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# Expression and Distribution of Free Zinc in Penile Erectile Tissue

Beom Seok Kang[1], Sang Won Suh[1], Dae Yul Yang[2], Bo Young Choi[1], Won Ki Lee[3]

[1]*Department of Physiology, Hallym University College of Medicine, Chuncheon,* [2]*Department of Urology, Kangdong Sacred Heart Hospital, Seoul,* [3]*Department of Urology, Hallym University Chuncheon Sacred Heart Hospital, Chuncheon, Korea*

**Purpose:** Several studies have shown that zinc has a significant influence on erectile function. However, no studies evaluating the cellular distribution of free zinc in penile erectile tissue have been performed. Therefore, this study aimed to test whether free zinc is present in penile tissue and whether it may be involved in the electrical stimulation (ES)-induced penile erection.
**Materials and Methods:** The subjects for this study were 26 young (8-week-old) male C57BL/6J mice. After the cavernous nerve was exposed through a midline stomach incision, 14 mice received ES of the cavernous nerve (ES group), whereas 12 mice did not (control group). Intracavernous pressure (ICP) (consisting of 10 V at a duration of 1 min, frequency of 12 Hz and a pulse width of 1 m/s) was recorded during ES. Immediately after ICP was recorded, penile tissues were harvested for histological and biochemical analysis, including analysis of zinc transporter 3 (ZnT3) and intracellular free zinc levels.
**Results:** The expression of neuronal nitric oxide synthase (nNOS) and endothelial NOS (eNOS) in penile tissue was significantly greater in the ES group than in the control group (p=0.036 and 0.016, respectively). And then, ZnT3 and intracellular free zinc were present in the penile tissue of both groups. However, ZnT3 immunofluorescence in the ES group was more intense in the dorsal nerve bundle (22% increase, p=0.032). The ES group also showed higher intensity N-(6-methoxy-8-quinolyl)-para-toluenesulfonamide (TSQ) fluorescence signals indicative of intracellular free zinc level in the penile tissue compared to the control group (49% increase in dorsal nerve bundle, p=0.001; 50% increase in corpus cavernosum, p=0.001).
**Conclusions:** The results of the study supported the expression and distribution of free zinc in penile tissue and increased levels after penile erection. Therefore, this study provides anatomical evidence for the potential role of free zinc in penile erection.

**Keywords:** Electrical stimulation; Endothelial nitric oxide synthase; Neuronal nitric oxide synthase; Penile erection; Zinc



## INTRODUCTION

Penile erection, a response that occurs during sexual arousal, is a neurovascular phenomenon that depends upon interactions between smooth muscle cells, neuronal cells and vascular endothelial cells [1,2]. It is mediated mainly by the nitric oxide/cyclic guanosine monophosphate (NO/cGMP) pathway. Although several vasodilators are involved in this process, NO is the principal one [3,4]. In the penis, stimulation of para-

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**Correspondence to:** Bo Young Choi https://orcid.org/0000-0002-9579-3503
Department of Physiology, Hallym University College of Medicine, 1 Hallymdaehak-gil, Chuncheon 24252, Korea.
**Tel:** +82-33-248-2596, **Fax:** +82-33-256-3426, **E-mail:** bychoi@hallym.ac.kr
**Correspondence to:** Won Ki Lee https://orcid.org/0000-0001-7172-0636
Department of Urology, Hallym University Chuncheon Sacred Heart Hospital, 77 Sakju-ro, Chuncheon 24253, Korea.
**Tel:** +82-33-240-5161, **Fax:** +82-33-256-3426, **E-mail:** trupus@hallym.ac.kr

sympathetic nerves inhibits noradrenalin release and encourages the release of acetylcholine (Ach), which binds to muscarinic receptors in endothelial cells; this increases endothelial NO synthase (eNOS) activation and subsequently, NO production [5].

NO is formed from the L-arginine by the enzymatic action of eNOS, neuronal NOS (nNOS), and inducible NOS (iNOS). All three NOSs are present in the penis, eNOS and nNOS are the essential composite active NOS enzymes expressed in penile tissues [6]. The intracellular mechanisms for relaxing the cavernosal smooth muscle are caused by reduced $Ca^{2+}$ concentration in the soluble guanylate cyclase (sGC)/cGMP and adenylate cyclase/cyclic adenosine monophosphate pathways by NOS. This interaction induces vasodilation of penile arteries, leading to increased blood flow and penile erection.

Zinc is the most important trace elements. It is present in all organs and fluids in the body. The zinc is composed of approximately 1.5 to 2.0 g or about 0.003% of the total weight of the human body. Zinc is involved in immune function, synthesis of DNA and protein, enzyme activity, cell division, tissue growth, wound healing, bone mineralization, cognitive function, and sperm motility. In the nervous system, zinc has a role as a neuromodulator or cofactor, and it is highly concentrated in the synaptic vesicles of some glutamatergic nerve terminals [7]. The release of this vesicular zinc creates signaling pathways that impact physiological function, including synaptic plasticity, long-term potentiation, and NOS activity [8-10].

In terms of eNOS and nNOS which are the key factor in penile erection, they are known to be active only as a homodimer. The dimer interface of nNOS and eNOS is formed between two N-terminal heme-binding oxygenase domains and is further stabilized by the coordination of zinc bound to two cysteine thiols from each monomer [11-14]. NO and NOS containing zinc are involved in the cardiovascular or renal physiology, including vasodilator effects [15]. In addition, it is broadly known that zinc also plays an important role in male sexual function, such as in the testicular, prostate glandular epithelium, generation of testosterone, erectile function, and sexual behavior [7,16-19]. However, although several studies have examined the various roles of zinc in male health, there are no studies to date that evaluate the cellular expression and distribution of free zinc in penile erectile tissue.

Therefore, the present study examined the presence of free zinc in mouse penile tissue and whether it may be involved in the electrical stimulation (ES)-induced penile erection.

## MATERIALS AND METHODS

### 1. Ethics statement about experimental animals

The present study was performed in accordance with the protocols of the Guidelines for the Use and Care of Laboratory Animals, allowed by the National Institutes of Health. Animal studies were approved by Institutional Animal Care and Use Committee (IACUC) at Hallym University (protocol # Hallym 2019-2; Date of approval: April 29, 2019). This study was written up according to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines [19]. We used twenty-six C57BL/6J male mice (aged 8 weeks, weight 20–25 g), which were bought from Daehan Biolink (DBL., Eumseong, Korea). Mice were housed three to four mice per cage under conditions of temperature-(20°C±2°C) and humidity-controlled (55%±5%). Food (Purina rodent chow 38057) and water were provided to the mice ad libitum and cages were changed weekly. The lights of the room were managed automatically; they were switched on and off in a 12-hour cycle (on at 6:00 a.m. and off at 6:00 p.m.).

### 2. Electrical stimulation and measurement of erectile function

Mice were anesthetized by inhaling a 25:75 mixtures of nitrous oxide and oxygen and 3% isoflurane. The cavernous nerve was exposed through a midline stomach incision. Fourteen mice received ES of the cavernous nerve (ES group), whereas 12 mice did not (control group). In the ES group, we inserted bipolar platinum wire electrodes around the cavernous nerve. Stimulation parameters were 10 V at a duration of 1 minute, a frequency of 12 Hz and a pulse width of 1 m/s (Fig. 1A). During expansion, the maximal intracavernous pressure (ICP) was observed. And then, the total ICP was confirmed through the area under the curve from the beginning of electric stimulation to a point 20 seconds after stimulus termination [20]. Mice were euthanized after ICP measurement using isoflurane as an anesthetic.

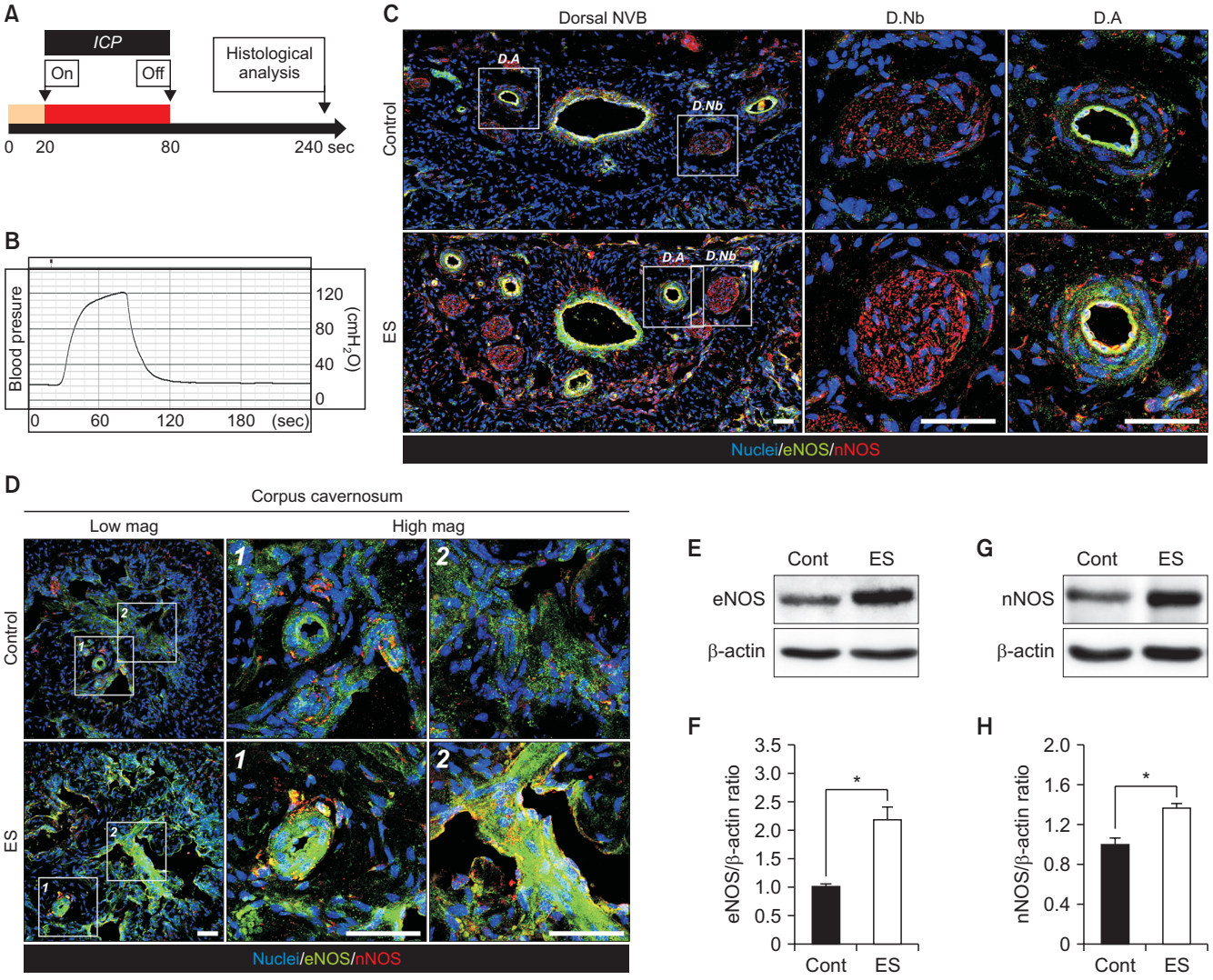


Fig. 1. Both neuronal nitric oxide synthase (nNOS) and endothelial NOS (eNOS) were increased in the penile tissue after electrostimulation-induced erection. (A) Timeline showing the experimental design for the penile erection after electrical stimulation (ES) of the cavernous nerve. (B) Physiograph represented the intracavernous pressure (ICP) of mice during ES of the cavernous nerve. Representative images showing sections of the dorsal nerve bundle (D.Nb) and dorsal artery (D.A) (C), and corpus cavernosum (D) immunostained for eNOS (green) and nNOS (red). Nuclei are counterstained with DAPI (blue). Scale bar, 50 μm. Western blot analysis of eNOS (E) and nNOS (G) in the penile tissue of control and ES group. Quantification of eNOS (F) and nNOS (H) protein levels from the penile tissue. Data are mean±SEM (n=3 per group). *p<0.05 *vs*. the control group (unpaired Student's t-test).

## 3. Penis sample preparation

After ICP measurement, the harvested penis was fixed for 1 hour in 4% paraformaldehyde. After fixation, we soaked penile tissue overnight into a 30% sucrose solution for cryoprotection. Penile tissue was frozen for 10 minutes and then cut with a cryostat at 12 μm thickness. The sections were placed on the gelatin coated slides (Fisher Scientific, Pittsburgh, PA, USA) and stored in a deep freezer at -80°C until used for immunofluorescence and immunohistochemistry staining.

## 4. Immunofluorescence analysis

To analyze the expression of eNOS, nNOS, zinc transporter 3 (ZnT3), and synaptophysin, we performed immunofluorescence staining. ZnT3 is one of the zinc transporters localized in the synaptic vesicles of zinc secreting neurons [21], and synaptophysin is an abundant polytopic synaptic vesicle protein [22]. Antibodies against synaptophysin and ZnT3 were used to determine the cellular localization and changes in the protein expression levels of ZnT3 on penile tissue.

Some of the primary antibodies used in this study were goat anti-nNOS (diluted 1:1,000; Abcam, Cam-

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bridge, UK), rabbit anti-eNOS (diluted 1:100; Invitrogen, Grand Island, NY, USA), rabbit anti-ZnT3 (diluted 1:200; Synaptic Systems, Göttingen, Germany), and mouse anti-synaptophysin (dilution 1:200; Cell signaling technology, Danvers, MA, USA). Following incubation in 0.01 M PBS containing 0.3% TritonX-100 (PBS-T), we left it in the shaker in a 4℃ incubator overnight. And then, penile tissue washed for 10 minutes through 0.01M PBS. To visualize the primary antibody, a corresponding fluorescent-conjugated secondary antibody was used: Alexa fluor 488 and 594 (both dilute 1:250; Invitrogen, Grand Island, NY, USA). The tissues were counterstained with DAPI (diluted 1:1,000; 4,6-diamidino-2-phenylindole; Invitrogen, Carlsbad, CA, USA). After placing the fluorescence-stained tissues on the gelatin-coated slide, it was dried in a dry oven and covered with dibutyl phthalate polystyrene xylene (Sigma-Aldrich, St. Louis, MO, USA). We identified fluorescence signals through a confocal microscope (LSM 710; Carl Zeiss, Oberkochen, Germany) with a sequential scanning mode for Alexa 594, 488, and DAPI. And then, stacks of images (512×512 pixels) from consecutive slices of 0.56 μm in thickness were conducted by averaging 20 to 25 scans per slice, and slices were proceeded using ZEN 2 (blue edition; Carl Zeiss). Images were taken from the penile cross-section. Through the ZEN 2 software, we were confirmed the quantification of mean intensity.

### 5. Zinc staining (TSQ method)

To analyze the expression of intracellular free zinc, we detected N-(6-methoxy-8-quinolyl)-para-toluenesulfonamide (TSQ) staining [23]. The penile tissues were harvested after ICP measurement and then immediately frozen in powdered dry ice. Penile tissue was sectioned at 10 μm thicknesses in a -15℃ cryostat. The sections were placed on gelatin-coated slides, air dried, dyeing in a solution of 4.5 mmol/L TSQ (Enzo Life Science, Enzo Biochem, Inc., Farmingdale, NY, USA) for 1 minute, and then washed for 1 minute in saline. The sections were confirmed with a 500 nm long-pass filter using an INFINITY3-1 CCD-cooled digital color camera (Lumenera Co., Ottawa, ON, Canada) and a microscope (Olympus upright microscope, epi-illuminated with 360 nm UV light) with the INFINITY Analyze software (release version 6.0). We performed the quantification of average intensity through the ImageJ (National Institues of Health, Bethesda, MD, USA).

### 6. Western blot

The penile tissue was homogenized in RIPA buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.4), 1% Nonidet p-40, 0.5% sodium deoxycholate, 0.1% SDS. The homogenate was separated at 14,000×*g* for 20 minutes at 4℃ through a centrifuge. After centrifugation, the supernatant was separated and preserved at -80℃. Through the Bradford protein assay, the protein concentration was confirmed. The proteins were diluted in SDS electrophoresis sample buffer, separated on a 6% or 8% SDS-PAGE gel, and then transferred to PVDF membranes (Millipore, Billerica, MA, USA). Nonspecific binding was blocked using a mixture of Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) and 5% skim milk for 1 hour at room temperature. And then, the membrane was incubated overnight with rabbit anti-nNOS (diluted 1:500; Abcam) and rabbit anti-eNOS (diluted 1:1,000; Invitrogen, Grand Island, NY, USA) in TBS-T and 5% skim milk at 4℃. The membrane was washed 3 times for 10 minutes using TBS-T. And, we incubated for 1 hour with secondary anti-rabbit IgG (diluted 1:5,000; Invitrogen) conjugated with horseradish peroxidase. Immunoreactivity was confirmed with increased chemiluminescent autoradiography (ECL kit; GE Healthcare, Amersham, UK), according to the manufacturer's instructions. The immunoreactivity assessments was normalized to beta-actin loading control and evaluate by densitometry using ImageJ.

### 7. Statistical analysis

Data were expressed as the mean±SEM. Comparisons between the control and ES groups were confirmed using a two-tailed unpaired Student's t-test and a nonparametric Mann–Whitney U-test with SPSS Statistics v25 (IBM Corp., Armonk, NY, USA). A p-value less than 0.05 was considered to be statistically significant ($p<0.05$).

## RESULTS

### 1. Electrical stimulation of cavernous nerve and expression of nNOS and eNOS in the penile tissue

The ICP was increased during ES of the cavernous nerve (Fig. 1B). To test whether ES increased the expression of eNOS and nNOS in the penile tissue, we used immunofluorescence staining and western blotting. Immunofluorescence analyses showed that eNOS

and nNOS were higher in the corpus cavernosum and dorsal nerve bundle (D.Nb) of penile tissue in the ES group than in the control group (Fig. 1C, 1D). Western blot also showed a significant increase in the level of each of these proteins in the penile tissue in the ES group compared to the control group (eNOS, p=0.036; nNOS, p=0.016; Fig. 1E-1H).

### 2. Electrical stimulation-induced changes in protein expression of ZnT3

In both groups, immunofluorescence staining confirmed the expression of synaptophysin and ZnT3 in the D.Nb of penile tissue. Double labeling with anti-ZnT3 antibodies and antibodies directed against synaptophysin revealed that ZnT3 staining overlapped in synaptophysin staining, indicating that these proteins were localized in the presynaptic nerve terminals (Fig. 2A). There were no important differences in synaptophysin immunoreactivity between the control and ES groups (control, 15,843.73±329.63; ES, 14,767.56±442.9; average gray-scale intensities; p<0.095; Fig. 2B). However, interestingly, more intense ZnT3 immunofluorescence was observed in the D.Nb of the penile tissue of the ES group compared to that of the control group (control, 7,832.15±536.5; ES, 10,077±541.11; a 22% increase; average gray-scale intensities; p=0.032; Fig. 2C). These findings suggest an upregulated expression of ZnT3 in the presynaptic terminals of D.Nb after ES of the cavernous nerve.

### 3. Increased level of intracellular free zinc in the penile tissue after electrical stimulation-induced erection

To investigate the level of intracellular free zinc in the penile tissue after ES of cavernous nerve, we stained penile sections of the control and ES groups with TSQ stating, zinc-specific fluorescent dye. TSQ fluorescence signals were observed in both groups (Fig. 3A, 3C). However, the ES group showed higher intensities of TSQ fluorescence signals in the penile tissue compared to the control group (D.Nb: control, 24.1±2.01; ES, 47.4±2.86, a 49% increase, corpus cavernosum: control, 25.37±0.87; ES, 50.52±1.22, a 50% increase; average gray-scale intensities; each p=0.001; Fig. 3B, 3D). Collectively, these results indicate upregulation of intracellular free zinc levels in the penile tissue after ES.

## DISCUSSION

Zinc, which is existent in all organs and fluid of the body, is one of the most important trace elements. It is noted that NO and NOS are the most important factor in physiology of penile erection. However, there have been no studies to date that evaluate the expression

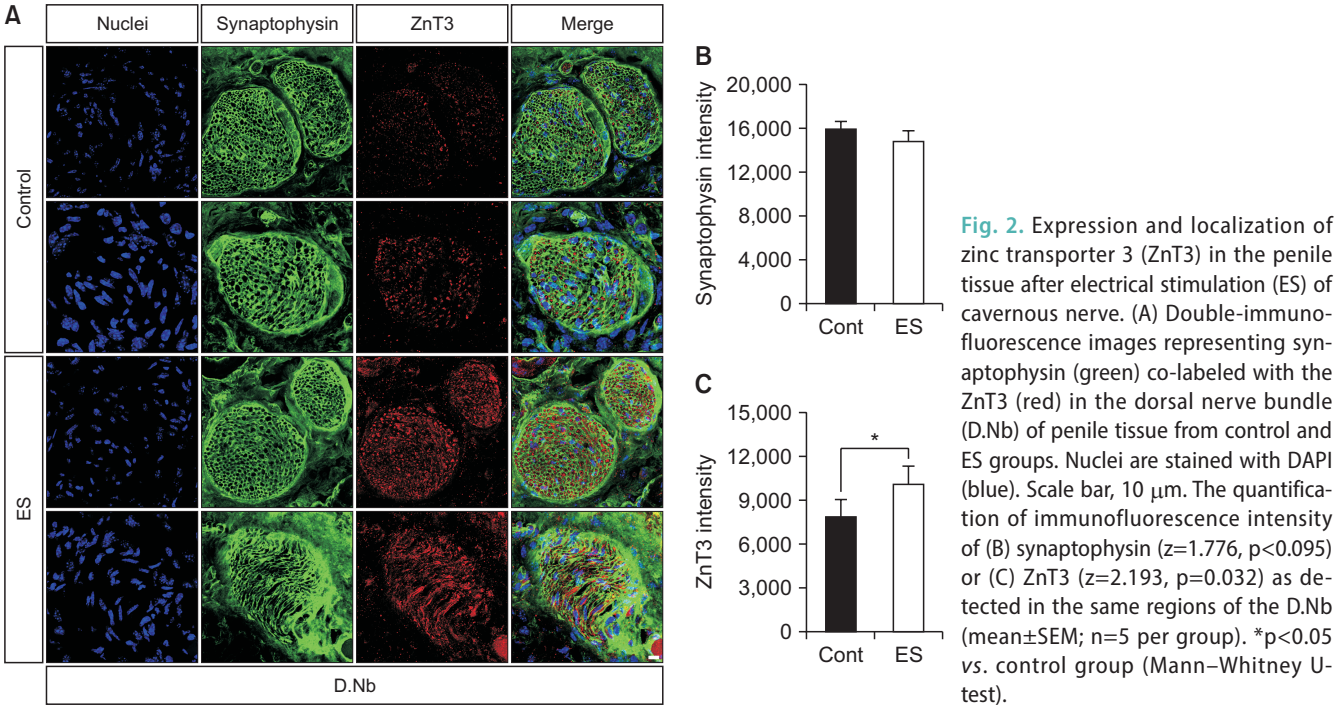


Fig. 2. Expression and localization of zinc transporter 3 (ZnT3) in the penile tissue after electrical stimulation (ES) of cavernous nerve. (A) Double-immunofluorescence images representing synaptophysin (green) co-labeled with the ZnT3 (red) in the dorsal nerve bundle (D.Nb) of penile tissue from control and ES groups. Nuclei are stained with DAPI (blue). Scale bar, 10 μm. The quantification of immunofluorescence intensity of (B) synaptophysin (z=1.776, p<0.095) or (C) ZnT3 (z=2.193, p=0.032) as detected in the same regions of the D.Nb (mean±SEM; n=5 per group). *p<0.05 *vs*. control group (Mann–Whitney U-test).

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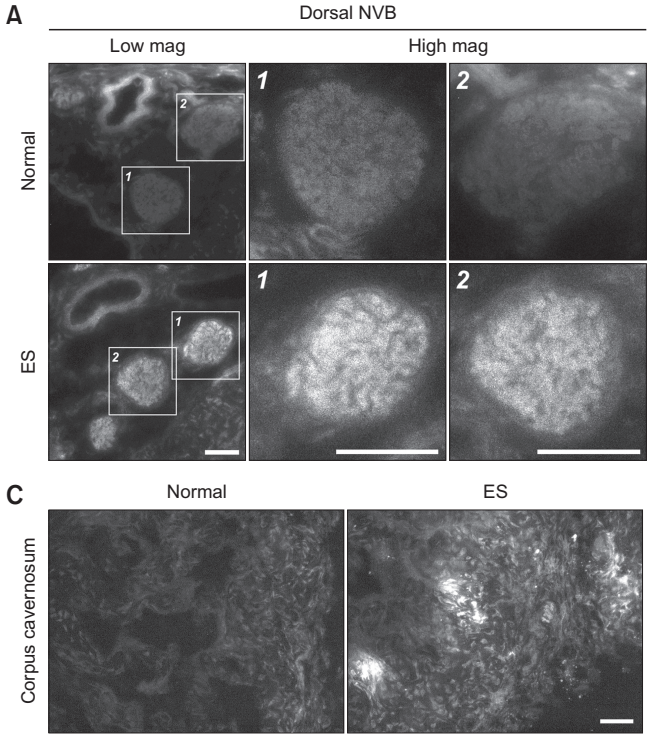


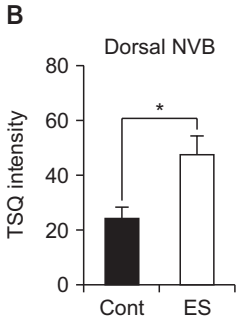


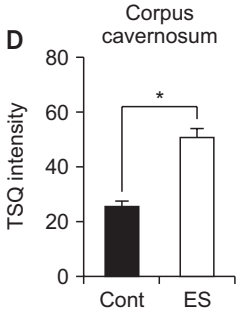


**Fig. 3.** Intracellular free zinc levels were elevated in the penile tissue after electrical stimulation (ES) of the cavernous nerve. Representative images displaying regions of the dorsal nerve bundle (D.Nb) (A) or corpus cavernosum (C) stained with TSQ to identify the intracellular free zinc level. Scale bar, 50 μm. The quantification of TSQ fluorescence intensity in the (B) D.Nb (z=2.558, p=0.01) or (D) corpus cavernosum (z=2.558, p=0.01) of penile tissue from control and ES groups. Data are mean±SEM (n=4 from control group, n=6 from ES group). *p<0.05 *vs.* control group (Mann–Whitney U-test).

and distribution of zinc in penile erectile tissue, although zinc has traditionally been known to be a main factor in sexual function including penile erection [7,11-14,24,25].

In this study, we first demonstrated that free zinc is present in mouse penile tissue and that its expression and distribution increase after ES-induced erection. Using the zinc-specific histofluorescence TSQ and ZnT3 immunofluorescence, we displayed that ES of the cavernous nerve markedly augments the ZnT3 expression and intracellular free zinc levels in the D.Nb and corpus cavernosum of the penis.

Under the erectile condition, it is caused by the release of NO through nNOS from parasympathetic and non-adrenergic non-cholinergic nerve [3,26]. Stimulation of parasympathetic nerves inhibits noradrenalin release and encourages the release of Ach, which binds to muscarinic receptors in endothelial cells and increases eNOS activation [5]. It is noted that these NOSs are active only as a homodimer [11]. The dimer interface of nNOS and eNOS is formed between two N-terminal heme-binding oxygenase domains and is further stabilized by the coordination of zinc bound to two cysteine thiols from each monomer [11-14,27]. A zinc-thiolate cluster is formed by a zinc ion which is tetrahedrally coordinated to two CysXXXXCys motifs at the NOS dimer interface [15]. Moreover, several studies have demonstrated that NO production leads to the release of vesicular zinc from presynaptic terminals to extracellular space [28,29]. Here, we found that ES of the cavernous nerve induced the increased expression of nNOS and eNOS from nerves and endothelial cells, thereby causing the release of NO in the penis. Furthermore, using a specific antibody to ZnT3 and zinc-specific TSQ fluorescent sensor to detect vesicular zinc, we observed that the expression of ZnT3, which is a transporter of zinc into synaptic vesicles of zinc-secreting neurons, and level of vesicular zinc had improved in penile erectile tissue. We speculate that the increase in the expression of these proteins and levels of vesicular zinc give rise to the release of NO and zinc in the penis.

NO activates sGC, which is a major receptor for NO. It leads to the relaxation of smooth muscle cells by increasing intracellular cGMP, which inhibits the influx of calcium into the cells, primarily *via* the activation of potassium channels, thereby reducing the concentration of intracellular calcium [30]. Myosin light-chain (MLC) kinase activity is regulated by the intracellular calcium concentration. With higher calcium levels in the cell, it goes on to phosphorylate regulatory MLC. In contrast, MLC is dephosphorylated by MLC phosphatase, which leads to smooth muscle relaxation when

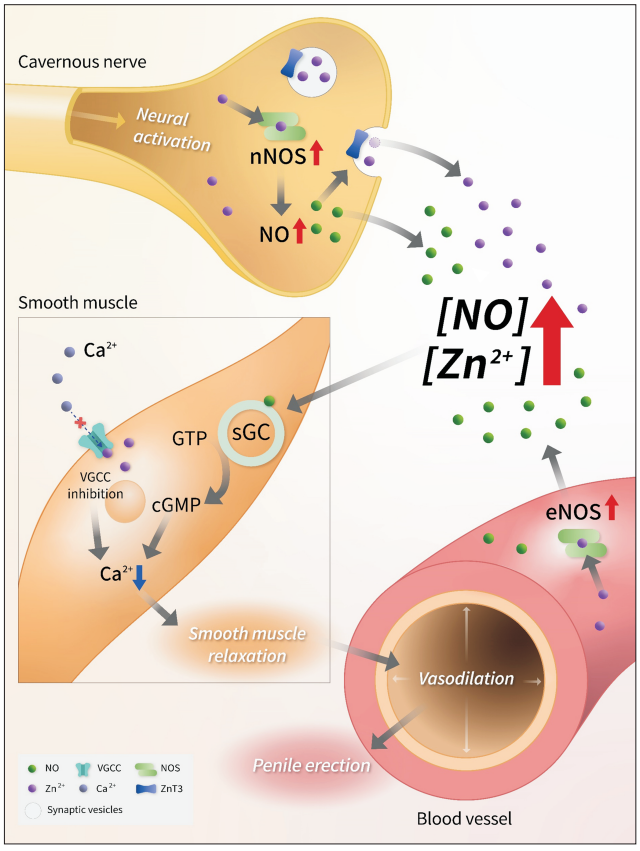


Fig. 4. Schematic illustration showing the proposed mechanisms by which $Zn^{2+}$ participate in penile erection. (1) ES of cavernous nerve induces neuronal nitric oxide synthase (nNOS) and endothelial NOS (eNOS) activation from nerves and endothelial cells, thereby causing the release of NO and $Zn^{2+}$ in the penis. (2) NO and $Zn^{2+}$ enter the smooth muscle cells. NO binds to soluble guanylyl cyclase catalyzing the conversion of guanosine 5′-triphosphate (GTP) in 3′-5′–cyclic guanosine monophosphate (cGMP). Intracellular $Zn^{2+}$ blocks the influx of calcium by inhibition of voltage-gated calcium channels (VGCC). (3) This interaction results in vasodilation of arteries, leading consequently to increased blood flow and the rigidity of penile erection.

calcium levels are low in the cell [31]. In addition, a recent study demonstrated that increased intracellular zinc causes vasorelaxation by acting as a pore inhibitor of L-type voltage-gated calcium channels in smooth muscle cells [32]. As observed in the present study, free zinc was present in the corpus cavernosum of the penile tissue and its levels were higher in the penile erectile tissue after ES. The corpus cavernosum is composed of an endothelial-lined sinusoidal structure surrounded by smooth muscle cells. Therefore, we suggest that increased free zinc in the corpus cavernosum of penile erectile tissue may affect the relaxation of smooth muscle (Fig. 4). Finally, smooth muscle relaxation causes vasodilation, which can lead to an increase in blood flow. It is possible that increased blood flow into the penis further triggers the production of NO by eNOS in the endothelial cells [33].

Nevertheless, the present study has some limitations that need to be addressed. Although our study focused on the expression and distribution of zinc during penile erection, voltage-dependent changes of free zinc may occur whether or not the penis is erect. In addition, this study showed increased levels of free zinc in penile tissue after ES of cavernous nerve, but we cannot exclude the possibility that ES release different nerve factors and activate different mechanisms that could be related to the zinc presence and function.

## CONCLUSIONS

To our knowledge, this study is the first to confirm the expression and distribution of free zinc in the penile tissue and the increased level of free zinc in the penile erectile tissue. Our findings provide anatomical evidence for the potential roles of free zinc in penile erection. The results of this study suggest that zinc may serve as an important modulator for penile erection. Further correlation with functional studies and confirmation of the associations observed in erectile dysfunction are needed to clarify these associations.

### Conflict of Interest

The authors have nothing to disclose.

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### Author Contribution

Conceptualization: WKL. Data curation: BSK, BYC. Formal analysis: BSK, BYC, WKL. Funding acquisition: WKL. Supervision: DYY, BYC, WKL. Writing – original draft: BSK, BYC, WKL. Writing – review & editing: SWS, BYC, WKL.

## Data Sharing Statement

The data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

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