ENDOCRINOLOGY

Effect of Late Androgen Replacement Therapy on Erectile Function Through Structural Changes in Castrated Rats

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ABSTRACT

Background: Because androgen replacement therapy (ART) is not performed immediately after the onset of androgen deficiency, the treatment is considered to be late.

Aim: To investigate the effects of late ART, starting 4 weeks after castration of rats, on erectile function and structural changes in the corpus cavernosum.

Methods: Rats were subjected to ART for 4 (Late-ART [4w]) or 8 (Late-ART [8w]) weeks. In either case, rats were assigned to the following groups: castrated (Cast), castrated with subcutaneous administration of testosterone (3 mg/kg/day; Cast+T), and sham (Sham). Cast + T rats received daily subcutaneous doses of testosterone starting 4 weeks after castration for 4 or 8 weeks whereas Sham and Cast rats received only the vehicle.

Outcomes: Erectile function was assessed by evaluating intracavernosal pressure (ICP) and mean arterial pressure (MAP) after electrical stimulation of the cavernous nerve, corporal veno-occlusive function using dynamic infusion cavernosometry, and histology using Masson's trichrome staining.

Results: No increase in the ICP was observed in Cast+T rats in the Late-ART (4w) group (0.47 ± 0.02, $P > .05$), whereas, in Cast+T rats in the Late-ART (8w) group, there was a significant increase in the ICP/MAP ratio (0.60 ± 0.02, $P < .05$), drop rate, and smooth muscle/collagen ratio.

Clinical Translation: The present study provides scientific evidence for the effect of late ART on erectile function.

Strengths and Limitations: This study provides insights into the influence of late ART on erectile function through improvements in the structure of corpus cavernosum. The major limitation of this study is the difference in the time required for healing between the humans and rats, which might have a bearing on the translational relevenace of the results.

Conclusions: Late ART could improve erectile function. However, as improvement requires a considerable time period, it is necessary to persist with therapy patiently for optimal results. **Kataoka T, Hotta Y, Yamamoto Y, et al. Effect of Late Androgen Replacement Therapy on Erectile Function Through Structural Changes in Castrated Rats. Sex Med 2021;9:100348.**

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Key Words: Testosterone; Erectile dysfunction; Corporal veno-occlusive function; Histological change

INTRODUCTION

Androgens are essential for male physical activity and normal erectile function.[1−5] Age-related androgen deficiency, known as late-onset hypogonadism (LOH), is a risk factor for erectile dysfunction (ED).[1,2] In recent years, metabolic syndrome and diabetes mellitus have also been reported to be associated with the development of androgen deficiency.[5−7] Androgen replacement therapy (ART) is used in the treatment
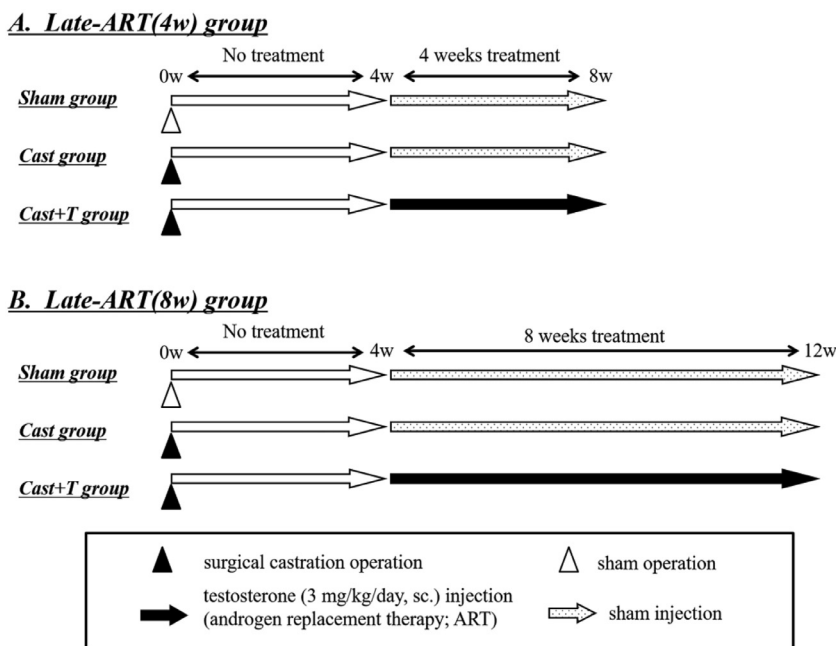
Figure 1. Experimental design. Two intervention periods were set—Late-ART (4w) and Late-ART (8w; Figure 1). For each period, rats were assigned to the following groups: castrated (Cast), castrated with subcutaneous testosterone (3 mg/kg/day) (Cast+T), and sham (Sham). Sham rats underwent sham laparotomies and the incision was sutured. In the Cast+T group, rats received daily subcutaneous doses of testosterone (3 mg/kg/day) starting 4 weeks after the operation for 4 (Late-ART [4w]) or 8 weeks (Late-ART [8w]). At the end of these periods, rats were subjected to erectile function testing in vivo and in vitro.

of androgen deficiency. Several studies have reported that ART improves the symptoms of LOH and ED.[8−10] We also demonstrated that administration of testosterone to castrated rats suppressed inflammation-dependent pathological damage to the corpus cavernosum (CC).[11] However, some patients receiving ART drop out because they find the therapy to be ineffective.

It has been demonstrated that deficiency of testosterone causes structural changes in the CC. Castrated rats show loss of smooth muscle and fibrosis through apoptosis of trabecular smooth muscle cells, which contributes to impaired veno-occlusion.[12] These structural changes lead to intractable ED. Several studies have demonstrated the effect of ART on ED in animal studies.[13−15] However, in most of these animal studies, ART was performed immediately after castration. These studies showed that testosterone maintained the erectile function, but did not improve the ED. Clinically, the inception of ART is considered to be late because the therapy is not performed immediately after the onset of androgen deficiency.

We hypothesized that, during late-ART, erectile function recovers only after a considerable length of time has passed. Thus, in this study, we investigated the effects of late-ART on erectile function and structural changes in the CC starting 4 weeks after the castration of rats to have a better understanding of the effect of ART in humans.

METHODS

Animals

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 laboratory chow and normal water.

Treatment Protocols

The intervention was performed for two different periods: Late-ART (4w) and Late-ART (8w; Figure 1). In each period, rats were assigned to the following groups: castrated (Cast), castrated with subcutaneous administration of testosterone (3 mg/kg/day; Cast+T), and sham (Sham). Rats in the Cast and Cast+T groups were castrated as reported previously.[11] In the Sham group, rats underwent sham laparotomy and the incision was sutured. In the Cast+T group, rats received daily doses of testosterone (3 mg/kg/day) subcutaneously starting 4 weeks after the operation for 4 (Late-ART [4w]) or 8 weeks (Late-ART [8w]). The testosterone solution contained testosterone propionate (Wako Pure Chemical Industries, Osaka, Japan) dissolved in a vehicle composed of sesame oil (Nacalai Tesque, Kyoto, Japan)

and 0.01% benzyl alcohol (Wako Pure Chemical Industries). The Sham and Cast groups received injections of the vehicle on the same schedule.

Tissue Sample Collection

After the measurement of erectile response, penile shaft samples were embedded in optimal cutting temperature compound (Sakura Finetek Japan, Tokyo, Japan) and frozen in liquid nitrogen for Masson's trichrome staining. All the samples were stored at −80°C until the analysis.

Real-time quantitative polymerase chain reaction (qRT-PCR). qRT-PCR analysis was performed as previously reported.[16,17] Total RNA was extracted from CC samples using TriPure Isolation Reagent (Roche, Pleasanton, CA, USA) according to the manufacturer's instructions. Using a ReverTra Ace-α kit (Toyobo, Osaka, Japan), 1 μg of total RNA was reverse-transcribed into complementary DNA, which served as the template for qRT-PCR, using the KAPA SYBR Fast qPCR Kit (Roche). The primer sequences were designed as follows: forward: 5'-ACCCTCCCATGGCACATTTT-3' and reverse: 5'-TTGGTTGGCACACAGCACAG-3' for androgen receptor (AR); and forward: 5'-TCCCTCAAGATTGTCAGCAA-3' and reverse: 5'-AGATCCACAACGGATACATT-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Amplification and detection were performed using the CFX96 Real-time System (Bio-Rad, Hercules, CA, USA). Target gene expression was quantified relative to GAPDH expression using the comparative C_T method. All measurements were performed in triplicate.

Examination of Erectile Function

Intracavernous pressure (ICP) was measured after electrical stimulation as reported previously[16,17]. Briefly, rats from each group were anesthetized using isoflurane (Mylan, Canonsburg, PA, USA). Under inhalational anesthesia, with 4% isoflurane for induction and 1.0%−1.5% isoflurane for maintenance, the carotid artery was cannulated for continuous monitoring of the mean arterial pressure (MAP) and the left crus of the CC was cannulated using a 23-G needle for continuous monitoring of the ICP. The pressure transducer was connected through an amplifier to a data acquisition board (PowerLab 2/26, ADInstruments Pty. Ltd., New South Wales, Australia). Stainless steel bipolar wire electrodes (Unique Medical, Osaka, Japan) and a pulse generator (Nihon Kohden, Tokyo, Japan) were used for penile stimulations with the following parameters: 1 minute at 10 V, 20 Hz, and a square wave duration of 5 ms. Erectile function was evaluated using the maximum ICP/MAP ratio.

Examination of Dynamic Infusion Cavernosometry (DIC)

Dynamic infusion cavernosometry (DIC) was performed as reported previously.[12] After measurement of the ICP, the right crus of the CC was cannulated using a 23-G needle for attaching to an infusion pump (LC-9A, Shimadzu, Kyoto Japan). Papaverine (2 mg in 0.1 mL) was administered through the infusion cannula and after 10 minutes, the infusion started. At the start, the infusion rate was 0.05 mL/min, which was increased at the rate of 0.05 mL/min until the ICP was 100 cm H_2O. After this pressure, the infusion was terminated and the change in ICP over the subsequent 30 seconds was recorded as the drop rate.

Biological Parameters

After measuring the erectile function, the bioavailable testosterone (bio-T) levels in blood samples obtained from the vena cava were measured. The samples were coagulated and centrifuged at 800 $\times$ g for 20 min at 4°C. The serum samples were stored at −80°C until analysis.

Bioavailable testosterone was measured as reported previously.[11,16,17] In brief, dexamethasone was used as the internal standard working solution, and concanavalin A was added to the serum samples to remove sex hormone-binding globulin. To improve their detectability, testosterone and the internal standard were derivatized using picolinic acid. The prepared samples were subjected to ultra-performance liquid chromatography-tandem mass spectrometry (Aquity UPLC-MS/MS; Waters, Milford, MA, USA), using an Inertsil ODS-3 column (2.1 $\times$ 50 mm; GL Sciences, Tokyo, Japan) for separation in a water:acetonitrile (40:60, v/v) mixture that contained 0.1% acetic acid as the mobile phase.

Histological Evaluation of Stained Sections

Frozen penile tissue sections (7-μm thick) from each group were adhered to charged slides and air dried overnight. The sections were then stained using Masson's trichrome staining as described previously,[11,16] examined microscopically, and photographed using an Eclipse Ti camera (Nikon, Tokyo, Japan). The images were subjected to morphological and quantitative analyses using the Adobe Photoshop CS4 extended software (Adobe System, San Jose, CA, USA) to quantify the amount of smooth muscle (SM, stained red) and collagen (stained blue) inside the tunica albuginea. The analysis was performed in a blind fashion.

Statistical Analyses

Results are expressed as means $\pm$ standard error of the mean (SEM). Statistical significance was determined using 1- or 2-way analysis of variance and Bonferroni's multiple t test in the SPSS Statistics ver. 26 (IBM, NY, USA). P-values < .05 were considered statistically significant.

RESULTS

Biological Parameters

In Late-ART (4w), Cast rats had significantly lower levels of bioavailable testosterone than their Sham counterparts (Sham, 1.81 $\pm$ 0.17 ng/mL; Cast, 0.15 $\pm$ 0.02 ng/mL, P < .01). However, the Cast+T and Sham rats had similar levels of bioavailable

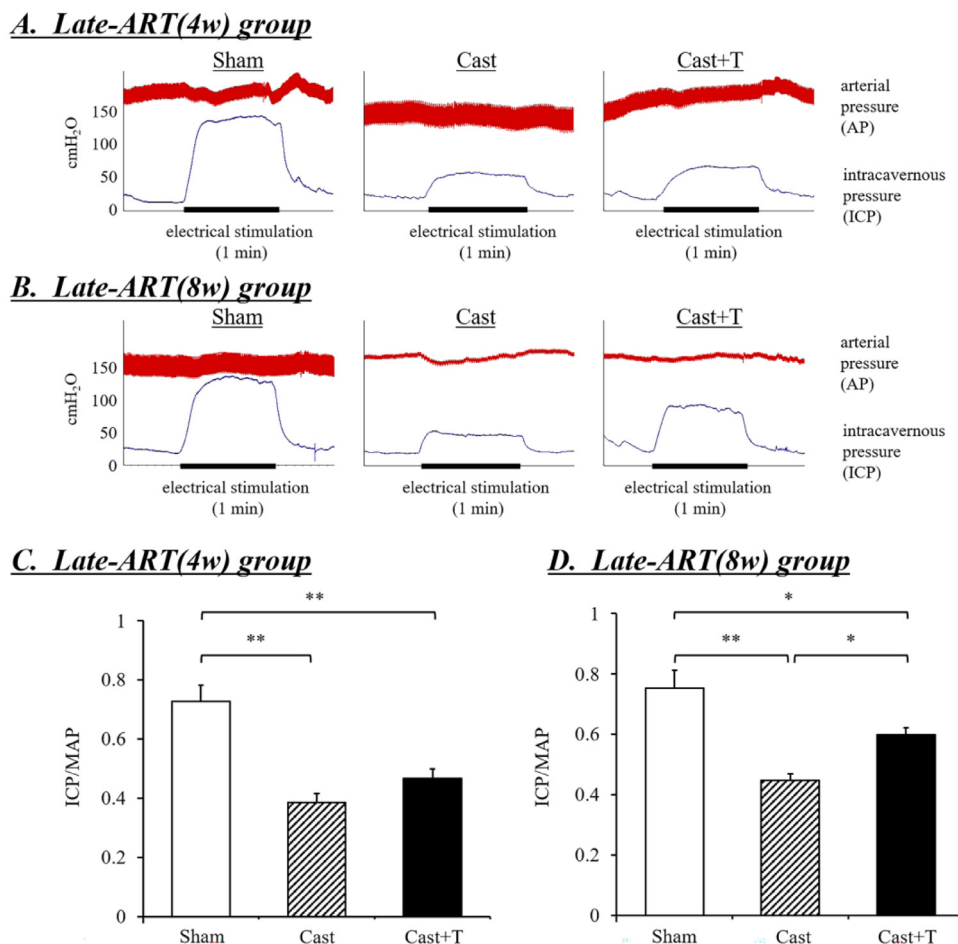## A. Late-ART(4w) group
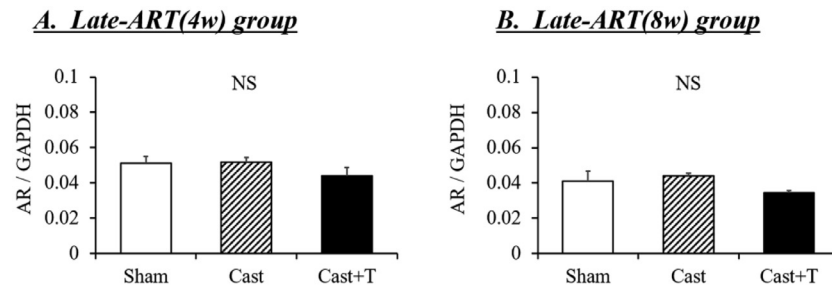
B. Late-ART(8w) group

Figure 2. Androgen receptor (AR) mRNA expression in the corpus cavernosum of rats. A. In the late-ART (4w) group; B. In the late-ART (8w) group. Target gene expression was quantified relative to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), using the comparative CT method. Data are reported as means ± standard error of the mean (n = 4 per group). N.S. means not significant using Bonferroni's multiple t-test.

testosterone (Cast+T, 2.01 ± 0.26 ng/mL, $P >$ .05). Similarly, in Late-ART (8w), Cast rats had lower levels of bioavailable testosterone than Cast+T rats (Sham, 1.73 ± 0.39 ng/mL; Cast, 0.16 ± 0.03 ng/mL; Cast+T, 1.98 ± 0.27 ng/mL).

AR mRNA expression in the CC is shown in Figure 2. There were no differences among the groups in each periods ($P >$ .05).

Erectile Function

Representative tracings of the ICP and arterial pressure changes during electrical stimulation of the cavernous nerve in the Late-ART (4w) group are shown in Figure 3A. The changes in the ICP in the Late-ART (8w) group are shown in Figure 3B. The ICP values in the Cast group appeared to

A. Late-ART(4w) group

B. Late-ART(8w) group

C. Late-ART(4w) group

D. Late-ART(8w) group

Figure 3. (A-B) Representative tracings of changes in the intracavernous pressure (ICP) and arterial pressure during electrical stimulation of the cavernous nerve in Sham, Cast, and Cast+T rats in the Late-ART (4w) and Late-ART (8w) groups. (C-D) Evaluation of the erectile function based on the ICP/mean arterial pressure (MAP) ratio. Data are reported as means ± standard error of the mean (n = 7−10 per group). *$P <$ .05, **$P <$ .01 vs each group as determined using analysis of variance and Bonferroni's multiple t-test.
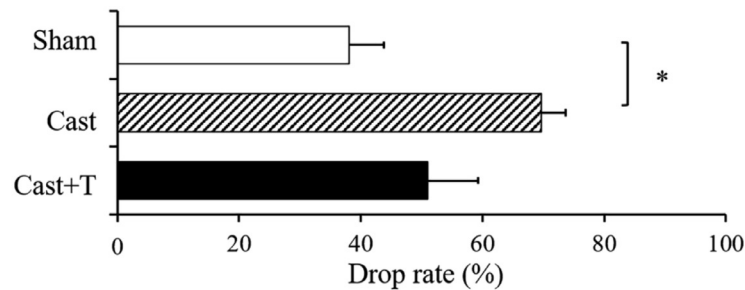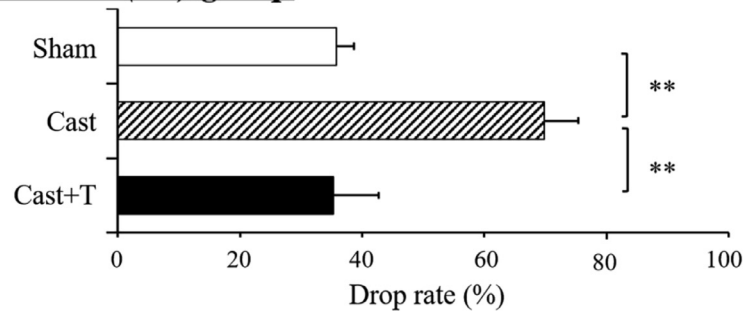
Figure 4. (A-B) Corporal veno-occlusive function as determined using the drop rate in dynamic infusion cavernosometry (DIC). Data are reported as means ± standard error of the mean (n = 7 per group). *P < .05, **P < .01 vs each group using analysis of variance and Bonferroni's multiple t-test.

be significantly lower than this in the Sham group for both the periods. Testosterone administration did not change the ICP in Cast+T rats in the Late-ART (4w) group, whereas it slightly increased the ICP in Cast+T rats in the Late-ART (8w) group. The ICP/MAP ratios for different stimulation frequencies are shown in Figure 3C and D. The Cast rats in the Late-ART (4w) group exhibited significantly lower ICP/MAP ratios than the Sham rats (Sham, 0.73 ± 0.06 ng/mL; Cast, 0.39 ± 0.03, P < .01). The Cast+T rats in the Late-ART (8w) also had a lower ICP/MAP ratio than the Sham rats (Sham, 0.75 ± 0.06 ng/mL; Cast, 0.44 ± 0.02, P < .01). Testosterone administration for 4 weeks did not increase the ICP in Cast+T rats in the Late-ART (4w) group (0.47 ± 0.02, P < .05); however its administration for 8 weeks significantly increased the ICP/MAP ratio in Cast+T rats in the Late-ART (8w) group (0.60 ± 0.02, P < .05), although the ratio was significantly lower than that for Sham rats (P < .05).

Dynamic Infusion Cavernosometry

The results of DIC in rats are shown in Figure 4A and B. The drop rate in Cast rats (69.6% ± 4.0%) was significantly higher than that in Sham rats (38.0% ± 5.8%, P < .05) in the Late-ART (4w) group. In Cast+T rats, the drop rate (50.9% ± 8.3%) did not decrease (P > .05 vs Cast). The drop rate in Cast rats (69.9% ± 5.5%) was also significantly lower than that in Sham rats (35.8% ± 2.9%, P < .05) in Late-ART (8w) group and that

in Cast+T rats (35.3% ± 7.4%) was significantly lower than that in Cast rats (P < .01).
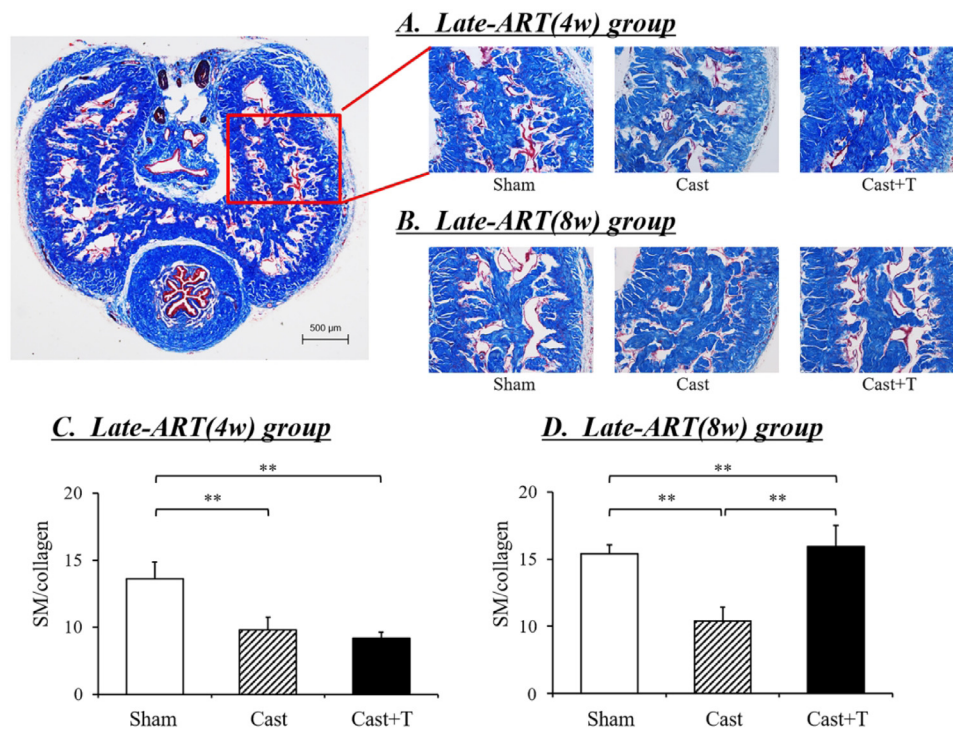
Histological Evaluation

The histological structure of the CC is shown in Figure 5A and B and changes in the SM/collagen ratio are shown in Figure 5C and D. The SM/collagen ratio in the Cast rats (9.8% ± 1.0%) was significantly lower than that in Sham rats (13.6% ± 1.2%, P < .01) in the Late-ART(4w) group. In Cast+T rats, the ratio (9.2% ± 0.5%) did not increase (P > 0.05 vs Cast). The SM/collagen ratio in Cast rats (10.4% ± 1.0%) was also significantly lower than that in Sham rats (15.4% ± 0.7%, P < .05) in the Late-ART (8w) group and that in Cast+T rats (15.6% ± 1.6%) was significantly higher than that in Cast rats (P < .01).

DISCUSSION

In this study, we administered testosterone for 4 or 8 weeks to castrated rats starting 4 week after castration. We previously reported that 4 weeks after castration the rats developed ED because of increased inflammation and structural changes.[11] The late ART of the castrated rats for 4 weeks did not change the erectile function, however an improvement was observed in the case of 8-weeks treatment. A much longer ART could have an even greater effect on erectile function.

In most of the previous animal studies, ART was administered immediately after castration. It was observed that the testosterone

Figure 5. Masson's trichrome staining of rat penile tissue. (A-B) Representative specimens stained with Masson's trichrome stain. (C-D) Histological evaluation of the corpus cavernosum used to calculate the area ratios of the smooth muscle (SM) and collagen fiber by computerized image analysis. Data are means ± standard error of the mean (n = 6 per group). $^{**}P < .01$ vs each group using analysis of variance and Bonferroni's multiple t-test.

administration maintained the erectile function, but did not improve it. The effects of delayed start of ART were reported previously.[18,19] Baba et al. demonstrated that 4-week testosterone replacement restored the erectile function in castrated rats 8 weeks after castration. However, the serum testosterone levels (control: 390.8 ± 22.6 pg/mL, castrated+T: 4448.4 ± 523.6 pg/mL) were extremely high. Huh et al. showed the testosterone levels only in the ART group, not in the control group. Taking into account the risk of side effects, we evaluated the effect of administering small doses of testosterone daily in improving erectile function. In this study, serum testosterone levels in Cast+T rats were similar to those in Sham rats. However, AR mRNA expression was not changed in any group. Therefore, a longer treatment period was required, and it is considered that such treatment can be safely performed.

To evaluate the improvement in the erectile function, we investigated the veno-occlusion in the rats by measuring the DIC. Davila et al. reported that testosterone deficiency causes corporal veno-occlusive dysfunction (CVOD) in rats.[12] We also observed CVOD in castrated rats. The late ART for 4 weeks did not improve the corporal veno-occlusive function. However, this function was improved in the case of late ART for 8 weeks. The results presented here indicate that these functional disorders might be responsie for the long time required for improvement of the erectile function.

We also investigated the histological changes after testosterone administration to evaluate the improvement in the corporal veno-occlusive function. The SM/collagen ratio was decreased in Cast rats when compared with their Sham counterparts, as determined by Masson's trichrome staining. Loss of smooth muscle and fibrosis have also been reported in castrated rat.[11,20−22] These effects of testosterone suggest that testosterone deprivation results in the apoptosis of trabecular smooth muscle cells and increased development of extracellular matrix.[23] Interestingly, Wang et al. reported that castration induced apoptosis of CC smooth muscle by regulating autophagy.[22] The loss in smooth muscle and fibrosis in the CC are thought to be related to ED.[24] As in the case of erectile and corporal veno-occlusive functions, the late ART for 4 weeks did not change the SM/collagen ratios. Because restoration of the normal histology takes time, it is considered that ART will take a considerable amount of time to become effective. On the contrary, the late ART for 8 weeks improved the SM/collagen ratio. It is suggested that the improvement of tissue structure improved the corporal veno-occlusive functions and improved the erectile function.

The major limitation of this study is the difference in the time required for healing between humans and rats, which might reduce the translational relevance of the results. Although the time required for rats cannot be directly applied to humans, Sengupta reports that 16.4−16.7 days in rats

equals approximately 1 human year.[25,26] Thus, the ART for 8 weeks could be similar to approximately 4 human years. Additionally, in this study, we used 12 week-old castrated rats. As these rats were young, the therapeutic effect may have been easier to obtain than if they were old rats, and we believe that studies should be conducted using old rats in the future. However, continuing treatment with ART could improve the ED in any animal species.

CONCLUSIONS

In this study, we found that late ART could improve erectile function; however, considerable time is required for the improvement. It is, therefore, necessary to continue with the therapy patiently to have beneficial effects.

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Conflict of Interest: The authors report no conflicts of interest.

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