

ARTICLE



Testosterone improved erectile function by upregulating transcriptional expression of growth factors in late androgen replacement therapy model rats

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We previously showed that castration of rats reduced erectile function over time; when testosterone replacement therapy was started 4 weeks after castration, erectile function improved. In this study, we examined the mechanism of improvement in erectile function following testosterone replacement therapy in rats. Thirty 12-week-old rats were divided into castrated (Cast), castrated with subcutaneous administration of testosterone (Cast + T), and sham (Sham) groups. Erectile function and mRNA and protein expression were evaluated in the rats by using standard methods. To assess erectile function, we measured the intracavernosal pressure, mean arterial pressure, mRNA expression of endothelial growth factors, and protein expression of endothelial nitric oxide synthase (eNOS). The intracavernosal pressure/mean arterial pressure ratio was significantly lower in the Cast group, and testosterone administration significantly improved ($P = 0.017$). Compared to the Cast group, the Cast+T group exhibited significantly increased mRNA expressions of vascular endothelial growth factor A (VEGF-A), intercellular adhesion molecule 1 (ICAM-1), transforming growth factor- β (TGF- β), nerve growth factor (NGF), α -smooth muscle actin (α -SMA), caveolae associated protein 1 (Cavin-1), Cavin-2, Cavin-3, sirtuin 1 (Sirt-1), sphingosine-1-phosphate 1 (S1P1), S1P2, and S1P3 and eNOS protein expression. Testosterone replacement therapy improved erectile function in castrated rats by increasing growth factors and eNOS protein.

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INTRODUCTION

Testosterone plays an important role in sexual function, such as erection and ejaculation, and the development of libido. However, blood testosterone in men decreases with age; by the age of 80 years, the testosterone secretion rate is approximately half that of young men [1]. Decreased testosterone has been reported to induce apoptosis in smooth muscle cells [2] and reduce nitric oxide (NO) expression in the endothelium and neurons [3]. It also causes pathogenic changes, such as the promotion of fat cell accumulation [4, 5] and placement of elastic fibers and connective tissue in the corpus cavernosum structure, which contribute to erectile dysfunction (ED) [6]. These factors cause sexual function to decline as testosterone declines, leading to the development of ED [7].

A meta-analysis study demonstrated that testosterone replacement therapy improved sexual function in patients with decreased sexual function due to testosterone deficiency [8]. In animal experiments, erectile function was restored by testosterone replacement therapy in castrated rats [9, 10]. However, because testosterone replacement therapy is performed immediately after castration in most animal experiments, this therapy may not

improve but rather maintain erectile function. In clinical practice, testosterone replacement therapy is administered to patients with testosterone deficiency and impaired erectile function. Therefore, in animal experiments, it is necessary to wait for a period after castration until erectile function declines before beginning testosterone replacement therapy to determine the effects of this therapy. We previously showed that castration of rats continued to reduce erectile function over time, with a significant decrease compared to in the control group at 4 weeks [11]. Additionally, when testosterone replacement therapy is started at 4 weeks after castration, erectile function improves with the improvement of the corpus cavernosum tissue structure over time, with significant effects observed at 8 weeks compared to in the castration group [12]. Studies are also needed to clarify the mechanism of improvement of erectile function following testosterone replacement therapy. We predicted that testosterone replacement therapy affects growth factors that control the corpus cavernosum structure. In this study, we examined the mechanism of improvement in erectile function by testosterone replacement therapy in rats.

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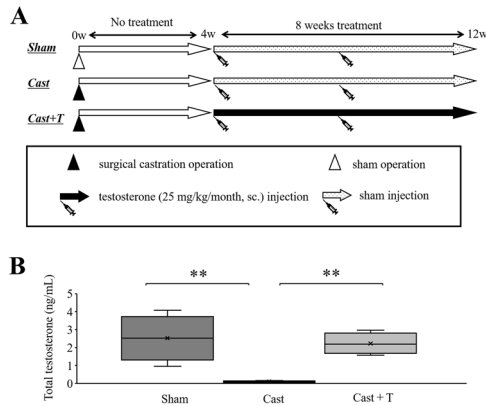


Fig. 1 Treatment protocols and testosterone levels. **A** Experimental design. Rats were assigned to the following groups: castrated (Cast), castrated with subcutaneous testosterone (Cast + T), and sham (Sham). Sham rats underwent sham laparotomies, and the incision was sutured. In the Cast+T group, rats were administered daily subcutaneous doses of testosterone undecanoate (25 mg/kg/month) starting 4 weeks after the operation for 8 weeks. After this period, the rats were subjected to erectile function testing in vivo and in vitro. **B** Total testosterone levels in serum. Data are presented as a box-and-whisker plot ($n = 4$). $**P < 0.01$ vs. each group using analysis of Tukey test.

MATERIAL AND METHODS

Animals and treatment protocols

Thirty 12-week-old male Wistar/ST rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan). All experimental protocols were approved by the ethics review board of Nagoya City University and conducted in accordance with our institutional standards for the care and use of animals (H25-P-09). The rats were kept in a temperature- and humidity-controlled room, with a 12-h/12-h light/dark cycle and free access to normal water.

Treatment protocols

Rats were assigned to the following groups: castrated (Cast, $n = 10$), castrated with subcutaneous administration of testosterone (Cast + T, $n = 10$), and sham (Sham, $n = 10$) (Fig. 1A). Rats in the Cast and Cast+T groups were castrated as reported previously [11, 12]. In the Sham group, rats underwent sham laparotomy, and the incision was sutured. In the Cast + T group, rats were administered monthly doses of testosterone undecanoate (25 mg/kg; Matrix Scientific, Elgin, SC, USA) subcutaneously starting 4 weeks after the operation for 8 weeks. The testosterone solution contained testosterone undecanoate dissolved in a vehicle composed of sesame oil (Nacalai Tesque, Kyoto, Japan) and 0.01% benzyl alcohol (Wako Pure Chemical Industries, Osaka, Japan). The Sham and Cast groups were injected with the vehicle on the same schedule. In this study, because of castration surgery, we could distinguish by the morphology of the rats, and we did not perform a blind test.

Blood samples and measurement of biological parameters

Blood samples were obtained from all rats via the vena cava. After coagulation and centrifugal separation at $800 \times g$ for 20 min at 4°C , serum samples were stored at -80°C until analysis. The serum testosterone level was measured using a Testosterone ELISA kit (ALPCO, Salem, NE, USA). The absorbance was measured using a Nivo 3S (PerkinElmer, Waltham, MA, USA).

Examination of erectile function

Intracavernosal pressure (ICP) was measured by electrical stimulation as previously reported [11–13]. Rats from each group ($n = 6$) were anaesthetized using isoflurane with an inhalation anesthesia apparatus (Nakazawa Seisaku-sho, Funabashi, Japan). The mean arterial pressure (MAP) was measured from the carotid artery, and the ICP was measured from the left crus of the corpus cavernosum using a 23-G needle. A data acquisition board (PowerLab 2/26, ADInstruments Pty. Ltd., New South Wales, Australia) with an amplifier was used to monitor these pressures via a pressure transducer. The cavernous nerve was electrically stimulated

Table 1. Primer sequences for real-time quantitative polymerase chain reaction (qRT-PCR).

mRNA	Sequence
VEGF-A	Forward 5'-GCCCTGAGTCAAGAGGACAG-3'
	Reverse 5'-CAGGCTCCTGATCTTCCAG-3'
ICAM-1	Forward 5'-ATCTGTGTCCCCCTCAAAGTC-3'
	Reverse 5'-CCATCAGGGCAGTTTGAATAGC-3'
TGF- β 1	Forward 5'-TGAGTGGCTGTCTTTGACG-3'
	Reverse 5'-TTCTCTGTGGAGCTGAAGCA-3'
NGF	Forward 5'-CATGGTACAATCTCTTCAAC-3'
	Reverse 5'-CCAACCCACACACTGACACTG-3'
α -SMA	Forward 5'-GGAGATGGCGTGACTCACA-3'
	Reverse 5'-CGCTCAGCAGTAGTCACGAA-3'
Cavin-1	Forward 5'-GGTGAATGAGAAGCAAGTGT-3'
	Reverse 5'-GGTGAAGCTGGCCTCCAGA-3'
Cavin-2	Forward 5'-CCTACAGCCACCACAGTGC-3'
	Reverse 5'-GGTCTCGCATCAGATCCTC-3'
Cavin-3	Forward 5'-GTGGACTGAGGGCAGGTGA-3'
	Reverse 5'-GTGTTCCCGCTCCGTGT-3'
Sirt-1	Forward 5'-TGTTTCTGTGGGATACCTGA-3'
	Reverse 5'-TGAAGATGGTCTTGGGTCTTT-3'
S1P1	Forward 5'-TTCTGCGGGAAGGAAGTATG-3'
	Reverse 5'-TGTCGCCGTTGTAGTTTC-3'
S1P2	Forward 5'-CCATTGCCATCGAGAGACAAG-3'
	Reverse 5'-CAACATTGACAGCTTTTGTACT-3'
S1P3	Forward 5'-GGGAGGGCAGTATGTTCTGTA-3'
	Reverse 5'-AAGTTCTCCAGGCAGTTCCA-3'
GAPDH	Forward 5'-ATGATTCTACCCACGGCAAG-3'
	Reverse 5'-CTGGAAGATGGTGTGGGTT-3'

The thermal cycler conditions were as follows: 2 min at 50°C , 10 min at 95°C , 40 cycles of 15 s at 95°C and 1 min at 60°C , 15 s at 95°C , 15 s at 60°C , and 15 s at 95°C to analyze the dissociation curve. Primer specificity was verified via analysis of the dissociation curve.

VEGF-A vascular endothelial growth factor A, ICAM-1 intercellular adhesion molecule 1, TGF- β 1 transforming growth factor- β , NGF nerve growth factor, α -SMA α -smooth muscle actin, Cavin Sirt-1, sirtuin 1, S1P sphingosine-1-phosphate/caveolae associated protein, GAPDH glyceraldehyde-3-phosphate dehydrogenase.

using stainless steel bipolar wire electrodes (Unique Medical, Osaka, Japan) connected to a pulse generator (Nihon Kohden, Tokyo, Japan). The parameters were 1 min at 5 V, 1–16 Hz, and a square wave duration of 5 ms. The erectile function of rats was evaluated using the maximum ICP/MAP ratio.

Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (qRT-PCR) analysis was performed as previously reported [13]. Total RNA was extracted from the corpus cavernosum samples using the TriPure Isolation Reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions ($n = 4$). RNA concentration and quality were measured using spectrophotometric analysis at 260 and 280 nm. Total RNA (1 μg) was reverse transcribed to complementary DNA (cDNA) using a ReverTra Ace- α kit (Toyobo, Osaka, Japan). cDNA (1 μL) was used for reverse transcription-polymerase chain reaction (RT-PCR) using KAPA SYBR Fast qPCR Kit (Roche, Basel, Switzerland). The reaction cycle consisted of incubations for 50°C for 2 min, 95°C for 10 min, 40 cycles each of 95°C for 15 s and 60°C for 1 min, and 95°C for 15 s using a CFX96 Real-time System (Bio-Rad, Hercules, CA, USA). The forward and reverse PCR primers are shown in Table 1. Target gene expression was quantified relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression by using the comparative cycle threshold (CT) method.

Western blot analysis

Western blot analysis was performed as previously reported [13]. Total protein was extracted from the corpus cavernosum using PRO-PREPTM (iNtRON Biotechnology, Gyeonggi-do, Korea) according to the manufacturer's instructions ($n = 4$). The protein concentration was quantified using Pierce™ BCA Protein Assay Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Separated total protein (25 μ g) was transferred onto polyvinylidene difluoride membranes (Merck, Kenilworth, NJ, USA). Mouse monoclonal anti-endothelial nitric oxide synthase (eNOS) (1:100; Abcam, Cambridge, UK), mouse monoclonal anti- β -actin (1:5,000; Sigma), and anti-mouse immunoglobulin G conjugated with horseradish peroxidase (1:20,000; GE Healthcare, Little Chalfont, UK) were used for detecting protein. The protein expression was evaluated with the bands using enhanced chemiluminescence (Amersham Biosciences, Amersham, UK) and ImageQuant LAS 4000 mini (GE Healthcare). Pictures were analyzed using the ImageJ 1.43 u software (NIH, Bethesda, MD, USA).

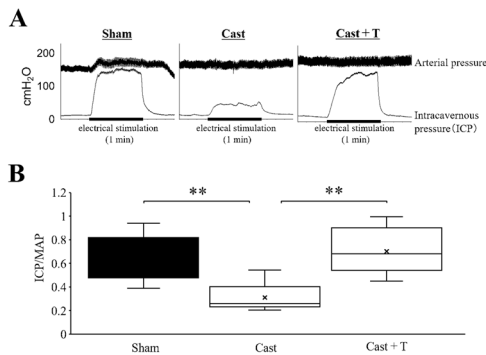


Fig. 2 Erectile function evaluation. **A** Representative tracings of intracavernous pressure (ICP) and arterial pressure changes during electrical stimulation (16 Hz) of the cavernous nerve in Sham, Cast and Cast+T rats. **B** Erectile function according to the ICP/mean arterial pressure (MAP) ratio. Data are presented as a box-and-whisker plot ($n = 6$). * $P < 0.05$, ** $P < 0.01$ vs. each group using analysis of Tukey test.

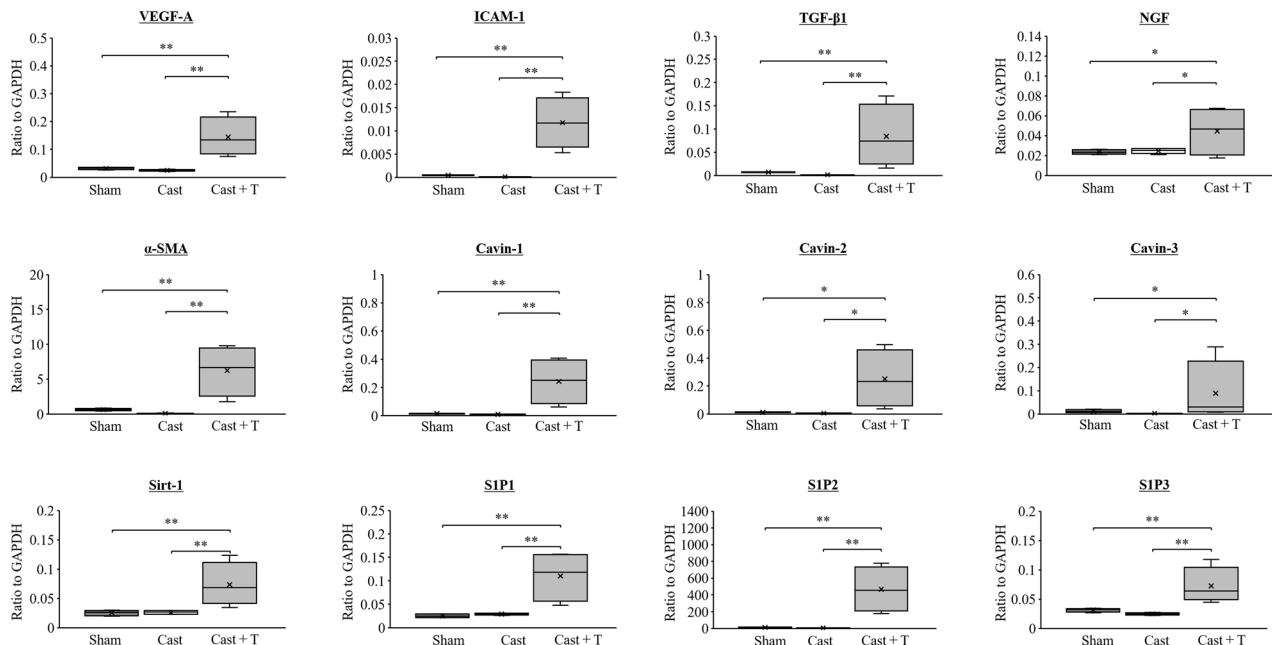


Fig. 3 Expression of mRNA in the corpus cavernosum of rats. Target gene expression was quantified relative to the expression of β -actin using the comparative CT method. Data are presented as a box-and-whisker plot ($n = 4$). * $P < 0.05$, ** $P < 0.01$ vs. each group using analysis of Tukey test.

Statistical analyses

Data were expressed as the mean \pm standard error of the mean. Statistical significance was determined by a one-way analysis of variance following Tukey comparison test using in EZR on R commander ver. 1.41 [14]. P values < 0.05 were considered to indicate statistically significant results.

RESULTS

Biological parameters

Figure 1 shows the serum total testosterone levels after testosterone administration. Cast rats showed significantly lower levels of serum total testosterone compared to their Sham counterparts (Sham, 2.52 ± 0.64 ng/mL; Cast, 0.10 ± 0.02 ng/mL, $P = 0.0057$). However, Cast+T and Sham rats exhibited similar levels of testosterone (Cast + T, 2.22 ± 0.24 ng/mL, $P = 0.0012$).

Erectile function

Figure 2A shows the representative tracings of the ICP and arterial pressure changes during electrical stimulation of the cavernous nerve. ICPs in the L-OHP group were lower than those in the Sham group. Figure 2B shows the ICP/MAP ratios for different stimulation frequencies. The Cast group (0.31 ± 0.05) exhibited significantly lower ratios than the Sham group (0.67 ± 0.08 ; $P = 0.0069$) at 16 Hz. The Cast+T group (0.70 ± 0.07) showed significantly higher ratios than the Cast group ($P = 0.017$).

mRNA expression analysis

Figure 3 shows the mRNA expression levels in the corpus cavernosum. The expression of vascular endothelial growth factor A (VEGF-A: $P = 0.0082$ and $P = 0.0058$), intercellular adhesion molecule 1 (ICAM-1: $P = 0.0018$ and $P = 0.0014$), transforming growth factor- β (TGF- β : $P = 0.0049$ and $P = 0.0035$), and nerve growth factor (NGF: $P = 0.0133$ and $P = 0.0164$) were higher in the Cast+T group than in the Sham and Cast groups. The expression of α -smooth muscle actin (α -SMA: $P = 0.0095$ and $P = 0.0060$), caveolae associated protein 1 (Cavin-1: $P = 0.0098$ and $P = 0.0096$), Cavin-2 ($P = 0.0311$ and $P = 0.0301$), and Cavin-3 ($P = 0.0491$ and $P = 0.0436$) were higher in the Cast+T group than in the Sham and Cast groups. Further, the expression of sirtuin 1 (Sirt-1: $P = 0.0063$ and $P = 0.0070$), sphingosine-1-phosphate 1

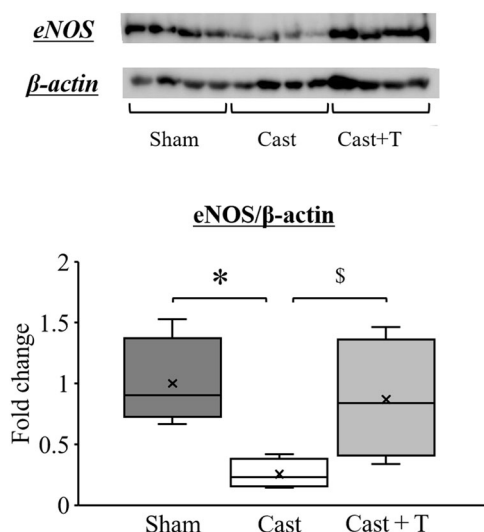


Fig. 4 Western blot analysis of the expression of nitric oxide synthase in the corpus cavernosum of Sham, Cast and Cast+T rats. Representative immunoblots of eNOS. Densitometric quantification of the corresponding bands. The data were normalized to β -actin expression and presented as fold-changes compared to in the sham group. Data are presented as a box-and-whisker plot ($n = 4$). * $P < 0.05$, $^S P < 0.10$ vs each group using analysis of Tukey test.

(S1P1: $P = 0.0048$ and $P = 0.0063$), S1P2 ($P = 0.0068$ and $P = 0.0062$), and S1P3 ($P = 0.0072$ and $P = 0.0031$) were higher in the Cast+T group than in the Sham and Cast groups.

eNOS protein analysis

Figure 4 shows the eNOS protein expression levels. The eNOS protein level in the Cast group was significantly lower than that in the Sham group ($P = 0.0424$). eNOS expression tended to be higher in the Cast+T group than in the Cast group ($P = 0.0933$).

DISCUSSION

We investigated a mechanism for improving erectile function using testosterone replacement therapy in castrated rat models. Castrated rats were treated with testosterone undecanoate for 8 weeks. Based on blood testosterone measurement using enzyme-linked immunosorbent assay, the blood testosterone level was significantly reduced by castration. In addition, testosterone administration significantly increased the blood testosterone level compared to in the Cast group and was similar to that in the Sham group, indicating that the testosterone dose was appropriate for testosterone replacement therapy.

According to a previous study, testosterone administration improved the corpus cavernosum structure, with consequent improvements in the erectile function of castrated rats; [12] therefore, in this study, we investigated the mRNA expression of growth factors involved in cell proliferation. VEGF-A is an important growth factor that acts specifically on vascular endothelial cells during angiogenesis and promotes lumen formation and the migration of endothelial cells [15, 16]. Testosterone has been reported to stimulate the secretion of VEGF and potent angiogenesis and vasodilators in castrated animal glandular epithelial cells [17, 18].

We observed a significant increase in VEGF mRNA expression in the Cast+T group compared to in the Cast group. Thus, testosterone replacement therapy appears to increase VEGF and improve the corpus cavernosum structure. In addition, ICAM-1 has been reported to activate angiogenesis [16, 19]. ICAM-1 is an intercellular adhesion molecule in vascular endothelial cells that is induced and expressed in endothelial cells by VEGF [20]. Based on

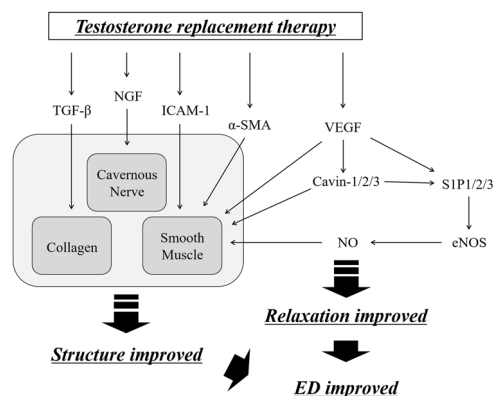


Fig. 5 Summary of the mechanisms that are improved ED after testosterone replacement therapy.

our results, ICAM-1 mRNA expression was significantly increased in the Cast+T group compared to in the Cast group. Thus, testosterone replacement therapy may improve the corpus cavernosum structure by increasing ICAM-1. Furthermore, the increased expression of factors constituting the corpus cavernosum, such as TGF- β and NGF, was observed following administration of testosterone.

With increases in the levels of these growth factors in the corpus cavernosum, increases in α -SMA, Cavin, Sirt-1, and S1P were also observed. In addition, eNOS protein was increased following testosterone administration. The caveolae has been reported to play an important role in signal transduction [21] and to mediate increases in endocytosis, which is associated with activation of NO production by eNOS [22]. Caveolae is a target for the S1P1 receptor and contributes to S1P-induced eNOS activation [23]. Studies using a knockout mouse model showed that Cavin-1 is required for formation of the caveolae structures [24], and Cavin-2 and Cavin-3 are also associated with caveolae [25, 26]. Loss of Cavin-3 reduces Cavin-1 in smooth muscle of blood vessels and bladder by approximately 40% and the amount of caveolae in smooth muscle by 40–45% [27]. According to our results, the mRNA expression levels of Cavin-1, Cavin-2, and Cavin-3 were significantly increased in the Cast+T group compared to in the Cast group. Therefore, testosterone replacement therapy may improve erectile function by increasing the expression of Cavin-1, Cavin-2, and Cavin-3, forming and regulating caveolae, and increasing eNOS.

In addition, testosterone replacement therapy increased Sirt-1 mRNA expression. Sirtuins have been reported to be involved in NO production via eNOS [28, 29], and the increase in Sirt-1 may have increased eNOS. S1P is involved in controlling NO production [23, 30–32]. S1P is produced in endothelial cells and is involved in numerous processes such as cell proliferation, apoptosis suppression, and neurotransmitter release [30]. It has been reported that S1P acts on the S1P receptor present in vascular endothelial cells to activate eNOS and induce NO production [23, 31, 32]. Additionally, VEGF specifically induces the expression of S1P1 receptor, enhances the intracellular signal transduction response to S1P, enhances S1P-mediated vascular relaxation, and significantly stimulates angiogenesis synergistically with S1P [33, 34]. In this study, the mRNA expression levels of S1P1, S1P2, and S1P3 were significantly increased in the Cast+T group compared to in the Cast group. Based on these results, testosterone replacement therapy appears to activate eNOS by increasing the expression of growth factors and inducing NO production to improve erectile function.

Our results are limited to the mRNA level, and further studies are needed to directly measure NO production at the protein level. In addition, it is unclear whether the blood concentration in rats represents that in humans. This study investigated the effects of

testosterone on castrated rats; however, in humans, testosterone is rarely administered to patients who have undergone castration surgery. Since the results of this experiment show the effect of testosterone administration on a rapid decrease in testosterone in the castrated rats, it is necessary to examine with another testosterone deficiency animal model. However, testosterone replacement therapy may be effective for treating ED (Fig. 5). In addition, ED is rarely treated with testosterone alone in older men, with phosphodiesterase-5 (PDE-5) inhibitors often used in combination. It has also been reported that testosterone changes the expression of PDE-5 [35], and it will be useful to investigate the effects of the combined use of testosterone and PDE-5 inhibitors in the future.

CONCLUSION

Testosterone replacement therapy improved the erectile function of castrated rats by increasing growth factors and eNOS protein.

DATA AVAILABILITY

The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

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AUTHOR CONTRIBUTIONS

Conceptualization, TK and KK; Data curation, TK, IH, TM, YH, and YM; Formal analysis, TK and IH; Funding acquisition, TK, YH, and KK; Investigation, TK, IH, and TM; Methodology, TK and YH; Project administration, TK; Supervision, YH, AS, YM, YFH, and KK; Writing-original draft, TK and KK; Writing-review & editing TK and KK.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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