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The Effect of *Salvia Officinalis* Extract on p53 and Creatine Kinase Levels in Downhill Running: A Crossover Randomized, Double-Blind, and Placebo-Controlled Study

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Abstract

Background: Various nutritional supplements may play a role in reducing cell stress to intense exercise. In the present study, the effect of *salvia officinalis* extract on serum levels of p53 and CK after a downhill running was investigated

Methods: 14 healthy men (age, 24.4±3.5 yr; body mass index, 22.46±1.5 kg/m^2) were randomly placed in two conditions of sage and placebo. Subjects took 500 mg capsules of sage extract (two daily) or placebo for two weeks. After 14 days, subjects performed downhill running at 12% downhill grade at about 70% of the maximum heart rate until volitional exhaustion (30 minutes). Blood sampling was performed before and immediately after the running workout for the measurement of the serum p53 and CK concentrations.

Results: The results showed that in the sage condition, the changes in p53 levels in the before exercise compared to the after running were not statistically significant ($p \geq 0.14$). In the placebo condition, p53 levels in the post- running increased significantly compared to the pre- running ($p \leq 0.001$). Also, CK levels in the post-running only in the placebo condition increased significantly compared to the pre- running ($p \leq 0.007$).

Conclusion: The findings of the present study showed that downhill running as eccentric contraction may lead to cell apoptosis and muscle damage by increasing p53 and CK levels, but short-term sage supplementation is likely inhibiting increased apoptosis and muscle damage marker in serum induced by acute exercise.

Keywords: Eccentric contractions, Cellular programming death, Muscle damage, Sage

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1. Introduction

Acute and progressive exercise is inevitable in order to adapt to training in many sports. Exercise is a type of physiological stress that affects the concentration of various cytokines, hormones, growth factors and oxidative status. It was previously thought that these disorders were mainly due to inflammatory processes and necrosis, but some studies have shown that apoptosis plays an important role in different types of tissues (1). Intense exercise can lead to apoptosis by increasing factors such as glucocorticoids, reactive oxygen species (ROS), intracellular calcium, and inflammatory factors (2). Apoptotic signaling induces apoptosis through cytokines/Fas-driven, mitochondrial-driven and endoplasmic/sarcoplasmic reticulum /Ca^{2+}-driven pathways (1). Although the exact mechanisms of exercise-induced apoptosis are unclear, exercise increases in mitochondrial-mediated apoptotic pathways including pro-apoptotic protein levels such as Bax, Bax/Bcl-2 ratio, initiator caspases (i.e., caspase-8, caspase-9, and caspase-12), leads to the activation of effector caspases (i.e., caspase-3, caspase-6, and caspase-7) responsible for DNA fragmentation (3, 4). DNA damage leads to the accumulation of p53 protein, the specific biological roles attributed to p53 are very complex, but p53 physiological levels eventually improves the DNA status (5). However, if this process is not performed properly or the DNA defects level is high, the p53 protein initiate the transcription of apoptotic contributing factors (6). p53, is a general sensor for detecting DNA damage (7) and transcribing genes to stop cell cycle (p21, GADD45) and apoptosis

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(eg, Bax, Fas, IGF-bp3, APAF-1, Bad) (6). p53 is the major cellular responder for intrinsic stress signals that can impair telomere function by suppressing factors affecting telomere composition, thereby indirectly leading to the development of cell apoptosis (5, 8).

On the other hand, evidence suggests that taking some herbal remedies may play a role in reducing stress and cell damage (9). Recent research has focused on the polyphenolic antioxidants found in some spices and herbs, such as *salvia officinalis* (sage), rosemary, and thyme. Replacement of synthetic antioxidants with natural compounds, if effective, may have a positive effect on the treatment of various human injuries (9). Sage is one of the largest species of the mint family, which has more than 900 species in the world and is widely used in traditional medicine around the world due to its biological activities, including antibacterial, antispasmodic, hemostatic, cytotoxic and anti-cancer (10). In addition, sage are used to treat of various disorders such as tuberculosis, psoriasis and eczema (11). Sage contains several active compounds such as toyon, cineole, borneol, pinene, flavonoids, saponins, glycosides, resins, vitamin C, vitamin E, tannins, gums and diterpene (10, 12). Among the components of sage, carnosol, rosmanol, epipresmanol, isorosmanol, galdosol, and carnosic acid have remarkably strong anti-inflammatory and antioxidant activity similar to α-tocopherol (12, 13). Rosmarinic acid and carnosol are the main constituents of all phenolic antioxidant extracts isolated from sage (14). In addition, a study by Jantova et al. (2014) showed that sage extract in culture medium leads to increased apoptosis (caspase 3, caspase 8 and caspase 9) in cancer cells (15).

Although not always obvious, eccentric muscle contractions are an integral part of most movements during sport activities (16). Acute eccentric exercise reduced muscle function, induce soreness, mechanical tearing, metabolic stress, the local and systemic inflammatory and elevate muscle Bax protein levels (17) and caspase-3 serum (18). Furthermore, eccentric exercise research indicates that ROS is evident following muscle damaging exercise (19). Eccentric exercise result in significantly greater muscle damage. When muscle is damaged by intense and unaccustomed exercise including muscle-group targeted eccentric resistance exercise, downhill running and plyometric jumping, there is disruption of the sarcolema allowing muscle proteins such as creatine kinas (CK) and myoglobin to be released from the cell into the blood stream (20).

To the best of our knowledge, however, the effects of sage extract on exercise-induced apoptosis and muscle damage have not been studied following an acute exercise session. Therefore, the aim of the present study was to investigate the effect of sage extract on serum levels of p53 and kinase CK following a downhill running session in young recreational athletes. We tested the hypothesis that sage extract can modify exercise-induced apoptosis and damage.

Methods

Subjects

Fourteen healthy untrained males volunteered to participate in the study. Following an explanation of all procedures, possible risks, and benefits, each volunteer gave his informed consent before participation in this research. The inclusion criteria for this study were as follows: (1) men aged 20 to 30 years; (2) normal, defined as a BMI 18.5 to 25, kg/m^2; (3) lack of regular exercise training in the previous year. As per the self-reported physical activity data based on the International Physical Activity Questionnaire (IPAQ), (21) subjects were recreationally active (i.e. performed physical activity 1–2 days per week) but non-trained (i.e. no regular training for at least 1 year). Exclusion criteria for this study were as follows: (1) use of androgens/drugs or nutritional supplements within the previous 6 months; (2) any disease or other condition that restricts ability to exercise safely; (3) taking medicine, cigarettes and tobacco. We excluded females' subjects because of gender differences in exercise-induced apoptosis due to eccentric exercise (17). Table 1 shows the physical characteristics of the participants. The Institutional Review Board of the University approved the research protocol.

Table 1. Physical characteristic of the subjects

Variables	Mean± SD
Age (y)	24.4±3.5
Weight (kg)	70.1±7.9
Height (cm)	176.7±6.7
Body Fat (%)	18.9±5.1
BMI (kg.m$^{-2)}$	22.46±1.5

Experimental design

This trial was a randomized, double-blind, placebo-controlled, crossover. The subjects were completed two familiarization sessions. During these sessions, the subjects were introduced to the research protocol and how to perform the tests, and the physiological characteristics of the subjects were also measured. The subjects were randomly assigned into one of two exercise condition: sage extract (SA) or placebo (PL). Subjects ingested 2 × 500 mg capsules (total 1000 mg of sage extract) of concentrated GOLDARU SALVIGOL extract or a visually identical placebo for 14 days. Each 500 mg SALVIGOL capsule contained dry extract of young shoots of sage (Goldaru Company, Health Ltd., Isfahan, Iran). Each placebo capsule contained 500 mg the combination of maltodextrin and dark flour. Two capsules were consumed after lunch and dinner. The two experimental conditions (sage extract and placebo) were separated by a 3-week washout period (22). Consumption of beverages such as green tea and/or any supplements that included polyphenols were prohibited throughout the study. Prior to downhill running tests, subjects walked on the treadmill at a starting speed of 1.5 km/h at 0° gradient for 5 min (as warm-up), this speed was then gradually increased to the determined running speed. Subjects ran at 12% downhill grade; this speed was then gradually increased until reaching about 70% of each subject's maximum heart rate (calculated as 220 – age). This speed was then maintained to 30 min continuous until volitional exhaustion. Heart rate was

recorded using a pulse oximeter connected to a treadmill. Park et al. (23) reported an increase in exercise-induced apoptotic markers with a similar protocol. The duration of the downhill continuous until volitional exhaustion (24). The subjects were encouraged to adhere to their normal dietary patterns throughout the study. Subjects were instructed to abstain from drinking alcohol and caffeine for 24 hours before the assessment. Twenty-four-hour diet recall were used to assess diet and subjects were instructed not to change their eating. Dietary intake is shown in Table 2.

Table 2: Comparison of dietary intake variables between sage extract and placebo condition.

Variable	*mean ± SD*		P-values
	Sage extract	Placebo	
Energy intake (Kcal)	2696.1±416.7	2719.3±612.7	0.234
Protein (g)	78.2±42.7	82.5±61.7	0.326
Carbohydrate (g)	401.2±93.5	382.2±96.4	0.176
Fat (g)	86.5±73.8	95.6±81.1	0.204

(P ≤ 0/05).

Blood samples

Blood samples (about 7 ml of blood from an antecubital vein) were collected from all subjects before and after the running. Samples were centrifuged at 3000 rpm for 15 minutes and serum was separated from blood cells and stored at -20 °C until analyzed. p53 protein was measured using Bioassay Technology laboratory kits by ELISA method. The intra- and interassay coefficients of variance was less than 10%. CK levels were measured using Pars Azmoun Company kits (Tehran, Iran) with a unit sensitivity and an auto-analyzer.

Statistical analysis

The assumption of a normal distribution for all data was verified using the Shapiro–Wilk test, and all data were normally distributed. Data were analyzed using two-way analysis of variance (2 time × 2 condition) with repeated measures. When the main effect or interaction was significant, the paired *t*-test with *Bonferroni* correction was used to identify significant differences among the mean values. All data were reported as mean ± SD. All statistical analyses were conducted using the statistical package for Social Sciences (SPSS, Version 22.0).

Results

All subjects completed 30-minute exercise test. Changes of means and standard deviations for p53 and CK responses to running exercise after PL or SA condition are presented in Table 3.

Table 3: Serum level responses of p53 and CK to downhill running.

	Time	Condition	
		SA	PL
p53 (ng/l)	Before running	482.04 ± 47.90	487.02 ± 78.09
	After running	514.09 ± 87.39	590.88 ± 56.48*#
CK (U/L)	Before running	65± 13.91	56.70 ± 14.81
	After running	68.20 ± 12.53	74.10 ± 19.68*

. #: SA versus PL (p< 0.05); *: After versus Before (p< 0.05). SA, sage; PL, placebo.

Regarding p53, statistically significant time effects (F= 30.11, p = .0001, η2 =.59) and interaction (F=8.40, p=.008, η2 =.57) were found, however condition effect did not reach statistical significance (F= 3.13, p = .08, η2 =.41). Changes in serum p53 did not reach statistical significance for SA condition (482.04 ± 47.90 vs. 514.09 ± 87.39 ng/l by 6.64 ± 3.82%; p = 0.14). p53 increased (487.02 ± 78.09 vs. 590.88 ± 56.48 ng/l by 21.32 ± 4.07%; p = 0.001) after downhill running protocol in the PL condition, compared to before exercise. Immediately after running, there was significant differences between the PL and the SA in p53 (p = 0.01) concentrations.

Regarding CK, statistically significant time effects (F= 10.42, p = .0005, η2 =.63) and interaction (F=5.58, p=0.02, η2 =.51) were found, however condition effect did not reach statistical significance (F=0.58, p=.45, η2 =.27). In the SA condition, CK levels were slightly higher (not significantly) compared to before downhill running exercise (65.01 ± 13.91 vs. 68.20 ± 12.53 U/L by 4.9 ± 2.3%; p = 0.39). CK increased (56.70 ± 14.81 vs. 74.10 ± 19.68 U/L by 30.68 ± 13.74%; p = 0.007) after downhill running protocol in the PL condition, compared to before exercise. Immediately after running, there was no significant differences between the PL and the SA in CK (p = 0.43) concentrations.

Discussion

Herbal remedies have been used for thousands of years to treat various ailments and offer many health benefits, as well as the healing and restorative abilities of various types of herbs. One of the major reasons for the increase is the ease of using plants and ensuring an absolute result. Increased levels of oxidative stress and inflammatory factors are major causes of apoptosis and cellular damage during acute exercise, and athletes are looking for a substance that minimizes cell damage. Several clinical and experimental studies indicate that sage has anti-oxidant and anti-inflammatory properties (12). Therefore, we tested the possible effect of sage on the marker of exercise-induced apoptosis and cell damage in a severe eccentric exercise. The results showed that sage reduced exercise-induced apoptosis and damage muscle markers in serum induced by a single bout of eccentric exercise.

It is been well established that acute exercise may increase apoptosis markers and damaging muscle. Our results agree with those recently reported in the literature (20, 25). The downhill running exercise proposed in the current study was able to increase serum p53 and CK. The possible mechanisms of the effect of eccentric exercise on apoptosis are not fully understood. However, the present study found that 30 minutes of eccentric exercise was sufficient to stimulate apoptosis signaling (increased p53 levels). Eccentric exercise has been shown to increase 3-muscle caspase (2) and Bax concentration (23). In addition, serum caspase-9 and p53 increased immediately after intense resistance

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activity in young men (26). Also, the results of our study are consistent with the study of Rahimi et al. (2015), which was performed on young athletes using progressive endurance exercise to fatigue, in which acute exercise increased blood p53 compared to before exercise (27). Sheikholeslami-Vatani et al. (2018) showed that the concentrations of caspase-9 and caspase-3 increased significantly after a downhill session in the placebo condition (18). The increase in p53 in this study is also consistent with the results of another study that showed elevated serum levels of p53 and caspase-9 after resistance activity in young men (28). Another study reported that p53 concentration increased in young men immediately and three hours after a session of resistance exercise (25).

Another result of the present study was that a 21.32% increase in p53 was significant only in the PL condition, while in the SA this increase was only 6.64%. When a cell is exposed to severe stress, p53 can activate numerous genes which increase ROS generation, thus leading to apoptosis (5, 29). ROS generated by severe stress can further activate p53 in a positive feedback loop (5). Salvia seems to have prevented an increase in p53 during eccentric exercise. To our knowledge, no studies to date have examined the effects of sage supplementation on the levels of markers of apoptosis after eccentric exercise. Previous studies have shown the anti-cancer, antioxidant and anti-inflammatory effects of sage (15, 30, 31). Salvia extract showed anti-apoptotic and growth inhibitory effects on cancer cell lines (32). Salvia extract also increases the release of TNF-α and nitric oxide from macrophages, thus increasing its cytotoxic effect (33). These effects may be due to the presence of several cytotoxic and anti-cancer compounds in sage (12). Among terpenes and terpenoids isolated from sage, caryophyllene and α-homolen have been shown to inhibit the growth of tumor cells in breast and colon cancer (34). The anti-cancer effects of sage appear to be due to inhibition of mitogen-activated protein kinase, suppression of ROS and nuclear transcription factor-kappa B (NF-κB), and decreased expression of the anti-inflammatory gene cyclooxygenase-2 (35). It also inhibits several stages of angiogenesis (proliferation, migration, adhesion, and tube formation) in endothelial cells (30). In addition, evidence from several studies shows that sage has strong antioxidant activity. Horvatova et al. (2016) showed that consumption of sage extract has a positive effect on the resistance of rat liver cells to oxidative stress and may have potential for liver protection (36). This protects hepatocytes against oxidative stress and DNA damage caused by hydrogen peroxide by increasing glutathione peroxidase activity (37). The most effective antioxidant compounds are sage carnosol, rosmarinic acid and carnosic acid (38). In addition to rosmarinic acid, other sage flavonoids, especially quercetin and rutin, have potent antioxidant activity (39).

On the other hand, flavonoids and terpenes are compounds that probably contribute to the anti-inflammatory action of this plant. Mansourabadi et al. (2015) reported that flavonoids extracted from sage reduce inflammation in mice and have a dose-dependent analgesic effect (40). Osakabe et al. (2004) showed the topical application of rosmarinic acid in inhibiting epidermal inflammation (41). Also, Mannol, carnosol and ursolic acid are terpenes that have anti-inflammatory potential (42). The original contribution of the present study is the protective effect of sage shown in vivo against apoptosis induced by acute exercise. Although the present study was not designed to investigate the possible mechanism of sage effect, but overall, it seems that due to the direct antioxidant/anti-inflammatory role of sage, this herbal medicine may reduce ROS production and DNA damage, which prevented the increase of p53 levels. Indeed, p53 can activate numerous genes which increase ROS generation, thus leading to apoptosis (5, 29). ROS generated by severe stress can further activate p53 in a positive feedback loop (5). Additionally, pro-inflammatory signals upon stress could establish self-perpetuating pro-inflammatory cycles leading to DNA damage (43). Due to the limited number of studies in this field, more research is needed to investigate the effect of sage on apoptotic markers.

CK is often used as a clinical marker of muscle damage (44). In the present study, CK levels in the SA condition did not increase significantly compared to the before exercise, but in the PL condition there was a significant increase. The sage appears to attenuate muscle damage by inhibiting CK in the acute exercise. Previous studies supports the role of the skeletal muscle intermediate filaments as a stress-transmitting and stress-signaling network (16). Cytoskeletal proteins help mitochondria not only in their movement and proper cellular positioning, but also to maintain their biogenesis, morphology, function, and regulation of energy fluxes (16). The functionality of these cytoskeletal proteins may thus influence the mitochondria functions, including the regulation of Ca^{2+} signals and apoptosis (45). Eccentric exercise-induced muscle damage can lead to calcium imbalance in and around muscle cells, resulting in activation of calcium-dependent calpains (46). Through this pathway, caspase-12 is activated, causing a caspase cascade, resulting in activation of caspase-3, independent of cytochrome C and Apaf-1 (47), which ultimately causes DNA damage and an increase in p53. Furthermore, activation of inflammatory cells such as neutrophils and lymphocytes during exercise due to muscle tissue damage can increase the production of superoxide, which can cause direct damage to DNA (48). Although more study is needed, our results suggest that cell protection by sage is associated with attenuate of p53 and CK.

Only one study in rodents examined the effect of sage consumption on CK levels (31). In this study, it was shown that mice that consumed sage extract for six weeks (daily 100 mg / kg) had a significant reduction in CK compared to the control condition, while receiving a daily dose of 150 mg / kg of sage extract did not cause a significant change in serum concentration of CK. These results suggest that the dose of sage may affect CK levels. It will be of interest to do additional studies on

different dose sage in combination with acute eccentric exercise at human studies.

This study was not without limitations. In this study markers of oxidative stress and inflammatory were not measured. Although, traces from cellular apoptosis can be detected from the circulation by measuring serum markers (49, 50), tissue biopsies were not obtained. Finally, we only considered p53, as a general sensor for detecting DNA damage and apoptosis. Thus, future studies, should address the effect sage on different tissues, extrinsic and intrinsic cell factors/pathways affecting exercise-induced apoptosis and apoptosis inhibitor.

Conclusion

Although more work is warranted to describe accurately the effects of sage on the assessed factors in this study, our data suggest that two weeks of sage extract supplementation inhibited increased p53 (as apoptosis marker) and CK (as muscle damage) induced by downhill running. The exact mechanism/s by which the sage extract exerts its cellular protection effect against exercise-induced apoptosis is unknown, however our results suggest that cell protection by sage is possibly due to the direct antioxidant/anti-inflammatory role of sage. Further experiments are necessary to determine the protective effect and possible mechanisms of sage supplementation after eccentric exercise.

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