The effect of Boldenone and Aerobic Training with Jujube Extract and Gallic Acid on Glutathione Peroxidase and Catalase in Heart Tissue of Male Wistar Rats

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Abstract

The aim of this study was to investigate the effect of boldenone and aerobic training with Jujube extract and Gallic acid on glutathione peroxidase and catalase in heart tissue of male Wistar rats. Thus, 42 male Wistar rats aged 8-12 weeks (weight 195±7.94g) were randomly divided into 7 groups: control, boldenone (5mg/kg), jujube (Ziziphus jujuba) extract + boldenone, Gallic acid + boldenone, endurance training + boldenone, endurance training + Ziziphus jujuba extract + boldenone, and endurance training + Gallic acid + boldenone. Endurance training protocol was performed 5 days a week for 8 weeks, each session 60 minutes at 25 to 30 meters per minute. Injection once a week, on an appointed day, and in the hamstrings was conducted in depth. After anesthesia, autopsy was performed and the cardiac tissue isolated. Data were analyzed using t-test, one way ANOVA and Scheffe post hoc at the P < 0.05. The results showed that there was a significant differences between catalase in the various groups (P = 0.000). Catalase expression in boldenone + training + Gallic acid increased significantly compared to the boldenone (P = 0.000) and control (P = 0.000) group. Also, there was a significant differences between glutathione peroxidase in the various groups (P = 0.000). Glutathione peroxidase expression significantly increased in boldenone + training + jujube compared to the boldenone (P = 0.000) and control (P = 0.000) group. According to the findings, it seems that boldenone injected with jujube extract/Gallic acid and performing endurance training for 8 weeks have beneficial effects on antioxidant system.

Keywords: Boldenone, Jujube, Gallic acid, Aerobic training, Heart tissue, glutathione peroxidase and catalase

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Introduction
Anabolic androgenic steroids, as compounds derived from testosterone, are used by athletes to improve physical performance and to increase muscle mass and strength [1, 2]. Effects of anabolic androgenic steroids abuse on cardiovascular system, including increased blood pressure, thrombosis, myocardial infarction, heart failure, atrial and ventricular fibrillation, and electrophysiological disorders have been reported [3-5]. In fact, high doses of physiological anabolic androgenic steroids during exercise increases cardiac damage. However, the molecular mechanisms by which anabolic androgenic steroids impairs the beneficial effects of exercise on the physiology and function of the heart largely remain unknown.

Some studies showed that taking anabolic steroids increases oxidative stress and lowers levels of antioxidant capacity [6]. Anabolic steroids increase the activity of the liver in children. This effect reduces the capacity of antioxidant [7]. Boldenone (1-dehydro derivative of testosterone) is prohibited due to its adverse effects on the human body. This steroid directly is used to build muscles [8]. Ehab et al examined the consumption of boldenone anabolic steroid and anti-oxidant enzyme activity in rabbits, that there was significant reduction in the capacity of antioxidant in the boldenone anabolic steroid consumer group in comparison with the control group [6]. Frankenberg et al also examined effect of nandrolone on oxidants homeostasis level in the liver, heart, and kidney of Wistar male rats. Their results showed that the levels of catalase and glutathione peroxides in the heart, kidney and liver in nandrolone consumer group declined [9]. Glutathione peroxides is general family name of enzymes with peroxidase activity that their biologic role is to protect the organism against oxidative damage. Catalase is also an enzyme that is found almost in all organisms and in most organs of the body. This enzyme is one of the most important enzymes in protecting cells against the contamination of the oxide by hydroxide [10]. Exercises often with the use of anabolic androgenic steroids are associated to real life [11, 12]. Although, heart antioxidant system response to such association is not clear. Chaves et al showed that high doses of nandrolone disrupts effects of heat protection and also SOD and GPX activities resulted from exercises [3]. Similarly, the recent study showed that high doses of testosterone disrupt catalase activity in heart of mice [13]. Peyet al examined the effect of stanozol long-term using on enzyme anti-oxidant activity and markers of oxidative stress after 8-week treadmill training and stated that the levels of glutathione peroxides and catalase activity in group exercises and steroid were increased [14]. Because of oppressive effect of body anti-oxidation system and oxidative progress of fats by extreme sports reason, coaches and athletes seek to use anti-oxidative supplement. A large number of anti-oxidative compounds, natural or artificial are introduced for the treatment or prevention of diseases associated with the bases. Nowadays, some artificial anti-oxidations are used which are poisonous, risky, and carcinogens. Thus, it has been suggested that herbal anti-oxidation be used. Jujube by scientific name of Ziziphus Jujuba is one of the anti-oxidative herbs with confirmed anti-oxidation feature and high amount of anti-oxidation compounds. Jujube contains fatty acids and alfatoxopherol betacarotene and phenolic compounds [15]. Studies have shown that jujube polysaccharide can increase the activity of glutathione peroxide and decrease fatigue caused by sports [16, 17]. Gallic acid or 3-hydroxyl benzoic acid is also a phenolic acid found in various plants, including oak, tea, sumac seeds, grapes and apples. Gallic acid prevents cellular damage by reducing the oxidative stress. Its antioxidant properties protect the cells against oxidative damage [18]. San et al. investigated the anti-genic effect of Gallic acid in rats and its effects on the oxidant and antioxidant parameters and showed that the levels of catalase and glutathione peroxides have had significant increase [19]. Padmaet al. also investigated the therapeutic effect of Gallic acid and quercetin in mice and the association with cardiac problems, and mentioned that the
consumption of Gallic acid and quercetin increase the performance of antioxidant enzymes such as glutathione peroxide and catalase [20]. Many studies have shown that short duration exercise increases antioxidant enzyme activity in heart. Nonetheless, few studies are available for the effect of regular long-term exercise along with prescribing anabolic androgenic steroids on the activity of antioxidant enzymes in heart. Also, there is no study about the relation of exercise and training time along with supplementation with jujube, Gallic acid and boldenone anabolic steroids and the expression of glutathione peroxide and catalase in heart tissue. Therefore, the current study sought to examine the impact of boldenone supplementation with and without alcoholic extract of jujube and Gallic acid, during a period of endurance training on gene expression, glutathione peroxide and catalase in heart tissue of male Wistar rats.

**Methods**

Sample includes 42 male Wistar rats obtained from Damghan Applied Sciences Institute aged 8-12 weeks and weighed 94.7±195 grams. Then, they were randomly divided into 7 groups, each with 6 rats as follows:

- **Group I:** control (n = 6)
- **Group II:** boldenone group 5 mg/kg body weight (n = 6)
- **Group III:** jujube extract + boldenone injection 5 mg/kg body weight (n = 6)
- **Group IV:** Gallic acid + boldenone injection 5 mg/kg body weight (n = 6)
- **The fifth group:** training + boldenone injection 5 mg/kg body weight (n = 6)
- **Sixth group:** training + jujube extract + boldenone injection 2 mg/kg body weight (n = 6)
- **Group VII:** training + Gallic acid + boldenone injection 5 mg/kg body weight (n = 6)

Studied groups were kept in special rodents' metal cages with PVC lace caps and their floor covered by clean wood chips. Room temperature was 22±1.4 °C, with a moisture equivalent of 65 to 75%. The samples were kept according to the cycle of 12 hours of sleep and 12 hours of awareness, and availability of tap water and special mice food (Gorgan factory, Gorgan, Iran). Graded insulin syringe was used for injection of medicine in the muscle posterior cance muscle deeply once a week (at 11 a.m.). Control group also received physiological normal saline or a solution of 0.09% sodium chloride.

**Endurance training protocol**

Training group rats exercised on treadmill 5 days a week (Saturday to Wednesday) for 8 weeks. Protocol included 10 days familiarizing animals with environment and treadmill device that was done 15 minutes with speed of 5 to 15 m/min and a zero percent slope. The duration and speed of Exercise were increased weekly until in the fourth week it reached to 50 minutes with the speed of 25 m/min. With a total of 10 minutes warm-up and 3 mins cooling, the whole time of training was 63 minutes after the eighth week. In order to warm-up each training session began with an initial speed of 10 meters/min of which gradually increased by 3 meters/min every 2 minutes. From the fourth week on, in minute 10 device speed reached to 25 m/min and in the last week in min 10 to 30 m/min. A 3 minute cool down was also considered after all trainings. To force cases to run only a plastic bar (instead of electric shock device) was used.

**How to prepare and inject boldenone hormone**

High-purity boldenone hormone (Medich, Germany) containing 250mg/ml saponin was purchased from market. It was injected once a week in a given hour and day in the muscle (hamstring).

**Jujube extract and Gallic acid supplementation**

Jujube fruit was washed and then dried at temperature of 40°C for a week. Then, the pits of the fruit were ground to a powder. Powder was extracted in 70% ethanol. Extract was condensed inside the semi-solid materials by means of rotating steam in 50°C. Extracts were solved in 600 mg/kg distilled water and was used by rats
orally at a dose of 600 mg/kg of body weight. Industrial Gallic acid under STIGMA commercial name was purchased from American market. Gallic acid was given to the appointed samples 5 mg per 100 g of body weight during 8 weeks (7 days a week). Gallic acid was given after the training that mice were thirsty.

**Tissue sampling and measuring changes in gene expression in heart tissue**

After 56 days, animals were kept in fasting for 12 hours, then weighed and became unconscious. Anesthesia was done using a desiccator with a piece of cotton soaked in chloroform (Merck, Germany). After anesthesia, autopsy was performed with fixing the animal on rodent's surgery board and immediately heart tissue was removed. Then, the corpse body was fixed in formalin 10% and then prepared for measurement of gene expression changes. In this research, the ethical principles about how to work with laboratory animals, including the availability of food and water conditions, proper maintenance, and lack of coercion in exercises were all considered. All tests were based on Declaration of Helsinki, confirmed by ethics committee in Ayatollah Amoli branch of Islamic Azad University.

**Purification of Total RNA:**

The obtained tissue, after weighing in buffer phosphate with (RMFS %1), was homogenized by homogenizer and all RNAs were purified using purification kit Tripur Isolation Reagen Roche-Cat (No. 11667157001-Germany) (based on kit structures). Then purified RNA was solved in 50 microliter water (RNase free) and the quality of purified RNA purification was evaluated by the spectrophotometer at 260 nm wavelength (260 nM). Also purified RNA was stained and colored in electrophoresis agarose gel 8.0% and by indium bromide in order to check and confirm the quality and quantity of RNA. After being assured of the accuracy of evaluation, quality and quantity of purified RNA, we approached the next stage.

**Production of cDNA**

One µg of purified RNA was used to produce cDNA (using kit AccuPower CycleScript RT PreMix (dN6) (Bioneer-Cat. No. k-2044-Korea). In order to produce cDNA from Master Mix following stages was used:

RNase Free Water 20 µl

Random Primer (50 mM) 1 µl

According to the instructions of the used kit, firstly, the mixture incubated at 42 °C for 60 minutes and then for 5 minutes at 95 °C.

The primer (initiator):

GAPDH:

F: 5'ACCACAGTCCATGCCATCAG3'
R: 5'TCCACCCACCCTGTGCTGTA3'

Initiator for glutathione peroxidase is like below:

F: 5'-CAGTTCCGACATCGAGAAT-3'
R: 5'AGACGGGTGAGCCTTCT-3'

Initiator for catalase is like below:

F: 5'-TTTTCACCAGCAGATGGCA-3'
R: 5'-AAGGTGTGTGAGCCATAGCC-3'

After the preparation of cDNA as a sample pattern by real time PCR method using ABI 7500 device, expression amount of catalase and glutathione peroxidase was measured based on device structures. To investigate the effect of independent variables on weight dependent variable, inter-group test and on-way ANOVA analysis was used for changes within the group. Scheffe post-hoc test was used for evaluation of differences between groups. All statistical operations was conducted through SPSS software version 22 at the significance level P<0.05.

**Results**

Average and standard deviation of mice weight in the different groups is shown in Table 1. Activity, endurance, and taking boldenone
supplement along with jujube and Gallic acid caused significant changes in male Wistar rats body weight in research groups (p = 0.007) (Table 1). Statistical analysis using paired t-test showed that the weight of mice in endurance training group, boldenone, jujube extract and Gallic acid in posttest stage has significant increase compared with pre-test level in interventional group compared with control group. Also, the results showed that weight of mice in training endurance group+ boldenone has significant changes compared to boldenone groups + jujube and boldenone + Gallic acid (Table 1).

The results showed that there is significant difference between Wistar male rats catalase average in the different groups (p = 0.001). The results of Scheffe post hoc test showed that the expression of catalase in the group boldenone + training + Gallic acid compared to boldenone group (p = 0.018) and control group (p = 0.001) has significant increase (Figure 1).

Also the results showed that there is no difference between Glutathione peroxidase averages of Wistar male rats in different groups (p = 0.001). The results of Scheffe post hoc test showed that glutathione peroxidase expression in boldenone group+ training+ jujube has significant increase comparing with boldenone group (p = 0.002) and control group (p = 0.003) (Figure 2).

Table 1. The average and standard deviation of research variables in different groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Variable</th>
<th>control</th>
<th>boldenone (5mg/kg)</th>
<th>Jujube-boldenone</th>
<th>Gallic acid-boldenone</th>
<th>Endurance training-boldenone</th>
<th>Endurance training-jujube-boldenone</th>
<th>Endurance training-gallic acid-boldenone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice weight</td>
<td>First week</td>
<td>184±12.4</td>
<td>229±29.2</td>
<td>245±38.2</td>
<td>243±31.8</td>
<td>161±8.6</td>
<td>217±16.1</td>
<td>241±43.3</td>
</tr>
<tr>
<td></td>
<td>Week 8</td>
<td>276±28.9</td>
<td>288±46.4</td>
<td>286±40.6</td>
<td>284±35.0</td>
<td>251±2.5</td>
<td>200±50.9</td>
<td>313±32.2</td>
</tr>
</tbody>
</table>

Figure 1. Changes of Wistar male rats' catalase in different groups after intervention

# Significant difference compared with control group
* Significant difference compared with boldenone group
Figure 2. Changes of glutathione peroxidase enzyme expression of Wistar male rats in different groups after intervention period
¥ Significant difference between training group + boldenone + jujube and control group
≈ Significant difference between training group + boldenone + jujube and boldenone group

Discussion
The present study is the first study that determined the impact of a period of aerobic exercise and consumption of boldenone along with the aqueous extract and Gallic acid on gene expression of glutathione peroxidase and catalase in Wistar male rats' heart tissue. GPX and CAT enzymes are anti-oxidative, which play role in clearing free radicals and reducing their damage [10]. The results of this study showed that boldenone injection had not a significant effect on the expression with control group. This result is not in line with the findings of Ehab et al., Peyet al and Barakat et al that showed an increase [6, 14, 21] and the results of Mayada et al that showed a reduction in the activity of anti-oxidation enzymes along with the prescribed boldenone [22]. For ability of boldenone to create peroxidation, Langford's et al claimed that testosterone increases HSL activity and stimulates rat's heart lipolysis myostatis, increases the access of fatty acids long chains for the synthesis of ATP which, in turn, increases oxygen consumption and thus support increasing production of ROS [23]. ROS operates like a messaging cell, and depending on the amount of production and accumulation, it causes the activation or inhibition of messaging pathways [24]. The results of this study showed that boldenone injection had not a significant effect on the GPX and CAT expression compared with the control group. In the present study, the rate of ROS production and the status of oxidative were not measured, though, boldenone injection in the boldenone group compared to the control group alone caused no certain change on the rate of ROS production, and was effective itself in the amount of anti-oxidation enzyme expression. On the other hand, it is probable that the boldenone injection for 8 weeks could have impact on the gene expression of other antioxidants such as SOD and glutathione reductase, which in the same vein, Delgado et al observed that 8 weeks consumption of stanozol causes a significant increase in the activity of the SOD enzymes and glutathione reductase and no change in activity of CAT and GPx [7]. Although, they did not evaluate gene expression.
In the present study, expression of catalase only in the group boldenone + training + Gallic acid increased significantly compared to the control group and boldenone group. In addition, the expression of GPx in group boldenone + training + Gallic acid had insignificant increase and in the group boldenone + training + extract had significant increase compared with group boldenone and control group. The results of this study showed the potential impact of three factors boldenone, endurance training, and antioxidant supplements together and not alone on the expression of antioxidant enzymes. It seems that no study investigated the synergy impact of these three factors simultaneously on the expression of genes, and even the activity of antioxidant enzyme.

Another result of the present study is the insignificant increase of CAT and GPx expression in group boldenone + training compared to the control group and the group boldenone. Some studies on male rats showed that endurance training cause insignificance increase of GPx [25]. Similar with the findings of the present study, some studies also reported that when training and nandrolone are combined together have no effect on heart tissue GPx [26-28]. On the other side, a number of studies showed that exercise on the treadmill for 8 weeks significantly increased the activity of GPx in male mice, although gene expression was not measured in these studies. In spite of the present study, Bravati et al reported that 10 mg/kg nandrolone injection during 6 weeks increases MDA/GPX relation while endurance training could prevent this increasing effect [29]. Perhaps this difference is because of the kind, amount, and consumption time of antioxidant enzyme or difference in consumption dose and sample study.

In the present study, supplementation with jujube and Gallic acid alone had no effect on the expression of antioxidant enzyme gene in mice male that receive boldenone injection. Some antioxidant supplements such as Gallic acid and jujube have anti-oxidation and anti-inflammatory properties due to phenolic compounds and collect free radicals. No study has investigated the effect of these two types of supplements on the status of oxidation-antioxidation following AASs injection, but a study showed that in addition of boldenone, propolis (a resinous product collected by honey bees) increases the antioxidant enzymes in the liver and kidney [21]. Taati et al showed that the activity of liver enzymes, SOD and GPx in ethanol group, in comparison to the control group were reduced significantly, while the extract of the fruit of jujube (200 mg) only increased the activity of enzyme GPx (30). Also, the results of Afzalpour et al showed that in the group that did not receive the jujube fruit, TAC was reduced after severe resistance and endurance training, but, in jujube group TAC was reduced after severe training. In jujube and sever endurance group TAC was increased after 3 weeks of taking jujube. Thus, one session of severe endurance will suppress TAC but taking jujube for 3 weeks decreases its negative effect [15]. Fangling and Cailian investigated the impact of the jujube polysaccharide on some indices of blood biochemical in male mice and found that jujube can increase superoxide dismutase and glutathione peroxidase activity [17]. Also, Yeh et al showed that the consumption of 14 days of the Gallic acid and phenolic acid, with a dose of 100 mg/kg significantly increases the gene expression of antioxidant enzymes, such as SOD, CAT and GPx in male rat heart tissue [31]. Previous studies have revealed that jujube contains various chemical constituents including triterpenic acids, flavonoids, cerebroside, amino acids, phenolic acids, mineral constituents, and polysaccharides and phytochemical which have antinecancer, anti-inflammatory, anti-obesity, immune-stimulating, antioxidant, hepato-protective, and gastrointestinal protectve activities and inhibit cell formation in macrophages [32]. Sen et al in their research investigated the impact of two doses of Gallic acid (100 and 200 mg/kg) on oxidant and antioxidant indices level in the tissues of stomach that the induction of gastric ulcers with the use of aspirin were added to the pyloric model. The results showed that treatment
with both doses of Gallic acid increased in SOD, CAT, GPx and glutathione reductase and decreased activity of the myeloperoxidase enzyme and lipid peroxidation in the tissues of rat stomach [19]. According to the results of the present study, it seems that boldenone with dose of 5 mg/kg of body weight can be used once a week without any damaging effects on the body.

Conclusion
The study showed that injecting boldenone along with consumption of aqueous extract/Gallic acid and endurance training for 8 weeks have beneficial effects on the anti-oxidation system of male mice, and can increase gene expression of the catalase and glutathione peroxidase enzyme. Thus, it could be a useful strategy to strengthen anti-oxidation system and prevent the disorders arising from the abuse of AASs. Furthermore, combination of aqueous extract/Gallic acid can be used along with regular aerobic activity to increase anti-oxidation enzymes of people who consume boldenone and reduce possible problems following abuse of this hormone. However, the effect of different doses of boldenone on the status of oxidation and gene expression of antioxidant enzymes accompanying with measuring free radicals and oxidation pressure indicators may better explain the results.

Conflicts of Interest
Authors have no conflict of interests.

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References


