



Argonaute 2 restored erectile function and corpus cavernosum mitochondrial function by reducing apoptosis in a mouse model of cavernous nerve injury

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Purpose: To determine whether the overexpression of the Argonaute RNA-induced silencing complex catalytic component 2 (Ago2) improves erectile function in mice after cavernous nerve injury (CNI).

Materials and Methods: Lentiviruses containing *Ago2* open reading frame (ORF) mouse clone (Ago2 O/E) were used to overexpress Ago2, and lentiviruses ORF negative control particles (NC) were used as a negative control. Three days before preparing the CNI model, we injected lentiviruses into the penises of 8-week-old male C57BL/6 mice. Animals were then divided into four groups: the sham operation control group and the CNI+phosphate-buffered saline, CNI+NC, and CNI+Ago2 O/E groups. One week later, erectile function was assessed by electrically stimulating cavernous nerves bilaterally and obtaining intracavernous pressure parameters. Penile tissue was also collected for molecular mechanism studies.

Results: Ago2 overexpression improved erectile function in mice after CNI-induced erectile dysfunction (ED). Immunofluorescence staining and Western blot analysis showed that under Ago2 overexpressing conditions, the contents of endothelial cells, pericytes, and neuronal cells increased in the penile tissues of CNI mice, and this was attributed to reduced apoptosis and ROS production. In addition, we also found that Ago2 overexpression could restore penile mitochondrial function, thereby improving erectile function in CNI-induced ED mice.

Conclusions: Our findings demonstrate that Ago2 overexpression can reduce penile cell apoptosis, restore penile mitochondrial function, and improve erectile function in CNI-induced ED mice.

Keywords: Apoptosis; Argonaute proteins; Mitochondria dysfunction; Nerve regeneration; Reactive oxygen species

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INTRODUCTION

Erectile dysfunction (ED) is a common problem worldwide, affecting more than half of middle-aged and older men [1]. Recent studies indicate that cavernous nerve injury (CNI)-induced ED occurs in as many as 85% of patients after radical prostatectomy and other pelvic surgeries [2,3]. Most first-line drugs used to treat ED are phosphodiesterase type 5 inhibitors (PDE5I), which enhance the nitric oxide (NO)-cyclic guanosine 3',5'-monophosphate pathway. However, these drugs require a certain amount of endogenous NO [3], and because of severe damage to blood vessels and nerves, the NO secreted by endothelial and neuronal cells is insufficient to meet the demands of penile erection promoted by PDE5I [4]. Therefore, drugs that promote vascular and nerve regeneration are required to treat CNI-induced ED effectively.

Oxidative stress is an imbalance between reactive oxygen species (ROS) and antioxidant defenses and can lead to cell and tissue damage [5]. Numerous studies have shown that oxidative stress is associated with different types of ED, such as in hypertension, diabetes, peripheral pathologies (such as cavernous nerve damage from pelvic surgery or pelvic fracture), and radiation-induced ED [6-8]. A literature survey on the relationship between CNI-induced ED and oxidative stress revealed that oxidative stress plays an important role in CNI-induced ED and different methods of CNI lead to varying degrees of oxidation stress in rat models [6,8]. In addition, Zhao et al. [9] showed that oxidative stress can inhibit cavernous nerve regeneration and induce major pelvic ganglion (MPG) neuronal apoptosis after CNI. Furthermore, antioxidant-like drugs like resveratrol and MitoQ may protect corpus cavernosum under androgen-deprived conditions by stimulating endogenous antioxidant systems [10]. Despite these efforts, the effectiveness of antioxidant therapy alone for treating ED appears limited, and therefore, additional drug candidates that simultaneously promote neurovascular regeneration and modulate oxidative stress are needed.

The catalytic component 2 (Ago2) of argonaute RNA-induced silencing complex is a member of the argonaute family that participates in RNA interference and DNA repair [11]. Zhang et al. [12] showed that Ago2 exists inside mitochondria and may serve as a key mitochondrial translation initiation factor to promote ribosome-mRNA interactions. In addition, the overexpression of mitochondria-targeted Ago2 in mouse embryonic fibroblasts enhanced the effects of mitoRNAi at the RNA level [13], and recently, Liu et al. [14] showed that the exogenous delivery of Ago2 protein can improve erectile function in streptozotocin-induced type-1diabetic mice. However, to our knowledge, no study has addressed the role of

Ago2 in neurological disease (especially CNI-induced ED).

Consequently, we aimed to determine whether increasing Ago2 expression via the lentiviral infection of Ago2 clone particles might promote erectile function in CNI-induced ED mice. We hypothesized that Ago2 protein might improve nerve regeneration and mitochondrial function, reduce cell apoptosis and oxidative stress, and thereby ameliorate erectile dysfunction in CNI-induced ED mice.

MATERIALS AND METHODS

1. Ethics statement and study design

All experimental and animal care procedures were approved by the Institutional Animal Care and Use Committee (assurance number: INHA 230502-871) of Inha University. Eight-week-old male C57BL/6 mice were purchased from Orient Bio, and their health and behavior were monitored daily as previously described [15]. All mice were anesthetized with an intramuscular injection of ketamine (100 mg/kg; Yuhan Corp.) and xylazine (5 mg/kg; Bayer Korea). Mice were euthanized by 100% CO₂ replacement, and death was confirmed by heartbeat and respiration cessation. No mouse died during any experimental procedure, and all experiments were performed in a blind manner (One researcher prepares the model and performs the injection, and another researcher evaluates the final functionality.). To investigate the efficacy and mechanism whereby Ago2 restores erectile function in CNI-ED mice, we injected lentiviruses containing open reading frame (ORF) negative control particles (NC, PS100092V; Origene Technologies) or lentiviruses containing an ORF mouse clone of *Ago2* (Ago2 O/E, MR227026L3V; OriGene Technologies) into the penises of CNI-induced ED mice at three doses (5×10³, 1×10⁴, or 5×10⁴ IFU/mouse). Three days later, we prepared the mouse CNI model and divided mice into four groups as follows (n=7/group): the sham operation group, the CNI+phosphate-buffered saline (PBS) group, and the CNI+NC and CNI+Ago2 O/E groups. After infection with lentivirus containing the Ago2 O/E, no mice died or showed any side effects. One week later, erectile function was assessed, and penises were harvested for molecular studies.

2. CNI model preparation

To perform CNI, a non-serrated hemostat (Karl Storz) was applied with full tip closure bilaterally to the both side of cavernous nerves 1 mm distal to the ganglion for 2 minutes, as described previously [16]. CNI mice were then randomly divided into three groups for further injection.

3. Measurement of erectile function

For erectile function measurement, a bipolar platinum wire electrode (BIOPAC Systems) was used to stimulate cavernous nerve using the following parameters: voltage 5 V, frequency 12 Hz, pulse width 1 ms, and duration 60 seconds. Maximal intracavernous pressure (ICP) was recorded during tumescence, and total ICP values were determined from the area under the ICP curve from stimulus onset to 20 seconds after stimulus termination. Systemic blood pressure was estimated using a non-invasive tail-cuff system (Visitech Systems). Maximum and total ICP to mean systolic blood pressure (MSBP) ratios were determined to calibrate variations in systemic blood pressure as described previously [17]. Measurements of erectile function were performed in a blinded manner.

4. TUNEL assay

TUNEL assays were performed using an ApopTag Fluorescein *In Situ* Apoptosis Detection Kit (Chemicon). Nuclear staining was performed by mounting samples in a DAPI solution (H-1200-10; Vector Laboratories). Digital images were obtained using a confocal fluorescence microscope (K1-Fluo; Nanoscope Systems), and numbers of TUNEL-positive cells were quantified in a blinded manner using ImageJ software (National Institutes of Health [NIH] 1.34; <https://imagej.net/ij/nih-image/>).

5. ROS detection

Hydroethidine (Molecular Probes), an oxidative fluorescent dye that detects intracellular superoxide anions, was used to assess ROS production *in situ*, and nitrotyrosine (1:50; Millipore) staining to detect peroxynitrite, as previously described [18]. Frozen sections of penile tissues (12 μ m) were stained with nitrotyrosine (1:50; Upstate Technology) and the corresponding second antibody. Samples were incubated with hydroethidine (1:10,000; Molecular Probes) for 30 minutes at room temperature in subdued light. Immunopositive areas of ethidium bromide and nitrotyrosine immunopositive from four separate experiments were measured. To estimate mitochondrial ROS (MitROS) contents, frozen sections of penile tissues (12 μ m) were stained with MitoSOX Red (1 μ M; Invitrogen) for 30 minutes at 37°C in subdued light. Images were visualized using a confocal fluorescence microscope (Nanoscope Systems), and immunopositive areas from four separate experiments were measured in a blinded manner.

6. Adenosine triphosphate (ATP) assay

ATP levels in penile tissues were determined using an ATP assay kit (Abcam). Briefly, 10 mg tissue homogenates

of mice in each group were lysed in 100 μ L of ATP analysis buffer and centrifuged at 13,000g for 5 minutes at 4°C. ATP levels were then measured in supernatants at 570nm using a microplate spectrophotometer (BioTek Instruments).

7. Histologic examination

Harvested penile tissues were fixed in 4% paraformaldehyde for 24 hours at 4°C. Frozen tissue sections (12 μ m) were blocked with 1% bovine serum albumin (Sigma-Aldrich) for 1 hour at room temperature and then incubated with the primary antibodies against CD31 (1:50; Millipore), NG2 (1:50; Millipore), Ago2 (1:50; Abcam), Neurofilament (NF, 1:50; Sigma-Aldrich), and neuronal nitric oxide synthase (nNOS, 1:50; Santa Cruz Biotechnology) at 4°C overnight. After washing with PBS, samples were incubated with corresponding second antibodies for 2 hours at room temperature. Images were visualized using a confocal fluorescence microscope (Nanoscope Systems), and stained areas from four separate experiments were measured in a blinded manner.

8. Western blot

For immunoblot analyses, a total of 30 μ g/lane of proteins were separated on 8%–15% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. After blocking membranes with 5% nonfat dry milk (BD) for 1 hour at room temperature, membranes were incubated with primary antibodies against Ago2 (1:2,000; Abcam), nNOS (1:500; Santa Cruz Biotechnology), nerve growth factor (NGF, 1:500; Abcam), brain derived neurotrophic factor (BDNF, 1:500; Cell Signaling), neurotrophin-3 (NT-3, 1:500; Novus Biologicals), Bax (1:1,000; Santa Cruz Biotechnology), Bcl-2 (1:1,000; Santa Cruz Biotechnology), phospho-c-JUN (1:1,000; Cell Signaling), c-JUN (1:1,000; Cell Signaling), phospho-JNK (1:1,000; Cell Signaling), JNK (1:1,000; Cell Signaling), inducible nitric oxide synthase (iNOS, 1:1,000; Abcam), p47phox (1:1,000; Santa Cruz Biotechnology), MT-ND1 (1:1,000; Novus Biologicals), Cytochrome c oxidase I (COX1, 1:1,000; Abcam), and β -actin (1:5,000; Santa Cruz Biotechnology) at 4°C overnight, and then incubated with appropriate HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 hour at room temperature. Signals were detected using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). The results were quantified by densitometry from four separate experiments in a blinded manner.

9. Statistical analysis

The analysis was conducted with GraphPad Prism version 8 (GraphPad Software). Results are expressed as the mean \pm standard error of mean of at least four independent

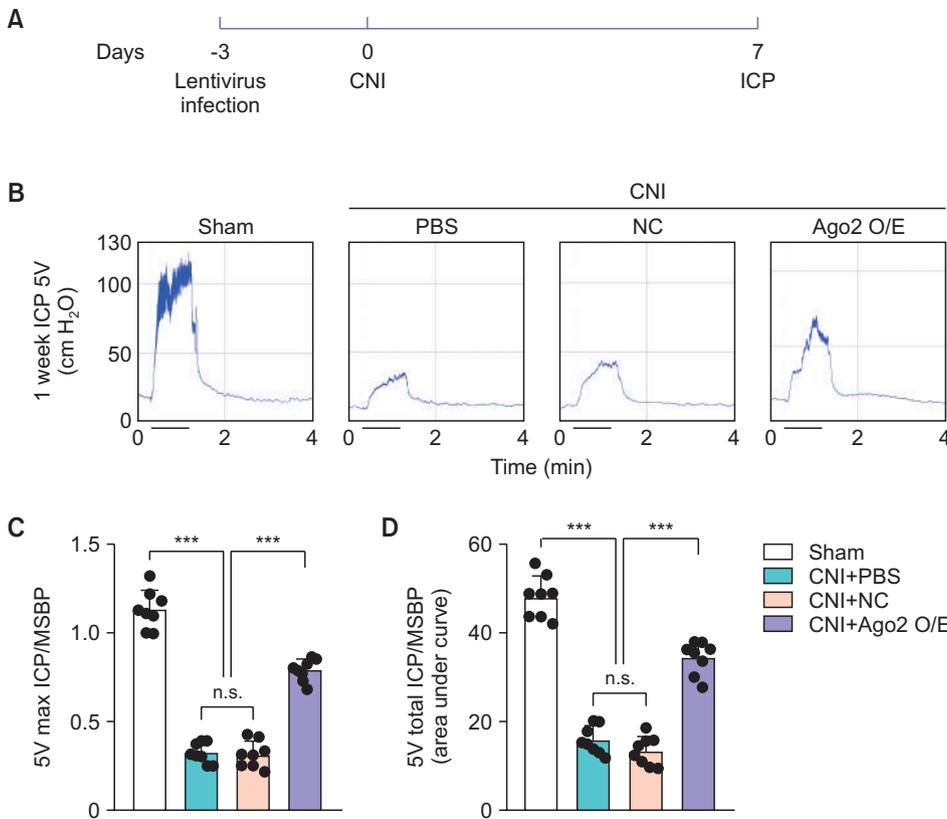


Fig. 1. Ago2 overexpression improved erectile function in CNI mice. (A) Schematic of the experimental procedure. (B) Representative ICP responses of sham (7 days) and CNI (7 days) mice stimulated at 10 days after an intracavernous injection with PBS (20 μ L), lentiviruses containing ORF negative control particles (NC, 5×10^4 IFU/mice), or lentiviruses containing an ORF mouse clone of *Ago2* (Ago2 O/E, 5×10^4 IFU/mice). (C, D) Ratios of mean maximum and total ICPs (area under the curve) to MSBP were calculated for each group and results are presented as mean \pm standard error of mean (n=8). ***p<0.001. CNI, cavernous nerve injury; ICP, intracavernous pressure; PBS, phosphate-buffered saline; ORF, open reading frame; IFU, infectious units; MSBP, mean systolic blood pressure; n.s., not significant.

experiments. Unpaired t-tests were used to compare two groups, and a one-way ANOVA followed by Tukey’s post hoc test was used to compare three or more groups. Statistical significance was accepted for p-values <0.05.

RESULTS

1. Ago2 improved erectile function in CNI mice

Ago2 is known to improve erectile function in diabetic mice [14]. To investigate whether Ago2 overexpression has beneficial effects on erectile function in CNI mice, we first evaluated Ago2 expression in penile tissues at different times (1, 3, 5, 7, and 14 days after surgery). We found that the expression of Ago2 protein exhibited an upward trend within the first 3 days in CNI mice and then returned to sham levels (Supplementary Fig. 1). We then overexpressed Ago2 in CNI mice by intracavernosal injection of lentiviruses containing an Ago2 O/E at different doses (5×10^3 , 1×10^4 , or 5×10^4 IFU/mice). The expressions of Ago2 in the 1×10^4 and 5×10^4 infection groups were significantly higher than in the CNI+NC group (NC, 5×10^4 IFU/mice), while the change in the 5×10^3 group was slight (Supplementary Fig. 2A, B). Therefore, we overexpressed Ago2 at a dose of 1×10^4 in CNI mice (Supplementary Fig. 2C, D) and assessed erectile function one week later. A schematic of the experimental

procedure is shown in Fig. 1A. During electrical stimulation, the ratios of maximum and total ICP to MSBP were significantly reduced in CNI+PBS and CNI+NC groups than in the sham group. However, these erection parameters were improved in the CNI+Ago2 O/E group and reached 70% of that in the sham group (Fig. 1B-D). No detectable differences in MSBP were observed between the sham operation control group and the CNI+PBS, CNI+NC, and CNI+Ago2 O/E groups. These results suggest that Ago2 overexpression might improve erectile function in CNI mice.

2. Ago2 overexpression increased cavernous endothelial cell, pericyte, and neuronal cell contents in CNI mice

To determine the effects of Ago2 on neurovascular regeneration in CNI mice, we performed immunofluorescent staining for CD31 (an endothelial cell marker), NG2 (a pericyte marker), neuronal NOS (nNOS, a neuronal cells), and neurofilament-2000 (NF, a neuronal cells) in the corpus cavernosum and dorsal nerve bundles (DNB) of penile tissues. We found that the numbers of CD31-positive endothelial cells (Fig. 2A, B), NG2-positive pericytes (Fig. 2A, C), and nNOS, NF-positive neuronal cells (Fig. 3A-C) were significantly lower in the CNI+PBS and CNI+NC groups than in the sham group. However, these neurovascular contents

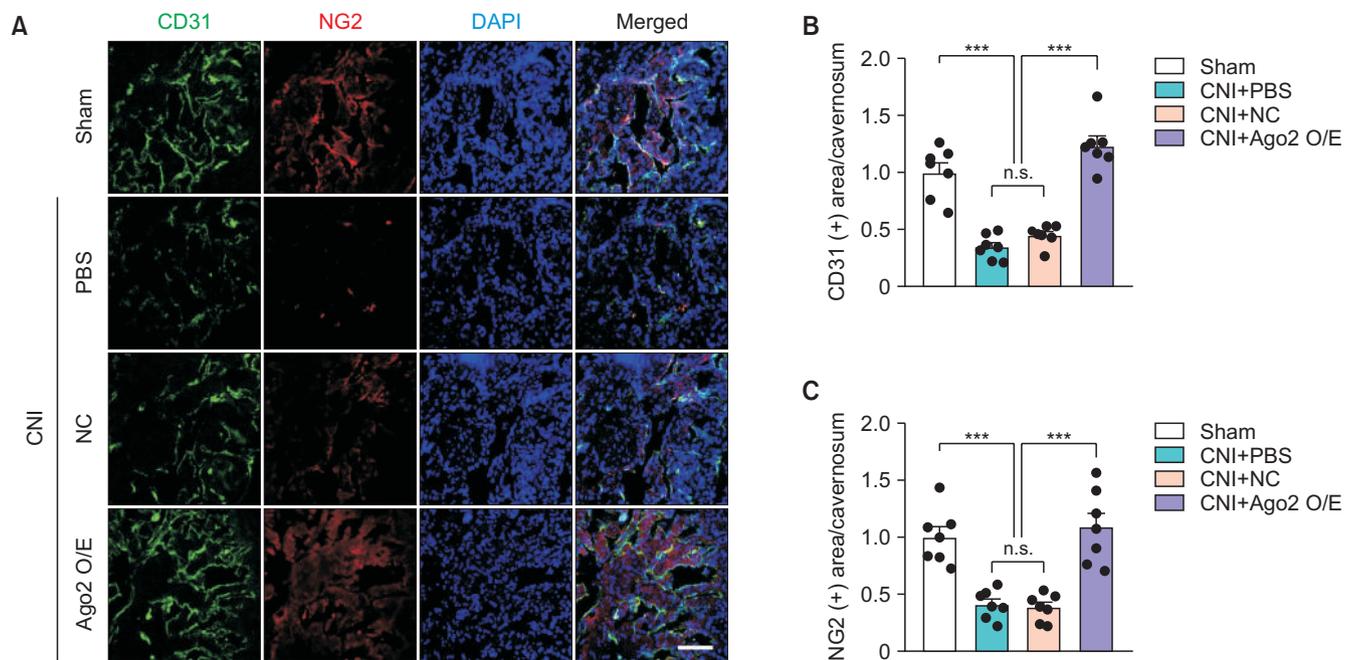


Fig. 2. Ago2 overexpression increased cavernous endothelial and pericyte numbers in CNI mice. (A) Immunofluorescence staining for CD31 (an endothelial cell marker, green) and NG2 (a pericyte marker, red) in cavernous tissues from sham, CNI mice after an intracavernous injection with PBS (20 μ L), lentiviruses containing ORF negative control particles (NC, 5×10^4 IFU/mice), or lentiviruses containing an ORF mouse clone of *Ago2* (*Ago2* O/E, 5×10^4 IFU/mice). Nuclei were labeled with DAPI (blue). Scale bar=100 μ m. (B, C) Quantification of CD31 and NG2 immunopositive areas in cavernous tissue was performed using ImageJ software relative to the sham control. Results are presented as mean \pm standard error of mean (n=7). ***p<0.001. The relative ratio in the Sham group was defined as 1. CNI, cavernous nerve injury; PBS, phosphate-buffered saline; ORF, open reading frame; IFU, infectious units; DAPI, 4,6-diamidino-2-phenylindole; n.s., not significant.

were dramatically higher in the CNI+Ago2 O/E group (Fig. 3A-C). In addition, western blot analysis showed that the expressions of Ago2, nNOS, and neurotrophic factors (BDNF and NT-3) were also significantly lower in the CNI+PBS and CNI+NC groups than in the sham group, while the expressions of these proteins in the CNI+Ago2 O/E group were similar to those in the sham group (Fig. 3D-H). These results indicate that Ago2 overexpression can induce neurovascular regeneration and thereby facilitate erection in CNI mice.

3. Ago2 overexpression decreased apoptosis in CNI mice

A TUNEL assay was conducted on cavernous tissues collected after ICP studies to explore how Ago2 induces neurovascular regeneration. We found that apoptosis was markedly higher in the CNI+PBS and CNI+NC groups than in the sham group. However, the apoptosis in the Ago2 O/E group was similar to that in the sham group (Fig. 4A, C). Furthermore, western blot showed that apoptosis factors, such as Bcl-2, Bax, and phosphorylated c-Jun and JNK, were at sham levels in the Ago2 O/E group (Fig. 4B, D-G). These studies suggest that Ago2 can help reduce cell death after nerve injury, thus preserving cavernous vascular and neuronal cell content.

4. Ago2 overexpression reduced ROS production in CNI mice

A previous study showed that Ago2 can reduce ROS production in diabetic mice [14]. Here, we performed immunofluorescence staining to observe ROS production, as determined by the generation of peroxynitrite (nitrotyrosine) and superoxide anions (hydroethidine). Imaging data indicated that nitrotyrosine and hydroethidine levels were significantly higher in the CNI+PBS and CNI+NC groups than in the sham group, but at the sham level in the Ago2 O/E group (Fig. 5A-C). In addition, western blot revealed that the expressions of p47phox (an active catalytic subunit of NADPH oxidase) and iNOS in Ago2 O/E group were at sham levels (Fig. 5D-F). These results indicate that Ago2 overexpression can reduce penile ROS production in CNI mice.

5. Ago2 overexpression restored penile mitochondrial function in CNI mice

Many studies have indicated that Ago2 directly influences mitochondrial translation and that loss of Ago2 enhances mitochondrial oxidation [12,19]. Therefore, we stained tissues with MitoSOX, a fluorescent dye designed for mitochondrial superoxide. MitoSOX immunopositive areas were significantly greater in the CNI+PBS and CNI+NC groups than

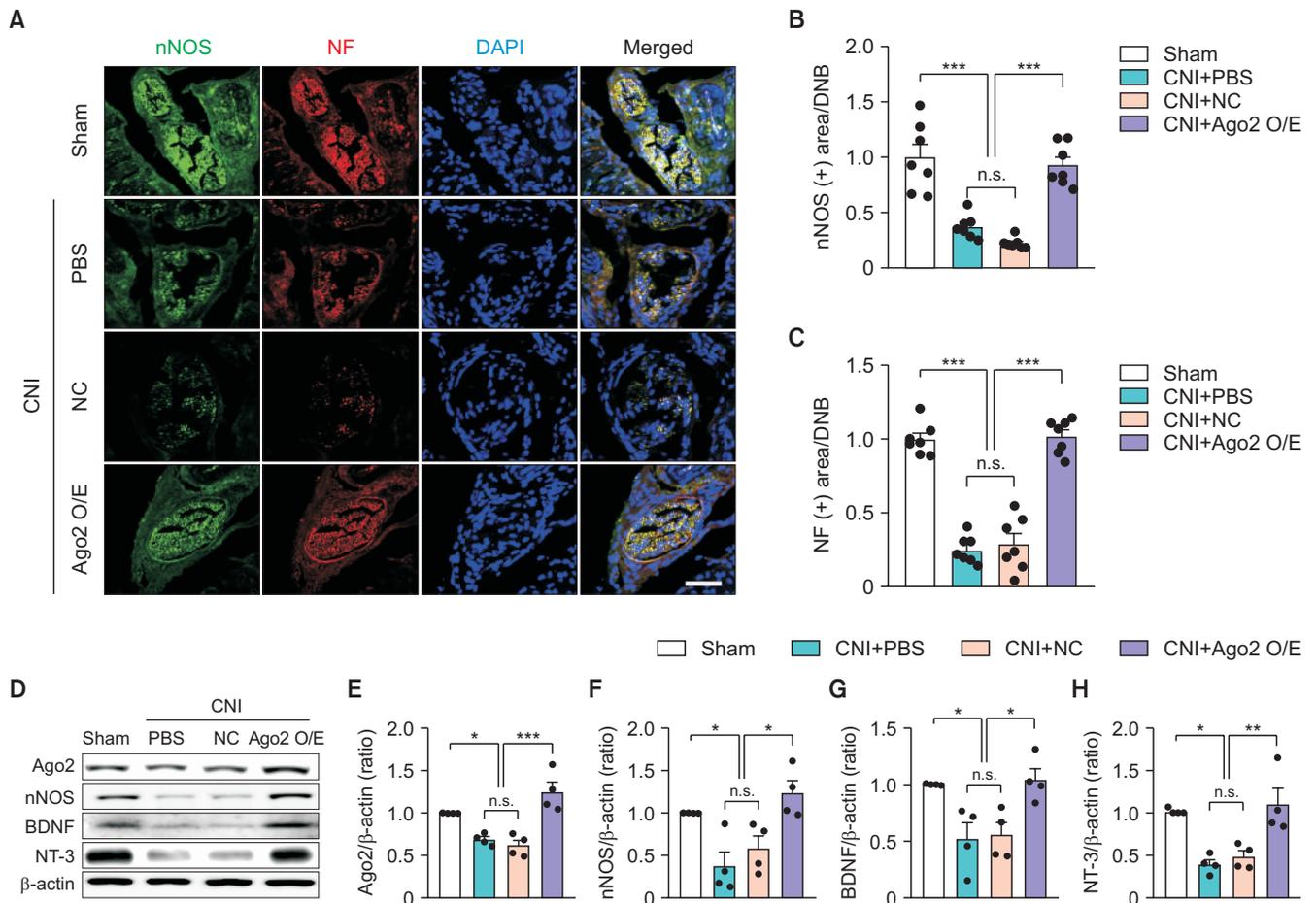


Fig. 3. Ago2 overexpression induced nerve regeneration in CNI mice. (A) Immunofluorescence staining for nNOS (green) and NF (red) in DNB from sham, CNI mice after an intracavernous injection with PBS (20 μ L), lentiviruses containing ORF negative control particles (NC, 5×10^4 IFU/mice), or lentiviruses containing an ORF mouse clone of *Ago2* (*Ago2* O/E, 5×10^4 IFU/mice). Nuclei were labeled with DAPI (blue). Scale bar=25 μ m. (B, C) Quantification of nNOS and NF immunopositive areas in cavernous tissue was performed using ImageJ software, and results are presented as mean \pm standard error of mean (SEM) (n=7). ***p<0.001. (D) Representative western blots for Ago2, nNOS, BDNF, and NT-3 in cavernous tissues of mice in the groups mentioned above. β -Actin was used as the internal control. (E-H) Normalized band intensity values were quantified using ImageJ software relative to the sham control. Results are presented as mean \pm SEM (n=4). *p<0.05; **p<0.01; ***p<0.001. The relative ratio in the Sham group was defined as 1. CNI, cavernous nerve injury; nNOS, neuronal nitric oxide synthase; NF, neurofilament; DNB, dorsal nerve bundles; PBS, phosphate-buffered saline; ORF, open reading frame; IFU, infectious units; DAPI, 4,6-diamidino-2-phenylindole; BDNF, brain-derived neurotrophic factor; NT-3, neurotrophin-3; n.s., not significant.

in the sham group but at the sham level in the *Ago2* O/E group (Fig. 6A, B). Next, mitochondrial function was assessed by measuring energy synthesis (ATP levels in mitochondria) and the expressions of mitochondrial proteins (MT-ND1 and MT-CO1). ATP levels (Fig. 6C) and mitochondrial protein expression (Fig. 6D-F) in the CNI+PBS and CNI+NC groups showed the same downward trend compared to the sham group but remained at sham levels in the *Ago2* O/E group. Taken together, these results demonstrate that *Ago2* overexpression can restore penile mitochondrial dysfunction in CNI mice.

DISCUSSION

Ago2 is known to affect gene expression and signaling pathways, and loss of *Ago2* may impair cell survival and inhibit human umbilical vein endothelial cells [20] and the angiogenesis of hepatocellular carcinoma [21], which suggests that *Ago2* plays a critical role in angiogenesis. In addition, *Ago2* protects neurovascular units from damage caused by systemic inflammation [22]. Our recent studies show that *Ago2* plays an important role in restoring erectile function in type 1 diabetic mice [14]. However, the biological function and specific mechanism of *Ago2* in the peripheral nervous system remain unclear, and therefore, we hypothesized that *Ago2* might beneficially influence neurogenic ED. Interest-

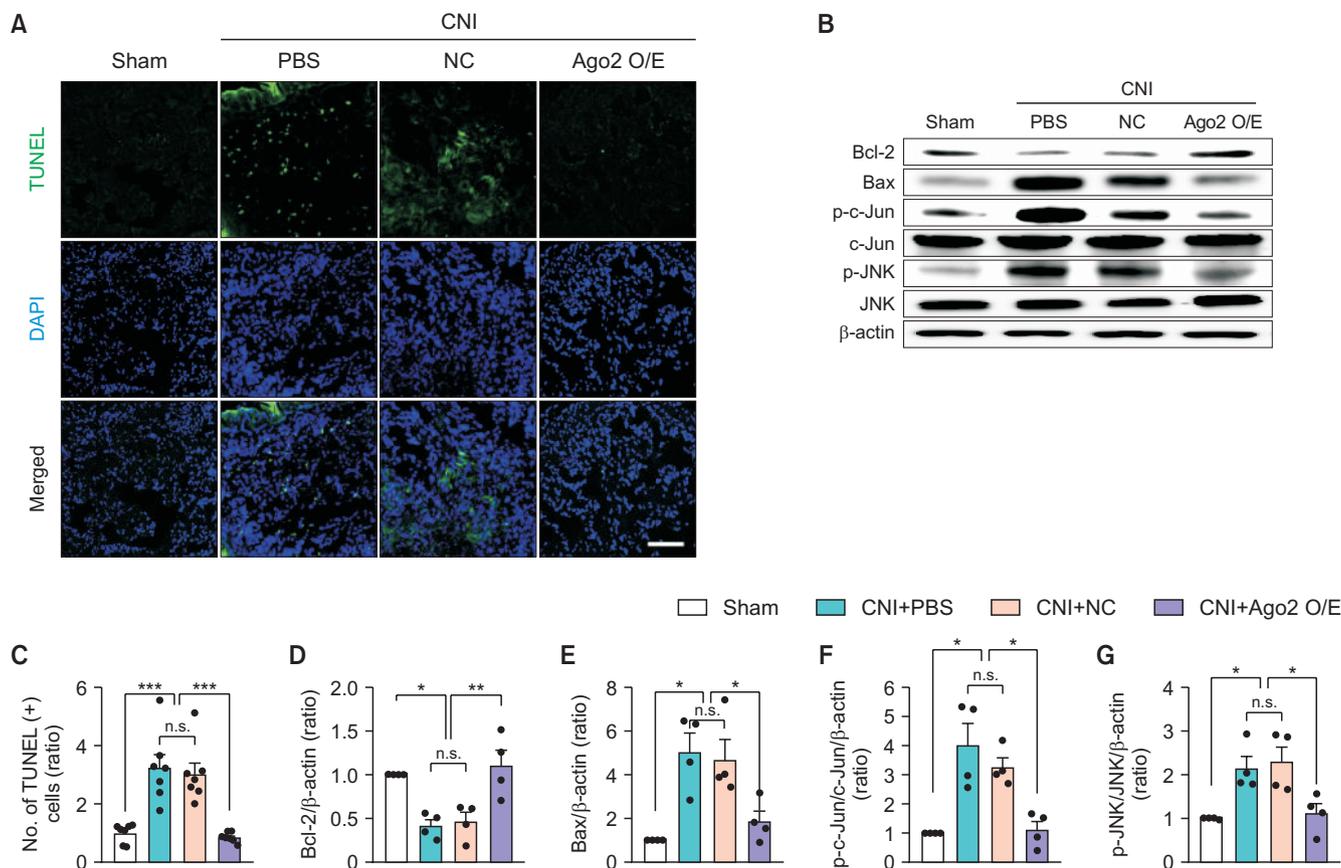


Fig. 4. Ago2 overexpression reduced apoptosis in CNI mice. (A) TUNEL assay of cavernous tissues from sham, CNI mice after an intracavernous injection with PBS (20 μ L), lentiviruses containing ORF negative control particles (NC, 5×10^4 IFU/mice), or lentiviruses containing an ORF mouse clone of *Ago2* (*Ago2* O/E, 5×10^4 IFU/mice). Nuclei were labeled with DAPI (blue). Scale bar=100 μ m. (B) Representative western blots for Bcl-2, Bax, phosphor-c-Jun/c-Jun (p-c-Jun/c-Jun), and phosphor-JNK/JNK (p-JNK/JNK) in cavernous tissues of mice in the groups mentioned above. β -Actin was used as the internal control. (C) Quantification of the number of apoptotic cells in cavernous tissue was performed using ImageJ software relative to the sham control. Results are presented as mean \pm standard error of mean (SEM) (n=7). ***p<0.001. (D-G) Normalized band intensity values were quantified by ImageJ software and results are presented as mean \pm SEM (n=4). *p<0.05; **p<0.01. The relative ratio in the Sham group was defined as 1. CNI, cavernous nerve injury; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling; PBS, phosphate-buffered saline; ORF, open reading frame; IFU, infectious units; DAPI, 4,6-diamidino-2-phenylindole; n.s., not significant.

ingly, in our initial experiments, the expression of Ago2 in the penile tissues of CNI-induced ED mice peaked at 3 days and then fell back to normal levels. Accordingly, we speculate that the amount of endogenous Ago2 present after nerve injury was insufficient to respond adequately to the needs of injured blood vessels and nerves.

In the present study, we used lentiviruses containing the *Ago2* O/E, which provided consistent and robust *Ago2* overexpression, to better visualize the role of *Ago2* in CNI-induced ED. We found that erectile function in CNI-induced ED mice was significantly improved by *Ago2* overexpression, and although the specific mechanisms have not been elucidated, several emerging studies suggest potential interactions between *Ago2* and ROS regulation [14,23]. Furthermore, oxidative stress is known to ameliorate neuronal damage and cell apoptosis [9,24]. Cheloufi et al. [25] demonstrated that *Ago2* plays an important role in the maturation of erythro-

cytes (essentially oxygen carriers), which suggests that *Ago2* may play a role in regulating oxygen stress. Interestingly, we also found that *Ago2* overexpression can reduce the production of ROS in penile tissues, which is consistent with these previous findings [14,23]. However, this study could not demonstrate whether these neurovascular regenerate effects (Figs. 2, 3) of *Ago2* are direct or through other pathways (such as RNA interference, DNA repair), which requires further gain or inhibition studies to elucidate.

Mitochondria are the powerhouse of cells and key organelles involved in energy production through oxidative phosphorylation [26]. However, their dynamic role is not limited to ATP synthesis as it also includes ROS generation [27]. As the main source of endogenous ROS, mitochondria can maintain the balance of intracellular ROS [27,28], and thus, impaired mitochondrial function may lead to ROS imbalance, leading to oxidative damage, apoptosis, and tissue dam-

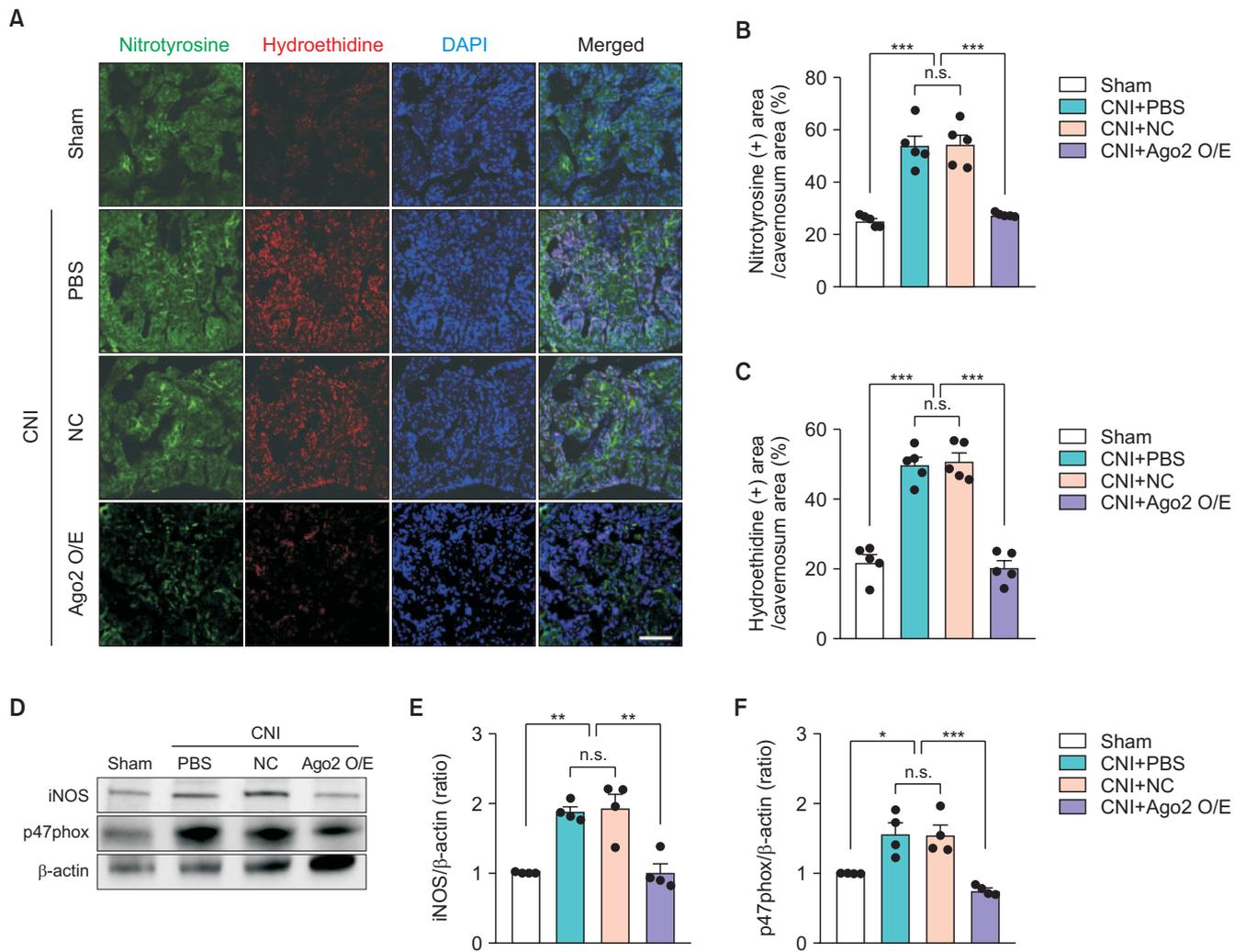


Fig. 5. Ago2 overexpression reduced ROS production in CNI mice. (A) Immunofluorescence staining for nitrotyrosine (green, peroxy-nitrite) and *in situ* detection of hydroethidine (red, superoxide anion) in cavernous tissue from sham, CNI mice after an intracavernous injection with PBS (20 μ L), lentiviruses containing ORF negative control particles (NC, 5×10^4 IFU/mice), or lentiviruses containing an ORF mouse clone of *Ago2* (Ago2 O/E, 5×10^4 IFU/mice). Nuclei were labeled with DAPI (blue). Scale bar=100 μ m. (B, C) Quantification of nitrotyrosine and hydroethidine immunopositive areas in cavernous tissue was performed using ImageJ software relative to the sham control. Results are presented as mean \pm standard error of mean (SEM) (n=5). ***p<0.001. (D) Representative western blots for iNOS and p47phox in cavernous tissue with the same groups as mentioned above. β -Actin was used as the internal control. (E, F) Normalized band intensity values were quantified using ImageJ software relative to the sham control. Results are presented as mean \pm SEM (n=4). *p<0.05; **p<0.01; ***p<0.001. The relative ratio in the Sham group was defined as 1. CNI, cavernous nerve injury; PBS, phosphate-buffered saline; ORF, open reading frame; IFU, infectious units; DAPI, 4,6-diamidino-2-phenylindole; n.s., not significant.

age [29]. Zhan et al. [30] showed that Ago2 levels are reduced in the mitochondria of diabetic cardiomyocytes and that overexpression of mitochondrial Ago2 alleviated diabetes-induced cardiac dysfunction. In this study, we found that mitochondrial ROS was significantly increased (as determined by MitoSOX staining), and mitochondrial function was significantly decreased, based on ATP levels and mitochondrial protein expression, in CNI-induced ED. However, these changes were returned to normal levels after Ago2 overexpression. Unfortunately, because MitoSOX staining indicated the presence of oxidative stress in the mitochondria of all

cells in penile tissue, we could not identify the cell types in which oxidative stress predominated. Further *in vitro* experiments are needed to demonstrate in which type of cells Ago2 overexpression plays main effect in the development of mitochondrial function. In addition, although we demonstrated that Ago2 overexpression can improve erectile function in CNI-induced ED mice by reducing oxidative stress and apoptosis and enhancing mitochondria, the mechanisms involved were not elucidated. While our findings support the potential of Ago2-based therapies, since our study focused on the effects of Ago2 in mice, these effects of Ago2 need to be

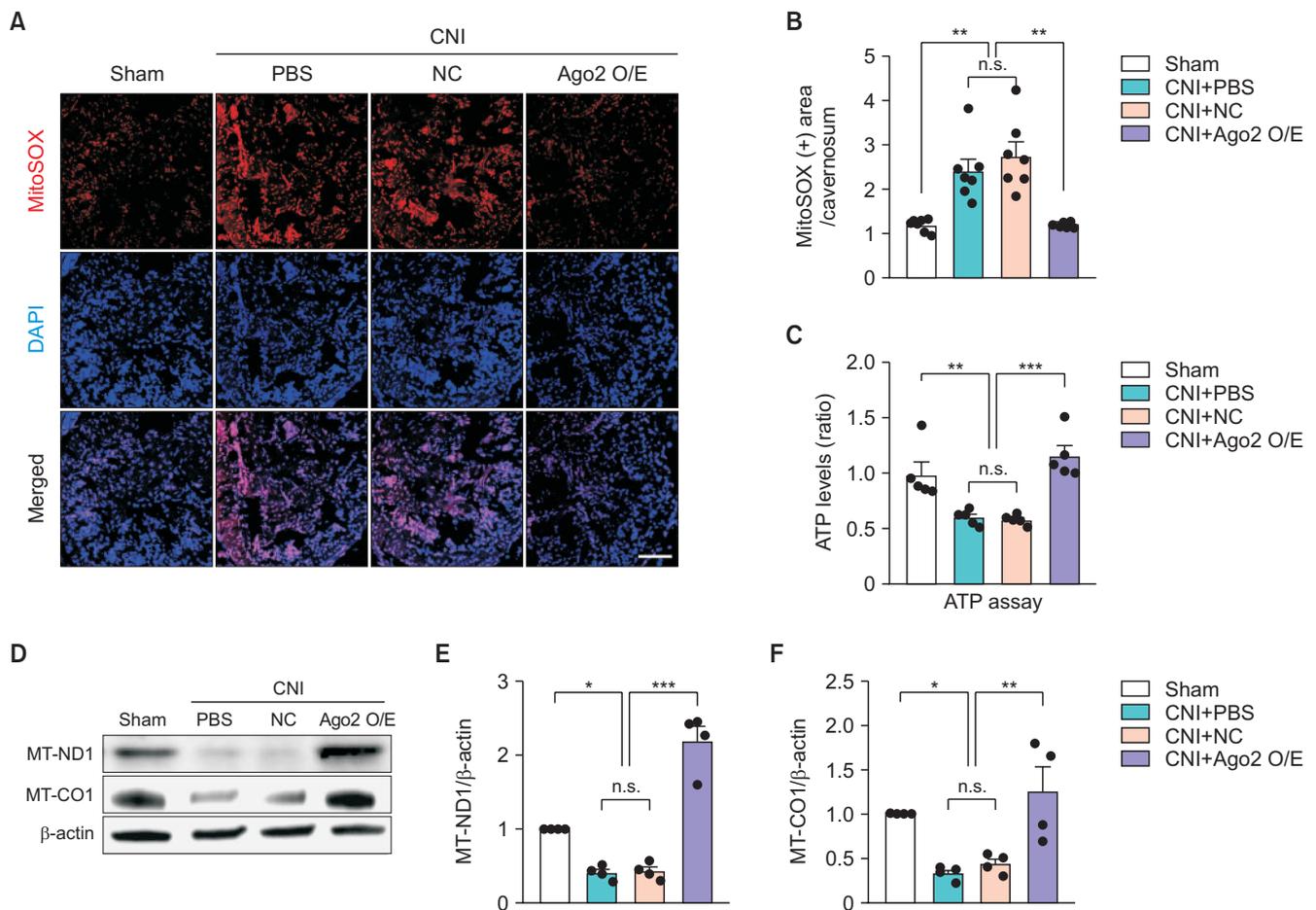


Fig. 6. Ago2 overexpression restored mitochondrial dysfunction in CNI mice. (A) Immunofluorescence staining with MitoSOX red (for mitochondria superoxide) in cavernous tissues from sham, CNI mice after an intracavernous injection with PBS (20 μ L), lentiviruses containing ORF negative control particles (NC, 5×10^4 IFU/mice), or lentiviruses containing an ORF mouse clone of *Ago2* (*Ago2* O/E, 5×10^4 IFU/mice). Nuclei were labeled with DAPI (blue). Scale bar=100 μ m. (B) Quantification of MitoSOX immunopositive areas in cavernous tissue was performed using ImageJ software and results are presented as mean \pm standard error of mean (SEM) (n=7). **p<0.01; ***p<0.001. (C) Ratios of mitochondria ATP levels in cavernous tissue were determined using an ATP assay kit and the groups mentioned above (n=5). **p<0.01; ***p<0.001. (D) Representative western blots for MT-ND1 and MT-CO1 in the cavernous tissues of mice in the same groups. β -Actin was used as the internal control. (E, F) Normalized band intensity values were quantified using ImageJ software relative to the sham control. Results are presented as mean \pm SEM (n=4). *p<0.05; **p<0.01; ***p<0.001. The relative ratio in the Sham group was defined as 1. CNI, cavernous nerve injury; PBS, phosphate-buffered saline; ORF, open reading frame; IFU, infectious units; DAPI, 4,6-diamidino-2-phenylindole; n.s., not significant.

confirmed in studies with human tissues or human-derived cells. Potential side effects, long-term effect and safety, and nonclinical toxicity testing of Ago2 overexpression need to be thoroughly investigated when considering translation to the clinical settings. Maybe neurectomy model will be better for long-term effect evaluation. It is known that ED can have multiple causes, and treatments that are effective for ED caused by CNI may not work for other types of ED. Therefore, combining Ago2 overexpression with other therapeutic targets may achieve higher efficacy in the treatment of different types of ED. Finally, systematic studies on whole gene expression profiles (such as RNA sequencing and single-cell RNA sequencing) on CNI-induced ED and other types of ED mice overexpressing Ago2 or not are required to detail the

Ago2's downstream targets and signaling pathways involved in neurovascular regeneration or oxidative stress modulation.

CONCLUSIONS

In summary, this is the first study to evaluate the short-term effect of Ago2 on erectile function in mice with CNI-induced ED. Our results suggest that protecting mitochondrial function in penile tissues through Ago2 overexpression, thereby rebalancing ROS, might be a therapeutic strategy for CNI-induced ED and be of considerable value for treating other neurogenic ED or peripheral nerve injuries.

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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SUPPLEMENTARY MATERIALS

Supplementary materials can be found via <https://doi.org/10.4111/icu.20240077>.

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