Testosterone improves erectile function through inhibition of reactive oxygen species generation in castrated rats

Rui Li, Xianghu Meng, Yan Zhang, Tao Wang, Jun Yang, Yonghua Niu, Kai Cui, Shaogang Wang, Jihong Liu, Ke Rao

Testosterone is of overwhelmingly importance in regulating erectile physiology. However, the molecular mechanisms are poorly understood. The purpose of this study was to explore the effect and mechanism of testosterone in castrated rats. Forty male Sprague-Dawley rats were randomized to 4 groups (control, sham-operated, castration and castration-with-testosterone-replacement). Reactive oxygen species (ROS) production was measured by dihydroethidium (DHE) staining. Erectile function was tested by recording intracavernous pressure (ICP) and mean arterial blood pressure (MAP). Protein expression levels were examined by Western blot. We found that castration reduced erectile function, and testosterone restored it. The activity of nitric oxide synthase (NOS) was reduced in castrated rats, and testosterone administration reversed this (each $p < 0.05$). The concentrations of testosterone, cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) were lower in castrated rats, and testosterone restored these decreases (each $p < 0.05$). The expression levels of cyclooxygenase-2 (COX-2), prostaglandin synthase (PTGIS), endothelial nitric oxide synthase (eNOS) and phospho-endothelial nitric oxide synthase (p-eNOS, Ser1177) were reduced in castrated rats compared with controls (each $p <0.05$). The expression levels of $p40^{phox}$ and $p67^{phox}$ were increased in castrated rats, and testosterone reversed this (each $p < 0.05$). Overall, testosterone can ameliorate erectile dysfunction (ED) after castration by reducing ROS production and increasing activity of the eNOS/cGMP and COX-2/PTGIS/cAMP signaling pathways.

Testosterone Improves Erectile Function through the Inhibition of Reactive Oxygen Species Generation in Castrated Rats

Running Title: Testosterone Improves Erectile Function in Castrated Rats

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Introduction

Testosterone is overwhelmingly important in regulating erectile physiology. Erectile dysfunction (ED) is a common symptom in hypogonadal men that can lead to decreased self-confidence, depression and other symptoms that seriously influence the quality of life [1]. A recent study revealed that testosterone yielded many benefits in the treatment of hypogonadism and ED [2]. However, the mechanism of how testosterone improves ED is not completely understood.

Endothelial cells produce and release nitric oxide (NO), which induces the activation of soluble guanylyl cyclase and accumulation of cyclic guanosine monophosphate (cGMP), resulting in the relaxation of smooth muscle and penile erection [3-5]. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a complex composed of $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $gp91^{phox}$, $p67^{phox}$ and a GTPase Rac1 or Rac2, is a crucial enzyme that can catalyze the production of reactive oxygen species (ROS). Recently, studies have reported that ROS play a major role in hypercholesterolemia-induced ED and diabetes-related ED pathogenesis [6-9]. In the process of hypercholesterolemia-induced and diabetes-related ED, increased oxidative stress leads to an imbalance between the limited antioxidant defenses and accumulation of ROS, which then induces endothelial dysfunction and decreases NO availability. Finally, increased oxidative stress from the NO/cGMP signaling pathway causes pathological ED [7, 8]. Although the effects of testosterone on the NO/cGMP signaling pathway have been documented over the last years, to the best of our knowledge, there are no comparative studies on the role of testosterone in ameliorating ROS in castration-induced ED. Hence, we hypothesized that the above-mentioned changes are present in castrated rats, and testosterone can improve erectile function by inhibiting

the production of ROS.

In addition, the reduction of cyclic adenosine monophosphate (cAMP) is a different mechanism underlying ED. Cyclooxygenase (COX) is an important enzyme in prostaglandin synthesis; COX-1 and COX-2 are two isoforms of the enzyme. COX-1 is constitutively expressed in cells, and COX-2 is expressed under certain anomalous conditions [10]. Both of these isoforms transform arachidonic acid into prostaglandin H_2 (PGH_2), which is further catalyzed into prostaglandin I_2 by prostaglandin synthase (PTGIS). Then, adenylyl cyclase is sensitized to produce cAMP. This activation causes smooth muscle relaxation and penile erection [11]. PGH_2 can also be converted to other prostaglandins with potent proinflammatory effects. Any factors affecting this pathway and leading to cAMP reduction may cause ED. Israel Pérez-Torres et al found that castration influenced arachidonic acid metabolism and reduced the expression of COX-2 in the kidneys of metabolic syndrome rats [12]. Similarly, Lin et al suggested that COX-2-10aa-PGIS gene therapy elevated erectile function following cavernous nerve injury to rats [11]. However, the role of the COX-2/PTGIS/cAMP signaling pathway has not been elucidated in castrated rats with ED.

The purpose of this study was to explore the effect of testosterone on the erectile process in castrated rats. We analyzed the function of testosterone and investigated the molecular mechanisms of castration-induced ED.

Materials and Methods

Castration Model and Treatment

In the experiment, 40 adult male, 8-weeks-old Sprague–Dawley rats weighing 200~250 g were obtained from Tongji Medical College, Huazhong University of Science and Technology. Rats were randomized into 4 groups: control, sham-operated, surgical castration, and castration-with-testosterone-replacement (n=10 for each group). The castration procedure was as follows. Briefly, rats were anesthetized by intraperitoneal injection (40 mg kg^{-1}) of pentobarbital sodium. A ventral midline incision was performed above the scrotum, and the abdominal wall was cut open. The spermatic cord was then separated, and the vas deferens and associated vasculature were identified and separately ligated. Next, the testicles were removed bilaterally. The rats in testosterone treatment groups received testosterone (Zhejiang Xianju Pharmaceutical Co., Ltd, Taizhou, Zhejiang, China, subcutaneous injection) at 100 mg kg^{-1} $month^{-1}$ for 1 month immediately after castration [13]. All procedures involving animals were performed in accordance with the guidelines by the Chinese Council on Animal Care and with approval from the Committees on Animal Experiments at Tongji Hospital (Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China; ID: TJ-A20131213).

In Vivo Assessment of Erectile Function

After 1 month of testosterone treatment, erectile function was assessed in all rats. The assessments were conducted according to the method reported by Li M et al [6]. First, the cavernous nerves were exposed and mounted onto stainless steel bipolar wire electrodes, which were connected to an electrical stimulator. The electrical stimulation parameters were 5 volts at

15 Hz with a square-wave duration of 1.2 ms for 1 min. Then, a PE-50 cannula (Becton Dickinson & Co., Sparks, MD, USA) was inserted into the left carotid artery to monitor the systemic mean arterial blood pressure (MAP). Finally, a 25-gauge needle was inserted into the right crura, connected to PE-50 tubing, and filled with 250 U mL^{-1} of a heparin solution. Both blood pressure and intracavernous pressure (ICP) were measured continuously with a data acquisition system (AD Instruments Powerlab/4SP, Bella Vista, NSW, Australia). The Max ICP/MAP was recorded for each rat. The animals were sacrificed via an injection of 20 mL of air, and the corporeal tissue of each rat was immediately collected. One third of the sample was fixed in 4% triformol and embedded in paraffin for further use. The remaining tissue was immediately frozen and stored at -80°C until analysis.

Measurement of Plasma Testosterone

Immediately after electrostimulation, blood was drawn by a PE-50 tube, which was inserted into the left carotid artery, to determine the testosterone levels. Whole blood was centrifuged at 1580 g for 15 min at 4°C. The testosterone level was determined by the clinical laboratory of Tongji Hospital. The remaining plasma was collected and stored at -80°C.

SDS-PAGE and Immunoblotting

The frozen penile tissues were homogenized in ice-cold RIPA buffer containing a protease inhibitor cocktail and sodium fluoride (NaF), followed by centrifugation at 12000 g for 15 min at 4°C. The protein concentrations were assayed using a BCA assay kit (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China). In total, 50 μg of protein was loaded onto a 10% sodium dodecyl sulfate-polyacrylamide precast gel and then transferred to a polyvinylidene

fluoride membrane. The membranes were blocked for 1 h at room temperature in a solution of 0.1% Tris-buffered saline and Tween-20 (TBST) with 5% (w/v) bovine serum albumin. The membranes were subsequently incubated with antibodies against $p40^{phox}$ (1:500, Bioworld, Nanjing, Jiangsu, China), $p67^{phox}$ (1:1000, Affinity, Zhenjiang, Jiangsu, China), endothelial nitric oxide synthase (eNOS, 1:1000, Abcam, Cambridge, MA, USA), phospho-eNOS at Ser1177 (p-eNOS, 1:1000, Cell Signal Technology, Beverly, MA, USA), COX-2 (1:500, Abcam, Hongkong, China), PTGIS (1:1000, Abcam, Hongkong, China) or β-actin (1:500, Multisciences, Hangzhou, Zhejiang, China) overnight at 4°C. After the membranes were washed three times in TBST for 1 h, they were incubated with secondary antibody in TBST at room temperature for 1.5 h. The membranes were again washed three times in TBST, and the signal was detected with an enhanced chemiluminescence detection system (Pierce, Thermo Fisher Scientific, Rockford, IL, USA).

Detection of ROS

The rat corpus cavernosum was quickly frozen, cut to a thickness of 8 μm at an optimized cutting temperature, and placed on glass slides. Fresh dihydroethidium (DHE) solution (1 μmol L^{-1}, Beyotime Institute of Biotechnology, Haimen, Jiangsu, China) was topically applied to each tissue slice, and the slices were incubated for 30 min at 37°C in the dark. Fluorescence images were captured with an Olympus BX51 fluorescence microscope (Olympus Corporation, Tokyo, Japan). The fluorescence intensity was analyzed using Image-Pro Plus software (Media Cybernetics Inc., Bethesda, MD, USA).

Determination of Nitric Oxide Synthase (NOS) Activity

The activity of NOS in the penile tissue was measured by an ELISA kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) according to the manufacturer's instructions. The assays were performed in duplicate, and the protein concentration was detected to normalize the data.

cGMP and cAMP Concentrations

The cGMP and cAMP concentrations in the penile tissue were measured by an ELISA kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) according to the manufacturer's instructions. The assays were performed in duplicate, and the protein concentration was detected to normalize the data.

Statistical Analysis

Parametric data are expressed as the mean ± SD. All statistical analyses were performed with SPSS15.0 software (SPSS, Inc., Chicago, IL, USA) using a one-way ANOVA test followed by Bonferroni multiple comparison post-test. Intergroup differences were considered to be significant when $p < 0.05$.

Results

Effects of Testosterone Treatment on Testosterone Concentration

Compared with the control rats, castrated rats demonstrated greatly lower plasma testosterone levels and body weight. Testosterone replacement restored the testosterone concentration, but the concentration was still lower than that of the control group (although this difference was not significant). There was no difference in plasma testosterone concentration between the control and sham-operated groups (Table 1).

Effects of Testosterone Treatment on Erectile Function

Fig. 1 provides a summary of the four groups. The Max ICP/MAP ratio in the castrated group was lower compared with that in the remaining three groups with 5 V stimulation. Testosterone therapy largely increased the Max ICP/MAP ratio compared with that of the castration group with electrostimulation ($p < 0.05$). However, the ratio was still lower than those of the control and sham-operated groups. There was no difference in the MAP between the four groups.

Effects of Testosterone Treatment on ROS Production in Penile Cavernous Tissue

ROS production was detected in the four groups. As shown in Fig. 2A and 2B, castration induced a dramatic increase in ROS production (detected by DHE fluorescence), which was attenuated by testosterone. Furthermore, to assess whether castration-induced ROS in the corpus cavernosum was associated with NADPH oxidase, the protein expression levels of the NADPH oxidase subunits $p40^{phox}$ and $p67^{phox}$ were analyzed. Western blot analysis indicated that the $p40^{phox}$ and $p67^{phox}$ levels were vastly higher in castrated rats than in control and sham-operated rats, and testosterone treatment greatly reduced protein expression ($p < 0.05$, Fig. 2C, 2D).

Effects of Testosterone Treatment on the NOS/cGMP Signal Pathways in Penile Cavernous Tissue

The expression levels of eNOS and p-eNOS (Ser1177) in the corpus cavernosum were measured by Western blot. The eNOS and p-eNOS (Ser1177) levels were dramatically reduced in castrated rats, and the ratio of p-eNOS (Ser1177)/eNOS was vastly lower than that in the normal control rats. Treatment with testosterone significantly increased the eNOS and p-eNOS (Ser1177) levels, as well as the p-eNOS (Ser1177)/eNOS ratio, in castrated rats ($p < 0.05$, Fig. 3A, 3B). In addition, to confirm the bioavailability of NO, an ELISA kit was used to detect the cavernous activity of NOS and cGMP concentration. As shown in Fig. 3C and 3D, the cavernous cGMP concentration and activity of NOS were immensely lower in the castrated rats compared with that in the control and sham-operated rats (each $p < 0.05$), indicating that the cGMP-protein-kinase-G axis mediates this inhibitory effect of NO. Testosterone treatment significantly inhibited the castration-induced reduction in cavernous cGMP and NOS activity ($p < 0.05$).

Effects of Testosterone Treatment on COX-2/cAMP Signal Pathway in Penile Cavernous Tissue

The expression of COX-2 and PTGIS was determined in the four groups. Cavernous COX-2 and PTGIS protein expression were significantly lower in the castration group than in the control and sham-operated groups (each $p < 0.05$). The cavernous COX-2 and PTGIS protein levels were increased after 1 month of treatment (each $p < 0.05$, Fig. 4A, 4B). Compared with the control and sham-operated rats, the cAMP concentration was largely lower in the penile tissue of the castration rats ($p < 0.05$, Fig. 4C). Testosterone treatment significantly inhibited the castration-

induced reduction in cavernous cAMP ($p < 0.05$, Fig. 4C).

Discussion

Testosterone replacement therapy has been widely studied and has been clinically used for ED. However, the underlying molecular mechanisms of exogenous testosterone administration are not fully understood and are worthy of detailed study.

ROS play an important role in various diseases, including cancer, obesity, and ED [12, 14, 15], via reactive elements produced by the reduction of O_2 with a single electron (superoxide), two electrons (hydrogen peroxide) or three electrons (hydroxyl radical) [16]. A recent study reported that the penile levels of ROS were significantly increased and the activity of eNOS/cGMP was reduced in diabetes-related ED [7]. However, there are no correlative studies in a castrated rat model. Excessive ROS production or the failure of oxidant cleaning systems can obstruct cellular function through the oxidation of proteins, lipids and DNA [17]. In our study, we found that the level of ROS was obviously increased and that the levels of the NADPH oxidase subunits $p40^{phox}$ and $p67^{phox}$ were also increased in the castrated rat model. The up-regulation of $p40^{phox}$ and $p67^{phox}$ resulted in increased levels of ROS in the corpus cavernosum. Therefore, the increased production of ROS, which are activated by enzymes as a result of their shape (especially NADPH oxidase), might be a key mechanism underlying castration-induced ED.

Several studies have revealed that testosterone is crucial for exerting antioxidant effects through decreasing ROS. Hwang et al demonstrated that testosterone supplementation can reduce oxidative damage in Leydig cells [18]. However, the effect of testosterone on ROS levels in the corpus cavernosum of castrated rats is still unclear and needs to be clarified. We found that testosterone treatment reduced ROS and the expression of $p40^{phox}$ and $p67^{phox}$ and improved

erectile function. The decrease in NADPH oxidase led to a subsequent reduction in ROS. Thus, preventing the generation of ROS by interfering with the enzymes that produce them, especially NADPH oxidase, may be a more valid measure for combating oxidative stress than eliminating ROS after their formation.

The NOS/cGMP pathway, the principle erectile pathway, has been shown to have an effect on androgen. A recent study indicated that low testosterone levels in men were associated with impaired endothelial function and NO bioavailability [19, 20]. The role of testosterone on the expression of NOS isoforms has been shown in the penile tissue [21-23]. Replacement with 5α-DHT and testosterone restored erectile function as well as NOS expression in the corpora cavernosa of castrated animals [21, 24]. However, the mechanism of testosterone's ability to improve the NOS/cGMP pathway is not fully understood. In this study, we discovered that the expression of eNOS and the concentration of testosterone and cGMP were reduced in castrated rats, and treatment with testosterone restored this expression and concentration. Numerous studies have concluded that augmenting the generation of ROS is one of the major causes of decreased NO bioavailability [25-27]. Hence, according to our study of ROS, we believe that treatment with testosterone ameliorates ED by reducing the expression of the NADPH oxidase subunits $p40^{phox}$ and $p67^{phox}$. The reduction subsequently triggers a decrease in ROS, improvement in endothelial cell function and increase in NO. Finally, these changes lead to elevated cGMP and smooth muscle relaxation in the corpus cavernosum.

In males, testosterone is essential for fertility, puberty, sexual motivation, and sexual performance [28]. Testosterone production is predominantly regulated through the interaction of

luteinizing hormone/human chorionic gonadotropin with specific receptors [29, 30], resulting in increased intracellular cAMP levels. Recent studies indicated that cAMP plays an important role in erectile physiology through the COX-2 pathway [11, 31]. COX-2 and PTGIS, which regulates the production of inflammatory mediators, are key enzymes in the process of cAMP activation. Prostaglandin E, which is catalyzed by COX-2 and PTGIS, binds to pathognostic receptors on the smooth muscle and is thought to enable the relaxation of smooth muscle by activating cAMP-dependent pathways [31]. The lack of testosterone decreased the expression of COX-2 and PTGIS, which in turn resulted in reduced cAMP levels in the corpus cavernosum. Then, obstructing the activation of cAMP-dependent protein kinase (PKA) subsequently caused a dysfunction in smooth muscle relaxation and ED. In our study, we found that the expression levels of COX-2 and PTGIS were reduced in castrated rats compared with control rats. The concentration of cAMP is lower in castrated rats than in age-matched control rats. Treatment with testosterone markedly enhanced the expression of COX-2 and PTGIS and increased the concentration of cAMP. These results imply that the COX-2/PTGIS/cAMP signaling pathway may be another mechanism responsible for castration-induced ED.

This study has a few limitations. The possible COX-2/PTGIS/cAMP signal pathway needs to be further verified. In addition, testosterone effects were evaluated in the short-term in our study; the long-term effects of testosterone are required in future studies. Finally, the lack of long-term effects of testosterone has limited its clinical application.

Conclusions

In conclusion, testosterone reduced ROS production and increased eNOS expression in castrated rats. Testosterone activated the COX-2/PTGIS/cAMP signaling pathway and increased the production of cAMP. Testosterone improved erectile function in castrated rats under the joint action of the above-mentioned factors. Therefore, this study may provide a novel mechanism to explain the molecular mechanisms of castration-induced ED. Further studies are needed to explain the precise mechanisms involved.

Conflict of Interest

The authors declare no conflicts of interest.

Acknowledgements

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Table 1(on next page)

Table1 Body weight and plasma testosterone levels in the four groups

* $p < 0.05$ vs the castration group; # $p < 0.05$ vs castration-with-testosterone-replacement group. Data were expressed as the mean ± SD. Co=control; So=sham-operated; Ca=castration; Ct=castration-with-testosterone-replacement; N = number of analyzed samples.

1

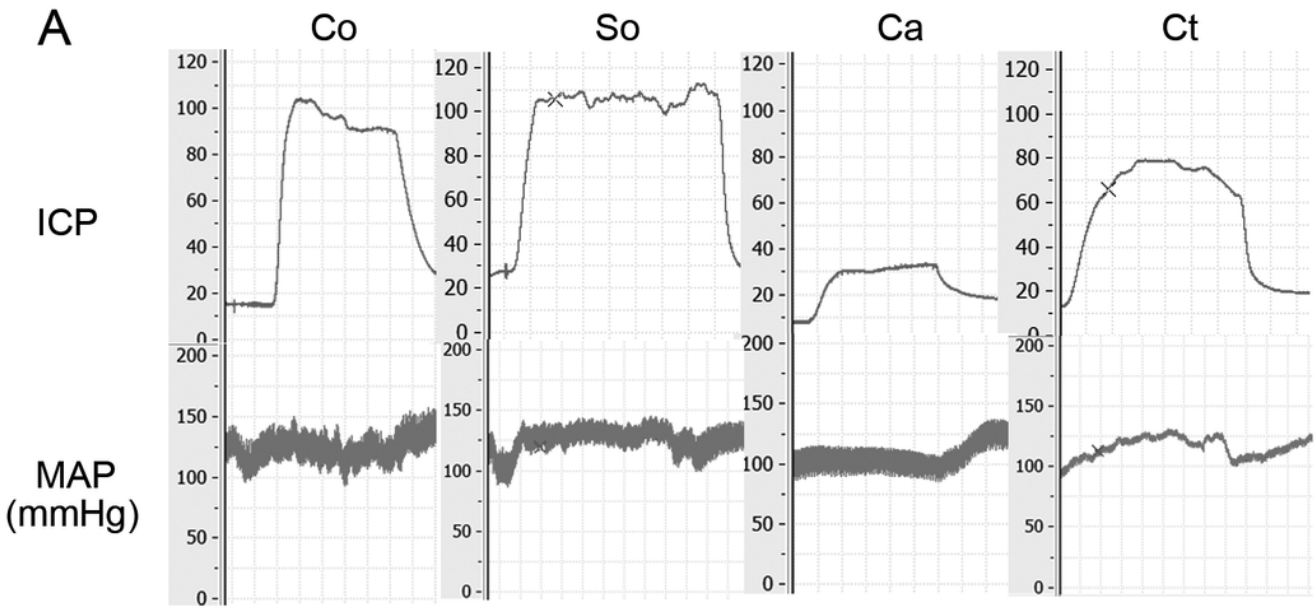
Group	N	Body weight, g		Plasma testosterone, ng/mL
		Initial	Final	
Co	10	224.7±4.7	407±37*#	4.18±0.27*
So	10	225.1±2.5	409±26*#	4.06±0.19*
Ca	10	224.5±3.4	340±39	0.51±0.09
Ct	10	225.4±2.0	343±44	3.93±0.12*

2

1

Fig. 1 Testosterone treatment increased the Max ICP/MAP during electrical stimulation of the cavernous nerve (5 V, 15 Hz, 1 min).

(A, B) Representative ICP and MAP tracing in the four groups. Bar graph depicting the ratios of the Max ICP/MAP. Data were expressed as the mean ± SD (n = 6~9 rats/group). Co = control; So = sham-operated; Ca = castration; Ct = castration-with-testosterone-replacement. * $p < 0.05$ vs the control group; # $p < 0.05$ vs the castration group; & $p < 0.05$ vs the castration-with-testosterone-replacement group.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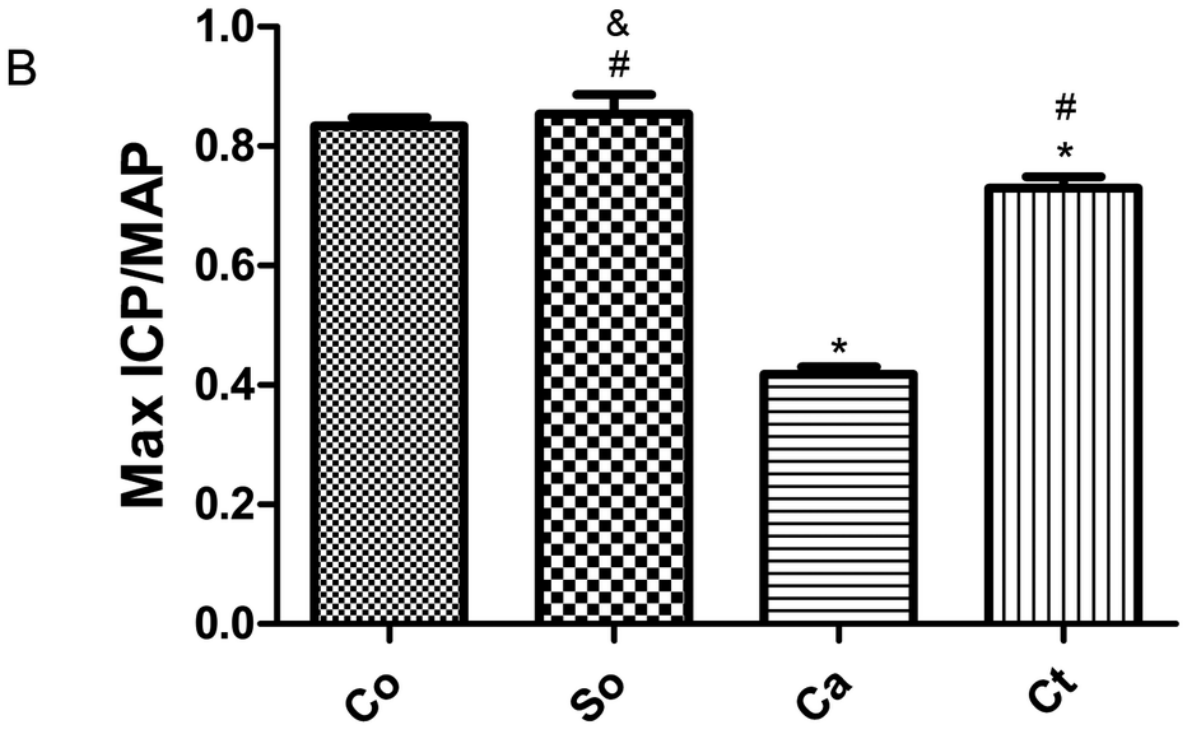
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&
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*
Co
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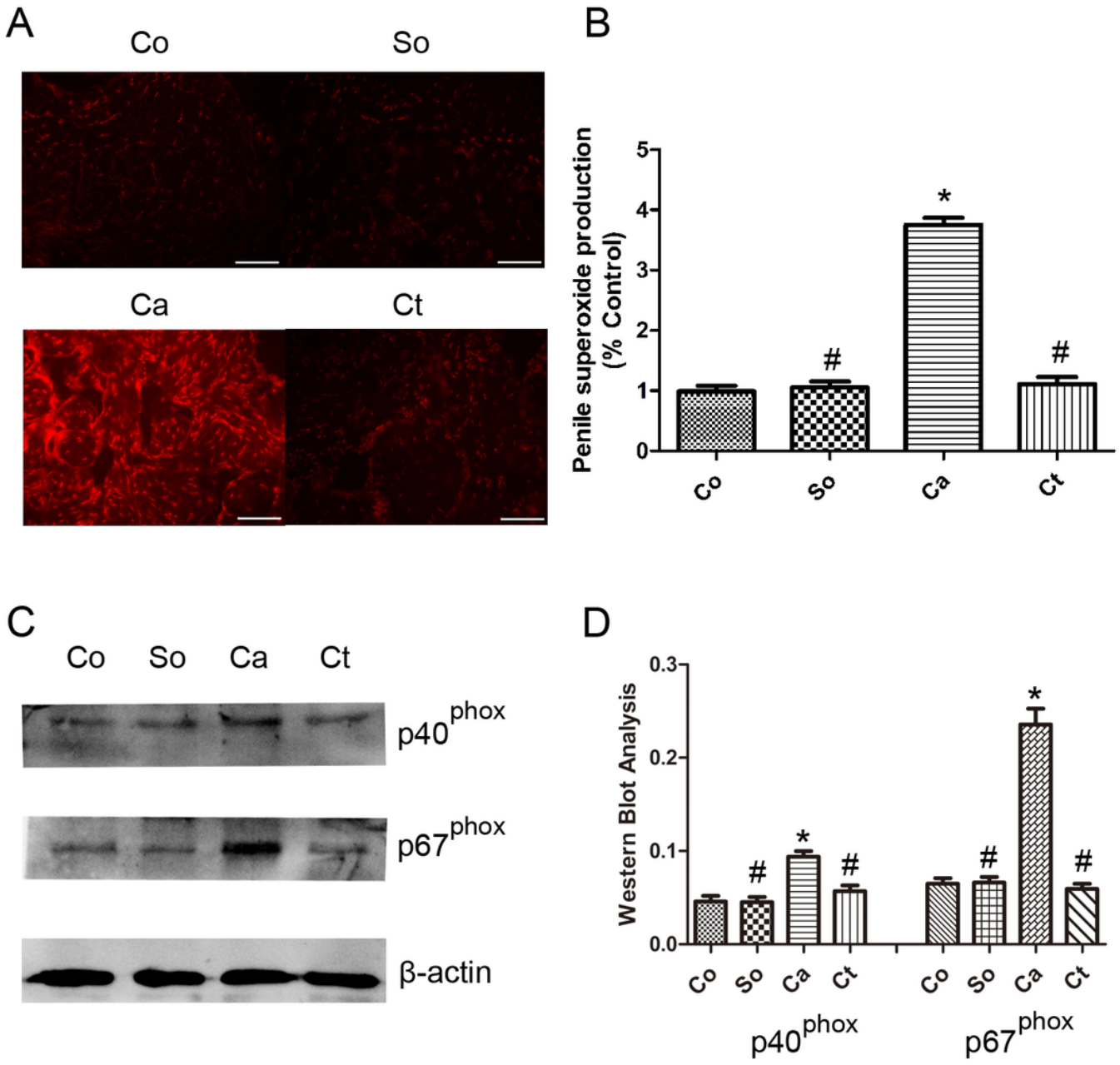
Ca
Ct

2

Fig. 2 Testosterone-induced changes in ROS and protein expression.

(A, B) Typical images of DHE in situ staining in corpus cavernosum from the four groups (red fluorescence; scale bars = 100 μm; time of exposure, 600 ms). The intensity of red fluorescence was evaluated by Image-Pro Plus software. (C, D) Representative Western blot showing the expression of $p40^{phox}$ and $p67^{phox}$ normalized to β-actin. Data were expressed as the mean ± SD (n = 6~9 rats/group). Co = control; So = sham-operated; Ca = castration; Ct = castration-with-testosterone-replacement. * $p < 0.05$ vs the control group; # $p < 0.05$ vs the castration group. ROS, reactive oxygen species; DHE, dihydroethidium.


A
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Penile superoxide production
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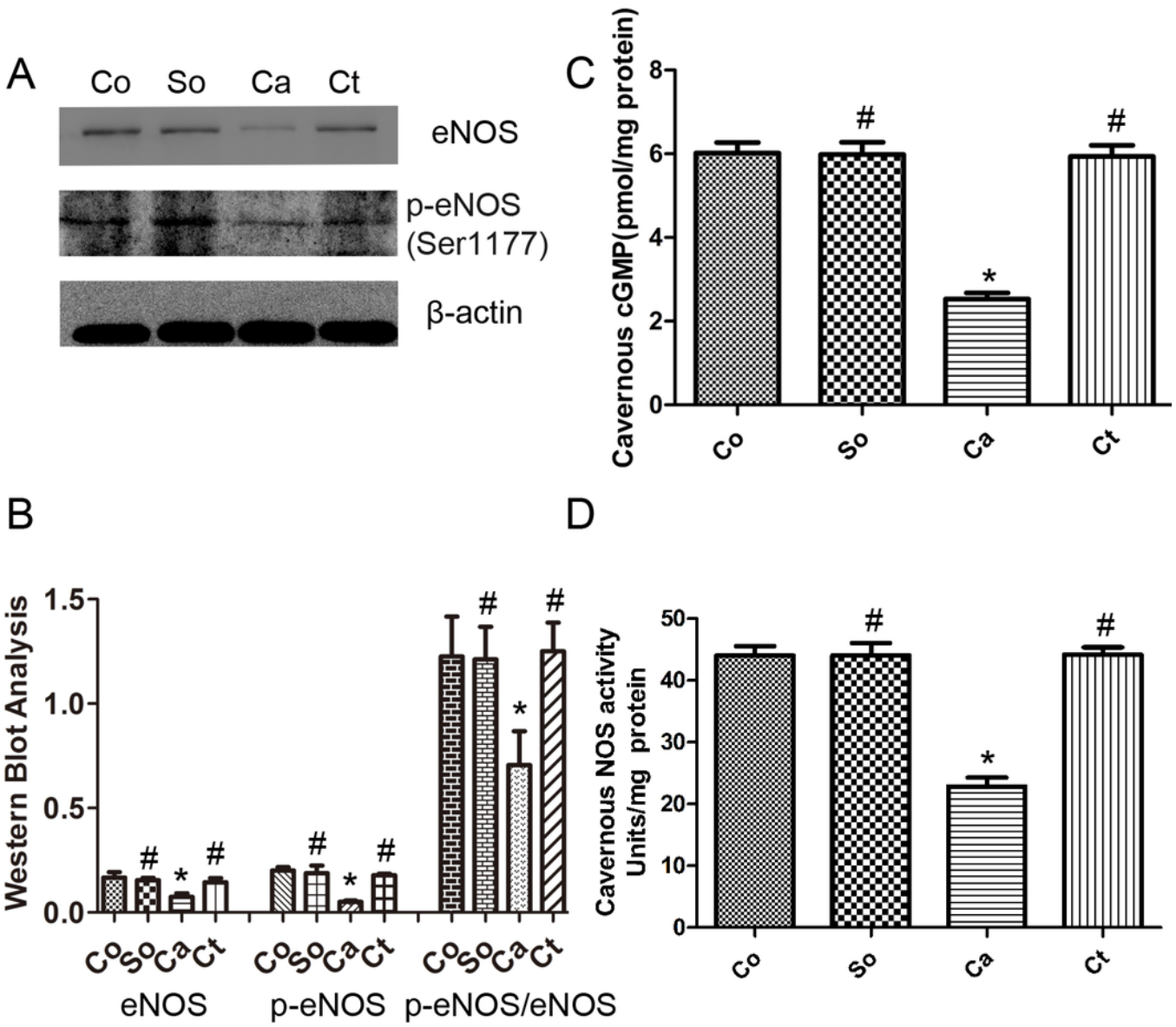
3

Fig. 3 Testosterone-induced increases in the NOS/cGMP signal pathway in penile tissue.

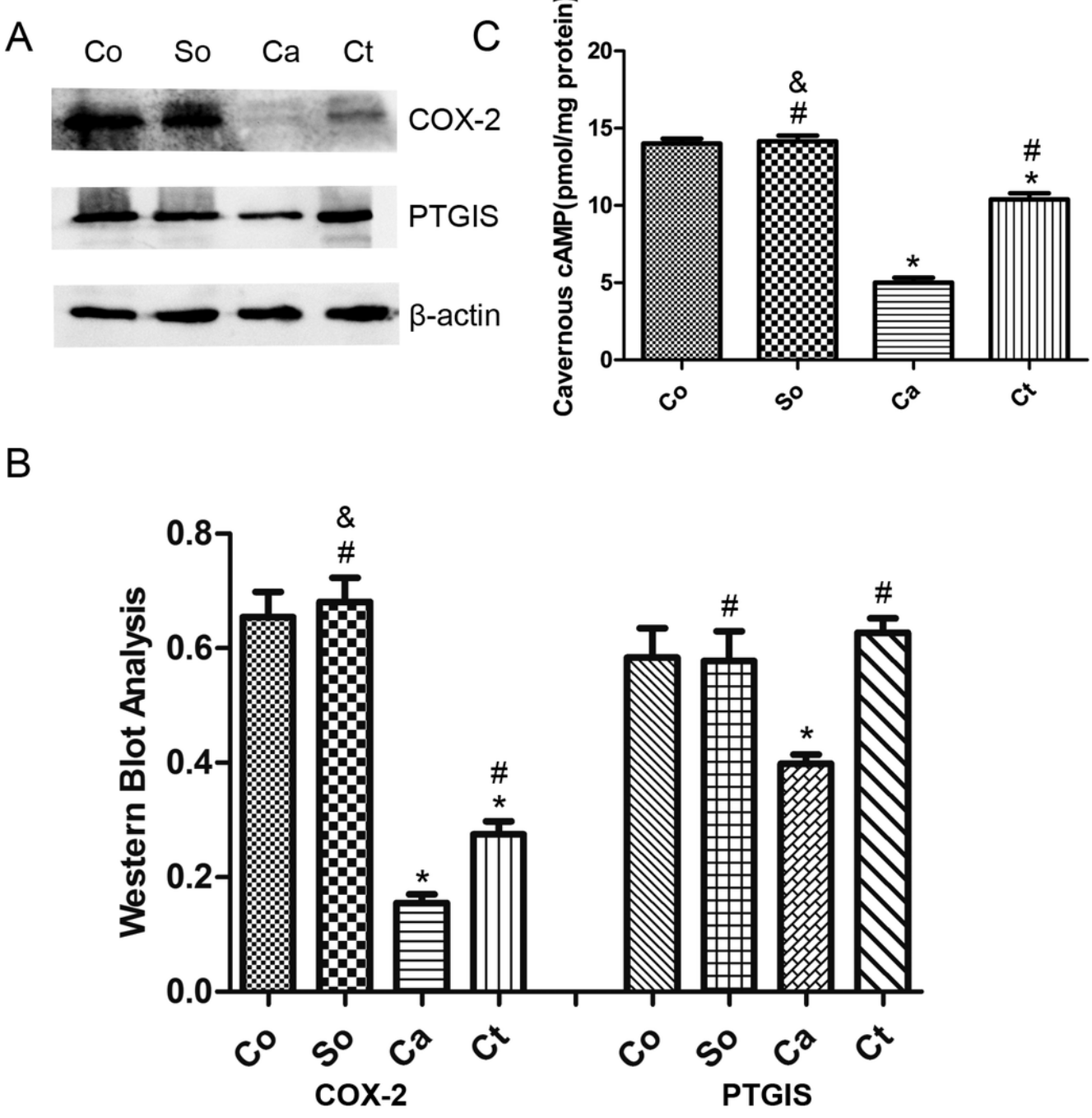
(A, B) Representative Western blot showing the expression of p-eNOS (Ser1177) and eNOS normalized to β-actin, as well as the p-eNOS/eNOS ratio. (C) The concentration of cGMP was detected in penile tissue. (D) The activity of NOS from the four groups. Data were expressed as the mean ± SD (n = 6~9 rats/group). Co = control; So = sham-operated; Ca = castration; Ct = castration-with-testosterone-replacement. * $p < 0.05$ vs the control group; # $p < 0.05$ vs the castration group.


A
Co
So
Ca
Ct
eNOS
p-eNOS
(Ser1177)
β-actin
C
Cavernous cGMP(pmol/mg protein)
8
6
4
2
0
#
#
*
Co
So
Ca
Ct
B
Western Blot Analysis
1.5
1.0
0.5
0.0
#
*
#
#
*
#
#
*
#
Co So Ca Ct
Co So Ca Ct
Co So Ca Ct
eNOS
p-eNOS
p-eNOS/eNOS
D
Cavernous NOS activity
Units/mg protein
50
40
30
20
10
0
#
#
*
Co
So
Ca
Ct

4

Fig. 4 Testosterone-induced increases in the COX-2/cAMP signal pathway in penile tissue.

(A, B) Typical Western blot showing the protein expression of COX-2 and PTGIS normalized to β-actin. (C) The concentration of cAMP was detected in the penile tissue. Data were expressed as the mean ± SD (n = 6~9 rats/group). Co = control; So = sham-operated; Ca = castration; Ct = castration-with-testosterone-replacement. * $p < 0.05$ vs the control group; # $p < 0.05$ vs the castration group; & $p < 0.05$ vs the castration-with-testosterone-replacement group.


A
Co
So
Ca
Ct
COX-2
PTGIS
β-actin
C
Cavernous cAMP(pmol/mg protein)
20
15
10
5
0
&
#
*
Co
so
Ca
Ct
B
Western Blot Analysis
0.8
0.6
0.4
0.2
0.0
COX-2
PTGIS