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The effects of testosterone replacement on penile structure and erectile function in castrated adult male rats

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The effects of testosterone replacement on penile structure and erectile function in castrated adult male rats

Directed by Professor Byung Ha Chung

The Doctoral dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree
of Doctoral Philosophy

Kyung Kgi Park

June 2016

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ABSTRACT

The effects of testosterone replacement on penile structure and erectile function in castrated adult male rats

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(Directed by Professor Byung Ha Chung)

Objectives: To evaluate the effects of testosterone on penile structure and erectile function in adult male rats.

Patients and Methods: Whole subjects were divided into two groups by observation period (group I (n=30), 12 weeks; group II (n=30), 20 weeks). Each group had three difference subgroups (10 rats each). Sham-operated control, surgical castration group and testosterone replacement for 4 week after 8 weeks of castration period. Group II also had a same kind of subgroup. Control, castration, and last subgroup had more longer castration period (16wks) and testosterone replacement at 4weeks before sacrifice. Erectile function was assessed by measuring intracavernosal pressure in response to cavernous nerve stimulation, and the expression of nNOS protein was determined by Western blot analysis. Serum testosterone values were measured in each animal by radioimmunoassay

Results: Serum testosterone levels and cavernousal smooth muscle contents and nNOS activity decreased significantly in castrated animals, whereas Testosterone undecanoate injection normalized the serum levels of testosterone and others. Total Intracaver-

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nosal pressure / mean arterial pressure was significantly decreased in untreated castrated rats. compared with intact controls (28.3 +- 3.5 and 78.9+-8.2, respectively). Erectile function was normalized in 4 weeks of androgen-replaced rats. The expression of nNOS was decreased in the corpus cavernosum of castrated animals compared with controls, whereas androgen replacement normalized the expression of nNOS. These results were consistently observed despite the period of androgen deprivation.

Conclusions: These data suggest that androgen regulates the expression of nNOS in rat penile corpus cavernosum and confirms the importance of androgens in the maintenance of erectile function. Howerver, irreversible structural and erectile functional changes are not found during study period.

Key words : Testosterone replacement, Erectile function, Castration

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The effects of testosterone replacement on penile structure and erectile function in castrated adult male rats

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I. INTRODUCTION

Penile growth is very important to male development. In human, masculinization of external genitalia is happened by 5 alpha-Dihydrotestosterone (5 a-DHT) during fetal period. During neonate and prepubertal period, length and girth are increased by growth of penile skin and corporeal tissue as a mesenchymal origin cell [1]. Serum testosterone level reached to adult level by increasing secretion of gonadotrophin releasing hormone (GnRH) in hypothalamus and pituitary gonadotropin stimulated by GnRH while in puberty. The secondary sexual characteristics are displayed by the continueouly increasing in testosterone. After that, level of serum testosteone are will continue to decline after the age of 40[2]. The lack of testosterone may result in any degree of development problem with the normal development of male life. Therefore, many researchers have had a lots of research about pubertal development failure due to depletion of androgen. However, there were not so many result about role of maintaining level of serum androgen in well developed male. To improved their erectile function, many studies with aged men with physiologic decreased level of serum testosterone are performed.

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However, it did not draw a lot of research for androgen deficiency in patients under the influence of a normal androgen. We confirmed that the continuous testosterone suppression performed shorten the length of the penis in patients that undergo continuous androgen suppression therapy(ADT) due to prostate cancer[3]. Adult animal model with a short period of testosterone suppression reduced the trabecular smooth muscle content and increased the interstitial connective tissue accumulation are also reported[4,5]. This result may be an evidence of the androgen also affects maintenance as well as growth of the penis. Therefore, the goal of this study was to evaluate the effects of long term testosterone deprivation on penile erection and structure using Rats model. And then confirm recovery of the changes in the erectile tissue and hemodynamic parameters of cavernosum caused by ADT with administration of testosterone.

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II. MATERIALS AND METHODS

1. Animal subjects and Treatment protocol

Sixty adult male Sprague-Dawley rats (11 weeks old) were used in the current study. Whole subjects were divided into two groups by observation period (group I (n=30), 12 weeks; group II (n=30), 20 weeks). Each group had three difference subgroups. First was sham-operated control (n = 10), second was surgical castration group (n = 10), and third was testosterone replacement for 4 week after 8 weeks of castration period. Group II also had a same kind of subgroup. Control (n = 10), castration (n = 10), and last subgroup had more longer castration period (16wks) and testosterone replacement at 4weeks before sacrifice (n = 10) (figure 1).

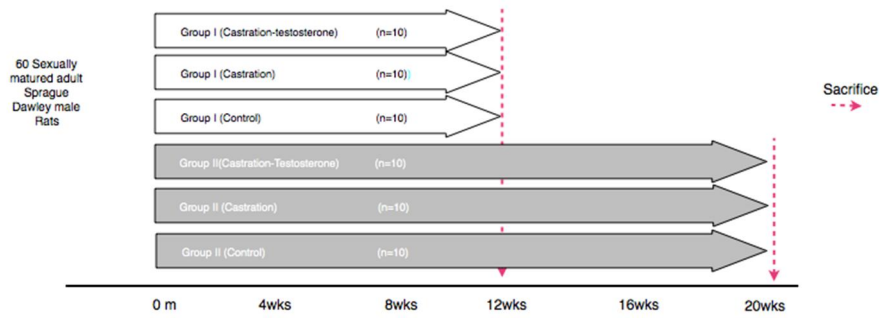

Figure 1. Study Diagram

Rats were castrated with vertical scrotal incision under ketamine anesthesia (90mg/kg intramuscular [IM]) and Xylazine analgesics (10mg/kg IM). Testosterone replacement was achieved by Monthly intramuscular injection of 100mg/kg of testosterone undecanoate (TU). After 4week of androgen treatment, rats were assessed for erectile

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function and blood was collected from the heart to determine circulating androgen levels by radioimmunoassay. Penile tissue was harvested for biochemical and histological analysis, as described below.

2. Measurement of Erectile Function

The Rats from each subgroup and their age-matched controls were anesthetized with ketamine (90 mg/kg) and Xylazine (10 mg/kg) intramuscularly. The animal was placed in the supine position, The penis was denuded of overlying skin and then stretched penile length (between base and tip) and girth (at mid-shaft) were measured by still and paper ruler, respectivel. The bladder and prostate were exposed through a midline abdominal incision. The major pelvic ganglion and cavernosal nerve were identified posterolaterally to the prostate on one side. Bipolar platinum wire electrodes were placed around the cavernous nerve. The penis was denuded of skin, and a 24-gauge needle filled with 250U/ml of heparin was inserted into one side of the corpus cavernous for monitoring of intracavernous pressure. A carotid artery was cannulated for measurement for systemic arterial pressure. Systemic arterial and intracavernosal blood pressure were measured with pressure transducers connected to a computerized system for data acquisition and analysis. Stimulation parameters were 10 V at a frequency of 12 Hz, a pulse width of 1 millisecond, and a duration of 1 minute. During tumescence, the maximal intracavernous pressure (ICP) was recorded. The total ICP was determined by the area under the curve from the beginning of cavernous nerve stimulation to a point 20 seconds after stimulus termination. The ratios of maximal ICP and total ICP to mean arterial pressure (MAP) were calculated to normalize for variations in systemic blood pressure.

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3. Tissue harvasting and histochemical staining

After the functional study was completed, rats were sacrificed with bilateral thoracotomy. A mid-portion of the penile segment was harvested for Histological assessment. Tissue samples were fixed in cold 2% formaldehyde and 0.002% picric acid in 0.1 M phosphate buffer, pH 8.0, for 4 hours followed by overnight immersion in buffer containing 30% sucrose. The specimens were then embedded in OCT Compound (American Master Tech Scientific, Lodi, CA, USA) and stored at -70°C until use. Sections were cut at 5 μm, mounted into charged slides and air dried for 5 minutes. Representative slides were stained with Hematoxylin & Eosin and Masson's trichrome for connective tissue and smooth muscle histology.

4. Western Blot Analysis

A portion of each penis was frozen, processed into tissue powder, and homogenized in phosphate buffer (10-mM phosphate [pH 7.4] and 150 mM NaCl) containing protease inhibitors (Thermo Fisher Sci- entific Inc., Waltham, MA, USA). The homogenates were centrifuged at 12,000 rpm for 20 minutes. The total protein concentration in the supernatant was measured by the Lowry micro-method. Equal amounts of protein (50 g) were electrophoresed through 16.5% polyacrylamide gels under denaturing conditions (12% SDS) and transferred onto nitrocellulose membranes. The membrane was blocked in 20-mM Tris-saline buffer (pH 7.2), containing 0.1% Tween 20 and 0.2% casein for 1 hour, and incubated with a nNOS (Santa Cruz Biotechnology, Dallas, TX, USA) or or b-actin (Abcam, Cambridge, U.K.) overnight. Before re- probing with an anti-actin antibody, the membrane was stripped in 62.5mM Tris-HCl, pH 6.7, 2% SDS, 10 mM 2-

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mercaptoethanol at 56°C for 30 minutes and then washed four times in 1x tris-buffered saline. Results were quantified by densitometry.

5. Statistical analysis

The differences of variables between subjects were evaluated by one-way analysis of variance followed by post hoc tests (GraphPad Prism ver. 5.00, GraphPad Software, San Diego, CA, USA). P values less than .05 was considered significant for analyses.

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III. RESULTS

Serum Testosterone

Serum testosterone level in both groups were significantly decreased after castration (group I: 1.30± 0.35; group II: 0.75± 0.23 ng/mL), which was restored to the control level (group I: 4.48± 0.76; group II: ng/mL) after testosterone replacement, respectively (P = 0.005). The mean body weights were not significantly different between any of the treatment groups (Table1).

Table 1. Physiologic parameters of subjects at end of the study. (n = 60)

	Weight (g)	MAP (cmH2O)
Group I		
Control	650±15.4	126.7±18.5
Castration (8wks)	629±10.7	127.4±19.8
Castration (8wks) + Tu Tx (4wks)	649±12.6	132.4±16.0
Group II		
Control	690±29.6	129.0±18.5
Castration (16wks)	645±15.3	132.5±14.8
Castration (16wks) + Tu Tx (4wks)	663±10.8	135.4±16.4

Data are presented as the mean ± standard deviation (n =10 per group). Group I was operated after 12 weeks, group II was done after 20 weeks. MAP: mean arterial pressure (2/3 diastolic blood pressure + 1/3, systolic blood pressure), Tu: testosterone undecanoate. Tx: treatment

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Gross appearance of penis and Erectile function study

Although there was no significant difference in total body weight, there were significant difference of penile length and girth. Mean ICP/MAP and total ICP/MAP were significantly lower in castration group and after 4 weeks of testosterone replacement, these parameters were significantly improved despite the period of castration. (Figure 2 and 3).

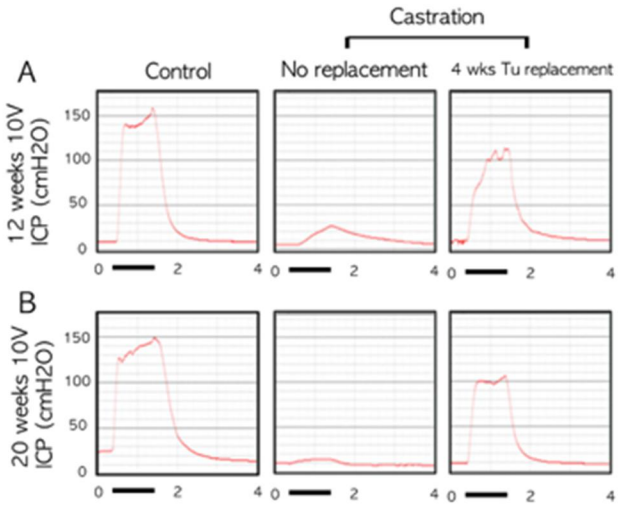

Figure 2. Testosterone replacement restores intracavernosal pressure of castrated Rats despite prolongation of castration period.

Lower solid bar indicate the electro-stimulus time. The X-axis is the elapsed time. ICP: intracavernous pressure, Tu: testosterone undecanoate, Results marked as A represents that ICP is restored after 4 week of testosterone replacement. Graph B also show good restoration of ICP despite double period of androgen deprivation.

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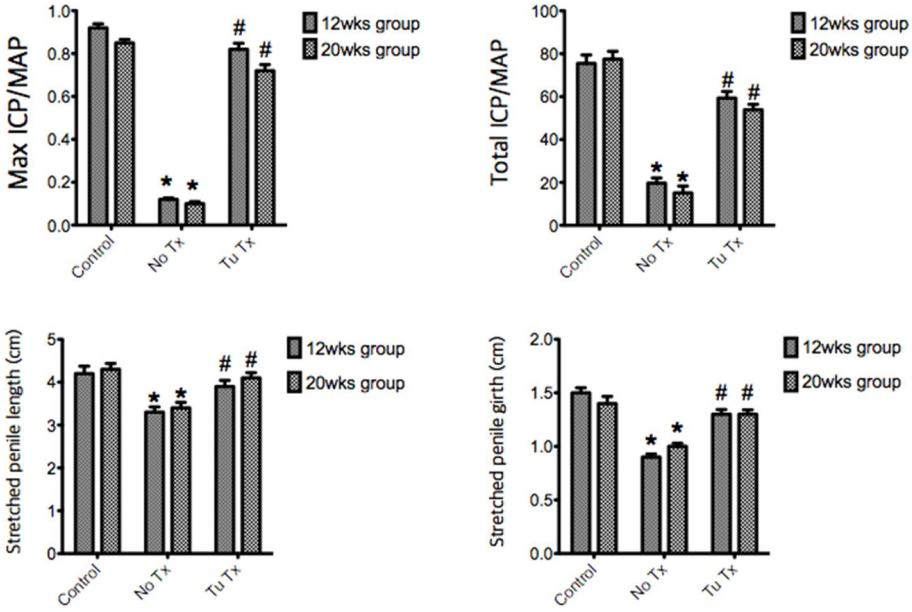

Figure 3. Testosterone replacement restores intracavernous pressure and gross morphology of penis in castrated rats despite prolongation of castration period.

12wks group was operated after 12 weeks, 20wks group was done after 20 weeks. There are no significant difference between 12wks and 20wks groups. However, significant different are shown within subgroups (12wks group and 20wks group). Astrix mark show that significant decrease compared to control. Sharp mark show significant increased compared to castration group. ICP: intracavernous pressure, MAP: mean arterial pressure (2/3 diastolic blood pressure + 1/3 systolic blood pressure), Tu: testosterone undecanoate. Tx: treatment

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Western blot analysis

Protein expression of neuronal nitric oxide synthase (eNOS) was significantly decreased and well restored their activity after 4 weeks of testosterone replacement in both group (Figure 4).

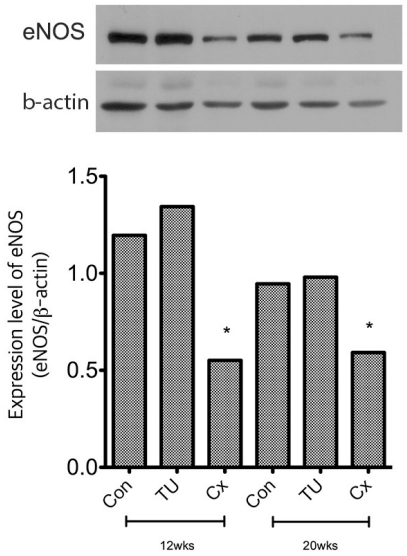

Figure 4. Western blot for neuronal nitric oxide synthase (eNOS)

12 weeks results represents that rats with 8wks of castration well restores their endothelial nitric oxide synthase (eNOS) activity after 4 weeks of replacement of testosterone. 20 weeks results also shows restoration of eNOS activity despite more long castration period (20wks). A astrix mark show that significant decrease compared to control. TU: Castration and replacement with testosterone undecanoate for last 4 weeks. Cx: castration withtout treatment

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Histochemical assessment of tissue structure

Masson's trichrome demonstrated significantly higher smooth muscle to collagen ration in control and testosterone replaced animals compared to the 8-week and 16-week of castration groups (Figure 5).

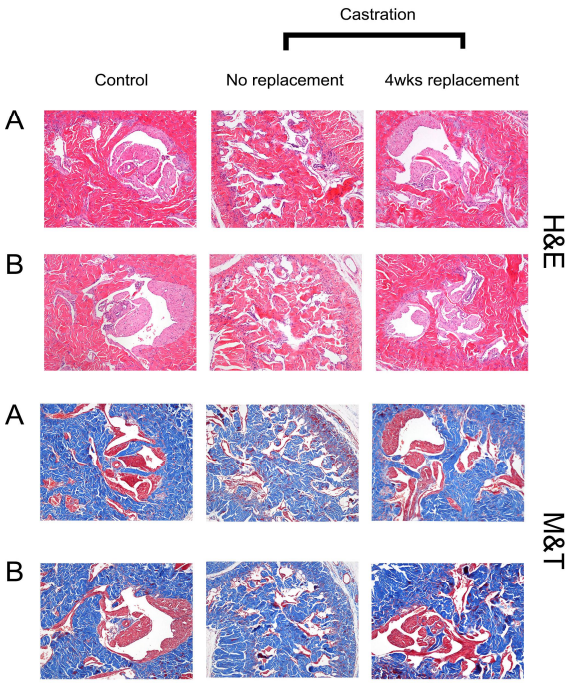

Figure 5. Smooth muscle contents in the corpus cavernosum

Representative images from rats in the control, castration, castration with testosterone replacement. A is 12weeks group, B is 20weeks followed up group. Smooth muscle and connective tissue are stained with red and blue, respectively, using the trichrome method. The smooth muscle content was higher in control and replacement group than castration groups. Magnification is x 100

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IV. DISCUSSION

This study showed that testosterone replacement could improve erectile function in adult male castrated rats despite period of deprivation of testosterone. Based on this study results, we could not find any irreversible change of penis. There are many parameters to identify erectability of penis. Of these, penile length and girth, ICP, nNOS activity, cavernosal smooth muscle content related to connective tissue and endothelial tissue could be restored by short term (4wks) testosterone replacement.

After the complete adult development, penile structure should need androgen to maintain their structure[6]. Structural, hormonal, neural and metabolic elements interplay to maintain erection. Of these, structural and neural integrity mediated by androgen. Lu and colleague reported that endothelial structures can be damaged by low concentration of testosterone [7]. Keast and colleague showed the critical role of testosterone for maturation and maintenance of terminal axon density and neuropeptide expression[8]. In recent study, Hwang and colleagues have showed androgen supports a maintaining of cavernousal endothelium and regulating the vascular endothelial growth factor (VEGF) [9]. Baba and colleagues have reported that androgen regulated the expression of nitric oxide synthase (NOS)informs in in there corpus cavernous of animal model. Several studies have shown that androgen deprivation results in a decrease level of testostosterone and ICP in response to nerve stimulation using NADPH-diaphorase straining as a marker of neural nitric oxide synthase(nNOS) expression. They also have reported testosterone injection could restore their erectile response to near normal values. Miranda and colleague studied about structural changes and their restoration related to androgen. Using adult male rats, significant decreased in the content of smooth muscle, sinusoidal space and total cavernous area after 2 month of castration. However, changes of struc-

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ture were restored with 1month of androgen replacement [10]. These results are similar with ours group 1 data (study was conducted during 8wks of ADT and 4 wks of replacement). These study period was relatively short to identify the irreversible change of penis in rats. Therefore, we have conducted a study based on more long period of ADT, however, there were restoration of penile erection and structure too. Afterward more long term study will be needed.

In human, Halioglu and colleague reported that short term androgen deprivation therapy (ADT) and external beam radiotherapy affects to shorten the penile length. However, these study based on mixed effect of ADT and Radiotherapy. Therefore it is difficulty to identify the sole effect of ADT. Our research team previously studied that the changes of penile length based on period of deprivation with androgen. The mean follow-up period was about 24 months. Penile length decreased until 15months after ADT and later, there was no significant changes of length despite of prolonged ADT[3]. In this study also showed the failure of the penile function and structure. Alternatively, we did not find any evidence of irreversible changes of penile function and structure, because relatively short period of abstinence of androgen. Some other researcher argued that, castration of the human male after attainment of sexual maturity does not result in a marked reduction in penile size, in contrast to that observed in animal models. Because human male produce testosterone in their adrenal gland as opposed to some animal model. [2,11]. However, as seen in the above paragraph, human male with LHRH agonist only could show shortening of their penis significantly as seen in various animal study.

Androgen would support their penile erection by maintaining the cavernousal structure and regulated the eNOS. However, we could not confirm that how long deprivation of androgen could change the penile erection irreversibly.

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V. CONCLUSION

Androgen would support their penile erection by maintaining the cavernousal structure and regulated the nNOS. However, we could not confirm that how long deprivation of androgen could change the penile erection irreversibly.

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ABSTRACT(IN KOREAN)

거세 남성 쥐에 시행한 남성 호르몬 보충이 성기의 구조와 발기력에 미치는 영향

<지도교수 정 병 하>

연세대학교 대학원 의학과

박 경 기

남성 쥐에서 남성 호르몬을 일정 기간 박탈 한 후 보충하였을때 성기 및 발기력의 보존에 미치는 영향을 확인하기 위해 성인 남성 쥐 60 마리를 대사으로 연구를 시행하였다. 8 주 거세군과 16 주 거세군으로 30 마리씩 나누어 각각 control 및 거세유지군 그리고, 마지막 4 주의 남성 호르몬 보충 군으로 10 마리씩 배정하였다. 발기력과 성기의 구조를 확인하기 위해 cavernousal pressure 측정 및 NOS 정량, 그리고 마지막으로 조직 염색을 통한 성기내부의 연부조직의 변화를 확인하였다. 결과는 8 주 와 16 주 거세군에서 동일하게 마지막 4 주간의 남성호르몬 보충 만으로 발기력이 호전되는 것을 볼 수 있었고 조직학적인 변화에서도 cavernosal

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muscle 이 퇴화되었다가 다시 회복되는 것을 확인 할 수 있었다. 신경근 말단에서 Nitric oxide 를 합성하여 발기를 유발하는 NOS 의 정량을 확인한 연구에서도 남성 호르몬 보충으로 다시 호전되는 것을 확인 할 수 있었다. 결론 적으로 남성 호르몬은 성기의 구조와 발기력을 유지하는데 중요한 역할을 하고 있음을 알 수 있었고 16 주간의 남성 호르몬 박탈은 성기의 비가역적인 변화를 주는데 충분하지 않음을 알 수 있었다.

핵심되는 말 : 남성 호르몬 보충, 발기, 거세