Low-dose testosterone alleviates vascular damage caused by castration in male rats in puberty via modulation of the PI3K/AKT signaling pathway

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Abstract. The aim of the present study was to investigate the effect of testosterone on glucolipid metabolism and vascular injury in male rats, and examine the underlying molecular mechanisms. A total of 40 male Sprague-Dawley rats were divided into a control group (n=10), high-fat-diet + castration group (n=10), high-fat-diet + castration + low dose testosterone group (n=10), and high-fat-diet + castration + high dose testosterone group (n=10). Hematoxylin and eosin staining was performed to evaluate the morphology of the thoracic aortic tissues. Immunohistochemical staining was used to detect biomarkers of the phosphoinositide 3-kinase (PI3K) signaling pathway. The mRNA and protein expression levels of PI3K, AKT, insulin receptor substrate-1 (IRS-1), glucose transporter type 4 (GLUT-4), nuclear factor (NF)-κB and tumor necrosis factor (TNF)-α in the aortas were determined using quantitative polymerase chain reaction and Western blot analyses, respectively. Apoptosis in the aortic tissues was detected using a TUNEL assay. Castration induced apoptosis in the animals fed a high-fat-diet, whereas low dose testosterone replacement ameliorated the apoptosis in the aorta. However, the levels of apoptosis were more severe following high-dose testosterone treatment. Low-dose testosterone induced upregulation in the levels of IRS-1, AKT, GLUT-4 protein, NF-κB, TNF-α and PI3K, compared with those in the animals fed a high-fat diet following castration. A high dose of testosterone resulted in a significant decrease in the levels of IRS-1, AKT, GLUT-4, NF-κB, TNF-α and PI3K. Compared with the rats in the high-fat diet + castration group, a low dose of testosterone induced upregulation in the mRNA levels of IRS-1, AKT and GLUT-4, and downregulation of the mRNA levels of NF-κB, TNF-α and PI3K. A high dose of testosterone resulted in a significant decrease in the levels of IRS-1, AKT and GLUT-4, and marked increases in the mRNA levels of NF-κB, TNF-α and PI3K, compared with the low dose group. Castration induced marked disorders of glucolipid metabolism and vascular injuries in the pubescent male rats. Low-dose testosterone treatment was found to ameliorate the vascular damage caused by castration via the PI3K/AKT signaling pathway.

Introduction

Improvements in quality of living and changes in diet have been identified as the major issues contributing to the escalation of obesity in children and adolescents (1). To date, obesity is considered to be associated with insulin resistance, abnormal glucose metabolism, dyslipidemia, inflammation and vascular damage (2). The increasing prevalence of peripubertal obesity has raised the concerns regarding the prevalence and severity of adolescent metabolic syndrome (3,4).

Epidemiological data indicates a higher incidence of diabetes mellitus and fatty liver in males than in females, which may be associated with the expression of endogenous sex hormones. In males, testosterone is important in the body composition, particularly of visceral fat (5). For example, increases in serum triglyceride and total cholesterol (TC) have been observed in men with testosterone deficiency (6). In addition, decreased plasma testosterone has been identified in obese adolescent males, compared with control individuals of the same Tanner stage, consistent with data in adult males, associating obesity and insulin resistance with hypotestosteronemia (7,8). Furthermore, testosterone replacement treatment can enhance the effect of insulin and contribute to glucolipid metabolism, whereas high doses of exogenous testosterone decrease insulin sensitivity (9,10).

Increasing attention has been focused on the association between androgen deficiency and a variety of cardiovascular diseases (11,12). For example, a decrease in endogenous testosterone is a risk factor for atherosclerosis in males (11). In addition, short-term administration of testosterone at physiological concentrations can ameliorate coronary heart disease and improve endothelial vascular function (13). To date, the roles of testosterone in the metabolism of glucolipids and cardiovascular
disease in obesity remain to be fully elucidated. In the present study, a male rat model subjected to a high-fat diet and castration was established, based on which the present study aimed to examine the effect of testosterone in these animals. The results may determine whether testosterone attenuates vascular injury and assist in elucidating the underlying mechanism.

Materials and methods

Animals. For the animal model, 40 male Sprague-Dawley rats (3-week-old, weighing 50-60 g) were provided by the Academy of Military Medical Sciences (Beijing, China). All rats were housed at a temperature of 26°C in a 12 h light/12 h dark cycle, and were provided with free access to water and food. Following adaptive breeding for 1 week, the animals were randomly divided into the following groups: Group 1, control group (n=10), in which rats were fed a high-fat diet containing 57% carbohydrate, 22% protein and 4% fat; group 2, high-fat-diet + castration group (n=10), which were fed a high-fat diet following castration; group 3, high-fat-diet + castration + low dose testosterone group (n=10), which were fed a high-fat diet following castration, and were administered with testosterone (Sigma-Aldrich, St. Louis, MO, USA) at a dose of 12.5 µg/kg/day (dissolved in sesame oil); group 4, high-fat-diet + castration + high dose testosterone group (n=10), which were fed a high-fat diet following castration, and received testosterone at a dose of 25 µg/kg/day. Castration was performed by removal of the testicles following anesthesia using 10% chloral hydrate (3.5 ml/kg; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) via intraperitoneal injection. All animals were fed for 6 weeks, and body weights were monitored weekly. The rats were sacrificed by cervical dislocation at the age of 10 weeks, and the thoracic aortas were obtained for subsequent experiments. All experimental protocols were approved by the Ethics Committee of the General Hospital of Tianjin Medical University (Tianjin, China).

Biochemical measurements. Prior to blood collection, the rats were maintained in a fasting state for 24 h. Blood samples (3-5 ml) were collected from an angular vein, followed by centrifugation at 1,680 x g and 4°C for 15 min. Subsequently, the levels of TC, high-density lipoprotein cholesterol (HDL-C), insulin, glucose and testosterone in the serum were determined using an Olympus AU400 Clinical Chemistry Analyzer (Olympus Corporation, Tokyo, Japan). Non-HDL cholesterol was defined as total cholesterol - HDL cholesterol. Serum glucose was determined using a glucose oxidase kit purchased from the China Institute of Atomic Energy (Beijing, China). Plasma lipids were measured using Hitachi 7170 automatic biochemistry analyzer (Hitachi, Tokyo, Japan). Homeostatic model assessment-insulin resistance (HOMA-IR) was calculated according to the following formula: HOMA-IR = fasting insulin (µU/ml) x fasting serum glucose (mmol/l) / 22.5.

Immunohistochemical staining. For immunohistochemical analyses, the paraffin-embedded sections (8 µm) were dewaxed for 10 min, followed by immersion in boiling citrate buffer solution (pH 6.4; Beijing Solarbio Science & Technology Co., Ltd.) for 10 min. Antigen retrieval was performed by microwave heating at a high heat for 10 min, a low heat for 10 min and cooled to room temperature. The sections were then immersed in distilled water in an orbital shaker for 5 min. Following antigen retrieval, the sections were incubated in 3% H2O2 solution (Beijing Solarbio Science & Technology Co., Ltd.) for 5 min at room temperature, and then blocked with 5% bovine serum albumin (Beijing Solarbio Science & Technology Co., Ltd.) for 1 h to avoid nonspecific staining. The sections were then incubated with the following primary antibodies: Polyclonal rabbit anti-rat IRS-1 (1:100; Abcam, Cambridge, UK; cat. no. ab131487), polyclonal rabbit anti-rat GLUT-4 (1:400; Abcam; cat. no. ab6542), polyclonal rabbit anti-rat AKT (1:800; Cell Signaling Technology, Inc.; Danvers, MA, USA; cat. no. 21926) and polyclonal rabbit anti-rat NF-κB (1:800; Cell Signaling Technology, Inc.; cat. no. 5970) at 4°C overnight. Subsequently, the sections were incubated with biotin-conjugated monoclonal goat anti-rabbit IgG (1:200; Vector Laboratories, Inc., Burlingame, CA, USA; cat. no. BA-1000) for 2 h at room temperature, and horseradish peroxidase (HRP) streptavidin (Vector laboratories, Inc.) for 1 h at room temperature. The images were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Spring, MD, USA).

TUNEL assay. The tissue sections (5 µm) obtained from the thoracic aortas were cut on a freezing microtome (Leica RM2255; Leica Microsystems, Wetzlar, Germany). The numbers and distribution of apoptotic cells within the aortic tissues were analyzed using an in situ Cell Death Detection kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s protocol. Subsequently, the sections were incubated with 40 µl reaction mixture containing terminal

| Gene       | Primer sequence (5′-3′)                     |
|------------|---------------------------------------------|
| NF-κB      | Forward: TGAGGCTGTGGTTGTTTGAGA             |
|            | Reverse: TTATGGCTAGGTTCTGTCTCTG            |
| TNF-α      | Forward: GCCCTCCTACATCGTATCCA              |
|            | Reverse: GCCTTGTTGTGTTGCTAGAC              |
| GLUT-4     | Forward: GTATGGTCCGAGATGCTATGG             |
|            | Reverse: CCTCTGGTTTTCAGGCCACTCT            |
| IRS-1      | Forward: GGCACCATCTCAAACATCT               |
|            | Reverse: GGGTCCCCACACCACACTG               |
| P3K        | Forward: CAAAGCCGAGAACCCTATGG              |
|            | Reverse: TGGACTTGGCCCATCTACAC              |
| β-actin    | Forward: CGTTGACATCCGTAAAGACC              |
|            | Reverse: AGAGCCACAAATCCACACA               |

NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; GLUT-4; glucose transporter type 4; IRS-1, insulin receptor substrate-1; P3K, phosphoinositide 3-kinase.
deoxynucleotidyltransferase (TdT) and digoxigenin-conjugated dUTP for 1 h at 37˚C. Subsequently, the sections were stained with DAPI (Roche Diagnostics GmbH, Mannheim, Germany) to stain the nuclei for 1 min at room temperature, followed by washing with phosphate-buffered saline. Finally, the TUNEL (Roche Diagnostics GmbH)-positive cells were observed under a fluorescent microscope (Olympus BX51TF; Olympus, Tokyo, Japan) at x200 magnification.

**Western blot analysis.** Protein extraction from the aortic tissues was performed using tissue lysis buffer (Solarbio, Beijing, China) containing 1% phenylmethanesulfonyl fluoride and protein was quantified using a BCA protein assay kit. The protein (25 µg) was separated on a 10% SDS-PAGE gel (Beijing Solarbio Science & Technology Co., Ltd.) and transferred onto polyvinylidene fluoride membranes (Merck Millipore, Darmstadt, Germany). Subsequently, the membrane was blocked with 5% nonfat dried milk for 2 h at room temperature, followed by washing with phosphate-buffered saline. Finally, the TUNEL (Roche Diagnostics GmbH)-positive cells were observed under a fluorescent microscope (Olympus BX51TF; Olympus, Tokyo, Japan) at x200 magnification.

Western blot analysis. Protein extraction from the aortic tissues was performed using tissue lysis buffer (Solarbio, Beijing, China) containing 1% phenylmethanesulfonyl fluoride and protein was quantified using a BCA protein assay kit. The protein (25 µg) was separated on a 10% SDS-PAGE gel (Beijing Solarbio Science & Technology Co., Ltd.) and transferred onto polyvinylidene fluoride membranes (Merck Millipore, Darmstadt, Germany). Subsequently, the membrane was blocked with 5% nonfat dried milk for 2 h at room temperature and then incubated with rabbit primary antibodies against NF-κB, TNF-α and polyclonal rabbit anti-rat β-actin (1:1,000; Cell Signaling Technology, Inc.; cat. no. 4967), GLUT4 and IRS1 (1:2,000; Abcam) and polyclonal rabbit anti-rat PI3K (1:1,000, Sigma-Aldrich; cat. no. 5295) overnight at 4˚C. The membrane was washed with 0.1% PBST three times for 5 min. Subsequently, the membrane was incubated with HRP-labeled goat anti-rabbit IgG (cat. no. GGHL-15P; ICL, Inc., Portland, OR, USA) secondary antibodies for 1 h at room temperature. The same membranes probed for β-actin served as an internal standard. The relative density of protein to β-actin was analyzed using ImageJ software (version 1.43; National Institutes of Health, Bethesda, MA, USA).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from the aortic tissues using TRizol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Synthesis of cDNA was performed using a TransScript First-Strand cDNA system (TransGen Biotech, Beijing, China), according to the manufacturer's protocol. The reaction mixture consisted of 10 µl of 2X TransStart Top Green qPCR SuperMIX, 0.4 µl forward primer, 0.4 µl reverse primer, 0.4 µl passive reference dye, 1.0 µl cDNA template and 20 µl ddH₂O. qPCR was performed using a TransScript Top Green qPCR SuperMix kit (TransGen Biotech) on a BioRad CFX96 qPCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the primers listed in Table I. The PCR amplification was conducted using the following conditions: 94˚C for 30 sec, followed by 40 cycles of 94˚C for 5 sec, 60˚C for 15 sec and 72˚C for 10 sec. The relative quantification of each gene was normalized to β-actin using the 2-ΔΔCq method (14).

![Figure 1. Serum levels of (A) TC, HDL and NHDL, (B) testosterone, (C) GLU, INS, HOMA-IR and E2 in rats. The data are presented as the mean ± standard deviation, based on three independent experiments. *P<0.05, compared with the control group; †P<0.05, group 3 compared with group 2, ‡P<0.05, group 4 compared with group 2. TC, total cholesterol; HDL, high density lipoprotein; NHDL, non-high density lipoprotein; GLU, glucose; INS, insulin; HOMA-IR, homeostatic model assessment-insulin resistance; E2, estradiol.](image)
Statistical analysis. Statistical analysis was performed using SPSS 17 software (SPSS, Inc., Chicago, IL, USA). All data are expressed as the mean ± standard error of the mean. The inter-group differences were analyzed using analysis of variance. Student’s t-test was performed to analyze the measurement data. P<0.05 was considered to indicate a statistically significant difference.

Results

Biochemical parameters. Compared with control (group 1), significant increases were found in the plasma levels of glucose, insulin, HOMA-IR, TC and non-HDL in group 2 (P<0.05; Fig. 1). A significant decrease was revealed in the levels of HDL and testosterone in the plasma of group 2, compared with the normal control (P<0.05). In the animals subjected to low dose testosterone replacement (group 3), significant reductions in plasma TC and non-HDL were observed, compared with group 2 (P<0.05). The plasma levels of glucose, insulin, TC and non-HDL were further increased following exposure to high dose testosterone (group 4), compared with group 2 (P<0.05).

In the scatter diagram of testosterone and the metabolism of the associated parameters, a continual decrease was identified in the plasma levels of glucose, insulin, HOMA-IR, TC and non-HDL as plasma testosterone increased in groups 1, 2 and 3, respectively (Fig. 2). However, an increasing trend was
noted in these parameters as plasma testosterone in group 4 increased. Plasma HDL increased as plasma testosterone in group 2 increased. Plasma HDL increased as plasma testosterone in group 4 increased.

**Morphological changes in thoracic aorta tissues.** As shown in Fig. 3, the inner elastic lamina and endothelial cells in the thoracic aorta tissues were intact in the control group, with no necrosis or mucoid degeneration (Fig. 3A). However, an
irregular lining, with partial endothelial cell loss, necrosis and mucoid degeneration was observed in group 2, together with wrinkling and deformation of the elastic lamina (Fig. 3B). In group 3, a significant reduction was observed in necrosis and mucoid degeneration, compared with group 2 (Fig. 3C). However, in the thoracic aorta tissues obtained from rats treated with high-dose testosterone replacement, severe necrosis and mucoid degeneration were noted, together with deformation of the elastic lamina and endothelium (Fig. 3D).

**Immunohistochemical staining.** Almost no staining of IRS-1 or GLUT-4 were detected in group 2 in the immunohistochemical analysis. However, the expression levels of IRS-1 and GLUT-4 were marked in group 3. Of note, the expression of IRS-1 in group 3 was significantly higher, compared with that in the normal group. In group 4, the lowest levels of IRS-1 and GLUT-4 were detected, as shown in Fig. 4. Castration in combination with a high-fat diet contributed to increases in the expression levels of TNF-α and NF-κB. In addition, treatment with a high dose of testosterone further increased the expression levels of TNF-α and NF-κB. In the castration + low-dose testosterone group, there was a decrease in TNF-α and NF-κB staining, compared with the castration + high-fat diet group.

**TUNEL staining.** The results of the TUNEL staining are shown in Fig. 5. Compared with group 1, a significant increase was observed in cell death in group 2. In addition, a significant decrease in cell death was observed in group 3, compared with group 2. By contrast, a significant increase in cell death was observed in group 4, compared with group 2. This indicated that testosterone at a physiological dose attenuated cellular apoptosis, whereas a high dose of testosterone aggravated the apoptosis.

**Western blot analysis.** As shown in Fig. 6, the protein levels of IRS-1, AKT and GLUT-4 in group 2 were lower, compared with the levels in group 1. Following exposure to low-dose testosterone, the protein expression levels of IRS-1, AKT and GLUT-4 in group 3 were markedly increased, compared with group 2. However, their levels of expression remained lower, compared with the normal control (group 1). By contrast, exposure to a high dose of testosterone resulted in significant decreases in the levels of IRS-1, AKT and GLUT-4 in group 4, compared with those
Figure 6. Low-dose testosterone upregulates the expression levels of IRS-1, AKT, GLUT-4 protein, NF-κB, TNF-α and PI3K, compared with those of animals on a high-fat diet following castration. High doses of testosterone resulted in a significant decrease in the levels of IRS-1, AKT, GLUT-4, NF-κB, TNF-α and PI3K, compared with animals fed a high-fat diet following castration. Error bars represent the standard deviation. *P<0.05, group 2 compared with group 1; #P<0.05, group 3 compared with group 2; ∆P<0.05, group 4 compared with group 2. Group 1, control; group 2, high-fat-diet + castration; group 3; high-fat-diet + castration + low-dose testosterone group 4, high-fat-diet + castration + high-dose testosterone; IRS-1, insulin receptor substrate-1; GLUT-4, glucose transporter type 4; TNF-α tumor necrosis factor-α; NF-κB, nuclear factor-κB; PI3K, phosphoinositide 3-kinase.

Figure 7. mRNA levels of (A) NF-κB, TNF-α and PI3K, and (B) IRS-1, AKT and GLUT-4. Compared with the rats subjected to castration and fed a high-fat diet, low-dose testosterone induced upregulation of the mRNA levels of IRS-1, AKT and GLUT-4, and downregulation of the mRNA levels of NF-κB, TNF-α and PI3K. However, high-dose testosterone resulted in a significant decrease in the levels of IRS-1, AKT and GLUT-4, and a significant increase in the mRNA levels of NF-κB, TNF-α and PI3K, compared with the low dose group. Error bars represent the standard deviation. *P<0.05, group 2 compared with group 1; #P<0.05, group 3 compared with group 2; ∆P<0.05, group 4 compared with group 2. Group 1, control; group 2, high-fat-diet + castration; group 3; high-fat-diet + castration + low-dose testosterone group 4, high-fat-diet + castration + high-dose testosterone; IRS-1, insulin receptor substrate-1; GLUT-4, glucose transporter type 4; TNF-α tumor necrosis factor-α; NF-κB, nuclear factor-κB; PI3K, phosphoinositide 3-kinase.
in group 2. In addition, significant upregulation in the levels of NF-κB, TNF-α and PI3K were observed in group 2, compared with the normal control. High-dose testosterone caused further increases in the levels of NF-κB, TNF-α and PI3K, compared with those in group 2, and a low dose of testosterone caused decreases in the levels of NF-κB, TNF-α and PI3K in group 3, compared with group 2. However, the levels of NF-κB and PI3K in group 3 remained higher than in the normal control.

RT-qPCR. As shown in Fig. 7, the mRNA expression levels of IRS-1, AKT and GLUT-4 in group 2 were lower than those in the normal control. Following exposure to low-dose testosterone, the mRNA expression levels of IRS-1, AKT and GLUT-4 in group 3 were markedly increased, compared with those in group 2. However, the mRNA levels of IRS-1, AKT and GLUT-4 in group 3 remained lower than those in the normal control group. Exposure to a high dose of testosterone resulted in significant decreases in the mRNA levels of IRS-1, AKT and GLUT-4 in group 4, compared with group 3. Furthermore, upregulation in the mRNA levels of NF-κB, TNF-α and PI3K were observed in group 2, compared with the normal control. A further increase was observed in the mRNA expression levels of NF-κB, TNF-α and PI3K following exposure to high-dose testosterone, compared with group 2. Whereas, low-dose testosterone treatment caused a decrease in the mRNA levels of NF-κB, TNF-α and PI3K mRNA in group 3. This indicated that the loss of testosterone induced disorder of the PI3K signaling pathway, and contributed to inflammation. A low dose of testosterone may alleviate disorders of PI3K and inflammation, whereas, a high dose of testosterone may aggravate insulin resistance and inflammation.

Discussion

Testosterone, the most abundant androgen in males, is synthesized and secreted by Leydig cells and regulated by pituitary-derived luteinizing hormone (15). To date, testosterone has been used in clinical practice due to its biological properties in the maintenance of normal sexual differentiation, puberty development and anabolism (16). In the present study, the roles of testosterone in glucolipid metabolism and cardiovascular diseases were investigated.

In the early stage of puberty, testosterone is regularly secreted by the testis under the stimulation of luteinizing hormone. The association between endogenous androgen and lipids is complex as it involves various factors, including as age, gender, body mass index and waist-hip ratio. For example, Botella-Carretero et al found that the plasma levels of testosterone of obese patients were higher following bariatric surgery, compared with baseline levels (17). In addition, a linear trend towards lower TC, low triglycerides and higher HDL-C have been reported with the increase of serum testosterone (18). However, other studies have shown that testosterone replacement induces a decrease in plasma HDL and an increase in TC (19,20). In the present study, testosterone was negatively correlated with TC and non-HDL, and was positively correlated with HDL. Adverse effects were observe from the supraphysiological testosterone concentration on plasma lipids. On this basis, an appropriate dose of testosterone is required in clinical practice.

Previously, testosterone was considered to be associated with the occurrence of coronary heart disease (21). However, recent data has revealed that testosterone is negatively correlated with the occurrence of coronary heart disease (22). In addition, testosterone is important in anti-atherosclerosis. It is known that the vascular endothelium is the barrier between circulating blood and vascular smooth muscle, and it is the major component of endocrine organs. It has been reported to be involved in the synthesis and secretion of several vasoactive substances associated with the regulation of vasomotor function. Testosterone can promote the secretion of nitric oxide from vascular endothelial cells, which affects vasomotor function (23). The results of the present study revealed that testosterone alleviated injury to the morphology of the thoracic aorta induced by hypoadrogenism.

Increasing attention has been focussed on the roles of insulin resistance in metabolic syndrome (22). For example, epidemiological data indicates that the incidence of type 2 diabetes in males is higher than in females, which may be affected by endogenous hormones (24). Increasing evidence has revealed that hypotestosteronemia is associated with increased risks of developing metabolic syndrome and diabetes, as well as insulin resistance (25). Previous studies have shown that testosterone replacement can ameliorate the pathological components of metabolism syndrome, however, adverse effects may occur in the presence of excessive administration (26,27). To investigate the potential mechanism underlying how testosterone is involved in insulin resistance, the present study investigated the activity of PI3K/AKT, a major component in the insulin signaling pathway for glucose transport. In the present study, rats subjected to castration exhibited upregulated expression of PI3K and downregulated expression levels of IRS-1, GLUT-4 and AKT. In the animals treated with testosterone, the levels of IRS-1, GLUT-4 and AKT were upregulated, together with downregulation in the levels of NF-κB and TNF-α. On this basis, the present study hypothesized that testosterone may regulate glucolipid metabolism through modulation of the PI3K signaling pathway.

In conclusion, the present study demonstrated that castration induced marked disorder of glucolipid metabolism and vascular injuries in male pubescent rats. Low-dose testosterone replacement treatment ameliorated the damage caused by castration via the PI3K/AKT signaling pathway. However, high-dose testosterone induced severe adverse effects, indicating an appropriate dose of testosterone is necessary. Testosterone deficiency and overdose induced disorder of glucolipid metabolism and vascular injuries in male pubescent rats. Thus, the dosage of testosterone used must be appropriate for the treatment of patients with testosterone deficiency and testosterone overdose should be avoided.

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