

## Original Article

# Panaxnotoginsengsaponin improves erectile function by inducing autophagy, inhibiting apoptosis, and increasing connexin 43 phosphorylation

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**Abstract:** Erectile dysfunction (ED) is a common clinical condition that is treatable by phosphodiesterase type 5 inhibitor; however, this treatment is less effective in cases of organic ED, especially in terms of reversing pathological changes. The present study investigated whether *Panaxnotoginsengsaponin* (PNS) could improve erectile function in a rat model of ED. We found that expression of the autophagy protein Beclin-1 was downregulated, whereas that of the apoptosis markers P62 and cleaved caspase-3 was upregulated in corpus cavernosum smooth muscle cells of ED rats. The phosphorylation of the gap junction protein connexin (Cx) 43 was also decreased in these animals. These effects were abrogated by treatment with PNS alone or in combination with the autophagy inducer rapamycin, both of which stimulated autophagy and suppressed apoptosis. The combination of PNS and the autophagy inhibitor 3-methyladenine (3-MA) had the opposite effects, and neither rapamycin nor 3-MA altered the phosphorylation status of Cx43. These results indicate that PNS can improve erectile function, providing a potential new treatment strategy for ED.

**Keywords:** Cx43, smooth muscle cell, 3-MA, rapamycin, *Panaxnotoginsengsaponin* (PNS)

## Introduction

Erectile dysfunction (ED) is a common clinical condition characterized by the inability to develop or maintain erection of the penis for satisfactory sexual intercourse for a period of at least 3 months. Sildenafil citrate is a phosphodiesterase type 5 inhibitor prescribed for the treatment of ED that has a response rate of 74% [1]. However, the rate is lower for organic ED patients. As such, there is a need for more effective therapies to treat these cases.

Autophagy is a process that maintains homeostasis of the intracellular environment. The activities of autophagy-related enzymes and lysosome morphology change with age in most tissues. For example, giant and incidental

autophagy decrease in aging males [2]. Age-related changes in autophagosomes are also observed in many cell types, including cardiomyocytes, skeletal muscle fibres, and others [3]. It has been suggested that the age-related decrease in autophagic activity is related to ED [4]. Therefore, therapeutic strategies targeting the autophagy pathway may be an effective treatment for ED.

The contraction/relaxation of corpus cavernosum smooth muscle cells (CCSMCs) plays an important role in penile erection. Gap junctions (GJs) are critical structures for the exchange of molecules and energy between cells, which is necessary for CCSMC contraction/relaxation [5, 6]. Connexin (Cx)43, the most widely distributed among Cx family proteins, is a marker for

the level of GJ intercellular communication [7, 8]. Under physiological conditions, the urethral sponge activates endothelial nitric oxide synthase (eNOS) and generates nitric oxide (NO), leading to an increase in cyclic guanosine monophosphate levels in CCSMCs and ultimately, to penile erection by rapid signal transfer between cells via GJs [9]. Patients with organic ED have a lower Cx43 expression than those with normal erectile function, indicating that a reduction in the number of GJs may underlie this condition [7]. On the other hand, the cavernous sinus of the penis, which is a cavity lined with SMCs and endothelial cells, is also important for penile erection [10]; excessive apoptosis of the SMCs decreases the ratio of smooth muscle/collagen, which could also lead to ED.

Saponin is an abundant bioactive compound derived from *Panaxnotoginseng* that has demonstrated vasodilatory and smooth muscle-relaxing effects and also protects endothelial cells and SMCs. *P. notoginseng*saponin (PNS) has been implicated in autophagy induction and apoptosis suppression [11, 12]. However, few studies have examined whether PNS is effective for treating ED. To address this issue, we used a rat model of ED to investigate the effect of PNS on autophagy and GJs in CCSMCs.

## Materials and methods

### *Animals and reagents*

Male SD rats were provided by Zhejiang Chinese Medical University Laboratory Animal Center (China). Antibodies against  $\beta$ -actin, Cx43, phosphorylated p-Cx43, Beclin-1, and P62 were from Santa Cruz Biotechnology (USA); microtubule-associated light chain 3 (LC3), GAPDH, caspase3 and cleaved caspase3 were from cell signaling technology (USA). Monodansylcavenderine (MDC), 3-methyladenine (3-MA), PNS and rapamycin were purchased from Sigma-Aldrich (St. Louis, MO, USA); and bicinchoninic acid (BCA) kit were from Sangon Technology (China).

### *Animal experiments*

All mouse experiments were performed in accordance with approved protocols from Shanghai Medical Experimental Animal Care

Commission. Rats with normal erectile functioning were clipped on both sides of the cavernous nerve, then divided into the following five groups: control, sham operation, and bilateral cavernous nerve transection (BCN) groups treated with high, medium, and low concentrations of PNS (150, 100, and 50  $\mu$ g/kg, respectively). Before PNS administration, each rat was injected with 100  $\mu$ g/kg apomorphine (APO) and observed for 30 min, and the time of erection was recorded. Glans penis congestion and penis growth were recorded as an erection. Rats exhibiting no erection were selected as ED models.

### *Cell culture*

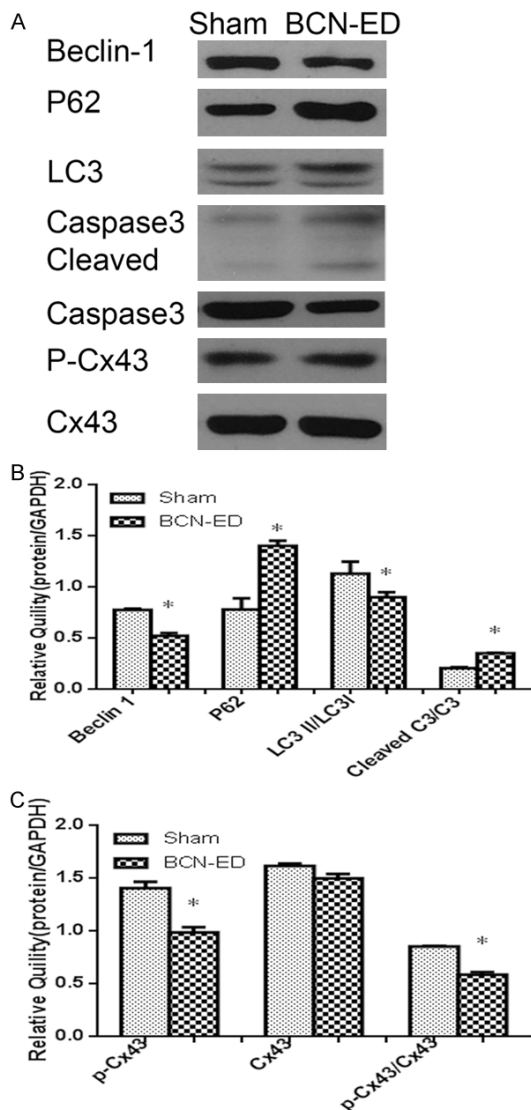
Primary CCSMC cells were isolated from the cavernosum of rats 2 weeks post-surgery and enzymatically digested, then cultured in Dulbecco's modified Eagle's medium. Cells were treated with PNS (100 mg/kg), 3-MA (5 mM), or rapamycin (100 nM).

### *Western blotting*

CCSMCs were lysed in lysis buffer (#78501; Thermo Fisher Scientific, Waltham, MA, USA) containing a protease inhibitor cocktail. Lysates were collected by centrifugation at 12,000 $\times$ g for 10 min at 4°C. Total protein concentration was determined with the BCA kit. Proteins were separated by 8%-14% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad, USA) that was blocked in 5% milk in phosphate-buffered saline with 0.1% Triton X-100 (PBST) for 1 h, then probed with primary antibodies overnight at 4°C. The membrane was washed in PBST and incubated with appropriate secondary antibodies for 1 h at room temperature and washed three times in PBST. Protein bands were visualized by enhanced chemiluminescence using a kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions.

### *GJ analysis with the scrape-loading and dye transfer (SLDT) assay*

The SLDT assay was used to detect GJs between smooth muscle cells. After three washes in PBS, cells were stained with 1% rhodamine and 1% Lucifer Yellow and wounds was stretched on the culture plate. After incubation



**Figure 1.** A. Western blot analysis of autophagy and apoptosis-related protein expression in rats with normal erectile functioning. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. The semi-quantitative analysis of bands from western blot assay. B, C. Data are the mean values  $\pm$  SD.  $P < 0.05$  was considered statistically significant. \* $P < 0.05$  vs Sham group.

for 10 min, cells were fixed with 4% paraformaldehyde and GJs were visualized by fluorescence microscopy (OLYMPUS IX71, Japan).

#### MDC assay

Cells were incubated with 0.05 mM MDC in PBS at 37°C for 10 min, then washed four times with PBS and fixed with 4% paraformaldehyde. Cells were visualized by fluorescence

microscopy at excitation/emission wavelengths of 492/525 nm.

#### Statistical analysis

All data are presented as the mean  $\pm$  SD. Statistical significance was determined by paired or unpaired Student t test in cases of standardized expression data. One-way ANOVA was performed for multiple group comparisons and comparisons between two groups were conducted using the least significant difference method.  $P < 0.05$  was considered significant.

#### Results

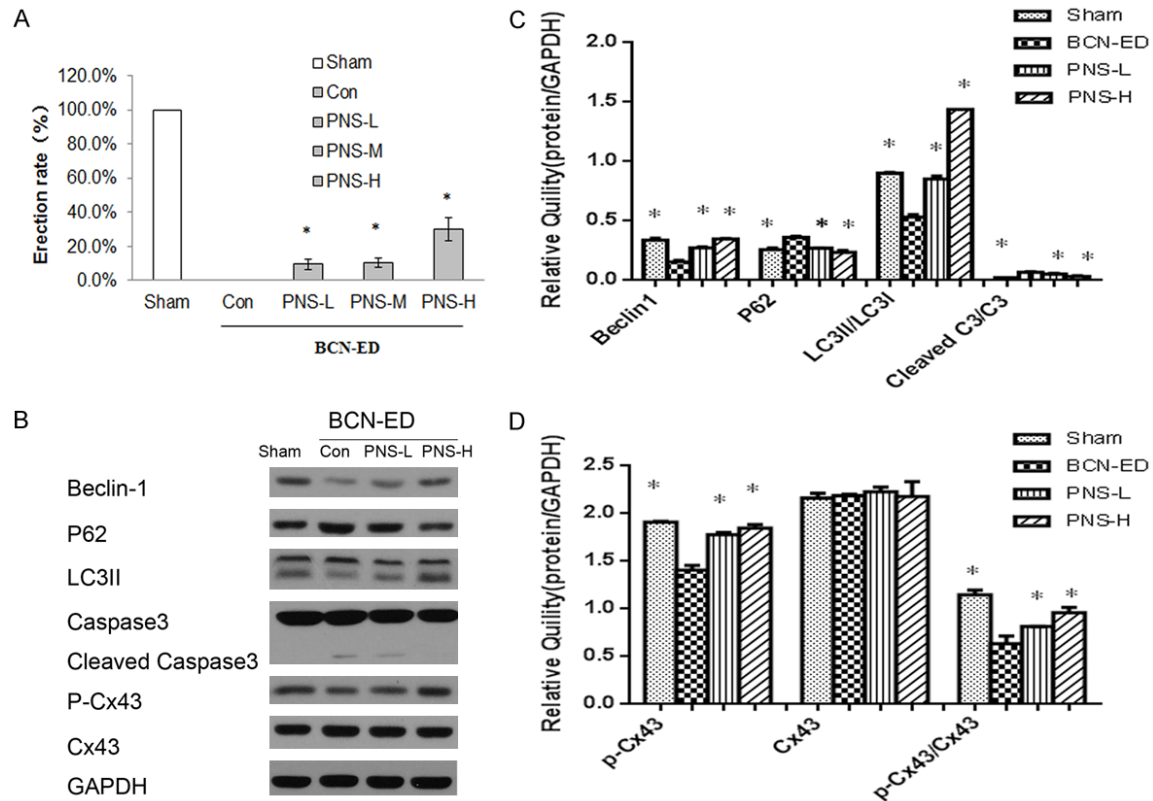
##### *Autophagy is decreased and apoptosis is increased in rats with ED*

Rats were injected with APO (100 mg/kg) 2 weeks after nerve transection and the number of rats exhibiting penile erection was recorded. Rats in the control and sham groups achieved erection whereas those in the BCN group showed ED, indicating that the model was successfully established.

The expression of the autophagy markers LC3-II/I and Beclin-1 were downregulated whereas that of apoptosis-associated proteins p62 and cleaved caspase-3 was upregulated in ED rats, as determined by western blotting. On the other hand, Cx43 phosphorylation was decreased in these animals, suggesting that they had fewer GJs (**Figure 1**).

##### *PNS improves erectile function by inducing autophagy, inhibiting apoptosis, and increasing the number of GJs*

After 2 weeks of PNS treatment, rats were injected with APO (100 mg/kg) and penile erection was evaluated. We found that PNS relieved ED in dose-dependent manner; erection rates in the PNS-L, -M, and -H groups were 10%, 10%, and 30%, respectively (**Figure 2A**). Western blot analysis revealed that LC3-II/I and Beclin-1 were upregulated whereas P62 and cleaved caspase-3 were downregulated in PNS-treated rats. Additionally, PNS increased GJ abundance, as evidenced by increased phosphorylation of Cx43 (**Figure 2B**). These results indicate that PNS improves erectile function by increasing the number of GJs and promoting cell survival through induction of autophagy and inhibition of apoptosis.



**Figure 2.** Effect of PNS on erectile function of rats and the expression of autophagy- and apoptosis-related protein. A. Effect of PNS on erectile function in rats. B. Western blot analysis of autophagy- and apoptosis-related protein expression. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. C, D. The semi-quantitative analysis of bands from western blot assay. Experimental groups were as follows: Sham, sham operation; Con, control; BCN, and bilateral cavernous nerve transection; PNS-H and PNS-L, high (150  $\mu\text{g/kg}$ ) and low (50  $\mu\text{g/kg}$ ) concentrations of PNS, respectively. Data are the mean values  $\pm$  SD.  $P < 0.05$  was considered statistically significant. \* $P < 0.05$  versus BCD-ED group.

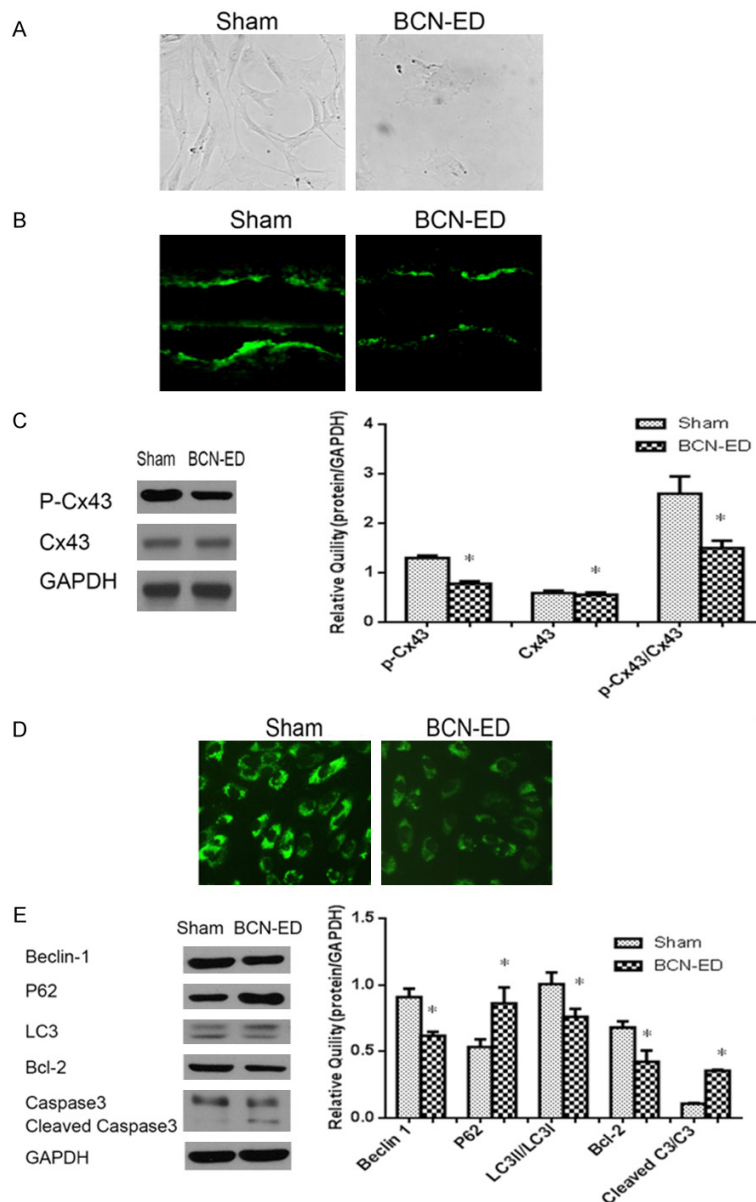
*Autophagy and GJ number are decreased and apoptosis is increased in CCSMCs from ED rats*

Primary CCSMCs were isolated from rats. After three passages, cells from normal rats retained their morphology and biological activity, while those from ED rats had an irregular shape (Figure 3A), which corresponded to a reduction in GJ formation, as determined by the SLDT assay (Figure 3B) and the downregulation of p-Cx43 (Figure 3C). The MDC assay revealed fewer autophagosomes in BCN as compared to sham rats, which was associated with lower levels of LC3-II/I and Beclin-1 and higher levels of P62 and cleaved caspase-3 (Figure 3D, 3E). These results confirm the *in vivo* observation that ED is associated with a reduction in autophagy and GJ abundance and increase in apoptosis.

*PNS and rapamycin treatment increases autophagy and GJ number and inhibits apoptosis in CCSMCs from ED rats*

We investigated whether PNS would have the same effects on CCSMCs as observed *in vivo* and in combination with other agents. Results of the SLDT assay demonstrated that GJs increased in number in BCN-ED groups upon treatment with PNS and rapamycin (Figure 4A); the expression level of LC3II/I, and Beclin-1 were upregulated, and P62 was downregulated in PNS and rapamycin treated group. While the downregulated cleaved caspase-3 suggested that the apoptosis decreased after PNS and rapamycin treated (Figure 4B). The opposite result was observed in 3-MA group. However, although p-Cx43 level was upregulated by PNS, rapamycin and 3-MA treatment had no effect on CX43 phosphorylation (Figure 4C).





**Figure 3.** Autophagy and GJ are decreased and apoptosis is increased in CCSMCs from ED rats. A. In vitro culture of CCSMCs from normal and ED rats at 48 h (200×). B. MDC staining of autophagosomes in Sham and BCN-ED rats. C. Western blot analysis of GJ protein (Cx43) expression in Sham and BCN-ED rats. D. Detection of GJs in CCSMCs by the SLDT assay. E. Western blot analysis of autophagy- and apoptosis-related protein expression. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control for western blot analyses. Data are the mean tumor volumes  $\pm$  SD.  $P < 0.05$  was considered statistically significant. \* $P < 0.05$  versus BCD-ED.

Together, these results indicated that PNS improve cell viability by inducing autophagy and inhibiting apoptosis and upregulate Cx43 phosphorylation, which increased the gap junction of CCSMCs from ED rat by in vitro.

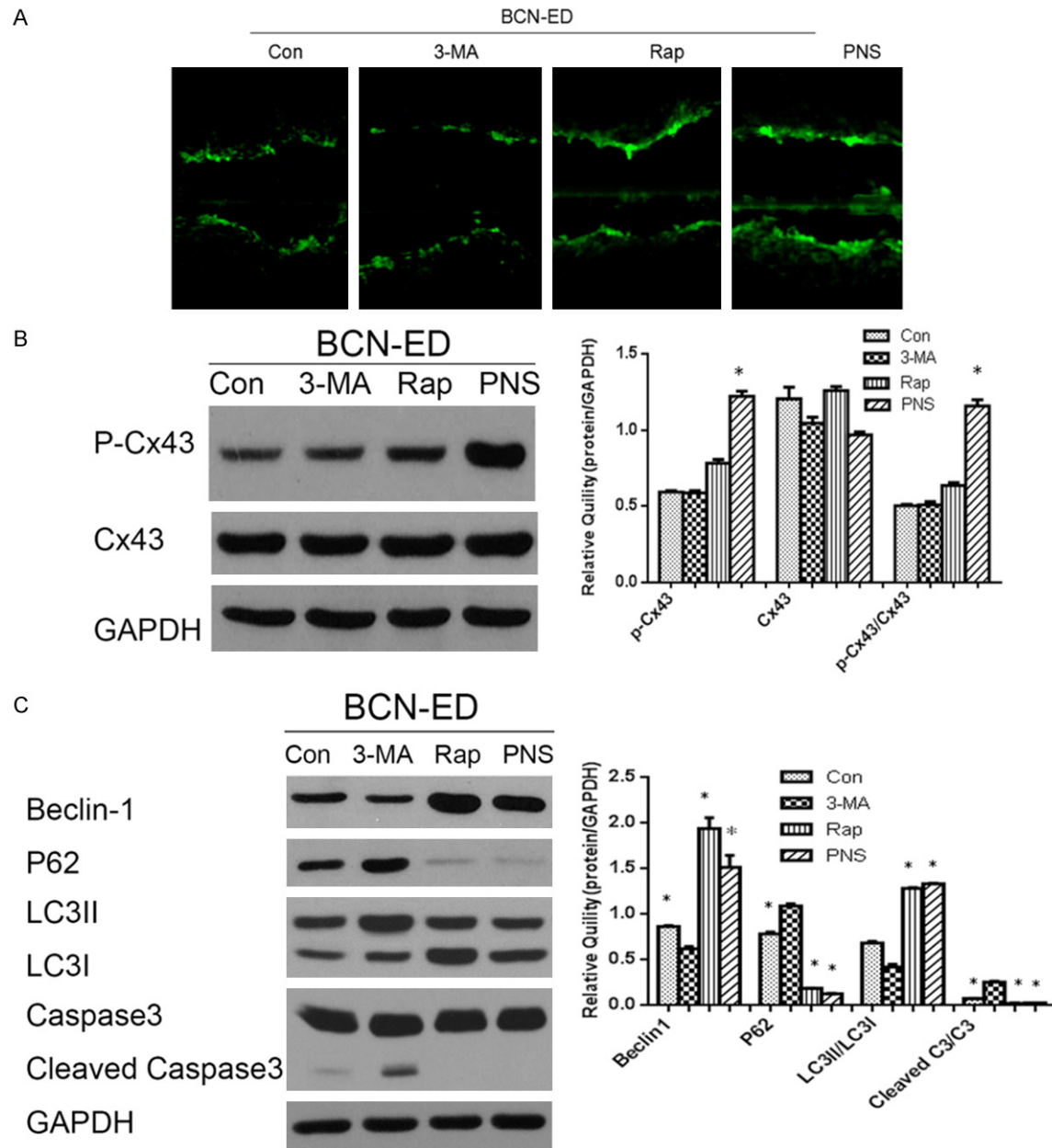
*3-MA inhibited autophagy of CCSMCs induced by PNS, enhanced apoptosis, but does not affect the phosphorylation of Cx43*

CCSMCs were treated with PNS, 3-MA and 3-MA and PNS combination, and then the SLDT, MDC and Western blot assays were accessed. SLDT assay showed that the gap junction, as well as the expression of autophagy-reducing proteins were significantly up-regulated in PNS group (Figure 5A and 5B). In the present of 3-MA and PNS combination, the level of autophagy was reduced and gap junction decreased slightly, compared with PNS treatment alone; however, the levels of p-Cx43 were not different with those exposed to PNS alone (Figure 5C). Together, these results suggested that *in vitro*, 3-MA inhibits autophagy induced by PNS, enhanced apoptosis of CCSMCs. But phosphorylation of Cx43 was independent of autophagy signaling pathway.

## Discussion

*Panaxnotoginseng* is a perennial herb of the Araliaceae family that is used in Chinese medicine. The major active PNSs are the ginsenosides Rb1, Rg1, and Rd and the notoginsenoside R1 [11, 13]. PNS has been shown to increase blood circulation and relieve swelling and pain in clinical trials [13]; it can also prevent vascular restenosis

after balloon injury, improve microcirculation, and promote the normal expansion/contraction of vascular smooth muscle [14]. PNS was also found to stimulate the repair of damaged blood vessels by inducing expression of vascular

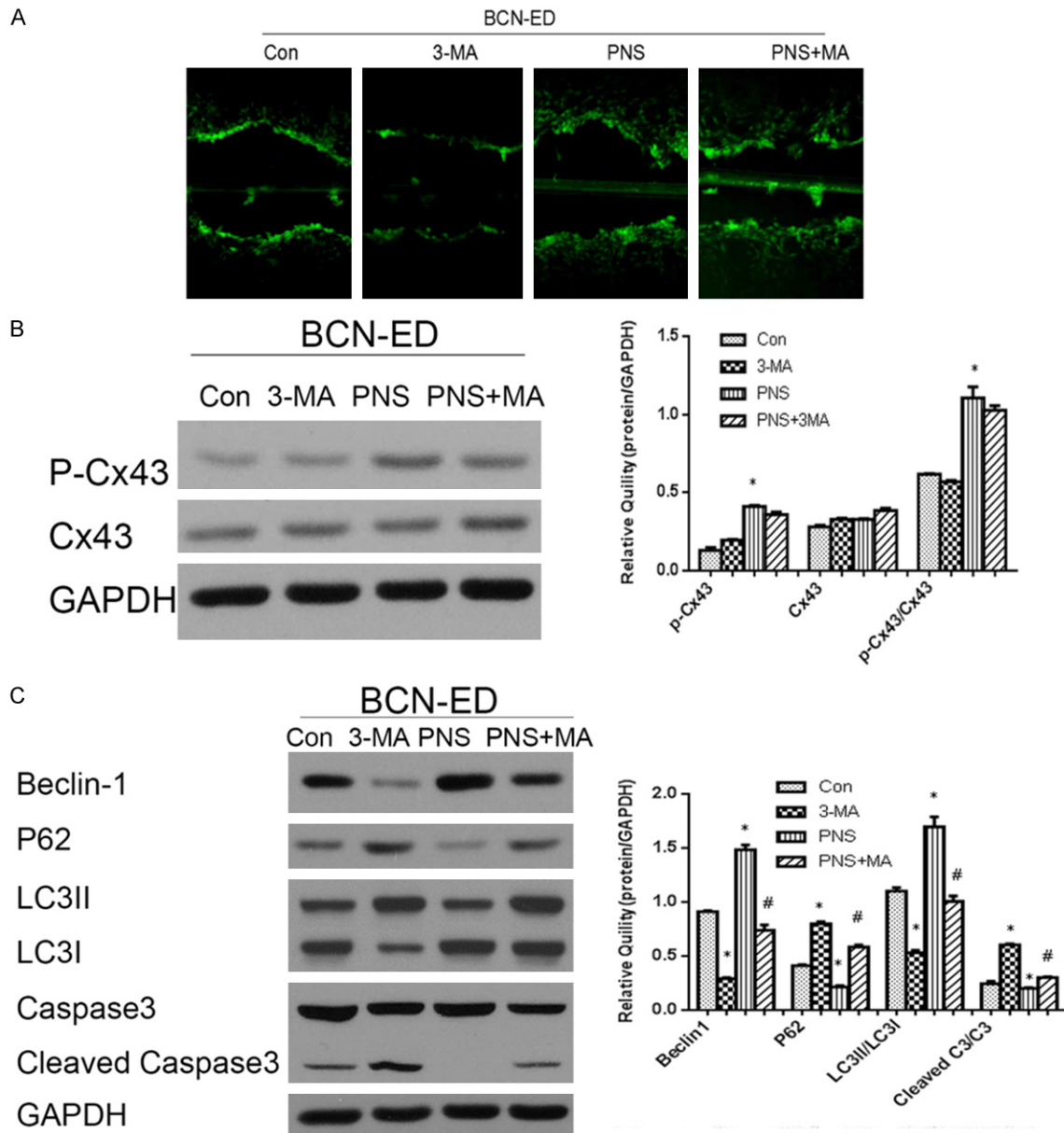


**Figure 4.** (A) Change in GJ abundance in CCSMCs following 3-MA, rapamycin (Rap), and PNS treatment, as determined by the SLDT assay. (B, C) Western blot analysis of autophagy-related protein (B) and Cx43 (C) expression following 3-MA, rapamycin (Rap), and PNS treatment. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. Data are the mean values  $\pm$  SD.  $P < 0.05$  was considered statistically significant. \* $P < 0.05$  versus BCD-ED.

endothelial growth factor and proliferation of vascular endothelial cells [15]. It was previously suggested that PNS could alleviate ED by stimulating eNOS activity in cavernosa cells and thereby increasing NO production and vasodilation [16]. PNS has also been found to block apoptosis by inducing autophagy in normal cells [17, 18]. This is consistent with our find-

ings that the decrease in LC3-II/I and Beclin-1 and increase in p62 and cleaved caspase-3 expression in the corpus cavernosum of ED rats were abrogated by PNS treatment.

Damaged organelles and proteins are sequestered in autophagosomes that fuse with lysosomes to form autophagolysosomes, the con-



**Figure 5.** (A) Change in GJ abundance in CCSMCs following 3-MA and PNS treatment, as determined by the SLDT assay. (B, C) Western blot analysis of autophagy-related protein (B) and Cx43 (C) expression following 3-MA and PNS treatment. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. Data are the mean values  $\pm$  SD.  $P < 0.05$  was considered statistically significant. \* $P < 0.05$  versus BCD-ED, # $P < 0.05$  versus PNS.

tents of which are degraded in order to maintain cellular homeostasis. Beclin-1 is an important regulator of autophagy that forms a complex with Atg1/ULK1 in conjunction with phosphatidylinositol 3-kinase (PI3K). Cells in a high-energy state activate mammalian target of rapamycin complex (mTORC) 1; mTOR binds to the autophagy complex, resulting in inhibition of Beclin-1 activity and suppression of autophagy. Conversely, during energy starvation, mTORC1

dissociates from the Atg1/ULK1 complex, thereby releasing Beclin-1 and stimulating autophagy [18]. It was previously reported that PNS treatment increased Beclin-1 level and the number of autophagosomes, thereby protecting cells by inducing autophagy [19]. In a rat model of diabetes-associated ED, apoptosis of corpus cavernosum cells was markedly increased; PNS was found to stimulate B cell lymphoma-2 expression by suppressing tumor

necrosis factor- $\beta$  and caspase-3 levels and consequently, the mitochondrial apoptosis pathway [20].

There are few reports on the relationship between Cx43 phosphorylation and autophagy. We addressed this in the present study using 3-MA, an autophagy inhibitor that suppresses the activity of triphosphatidylcholine kinase (Class III PI3K) [21]. In contrast, rapamycin promotes autophagy by blocking the mTOR receptor [21]. In this study, we found that combined PNS and rapamycin treatment increased autophagy and decreased apoptosis, whereas the opposite was observed when PNS was combined with 3-MA. These results demonstrate that the PNS counters ED by stimulating the autophagy pathway. However, although Cx43 phosphorylation was upregulated by PNS, it was unaffected by rapamycin and 3-MA, suggesting that the effects of PNS are also exerted via a mechanism involving Cx43 and GJs that is independent of autophagy activation.

In conclusion, the results of this study demonstrate that PNS effectively reversed the increase in apoptosis, decrease in autophagy, and reduction in GJs associated with ED. Therefore, PNS is a promising alternative to sildenafil citrate for the treatment of organic ED in humans.

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## Disclosure of conflict of interest

None.

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