Journal of Environmental Sciences Studies

Journal home page: www.jess.ir

Effect of Thymus Extract Supplementation on Endurance Exercise Performance, Liver and Oxidative Enzymes in Rats

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Received: (2022-01-30)
Accepted: (2023-08-10)

Abstract

In this study was to determine the influence of *Thymus migricus* extract supplementation on mitochondrial oxidative enzyme activity and the endurance exercise performance. Twenty rats were divided into 2 groups. They received thyme hydro-ethanolic extract dissolved in distilled water to the desired concentration (400 mg/kg.day). All rats in both groups were subjected to endurance running on the treadmill 5 days/wk with maximum speed and duration of 1 h/day at 27 m/min for 8 wks. At the end of the training period, treadmill running to fatigue at 36 m/min speed was applied to determine the endurance capacity. 24 hours after the end of the endurance capacity test, the rats were decapitated. The activities of cytochrome c oxidase and citrate synthase in the soleus tissue were determined spectrophotometrically. Findings showed that there was no significant difference in liver enzymes between the two groups, thyme supplementation significantly down-regulated the activity of citrate synthase (35%) and cytochrome c oxidase (24%) within the soleus muscle compared to the non-supplemented group (respectively, t_{14}=4.61, p=0.0004; t_{14} =4.19, p=0.001). The results of the t test revealed that the exhaustive running time of rats in the thyme group was significantly prolonged compared with the non-supplemented group (t_{18}= 4.05, P=0.001).

Keywords: Endurance exercise, Performance, Mitochondrial enzyme activity, Liver Enzymes, Thymus migricus.

1. Introduction

In untrained or recreationally trained participants, certain muscular adaptations involving elevated citrate synthase (CS) activity, mitochondrial density, capillary-to-fiber ratio, and fiber cross-sectional area have been evoked to be involved in the improved endurance performance [1]–[4]. The available evidence shows that changes in mitochondrial proteins such as 1 q25.2-25.3. Cyclooxygenase (COX) (known as prostaglandin-endoperoxide synthase (PTGS)- an enzyme; specifically, a family of isozymes, EC 1.14.99.1; responsible for formation of prostanoids, including thromboxane and prostaglandins such as prostacyclin, from arachidonic acid or citrate synthase after 10–12 weeks of exercise training are highly indicative of changes in mitochondrial mass/content and improvements in exercise capacity. However, mitochondrial

enzymes do not respond in a uniform pattern to endurance training. In response to the same exercise stimulus in rats, enzymes involved in the oxidation of fatty acids develop by nearly two-fold [5], while enzymes of the citric acid cycle barely increase by up to 50% [6].Since mitochondrial biogenesis such as elevated enzyme activities plays a significant role in response to the endurance exercise, some scientists attempt to use various supplements to increase biogenesis and exercise performance based on different mechanisms [7]–[10].For example, resveratrol as an antioxidants has previously been shown to increase mitochondrial copy number, CS activity and the mRNA content of oxidative proteins in skeletal muscle [11], [12]. Although the effect of antioxidant support after exercise is unclear and depends mainly on its type and dosage, its positive effects have been reported [3].Most of the studies on this topic have been carried out on plant-derived active substances with antioxidant effects such as flavonoids, proanthocyanidin, resveratrol [13]–[16]. Although many studies have investigated the hepatoprotective effects of herbal extracts no studies have been carried out on the effects of total plant extracts on endurance exercise performance as well as liver and oxidative enzymes.One of the endemic species of thyme is; *Thymus migricus* Klokov&Desj.-Shost. It grows in Iran, Turkey and Armenia. Its major active ingredients like all thyme species are thymol, carvacrol and linool [17]. It used in traditional medicine widely in Iran and Turkey, along with other types of thyme taxa. This plant is a member of Lamiaceae family recorded as the native herb of the Mediterranean used traditionally as food additive and its main essence (thymol) is widely used in several medicinal products [18]. In the traditional medicine in different countries as well as modern medicine, its essence is used for the treatment of respiratory tract infections, dry cough, seizures, smooth muscle spasm, bloating [19], high blood pressure, rheumatism, skin fungus, and headaches [20]. Thyme extract has anti-inflammatory [18], disinfectant, anti-worm, appetizing, sedative and sexually stimulating effects[20] and has also been used to relieve early child bearing and fracture pain [21]. As far the antioxidant characteristics of thyme, these are due to its phenolic compounds such as flavonoids and phenolic acids as well as thymol and gamma-terpinene [18], it may improve endurance to exercise performance and enhance the activity of mitochondrial oxidative enzymes and affect metabolic and hematological parameters especially ALT and AST enzymes.Alterations in cytochrome c oxidase and citrate synthase activity, two of oxidative enzymes may be an essential factor in etiology of several herbal supplements like thyme in improving exercise performance [22]. The aim of our study was to examine the effect of 8-week thyme extract supplementation on the cytochrome c oxidase and citrate synthase activity and in rat soleus and ALT, AST in muscle and their Liver Enzymes and its effect on endurance exercise in trained rats.

2. Materials and Methods

A total number of 20 male Wistar rats, 8 weeks old were used in this study. Rats were purchased from Pasteur Institute (Tehran, Iran). Animals were left for adaptation for 3 days before any experimental intervention. All rats were cared for in the animal facility at the Tabriz University of medical sciences. All aspects of this experimental protocol were approved by the University's Institutional Animal Care and Use Committee in compliance with Author Guidelines of the International Association of Veterinary Editors' Consensus on Animal Ethics and Welfare. Housed individually in regular cages, the rats were maintained in low-stress conditions, low noise, 12:12-h light-dark cycle, 22°C temperature, and 50% humidity. They were divided randomly into two groups and received standard rat chow containing protein with all the essential amino acids (30%), carbohydrates (40%) and lipids (30%) as well as either water ad libitum (non-supplemented group: n = 10) or thyme hydro-alcoholic extract dissolved in distilled water to the desired concentration (400 mg/kg) according to their daily water consumption (30 ml) (supplemented group: n = 10). All rats in both groups were subjected to endurance running on a motorized treadmill 5 days/week. First, the rats started running for 10 min/day at 10 m/min and 10% grade. The speed and duration were progressively increased during the next weeks until each rat was running continuously for a period of 1 h/day at 27 m/min for 8 weeks. Throughout the experiment, there were no significant differences in the mean food consumption, (about 20 g per day per rat) nor in the body weights of the animals in the two experimental groups. Twenty-four hours after the last endurance performance test, the rats were subjected to decapitation. The blood was drained from the neck and collected into heparinized glass test tubes. Plasma was gained after centrifugation. The soleus muscles were rapidly excised, frozen, and kept at -280°C.

2.1. Treadmill Performance (maximal endurance capacity)

Endurance capacity was assayed by treadmill running to fatigue after the end of the training period. The rats were compelled to run on a motorized treadmill (5/lane) at a speed of 36 m/min and a grade of 8% until they

got exhausted [23]. They were removed from the treadmill when they showed inability to maintain the appropriate speed despite continuous hand prodding for 1 min. The running time was recorded at the moment.

2.2. Hydro-alcoholic Extract Preparation

A 30% (W/V) plant material was made in methanol/water (80:20, v/v) at 25°C, stirred 150 rpm for 1 h and filtered through Whatman No. 4 paper. The extra hydro-alcoholic mixture was added to the remanent for extracting the residue. The pooled extracts were dried at 35°C under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland) and then further lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA).

2.3. Spectrophotometric Assays

Since researchers have considered an increase in mitochondrial enzyme activity as a marker of mitochondrial biogenesis [24] we examined two of the important enzymes involved in the Krebs cycle, including citrate synthase and cytochrome C oxidase. The complex IV (cytochrome c oxidase) activity was measured in mitochondrial particles prepared by sonicating, under nitrogen atmosphere, 1 mg of rat soleus muscle dissolved in 1 mL of 50 mM phosphate buffer (pH7.2). The assay mixture contained 1.2 mMantimycin A in 3 ml of 50 mM phosphate buffer (pH7.2) at 30 °C. The soleus sample (25 µg) was added to the assay mixture and the reaction was initiated by adding 30 µM of reduced cytochrome c. The reaction was measured by following the decrease in absorbance of cytochrome c at 540–550 nm using a diode array spectrophotometer [25]. The activity was calculated using an extinction coefficient of 19 $mM^{-1}Cm^{-1}$for reduced cytochrome c. The special activity of the enzyme is expressed as nmol of cytochrome c oxidized/min per mg of muscle protein. The cyanide-insensitive rate of cytochrome c oxidation was measured and subtracted.Citrate synthase activity (CS) was measured with the method of Srere as described previously [26] with modification for microplate analysis. The portions of muscle sample were thawed, weighed, and homogenized for 20 seconds with an electric adaptable homogenizer (VDI 25; VWR International) on ice in 20 volumes (1:20 weight/volume) of homogenizing buffer (100 mM KPO_4 + 5 mM EDTA + 5 mM ethylene glycol tetraacetic acid, pH = 7.4). Homogenates were subsequently vortexed, subjected to three freeze–thaw cycles, and further diluted to a 1:400 ratio. Citrate synthase activity was measured by following the production of the 4 mercaptide ion spectrophotometrically at a wavelength of 412 nm. Twenty microliter of homogenate was added in triplicates for each sample to a 96-well plate with each well containing 170 µL of reaction mixture (100 mM Tris + 30 mM acetyl CoA + 10 mM 5.5′-dithiobis [2-nitrobenzoic acid]). Baseline reactivity was measured to account for endogenous thiol and deacetylase activity and subtracted from enzyme activity initiated by the addition of 10 mMoxaloacetic acid. Net citrate synthase activity was expressed as pmol × min^{-1} × g^{-1} wet weight. Homogenate protein concentration was assessed using the BioRad protein assay with bovine serum albumin as the standard and measured spectrophotometrically at 595 nm. Citrate synthase activity was then declared as pmol × min^{-1}× mg protein^{-}

2.4. Liver Enzymes

Transaminase enzymes including alanine aminotransferase (ALT) and aspartate amino transferase (AST) were measured to evaluate liver function using biochemical calorimetric methods.

2.5. Statistical Analysis

Sample size was determined by PASS Sample Size Software**.** The data obtained was analyzed using SPSS (version22) through the independent t student test. The results were expressed as mean ± S.E. Significant level was considered as $P < 0.05$.

3. Results and disscusion

The levels of Weight, lipid profile of groups and the levels of serum liver enzymes are shown in Table 1. It shows that there was no significant difference in liver enzymes between the two groups. This result is also seen in the weight ($P < 0.05$). It was also observed that thyme supplementation reduce some lipid indices compared to the control group.

Table 1. The effect of thyme supplementation on weight and some metabolic parameters in rats

Index	Non- Supplemented (n=10)	Thyme-Supplemented (n=10)
Initial Weight (g)	245.75±6.04^{ns}	260.15±3.09
Final Weight (g)	298.75±3.49 ^{ns}	294.50±9.79
Aspartate Transferase (U/L)	205.50±9.79 ^{ns}	194.75±7.50
Alanine Amino Transferase (U/L)	54.62±3.50*	60.87±4.02
TAG(mg/dl)	59.87±4.21*	51.87±5.01
Total Chlostrol (mg/dL)	67.12±4.44*	60.12±5.09

ns: non-significant, U/L: Unit/Liter and mg/dL: milligram/deciLitre

The activity of citrate synthase and cytochrome c oxidase as well as endurance running performance was measured in rats supplemented or non-supplemented with thyme extract. As shown in Fig. 1a, the activity of complex IV significantly diminished (around 30 %) in soleus muscle of rats treated with thymus extract relative to the non-supplemented group ($t_{10.012}$= 4.62, P=0.001). Approximately, the same pattern of significant variation (24% decrease) was found when we compared the activity of citrate synthase between two groups, Fig. 1b ($t_{8.11}$= 4.23, P=0.003).

Table 2. The effect of thyme extracts supplementation on (a) cytochrome c oxidase, (b) citrate synthase activity, and final endurance exercise performance.

Index	**Non-Supplemented (n=10)**	**Thyme-Supplemented (n=10)**
Cytochrome C oxidas activity (mU/ml)	3.057±0.12*	2.33±0.25
Citrate synthase activity (mU/ml)	18.61±2.5**	12.02±1.2
Mean final endurance (Minutes/L)	10.88±3.25	51.1±4.5**

Significant difference between Control and experimental group was marked with * (P < 0.05) or ** (P < 0.01).

In addition, the exhaustive running time of the rats in supplemented group was significantly prolonged (over three times longer) as compared to the non-supplemented group, Fig. 2 (t_{18}= 4.05, P=0.001).The present study has revealed that thyme supplementation significantly reduces the activity of citrate synthase (35%) and cytochrome c oxidase (24%), which are important for improving muscular endurance [27], compared to the non-supplemented group (respectively, t_{14}=0.0004 and t_{14}=0.001). These findings suggest that thyme supplementation may restrict the inducing effect of exercise training on citrate synthase and cytochrome c oxidase activity and mitochondrial biogenesis (Figs. 1, 2). This finding was consistent with earlier ones where different kinds of antioxidant supplements have been utilized, in which PGC-1α, NRF-1, Tfam and cytochrome c have been revealed to be down-regulated [28], [29].Regarding down-regulation of mitochondrial enzyme activities in the thyme extract supplemented group, as mentioned in some of the previous studies, it is possible that chronic intake of such a powerful antioxidant like thymus extract may hamper, and even inhibit the improving effect of exercise on physiological adaptations like mitochondrial biogenesis due to reduced reactive oxygen species (ROS). Based on the evidence currently available, it seems that appropriate ROS levels act as a biological messenger, initiating cellular signaling cascades via the process of electron transfer to promote adaptive responses within the body [30]. In fact, levels of exercise producing oxidative stress can result in the activities of enzymes and the activation of MAP kinases (p38 and ERK1/2), NF-κB, protein kinase B (Akt), mammalian target of rapamycin (mTOR), p70 ribosomal S6 kinase (p70s6K) pathway [31]. It is possible that the dosage of thyme extract used in the present study was high and alleviated the favorable effects of moderate exercise-induced ROS in producing beneficial cellular adaptation.In contrast to the activity of mitochondrial oxidative enzymes, our findings indicate that running performance of thyme extract supplemented group was significantly better than non-supplemented group, more than five times longer (t_{18}= 4.05, P=0.001). This challenging result suggests that thyme supplementation could elevate the exercise tolerance. Although there are studies examining the effects of some compounds with polyphenolic structure on the endurance performance, no such study has been conducted with any total plant extract [23], [32], [33]. The effect of thyme extract on endurance performance was investigated for the first time here and positive results were obtained. Such an improvement in performance seems to be related to cardiovascular, chemo-preventive, immunological, or other adaptations [31] rather than mitochondrial biogenesis (due to lower mitochondrial enzymes activities in supplemented group). In this regard, some researchers have also shown that PGC-1α KO mice have

regular voluntary running activity in spite of diminished basal mitochondrial respiratory activity [34] indicating that biogenesis may be helpful but not necessary in promoting endurance running performance. These contrasting findings could be related to compensatory accommodations in various tissues because of reduced mitochondrial enzyme activities.Furthermore, thyme supplementation has led to decreased hematocrit and mean corpuscular volume, both important in determining blood viscosity (not reported). It is well accepted that low blood viscosity has direct correlation with cardiac output, oxygen delivery to the tissue, maximum oxygen consumption, lipid oxidation during exercise, and endurance performance [35]. In this way, thyme extract supplementation can help athletes to improve their endurance capacity. On the other hand, in vitro studies have shown flavonoids are adenosine A1 receptor antagonist (such as caffeine, methyl xanthine, theophylline) which could be partly responsible for their stimulating and ergogenic effects [36]. A1 receptors have an inhibitory effect on most of the body tissues. In the brain for example, these receptors reduce metabolic activity and induce lethargy and sluggishness by inhibiting cholinergic receptors, decreasing the release of synaptic vesicles in the presynaptic and stabilizing magnesium on N -methyl-D-aspartate receptor. In the heart tissue, A1 and A2 adenosine receptors regulate myocardial oxygen consumption and coronary blood flow. Stimulating A1 receptor via decreasing the ability to conduct electrical impulses and repression of pacemaker cells ultimately declines heart rate and function. So, we can say that adenosine A1 receptors have an inhibitory effect on sympathetic tone and thyme extract may inhibit these receptors by increasing sympathetic activity [37] and may lead to improved exercise performance. However, for further clarification precise mechanisms by which thyme extract improves endurance exercise performance needs to be investigated at length. The liver is an important member of the natural maintenance of blood glucose and maintenance of blood sugar during exercise is important. An increase in liver enzymes, including alanine amino transferase (ALT), which is commonly associated with liver damage and inflammation [8]–[10], causes liver inflammation, which over time, disturbs the normal balance of metabolism. Intense exercise may act as a stress and increase liver enzymes and ALT, AST may increase during exercise so thyme supplement reduces this effect. The consumption of thyme does not allow these enzymes to increase. (Table 1). In addition, during the last weeks of present study, some rats of both groups (mostly non-supplemented group) did not cooperate in the training after the early stages of practice and were reluctant to continue for the rest of the training period even at low-intensities. This reluctance may be a sign of over-training in non-supplemented rats, which could be the evidence of increased oxidative stress. This condition was much lower in the thyme supplemented group. Taking into consideration that the above mentioned assumption is correct, it is possible that supplementation with thyme; as a strong antioxidant; helps in the antioxidant system of the rat body to prevent the harmful effects of too much oxidative pressure and subsequent over-training. It is necessary to prove by measuring indices likes levels of lactate, ammonia, urea, creatine kinase, the ratio of testosterone to cortisol and lipid peroxidation in basal resting condition[38] during the similar training periods and before beginning intense exercises.

4. Conclusion

Our findings suggest that prolonged antioxidant supplementation could potentially impair the endogenous metabolic and redox status of skeletal muscle in trained rats and prevent some of the beneficial adaptations to exercise training. Antioxidants attenuate mitochondrial biogenesis in the long-term, in particular, COX IV and citrate synthase activity possibly through an alternate, unknown mechanism. Our study contributes towards the comprehension of how antioxidants may interfere with adaptations to exercise, and the results suggest that high dosages of thyme extract should be digested with caution [27].Although thyme extract supplementation could not increase mitochondrial enzyme activities as a marker of mitochondrial biogenesis, the data revealed that the extract supplementation could elevate the exercise tolerance. Also the consumption of thyme does not allow liver enzymes to increase. However, more work is needed to understand the molecular mechanisms that may be activated by the presence of thyme extract in the diet to improve endurance performance. Furthermore, it is suggested that the samples should be taken at different times (immediately, 24 h and 48 h following exercise) and use lower dosages of thyme extract to better understand the effect of this extract on oxidative stress, lipid peroxidation, antioxidants levels and markers of mitochondrial biogenesis. We were not able to do this due to some local difficulties.

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