ORIGINAL ARTICLE

Chokeberry juice affects membrane lipid status and cellular antioxidant enzymes in healthy women with aerobic training activity

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Abstract

The present study examined the effects of aerobic training alone or combined with chokeberry juice on membrane lipid status and activities of antioxidant enzymes in non-athlete women. Participants were randomly assigned into the training group performing aerobic training three times per week; the chokeberry-training group followed the same training regime and additionally consumed 100 ml of chokeberry juice per day and the control group neither trained nor consumed the juice.

Blood samples were collected at baseline and the end of the eight-week-long intervention. Membrane fatty acids' composition was analyzed by gas-liquid chromatography, while the activities of antioxidant enzymes were measured by spectrophotometry.

As a result, the n-3 fatty acids' content was significantly higher in the chokeberry-training (median (interquartile range) of 5.96 (1.65) %) compared with the control group (5.12 (0.87) %), while saturated fatty acids' content was lower in the chokeberry-training (40.14±1.19 %) than in the training group (42.59±2.29 %). We detected significantly higher activity of superoxide dismutase in the training (2224 (2170) U/gHb) compared with the chokeberry-training (1252 (734) U/gHb) and control group (1397 (475) U/gHb).

Our study indicates that supplementation with chokeberry juice may induce favorable changes in cell fatty acid composition and antioxidant response in women performing aerobic training.

Keywords aerobic training • antioxidant enzymes • chokeberry juice • fatty acids • healthy women.

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Introduction

Moderate-intensity exercise has multiple health benefits, and it is, thus, highly recommended for the prevention of cardiovascular diseases. Still, exercise, specifically intense or long-lasting can lead to excessive production of free radicals and reactive oxygen species (ROS) and negatively affect pro-oxidant–antioxidant balance (He et al., 2016). This, in turn, causes the oxidation of cell components, such are lipids and fatty acids. Fatty acids in cell membranes are highly susceptible to oxidative damage, especially those with a high number of unsaturated bonds (Jimenez, Winward, Walsh & Champagne, 2020). The extent of oxidative damage depends on exercise intensity and duration, as well as on the host's defense system. Moderate exercise induces the production of low levels of ROS that can activate signaling pathways involved in cellular responses beneficial for health (Ristow & Zarse, 2010). In addition, regular training can up-regulate antioxidant defense and attenuate oxidative stress in diseases

such are cardiovascular ones (Jackson, 2008). Cardiovascular benefits of aerobic exercise can be exerted in minutes-hours or even several days after training (Bolli, 2000). Although some authors indicated that antioxidant supplementation may blunt these cardiovascular benefits, others underlined inconsistency of data and varying effects of antioxidants combined with training, depending on compounds, doses, and characteristics of exercise (Gliemann et al., 2013; Mankowski, Anton, Buford & Leeuwenburgh, 2015). Thus, a variety of dietary antioxidant supplements have been developed aiming to ameliorate excessive production of free radicals during exercise and upgrading the physical outcomes of the training (Williams, Strobel, Lexis, & Coombe, 2006). Researchers have demonstrated the impact of natural antioxidants in reducing exercise-induced oxidative stress, as well as improving performance and resistance to injury. Among these, polyphenols take an important place, as they express the potential to attenuate oxidative stress caused by both acute and chronic exercise (McAnultya et al., 2004; Panza et al., 2008). One of the promising polyphenol-rich foods is chokeberry juice, with beneficial effects demonstrated in human intervention trials, specifically those on oxidative status (Broncel et al., 2010; Kardum et al., 2014a; Pilaczynska-Szczesniak, Skarpanska-Steinborn, Deskur, Basta & Horoszkiewicz-Hassan, 2005). Additionally, chokeberry acts health promoting not only in subjects at high risk of cardiovascular disease but in healthy subjects as well (Kardum et al., 2014b). Three months-long supplementations with chokeberry juice beneficially affected cellular antioxidant status and membrane fatty acid composition in apparently healthy females (Kardum et al., 2014b). The beneficial effects of chokeberry extract supplementation on redox status have been demonstrated in a recent study with active handball players as volunteers (Cikiriz et al., 2021). On the other side, the lack of antioxidant effects has been reported in a study that investigated the impact of chokeberry juice supplementation on oxidative balance in young footballers (Stankiewicz et al., 2021).

Taking into account all these facts, the objective of our study was to investigate the effects of a type of aerobic training alone or in combination with chokeberry juice consumption, on cellular oxidative status, measured as membrane fatty acid composition, and the activities of antioxidant enzymes, in healthy non-athlete women.

Method

Subjects and study design

We included 28 healthy women with a mean age of 25.1±2.8 years, body height 168.2±5.8 cm, and body weight of 60.4±9.7 kg and randomly assigned them into three groups. The participants were non-athletes assuming they undertook less than 30 minutes of intense or 60 minutes of moderate activity per week in the preceding three months. This study was approved in advance by the Ethical Committee of the Medical Clinical Center in Zemun (Belgrade, Serbia) and was undertaken according to the Helsinki Declaration. Each participant voluntarily provided written informed consent before participating. The exclusion criteria were: the presence of chronic diseases, body mass index ≤ 18 or ≥ 30 kg/m2, food allergy or intolerance to juice components, irregular dietary pattern, pregnancy, or breast feeding.

The first subject group performed training; the second group trained and consumed 100 ml of chokeberry juice per day, and the third group was defined as the control group neither training nor consuming chokeberry juice. Both training and chokeberry-training groups were instructed to do the same training program.

Training

A commercial aerobic dance exercise program named body combat (Les Mills, New Zealand) was performed by participants 3 times a week, for 60 minutes, in the evening hours, at three exercising facilities located in Belgrade (Serbia) certified by Les Mills. Body combat is defined as a vigorous or moderate-to-high-intensity aerobics class. More precisely, it is a form of interval training with a regular exchange of moderate and high-intensity bouts, like a high interval intermittent training (Jung, Bourne & Little, 2014).

The certified instructors performed the same choreography in all three facilities and encouraged the participants to exercise at the highest possible intensity they could. The planned number of training sessions was 24, and participants were allowed to miss a maximum of 2 of them. When they were unable to attend the training in their term, they could do it in the scheduled session on the following day.

Chokeberry juice

Participants in the chokeberry-training group consumed polyphenol-rich chokeberry juice and they were advised to keep it in a refrigerator after opening

it. The content of total phenolics was 648.4 mg of gallic acid equivalents per 100 mL, while proanthocyanidins, anthocyanins, and phenolic acids were the main phenolic subclasses. The detailed characterization and quantification of phenolic compounds present in the juice have been previously published by Tomic et al. (2016). The chokeberry juice was donated from Rheapharm d.o.o., Belgrade, Serbia.

Laboratory assays

Blood samples were collected after an overnight fast at baseline and the end of the 8-week-long intervention. Biochemical parameters were determined on the same day the samples were collected, using a clinical chemistry analyzer Cobas c111 (Roche Diagnostics, Basel, Switzerland) and Roche Diagnostics' kits according to the manufacturer's instructions. Erythrocytes were isolated, washed out with cold isotonic saline, divided into aliquots, and stored at -80ºC for further analysis of the fatty acid profile and antioxidant enzyme activities.

Analyses of fatty acid composition

Firstly, lipids were extracted using the organic solvents chloroform and isopropanol as previously described (Rose & Oklander, 1965). Further, phospholipids were separated by a thin layer of chromatography with a mixture of petroleum ether, diethyl ether, and glacial acetic acid. According to the modified procedure of Cristopherson and Glass (1969), direct transesterification of fatty acid was carried out, followed by the evaporation of hexane extracts under a stream of nitrogen. The final residue was dissolved in 10 µL of hexane and 1 µL was injected into the Shimadzu chromatograph GC 2014. The chromatograph was equipped with a flame ionization detector and Rtx 2330 column (60m x 0.25mmID, 0.2µm, Restek, Bellefonte, Pennsylvania).

Adequate separation of methyl esters was obtained over 50 minutes with a temperature of 140°C held for 5 minutes, then increased to 220°C at a rate of 3°C/min and held at final temperature for 20 minutes. The identification was made by comparing peak retention times with standard mixtures and the contents of fatty acids from C16:0 through C22:6n-3 were expressed as a percentage of total fatty acids identified. The content of total saturated fatty acids (SFA) was calculated by summing the contents of C16:0 and C18:0 percentages, while the content of monounsaturated fatty acids was calculated from C16:1n-7, C18:1n-9, and C18:1n-7 percentages. Finally, the content of total polyunsaturated fatty acids (PUFA) was calculated from the percentages of C18:2n-6, C20:3n-6, C20:4n-6, C22:4n-6, C20:5n-3, C22:5n-3, and C22:6n-3.

Analyses of antioxidant enzymes

The activity of superoxide dismutase was determined using a commercial kit Ransod (Randox, Crumlin, UK), based on superoxide anion production in xanthine-xanthine oxidase system and its further reaction with 2-(4-iodophenyl)-3-(4-nitrophenol)-phenyltetrazolium chloride, resulting in the formation of red formazan dye, and absorbance measurement at 505 nm and 37°C for 3min.

The glutathione peroxidase activity was determined with the use of the commercial kit Ransel (Randox, Crumlin, UK). In the presence of cumene hydroperoxide, glutathione peroxidase from samples catalyzed the oxidation of glutathione, while glutathione reductase further reduced oxidized glutathione with the consumption of coenzyme NADPH+H+.

The determination of catalase activity was based on its ability to degrade hydrogen peroxide, as previously described (Aebi, 1984). A decrease in H2O2 absorbance was measured at 230 nm for 3 minutes and this change was used for the determination of catalase activity.

The activities of enzymes were expressed in U/gHb, and the cyanmethemoglobin method with Drubkin's reagent was applied for the assessment of hemoglobin (Hb) concentration (Van Kampen & Zijlstra, 1961).

Dietary intake

The participants were explicitly instructed to continue with their normal lifestyle, particularly regarding their diet. The training and control groups were instructed not to include any antioxidant-rich food or supplements that they haven't habitually consumed. The chokeberry-training group was allowed to consume only chokeberry juice as additional polyphenol-rich food, as explained in the study protocol. We then evaluated participants' dietary intake using a validated 24-hour dietary recall questionnaire on three separate days (1 working day at the beginning of the study, one weekend day during the study, and one working day at the end of the study). A well-trained and experienced interviewer in dietary intake assessment collected information on food type, preparation methods, recipes, and

commercial products from each participant in a face-to-face interview. For estimating the portion sizes, we provided pictures of various foods, dishes, and beverages, as previously reported (Gurinović et al., 2016). Nutrient calculations were performed using the Serbian Food composition database.

Statistical analysis

Before comparisons, the normal distribution was evaluated by the Shapiro-Wilk test. For normally distributed variables a student's paired t-test was applied, and data are presented as mean and standard deviation (SD). Wilcoxon Signed Rank test was used to compare non-normally distributed variables within each group, and data are presented as the median and interquartile range (IQ). To identify differences between groups and other parameters at baseline, one-way analysis of variance (ANOVA) and Kruskal-Wallis tests were applied for normally and non-normally distributed data, respectively. Finally, to compare the intervention effects between the groups, data were analyzed using an analysis of covariance (ANCOVA), with baseline values as covariables, and Bonferroni as a post hoc test. Analysis was performed using the SPSS software (ver. 20.0) and p values <0.05 were considered statistically significant.

Results

Characteristics of the subjects, dietary intake, and biochemical parameters

Participants were well matched by age, body weight, and height with no difference between study groups. There were no significant changes in body weight at the end of the intervention period (Table 1). By comparing the dietary intakes of all three study groups (as a mean of three 24 h dietary recalls), we confirmed that participants had a uniform diet during the intervention period.

The values of total cholesterol significantly decreased in all three study groups: training (p=0.007), chokeberry-training (p=0.034), and control (p=0.002), compared with the baseline. A Similar was observed for blood glucose, with a significant decrease just in the training group (p=0.017). The post-intervention level of low-density lipoprotein (LDL) cholesterol was significantly higher in the chokeberry-training group compared with the training group (p=0.04). Still, the values of the total, LDL, and high-density lipoprotein (HDL) cholesterol, as well as fasting blood glucose and triglycerides were in reference ranges, indicating that the participants were in good cardiovascular health (Table 1).

Table 1. Characteristics of subjects at baseline and after 8 weeks of intervention

Variable	Training		Chokeberry-training		Control	
	Baseline	8 weeks	Baseline	8 weeks	Baseline	8 weeks
Age (years)	25.7±2.8		24.5±2.4		25.1±3.5	
Body height (cm)	170.8±4.9		166.0±5.7		167.7±7.3	
Body weight (kg)	60.9±6.6	60.2±5.3	60.8±11.8	60.9±11.8	59.1±11.1	58.6±11.0
Energy (kcal)	1899±340		1685±306		1919±351	
CHO (g/day)	195±60		176.29±52.26		192.97±54.21	
Protein (g/day)	70.72±11.90		67.66±17.42		73.18±14.44	
Fat (g/day)	92.46±21.15		78.21±20.99		94.69±14.50	
Glucose (mM)	4.60 (0.58)	4.06 (0.65)*	4.58 (0.51)	4.24 (0.41)	4.46 (0.40)	4.09 (0.59)
TG (mM)	0.61 (0.70)	0.66 (0.65)	0.64 (0.29)	0.71 (0.25)	0.71 (0.32)	0.63 (0.28)
Total chol (mM)	4.80±0.79	4.29±0.77**	4.97±0.54	4.69±0.52*	4.49 ± 0.65	4.09±068**
LDL-chol (mM)	2.16±0.58	2.08±0.52	2.29±0.48	2.50±0.48*;†	1.90±0.26	2.05±0.33*
HDL-chol (mM)	1.73±0.23	1.73±0.19	1.74±0.39	1.71±0.32	1.70±0.37	1.64±0.40
UA (μM)	280.3±51.3	268.2±46.3	228.5±62.6	233.7±53.3	252.1±68.1	266.2±31.8
ALT (U/L)	14.5 (4.7)	14.0 (5.7)	11.7 (6.8)	14.2 (5.8)	11.6 (3.4)	11.0 (4.1)
AST (U/L)	20.5 (6.5)	19.6 (3.6)	18.1 (4.6)	19.0 (6.5)	18.1 (3.0)	16.4 (2.6)

Note: Data are presented as mean ± standard deviation or as median (interquartile range) *p<0.05** p<0.01 compared with baseline; † p<0.05 compared with training group

ALT- alanine aminotransferase; AST – aspartate aminotransferase; chol – cholesterol; CHO – carbohydrate; HDL – high-density lipoprotein; LDL – low-density lipoprotein; TG – triglycerides.

Effects on fatty acid profile

The paired t-test revealed a significant decrease in the levels of total SFA (p=0.026), palmitic acid (p=0.047), and n-6/n-3 ratio (p=0.034) in the chokeberry-training group (Table 2). In the same group, there was a significant increase in the content of total PUFA (p=0.009), n-3 PUFA (p=0.003), docosahexaenoic (C22:6n-3; p=0.002), and arachidonic acid (C20:4n-6; p=0.012). Between groups comparisons at the end of the intervention confirmed these findings. The chokeberry-training group had a significantly higher content of total n-3 PUFA (p=0.019) and docosahexaenoic acid (p=0.011) than the control group, while the n6/n3 ratio was significantly lower (p=0.049). When compared with the training group, the chokeberry-training group had significantly lower concentrations of SFA (p=0.017) and palmitic acid (C16:0; p=0.021). We found no significant differences in fatty acid profile between groups at the baseline (Table 2).

Table 2. Fatty acid composition of membrane phospholipids at baseline and after 8 weeks of intervention

Variable	Training		Chokeberry-training		Control	
	Baseline	8 weeks	Baseline	8 weeks	Baseline	8 weeks
SFA (%)	42.78±2.32	42.59±2.29	41.95±1.68	40.14±1.19*;†	41.36±3.58	40.91±1.57
16:0	21.36 (2.46)	21.54 (1.46)	20.77 (1.79)	19.92 (0.78)*;†	20.56 (1.87)	20.25 (3.18)
18:0	21.35 (2.19)	20.71 (3.48)	20.81 (1.39)	20.36 (1.89)	19.86 (2.41)	20.59 (1.21)
MUFA (%)	14.69 (1.16)	14.70 (3.08)	14.50 (1.73)	14.76 (1.25)	15.19 (1.72)	15.00 (2.57)
16:1n-7	0.20 (0.08)	0.21 (0.09)	0.24 (0.06)	0.20 (0.09)	0.20 (0.07)	0.21 (0.08)
18:1n-9	12.88 (1.07)	12.82 (1.36)	12.62 (1.48)	12.91 (1.39)	13.25 (1.65)	13.20 (1.91)
18:1n-7	1.58 (0.17)	1.60 (0.29)	1.62 (0.09)	1.60 (0.29)	1.56 (0.27)	1.57 (0.52)
n-6 PUFA (%)	36.86±1.99	37.91±2.25	38.09±1.92	38.99±1.02	37.92±3.22	38.56±2.06
18:2n-6	14.20 (2.84)	14.02 (1.20)	13.71 (1.56)	13.53 (1.27)	14.19 (1.03)	13.89 (2.18)
20:3n-6	1.53 (0.46)	1.50 (0.36)	1.62 (0.32)	1.67 (0.24)	1.80 (0.49)	1.96 (0.34)
20:4n-6	17.18±1.48	17.57±1.60	18.27±1.04	19.07±1.43*	17.50±2.39	18.54±1.40
22:4n-6	4.46±0.68	4.53±0.55	4.60±0.82	4.84±0.63	4.72±0.63	4.72±0.42
n-3 PUFA (%)	5.37 (0.89)	6.20 (1.16)	5.42 (1.41)	5.96 (1.65)**; ‡	5.14 (2.24)	5.12 (0.87)
20:5n-3	0.22 (0.06)	0.22 (0.10)	0.18 (0.09)	0.24 (0.10)	0.20 (0.10)	0.19 (0.06)
22:5n-3	1.36 (0.42)	1.47 (0.28)	1.42 (0.45)	1.46 (0.47)	1.57 (0.13)	1.63 (0.30)
22:6n-3	3.72 (1.30)	1.57 (0.13)	1.63 (0.30)	4.40 (1.41)**;‡	3.48 (1.84)	3.42 (1.06)
Total PUFA (%)	42.05 (3.08)	4.46 (0.80)	3.74 (1.41)	45.31 (1.72)**	44.64 (5.19)	45.21 (2.46)
n-6/n-3 ratio	6.82±0.90	43.58 (2.28)	43.03 (2.14)	6.34±0.99*;‡	6.98±1.34	7.09±1.10

Note: Data are presented as mean ± standard deviation or as median (interquartile range)
*p<0.05** p<0.01 compared with baseline; † p<0.05 compared with training group; ‡ p<0.05 compared with control group MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; SFA – saturated fatty acids

Effects on antioxidant enzymes

Significantly higher superoxide dismutase activity was observed at the end of the intervention in the training group compared with the other two groups (p=0.023, Table 3).

Table 3. Activities of antioxidant enzymes at baseline and after 8 weeks of intervention

Variable	Training		Chokeberry-training		Control	
	Baseline	8 weeks	Baseline	8 weeks	Baseline	8 weeks
GPx (U/gHb)	49.7±12.7	48.3±11.7	54.1±13.4	47.7±9.3	55.1±12.7	56.6±16.8
SOD (U/gHb)	1958.9 (2555.9)	2223.8 (2169.6)	1677.9 (1470.5)	1251.8 (734.3) †	1769.6 (1767.1)	1397.2 (475.2) †
CAT (kU/gHb)	61.1 (9.5)	65.9 (27.3)	66.6 (6.4)	62.7 (16.8)	64.4 (32.8)	68.3 (34.5)

Note: Data are presented as mean ± standard deviation or as median (interquartile range) † $p<0.05$ compared with training group GPx – glutathione peroxidase; SOD – superoxide dismutase; CAT – catalase; Hb – hemoglobin

Discussion

The key findings of this study were observed in the group that performed aerobic training in combination with chokeberry juice supplementation. At the end of the 8-week-long intervention, a significant increase in the level of n-3 PUFA and a decrease in the n-6/n-3 ratio were observed in the chokeberry-training group, compared with both the baseline and the control group. In the same group, the content of total SFA and palmitic acid significantly decreased in comparison with the baseline and the training group. Finally, compared with the baseline, the level of total PUFA significantly increased after 8 weeks of supplementation with chokeberry juice and performing aerobic training.

Our results on the level of total and n-3 PUFA in the chokeberry-training group were in accordance with previously reported intervention studies with chokeberry juice. Three months-long supplementations with chokeberry juice resulted in a significant increase in the level of erythrocytes' PUFA in healthy females (Kardum et al., 2014b). Additionally, chokeberry juice enriched with dietary fibers increased membrane n-3 PUFA' status in obese women after 4 weeks of consumption (Kardum et al., 2014c). Among fatty acids in membrane phospholipids, PUFA is the most prone to oxidative damage, since they have a high number of unsaturated bonds, as the main site of free radicals' attack. Thus, the relative content of PUFA can be used as an indirect measure of the extent of membrane lipid peroxidation. In accordance, the observed increase in the levels of total and n-3 PUFA in the chokeberry-training group could indicate favorable effects against oxidative damage of membrane lipids. Considering that we observed no effect in the training group, we suggest chokeberry juice as responsible for changes in n-3 PUFA' level.

An increase in levels of total n-3 PUFA and docosahexaenoic (C22:6n-3) fatty acid in the chokeberry-training group implies positive effects of chokeberry juice on cardiovascular health in females performing the aerobic activity. As Mozaffarian and Wu explained, n-3 PUFA exerts multiple cardiovascular-related physiological effects (Mozaffarian & Wu, 2011). In addition, there is a negative correlation between the levels of n-3 PUFA in membranes and the overall risk of cardiovascular diseases, as previously reported (Piñeiro-Corrales, Lago Rivero & Culebras-Fernández, 2013). An increase in n-3 PUFA content in the chokeberry-training group was accompanied by a decrease in the n-6/n-3 ratio, which was significant in comparison with the baseline and the control group. We find this result noteworthy since the decrease in this parameter is associated with the suppressive effect on further pathogenesis of cardiovascular and other diseases, mediated by the production of favorable anti-inflammatory eicosanoids from n-3 PUFA (Simopoulo,s 2008).

Further on, we detected a significant decrease in the level of total saturated fatty acids in the chokeberry-training group, when compared with both the baseline and the training group. Saturated fatty acids are positively associated with cardiovascular risk factors, such as high levels of plasma triglycerides and blood pressure, suggesting that a

decrease in the content of these fatty acids acts beneficially on cardiovascular health (Kim, Jeon & Lee, 2015).

Contrary to our findings, a recent study reported a weak impact of chokeberry juice supplementation on fatty acid composition in serum phospholipids of handball players (Petrovic et al., 2016). However, unlike our participants, these were professional players, who adapted to the stress they were regularly exposed to, and changes, thus, were less likely to occur. Also, the supplementation period was twice as shorter as in our study.

Another important finding of our study was significantly higher activity of superoxide dismutase in the training group compared with the other two groups. The group of authors reported the same effect of aerobic training in an animal model and suggested the up-regulation of genes for superoxide dismutase as a potential mechanism of action (Marini, Abruzzo, Bolotta, Veicsteinas & Ferreri, 2011). Other researchers indicated that training in humans could lead to increased auto-oxidation of hemoglobin and the production of superoxide anions (Bolli, 2000). Excessive production of superoxide anions could, in turn, stimulate and enhance the activity of an enzyme that neutralizes them, i.e., superoxide dismutase. Since we observed lower activity of superoxide dismutase in the chokeberry-training group, we propose that chokeberry juice might have counteracted training-induced production of superoxide anions, i.e., attenuated training-induced oxidative stress. Similarly, a previous study reported a decrease in the activities of superoxide dismutase and glutathione peroxidase in the rowers supplemented with chokeberry juice (Pilaczynska-Szczesniak et al., 2005). In accordance, we detected a marginally significant decrease in glutathione peroxidase activity in the chokeberry-training group compared with the baseline.

There are several limitations of our study regarding the analytical methods applied. The addition of other oxidative stress markers could explain in more detail the biological mechanisms underlying here observed effects. Previous studies (Broncel et al. 2010; Pilaczynska-Szczesniak et al. 2005), however, suggested that the effects of chokeberry juice on oxidative status are mostly exerted by the modulation of antioxidant enzymes' activities, which is why we focused on these parameters specifically.

Conclusion

Altogether, our results show the beneficial effects of chokeberry juice consumption on cell fatty acid composition and enzymatic antioxidant defense in females performing aerobic training, supporting its use in maintaining cardiovascular health. Still, further research is required to confirm our findings and improve our knowledge of chokeberry juice's effects on exercise-mediated oxidative stress.

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All the authors contributed to the: conception and design of the study, data collection and interpretation, and manuscript preparation. Accordingly, they all approved the final version of the paper. None of the authors declare a conflict of interest.

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