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Researching the Effects of Ellagic Acid on Depletion Exercise

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Authors' contributions

This work was carried out in collaboration between all authors. Author EK designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors FKC and MA designed the study and managed the analyses of the study. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

Introduction: The purpose of this study was to investigate whether exhaustion exercise causes endothelial damage and to investigate the effect of ellagic acid on this potential damage.
Methods and Materials: The study was carried out on 32 male and adult Spraque - Dawley rats at Experimental Animal Research and Research Center of Afyon Kocatepe University. The experimental animals were equally divided into four groups. Swimming exercises will be performed as acute exercises for once and experimental animals are made to swim in groups including two rats following the completion of the study and before the decapitation. At the end of procedures that last for four weeks, total oxidant (TOS), total antioxidant (TAS), nitric oxide (NO) and Asymmetric

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dimethyl Arginine (ADMA), TNF α, INF γ and IL6 plasma and tissue levels was assayed from the blood samples to be taken from experimental animals with decapitation.
Results: The data obtained as a result of the studies were evaluated by using SPSS 20.0 statistical program. Endothelial damage markers and cytokine levels were significantly ($p<0,05$) increased in swimming groups and ellagic acid significantly ($p<0,05$) decreased these parameters.
Conclusion: Ellagic acid may have protective effect against damage that may occur during exhaustion exercise.

Keywords: Rat; ellagic acid; endothelial damage.

1. INTRODUCTION

Throughout the grape fruits, there are high concentrations of phenol and flavonoid compounds containing gallic acid, routine, isoquercitrin and ellagic acid. The most important of these compounds is ellagic acid, which is reported to prevent tumor growth on animal studies in laboratory conditions. The maximum amount of ellagic acid is in black raspberry [1,2].

Ellagic acid not only is a phenolic acid with a strong anticarcinogen and antimutagenic effect but also have antibacterial and antiviral effect [3,4]. As a result of some chemical processes of ellagitannin, ellagic acid is formed [5]. Ellagic acid should be in a suitable form for detection and use by a cell. This form is a result of the merger with the sugar molecules [6]. Ellagic acid, which is found in most red (*Rubus ideaus*) and black *(Rubus occidentalis*) raspberries in all fruits and vegetables, shows an anticarcinogenic effect by inactivating the chemicals that cause cancer in the body [7]. Several studies conducted on humans have reported an increase in the amount of free radicals during exercise [8]. Increased oxygen consumption during exercise causes an increase in free radical production [9]. Primary sources leading to free radical formation are prostanoid metabolism, electron leakage from mitochondria during aerobic respiration, catecholamines, xanthine oxydase, NADPH oxidase enzymes. Secondary sources are phagocytic cells, degradation of iron-containing cells and excessive calcium accumulation.

Exercise can lead to an increase in radical formation through many different mechanisms [10,11].

Acute exercise leads to the formation of free radicals depending on the severity, duration and type of exercise. Severe physical exercise is known to increase the production of free radicals in skeletal muscles and other tissues. Studies have shown that increasing intensive exercise increases the production of free radicals, while regular exercise strengthens the antioxidant defense mechanism [12,9].

The endothelium is an active structure that takes part in blood pressure, clotting and inflammatory events and provides homeostasis. The most important element showing endothelial function is NO with two atom molecules. Endothelial dysfunction is characterized by a decrease in the bioavailability of vasodilating agents; the most obvious one is the reduction of NO (23). This imbalance causes a decrease in endothelium-dependent vasodilatation, which is characteristic of endothelial dysfunction. NO is involved in vascular tone, inflammation, coagulation and oxidation with other substances. If these are not properly regulated, they may lead to vascular structure deterioration, subclinical atherosclerosis and eventually cardiovascular disease. The endothelium protects vascular homeostasis by providing local mediators that regulate vascular tone, platelet adhesion, inflammation, fibrinolysis, and vascular proliferation. Disruption of endothelial function negatively affects these events and causes cardiovascular risk [12].

Advances in this area suggest that oxidative stress facilitates vascular dysfunction mechanisms.

In the case of oxidative stress, endothelial cells lose protective phenotypes and synthesize proinflammatory molecules [13]. In the light of this information, it was aimed to evaluate the effects of ellagic acid on oxidative stress and possible endothelial damage in rats with acute exhaustion model and to make a contribution to the literature.

2. MATERIALS AND METHODS

2.1 Experimental Animals

The study was performed on 32 Wistar Albino adult male rats at the experimental animals

center of Afyon Kocatepe University. This study was approved from ethical committee (ref no, AKU-HADYEK-450-15). The experimental animals were divided into 4 equal groups as 5 rats in each cage. 10 days prior to the study, the experimental animals under observation were allowed to adapt to the experimental environment. Animals were kept in 12 hours of darkness and 12 hours of light. During the study, all subjects were kept under equal environmental conditions and subjects were given standard rat feed and water ad libitum throughout the study.

The feeding was scheduled twice at 09.00 A.M and 7.00 P.M. Ellagic Acid provided from Sigma-Aldrich.

Group 1: General control group with no application.

Group 2: Acute swimming exercise control group.

Group 3: The group that was applied 15 mg / kg / day Ellagic Acid **(**EA) EA for 4 weeks and performed acute swimming exercises at the end of the application.

Group 4: The group that was applied 15 mg / kg / day Ellagic Acid (EA) for 4 weeks and was not performed acute swimming exercises at the end of the application.

2.2 Exercise Protocol

Acute swimming exercise was performed in a thermostat glass swimming pool with a height of 50 cm, width 50 cm and a length of 100 cm. The pool was made of heat-resistant glass, which allows the water to remain constant at 36.5 ± 0.5 ^{o}C. Swimming exercises were performed as one-off acute exercises and experimental animals were swum in pairs at the end of the study. In case the rats were suspended in water surface, they were directed to swim with a thin long rod. The initiation of movements without coordination and immobilization for 10 seconds under water were considered as exhaustion criteria of rats [14].

2.3 Termination of the Study

At the end of 4 weeks, the rats were anesthetized with sodium pentobarbital (40 mg / kg, i.p.) and then the blood samples taken from hearts and tissue samples were prepared for biochemical analysis. Blood samples were taken into EDTA and anticoagulated tubes and immediately centrifuged at 4000 rpm for 10 minutes. The plasma obtained separately from each sample was divided into 3 portions for ELISA and spectrophotometric analysis and stored at -20°C.

3. BLOOD AND TISSUE ANALYSIS

3.1 Preparation of Liver Tissue Homogenates

Liver tissue samples stored at -20°C were thawed to homogenize prior to ELISA studies. The homogenization process was optimized by modifying the homogenization method stated by Yildirim and Yurekli (2010). Approximately 0.5 g of liver tissue to be homogenized was weighed and taken into 5 ml pH 7.4 cold PBS buffer. The tissue sample taken in cold PBS buffer was homogenized in ice using the IKA Ultra Turrax T18 Basic homogenizer for about 1 minute. After homogenization process, the samples were sonicated at 450 sonifier for 20 seconds and waited 10 seconds at each end for sonication by using Geprüfte Sicherheit UP 50H Sonicator.

Homogenized tissue samples were separated equally into 2 ml ependorf godets and centrifuged at 15000 g for 10 minutes in refrigerated ultracentrifuge (+4°C). After that the supernatants were collected and separated for 3 portions. The portions were frozen again at -20°C for ELISA [15].

3.2 Total Protein Analysis from Tissue Homogenates

Tissue homogenates were diluted with sterile distilled water and then used for total protein analysis. 5 µl of diluted tissue homogenate was added to the ELISA plate wells. Then 100 µl of Coomassie Brillant Blue (CBB) reagent was added to homogenates and total protein analyzes were performed by spectrophotometric measurement at 630 nm wavelength using the Thermocycle ELISA Reader. The data obtained were corrected by a two-fold dilution coefficient.

3.3 Nitric Oxide Level Measurement

Nitric oxide levels were measured by using the ELISA kit. The principle of the technique is based on the reaction between the antigen and the antibody. The unlabeled antigen and the labeled

antigen compete to react with the antibody. The unsigned antigen is the substance to be determined. The enzyme is used in the preparation of the labeled antigen. Therefore, this method is called enzyme immunoassay. Alkaline phosphatase and Horseradish peroxidase are the most commonly used enzymes. After the completion of the reaction, separation is carried out and the enzyme activity is measured by adding substrate. The analyte concentration is determined from the relationship between the enzyme activity and the substance to be determined.

3.4 Total Antioxidant Capacity (TAC) Analysis

According to the method developed by Measurement Erel (2005), Diagnostics Total antioxidant status kit was used and 10 minutes after sample and reagents are mixed, kinetic reading was performed on the spectrophotometer. The method is based on the principle that the antioxidants present in the sample convert to the ABTS molecule by reducing the radical cation of dark green colored 2,2'-azinobis-3-ethylbenzothiazoline6-sulfonic acid (ABTS +) in the reagents used. Depending on the amount of antioxidant contained in the sample, as the reduction rate of ABTS + to ABTS increases, the dark green color begins to turn white. The change of absorbance values readings at the spectrophotometer at 660 nm depends on the total antioxidant levels found in the sample.The measurement is calibrated using a standardized antioxidant solution called Torolox Equivalent, a vitamin E analog and usually used in TAC assays. Measured TAC levels were expressed in mmol Trolox Equivalent / L [16].

3.5 Total Oxidant Capacity (TOC)

According to the method developed by Measurement Erel (2004), Rel Assay Diagnostics Total Oxidant Status kit was used and 10 minutes after sample and reagents are mixed, end-point reading was performed by using the spectrophotometer. The method is based on the conversion of oxidants in the sample to ferric (Fe + 3) form by oxidizing the ferrous (Fe + 2) ion complexes in the reagent used in the analysis.
The ferric ions, which are increased by the oxidation reaction, form a colored complex with the chromogen (which is colorless, but which creates a colored compound with effects such as oxidation) in the acidic environment of the reaction. The intensity of the resulting color varies depending on the total amount of oxidizing agents present in the sample. These changes are determined by spectrophotometer reading at 560 nm.

The TOC assay was calibrated with hydrogen peroxide (H_2O_2) and the results were expressed as hydrogen peroxide equivalent liter (μmol H_2O_2 Equiv./L) [16].

3.6 Measurement of Asymmetric Dimethyl Arginine Levels

ADMA levels were measured by colorimetric method using ELISA (mouse / rat) kit.

3.7 Measurement of Interferon Gamma (IFNγ) TNF-alpha, Interleukin 6 (IL-6) Levels

Proinflammatory cytokines IL-6, TNF-α and IFN-Rat were measured by using Rat ELISA kits and thermo biotech ELISA Reader in both tissue homogenates and serum samples.

3.8 Oxidative Stress Index

OSI value was accepted as % of TAK and TOD values. First, the TAC values was converted to mmol / L. OSI value was calculated according to Formula method. OSI (Arbitrary Unit)=TOS (mmol H_2O_2 Equiv./L)/TAK (mmol Trolox Equiv./L) (38).

3.9 Statistical Analysis

The data obtained as a result of the studies were evaluated by using SPSS 20.0 statistical program. The data are expressed as mean ± standard errors. The normality tests of the obtained data were done and variance analysis (Anova test) and Duncan test were used to determine the statistical differences between the groups. For statistical significance, $P < 0.05$ was chosen.

4. RESULTS

Biochemical analysis of blood and tissue samples were performed after four weeks of experimental study.

Experimental study was taken time for four weeks and biochemical analysis of blood and tissue samples were performed.

4.1 Plasma Nitric Oxide (NO) Levels and ADMA Levels

Plasma NO values of the subjects who were subjected to exhaustion exercise are shown in Table 1. Plasma NO levels were significantly higher in the exhausted exercise group than the control group ($p < 0.05$). In the EA + exercise group, NO levels were significantly lower than the exhaustion exercise group ($p < 0.05$). Plasma ADMA levels were not shown any statistical significance between the groups.

4.2 Plasma TAS and TOS Values

The plasma and tissue TAS and TOS values of the groups were significantly lower than the totaloxidant capacity control group ($p < 0.05$).

4.3 Plasma and Tissue Cytokine Levels (IL-6 TNFα INF-γ)

Plasma and liver tissue of exhausted exercise rats were given IL-6 and TNFα levels in Tables 3,4. These levels were significantly lower in the EA + exercise group than in the exercise group.

5. DISCUSSION

In this study, damage caused by exhaustion exercise applied to rats, its immune responses, changes in blood parameters and the protective effects of Ellagic acid on this possible damage were investigated.

Regular and continuous physical activities are considered important for health.

Table 1. Plasma NO, ADMA, the difference between groups with different letters in the same column is important

Groups	NO (µM)	ADMA (µmol/l)
Control	9.81±0.89[a]	0.34±0.02
EA	9.29±0.77[a]	0.34±0.01
Swimming	16.36±1.25[b]	0.36±0.02
EA+Swimming	12.62±0.25[c]	0.34±0.01

Table 2. Plasma TAS, TOS, ISO, levels, a, b, c: The difference between groups with different letters in the same column is important. P<0.05

Groups	TAS (mmolTroloxequivalents/L)	TOS (mmolTroloxEquiv./L)	OSI (ArbitraryUnite)
Control	1.163±0.094[a]	8.876±0.954[b]	6.70±0.14
EA	1.358±0.145[a,b]	6.803±0.941[a,b]	5.59±0.16
Swimming	1.604±0.142b'	5.891±0.087[a]	4.49±1.32
EA+Swimming	1.922±0.103	5.941±0.374[a]	4.11±0.50

Table 3. Plasma, INF γ, IL-6 TNFα a, b, c: Differences between groups with different letters in the same column are important P<0,05

Groups	IL-6 (ng/ml)	TNF-α (ng/ml)	INF-γ (ng/ml)
Control	2128±249[b]	*41.88±.,42*[a]	*106*±25[a]
EA	2137 ±333[b]	41.03±6.18[a]	112±32[a]
Swimming	2784±255[a,b]	61.60±8.39[b]	88±15[a]
EA+Swimming	2976±117[a]	57.50±4.20 [a,b]	126±26[a]

Table 4. Liver tissue, INF- γ, IL-6 TNFα a, b, c: The difference between groups with different letters in the same column is important P<0.05

Groups	TNF-α (pg/mg protein)	INF-γ (pg/mg protein)	IL-6 (pg/mg protein)	NO
Control	0.026±0.004[b]	0.071±0.006	3.38±0.32[b]	42.90±0.78[a]
EA	0.028±0.004[a,b]	0.070±0.009	3.06±0.57[a,b]	46.42±0.77[a]
Swimming	0.044±0.0007[a]	0.069±0.008	2.01±0.05[a]	51.72±1.05[b]
EA+Swimming	0.035±0.001[a]	0.071±0.005	2.42±0.24[a,b]	44.50±2.10a

However, irregular or acute exercises may lead to an increase in ROS (Reactive Oxygen Species) production and oxidative damage may occur in parallel with this increase [17].

As a result of severe exercises, the body's need for oxygen increases. This increase can be increased up to 20 times according to the resting level, and the oxygen use of the active muscle fibers may increase by 200 times [18]. Mitochondrial metabolic leakage due to excess oxygen consumption may lead to an increase in free radical production [19].

The metabolic rate is increased in proportion to the intensity of muscle activity. The severity and duration of exercise may cause differences in oxidative stress. However, if the increase in the production of free oxygen radicals exceeds the defense capacity of the cells during the exercise, oxidative damage occurs [19]. As a result of the free radicals formed, the polyunsaturated fatty acids in the membrane structure undergo oxidation and as a result, lipid peroxidation develops.

Oxidative stress occurs as a result of the increase in ROTs in the body and the decrease in antioxidant defense system. Oxidative stress has been shown to play an important role in the pathognomy of aging and many diseases including coronary heart disease. In our study, we measured the total oxidant and total antioxidant capacity of blood samples in order to determine whether exhaustion exercise causes oxidative stress in rats. As a result of our measurements, total oxidant capacity levels were found higher and total antioxidant levels were found lower compared to the control groups ($P<0.05$).

When the studies about the subject are examined, it was determined that swimming exercise led to an increase in lipid peroxidation in rats [20]. (2008) applied swimming exercises induced oxidative stress in rat tissues and supported our results.

Again, Biçer et al. [21] investigated the effect of zinc supplementation on lipid peroxidation and lactate levels in diabetic rats exposed to streptozotocin and acute swimming exercises. In their study on 80 male rats, when they evaluated the results of lipid proxidation products as a result of swimming exercises performed in the 4th week of the study, they determined that acute swimming exercise increased lipid peroxidation and free radical formation. Akil et al. [22] investigated the effect of selenium supplementation on lipid peroxidation and lactate levels in rats with acute swimming exercises. 32 Sprague-Dawley type male rats were divided into 4 groups. Group 1; Control, Group 2; selenium support, Group 3; Swimming control, Group 4; Selenium swimming group. According to the results of the study, MDA levels increased due to acute swimming exercise [22]. The presence of MDA reflects the degree of lipid peroxidation caused by reaction with free radicals. Therefore, it has been reported in various sources that maximal exercise leads to the formation of significant free radicals [22].

We also evaluated the levels of nitric oxide and asymmetric dimethylarginine. As a result of our measurements, we observed a significant increase in NO level of exhaustive exercise groups but we did not see any statistical significance in ADMA levels. According to the results, we can explain that free radical formation induced by exhaustion exercise is compensated by NO increase in the effect of oxidative stress. According to the result, we can explain that the free radical formation induced by exhaustion exercise is compensated by NO increase of the effect of oxidative stress.

This result suggested that NO may also act as an antioxidant [23]. Also, it was shown that nitric oxide combines with superoxide and forms a toxic oxygen metabolite, peroxynitrite. This situation leads to tissue damage [24]. All these results suggest that NO is still a fully unexplained compound with both bioavailable and harmful effects.

In our study, we examined some cytokine levels and evaluated whether there were any changes in their levels with swimming training. Our results showed that TNF-α and IL-6 levels were significantly elevated in blood and liver tissue samples of swimming groups. It can be explained that the exhaustion exercise of our findings causes oxidative stress. Rabinovitch et al. (1996) suggest that some cytokines, such as IL-1β, TNF-α and IFN- γ, induce free oxygen radicals and cause toxic effects by producing lipid peroxidation and aldehyde.

In our study, perhaps the most remarkable result was obtained from the results of Ellagic acid and exercising group. According to this, it was found that increased TOS levels with ellagic acid supplementation were decreased significantly

and TAS levels were increased significantly. In the studies, we can think that the action mechanism of ellagic acid inhibits lipid peroxidation by acting on free radicals, blocking lipid chain reactions. We think that Ellagic acid does this effect with its antioxidant properties.

6. CONCLUSION

As a result, it was observed that oxidative stress developed after acute exhaustion exercise and ellagic acid could decrease that developing oxidative stress. At the same time, it has been determined that proinflammatory cytokine levels are accompanied by increased oxidative stress and that Ellagic acid reduces increased cytokine levels by showing anti-inflammatory effect.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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