, 4,6-diamidino-2-phenylindole; α-SMA, alpha-smooth muscle actin; n-NOS, neuronal nitric oxide synthase; Ri-ED, radiation-induced erectile-dysfunction.

(a marker of neural content) in the MPG, significantly improve neurological function, and approach the level of the control group. *Figure 5* shows that compared with the control group (10.17±2.64), the number of n-NOS-positive nerve fibers in the corpus cavernosum of the Ri-ED group (3.17±1.72) was significantly decreased (P<0.01). The number of positive nerve fibers (6.50±1.05) in the YS-10 group was significantly increased (P<0.05).

Western blot also showed that compared with the control group, the Ri-ED group had significantly lower

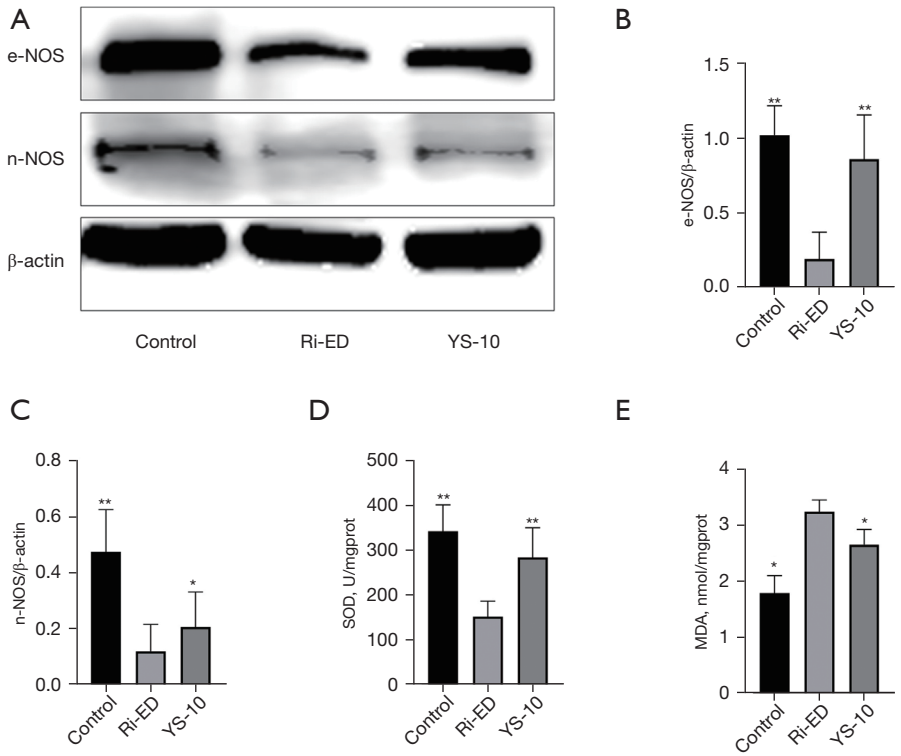

Figure 6 Changes of the protein content of n-NOS and e-NOS and expression of SOD and MDA content in the corpus cavernosum penis of each rat group (n=6). (A) Western blot showing the expression changes of n-NOS and e-NOS in penile tissues of rats. (B,C) n-NOS and e-NOS in the penile tissue of rats in each group. (D,E) SOD and MDA in the penile tissue of rats in each group; the columns indicate the mean standard deviation ($\bar{x}$±s). *, P<0.05 versus the Ri-ED group; **, P<0.01 versus the Ri-ED group. e-NOS, endothelial nitric oxide synthase; n-NOS, neuronal nitric oxide synthase; Ri-ED, radiation-induced erectile-dysfunction; SOD, superoxide dismutase; MDA, malondialdehyde.

levels of n-NOS (0.12±0.10 *vs.* 0.48±0.15) and endothelial nitric oxide synthase (e-NOS) (0.19±0.18 *vs.* 1.02±0.20) (P<0.01). The expressions of n-NOS (0.21±0.13) and e-NOS (0.86±0.30) in the YS-10 group were significantly higher than those in the Ri-ED group (P<0.05 and P<0.01, respectively), which were close to the level of the control group (*Figure 6A-6C*).

YS-10 improves oxidative stress indicators

Compared with the control group, SOD (153.49±34.06 *vs.* 343.73±58.57) in the corpus cavernosum of the Ri-ED group was significantly decreased, and MDA (3.25±0.21 *vs.* 1.80±0.31) was significantly increased (P<0.01). After YS-10 treatment, SOD (285.33±66.20) was significantly increased, and MDA (2.67±0.27) was significantly decreased (P<0.05) (*Figure 6D,6E*).

Discussion

Ri-ED is one of the most common complications of radiation therapy for prostate cancer (15,16). In our experiment, after rats received irradiation to the prostate, the ICP/MAP ratio was significantly decreased indicating the erectile function of the rats was dramatically impaired and the modeling of mimic Ri-ED was successful. To date, there is no sufficiently effective method to prevent or treat Ri-ED (17). Only a minority of patients respond positively to first-line drugs such as PDE5 inhibitors (18). In this study, the hemodynamic results showed a significantly higher ICP/MAP ratio in the YS-10 treatment group than that in the Ri-ED group, and the erectile function was indeed improved.

There are many factors affecting penile erection, including the smooth muscle of the corpus cavernosum

which controls the arteriovenous blood flow of the corpus cavernosum and is in an extremely important position (19). Our experiment confirmed that the content of α-SMA in the corpus cavernosum of the rats was increased after YS-10 treatment. The contraction and relaxation of smooth muscle is controlled by chemicals secreted by endothelial cells. Immunofluorescence staining showed that vWF in the corpus cavernosum in the YS-10 treatment group was significantly higher than that in the radiation injury group, which was similar to our previous results in the treatment of bilateral cavernous nerve injury (BCNI) ED with ICA II.

Increasing evidence has indicated that the expression levels of n-NOS and e-NOS are reduced in senile ED models, which may be due to the mediation of cavernosal smooth muscle relaxation through the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) signaling pathway (20-22). In our research, it was confirmed that the content of n-NOS and e-NOS in the corpus cavernosum of the YS-10 treatment group was significantly increased compared with those in the Ri-ED group. Chung's research also confirmed that icariside could upregulate the expression and activity of e-NOS in cells, which may be related to the activation of mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B Akt/e-NOS-dependent signaling pathways in human endothelial cells to stimulate angiogenesis.

The results of our experiment confirmed that after irradiation, rats showed dramatic erectile dysfunction and corresponding pathological changes, including a reduction in smooth muscle content, n-NOS-positive nerve fiber deficiency, and impaired neurological and endothelial function. The mechanism may be related to oxidative stress injury. YS-10 treatment could significantly reduce the level of local oxidative stress in the penis and reverse histopathological changes.

Radiation induces the production of ROS, and ROS aggregation can generate lipid peroxides (MDA, hydroxyl, etc.), which oxidize proteins and amino acids and cause tissue damage (23,24). We believe that although the pathogenic mechanism of Ri-ED is complex and may involve many factors, such as nerves, blood vessels, metabolism, and the endocrine system, oxidative stress plays an extremely important role. Our study showed that compared with the control group (Control), SOD content in the penile tissue of the rats in the radiation injury group was significantly decreased, and the MDA level was significantly increased, indicating that a large number of oxygen free radicals were produced in the tissue after radiation therapy, and that the body was in a state of peroxidation. YS-10 treatment could reverse the expression levels of SOD and MDA, suggesting that YS-10 could significantly improve the peroxidation state of tissues, inhibit the damage of oxidative stress, and thus improve penile erectile function. Similarly, in an experimental study of irradiation in male C57BL/6 mice, it was concluded that icariin could reduce radiation-induced oxidative stress by modulating endogenous antioxidant levels. This was consistent with the experimental results of icariside derivative YS-10 (25). In recent years, the pathological changes and molecular mechanisms of ED have become clearer, in which oxidative stress-induced smooth muscle and endothelial cell dysfunction is an important influencing factor in the development of ED.

Conclusions

YS-10 could improve the erectile function of the penis by inhibiting oxidative stress in the corpus cavernosum, thereby reducing the damage to vascular endothelium and nerves by radiation. The mechanism may be related to the improvement of radiation-induced oxidative stress. We will further explore the complete signaling pathway and key regulatory proteins in the future.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://tau.amegroups.com/article/view/10.21037/tau-22-376/rc

Data Sharing Statement: Available at https://tau.amegroups.com/article/view/10.21037/tau-22-376/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tau.amegroups.com/article/view/10.21037/tau-22-376/coif). The authors

have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were performed under a project license (No. 2020113) granted by Animal Ethics Committee of the Affiliated Hospital of Changchun University of Chinese Medicine, in compliance with Changchun University of Chinese Medicine (Jilin, China) guidelines for the care and use of animals.

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