·*Original Article*·

Changes in aortic endothelium ultrastructure in male rats following castration, replacement with testosterone and administration of 5α-reductase inhibitor

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Abstract

Aim: To investigate the relationship between low androgen level and ultrastructure of vascular endothelium. **Methods:** Forty-eight male Sprague-Dawley rats were randomly divided into four groups: group A, normal rats with sham castration; group B, castrated rats; group C, castrated rats given testosterone (T) undecanoate; and group D, intact rats treated with 5α-reductase inhibitor. After 10 weeks of treatment or castration, rats in different groups were killed and serum T, free T (FT) and dihydrotestosterone (DHT) were measured. The aortic endothelia were scanned under electron microcopy and the Vascular Endothelium Structure Score (VESS) was computed. **Results:** Serum T and FT concentrations of rats in group B were significantly lower than those of the other three groups ($P < 0.01$); DHT concentrations of group D rats were significantly decreased ($P < 0.01$) when compared with those of groups A and C. Rats in groups B and D rats (with low androgen levels) had obvious damage to their endothelial surfaces, which appeared crimpled, rough, adhesive and ruptured, and had high destruction of VESS. **Conclusion:** These results suggest that low concentrations of T and DHT are associated with ultrastructural damage of the aortic endothelia in male rats.

Keywords: endothelium; ultrastructure; testosterone; 5α-reductase inhibitor; castration

1   Introduction

Age and being male are two independent risk factors for coronary heart disease (CHD). It has been

shown that men are consistently twice as likely to die from CHD as their female counterparts [1]. Furthermore, it is recognized that changes in sex steroid hormone levels may be associated with variation in risk of cardiovascular disease [2]. Although androgen might play a role in the etiology of atherosclerosis, its precise role has yet to be elucidated [3]. To our knowledge, there has been no report on the effects of androgens on aortic endothelial ultrastructure. Therefore, this study sought to investigate whether low androgen hormone, as in castrated rats, and those treated with 5α-reduc-

tase inhibitor, has an impact of endothelial ultrastructural integrity.

2 Materials and methods

2.1 Experimental animals

The experiments were performed in conformity to the guidelines for the care and use of laboratory animals published by the US National Institutes of Health [4]. Forty-eight male Sprague Dawley rats from Zhejiang University Animal Laboratory Center were used in the study. The rats were 5 weeks old and had body weight of 125 g ± 23 g. They were divided into four groups. Group A: 12 rats were normal intact rats which underwent sham castration to serve as control; Group B: 12 rats were castrated, hence have very low androgen levels; Group C: 12 castrated rats given testosterone (T) undecanoate (50 mg/kg·month) by intramuscular injection for 10 weeks to replenish androgen; Group D: 12 rats which were raised with 5α-reductase inhibitor (4.5 mg/kg·day) administered intragastrically to inhibit dihydrotestosterone (DHT) production. All animals were housed in our animal facility with temperature set from 20ºC to 22ºC and a 12 h : 12 h light : dark cycle. Rats had free access to chow and water throughout the study. After a 10-week treatment described above, the rats were killed by intraperitoneal injection of ketamine (35 mg/kg).

2.2 Measurements of T, free testosterone (FT) and DHT by radioimmunoassay

T, FT and DHT were measured by RIA kits from ICN Biomedicals (MP Biomedicals, Orangebury, NY, USA).

2.2.1 Measurement of T

The assay set-up included blanks, non-specific binding (NSB), quality control samples, a series of standards ranging from 0 nmol/L to 55 nmol/L and test samples. The samples were then incubated with the primary anti-testosterone antibody at 37ºC for 2 h and followed by incubation with the second antibody for 60 min. Following the incubation, the supernatant was discarded and tubes dripped dry by inversion onto absorbent towels, and counted in a gamma scintillation counter for 1 min. A dose-response curve was constructed and unknown sample concentrations were interpolated from this curve.

2.2.2 Measurement of FT

For the measurement of FT, serum samples were first extracted and undergone chromatographic separation using in-house methods before assaying for T using the sample method as described above.

2.2.3 Measurement of DHT

Test samples 500 µL of the oxidant was added into 400 µL of quality control, and then the tubes were incubated at room temperature for 15 min. Following the incubation, 4 mL of 98% N-hexane and 2% ethanol mixture and 50 µL of DHT buffer were added to each tube. The tubes were mixed thoroughly and then centrifuged for 15 min at 2–8ºC. Then 2.5 mL of the supernatant was transferred into separate labeled tubes and extracts were dried via blowing of nitrogen gas. The dried extracts were reconsitituted with 250 µL of "0" standard DHT and tubes were mixed vigorously. The assay procedure for DHT was similar to that for T.

2.3 Electron microscopic scanning of aortic endothelial cell

Endothelia from renal arterial tissues were carefully dissected out using microsurgical scissors under the dissecting microscope. Then it was washed with normal saline and kept damp at all time. For the chemical fixation, these tissues were stored in 2.5% glutaraldehyde (pH 7.2–7.4) for 3–5 h, and then poached with 0.1 mol/L PBS for three times before putting them into a solution of osmium tetroxide (OsO_4-$HgCl_2$) for 2 h. Following this, the tissues were washed again. For the dehydration step, the tissues were serially immersed into solutions with gradually increasing concentrations of ethanol from 30%, 50%, 70%, 80%, 90% and 100%. The 100% ethanol was then replaced by 100% acetone and tissues were immersed in this solution for 15 min. Finally the tissues were immersed into xeno-penta-ester in preparation for immersion into liquid CO_2.

Drying in this method is of critical importance. Samples were desiccated in a controlled manner in order to maintain the same size and shape as with the original living material. The application of heavy metal salts: mounting, diode sputtering coated with gold-palladium (Sputtering Equipment E1020; Hitachi China Ltd, Beijing, China) was used to increase the electron density (scattering-power) of the specimen.

For each sample, ten separate regions were scanned and photographed by the scanning electron microscope (Leica-Stereoscan 260; Leica Instruments Ltd, Cambridge,

UK). Each photograph of the endothelium was then given a Vascular Endothelium Structure Score (VESS). Several parameters of the endothelial cells were assessed under the electron microscope, and they were a) array (shape), b) adhesive on surface, c) endothelial abruption or rupture and d) the connection condition between endothelium. So we score these items for the VESS. A VESS score composed of a) the cell array, b) the degree of adhesiveness, c) the degree of abruption or rupture, and d) the integrity of the connection in the endothelium. In addition, two independent researchers provided the score for each piece of observation. For each of these four parameters, a score on a scale of 0 to 10 was given with 0 = normal, 2 = close to normal, 4 = partial damage, 6 = when the destruction is not too obvious, 8 = obvious damage seen and 10 = when the damage was severe. For each sample, the average scores of the four parameters for the ten regions were computed. Hence, for each sample the VESS can have a minimum score of 0 (normal) or 40 (severely damage).

2.4 Statistical analysis

All results were expressed as mean ± SD. Statistical analyses were carried out using the SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA). One-way ANOVA was done where appropriate and significance was set at $P < 0.05$.

3 Results

Serum levels of T and FT in castrated rats (group B) were significantly lower than those in groups A, C and D ($P < 0.01$). DHT level in group B was significantly lower

when compared with those in groups A and C ($P < 0.05$). DHT level in group C was significantly higher when compared with those in groups B and D ($P < 0.05$). DHT level in group D was significantly lower compared with those in groups A and C ($P < 0.01$) (Table 1).

The aortic lumen of normal rats showed that the surface of cell was smooth and stretched freely. The structure was regular. The damage index or VESS in group A (7.15) was significantly lower than those in the other three groups. The endothelia in castrated rats group were severely crimpled, coarse, and protuberant. The connections between cells were ruined and many red blood cells were noted to adhere to the surface. The total damage index of VESS (32.20) was the highest among all groups. The mean value of the four parameters was 8.05 ± 2.11 and was significantly higher than those in groups A, C and D. The endothelia structures in rats which were supplemented with T undecanoate showed some recovery and were better than those in the castrated rats. However, lesions were still noticeable. The VESS was 12.3 which was still significantly higher than that in group A. On the other hand, the endothelia surface in rats raised with 5α-reductase inhibitor leading to low dihydrotestosteron was uneven. There were a lot of red blood cells adhering to it. The VESS (30.95) was significantly higher when compared with group A and group C (Figure 1 and Table 2).

The results from this study showed that the degree of aortic endothelial damage was highest in the castrated group, with those treated with the 5α-reductase inhibited coming up as the second worse case.

4 Discussion

Following castration, T, FT and DHT decreased dramatically as was shown in this study. After 10 weeks, the endothelia integrity was compromised. The VESS for shape, adhesion, rupture and connection was higher than that in control rats, indicating that the depletion of androgens could lead to substantial endothelial damages. It is also possible that endothelial damages could result from the depletion of both T and DHT. Support for this suggestion was provided by the observation of some degree of recovery from the damage when castrated rats were supplemented with T. Although the reversal was not complete, probably due to the inadequate amount of T supplementation, this observation concurred with those of other investigators [5]. However, the exact mecha-

Table 1. Serum testosterone (T), free testosterone (FT) and dihydrotestosterone (DHT) concentration. Two rats died in groups A and C, respectively. Group A was sham. Group B was castrated. Group C was also castrated but given testosterone undecanoate (50 mg/kg·month) by intramuscular injection. Group D was raised with 5α-reductase inhibitor (4.5 mg/kg·day). [a]$P < 0.01$, [b]$P < 0.01$, compared with that in groups A, C and D; [c]$P < 0.05$, compared with that in groups A and C; [d]$P < 0.05$, compared with that in groups B and D; [e]$P < 0.01$, compared with that in groups A and C.

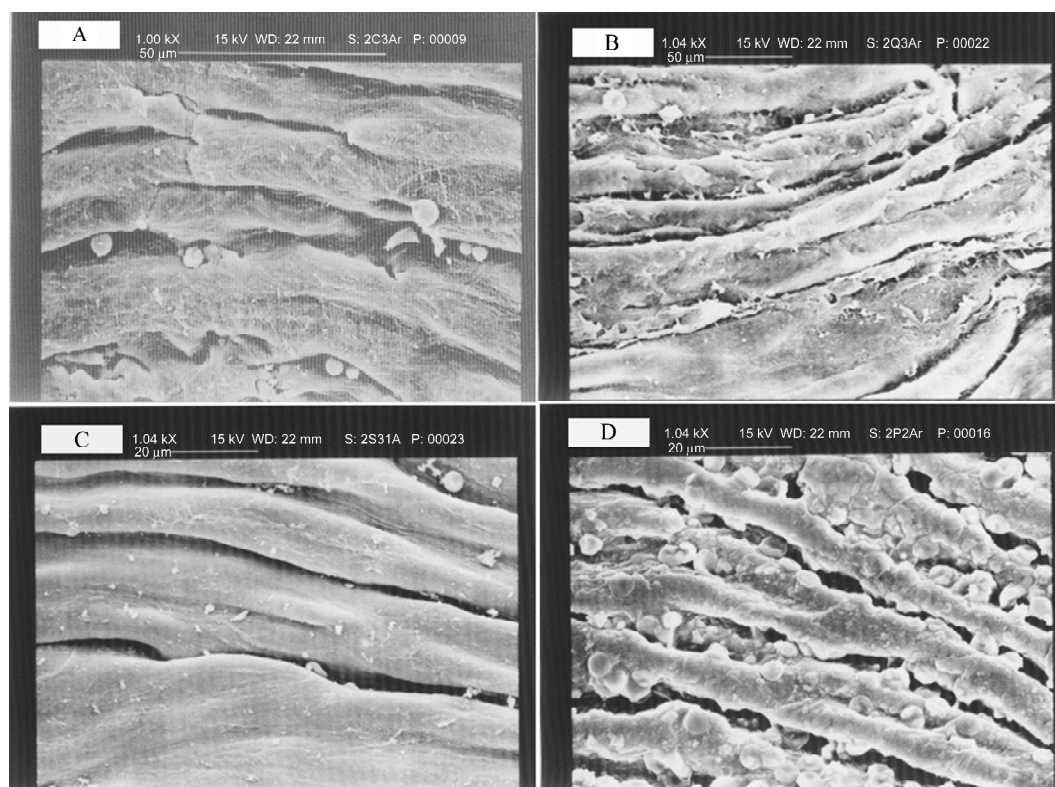| Group	n	T (nmol/L)	FT (pmol/L)	DHT (pg/mL)
A	10	20.17 ± 7.08	129.96 ± 48.82	165.21 ± 55.40
B	12	0.48 ± 0.10[a]	1.49 ± 0.28[b]	45.20 ± 10.71[c]
C	10	2.23 ± 0.70	10.80 ± 2.96	81.46 ± 68.84[d]
D	12	7.53 ± 6.80	46.46 ± 45.82	46.55 ± 15.69[e]

Figure1. The endothelial ultrastructure in different conditions. (A): Control, the surface of cell was smooth and its stretching was free. The structure presented regular, bar = 50 µm. (B): This group was castrated. The surface was broken and uneven. There were some fissure and hollow. The mucosa was exfoliated, bar = 20 µm. (C): This group was supplemented with testosterone undecanoate after castrated. The surface of cell was smooth and regular, bar = 20 µm. (D): This group was raised with 5α-redutase inhibitor. There was a lot of red cells adhering to the surface. Cells were disjunctive, bar = 20µm.

Table2. Vascular Endothelium Structure Score (VESS). Group A was sham. Group B was castrated. Group C was also castrated but given testosterone undecanoate (50mg/kg·month) by intramuscular injection. Group D was raised with 5α-reductase inhibitor. [a]P showed mean ± SD in group A compared with that in groups B, C and D. [b]P presented mean ± SD in group B compared with that in groups C and D. [c]P showed mean ± SD in group C compared with that in group D.

Group	Shape	Adhesion	Rupture	Connection	Total	Mean ± SD	[a]P	[b]P	[c]P
A	2.05 ± 0.47	2.20 ± 0.41	1.35 ± 0.51	1.55 ± 0.67	7.15	1.78 ± 0.40			
B	9.15 ± 0.54	5.05 ± 0.29	9.80 ± 0.40	8.20 ± 0.45	32.20	8.05 ± 2.11	0.000		
C	5.35 ± 0.65	2.15 ± 0.39	2.00 ± 0.33	2.80 ± 0.39	12.30	3.07 ± 1.56	0.248	0.001	
D	8.75 ± 0.45	9.00 ± 0.42	6.00 ± 0.43	7.20 ± 0.39	30.95	7.72 ± 1.41	0.000	0.773	0.001

nism for this androgen-induced impairment remains unclear. There is some evidence for androgen involvement in the vascular activities. T induces acute vasorelaxation and plays a role of vascular smooth muscle K channels in rat thoracic aorta and dilation vessel. It inhibits calcium-dependent elements of vascular contraction [6]. A study showed that supplemental T therapy in human with angina improved symptoms and reduced ob-jective measures of ischemia [7]. It was reported that T inhibited TNF-alpha-induced activation of the transcriptional nuclear factor-kappaB, which was critical for the inducible expression of VCAM-1, probably through the suppression of the nuclear translocation to enhance immune responses [8, 9]. Therefore, T may have function to protect vascular endothelial structure maybe through inhibiting inflammation medium, support dilation via se-

cretion of nitric oxide and modulating the calcium-dependent elements of vascular contraction [10].

In 5α-reductase inhibitor treated rats with resultant low DHT levels, there were a lot of red cells adhering to the endothelial surface. The vascular endothelial structure was apparently damaged. This was suggested that prolonged low DHT level might lead to increased blood viscosity which could induce thrombosis and endothelial destruction. Norata *et al.* [10] thought that DHT could positively regulate endothelial function through the control of the inflammatory response mediated by nuclear factor-kappaB in endothelial cells.

Evidence from this study suggests that low androgen levels can lead to impairment of the endothelial integrity and therefore, hypoandrogenism might be an important risk factor for cardiovascular disease.

Acknowledgment

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