

The Effect of Resistance Training with Boldenone Consumption, Ziziphus jujuba Extract and Gallic Acid on Annexin-5 and Caspase-3 Genes Expression in Cardiac Tissue of Male Wistar Rats

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Received: 10 October 2015

Accepted: 28 December 2015

Published online: 1 February 2016

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Competing interests: The authors declare that no competing interests exist.

Citation: Abbassi Daloi A. The effect of resistance training with boldenone consumption, Ziziphus jujuba extract and gallic acid on annexin-5 and caspase-3 genes expression in cardiac tissue of male Wistar rats. Rep Health Care 2016; 2 (1): 55- 65.

Abstract

Introduction: Herbal medicines have gained growing popularity and have been used worldwide as alternative and complementary medicine and food supplements. The aim of this study was to investigate the effect of resistance training with boldenone consumption, ziziphus jujuba extract and Gallic acid on annexin-5 and caspase-3 genes expression in cardiac tissue of male Wistar rats.

Methods: In this experimental study, 42 male Wistar rats aged 8-12 weeks with mean weight of 195 ± 7.94 g were randomly divided into seven groups: control, boldenone, jujube extract + boldenone, Gallic acid + boldenone, resistance training + boldenone, resistance training + jujube extract + boldenone, resistance training + Gallic acid + boldenone. Resistance training program included six weeks of climbing a ladder for 5 sessions, 3 sets, 5 repetitions and one-minute resting between sets with 50% 1RM in the first week and 100% 1RM in the final week. The drug was injected in the hamstring in depth once a week on an appointed day. After anesthesia, the autopsy was performed and cardiac tissue was dissected. Data were analyzed using paired sample t-test, One-way ANOVA and Scheffe post hoc at the $P < 0.05$.

Results: The findings demonstrated that there was a difference for the mean Annexin-5 gene expression in cardiac tissue among the groups ($P=0.002$). Annexin-5 gene expression significantly was reduced in jujube extract-boldenone group ($P=0.01$) and Gallic acid- boldenone group ($P=0.007$). In addition, there was a difference for the mean caspase-3 gene expression in cardiac tissue among the groups ($P=0.001$). Caspase-3 gene expression significantly showed reduction in Jujube extract- boldenone group ($P=0.003$) and resistance training- Gallic acid-boldenone group ($P=0.018$) compared to boldenone group.

Conclusion: According to the present findings, it seems that boldenone causes apoptosis in cardiac tissue, and probably resistance training with Jujube extract and Gallic acid could reduce the cardiac apoptosis.

Keywords: Boldenone, Ziziphus jujuba, Gallic Acid, Apoptosis, Resistance Training, Heart

Introduction

Testosterone and respective synthetic derivatives are widely used by athletes to increase muscle mass and improve physical performance. The administration of endogenous anabolic androgenic steroids induces cardiac hypertrophy, in vitro and in vivo (1, 2). Investigations have indicated that long-term use of high doses of anabolic

androgenic steroids can lead to an increase in interventricular septum thickness, cardiomyopathy, arrhythmia, heart failure and sudden cardiac death in power athletes (3- 5). Boldenone is a 1-dehydro derivative of testosterone, which its consumption has been banned due to its unfavorable effects on the human body (6); this steroid is used directly to build muscle. However, there is little

information about its adverse effects on various organs including cardiac tissue apoptosis. In recent studies, the identification of molecular mechanisms of cardiovascular protection profile has attracted more attention following the sporting activities. The potentially possible factors evaluated in previous studies on the myocardium include antioxidant defense (7), increased angiogenesis (8) and desired state of inflammation (9). Evidence suggests that exercise is associated with modifiable modulation of programmed cell death (apoptosis) of cardiac myocytes (10- 12). Apoptosis is a form of cell death that causes the loss of non-functional, abnormal or damaged cells as well as harmful cells. However, excessive apoptosis (for example, in pathological disease and in the use of testosterone and its derivatives) can lead to abnormal changes in the structure and function of the heart (13). More recently, the relationship among apoptosis with high doses of androgenic anabolic steroids and exercise has been evaluated experimentally in animal models. In this regard, Shokri *et al.* (14) reported a significant increase in apoptosis after administration of nandrolone and improvements following physical activity. Scientific studies indicate the effective role of Caspase-3 and Annexin-5 as pre-apoptotic agents in unstable reduction in heart function and myofibril damage (15). However, it is still unclear the confounding role of exercise training on the magnitude of changes in the activity of Caspase-3 and Annexin-5. Activation of Caspase-3 during apoptosis is associated with the expression of phosphatidylserine in apoptotic cells (16). Annexin-5 is a cytosolic protein and is involved in many physiological functions, including stabilization of membranous phospholipids, regulation of blood clotting and cell apoptosis or programmed cell death (17). The Annexin-5 has a great affinity for binding to phosphatidylserine and is used in the detection of apoptosis, in vitro and in vivo (18,

19). Sports and exercise depending on the type and intensity can make a positive impact on the physiology and morphology of cardiac tissue, including myocardial contractility, elevation in the left ventricular cavity size, increased wall thickness and improved heart mass, which is known as athlete's heart (20). Barauna *et al.* (21) reported that resistance training for four weeks causes cardiac hypertrophy without changes in heart function. Doustar *et al.* (22) also observed that training for four weeks does not cause much damage to the heart because the level of apoptosis did not change in their research. In contrast, Kwak *et al.* (23) found that 12 weeks of training significantly increases apoptosis in the left ventricle. Furthermore, the administration of high-dose androgenic anabolic steroids in conjunction with intense exercise in rats leads to cardiac hypertrophy, increased levels of inflammatory cytokines and significant stimulation of sympathetic nervous system (24). Therefore, the combination of these factors may predispose individuals to myocardial damage. However, cardiac muscle adaptation to exposure to high doses of anabolic androgenic steroids, the use of herbal supplements and their relationship with physical activity have been poorly understood. In recent years, medicinal plants with natural and synthetic compounds have been used to protect cells against some organ inflammation and toxicity (25). Species of the genus *Ziziphus* (family Rhamnaceae) are found in the vast areas of Asia, Africa and South America. Certain species called jujube (*Ziziphus jujuba*) has been taking as a medicinal plant from the distant past. Fruits, leaves and even roots of this plant have been used widely in East Asian countries, in the treatment of different diseases (26). In various studies, the presence of very diverse chemical compounds has been reviewed and approved in the jujube. In a study, it was found that the jujube fruit is rich in carbohydrates, fiber, protein, fat, essential vitamins and minerals (27). Jujube fruits compared with stems, roots

and leaves have further vitamins C and A, and are rich in linoleic acid (28). In addition, the jujube fruits are composed of flavonoids, triterpenoid saponins, alkaloids, sterols and lauric acid (29). Numerous health benefits have been proven to jujube, such as anti-inflammatory and anti-apoptotic properties (26, 30- 32). A study has shown that training with jujube consumption can prevent cardiovascular disease (33). Gallic acid (also known as 3, 4, 5-trihydroxybenzoic acid) is a type of phenolic acid found in various plants such as oak, tea, sumac, grape seed and apple. Gallic acid helps prevent cell damage by reducing oxidative stress. Its antioxidant properties protect cells against oxidative damages. In addition, it affects pre-apoptotic and apoptotic indicators by activating a signaling pathway (34, 35). Since there is little information on the effects of androgenic anabolic steroids on the cardiac tissue structure during resistance training and nutrition (the use of natural herbal supplements), so the present study was conducted to better understand the effects of androgenic anabolic steroids at high doses on apoptosis of heart muscle tissue when combined with exercise and supplements within a six-week period. Considering the widespread use of anabolic androgenic steroids by athletes and their side effects on the cardiac tissue, the results of the present research can contribute to a better understanding of athletes from the effects of these drugs. Therefore, it seems necessary to carry out further researches in this field. According to the mentioned issues, this study was conducted to investigate the effect of resistance training with boldenone consumption, *Ziziphus jujuba* extract and Gallic acid on annexin-5 and caspase-3 genes expression in cardiac tissue of male Wistar rats.

Methods

The statistical population of study included male Wistar rats purchased from Damghan

Institute of Applied Sciences, Iran. Among them, 42 male Wistar rats with age of 12.8 weeks and initial weight of 195 ± 7.94 g were selected as statistical samples that were selected by purposive sampling method according to weight and age. Then, they were divided randomly into seven groups with six rats in each group: Group I: group (control) without training + placebo injection (n=6); Group II: group with 5 mg Boldenone per kg of body weight (n=6); Group III: jujube extract + Boldenone injection of 5 mg per kg body weight (n=6); Group IV: Gallic acid + Boldenone injection of 5 mg per kg body weight (n=6); Group V: resistance training + Boldenone injection of 5 mg per kg body weight (n=6); Group VI: resistance training + jujube extract + Boldenone injection of 2 mg per kg body weight (n=6); Group VII: resistance training + Gallic acid + Boldenone injection of 5 mg per kg body weight (n=6). The study groups were subjected to PVC cages special for rodents with metal mesh lid and a floor covered with clean wood chips. The room temperature was $22 \pm 1.4^\circ\text{C}$ with humidity of 65 to 75 percent. The samples were kept under 12-hour sleep-wake cycle with free access to food and water. The used food was as compressed and prepared food for animals (Gorgan and Dasht Poultry & Livestock Food Factories Co.). The consumed water was urban treated water inside the drinking container made of PVC. The graded insulin syringe was used for the administration and injection of drug. The drug was injected in the hamstring in depth once a week on an appointed day and certain hours (11 am). The control group received physiological normal saline solution or sodium chloride solution 0.9%. The resistance training consisted of six weeks of climbing the 76-cm ladder with 47 steps and 19-cm width at an angle of 80 degrees with the rest site at the top (Gyro Tech Co., Iran). In order to determine the appropriate weights, the weight of rats was measured every four days. Each training session consisted of three sets with five

repetitions and one-minute resting between sets. The training was carried out after attaching a weight to the tail of the rat. In the first week, the weights attached to each animal's tail was calculated 50% 1RM on the day before the start resistance training, which increased by 10% per week and up to 100% in the last week. The animals were exposed to climb the ladder during the two weeks prior to training. The rats in case of refusal were forced to climb with manual stimulation. The final load was considered as the maximum carrying capacity of that session (36). The jujube fruits were washed and dried at 40°C for one week. Then the seeds were separated from the fruit and powdered. The extract was obtained by 70% ethanol from the fruit powder. The extract was concentrated in the semi-solid materials by means of a rotary evaporator at a temperature of about 50°C (25). The extract was dissolved in 600 mg per ml of distilled water and then was consumed by rats orally at a dose of 600 mg per kg of weight. Industrial substance of Gallic acid was purchased from the market (brand SIGMA, America). The groups receiving Gallic acid received 50 mg Gallic acid per 1 kg (5 mg per 100 g of rat weight) for eight weeks (7 days a week). Gallic acid was consumed after training, when the rats were thirsty, through a kind of serum. At the end of the study, the animals were kept fasting for 12 hours. Then the samples were weighed and anesthetized for sampling. Anesthesia was performed using desiccator containing cotton soaked in chloroform (Merck, Germany). After 40 to 50 seconds, the animal was properly anesthetized. After anesthesia, the dissection was carried out by fixing the animal on the surgical board for rodents, and the cardiac tissue was dissected immediately. The specimens were fixed in 10% formalin after separation and then were prepared for measuring changes in apoptosis. In this study, ethical principles were considered about how to work with laboratory animals, including the availability of food and water, proper living conditions and absence of

coercion in training. All experiments were carried out based on the Helsinki Declaration and were reviewed and approved by the ethics committee of Islamic Azad University, Ayatollah Amoli Branch in Iran. Expression of Annexin-5 and Caspase-3 genes was measured by real time-PCR technique and after quantitative analysis of gene expression values using $ct\Delta\Delta-2$ formula. The PCR reaction was performed using PCR master mix (Applied Biosystems) and SYBR Green in the ABI Step One (Applied Biosystems, Sequence Detection Systems, Foster City, CA) device according to the manufacturer's protocol. The sequence of primers used in the present study is presented in Table 1. In statistical analysis, paired sample t-test for intergroup changes and one-way ANOVA for intergroup changes and Scheffe post hoc for intergroup differences were used to study the effects of independent variables on the dependent variable of weight. Data were analyzed using SPSS version 22 and $P < 0.05$ was considered as significance level.

Results

Table 2 shows the mean and standard deviation of variables in different groups. The resistance training and Boldenone consumption could cause significant changes in weight of male Wistar rats among the study groups ($P=0.001$). The results showed the difference in the mean Annexin-5 of male Wistar rats among different groups ($P=0.002$). Scheffe post hoc test results indicated that the changes in Annexin-5 in the boldenone + jujube group ($P=0.01$) and boldenone + Gallic acid group ($P=0.007$) had a significant decrease compared to the Boldenone group. The Annexin-5 changes in the resistance training + Boldenone group ($P=0.144$), resistance training + Boldenone + jujube group ($P=0.09$) and resistance training + boldenone + Gallic acid ($P=0.21$) showed no difference compared to the boldenone group. In addition, there were no differences in annexin-5 changes among experimental groups compared to the

control group ($P=0.005$) (Figure 1). The results revealed that there is a difference for the mean Caspase-3 of male Wistar rats among different groups ($P=0.001$). The results indicated that Caspase-3 changes in the bldenone + jujube group ($P=0.003$) and Boldenone + resistance training + Gallic acid group ($P=0.018$) were significantly reduced compared to the Boldenone group. However, Caspase-3 changes in the resistance training +

Boldenone group ($P=0.97$), resistance training + Boldenone + jujube group ($P=0.135$) and Boldenone + jujube group ($P=0.003$) did not differ compared to the Boldenone group. In addition, there were no differences for Caspase-3 changes among the experimental groups than in the control group ($P=0.005$) (Figure 1).

Table 1. Primer sequence used by the studied variables

Variable	Sequence	Primers
GAPDH	5'ACCACAGTCCATGCCATCAC3'	Forward
	5'TCCACCACCTGTTGCTGTA3'	Reverse
Annexin-5	5'-CCTGATGGGTGTGTGATCTTGG-3'	Forward
	5'-TTTCTTCATCCGTCCCCAC-3'	Reverse
Caspase 3	5'-CGGGTGCCAGTCATTCATCT-3'	Forward
	5'-AACCGTCTTTGATTTCCACGC-3'	Reverse

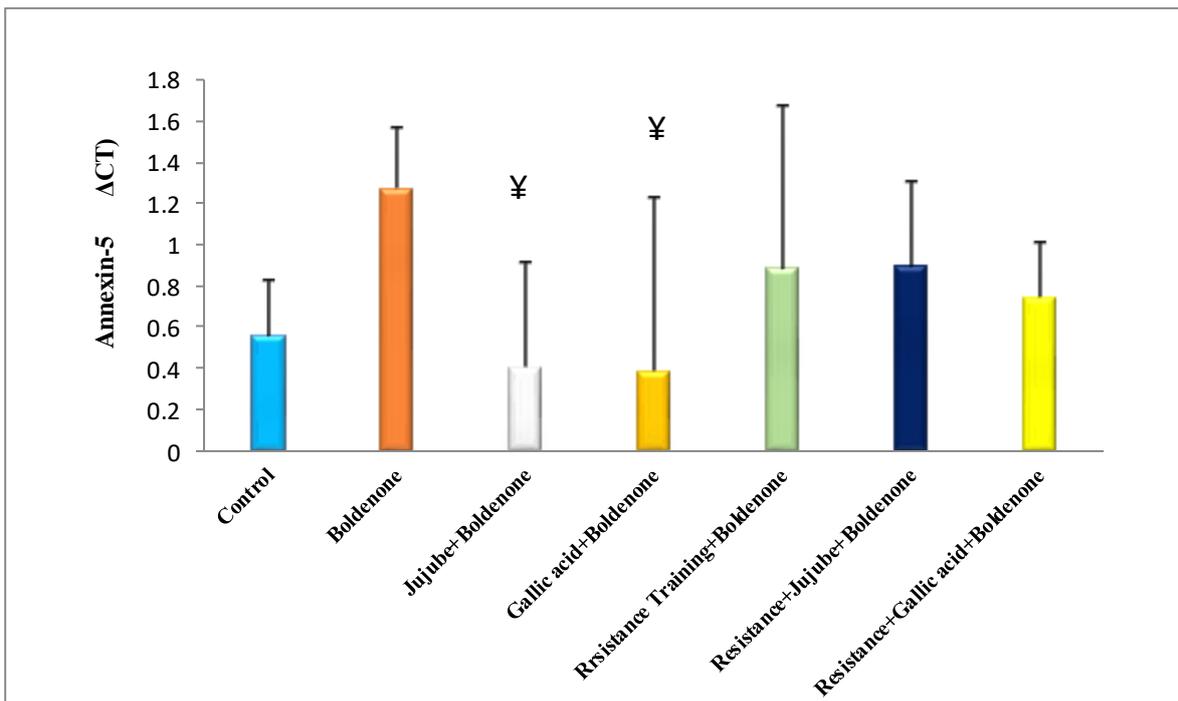
Table 2. Mean and standard deviation of study variables in different groups

Group	Variable	Weight		Annexin-5	Caspase-3
		First week	Eighth Week		
Control		184±12.4	277±18.6	0.55±0.27	0.96±0.46
Boldenone (5mg / kg)		229±29.2	283±33.5	1.27±0.30	2.02±0.69
Jujube+ Boldenone		245±31.2	294±39.7	0.40±0.21	0.48±0.16
Gallic acid+ Boldenone		243±31.8	297±37.3	0.38±0.29	0.92±0.76
Resistance training+ Boldenone		186±5.1	280±8.3	0.88±0.38	1.08±0.45
Resistance training+ jujube+ Boldenone		228±48.9	280±38.8	0.89±0.46	1.06±0.44
Resistance training+ Gallic acid+ Boldenone		240±31.04	287±18.3	0.74±0.14	0.72±0.28

Discussion

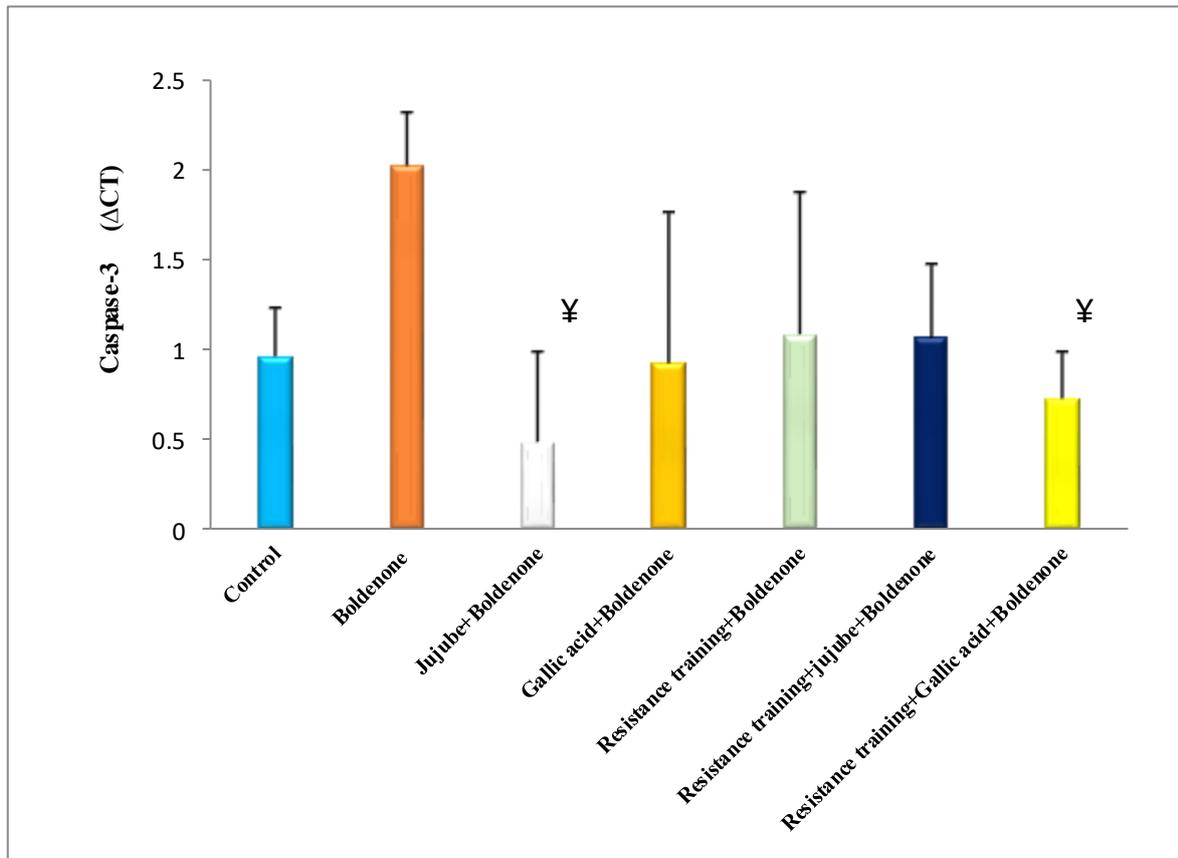
The results of present study showed that Boldenone caused apoptosis of cardiac muscle tissue by increasing the expression of Annexin-5 and Caspase-3 genes. Researchers have demonstrated that abuse of anabolic androgenic steroids is associated with structural changes and apoptosis of cardiac muscle tissue (4, 5, 14). The present results are consistent with previous findings so that apoptosis of cardiac muscle tissue was observed in the present study among the group

receiving Boldenone. Physiological and pharmacological mechanisms of action of anabolic androgenic steroids have not been clearly identified on the structure and function of the heart. The anabolic androgenic steroids are bound to androgen receptors in the heart and major arteries; physiological levels (e.g., testosterone) may have a positive effect on coronary artery through the release of endothelial nitric oxide and inhibition of vascular smooth muscle tone (37).



¥: Significant difference compared to Boldenone group

Figure 1. Trend of Annexin-5 changes in male Wistar rats among different groups after intervention period



¥: Significant difference compared to Boldenone group

Figure 2. Trend of Caspase-3 changes in male Wistar rats among different groups after intervention period

Animal studies have shown that abuse of anabolic androgenic steroids such as nandrolone in high doses may reverse the vasodilator response and may lead to further growth effects on the cardiac tissue as hypertrophic cardiomyopathy and subsequently cause programmed cell death (38). These effects are likely to be modified by cascades of secondary messenger membrane receptors that increase intracellular flow of Ca^{2+} and recall of Ca^{2+} from sarcoplasmic reticulum networks (39). Increased Ca^{2+} affects the mitochondrial permeability and leads to the release of apoptogenic factors, such as cytochrome C, apoptosis inducing factor and Caspase (9). It should be noted that the doses of anabolic androgenic steroids with sudden cardiac death, MI, and cardiomyopathy, ventricular reconstruction are related to apoptosis (40). These findings may explain the clinical observations that anabolic androgenic steroids without coronary thrombosis can lead to death or cardiac atherosclerosis. The results of this study showed that jujube extract reduced the apoptosis of cardiac muscle tissue and changes in apoptotic markers in cardiac muscle tissue were at a lower level so that the Annexin-5 and Caspase-3 genes expression was decreased significantly in the jujube extract group. Previous studies suggest the apoptotic effects and inhibition of apoptosis by jujube extract (30-32). However, the protective effect of *Z. jujuba* has not been examined so far on the apoptosis of heart tissue. In this regard, some studies have shown that *Z. jujuba* affects tissue destruction factors with a dose-dependent manner. The observations of Ebrahimi *et al.* (25) indicated that treatment with jujube at a dose of 600 mg per kilogram of body weight provides a significant improvement in carbon tetrachloride-induced liver necrosis and reduces the cellular portal inflammation. In the intervention group receiving 400 mg of jujube, cellular destruction and necrosis were observed to some extent. Also in the intervention group with 200 mg jujube, cell

necrosis and hepatic portal inflammation were seen at high level and were made improvement less than the other two groups (25). Abedini *et al.* (32) also showed that Jujube has effects on apoptosis by time- and dose-dependent approach. Therefore, apoptotic response and histological changes to consumption of jujube is probably dependent on the dose. Moreover, comparison of the results from different studies is difficult often due to compositions of extracts that are largely dependent on geographic location, variety, age of the plant, drying technique and extraction methods. In addition, the results of the present study demonstrated that Gallic acid could reduce the apoptosis of cardiac muscle tissue so that the level of Annexin-5 and Caspase-3 genes expression was reduced in the Gallic acid group; this reduction was significant only in the Annexin-5 gene expression. Gallic acid is a polyphenolic compound found in many plants, fruits and vegetables. It is reported that Gallic acid derivatives such as alkyl esters have many biological and pharmacological activity. Some studies have shown the influence of Gallic acid on cell apoptosis (34, 35). In the present study, the role of concentration and duration of administration of *Z. jujuba* and Gallic acid were considered as critical factors for reducing the cardiac tissue damage resulting from bldenone. The results of this study showed that the apoptotic effect of cardiac tissue was decreased in the *Z. jujuba* and Gallic acid group following administration of Boldenone. It seems that treatment with jujube extract and Gallic acid can reduce the effects of cardiac muscle tissue apoptosis induced by the Boldenone. The prescribed dosage of *Z. jujuba* and Gallic acid should not be ignored so that it is possible to obtain clearer results by changing the dosage of *Z. jujuba* and Gallic acid and their use in different doses. However, further investigations are needed in this area. Finally, the results of this study showed that administration of Gallic acid and jujube extract with resistance training could reduce

the damage to the cardiac tissue and cardiac muscle cells. However, the impact of Gallic acid in combination with resistance training had a significant effect on cardiac muscle cells Caspase-3. Exercise trainings result in improving the health and cardiovascular function, and are useful means for cardiovascular treatments (41). Abuse of androgenic anabolic steroids that are commonly applied to enhance sports performance among athletes is associated with cardiovascular disorders. Heart is an organ that is highly influenced by the administration of steroids (42). A few studies have studied the effects of training along with androgenic anabolic steroids on apoptosis. Shokri *et al.* (14) investigated the combined effect of 8 weeks of physical activity and high doses of nandrolone (10 mg/kg/week) on apoptosis (by assessing the levels of Caspase-3 and TUNEL). In this study, cellular apoptosis was observed following the administration of high doses of nandrolone and a significant reduction in the nandrolone group treated with training. They stated that the training increases the rate of changes in apoptosis induced by high doses of nandrolone in rats (14). Hassan and Kamal expressed that the heart benefits of exercise cannot be achieved fully because of the potential risks of using anabolic steroids androgenic (4). Abuse of androgenic anabolic steroids is clinically associated with sudden cardiac death, myocardial infarction and cardiomyopathy. Molecular mechanisms of these events are closely related to apoptosis (24). The results of Hassan and Kamal showed that the Caspase-3 activity, as a marker of apoptosis, was increased in cardiac muscle in the steroid group compared with other groups (4). The present results are consistent with some findings in animal studies that showed androgenic anabolic steroids lead to body tissue lesions (43, 44), especially cardiac apoptosis (4,14,43,44). However, the present results are inconsistent with the findings of Doustar *et al.* (22) and Kwak *et al.* (23); they observed that training for four weeks and

twelve weeks makes no change or an increase in apoptosis. Recent studies have shown that androgenic anabolic steroids indirectly mediate the processes of mitochondrial damage, apoptosis and sarcomeric dysfunction so that the activity of Caspase-3 in heart was decreased in the training group supplemented with steroids, and no apoptotic change was observed on histological examination. These findings suggest that training is able to reduce the level of apoptosis in rat cardiac muscle (4). However, due to the lack of similarity in administering the dose of anabolic androgenic steroids, training programs and the duration of experimental treatments in various studies, it is difficult to draw conclusions about the actual effect of specific dose of steroids in the cardiac tissue.

Conclusion

In general, the results of this study showed that boldenone makes apoptosis in cardiac muscle tissue. According to the findings of this research, it appears that resistance training with *Z. jujuba* extract and Gallic acid can probably cause reduction in apoptotic changes in cardiac tissue in rats receiving boldenone. However, regarding limited studies conducted in this regard, investigations on the relationship among physical activity, anabolic steroids and cardiac tissue apoptosis require further clarification.

Ethical issues

Not applicable.

Authors' contributions

The author contributed to the writing and revision of this paper.

Acknowledgments

We would like to appreciate all those who have collaborated in this study.

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