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Effect of testosterone on the mRNA expression of Wnt-2 and dickkopf1 (DKK1), collagen deposition and oxidative stress in the cardiac tissue in male rats

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ABSTRACT

Some studies suggest that misuse of androgenic-anabolic steroids may increase the risk of cardiovascular diseases in males. This study explored the effects of testosterone enanthate (TE) on the total antioxidant capacity (TAC) and malondialdehyde (MDA) levels as biomarkers of oxidative stress in the cardiac tissue of rats that were treated with TE. Also, we evaluated the levels of collagen deposition as a marker for cardiac fibrosis and the mRNA expression of the *Wnt-2 and dickkopf1 (DKK1)* as potential factors that may be involved in the increase of collagen deposition. In this study, 21 male Wistar rats were divided into three groups (n=7): CO: controls; T-T: normal rats that were treated with 25 mg/kg/day TE for 2 weeks and served as an androgen abuse model; V-T: these animals were treated with the sesame oil as a solvent of TE. At the end of treatment, the relative mRNA expression of *Wnt-2 and DKK1* in the ventricular tissue was determined by q-RT-PCR. The degree of collagen deposition in the myocardial tissue was evaluated by Masson's trichrome staining. Results showed that the mRNA expression of *DKK1* was down-regulated following excess androgen exposure (p=0.009) but *Wnt-2* mRNA expression wasn't affected (p=0.069). Increased collagen deposition was observed in the T-T group (p=0.000). The levels of MDA and TAC in heart tissue weren't altered significantly (p>0.05). These results suggest that the raised collagen deposition by exogenous testosterone may be mediated, at least in part, by the reduction of expression of *DKK1* mRNA. These findings may explain some structural alterations in the heart of some androgens abusers.

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Introduction

The unauthorized use and misuse of androgenic-anabolic steroids (AAS) in adolescents and young adults has become an important public health challenge worldwide and recently, using androgens to improve physical function has become common. Also, it is used as an anti-aging agent (1). It has been estimated that 6.4% of men and 1.6% of women use testosterone enanthate and testosterone propionate as performance-enhancing drugs (2).

In spite of the beneficial effects of testosterone, some studies reported that testosterone misuse is associated with the occurrence of premature cardiovascular events and an increase in cardiovascular complications such as myocardial hypertrophy, myocardial infarction, myocardial fibrosis, and myocardial apoptotic death (1, 3, 4). Levels higher than physiological values of testosterone and other androgens may increase the risk of cardiovascular diseases (CVD) and these effects are probably through changes in homeostatic mechanisms, changes in lipid profile, activation of proinflammatory and redox processes, reduction of nitric oxide bioavailability and effect on vasoconstrictor signalling pathways (5).

It has been indicated that testosterone may have pro-oxidant properties and high levels of testosterone may induce oxidative stress by disrupting the balance between reactive oxygen species (ROS) production and antioxidant defence mechanisms (6), another study reported that testosterone has protective effects against oxidative (7). But, the effects of steroids on oxidative stress parameters and antioxidant defence systems in heart tissue are not clear.

Unauthorized use of anabolic androgenic steroids increases the plasma concentrations of androgens, which may have direct toxic effects on the myocardium through androgen receptors in myocytes (8, 9). The results of some studies suggest that abusing anabolic steroids may increase the risk of CVD (10, 11). Also, several studies suggested that there is an association between long-term use of anabolic androgenic steroids and cardiac dysfunction including left ventricular systolic and diastolic dysfunction and increased left ventricular mass (12-14). Also, clinical and legal cases have reported myocardial fibrosis among users of AASs (15-18) and it has also been reported that AAS may alter collagen synthesis and its concentration in the myocardium (19). One study showed that after testosterone administration, myocardial hypertrophy was associated with increased androgen receptor expression (20). Although testosterone has numerous effects on myocytes and skeletal muscle myocytes, information on the molecular mechanisms by which testosterone affects cardiorespiratory parameters is very limited and unclear (21). Cardiac fibrosis refers to different processes such as fibroblast proliferation, collagen synthesis, and conversion of fibroblasts into a contractile "myofibroblast" phe-

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notype. Due to excessive deposition of the extracellular matrix, cardiac fibrosis is associated with interruption of the normal myocardial structure. It seems that myocardial matrix remodeling and fibrosis have axial roles in the development of ventricular dilatation and heart failure. Also, an increase in cardiomyocyte size and fibrosis is seen in cardiac hypertrophy (22-24). The process of fibrosis is a significant contributor to cardiovascular dysfunction and diastolic heart failure, so the increased collagen deposits are associated with cardiac dysfunction and under these conditions, the cell-cell pairing and membrane potential depolarization is disrupted (25, 26). The Wnt signaling pathway plays a vital role in some pathophysiological processes such as tumour formation and fibrosis and regulates the fibrosis process in various organs and it is closely related to the process of cell proliferation and differentiation (27). While under normal conditions, the canonical Wnt signaling is muted, its activation is observed in pathological conditions such as hypertrophy and ischemia (28). The secretory glycoproteins dickkopf 1 (Dkk1), a member of the Dkk family, is an antagonist of the canonical Wnt/β-catenin signalling pathway and plays different roles in physiological and pathological processes (29). Some studies have suggested the effects of testosterone on myocardial hypertrophy and stiffness (30, 31) and the involvement of the Wnt signaling pathway in the fibrosis and left ventricular regeneration (24, 32).

The pathophysiological mechanisms responsible for the effects of AAS misuse are not well understood but the effects of exogenous testosterone abuse on myocardial regeneration and worsening of cardiac dysfunction after myocardial infarction have been shown in both males and females (21). This study was designed to investigate the effect of testosterone enanthate (TE) on the malondialdehyde (MDA) and total antioxidant capacity (TAC) as two indirect indices of oxidative stress in heart tissue of male rats. Also, we evaluate the levels of collagen deposition as a marker for cardiac fibrosis and the expression of the genes *Wnt-2* and *DKK1* as the genes may be involved in the induction of collagen deposition in the cardiac tissue of normal male rats.

Materials and Methods

Chemicals

TE was prepared from Aburaihan, Iran. The RNA extraction kit and oligo (dT) primers were prepared from Denazist, Iran. RT-Enzyme was purchased from Thermo Scientific, USA. DNase enzyme was the product of Ferementase, USA. SYBR premix Ex Taq TM II was prepared by Biobasic, Canada. The other reagents used were of analytical grade. The Trichloroacetic acid (TCA), thiobarbituric acid, and butylated hydroxytoluene (BHT) were prepared from Sigma-Aldrich.

Animals and experimental design

This experimental study was performed on twenty-one male Wistar rats (200-220 g). The experimental protocols were approved by the Institutional Animal Care and Ethical Committee of Biological Sciences of Razi University (Approval ID: IR.RAZI.REC.1399.018).

Animals were housed under standard conditions with free access to tap water and standard rat pellet *ad libitume*. After 2 weeks of acclimatization, they were randomly divided into three groups (n=7) as follows: I) The CO group (controls). These animals had no treatment; II) The T-T group (testosterone-treated group). These animals were treated with TE 25 mg/kg (33) dissolved in sesame oil for 14 days. TE was injected subcutaneously; III) The V-T group (vehicle-treated group). These animals were treated with 0.1 ml sesame oil as the solvent of TE, for 14 days subcutaneously. All the injections were performed at 8:00 AM once a day.

After treatments, the blood samples (5mL) were collected from the hearts of fasted rats by cardiac puncture. The left ventricle of each rat was cut perpendicular to the apex-to-base axis and a sample from this tissue was frozen in liquid nitrogen and kept at -80 °C. 0.4 g from this tissue was homogenized separately in 0.1 mol Tris-HCl buffer, pH 7.4 using a homogenizer at 4°C. The homogenate was centrifuged for 15 min (at 9000 rpm). Then the supernatant was stored at-20°C for analysis of MDA as a marker for lipid peroxidation and TAC by a colorimetrically method.

Also, a sample from this tissue (the left ventricle) was fixed with paraformaldehyde 10% and embedded in paraffin and serial sections (5 µm) were prepared and stained using Masson's trichrome staining (34) using a Trichrome (Masson's) Stain Kit (Asia Pajhohes, Iran). The percentage of collagen deposition was determined by using Image J software (https://imagej.nih.gov/ij/docs/examples/stained-sections/index.html).

Hormonal assays

The serum concentrations of testosterone were determined by a rat testosterone ELISA Kit (Cusabio, Wuhan, China) based on the principle described by Tietz (35). The detection wavelength for reading the absorbance of samples was 450 nm and its sensitivity was 0.06 ng/mL.

The qRT-PCR analysis

Total RNA was extracted from the left ventricular tissues. The cDNA was synthesized by using total RNA, oligo (dT) primers and RT-Enzyme. The expression of *Wnt-2* and *DKK1* mRNA was measured by using the qRT-PCR method using a Corbett Research RG 3000 thermal cycler (CR CORBETT, Australia). For each reaction, 2µl cDNA, 3 µl water, and 6µl 2 X SYBR Green Master mixes (SYBR premix Ex Taq TM II) and primer pairs at 5 pM concentrations in a final volume of 12µl were mixed. The characteristics of primers are presented in Table 1. The reactions were initiated by denaturation at 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 60 °C for 30 s. A melting curve was produced (95 °C for 15 s, 60 °C for 1min, 95 °C for 15 s). The cycle threshold (Ct) was determined for each sample. The expression of the *wnt-2* and *DKK1* genes was normalized to GAPDH using the $2^{-\Delta Ct}$ method (36).

Assay of MDA and TAC in cardiac tissues

In order to estimation of MDA levels, the BHT (7.2%) was dissolved in ethanol (95%)and TCA (1.4M) was prepared in deionized distilled water and the TBA-TCA solution was prepared.100 µl from the supernatant prepared from the mentioned homogenate was mixed with 100 µl of the TCA and 100 µl TBA solution (20 mM) and

vortexed and heated in a 90 °C water bath for 15 min, Then it was cooled in a cold water bath for 10 min, and centrifuged at 2,000· g for 15 min. The absorbance of the

Table 1. The primers used in the qRT-PCR analysis.

Gene	Gene ID	Primer	Sequence	Product length (bp)
GAPDH	XM_039107008.1	Sense Antisense	5'-AAGTTCAACGGCACAGTCAAGG-3' 5'-CATACTCAGCACCAGCATCACC-3'	121
Wnt-2	XM_575397.8	Sense Antisense	5'-TTGGTTGGAATGTGGAAGTGTG-3' 5'-GAAGGTGGTAAAGGGTAAGAG-3'	173
DKK1	NM_001106350.1	Sense Antisense	5'-GGCTCTCTTCAGCCTCCCTCC-3' 5'-TGCCGCACTCCTCATCCTCC-3'	224

supernatant was measured at 531 nm (37)

The TAC in ventricular tissue was identified using a commercial kit (ZellBio GmbH, Wurttemberg, Germany) according to the manufacturer's instructions by a colorimetrically method at 490 nm. The intra- and inter-assay coefficients of variation were <3.4% and <4.2% respectively. The sensitivity of the kit was 0.1m M. The TAC levels were measured as the amount of antioxidants in the samples that were compared with ascorbic acid which was considered as a standard. The detection range was 0.125-2 mM.

Statistical Analysis

Data were shown as mean±SEM and analysed by one-way ANOVA followed by the Post hoc Tukey's test using SPSS (version 16.0). The *p-values* <0.05 were considered to show statistical significance.

Results

The measurement of testosterone and MDA and TAC levels

The results showed that the concentrations of testosterone significantly increased in the T-T (testosterone-treated) group (16.92 ± 0.36) when compared to the CO (control) group (2.60 ± 0.24) (p = 0.000) and the V-T (vehicle-treated) group (2.360 ± 0.2249) (p = 0.000). The testosterone level in the V-T group (2.360 ± 0.2249) wasn't significantly different from the CO group (p = 0.820) (Figure 1).

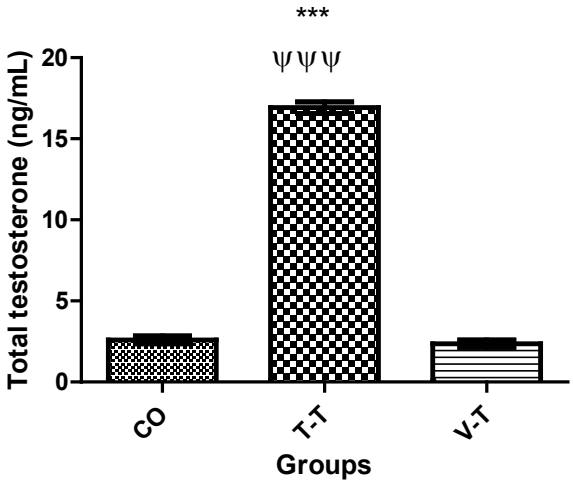

Figure 1. The total testosterone concentrations; Mean±SEM (N=7). One-way ANOVA followed by the Tukey's *post hoc* test. *P values* less than 0.05 were considered statistically significant. ***: $P < 0.001$ *vs.* the Control group; ΨΨΨ: $P < 0.001$ vs. the V-T group. The CO: control; T-T: testosterone-treated group; V-T: vehicle-treated group.

As is shown in Table 2, the mean of MDA levels in the T-T group wasn't significantly different from the CO group (p = 0.391). The MDA levels in the V-T group weren't significantly different from the CO group and T-T group (p = 0.851 and p = 0.700, respectively) (Table 2).

Also, the mean of TAC levels in the T-T group wasn't significantly different from the CO group (p = 0.499). The TAC levels in the V-T group weren't significantly different from the CO group and T-T group (p = 0.140 and p = 0.648, respectively) (Table 2).

Evaluation of collagen deposition

The percentage of collagen deposition was significantly higher in the T-T group (28.10 ± 2.48) in comparison with both CO (5.27 ± 1.43) and V-T (4.97 ± 1.33) groups (p = 0.000 and p = 0.000, respectively). The percentage of collagen deposition in the V-T group wasn't significantly different from the CO group (p = 0.993) (Figure 2).

The qRT-PCR analysis

The ventricular *wnt-2* mRNA expression in the T-T group (0.09168 ± 0.02934) wasn't significantly different from the CO group (0.03011 ± 0.006229) (p = 0.069). The *wnt-2* mRNA expression in the V-T group (0.03011 ± 0.004911) wasn't significantly different from the CO group and T-T group (p = 1.000 and p = 0.070, respectively) (Figure 3a).

In addition, the relative *DKK1* mRNA expression in the ventricular tissue of the T-T group (0.02754 ± 0.006308) was significantly decreased in comparison with the control group (0.06352 ± 0.004016)) (p = 0.001).

The relative *DKK1*mRNA expression in the T-T group was significantly different from the V-T group (0.05012 ± 0.005799) (p= 0.31). The relative *DKK1* mRNA expression in the V-T group wasn't significantly different from

Table 2. Effect of testosterone enanthate on the MDA and TAC levels as oxidative stress parameters (Mean±SEM, n=5). *

Parameter	Control	T-T	V-T
MDA (nmol/100g tissue)	20.20±0.8602	18.20±0.8602	19.40±1.327
TAC (mM)	0.79±0.04	0.73±0.01	0.68±0.05

* TAC amount was considered as the amount of antioxidant in the sample that was compared with ascorbic acid that acts as a standard. CO: control; T-T: testosterone-treated group; V-T: vehicle-treated group.

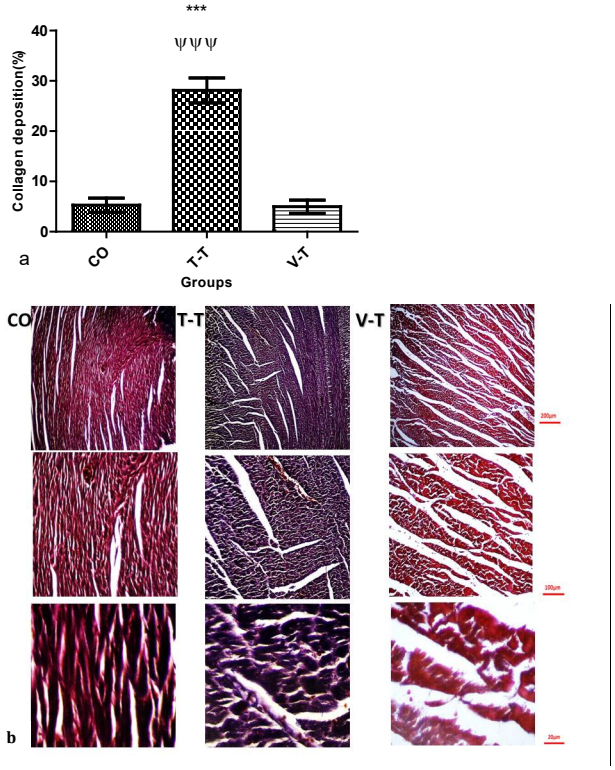

Figure 2. The degree of collagen deposition in the myocardial tissue was analyzed by the Image J software (Masson's trichrome staining); Means±SEM (N=3) (a). The myocardium of testosterone enanthate-treated rats has a maximal amount of blue-stained fibrous connective tissue lying (b). *P values* less than 0.05 were considered statistically significant. ***: $P < 0.001$ *vs.* the CO group; ΨΨΨ: $P < 0.001$ *vs.* the V-T group. The CO: control; T-T: testosterone-treated group; V-T: vehicle-treated group.

the CO group (p = 0.233) (Figure 3b).

Discussion

In the present study, the effects of TE on the TAC and MDA levels as two biomarkers of oxidative stress in the ventricular tissue of rats were determined. One strategy to evaluate a free radical-antioxidant balance in biological systems is the measurement of the TAC (38).

It is possible that oxidative stress via interference with mitochondrial function and increasing the mitochondrial permeability, disturbing the calcium homeostasis, induction of membrane lipid peroxidation, damage to DNA and activation of mitogen or stress-activated protein kinase (MAPK, SAPK), damage to oxidation-sensitive proteins such as kinases, transcription factors, metabolic enzymes, and induction of apoptosis cause disruption in cell signalling and impair the cardiac function (39). Our results showed that treatment with a dose of 25mg/kg TE did not significantly change the levels of MDA and TAC in heart tissue. In spite of our results, previous studies have reported the negative effects of anabolic steroids on oxidative stress responses in the liver and heart of rats (13, 40). However, one study showed that testosterone deficiency induced by the aging process or surgical castration declined the antioxidant status in the heart left ventricle samples and caused the elevation in the inflammatory parameters by the modulation of the heme oxygenase enzyme system. This enzyme is an important enzyme in the regulation of cellular oxidative stress and induction of heme oxygenase -1 can increase the antioxidant capacity of cells against oxidative stress (41).

Due to antioxidative efects of sex hormones, a reduction in the testosterone level may causes the oxidative stress (42). Also, one study by Pansarasan in gastrocnemius muscle tissue showed that treating rats with 5 mg/kg i.m, 6 days/week testosterone propionate increased mitochondrial superoxide dismutase compared to the control group and decreased cytochrome oxidase activity and testosterone increased basal lipid peroxidation and they concluded that administration of testosterone to untrained rats may decrease mitochondrial function and increase lipid peroxidation in muscle (43). Our findings weren't in line with their results. This difference may be due to the fact that these parameters have been investigated in different tissues because some studies have shown that the effect of testosterone on oxidative stress in different tissues depends on the type of tissue studied. For example, one study showed that testosterone has antioxidant effects on the nervous system of rats(7). More studies with higher doses of TE as well as other parameters of oxidative stress

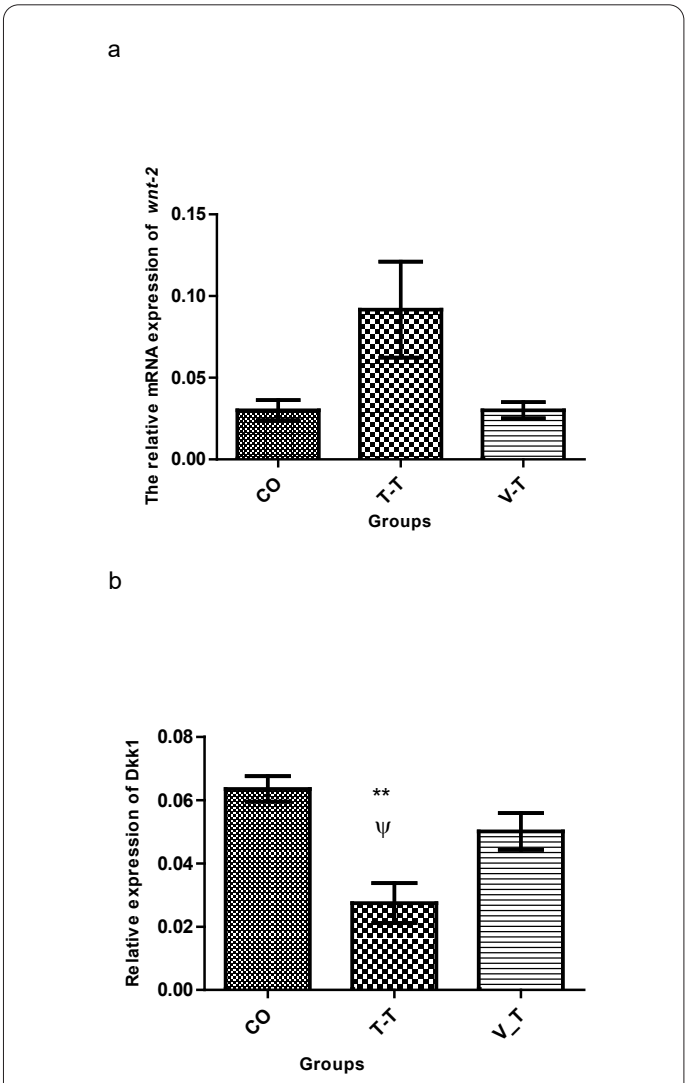

Figure 3. The relative mRNA expression of *wnt-2* (a) and *dickkopf1 (DKK1)* (b) genes normalized to GAPDH; Mean±SEM (N=5). *P values* less than 0.05 were considered statistically significant. **: $P < 0.01$ *vs.* the CO group; Ψ: $P < 0.05$ *vs.* the V-T group. The CO: control; T-T: testosterone-treated group; V-T: vehicle-treated group.

such as the levels of antioxidants enzymes (SOD and glutathione peroxidase) are necessary to determine the effect of TE on oxidative stress in the heart.

The current study showed that the collagen deposition level as a marker for fibrosis in the ventricular tissue of the T-T group was significantly increased. The effects of AAS on collagen synthesis and its concentration in the myocardium of the dog heart have been reported in a previous study by Takla et al, where they reported that methandienone has further enhanced exercise-stimulated increase in collagen concentrations in the dog heart (19). Our results are in accordance with their results. Under pathological conditions, the abnormal activation of cardiac fibroblasts by overproduction of extracellular matrix proteins causes scar tissue that replaces healthy myocardium and induces stiffening of the heart, and adverse tissue remodeling that may be associated with different forms of cardiac diseases (44). Our findings about increased collagen deposition in the T-T group are consistent with findings of an in vitro study that showed treatment with testosterone increased the collagen production in the fibroblasts by up to 20% (45).

Our results are in line with previous studies that showed cardiac tissue undergoes some structural alterations such as myocardial hypertrophy and myocardial stiffness and fibrosis following androgen abuse (46, 47). Also, one study by Melik et al showed that treating male rats with a dose of 100mg/100g testosterone for 14 and 90 days induced changes in the myocardial tissue and caused misshapen nuclei, disorganized myocardial fibers and leukocytic infiltrates(9). Here, an increase in the deposition of collagen in the ventricular tissue following treatment with TE suggests that the administration of testosterone may expose subjects to the risk of cardiac fibrosis. In this regard, one study found that in the subjects in them, the anabolic androgenic steroids use tests were positive at least once, the risk of cardiovascular disease was two to three times higher than the individuals whose all doping tests were negative (47). In spite of our findings, one study showed that castration of male rats reduced the contractility of isolated myocytes and increased the accumulation of collagen fibers around blood vessels in the heart. Also, the percentage of fibrotic tissue in the heart of these animals was high (48). It seems that their findings are in spite of our findings. This evidence shows that cardiac tissue may be directly affected by the alteration of testosterone levels because of its androgen receptors (8, 9).

Our findings showed that the relative mRNA expression of *wnt-2* in the cardiac tissue wasn't altered following treatment with TE. Also, our results showed that treatment with TE decreased the relative expression of *DKK1* mRNA which suggests that testosterone administration may affect the Wnt signaling in cardiomyocytes by down-regulation of *DKK1* mRNA expression. DKK1 is an endogenous inhibitor of Wnt/β-catenin signaling and can suppress the canonical signaling of wnt ligands by connecting to the LRP6 or Wnt co-receptor as well as by induction of endocytosis of the ternary Dkk1–LRP5/6–Kremen1 complex (49).

Our findings suggest that treatment with TE can induce cardiac fibrosis in male rats through an increase in collagen deposition, but a reversal of this fibrosis after stopping TE injection was not determined and more studies in this regard are needed because some studies have reported that collagen turnover in the extracellular matrix can reverse the fibrosis (26, 50). So, more studies are necessary to clear if stopping using testosterone cause reverses the observed elevation in collagen deposition or not. The Wnt signalling in cardiac tissue is complex and canonical wnt signalling can act as a master contributor to gene expression in cardiac tissue and other organs (26). Irregularity in the wnt may be involved in susceptivity to heart diseases such as hypertrophy, fibrosis, and ischemia (46). Specifically, the DKK1 levels can affect the expression of collagen I (26). However, testosterone can exert its effects on collagen deposition levels through other mechanisms and pathways such as TGF-β1/Smad signaling or/and alteration of the balance between matrix metalloproteinases and tissue inhibitors of metalloproteinases as well as the expression of different types of collagen (51, 52) and more research is needed. It seems that abnormal concentrations of testosterone can affect collagen deposition and fibrosis in the heart and maintenance of testosterone levels in the normal physiological range is important to prevent the development of structural changes in the heart tissue. Our results showed that the effects of TE in the raise of collagen deposition may be mediated, at least in part, by the reduction of expression of *DKK1* mRNA. Similarly, previous studies showed that chronic administration of AAS can affect the level of mRNA expression of catalase and the expression of ATP-sensitive potassium channel (K_{ATP} channel) subunits in male Wistar rat hearts (53, 54). More analyses are necessary for the evaluation of the level of mRNA and protein expression of the other molecules that are involved in the collagen deposition such as the genes specific to fibrosis (Tgf-β, Il-1β, Pdgfb), matrix (Acta2, Col1a2, Col3a1, Lox, Itgb1/6, Itga2/3) and members of the TGF-β superfamily (55).

Overall, the results obtained from the present study suggest that gene and collagen expression changes can be seen in the ventricular tissue of testosterone-treated rats. More studies are needed to investigate the cardiovascular effects of various AAS especially the long-term effects of their use.

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