ORIGINAL ARTICLE

Effect of herbal antioxidant-rich formula on improvement of antioxidant defense system and heat shock protein-70 expression in recreational female athletes: A randomized controlled trial

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Background: The aim of the present study was to evaluate the effect of natural antioxidant formula (blend of herbs: ginger root, cinnamon bark and raw almond fruit powder, rosemary leaf powder, and honey) on oxidative status, antioxidant enzyme activity, and relative heat shock protein (HSP-70) expression in recreational female athletes. **Materials and Methods:** Eighteen female participants trained for 4 weeks and randomly received either antioxidant formula (FormEX) (n = 8) or placebo (PlcEX) (n = 10) in a randomized controlled trial. Blood samples were obtained 1-h before, 1 h and 24 h postexercise to measure malondialdehyde (MDA), total antioxidant capacity (TAC), superoxide dismutase (SOD), glutathione peroxidases (GPx), and HSP70 mRNA expression. Data analysis was performed using 2 (treatment = grouping factor) ×6 (time = within-factor) repeated measurements analysis of variance or generalized estimating equations (GEE) test. We used the independent t-test to evaluate any significant differences for real-time polymerase chain reaction data. **Results:** Antioxidant formula increased the relative HSP-70 mRNA expression more than Plc-EX group in all time points (P = 0.001). The time main effect was significant with regard to TAC and SOD concentrations (P = 0.001 and 0.002, respectively). However, there were no statistically significant differences between groups for TAC, SOD, and MDA (P = 0.25, 0.06, and 0.38, respectively). Neither the time main effect for MDA nor time and intervention interaction was not statistically significant for MDA, TAC, and SOD (P = 0.19, 0.13, and 0.10, respectively). GEE results for GPx showed that there were no significant differences between the groups (P = 0.11). **Conclusion:** The results presented herein revealed that natural antioxidant rich formula had variable effects on oxidative status. However, in contrast to many antioxidant supplements, this formulation increases the HSP-70 mRNA expression which might improve the antioxidant ability of cells in the long-term period and exercise-induced adaptation.

Keywords: Exercise-induced adaptation, heat shock proteins, natural antioxidants

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INTRODUCTION

When reactive oxygen species are presented in physiological concentrations; they play an important role in the modulation of gene expression.[1] Exercise increases the antioxidant activity[2] via activation

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of specific signaling pathways such as antioxidant enzymes (AOEs), superoxide dismutase (SOD), glutathione peroxidases (GPx), and heat shock protein (HSP) family such as HSP-70.[1-4] This occurs in blood cells such as lymphocytes.[1] Some studies showed that supplementation with antioxidants such as vitamin[3,5] may suppress the adaptive stress signaling[6] and hormesis[5,7] or had no beneficial effects on exercise adaptation.[8]

There are some dietary ingredients, that initially act as oxidants and/or activators of the stress signaling pathways and thereby, inducing an adaptive stress response. These ingredients include polyphenols, carotenoids, rosmarinic acid, electrophile compounds, and enone structure (zerumbone, curcumin, and shogaol).[7,9,10]

Accordingly, it seems possible to develop a natural/herbal formulation rich in multihormetins, to activate hormetic pathways and improve antioxidant status.[9]

In view of the ability of hormetins such as ginger, cinnamon, and rosemary to increase antioxidant activity and HSP-70 level, and the ability of nuts such as almond in increasing the AOEs, and the lack of studies in this concern, we hypothesized that they might improve the exercise-induced hormesis.

Therefore, the purpose of this study was to ascertain whether a selective herbal antioxidant formula, in contrast to many other antioxidant supplements, could increase the exercise adaptation through hormesis pathways.

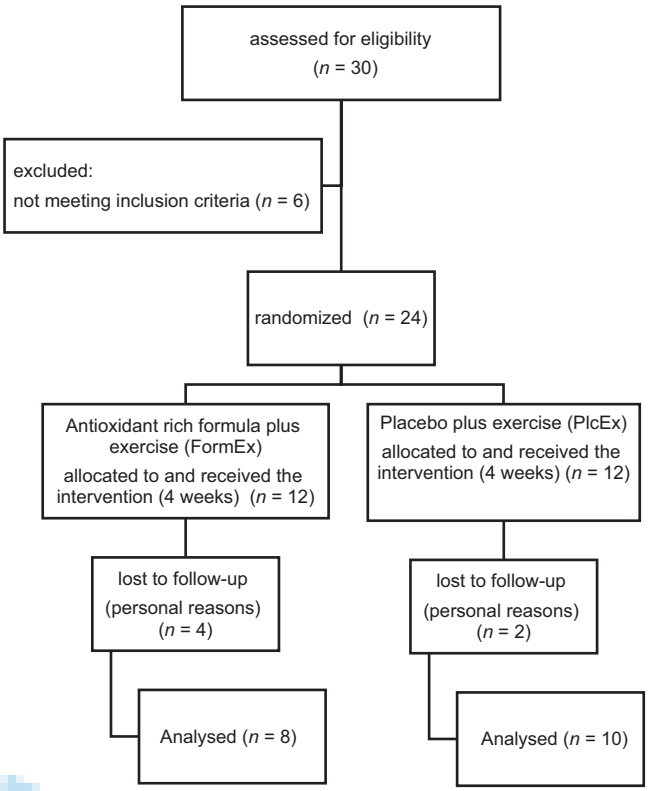

Diagram 1: Flow diagram of the progress

METHODS

Study design

This experimental study was a randomized, double-blind, placebo-controlled trial. Participants were randomly assigned to intervention or control group as indicated in Diagram 1. Randomization was done using Excel (random number generation), using Bernoulli distribution with $P = 0.5$.

Selection and description of participants

All female volunteers completed questionnaires on medical, diet and supplement history, and physical activity. Inclusion criteria included are as follows:[11] (1) Having no diagnosed cardiovascular, musculoskeletal disorders and history of medical events that might influence the study outcome, (2) Taking no herbal/nonherbal supplements 6 weeks before the test, and (3) Having no special dietary patterns.

The participants were fully informed about all experimental procedures and the risks and benefits associated with the study and provided written consent. The study was approved by the Ethical Committee at the Ahvaz Jundishapur University of Medical Sciences. The study was registered in primary registry in the WHO registry network of clinical trials (IRCT2016030526909N1).

The selected participants were randomly assigned to one of the two groups, namely, antioxidant-rich formula plus exercise (FormEx) and placebo plus exercise (PlcEx). Both groups followed a supervised exercise program for 3 sessions/week, 38 min/session, at 60%–65% VO_2 max. Height was measured without shoes using the stadiometer with a precision of 0.1 cm, and weight was measured in light clothing to the nearest 0.1 kg. Body fat percentage was calculated using bioelectrical impedance analysis (Quadscan 4000, Bodystat Ltd; Isle of Man, UK). A 3-day 24-h dietary recall (2 weekdays, and a weekend) was obtained from the participants 1 week before and within the last week of intervention protocol. The average daily nutrients, as well as antioxidants intake, were estimated by customized Nutritionist IV software (version 3.5.2, First DataBank; Hearst Corp, San Bruno, California, USA). The participants were asked to abstain from strenuous physical activity 48-h before blood sampling. The tests and blood sampling were carried out during the preovulatory phase, at the same time of day. The participants were asked about any side effects of the supplements. The study protocol is presented in Diagram 2.

Training protocol

Each training session consisted of exercise beginning with 10 min warm-up period till reaching heart rate (HR) = 120 and finishing with 5-min cool down and recovery period. The main part of each session consisted of 2 × 10 min exercise with target HR with 3-min active rest within each 10 min (maintaining HR at 100–120) [Diagram 3].[12]

VO_2 max and exercise test protocol

Participants performed one preliminary treadmill-based test at least 10 days before the main trial to determine VO_2 max and to calculate the workload for the exercise prescription and familiarization with the test as well. Bruce protocol is one of the most common methods for estimating VO_2 max on a treadmill. All measurements were performed in a sport science laboratory by an experienced assessor and recorded by one staff that was blinded to participants. Neither the participants nor the staff knows who is getting formula and who is getting the placebo.

Intervention and supplementation

A person with no clinical involvement in the trial packed the supplements in numbered boxes. The training coach, who was not aware of random sequences, assigned the participants to the numbered boxes of supplements. The materials were purchased from the local market and were authenticated by an expert botanist. The supplements were prepared by YashilDaruiSahand company-Iran based on recipe formulated and designed by the supervisor of the study and were distributed every week to keep the freshness of contents. The formula contained 2 g ginger root powder (*Zingiber officinale*; Zingiberaceae family), 2 g cinnamon bark (*Cinnamomum zylanicum*; Lauraceae family) and 30 g raw almond fruit (Prunus amygdalus var dulcis; Rosaceae family) powder, 2 g rosemary leaf powder (*Rosmarinus officinalis*; Lamiaceae family), and honey.[13,14] The placebo was similar in calorie content (197 Kcal), color, texture, and package to the formulas (219 Kcal). It contained materials with no antioxidant activity, which are roasted wheat flour, sugar syrup, essence, and artificial color. The ingredients were mixed thoroughly, and everyday supply was packed in little boxes. The texture of supplements was similar to peanut butter. The participants were instructed to keep the formula/placebo in a refrigerator and to use them as a snack between meals or as a breakfast. The administered dosage was determined by referring to previous clinical trials testing the effect of each of the components of the formula.

Blood collection and analysis

Fasting blood samples were obtained from an antecubital vein. Approximately 3 mL of that was added to tubes-containing ethylenediaminetetraacetic acid (EDTA) to determine SOD, GPX, and HSP-70 in plasma. The rest of the blood sample was added to EDTA free tubes and were centrifuged (3000 rpm) for 10 min, and the serum quickly frozen and stored at −70°C to determine total antioxidant capacity (TAC) and malondialdehyde (MDA).[15] Hemoglobin and hematocrit were tested by Sysmex KX-21N™ automated hematology analyzer, and values were used to estimate changes in plasma volume relative to preexercise.[16]

Isolation of total RNA

Total extraction of RNA was done by NucleoSpin® RNA XS kit (Macherey-Nagel, Germany) according to the standard procedure.

Real-time polymerase chain reaction and gene expression analysis

Real-time polymerase chain reaction (RT-PCR) was carried out using SYBR® Premix Ex Taq™II (TAKARA) in accordance with the manufacturer's instructions. The specific sequences of primers (5′ to 3′) used were ccctgggcatcgaaacc and ccctcgtagacctggatcatg. The housekeeping gene was glyceraldehyde-3-phosphate dehydrogenase. The threshold cycle (CT) was determined manually for each run. PCR efficiencies for each set of primers were determined using serial 10-fold dilutions of cDNA and resulting plots of CT versus the logarithmic cDNA dilution, using the efficiency equation (E): E =10 (−1/slope).

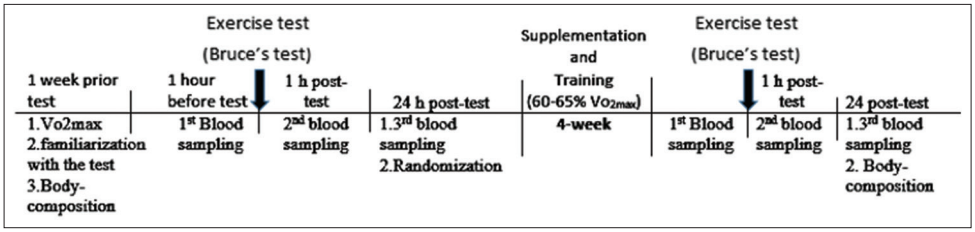

Diagram 2: The study protocol

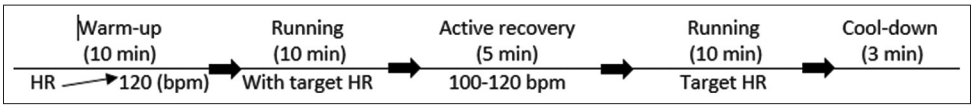

Diagram 3: Training protocol

Gene expression of the lymphocytes[14] compared to the controls was calculated using REST_ software 2009.[17] All participants were instructed to keep their normal diets.

Statistics

Data analysis was performed using SPSS version 16 statistical software (SPSS Inc., Chicago, IL, USA). Data were reported as means ± standard deviation (SD), and the value of $P < 0.05$ was considered as statistically significant. Normality of distribution was assessed using the Shapiro–Wilk test. Independent *t*-test and Mann–Whitney U-test were conducted initially to examine between the group differences. Data were analyzed using 2 (treatment = grouping factor) × 6 (time = within factor) repeated measurements analysis of variance (ANOVA) or generalized estimating equations (GEE) test. *Post hoc* analysis was carried out, where appropriate, using paired samples *t*-test or Wilcoxon test with Bonferroni correction. To evaluate the changes within groups in detail, paired *t*-test was used. We used independent *t*-test to evaluate any significant differences for RT-PCR data.

RESULTS

Eighteen female athletes completed the study [Diagram 1]. Baseline serum variables were measured before the intervention procedure. Baseline nutrition intake was similar between the two trial groups [Table 1].

The independent *t*-test showed that relative HSP-70 expression were not different between the groups before intervention ($P = 0.24$ and $P = 0.11$ for 1 h before (A) to 1 h after (B) and B to 24 h after exercise test, respectively), except for A to C ($P = 0.001$). However, the increased levels from after intervention from 1 h before (D) to 1 h after (E) t (12.14) = 8, $P = 0.001$, E to F t (9.44) = 8, $P = 0.001$ and from D to 24 h after exercise test (F) t (20.42) =8, $P = 0.001$ and A to D t (38.75) =8, $P = 0.001$ were statistically significantly higher in formula ingested group compared to the placebo group [Figure 1].

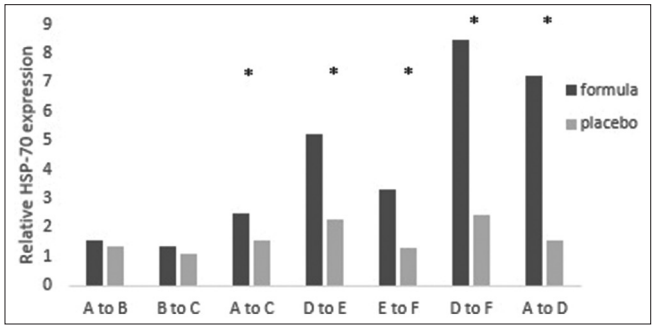

Figure 1: Increased levels of relative heat-shock protein-70 expression between the two time points in groups. Values are means ± standard deviation. Before intervention: (A): 1 h before, (B): 1 h after, (C): 24 h after exercise test. After intervention: (D): 1 h before, (E): 1 h after, (F): 24 h after exercise test. $P = 0.001$

Repeated measures ANOVA for MDA, TAC, and SOD are presented in Table 2. Neither the time main effect nor time and intervention interaction regarding MDA was significant. Moreover, there were no significant differences between the groups. Paired *t*-test results and significant values are shown in Figure 2. Means (with SDs in parentheses) and confidence intervals (CIs) (95%) for formula and placebo group before and for formula and placebo group after intervention were 0.27 (0.29), CI (−0.52, −0.02), 0.27 (0.35), CI (−0.52, −0.01), 0.08 (0.08), CI (−0.14, −0.01), 0.24 (0.22), CI (−0.40, −0.08), respectively.

The ANOVA test results [Table 2] showed that the time main effect was significant with regard to TAC concentrations; however, neither the intervention main effect nor time and intervention interaction was significant. Paired *t*-test results and significant values for TAC are shown in Figure 2. Means (with SDs in parentheses) and confidence intervals (95%) for formula and placebo group before and for placebo group after intervention were 0.27 (0.13), CI (−0.38, −0.16), 0.40 (0.33), CI (− 0.63, −0.16), 0.30 (0.24), CI (−0.48, −0.13), respectively.

Table 1: Participants characteristics and dietary information, (mean±standard deviation)

Variable	FormEx (*n*=8)	PlcEx (*n*=10)	*P*
Age (year)	23.25±5.14	22.70±4.37	0.75[β]
Weight (kg)	58.58±8.6	62.59±6.07	0.26[α]
Height (m)	1.65±0.05	1.65±0.03	0.76[α]
BMI (kg/m^2)	21.39±2.09	22.73±1.61	0.13[β]
Total body fat (%)	25.78±1.87	26.52±1.37	0.35[α]
Glucose (mmol/L)	5.35±0.28	5.13±0.29	0.13[α]
Cholesterol (mmol/L)	4.28±0.68	4.26±0.50	0.95[α]
Triglyceride (mmol/L)	0.95±0.33	1.11±0.78	0.72[β]
Creatine kinase (U/L)	74.25±54.79	84.50±26.88	0.06[β]
Lactate dehydrogenase (U/L)	352.12±96.17	323.30±54.76	0.46[α]
Hemoglobin (g/L)	136.87±11.64	134.70±5.31	0.60[α]
Hematocrit (%)	43.01±3.12	42.04±1.68	0.41[α]
VO_2 max (ml/kg min)	35.11±6.02	38.02±6.45	0.34[α]
Dietary information			
Energy (kcal/day)	2142.50±137.25	2069.50±193.86	0.38[α]
Carbohydrate			
Gram	308.83±20.32	283.90±35.17	0.08[α]
Percentage (%)	57.25±4.97	54.10±5.76	0.23[α]
Protein			
Gram	72.47±14.66	70.48±19.26	0.81[α]
Percentage (%)	13.50±2.56	13.40±3.47	0.94[α]
Fat			
Gram	71.27±14.02	76.22±17.33	0.52[α]
Percentage (%)	29.25±4.13	32.40±6.34	0.22[α]
Vitamin A (mcg)	1464.18±1122.07	1261.15±778.37	0.65[α]
Vitamin E (mg)	5.13±3.20	3.24±1.25	0.13[β]
Vitamin C (mg)	57.52±21.01	81.28±54.47	0.28[α]
β-carotene (mcg)	1117.42±1083.07	914.58±745.83	0.66[α]

[α]Data are tested by independent *t*-test; [β]Data are tested by Mann-Whitney U-test. FormEx=Antioxidant rich formula plus exercise; PlcEx=Placebo plus exercise; BMI=Body mass index

Table 2: Effect of time and intervention, and their interaction on measured variables assessed by repeated measurements analysis of variance

Variable	FormEx (n=8) 95% CI	PlcEx (n=10) 95% CI	Time main effect				Intervention main effect				Time and intervention interaction			
			$F(df_1, df_2)$	Effect size	Power	P	$F(df_1, df_2)$	Effect size	Power	P	$F(df_1, df_2)$	Effect size	Power	P
MDA	1.68-1.96	1.72-1.97	0.09 (5, 75)	0.13	0.34	0.42	0.82 (1, 15)	0.005	0.14	0.38	1.62 (5, 80)	0.09	0.39	0.19
TAC	1.39-1.83	1.56-1.94	18.18 (5, 75)	0.48	0.98	0.001	1.39 (1, 15)	0.06	0.15	0.25	1.74 (5, 75)	0.10	0.57	0.13
SOD	1183.53-1352.93	1087.23-1238.74	4.05 (5, 80)	0.20	0.94	0.002	3.85 (1, 16)	0.19	0.45	0.06	1.87 (5, 80)	0.10	0.61	0.10

MDA=Malondialdehyde; TAC=Total antioxidant capacity; SOD=Superoxide dismutase; CI=Confidence interval

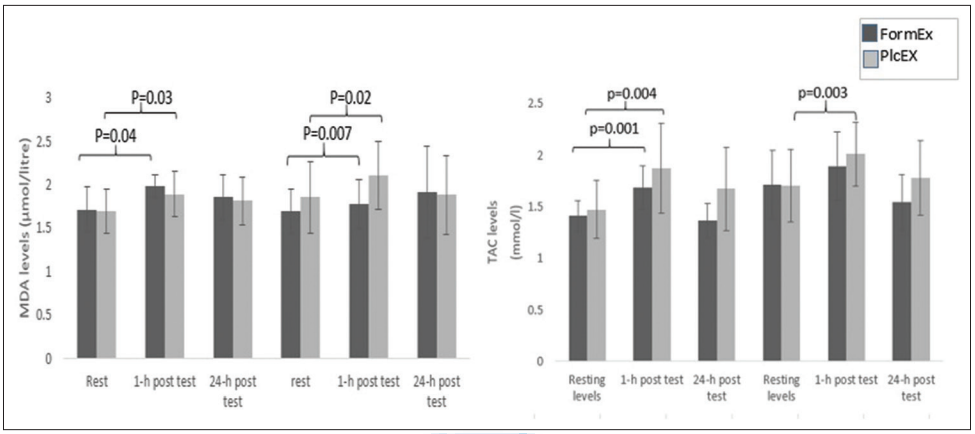

Figure 2: MDA and TAC levels at rest, 1 h and 24 h post exercise, before and after 4 weeks intervention. TAC: total antioxidant capacity. MDA: malondialdehyde. *P* values are according to the paired *t*-test

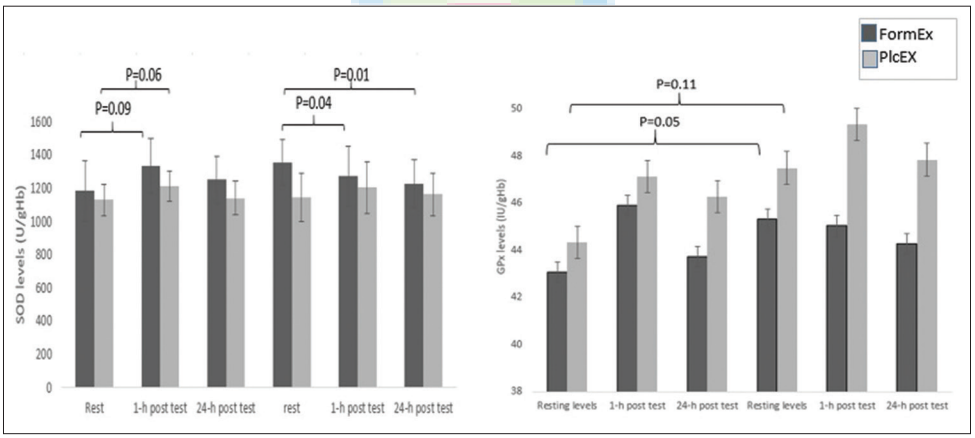

Figure 3: SOD and GPx levels at rest, 1 h and 24 h postexercise, before and after 4 weeks intervention. GPx: glutathione peroxidase. SOD: superoxide dismutase. *P* values are according to the paired *t*-test for SOD and Wilcoxon test for GPx

Repeated measures ANOVA showed that a significant main effect of time was significant for SOD. Neither intervention main effect nor time and intervention interaction was significant [Table 2].

Paired *t*-test results and significant values for SOD are shown in Figure 3. Means (with SDs in parentheses) and confidence intervals (95%) for formula and placebo group before and for formula group (before versus 1 h and 24 h after test) after intervention were 149.65 (217.06), CI (−331.12, 31.81), 80.97 (123.05), CI (−169.00, 7.05), −80.02 (90.94), CI (3.98, 156.05), 131.40 (113.94), CI (36.14, 226.66), respectively.

GEE results for GPx showed that there were no statistically significant differences between the groups (estimate: −2.53; SE: 1.61; CI: −5.68, 0.63, P = 0.11).

DISCUSSION

The main purpose of this study was to investigate the effect of natural antioxidant formula on exercise training adaptation and HSP70. No side effects of supplementation were reported. We showed that natural antioxidant supplementation differentially modulates oxidative stress-related parameters and the formula increased

mRNA expression of HSP70 in lymphocytes. The results demonstrated that lipid peroxidation increased 1 h after exercise, pre- and post-intervention in both groups evident by increased levels of MDA and then returned to pre-exercise levels after 24 h. The exhaustive exercise-induced increase in the plasma levels of MDA was consistent with those previously reported in studies carried out on rodents[18] and human.[19]

The baseline MDA levels demonstrated no significant differences between groups, before and after intervention, indicating neither supplementation nor prescribed exercise training affected resting MDA levels. In contrast to this study, Azizbeigi *et al*. showed that endurance training decreased the resting MDA levels significantly in untrained males.[20] It is speculated that the different training levels of participants of these two studies might be the explanation for different results.

Moreover, other studies reported that exercise training decreased MDA levels at rest.[21,22] Regarding the effect of natural antioxidant supplementation on resting MDA levels, Carrera-Quintanar *et al*. showed that supplementation with lemon verbena extracts combined with 21 days aerobic training program decreased the levels of oxidative stress markers such as MDA and protein carbonyls in plasma.[23]

Due to the adaptive response resulted from cumulative effects of repeated exercise bouts, the initial signal for stimulation leading to the long-term modulation, i.e., oxidant production, should occur after each individual exercise bout.[24] This fact and the increased levels of MDA observed in this study 1 h postexercise after supplementation in formula group, could explain the markedly increased levels of HSP mRNA expression, supporting higher training adaptation in the formula group compared to the placebo in the long-term period.

The fact that MDA levels did not change significantly by formula intake, suggests that MDA status was not the main reason for increased HSP70 mRNA expression and it might be proposed that the hormetic effect of formula ingredients was directly involved in the gene regulation of HSP70.

In the present study, TAC levels were significantly increased 1-h after exercise in both FormEx and PlcEx preintervention. The increased TAC levels, after exercise test may result from mechanisms to compensate for the increased MDA production during the exercise. The increased levels of TAC could explain the following reduction in MDA levels 24-h after exercise before intervention in both groups. The increased TAC level in formula group after intervention was insignificant. It is proposed that ingredients of this formula may exert prooxidant effects, acting to abolish the increment of TAC levels. Therefore, another possible explanation is that formula diminished the TAC increment, however, since relative mRNA expression of HSP-70 was increased in FormExmore than PlcEx group in all time points, it is speculated that prooxidant effect could potentially lead to decreased TAC levels, with subsequent adaptive increase in relative mRNA HSP-70 expression. However, the increased levels of HSP70 did not have the ability to improve TAC levels in short time duration, through increasing AOEs, 1–24 h postexercise test in the present study.

To the best of our knowledge, there has been no study investigating the effects of natural antioxidant-rich formula combined with aerobic exercise training on mRNA expression of HSP-70. In this study, the overall effect of training was increment of HSP-70 expression; however, the antioxidant-rich formula was capable to significantly increase the lymphocyte HSP-70 mRNA expression compared to the PlcEx. The increased level was particularly marked at rest and 24-h postexercise following supplementation in FormEx. Our findings were in consistent with Locke *et al*., who showed that a single bout of exhaustive treadmill running led to an elevated HSP-70 synthesis in lymphocytes.[25] In addition, Walsh *et al*., also showed that a single bout of exercise accelerated the synthesis of HSP-72 mRNA expression 2-h post-exercise in the skeletal muscle in human.[26]

Several studies have declared that when HSPs are located intracellularly, such as in lymphocytes, they act as chaperone-like proteins involved in folding of newly synthesized proteins and bind to denatured proteins as well.[27]

Dabidi Roshan *et al*. reported that ginger supplementation for 1 week increased plasma HSP-72 protein content immediately and 24-h after strength exercise, however, it was less than that of the placebo group.[28] Pinot *et al*. reported that preincubation of monocytes with the antioxidant quercetin, a flavonol compound in honey, increased the induction of HSP-70.[29]

We tested weather antioxidant-rich formula can lead to increased levels of enzymes necessary for exercise adaptation. The increased HSP-70 mRNA in lymphocytes could be a part of the cell response to increased oxidative stress induced by exercise and antioxidant-rich formula supplementation. However, surprisingly SOD levels decreased postintervention in the supplemented group. This is in contrast with Khassaf *et al*. showed that Vitamin C supplementation increased the baseline activities of SOD.[2] One potential explanation could be that, after supplementation, the increased level of MDA in FormEx after 1-h of exercise was significantly less than that of

PlcEx. Therefore, there was no need for increased levels of SOD, and the existed amount was capable to neutralize the MDA. Another explanation might, partially, be that the supplementation could directly diminish the lipid peroxidation.

SOD levels decreased 1-h postexercise only in FormEx after supplementation and remained unchanged 24-h thereafter. Although preintervention SOD levels increased 1-h postexercise in both FormEx and PlcEx, their increased level was marginally significant or insignificant, with no changes in resting levels after supplementation in both groups. Our findings regarding the effect of training on SOD levels was in contrast with Ristow *et al*., who showed that SOD RNA levels increased after exercise.[30] Whereas in the present study, neither training nor supplementation was effective on resting SOD levels. Azizbeigi *et al*., as well, showed the increased levels of SOD after 8 weeks training.[20] The mechanisms underlying the decreased levels of SOD are unknown in spite of elevated HSP-70 expression. It is plausible that there was no enough time to observe the effect of HSP-70 increment.

We observed that GPx level did not change 1-h/24-h postexercise neither in FormEx nor in PlcEx; however, the resting levels of GPx were increased after supplementation in FormEx. In contrast to this study, Cumming *et al*. showed that GPx level decreased after 11 weeks of endurance training, but antioxidant supplementation did not have any beneficial or detrimental effect on this variable.

It has already been shown that female rats have demonstrated a lesser extent of exercise-induced muscle oxidative damage and training adaptation of AOEs than their male counterparts, possibly due to the proposed antioxidant effect of estrogens.[31] We have also investigated the effect of antioxidant formula only in women. It is proposed to conduct similar intervention in both genders.

Limitations of the study

As a limitation, the mRNA levels measured in the present study did not reflect the protein levels of this variable; therefore, it is suggested to measure the protein levels of HSP-70 protein. In addition, the small sample size is another limitation of the current study, the significance of the results might be different with larger sample size. Longer duration would be warranted in the future works.

CONCLUSIONS

Our study showed that antioxidant-rich natural formula increased the relative expression of HSP-70 mRNA, but it had variable effects on markers of oxidation. Although mRNA generally has a short half-life, the increment of mRNA expression of HSP-70 had an upward trend 24-h postexercise. Therefore, we would favor the possibility that natural antioxidant rich formula increases the HSP-70 mRNA expression in female recreational athletes accompanied by exercise training at 60%–65% VO_2 max which is of practical relevance as it might improve the antioxidant ability of cells in the long-term period. Considering this fact, prescribing these herbs as a commercial formula for athletes, in the adaptation phase to exercise, will be helpful to increase HSP-70 expression and subsequently improvement of antioxidant status.

Acknowledgments

This article was written based on a dataset of PhD thesis (Sevana Daneghian) registered in Ahvaz Jundishapur University of Medical Sciences.

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Conflicts of interest

There are no conflicts of interest.

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