Effect of *Ginkgo biloba* Extract (EGb-761) on Recovery of Erectile Dysfunction in Bilateral Cavernous Nerve Injury Rat Model

Yi-No Wu, Chun-Hou Liao, Kuo-Chiang Chen, Shih-Ping Liu, and Han-Sun Chiang

OBJECTIVE To investigate whether the therapeutic effect of *Ginkgo biloba* extract (GBE) in a rat model can improve erectile dysfunction after bilateral cavernous nerve injury.

METHODS Forty-three male Sprague-Dawley rats underwent cavernous nerve crush injury and were randomized into 4 groups, including: vehicle only, high-dose GBE, medium-dose GBE, and low-dose GBE. Eight animals underwent sham operation. Four weeks later, erectile function was assessed by cavernous nerve electrostimulation, and penile tissue was collected for histologic analysis.

RESULTS Significant recovery of erectile function was observed in the high-dose GBE group in a dose-dependent manner as compared with the vehicle-only group ($P < .001$). The high-dose GBE group had a significant increase in neurofilament-1 expression ($P < .001$), preservation of neural nitric oxide synthase nerve fibers of the dorsal penile nerve ($P < .05$), and increased smooth muscle cell content ($P < .001$) compared with the vehicle-only group. In addition, high-dose GBE markedly augments the smooth muscle-to-collagen ratio ($P < .05$) and reduces the apoptotic index.

CONCLUSION Administration of GBE increases neuron survival and preserves the neural nitric oxide synthase nerve fiber and contents of the corpus cavernosum after bilateral cavernous nerve injury. These implications indicate the beneficial effects of GBE use in the repair of the cavernous nerve and recovery of erectile function after radical prostatectomy. UROLOGY 85: 1214.e7–1214.e15, 2015.
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Radical prostatectomy is one of the treatments for localized prostate cancer. However, injury to the cavernous nerve is a significant concern for patients with prostate cancer after radical prostatectomy because of erectile dysfunction secondary to denervation of the penis. Loss of innervation causes severe and irreversible morphologic changes in the penis, including induction of smooth muscle apoptosis and collagen induction or fibrosis in the corpus cavernosum.[1] Currently, therapies for erectile dysfunction such as phosphodiesterase type 5 inhibitors and several neurotrophic factors[2] are being investigated to identify agents that might have neuroprotective and regenerative functions after cavernous nerve injury.[2-4] Alternatively, therapeutic options such as gene therapy, stem cell, and platelet-rich plasma have also been applied for regenerating the cavernous nerve in animal models and have been shown to be successful in the recovery of erectile function.[5-7] Although many experimental non-pharmacologic agents and therapeutic methods are successful in crush injury involving the peripheral nerves, some of them remain controversial with regard to safety and ethics.

The *Ginkgo biloba* extract (GBE) is derived from dried G *biloba* leaves and contains 24% Ginkgo flavonol glycosides and 6% terpene lactones, such as ginkgolides

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Yi-No Wu, Chun-Ho Liao carried out the animal experimental studies, participated in the design of the study, immunoassays, and drafted the article. Kuo-Chiang Chen performed the statistical analysis. Shih-Ping Liu and Han-Sun Chiang conceived of the study, participated in its design and coordination, and helped to draft the article. All authors read and approved the final article.

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A, B, C, and J, and bilobalide. The standardized extract of *G biloba* leaves (EGb 761), found to mitigate neuronal damage and prevent neurodegenerative disease, has demonstrated its neuroprotective effects.[8] Hsu et al[9] reported that Schwann cells seeded with EGb 761 increase the total number of myelinated axons in nerve regeneration and improve functional recovery of peripheral nerves. In addition, the vasotropic and neuroprotective effects of GBE have been widely investigated and reported in several rat injury models, such as optic nerve crush injury, ischemia, and facial nerve crush injury.[10-12]

GBE has been previously observed to demonstrate both vasoprotective and neuroprotective features in several studies. However, to the best of our knowledge, the effects of GBE on the recovery of erectile function after bilateral cavernous nerve injury have never been investigated. The purpose of this study was to assess whether GBE could enhance the neuroprotective effect on erectile function after bilateral cavernous nerve injury in a rat model.

METHODS

Animals

Forty-three 12-week-old, male, Sprague-Dawley rats (weight, 450-600 g) were used in this study. All animals were supplied by BioLASCO Taiwan Co., Ltd. (Taipei, Taiwan), and the study procedures were approved by the Fu Jen Catholic University Animal Care and Use Committee (IACUC approval no.: A10137).

Experimental Design and Surgical Procedures

The animals were randomly assigned to 5 groups: sham (n = 8), vehicle only (n = 8), high-dose GBE (n = 8), medium-dose GBE (n = 11), and low-dose GBE (n = 8; SHUSYUN Drops; obtained from Yuan Shan Co., Ltd., Taiwan). For the surgical procedure, the animals were first anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg.) After the abdomen was shaved and wiped with an iodine-based solution, a lower midline abdominal incision was made. The prostate gland was exposed, and the posterolateral cavernous nerves and the major pelvic ganglion were identified, and all rats, except the sham group, were subjected to a bilateral cavernous nerve injury using a hemostat clamp (Roboz Surgical Instrument Co. Inc., Gaithersburg, MD) for 2 minutes before closing the abdomen. In the sham group, no further surgical procedure was carried out, and the abdomen was closed. The vehicle-only group received 0.9% saline by the intragastric route. Rats in the high-dose, medium-dose, and low-dose GBE groups received 5 mL/kg/d of 10%, 4%, and 2% GBE, respectively, by the intragastric route for 4 weeks.[7]

Measurement of Erectile Responses

Four weeks after injury, the cavernous nerves were exposed and isolated via a repeat midline abdominal incision, and the crura of the penis were identified. A 24G needle containing 50 U/mL of heparin solution was inserted into the right penile crus and connected to a polyethylene-50 tubing to measure intracavernous pressure (ICP) with an MP36 pressure transducer (BIOPAC Systems Inc, Goleta, CA) and BSL 3.7.3 software (BIOPAC Systems Inc). The cavernous nerves were stimulated using a bipolar, stainless steel electrode through monophasic rectangular pulses generated by a computer with a DS3 constant—current-isolated stimulator (AutoMate Scientific Inc., CA). The stimulus parameters were as follows: 7.5-mA amplitude, 20-Hz frequency, 0.2-ms pulse width, and 60-second duration. A real-time response of the erectile tissue was determined based on the maximum ICP, the change in ICP (ΔICP), the area under the ICP curve, and the ratio of change in ICP and the maximum ICP and mean arterial pressure (MAP; ΔICP/MAP; maximum ICP/MAP).[7]

Immunofluorescence Staining for the Dorsal Penile Nerve and Corpus Cavernosum

After measuring the erectile response, the animals were killed by administering a high dose of pentobarbital sodium solution. The tissue from the middle portion of the penis was obtained from all 43 rats. The freshly dissected tissue was formalin fixed (10% formaldehyde w/v) for 24 hours and, thereafter, dehydrated, postfixed, and embedded. Then, 5-μm-thick cross-sections were taken, and adhered to charged slides. These were deparaffinized in xylene for 10 minutes. The procedure was repeated twice for a total of 3 treatments and was followed by hydration of sections through graded alcohols. The slides were incubated for 20 minutes in 10% goat serum/2% bovine serum albumin/0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO). Thereafter, the slides were extracted and incubated overnight at room temperature with antineurofilament-1 (NF-1; Thermo Fisher Scientific, Cheshire, United Kingdom), rabbit antineural nitric oxide synthase (nNOS; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti—neuron-specific β-III tubulin, and anti—smooth muscle actin (SMA) primary antibodies (both from Abcam Inc., Cambridge, MA), followed by a 3-hour immersion in 1:400 dilution of the secondary antibody, conjugated with AlexaFluor 488 or Texas Red (Invitrogen). The slides were evaluated using fluorescence microscopy. For analysis of nNOS content, the ratio of the area of nNOS-positive fibers per nerve over the β-III-tubulin area of the nerve at a magnification of ×400 was calculated. For analysis of NF-1, the ratio of the distribution of NF-1 in the dorsal penile nerve was calculated at a magnification of ×400. All the nerve and corpus cavernosum computerized histomorphometric analysis was performed using ImageJ software 1.46r, download on 2012-09-21 (National Institutes of Health Bethesda, MD).

Masson Trichrome Staining

Penile tissue sections were fixed with 10% buffered formalin and stained using the Masson trichrome staining reagent kit according to the manufacturer's instructions (Muto Pure Chemicals, Tokyo, Japan). All computerized histomorphometric analyses of corpus cavernosum were performed using Olympus cellSens software (Olympus).

Transferase-mediated deoxyuridine triphosphate (dUTP)-biotin Nick End Labeling Staining

Quantification of apoptotic cells was performed by detecting deoxyribonucleic acid damage in situ using Apo-BrdU In Situ DNA Fragmentation Assay Kit (BioVision, Inc., CA) and counterstaining by 4′,6-diamidino-2-phenylindole (DAPI) in

Table 1. Intracavernous and mean arterial pressure measurements on cavernous nerve electrostimulation

Groups	Maximum ICP (cm H_2O)	ΔICP (cm H_2O)	AUC	ΔICP/MAP	Maximum ICP/MAP
Sham	141.72 ± 12.91*	110.59 ± 12.79*	4479.86 ± 948.83*	0.68 ± 0.10*	0.94 ± 0.09
Vehicle only	56.19 ± 9.01	33.00 ± 10.12	1059.71 ± 413.66	0.19 ± 0.06	0.38 ± 0.06
Crushed + high-dose GBE	127.39 ± 18.58*	99.76 ± 16.72*	3971.84 ± 1422.60†	0.59 ± 0.10*	0.85 ± 0.12*
Crushed + medium-dose GBE	93.78 ± 35.64†	72.37 ± 33.07†	3426.41 ± 1933.45†	0.43 ± 0.19†	0.62 ± 0.24†
Crushed + low-dose GBE	69.94 ± 8.46	43.12 ± 5.69	1879.86 ± 419.95	0.25 ± 0.03	0.47 ± 0.06

AUC, area under the curve; GBE, *Ginkgo biloba* extract; ICP, intracavernous pressure; ΔICP, maximum intracavernous pressure − minimum intracavernous pressure; MAP, mean arterial pressure.
* $P < .001$ vs vehicle-only group.
† $P < .05$ vs vehicle-only group.

paraffin-embedded tissue sections. The slides were evaluated using fluorescence microscopy.

Statistical Analysis

Data are presented as mean ± standard deviation. The difference between the means of multiple treatment groups was tested by the analysis of variance and the Scheffe post hoc test, with a statistical significance determined at $P < .05$. Statistical analysis was performed using SPSS, version 12.0 (SPSS Inc., Chicago, IL) for Windows.

RESULTS

Erectile Function

The maximum ICP was significantly lower in the vehicle-only group (56.19 ± 9.01 cm H_2O) compared with the sham group (141.72 ± 12.91 cm H_2O; $P < .001$). Similarly, the other measurements of erectile function—ΔICP, the area under the ICP curve, the ΔICP/MAP ratio, and the maximum ICP/MAP ratio—were significantly lower in the vehicle-only group ($P < .001$). However, these 5 parameters were significantly higher in the group treated with high-dose GBE than in the vehicle-only group ($P < .001$; Table 1; Fig. 1A); values for the high-dose (maximum ICP, 127.39 ± 18.58 cm H_2O) and the medium-dose GBE groups (maximum ICP, 93.78 ± 35.64 cm H_2O) reached statistical significance ($P < .05$; Table 1). However, there were mixed results for the recovery of erectile function in the medium-dose GBE group (Fig. 1B). These data appear to indicate the possible relationship between drug dosage and effective response threshold. We next explored the mechanisms by which GBE may improve the recovery of erectile function in rats after cavernous nerve injury.

Histomorphometric Analysis—NF-1 and nNOS Expression in the Dorsal Penile Nerve

Nerve fibers of the dorsal penile nerve were assessed for NF-1 expression by immunofluorescence staining. Representative images of each group are shown in Figure 2A. In line with the functional result, there was significant reduction of NF-1 in the dorsal penile nerve in the vehicle-only group as compared with the sham group ($P < .001$; Fig. 2B). The ratio in the high-dose GBE group was significantly higher than in the vehicle-only group ($P < .001$; Fig. 2B); however, differences between the vehicle-only and medium-dose GBE groups were not statistically significant. The nNOS-positive nerve fibers of the dorsal penile nerve were immunostained for β-III-tubulin to identify nerve fibers positive for nNOS and to quantify their nNOS content (representative images of each group in Fig. 2C). As shown in Figure 2D, compared with the sham group, the ratio of the area of nNOS/β-III-tubulin expression was significantly reduced in the vehicle-only ($P < .001$) and low-dose GBE groups ($P < .001$); however, there was significantly increased ratio of the nNOS/β-III-tubulin area ($P < .05$) in the high-dose GBE group compared with the vehicle-only group.

Histomorphometric Analysis—Smooth Muscle Cell and Collagen Expression in the Corpus Cavernosum

After treatment with high- and medium-dose GBE, an increase in smooth muscle cell content was detected as observed by the increased expression of SMA ($P < .001$; Fig. 3A,B). The ratio of smooth muscle and collagen in the corpora cavernosa was evaluated by Masson trichrome staining. A distinct decrease in the smooth muscle content and smooth muscle-to-collagen ratio within the corpora cavernosa in the vehicle-only group compared with the sham group indicated smooth muscle atrophy and possible progression of corpus cavernosum fibrosis after cavernous nerve injury (Fig. 3C,D). However, smooth muscle atrophy was minimal in the high-dose GBE group. These results showed that smooth muscle atrophy in the corpus cavernosum was effectively prevented by the administration of high-dose GBE.

Apoptosis of Corpus Cavernosum

Transferase-mediated dUTP-biotin nick end labeling staining showed nuclear co-localization with DAPI. Only cells positive for both transferase-mediated dUTP-biotin nick end labeling and DAPI were considered positive for apoptosis. Apoptosis was significantly more abundant in animals that underwent crush injury compared with sham animals. Treatment with high-dose GBE was able to reduce the apoptotic index significantly (Fig. 3E).

COMMENT

In the present study, we evaluated the effect of GBE on improving erectile function after bilateral cavernous

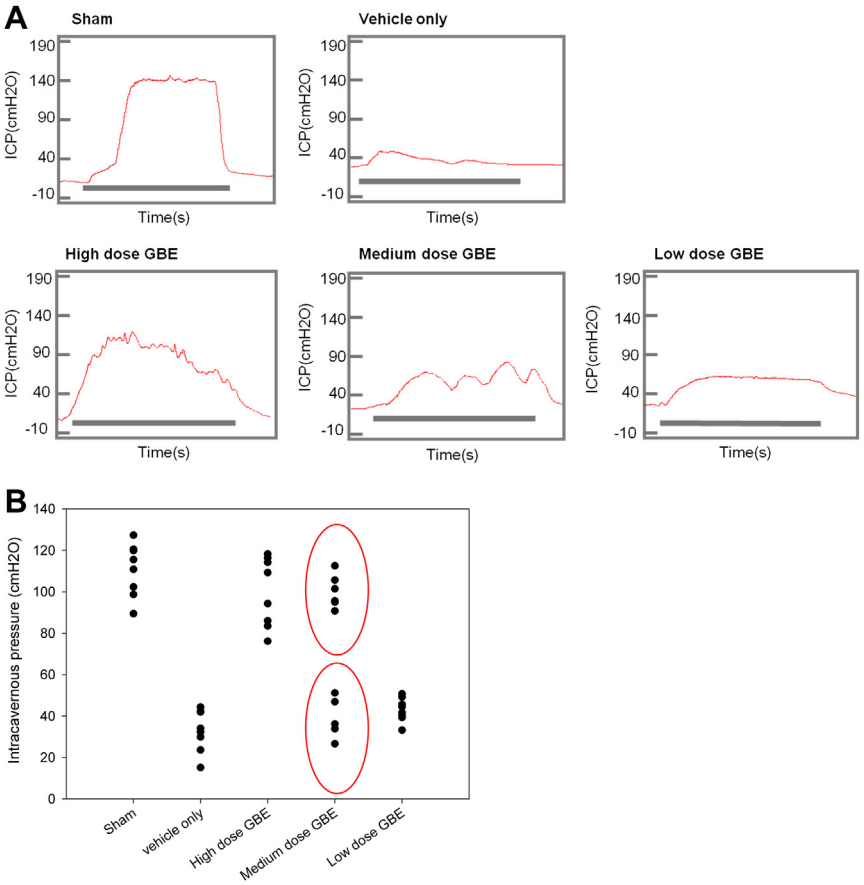


Figure 1. Electrostimulation of cavernous nerve at 4 weeks. **(A)** Recordings of intracavernous pressure in sham, vehicle-only, high-dose *Ginkgo biloba* extract (GBE), medium-dose GBE, and low-dose GBE groups. The x-axis depicts seconds and the gray bar represents 1 electrical stimulus lasting 60 seconds. **(B)** The effects and distribution of chronic treatment with GBE in increasing dosages on intracavernous pressure with electrical stimulation of the cavernous nerve. The medium-dose GBE group showed 2 different results, indicated by a red circle (n = 8 and 11, respectively). GBE, *Ginkgo biloba* extract; ICP, intracavernous pressure.

nerve injury in rats. We performed a histologic analysis to observe the expression of NF-1, the nNOS/β-III-tubulin ratio, and SMA content in the corpus cavernosum after daily administration of high-dose GBE for 4 weeks. Four weeks of daily treatment with high-dose GBE significantly improved erectile function, as indicated by the ICP and in comparison with the vehicle-only treatment. Histologically, immunofluorescence staining of nNOS, NF-1, and SMA in the dorsal penile nerve and corpus cavernosum revealed significant improvement in neuroregeneration and preservation of smooth muscle cell in the high-dose GBE group. This positive dose-response relationship further strengthens the association of the GBE effect on recovery of erectile function after bilateral cavernous nerve injury.

This study was preceded by a small number of studies investigating GBE in the treatment of nerve regeneration after sciatic nerve transection and facial nerve crush injury. Lin et al have shown the effect of GBE on promoting regeneration of peripheral nerve,[13] and a recent study by Jang et al[12] reports that intraperitoneal injection of GBE has been effective in promoting nerve regeneration in an experimental facial nerve crush injury rat model. In an earlier study, the extract of the leaf of G *biloba* was promoted in the treatment of chronic central nervous system diseases, such as Alzheimer disease, and cardiovascular disease.[14,15] In the field of clinical urology, GBE has usually been applied in antidepressant-induced sexual dysfunction, and a previous study showed GBE to be 84% effective as a treatment.[16] The present study is the first to examine the effect of GBE on neurogenic erectile dysfunction after cavernous nerve injury. Our findings show improvement in erectile function, on the functional assay, after treatment with high-dose GBE (EGb-761) in a bilateral cavernous nerve injury rat model. However, the mechanisms by which GBE improves erectile function are yet to be clarified.

Smooth muscle relaxation in corpus cavernosum is primarily an nitric oxide-cyclic guanosine monophosphate-mediated response, which includes NO synthesis by nNOS within the nerve terminals of the cavernous nerve.[17] Moreover, increase of nNOS expression in the dorsal penile nerve has also been an evaluation index for the recovery of erectile function in recent

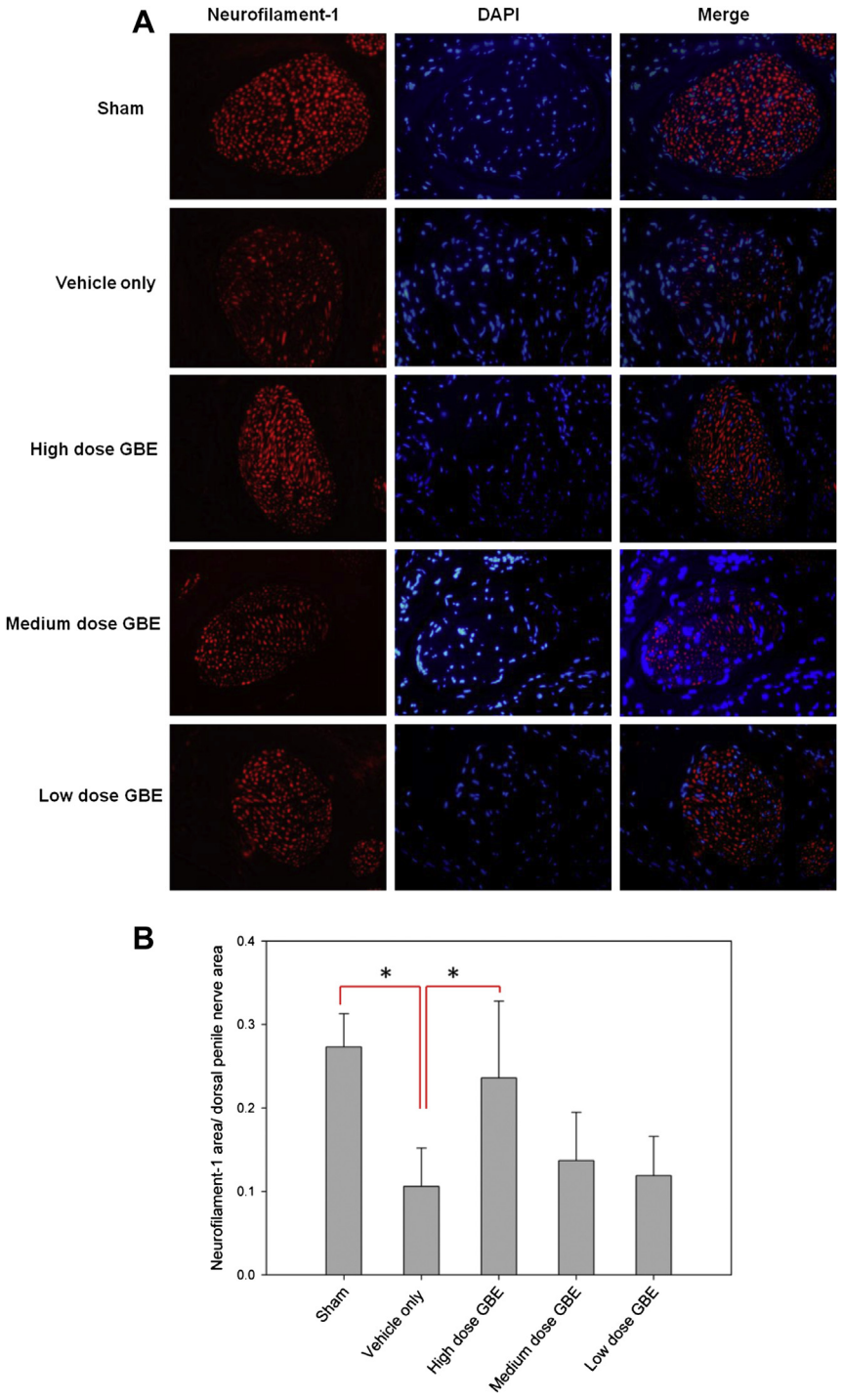
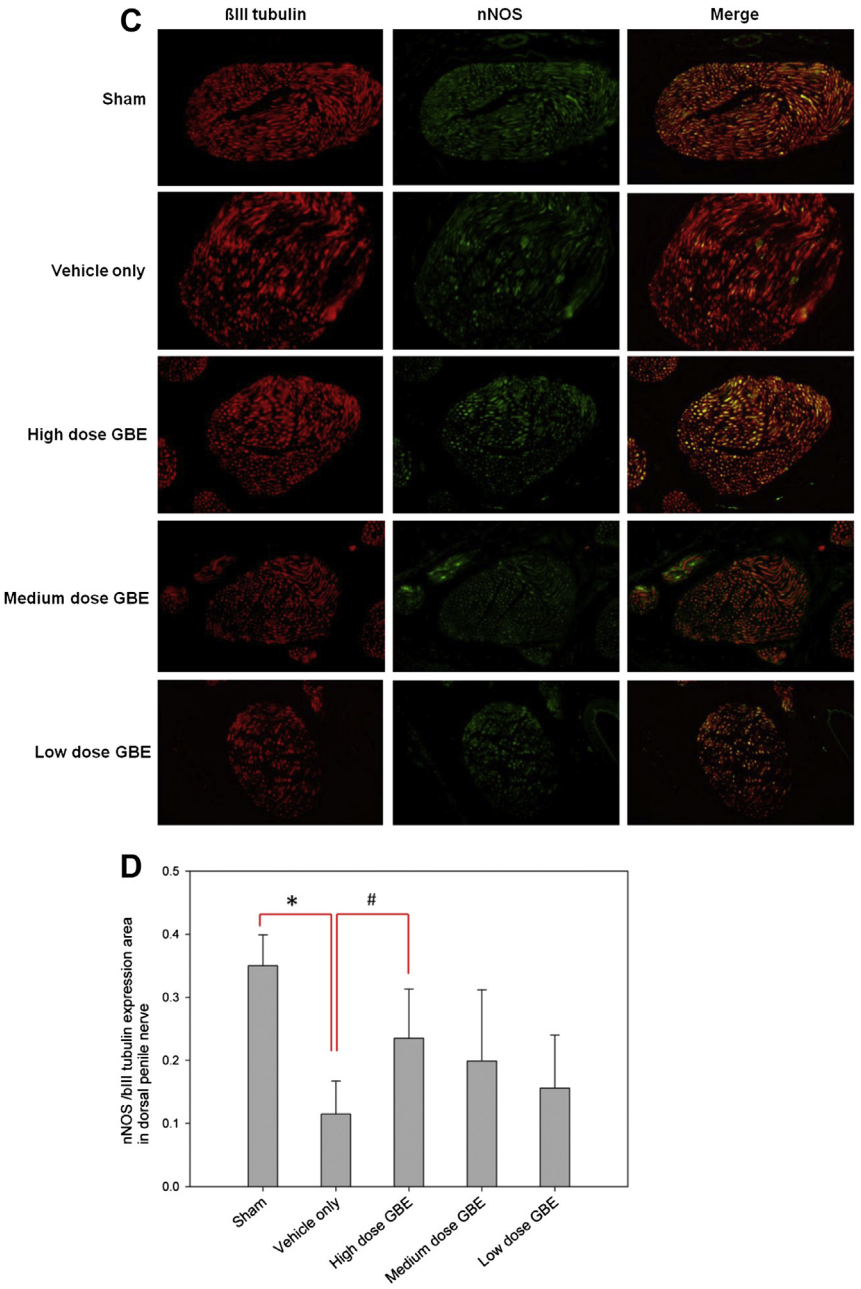


Figure 2. Immunofluorescence staining for NF-1 expression in the dorsal penile nerve. **(A)** Representative images of the dorsal penile nerve in each group (NF-1, red color; nuclear, blue color; original magnification, $\times$400). **(B)** Quantification of NF-1-positive nerve fiber of the dorsal penile nerves expressed as the area of NF-1-positive nerve fiber or dorsal penile nerve. Quantitative analysis shows that the number of NF-1-positive nerve fibers is dramatically reduced in the vehicle-only group as compared with the sham group; however, there is a significant increase in the number of NF-1-positive nerve fibers after high-dose *Ginkgo biloba* extract treatment. $^{*}P < .001$ vs vehicle-only group. Immunofluorescence staining for neural nitric oxide synthase (nNOS) in dorsal penile nerve. **(C)** Representative images of the dorsal penile nerve of each group. Original magnification $\times$400. **(D)** Result of nNOS-positive nerve fibers in the dorsal penile nerve quantified as the area of nNOS-positive nerve fibers/β-III tubulin. Quantitative analysis shows that the number of nNOS-positive nerve fibers is dramatically reduced in the vehicle-only group compared with the sham group; however, there is a significant increase in the number of nNOS-positive nerve fibers after high-dose *G biloba* extract treatment. $^{*}P < .001$ vs vehicle-only group; $^{\#}P < .05$ vs vehicle-only group. GBE, *Ginkgo biloba* extract; nNOS, neural nitric oxide synthase.



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studies in the cavernous nerve injury rat model.[18,19] The dorsal nerve of the penis arises as the last branch of the pudendal nerve. In animal studies, the dorsal nerve of the penis has been shown to contain sympathetic fibers, suggesting that communicating branches exist between the cavernous and the dorsal nerves.[20] In the present study, the nNOS level, which was expressed as a ratio to β-III-tubulin, improved after treatment with a high dose of GBE, suggesting its neurogenic potential. The enhancement in nNOS-immunoreactive cell numbers is associated with a better noncontact erection and might support cell survival in axotomized neurons.[21]

A neurofilament is a critical component of the axon cytoskeleton that is specific to neurons and plays a major role in determining axon caliber. Moreover, it is a protein marker for neurite outgrowth. Peripheral axotomy will induce neurofilament decrease, atrophy, and demyelination.[22] The bioactive ingredients of EGb-761 not only repair endothelial damage but also improve abnormal brain metabolism and neurotransmitter disturbance. The neuroregenerative action of GBE has been successfully shown in the treatment of glaucoma, where it exhibited enhanced axonal growth and density of retinal ganglion cells along with improvement in optic nerve function.[23] Therefore, the increased expression of the neurofilament in the present study provided the evidence of the neuroprotective effect of GBE in the dorsal penile nerve supplying the corpus cavernosum.

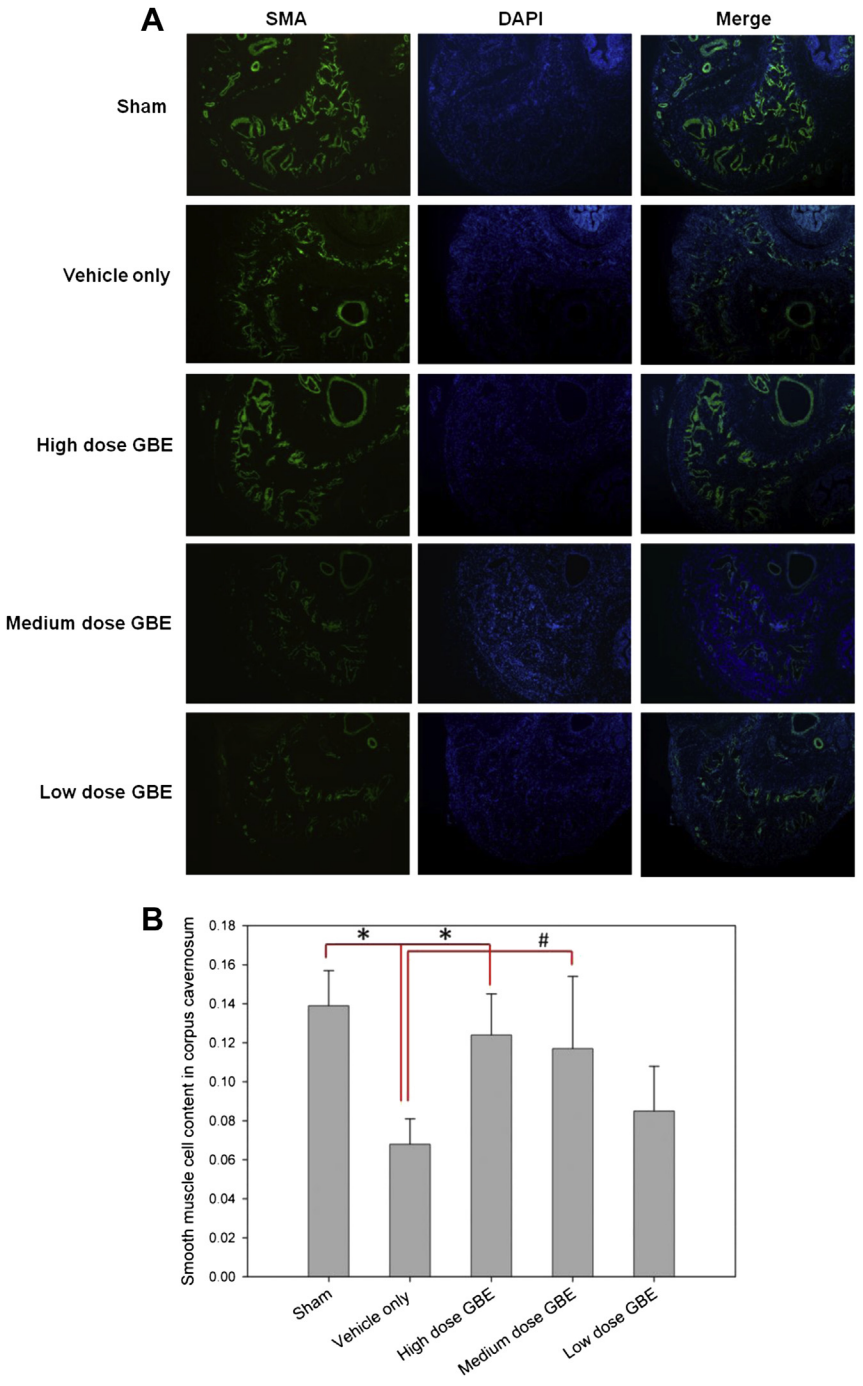

Figure 3. **(A)** Representative fluorescent images of α-smooth muscle actin–positive area in the rat penile corpus cavernosum (smooth muscle, green color; nuclear, blue color; original magnification ×50). **(B)** Smooth muscle cell content in the corpus cavernosum quantified as the α-smooth muscle actin–positive area or corpus cavernosum. $^{*}P<.001$ vs vehicle-only group; $^{\#}P<.05$ vs vehicle-only group. **(C)** Histologic analyses in the corpus cavernosum 4 weeks after surgery. Masson trichrome staining for smooth muscle and collagen in the corpus cavernosum. Smooth muscle was stained red, and collagen was stained blue; original magnification ×50 and ×400, respectively. **(D)** Fibrosis severity quantified as the smooth muscle cell area or collagen area. $^{*}P<.05$ vs vehicle-only group. **(E)** Transferase-mediated dUTP-biotin nick end labeling staining showed nuclear colocalization with 4′,6-diamidino-2-phenylindole (original magnification ×400). Only cells positive for both transferase-mediated dUTP-biotin nick end labeling and DAPI were considered positive for apoptosis. There is an increase in apoptosis in rat corpus cavernosum tissue after cavernous nerve crushing without treatment. GBE, *Ginkgo biloba* extract; SMA, smooth muscle actin; TUNEL, transferase-mediated deoxyuridine triphosphate-biotin nick end labeling.

The increased expression of SMA is consistent with the vasorelaxant effect attributed to G *biloba*, which is an indication of the increased functional ability to contract and relax as a direct response to the GBE.[24] In addition, a recent study has shown that GBE can induce relaxation of the corpus cavernosum and

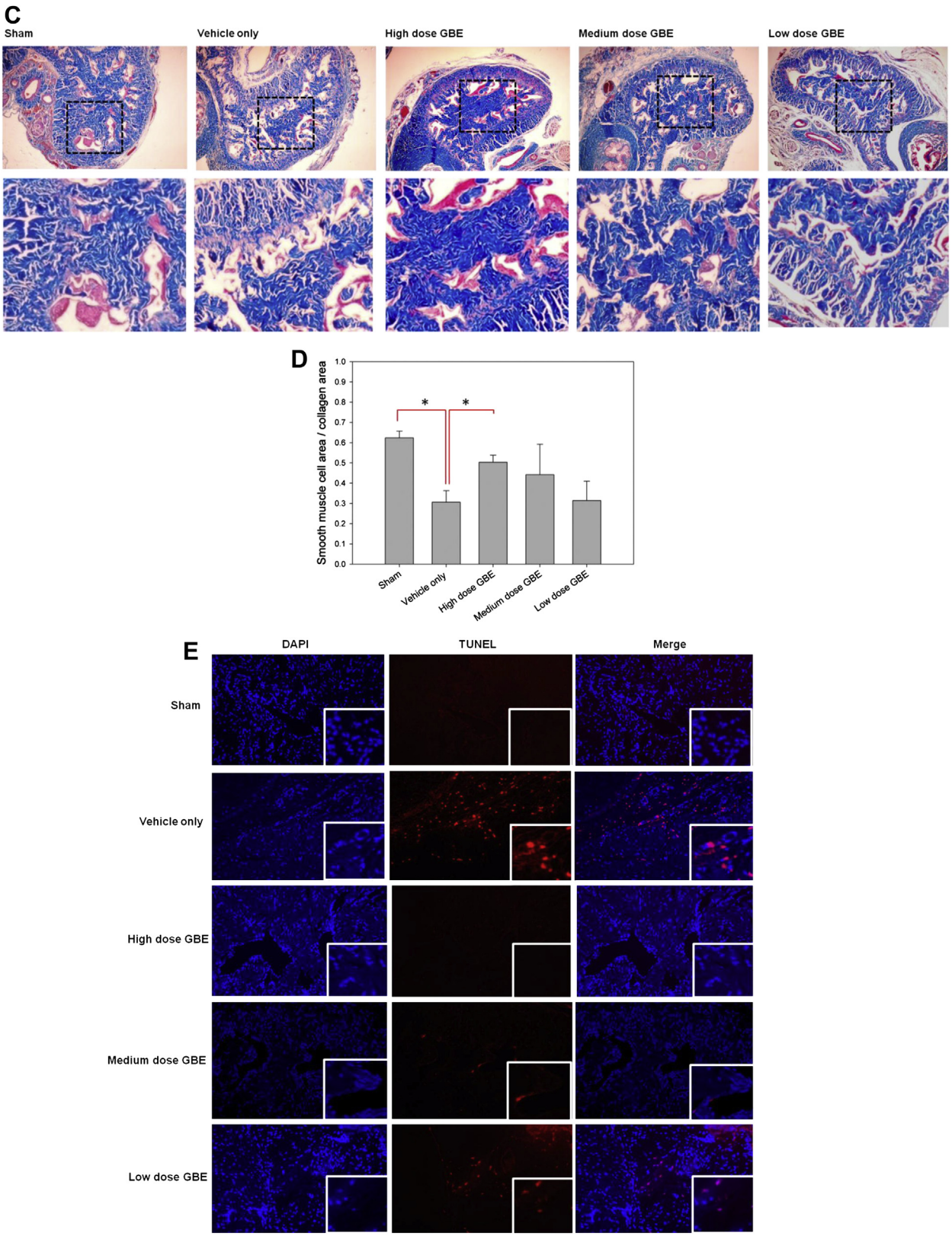

Figure 3. (*continued*).

enhancement of endothelial function.[25] In this present study, we also found that the group treated with a high-dose of GBE showed a statistically significant decrease in apoptosis of corpus cavernosum and increase in the content of smooth muscle cell compared with the vehicle group.

According to these findings, we believe that both the neuromodulatory and tissue preservation effects of GBE

may be associated with the antioxidant property and ability of its bioactive component. In additional, our findings are consistent with previous research studies in which GBE has shown neuroprotective and tissue-protective effects in a dose-dependent manner. This study not only supports other reported therapeutic effects of the GBE after crush injury of the peripheral nerve[10,12] but also provides some reasonable and basic research evidences for supporting the clinical use of GBE in erectile dysfunction. More evidence for its optimized dosage and mechanism of effect on recovery of erectile function is needed.

CONCLUSION

In summary, our study shows that the pharmacologic effect of GBE, EGb-761, on recovery of erectile function is dose dependent, and offers mechanistic insight into the beneficial effects of GBE in preserving erectile function after cavernous nerve injury. Furthermore, this work has clinical implications in treatment with GBE after radical prostatectomy.

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