ORIGINAL ARTICLE

Quercetin and caffeic acid phenethyl ester (CAPE) attenuate acute exercise-induced oxidative stress

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Summary. Flavonoids are naturally occurring antioxidant molecules that are abundantly existing in the human diet. We investigated effects of quercetin and caffeic acid phenethyl ester to redox balance in acute treadmill exercise. Malondialdehyde, superoxide dismutase activity, xanthine oxidase activity and total nitrite in blood, skeletal muscle and liver were analyzed in 58 male Sprague-Dawley rats that were exposed to a single bout of exercise. We found that exercise provoked the increment of analyzed parameters except for total nitrite which indicates nitric oxide. Quercetin and caffeic acid phenethyl ester decreased malondialdehyde levels in skeletal muscle and liver. However, they were ineffectual in preventing lipid peroxidation in blood probably due to limited repair and biosynthesis capability of erythrocytes. Since quercetin and CAPE successfully weakened oxidative stress in liver and skeletal muscle, they may be seen as promising in preventing acute exercise-induced oxidative injury.

Key words: Oxidative stress, quercetin, caffeic acid phenethyl ester, CAPE, acute exercise

«Quercetina e acido caffeico fenetil estere (CAPE) attenuano lo stress ossidativo indotto da esercizio fisico prolungato»

Riassunto. I flavonoidi sono naturalmente presenti nelle molecole antiossidanti che esistono in maniera preponderante nella dieta umana. Abbiamo studiato gli effetti di quercetina e dell'acido caffeico fenetil estere (CAPE) sull'equilibrio redox durante un esercizio fisico prolungato di routine. Sono stati analizzati la malondialdeide, l'attività della superossido dismutasi, l'attività della xantina ossidasi e i nitriti totali nel sangue, nel muscolo scheletrico e nel fegato di 58 ratti maschi Sprague-Dawley che erano stati coinvolti in un singolo round di esercizio fisico. Abbiamo scoperto che l'esercizio fisico ha provocato l'incremento dei parametri analizzati ad eccezione dei nitriti totali che indicano l'ossido nitrico. La quercetina e il CAPE hanno diminuito i livelli di malondialdeide nel muscolo scheletrico e nel fegato. Tuttavia, erano inefficaci nel prevenire la perossidazione lipidica nel sangue probabilmente per la limitata capacità di riparazione e biosintesi degli eritrociti. Dal momento che la quercetina e il CAPE hanno diminuito con successo lo stress ossidativo nel fegato e nel muscolo scheletrico, possono essere visti come elementi promettenti nella prevenzione del danno ossidativo indotto dall'esercizio fisico prolungato.

Parole chiave: Stress ossidativo, quercetina, acido caffeico fenetile estere, CAPE, esercizio fisico prolungato

Introduction

Oxidation processes in mitochondrial electron transport chain is vitally important for animal livings. Although consequences of oxidation like ATP synthesis, apoptosis, immune responses are critical for animation, concurring production of reactive oxygen species (ROS) can exhibit deleterious effects by attacking proteins, membrane lipids and nucleic acids (1).

In molecular perspective, loss of an electron

is known as oxidation while gain of an electron addresses to reduction. Thus, reductants are anti-oxidants and substances which oxidates are pro-oxidants. Due to coincidence of oxidation and reduction in livings, counterpoises of these processes constitute a "redox balance" (2). Even though cellular signaling is fundamental physiological role of ROS and in this way, they contribute to homeostasis (i.e. immunity, differentiation, autophagy, cell survival etc.) (3), disturbance of redox balance in behalf of oxidation causes an oxidative stress that is destructive to biological systems.

Primary source of ROS in eukaryotic cells is mitochondria. Main superoxide radical (O_2^-) generators are complex I and III of the electron transport chain (4) and occurring O_2^- is converted to hydrogen peroxide (H_2O_2), antecedent of highly reactive hydroxyl radical (OH^-) (5), with catalytic activity of superoxide dismutase (SOD).

Contribution of oxidative stress to perpetration of cancer, cardiovascular diseases, neurological pathologies, and psychiatric disorders (6) has risen considerations for health benefits of antioxidant molecules, relying on their enzyme activating or radical scavenging properties. Human diet is substantially rich in polyphenolic compounds and the most common subclass of this large family is flavonoids. Their anti-microbial, anti-diarrheal, anti-ulcer, anti-inflammatory, anti-hypertensive, anti-allergic, anti-hypercholesterolemic and several other effects have been reported (7). Flavonoids can be divided into six main groups: flavones, flavonols, flavanols, flavanons, isoflavones and anthocyanidins (8) and beneficial health effects are related to at least partly their antioxidant activity. Flavonoids' antioxidant properties depend on various mechanisms like scavenging ROS, inducting antioxidant enzymes, inhibiting oxidases, metal chelation (9).

Caffeic acid phenethyl ester (CAPE) is one of the active molecules of propolis, widely used substance in traditional medicine due to its antioxidant, anti-cancer and anti-inflammatory characteristics. CAPE is a polyphenolic compound that augments antioxidant enzyme activity, reduces tissue injury, attenuates ROS and inflammatory cytokine production, and induces apoptosis of cancer cells (10–12).

Quercetin, a flavonol, is the most common (7) and potent (13) molecule among flavonoids. Although there is a consensus in medical literature on its *in vitro* antioxidant activity, there are contradictory results about its effects on exercise performance (14–16). Nevertheless, in addition to anti-atherosclerotic (17, 18), anti-inflammatory (19), psychostimulant (20) effects of quercetin that arise from its antioxidant activity, it may be useful to prevent exercise-induced muscle damage through reported action to improve mitochondrial biosynthesis (20).

Increasing oxygen consumption during exercise results formation of ROS that causes muscle damage (21, 22). Relative ischemia/reperfusion state in the course of muscle contractions is also important in exercise-induced damage (23). With keeping in mind that mitochondria is the primary source of ROS at exercise (24), xanthine oxidase activity can be influential in terms of ischemia/reperfusion state as well. Ischemia/reperfusion conduces conversion of xanthine dehydrogenase to xanthine oxidase. Hypoxanthine transforms to xanthine and afterwards uric acid, meanwhile xanthine oxidase emanates O_2^- and H_2O_2 (25). Additionally, emerging nitric oxide (NO) due to increased blood flow and ischemia reacts with O_2^- to produce remarkably potent peroxynitrite ($ONOO^-$) (26). Reaction of $ONOO^-$ with carbon dioxide (CO_2) generates carbonate radical (CO_3^-) which is more toxic than OH^- (26). Thus, provoked increase on metabolic activity by exercise escalates CO_2 levels and so, may enhance oxidative injury.

In our present study, considering mentioned benefits of CAPE and quercetin, we aimed to investigate effects of these two natural antioxidant molecules on oxidative stress in an acute exercise model of rats.

Materials and methods

Materials

Quercetin (≥ 95% purity) and CAPE (≥ 97% purity) were purchased from Sigma (St. Louis, MO, USA).

Animals

Fifty eight adult (10-12 week-old) male Sprague-Dawley strain rats, weighing 250 – 350 mg, were pur-

chased from Selcuk University Experimental Medicine Research and Application Center, and study protocol was approved by Selcuk University Experimental Medicine Research and Application Center Animal Ethics Committee. Animals were housed in cages under controlled temperature (25 ± 2°C) and in 12 h light/dark cycle. They were fed with standard rat chow ad libitum.

Rats were randomly divided into six groups: Control (Con, *n*=10), Quercetin without exercise (Que, *n*=10), CAPE without exercise (CAP, *n*=10), Exercise control (ECon, *n*=8), Quercetin with exercise (EQue, *n*=10) and CAPE with exercise (ECAP, *n*=10). Physiological saline were given to Con and ECon rats intraperitoneally. Que and EQue rats were treated for 2 weeks with 50 mg/kg/day i.p. quercetin while CAP and ECAP rats were treated with 10 μmol/kg/day i.p. for same duration. Running exercise was performed the day after last supplementation. Rats in exercise groups were subjected to run on a treadmill for 30 min at 30 meters/min that constitutes a single bout exercise.

Homogenization and biochemical markers

Rats were sacrificed immediately by exsanguination under ether anesthesia following to exercise. A portion of obtained blood was taken to sterile EDTA-containing tubes. Rest of the blood was poured into sterile glass tubes left to coagulation at room temperature for 30 min. Thereafter, coagulated blood was centrifuged at 2000 rpm for 10 min and erythrocyte pellets were washed with physiological saline 3 times. 0.4 mL ice-cold water per 0.1 mL cell suspension was added to lyse erythrocytes. Then, to precipitate hemoglobin, suspension was treated with equal volume of ethanol/chloroform mixture [3/5 (v/v)] and shaken vigorously for 5 min. After centrifugation, supernatant was used for superoxide dismutase and xanthine oxidase analysis. Skeletal muscle (gastrocnemius) and liver tissues were homogenized with a homogenizer (Bandelin; UW2070) at 4°C in Tris-HCl buffer (pH 7.5, 0.2 mM) and homogenate was used for malondialdehyde (MDA) and total nitrite measurements. Also a portion of homogenate was centrifuged and supernatant was separated. Equal volume of ethanol/chloroform mixture [3/5 (v/v)] was added and cold-centrifuged at 3220 rpm, 6°C for 30 min. Protein was analyzed at all intermediary steps by the Lowry method (27).

Lipid peroxidation was assessed using thiobarbituric acid (TBA) reactivity (28). MDA is a product of lipid peroxidation and generated pink-colored complex by reaction of MDA with TBA was measured spectrophotometrically at 532 nm. 1,1,3,3 tetraethoxypropane was used for the standard curve.

Superoxide dismutase (SOD) activity was analyzed as described by Sun et al. which is based on the reduction of nitro blue tetrazolium (NBT) by superoxide generator xanthine – xanthine oxidase system (29). One unit of SOD was defined as the amount of enzyme causing 50% inhibition of NBT reduction.

Total nitrite was determined using Griess reaction (30). Due to difficulty of direct measurement of nitric oxide, nitrate was transformed to nitrite by cadmium and subsequently, total nitrite was measured spectrophotometrically at 540 nm as an indirect indicator of nitric oxide levels.

Xanthine oxidase (XO) activity was assessed by measuring absorbance increase spectrophotometrically at 293 nm during uric acid production from xanthine, according to the method of Prajda and Weber (31). One unit of XO was defined as the amount of produced 1 μmol uric acid per minute.

Statistical analysis

Statistical analysis was performed system using SPSS v.18 statistics software (Chicago, IL). One-way ANOVA and post hoc Tukey's multiple comparison tests were used for normally-distributing data whilst Kruskal-Wallis and post hoc Dunn's multiple comparison tests were used for data that is not normally-distributing (statistical significance $p < 0.05$)

Results

Malondialdehyde

Quercetin and CAPE, individually, decreased blood MDA levels in non-exercised rats (respectively, p=0.005 and p=0.003). Whilst exercise provoked an increase on blood MDA levels (p=0.006), administration of quer-

cetin and CAPE did not exhibit a significant effect (p>0.05). Liver and skeletal muscle MDA levels of non-exercised animals were changed with neither quercetin nor CAPE (p>0.05). Nevertheless, exercise increased MDA in liver and muscle tissues (respectively, p=0.000 and p=0.000). Liver MDA levels similarly decreased with either quercetin or CAPE (respectively, p=0.000 and p=0.000) and in skeletal muscle, individual administration of both antioxidants turned MDA levels back to status that was on pre-exercise (respectively, p=0.000 and p=0.000). MDA levels in blood, liver and skeletal muscle tissues were given in Table 1 and multiple comparison of groups for MDA is shown in Figure 1.

Superoxide dismutase

Quercetin and CAPE, individually, did not exhibit any significant effect on SOD activity of blood and liver tissue (p>0.05), but decreased activity on skeletal muscle (respectively, p=0.015 and p=0.043) in non-exercised animals. Exercise produced an increase in SOD activity of blood, skeletal muscle and liver tissues (respectively, p=0.000, p=0.000 and p=0.006). In exercised animals, SOD activity of blood and skeletal muscle decreased with either quercetin or CAPE administration (respectively, p=0.000 and p=0.004), but only quercetin achieved a significant decrease in liver tissue (p=0.002). CAPE was inefficient to create any change in liver SOD activity (p>0.05). SOD activities in blood, liver and skeletal muscle tissues were given in Table 2 and multiple comparison of groups for SOD is shown in Figure 2.

Xanthine oxidase

Administration of CAPE decreased XO activity of blood (p=0.001) whilst quercetin did not exhibit a significant effect in non-exercised animals (p>0.05). XO activity was influenced by neither quercetin nor CAPE in liver tissue (p>0.05). Quercetin decreased XO activity of skeletal muscle (p=0.01), but there was no significant change with CAPE (p>0.05). Exercise provoked increase of XO activity in blood, skeletal muscle and liver tissues (respectively, p=0.000, p=0.000 and p=0.000). XO activity of exercised animals decreased to levels of

Table 1. Malondialdehyde levels in blood, liver and skeletal muscle.

		Con	Que	CAP	ECon	EQue	ECAP
MDA	[1] Blood	0.96 ± 0.45	0.40 ± 0.22	0.26 ± 0.15	1.54 ± 0.39	1.15 ± 0.46	1.25 ± 0.19
[1] µmol/L	[2] Liver	6.67 ± 1.64	5.86 ± 1.40	5.64 ± 1.25	14.94 ±3.12	8.73 ± 2.18	9.96 ± 2.50
[2] nmol/g protein	[2] Muscle	8.27 ± 5.34	7.86 ± 3.81	7.71 ± 4.56	20.18 ±5.22	8.68 ± 3.85	5.28 ± 2.55

[a] *Data are expressed as mean ± SD*

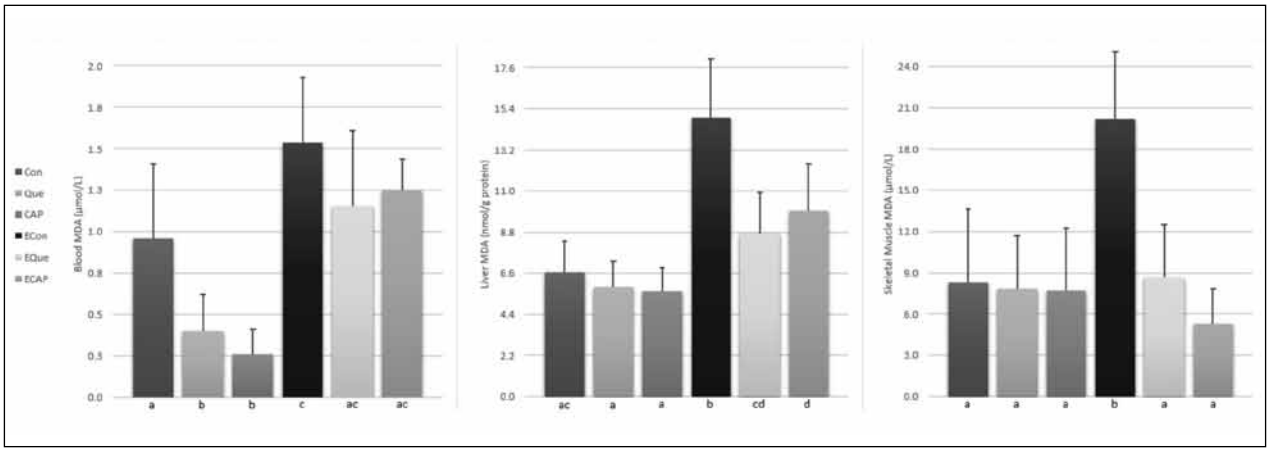

Figure 1. Multiple comparison of groups for MDA levels. Same letters are statistically non-significant for each tissue (p>0.05). Means and positive standard deviations are shown as µmol/L for blood and nmol/g protein for liver and skeletal muscle.

Table 2. Superoxide dismutase activities in blood, liver and skeletal muscle.

		Con	Que	CAP	ECon	EQue	ECAP
SOD	[3]Blood	2.38 ± 0.77	1.43 ± 0.50	1.64 ± 0.47	6.43 ± 1.83	3.70 ± 1.31	4.53 ± 1.00
[3]U/mL	[4]Liver	0.35 ± 0.14	0.32 ± 0.11	0.30 ± 0.10	0.76 ± 0.16	0.49 ± 0.15	0.58 ± 0.18
[4]U/mg protein	[4]Muscle	0.42 ± 0.18	0.19 ± 0.10	0.21 ± 0.08	0.68 ± 0.22	0.44 ± 0.14	0.40 ± 0.16

[a]*Data are expressed as mean ± SD*

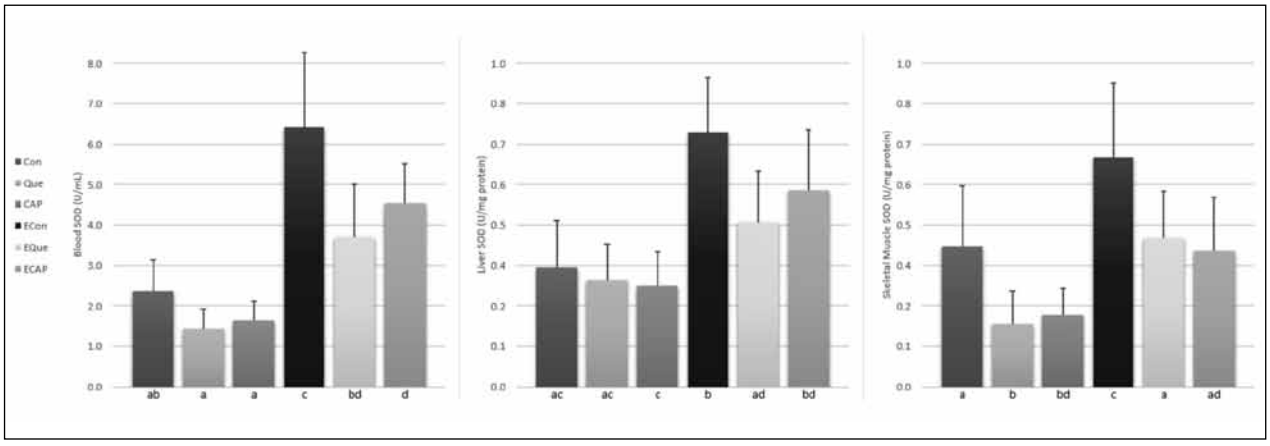

Figure 2. Multiple comparison of groups for SOD activities. Same letters are statistically non-significant for each tissue (p>0.05). Means and positive standard deviations are shown as U/mL for blood and U/mg protein for liver and skeletal muscle.

non-exercised controls in blood, skeletal muscle and liver tissues with quercetin administration (respectively, p=0.000, p=0.008 and p=0.009). CAPE had similar effects in decreasing XO activity for exercised animals (respectively, p=0.000, p=0.000 and p=0.000). XO activities in blood, liver and skeletal muscle tissues were given in Table 3 and multiple comparison of groups for XO is shown in Figure 3.

Nitric oxide

As an indicator of nitric oxide, total nitrite levels in blood, liver and skeletal muscle tissues were given in Table 4. In non-exercised animals, CAPE did not exhibit any significant effect on total nitrite levels of blood, skeletal muscle and liver tissues (p>0.05), but quercetin decreased total nitrite in blood (p=0.000). Exercise did not alter total nitrite levels (p>0.05) and also, there was no significant change with either quercetin or CAPE administration in exercised animals (p>0.05). Multiple comparison of groups for total nitrite is shown in Fig. 4.

Discussion

Acute exercise defines a single bout of exercise, and repeated sessions of exercise are termed as chronic exercise. Metabolic responses to acute exercise last for minutes or hours while that to chronic exercise continues for days or more (25). Exercise enhances production of reactive oxygen species by increasing oxygen consumption (21, 22, 32). Chronic exercise up-regulates endogen antioxidant systems, but acute exercise shifts the redox balance in favor of oxidative stress (33). Health-related exercise is regular (i.e. chronic) exercise and thus, development of oxidative stress by exercise is mainly relevant to acute exercise.

In our study, we preferred treadmill exercise instead of swimming for the model of acute exercise because swimming exercise may require pre-conditioning and induce additional stress and anxiety. We noted that exercise provokes increment of MDA levels, SOD activities, and XO activities in blood, skeletal muscle (gastrocnemius) and liver tissues. MDA is a product of

Table 3. Xanthine oxidase activities in blood, liver and skeletal muscle.

		Con	Que	CAP	ECon	EQue	ECAP
XO	[3]Blood	0.86 ± 0.07	0.71 ± 0.13	0.61 ± 0.18	1.41 ± 0.09	0.80 ± 0.07	0.88 ± 0.18
[3]U/mL	[4]Liver	2.71 ± 0.91	2.45 ± 0.73	2.67 ± 1.00	5.26 ± 1.13	3.63 ± 0.90	2.96 ± 1.01
[4]U/mg protein	[4]Muscle	5.10 ± 1.28	3.28 ± 1.10	3.75 ± 0.78	7.82 ± 1.10	5.81 ± 1.19	3.99 ± 1.39

[a]*Data are expressed as mean ± SD*

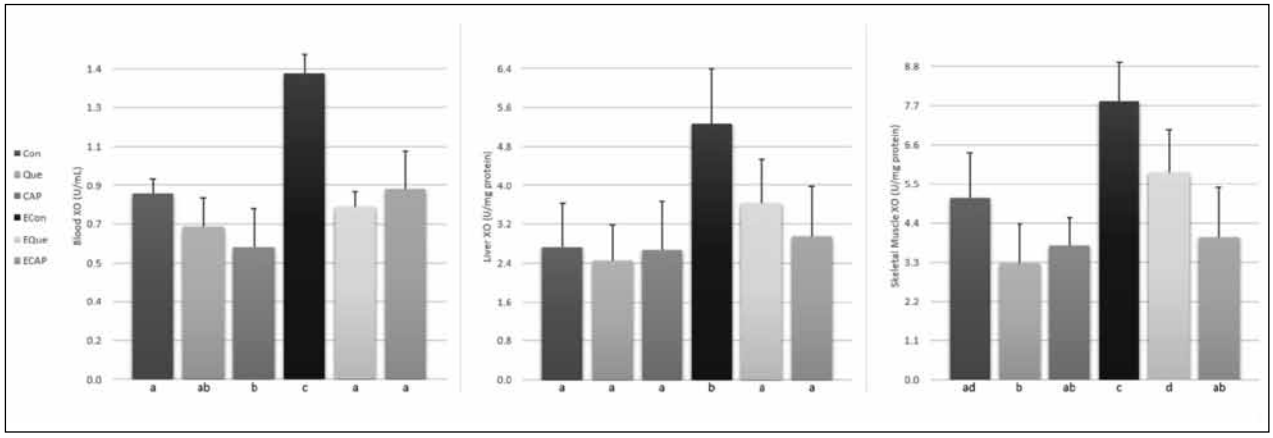

Figure 3. Multiple comparison of groups for XO activities. Same letters are statistically non-significant for each tissue (p>0.05). Means and positive standard deviations are shown as U/mL for blood and U/mg protein for liver and skeletal muscle.

Table 4. Total nitrite levels in blood, liver and skeletal muscle.

		Con	Que	CAP	ECon	EQue	ECAP
Total Nitrite	[5]Blood	55.32 ±7.62	31.90 ±5.25	42.05±12.50	43.52±10.31	41.91 ±9.14	37.15 ±6.37
[5]mmol/L	[6]Liver	6.10 ± 2.11	5.44 ± 1.99	4.80 ± 1.15	5.28 ± 1.07	7.05 ± 1.36	6.10 ± 1.86
[6]µmol/g protein	[6]Muscle	0.92 ± 0.55	0.52 ± 0.29	0.56 ± 0.29	1.42 ± 0.61	1.25 ± 0.62	0.78 ± 0.41

[a]*Data are expressed as mean ± SD*

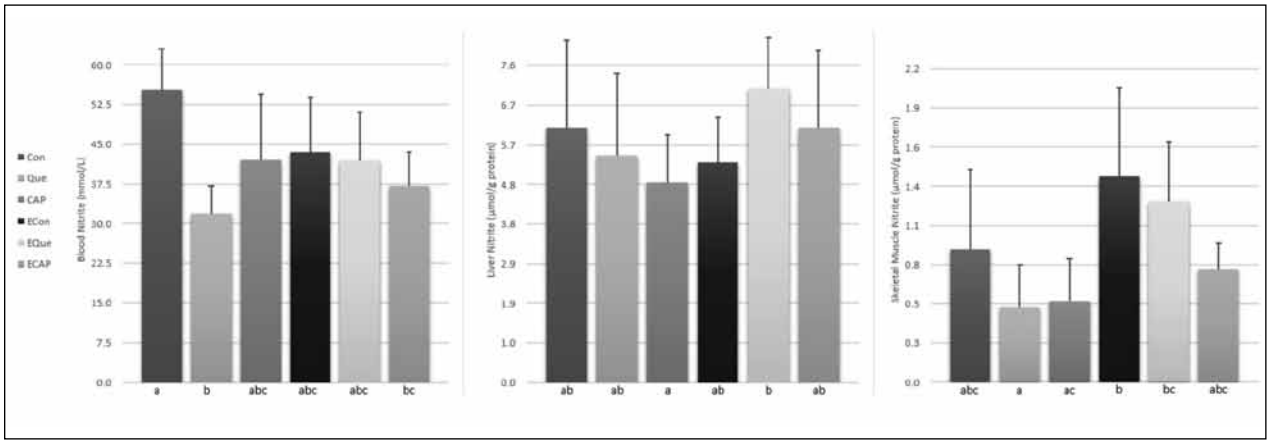

Figure 4. Multiple comparison of groups for total nitrite levels. Same letters are statistically non-significant for each tissue (p>0.05). Means and positive standard deviations are shown as mmol/L for blood and µmol/g protein for liver and skeletal muscle.

polyunsaturated fatty acid (PUFA) peroxidation and pertained to frameshift mutations, base pair substitutions, interstrand cross-links, and DNA – protein and protein – protein cross-links (34). In the present study, increase of MDA level and XO activity, a generator of superoxide radical, following to exercise indicates the oxidative injury and increased activity of SOD, primary step of antioxidant defense, demonstrates an antioxidant system activation against exercise-induced oxidative stress. XO activity correlates with lactate levels and so, it is thought to be related with oxidative injury during anaerobic exercise (33). Neutrophils are responsible for emanation of reactive oxygen species by the NADPH-oxidase pathway and exercise stimulates this system (35). By inhibiting IKB (Inhibitor of NF-kappa B), oxidative stress augments release of NFKB which is a major inflammatory and apoptotic transcription factor (36).

Antioxidants that are used in the present study, quercetin and CAPE, exhibited similar effects on analyzed oxidative parameters. Quercetin reduced skeletal muscle and liver MDA levels. It also attenuated blood, skeletal muscle, and liver SOD and XO activities, but it did not change blood MDA levels. According to those findings, quercetin suppressed oxidative injury that is generated by acute exercise, and created an exogenous antioxidant activity that relieves endogenous antioxidant defense system response, but it was ineffective for hampering cell damage in blood. Even though the administration of CAPE lessened the SOD activity, the decrement was not statistically significant, contrary to the effect of quercetin. Both antioxidants were not able to attenuate MDA in blood probably due to limited repair and biosynthesis capability of erythrocytes (37).

While chronic exercise is evident with changes in NO levels (38), several studies, of which results are in consistency with the present study, reported that acute exercise does not alter total nitrite levels (39-42). We also found that neither quercetin nor CAPE administration changes total nitrite in exercised rats. NO can be generated by activity of three isoforms of nitric oxide synthase (NOS) which uses L-arginine as substrate or reduction of nitrite (43). Exercise provokes the production of NO by stimulating NOS and nitrite reduction (43). Meanwhile, increased XO activity during exercise causes generation of uric acid, an endogenous antioxidant, which depletes NO to form 6-amino uracil (44, 45). Therefore, plasma total nitrite level is finely maintained in a stable range (42), and hence, we hypothesized that forming nitrite is counterpoised by NO scavenging activity of uric acid in our model of acute exercise. Also, considering the method which we used as an indirect indicator of NO, measures stable end product nitrite, it should be noted that actual NO production from NOS could not be determined.

Shen et al. demonstrated that CAPE attenuates skeletal muscle oxidative damage by inhibiting NFKB phosphorylation (46). On the basis of our results, decrement in XO activity and lipid peroxidation, which are in consistency with findings of Alp et al., we deduced that CAPE reduced exercise-induced oxidative injury (47).

Quercetin is reported to be inefficacious in ameliorating exercise-induced oxidative stress by several authors (48, 49), but above mentioned studies had been conducted on elite athletes and this may explain their controversial results. Already induced endogen antioxidant defense in chronic exercise may make exogenous antioxidants to appear like incapacitated in preventing oxidative stress. Reactive oxygen species are critically important for cellular signalization (50) and hence, quercetin can be considered a safe supplement for elite athletes under the name of naturally-occurring antioxidant because it does not disturb redox balance that had been established in chronic course.

Methodological diversities are the probable reasons for the variation in results of oxidative stress-related researches. Balci et al. reported that exercise has no effect on muscle MDA levels and SOD activity in an exhausting swimming exercise model (51), whereas Thirmulaia et al. noted that similar exercise increases MDA levels while decreasing SOD activity (52). Notable methodological differences in the latter study such as attached weights to tails, lesser initial weight of animals and longer duration of exercise may be the main source of different results. Also, one can see contrasting results in medical literature between swimming and treadmill exercises. For instance, Liu et al. (22) found that acute exercise increases MDA levels in liver, but not skeletal muscle, in a study that investigates effects of acute and chronic treadmill exercise on redox balance. Nevertheless, even low intensity pre-conditioning in acute exercised animals may affect the acquired results.

Conclusively, this study demonstrated that quercetin and CAPE, individually, attenuate acute exercise-induced oxidative stress in skeletal muscle and liver tissues, although a limitation of the study is that we did not analyze other antioxidative enzymes such as catalase and glutathione peroxidase which are part of endogenous antioxidant defense system. However, there are controversial reports in the medical literature and they may be related to both predictable and unpredictable situations such as pre-conditioning, animal weights and ages. Development of standardized procedures is essential to clarify this controversy and to pursue further studies that will give us a clear understanding of oxidative stress and its influences on well-being.

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