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Circulatory miR-133a and miR-145 are Associated with the Improving Impact of Combined Endurance Exercise and MitoQ on Cardiovascular Function in Patients with Hypertension

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HIGHLIGHTS

- MitoQ and endurance training (ET) improved blood pressure and cardiac function in patients with HTN.
- MitoQ and ET increased expression of miR-145 and miR-133a.
- Anti-inflammatory and antioxidant properties of ET and MitoQ linked to increased miRs-145 and 133a.

Abstract: We used mitoquinone (MitoQ), a mitochondria-targeted antioxidant, alone and combined with moderate ET (endurance training) in male patients with hypertension to determine their effects on blood pressure (BP), cardiac function, TOS (total oxidant status), and serum miR-145, miR-133a, and hs-CRP levels. Moderately HTN patients participated in groups of Placebo, MitoQ, ET, and MitoQ+ET. Serum was used to assess miR-133a, miR-145, TOS, and hs-CRP, and echocardiography was performed to assess cardiac performance. In the MitoQ+ET group, BP, left ventricular hypertrophy, and cardiac filling pressure decreased. Ejection fraction did not change significantly. Both MitoQ+ET and ET significantly reduced TOS and hs-CRP and significantly increased miR-145 and miR-133a in serum. Overall anti-inflammatory and antioxidant properties of ET and MitoQ were associated with miR-145 and miR-133a increase, and with cardiac function and BP improvements in patients with HTN. MitoQ+ET may potentially be used as an alternative therapy in HTN treatment.

Keywords: Hypertension; MitoQ; Endurance training; TOS; hs-CRP; miR-133a; miR-145.

INTRODUCTION

Hypertension (HTN) is one of the most common chronic diseases and affects almost 1.2 billion globally [1, 2]. It is estimated that hypertension will affect one-third of the adult population by 2025 [3]. This disease is the primary cause of congestive heart failure, stroke, and coronary artery disease (CAD), causing a heavy economic burden globally [1]. It also causes long-term organ damages, such as vasculopathy, nephropathy, and neuropathy [1].

One non-pharmacological intervention that may control, treat, and prevent hypertension is exercise [4, 5]. It has been reported that endurance training (ET) significantly reduces systolic blood pressure (SBP) and diastolic blood pressure (DBP) through the sympathetic nervous system [3] and the regulation of the renin-angiotensin system [4].

Oxidative stress is related to the high production of reactive oxygen species (ROS) and a reduction in the antioxidant defense system capacity [6]. Both oxidative stress and inflammation are related to endothelial dysfunction and elevation in blood pressure [6-8]. Previous studies have demonstrated that exercise training contributes to higher ROS production and promotes antioxidant defense systems to compensate for produced ROS [9] and adaptation to oxidative stress [10, 11]. A systematic review study reported that exercise training reduced hs-CRP levels, attenuated cytokine production, and ameliorated endothelial dysfunction [12].

microRNAs (miRNAs) are involved in post-transcriptional gene expression regulation. They are involved in a broad spectrum of diseases, including cancer, CVD, hypertension, inflammation, and other metabolic diseases [13, 14]. They also play regulatory roles in exercise-induced adaptations [15]. miR-133a has a pivotal role in the development and function of cardiac and skeletal muscles [16]. Recently it was reported that miR-9, miR-126, miR-133, miR-143, and miR-145 are downregulated in hypertensive individuals. Also, it was shown that there is a positive correlation between miR-133 and 24-h ambulatory blood pressure in hypertensive individuals [17] and that the action of ACE/AngII/AT1R is repressed by miR-145 [18]. Endurance training (14 w) significantly increased left ventricle miR-133 expression [19].

Mitochondria are the primary origin of ROS production [20] and may contribute to the pathophysiology of HTN. Mitoquinone (MitoQ) is an orally available mitochondria-targeted antioxidant that reduces lipid peroxidation in the mitochondria [21]. Its beneficial effects have been reported in cardiac ischemia/reperfusion injury [22], and reduced plasma oxidized LDL levels [23]. Therefore, this agent is expected to have beneficial effects on HTN patients' blood pressure.

ET impacts muscle recovery, mitochondrial biogenesis, energy metabolism, and inflammation [24]. However, the precise mechanism for the beneficial effects of ET and MitoQ on the cardiovascular system is not fully understood [25]. Recently we found that ET in combination with MitoQ improved BP and some cardiac factors in association with increase in the circulatory level of miR-126 and reduction of miR-27a [26], miR-21 and miR-222 [27]. In the present study, the impact of MitoQ with and without ET on blood pressure, cardiac function, miR-145, and miR-133a levels in serum was assessed in HTN individuals.

MATERIAL AND METHODS

Materials

The materials used included MitoQ (MitoQ Ltd., New Zealand), Placebo (In the same shape/size/color as MitoQ capsules produced by the school of pharmacy of KMU), Total oxidant status (TOS) assay kit (Kiazist, product (P) code KTOS-96, Iran), high-sensitivity CRP (hs-CRP) ELISA kit (LDN, P. code DM E-4600, Germany), RNA isolation kit (Norgen Biotek, P. code 17200, Canada), cDNA synthesis kit (Norgen Biotek, P. code 54410, Canada), cel-miR-39 (Norgen Biotek, P. code 59000, Canada), SYBR green (Ampliqon, P. code A325402, Denmark), universal primer (reverse) (Norgen Biotek, P. code 59000, Canada), and miR-133a and miR-145 primers (Metabion, Germany).

Subjects

In a randomized, double-blind clinical trial, fifty-two subjects with high blood pressure (between SBP/DBP 140/90 to 150/100 mmHg) in the age range of 40 to 55 were enrolled in the study. The subjects included participants of the Kerman Coronary Artery Disease Risk Factor Study (KERCADRS) and individuals referred to Shafa Hospital in Kerman, Iran. An informed consent form was read and signed by all participants. All protocols were approved by the Iranian registry of clinical trials (IRCT 20190228042870N1, link:

en.irct.ir/trial/37904) and by the university Ethics Committee (IR.KMU.REC.1398.353), and all protocols followed the guidelines of the Declaration of Helsinki.

HTN was defined according to the criteria of the European Heart Association: DBP ≥ 90 mm Hg and/or SBP ≥ 140 mm Hg. Individuals who reported complications such as lung, kidney, and liver diseases, and also, those with cancer, diabetes, Obesity (Body mass index; BMI ≥ 30 kg/m^2), and known cardiovascular diseases (e.g., valvular heart disease and heart failure) besides HTN were excluded from this study. A validated questionnaire was completed by face-to-face interview for collection of demographic information. The patient's physical activity (including type of activity, frequency, and duration), was questioned using a validated questionnaire being used in KERCADRS (global physical activity questionnaire, GPAQ) [28]. In this questionnaire the intensity of physical activity (PA) is measured based on metabolic equivalent of task (MET) units. MET is the use of energy in an adult individual while he or she is sitting. Less than 1500 METs per week is scored as having low, Moderate PA is between 1500 and 3000 METs and more than 3000 METs is assigned to intense PA. The baseline physical activity was low in 8(61.5%), 9(69.2%), 5(38.5%) and 4(30.8%) of subjects in the four groups of the study respectively (no significant difference among the groups). The rest of subjects in the studied groups were sedentary. The subjects were males with moderate HTN and were assigned randomly to four groups (n = 13 each) of placebo, MitoQ (20 mg/day, oral) [23], endurance training (ET), and MitoQ+ET (Figure 1). They performed moderate-intensity ET (intensity 40 to 60% VO2 peak, heart rate 120–140 b/min, 45 min duration, three sessions per week) for the length of six weeks. Blood samples were collected both at baseline and on day 43 (24 h post-intervention). After incubation at room temperature for 45 minutes, the blood samples were centrifuged (5000 rpm), and the serum samples were used for quantification of miRNAs 133a and 145, TOS, and hs-CRP. We controlled the participants' adherence to the supplementation protocol. A pack of fourteen MitoQ or placebo capsules were provided to each subject and after two weeks another pack was provided. The subjects were questioned about the daily use of the pills and if any of them was left, when they came back to receive the next pack, to ensure that all the pills had been consumed regularly and completely.

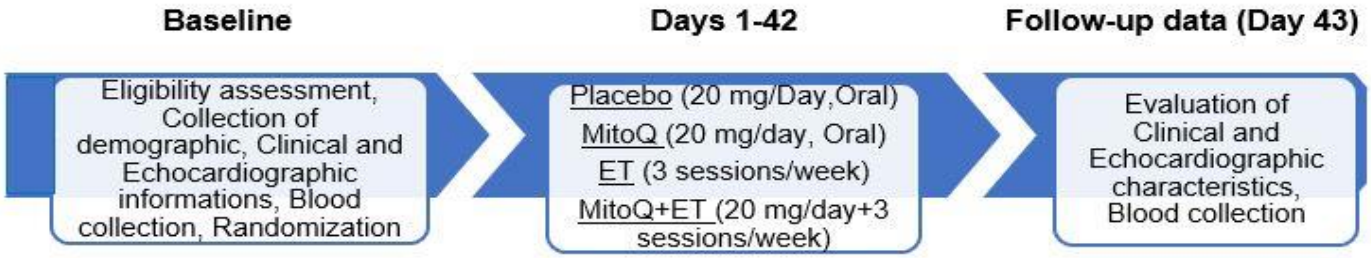

Figure 1. The study timeline and test procedures

Blood pressure measurement

An automated device (Omron, M6 Comfort, Japan) was used to measure BP following a 10-min rest twice at 30-min intervals in the morning. The formula MAP = DBP + 1/3 (SBP-DBP) was used to calculate the MAP (mean arterial pressure).

Modified Astrand-Ryhming cycle ergometer test

VO_2 peak was measured before the ET protocol started by a cardiopulmonary exercise test (CPET), in which the subjects cycled on an upright position cycle ergometer (Monark, Ergomedic 839 E, Sweden) coupled with a gas analyzer (Cortex, Metalyzer 3B, Germany) [29].

Endurance training protocol

The subject performed moderate-intensity ET for six weeks (3 sessions/week) by a cycle ergometer fitted with an electrocardiogram. The first training session was 15 minutes long and at 40% to 60% maximum output wattage. Approximately 2.5 min was added to every following training session until the ET length reached approximately 45 min. The ET intensity and duration remained at this level for the last two weeks. ET was performed under the supervision of a licensed physician, and BP was measured before and after training [30]. The participants' adherence to the exercise protocol was also recorded. Only two of participants did not return on time, each in two sessions of training, during six weeks exercise protocol. For these subjects another session was programed immediately at the day after the appointed time to complete their total number of training sessions.

Assessment of cardiac function by echocardiography

A two-dimensional mode ultrasound machine (Philips, EPIQ, USA) was used to assess parameters of cardiac function, including isovolumic relaxation time (IVRT), mean left atrial pressure (MLAP), and left ventricle ejection fraction (EF), at baseline and on day 43. Moreover, posterior wall and interventricular septum thickness were measured by echocardiography and scored as with left ventricular hypertrophy (LVH) (> 1.1 cm) or without LVH (< 1.1 cm). Simultaneous ECG was used for the determination of heart rate (HR).

Determination of serum total oxidant status (TOS)

First, 200 µL of TOS reagent (Kiazist, Iran) was added to 50 µL of serum into the plate wells and incubated at room temperature for 15 min to assess TOS levels. PBS was used as blank. The OD was obtained by a microplate spectrophotometer reader (BioTek instrument, Epoch, USA) at 560 nm. The standard curve was obtained using different concentrations of H_2O_2 against their OD, and the results were expressed as micromole per milliliter of serum [31]. The reaction mechanism was based on the oxidation of Fe^{+2} to Fe^{+3} by cellular oxidants and the production of a detectable chromogen, whose absorbance directly correlates to oxidant levels.

High-sensitivity C-reactive protein assay (hs-CRP)

An enzyme-linked immuno-sorbent assay (ELISA) kit was used to measure serum hs-CRP levels on an ELISA reader at 450 nm using a Power Wave microplate spectrophotometer (DRG instrument, Cat. No. ELM-2000, Germany). The serum hs-CRP levels were expressed as ng/mL.

miR-133a and miR-145 measurement by real-time PCR

A total RNA isolation kit was used to extract the total RNA from the serum. RL buffer was added to 150 µl of serum sample, and then it was loaded into the column specifically collecting the RNA. RNase-free water was used to wash the RNAs in the column. The isolated RNA concentration and purity were measured by NanoDrop (ND-2100, Thermo Fisher Scientific, USA). In order to normalize and reduce sampling errors, each sample received 3.5 µl cel-miR-39 (*Canorhabditis* elegans miR-39) during the extraction procedure as external control. Then 5µl of isolated RNA was used to synthesize cDNA, and the procedure was performed according to the microRNA cDNA synthesis kit. In order to perform real-time PCR, we used high ROX Real Q Plus Master Mix Green and newly obtained cDNA-specific miR-145 and miR-133a primers. The reaction mixture was amplified by StepOnePlus (Applied Biosystems, USA). We normalized the miR-145 and miR-133a relative expressions to cel-miR-39 as external control. The expression was calculated as fold change, $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT$ = [(CT gene - CT cel-miR-39)treatment - (CT gene - CT cel-miR-39)]CTL [32]. The forward primer sequences of miRs were as follows:

miR-133a; 5′-TTTGGTCCCCTTCAACCAGCTG-3′,

miR-145; 5′-GTCCAGTTTTCCCAGGAATCCCT-3′,

cel-miR-39; 5′-UCACCGGGUGUAAAUCAGCUUG-3′.

Statistical analysis

Data analysis was performed by GraphPad Prism (GraphPad v.8.4.3., San Diego, USA). We used two-way repeated measure ANOVA to assess the differences among the study groups, followed by Tukey's post hoc test for pairwise comparisons. Chi-square test was used for descriptive statistics (history of HTN, and level of physical activity). $P < 0.05$ was considered as significant.

RESULTS

General clinical and echocardiographic characteristics

At baseline, the groups were not significantly different in age, BMI, and heart rate (Table 1). Alcohol intake, physical activity level, family HTN history, and smoking were also not different among the groups (data not shown). BMI in the groups that performed ET significantly decreased ($P<0.05$) at follow-up compared to the baseline.

Table 1. Baseline and follow-up demographic/anthropometric and echocardiographic characteristics of the study groups.

Groups	**Placebo (*n* = 13)**		**MitoQ (*n* = 13)**		**ET (*n* = 13)**		**MitoQ+ET (*n* = 13)**	
Variable	**Baseline**	**Follow-up**	**Baseline**	**Follow-up**	**Baseline**	**Follow-up**	**Baseline**	**Follow-up**
Age (years)	49 ± 0.7	49 ± 0.7	49 ± 0.7	49 ± 0.7	48 ± 0.9	48 ± 0.9	47 ± 1.1	47 ± 1.1
MAP (mmHg)	108 ± 1.4	107 ± 1.5	110 ± 1.3	105 ± 1.0**	104 ± 0.6	99 ± 0.6**	111 ± 1.5	102 ± 1.1***
BMI (kg/m^2)	26 ± 0.5	26 ± 0.3	27 ± 0.5	26 ± 0.6	26 ± 0.4	25 ± 0.2*	27 ± 0.4	26 ± 0.3*
EF (%)	59.6 ± 0.4	59.6 ± 0.4	60 ± 0.02	60 ± 0.02	59.2 ± 0.5	60 ± 0.01	58.8 ± 0.6	60 ± 0.03
LVH	0.84 ± 0.22	1.0 ± 0.19	1.07 ± 0.13	0.84 ± 0.15	1.07 ± 0.07	0.92 ± 0.07	1.07 ± 0.17	0.76 ± 0.12*
PWT (cm)	0.87 ± 0.02	0.83 ± 0.02	0.85 ± 0.01	0.90 ± 0.02	0.90 ± 0.03	0.90 ± 0.02	0.90 ± 0.03	0.92 ± 0.03
IVRT (ms)	86 ± 1.4	88 ± 1.7	91 ± 2.1	90 ± 1.7	92 ± 1.3	87 ± 1.4	92 ± 2.4	87 ± 1.6
MLAP (mmHg)	9.1 ± 0.41	8.7 ± 0.35	9.9 ± 0.33	9.3 ± 0.29	9.4 ± 0.20	8.6 ± 0.23	9.6 ± 0.44	8.2 ± 0.31***##
Resting and/or peak HR (bpm)	76 ± 1.8	NA	76 ± 1.9	NA	72 ± 1.8	127 ± 2.5***	74 ± 2.0	132 ± 2.5***

Values are mean ± SEM. ∗ significantly vs baseline, # significantly vs MitoQ follow-up. MAP: mean arterial pressure, BMI: Body mass index, HR: heart rate (Peak HR; Maximum HR during training in groups with exercise), NA: not applicable (non-ET groups). EF: ejection fraction, LVH: left ventricular hypertrophy, PWT: posterior wall thickness, IVRT: isovolumic relaxation time, MLAP: mean left atrial pressure.

MitoQ and ET effects on cardiac function and BP

ET, MitoQ, and their combination caused a significant reduction in MAP compared to their respective baselines. The combination treatment decreased MLAP more effectively ($P < 0.001$). Table 1 shows that LVH significantly decreased in the combined group compared to its baseline ($P < 0.05$). However, interventions did not change EF, PWT, and IVRT significantly.

Effects of interventions on serum TOS and hs-CRP

Following six weeks of MitoQ supplementation and ET, the TOS levels in the serum were reduced significantly by the interventions (Figure 2). ET and its combination with MitoQ caused a significant reduction in hs-CRP compared to their baseline values ($P < 0.01$ and $P < 0.001$, respectively) (Figure 3). In both variables, the combination therapy was more effective compared to single interventions.

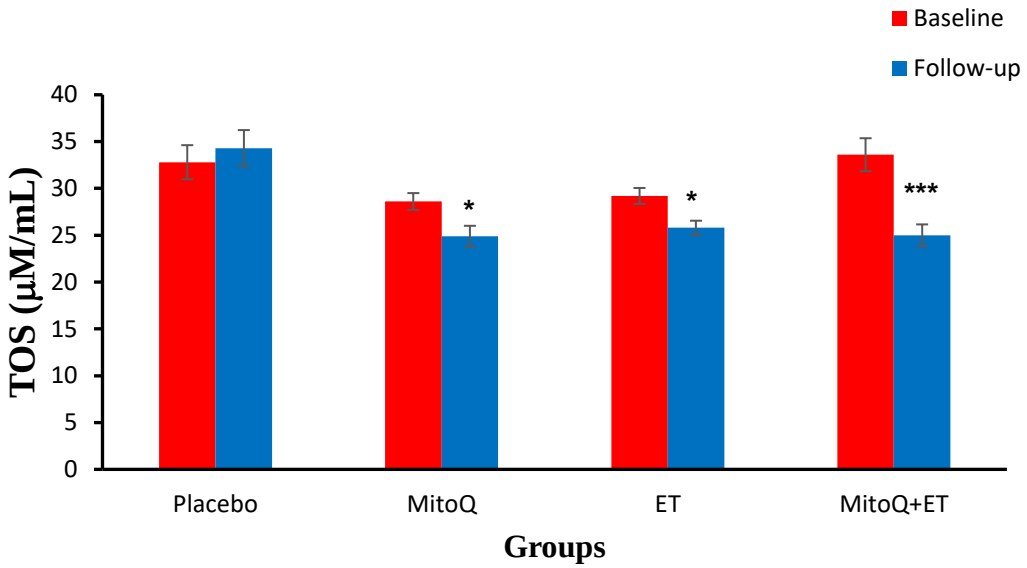

Figure 2. Serum total oxidant status (TOS) levels (mean ± SEM) at the beginning and end of the study in patients with HTN. $n = 13$ in each group. * $P < 0.05$, *** $P < 0.001$ *vs* related baseline. ET: endurance training.

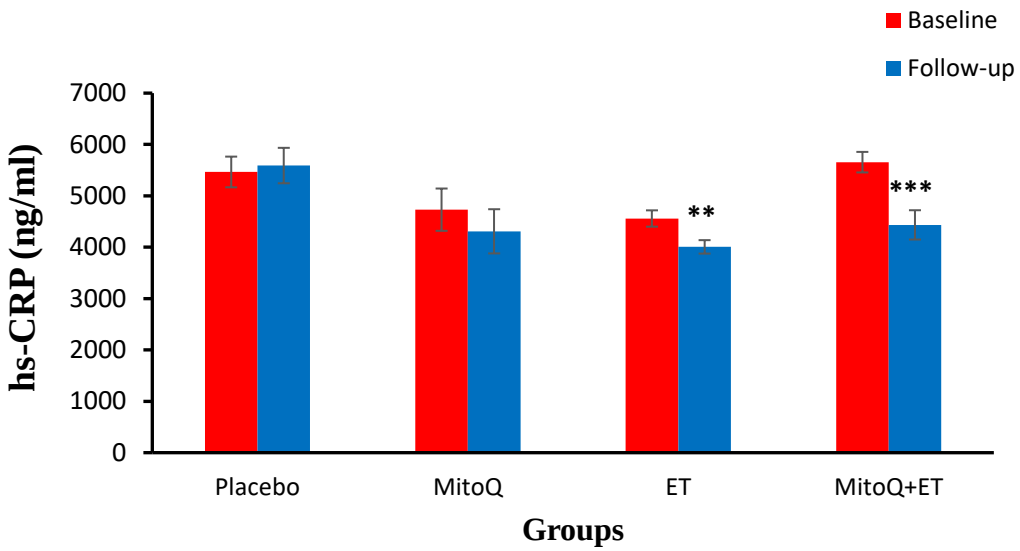

Figure 3. Initial and post-intervention serum high sensitive C-reactive protein (hs-CRP) levels (mean ± SEM) in patients with HTN. $n = 13$ in each group. ** $P < 0.01$, *** $P < 0.001$ *vs* related baseline. ET: endurance training.

Impact of intervention on expression of miR-145 and miR-133a

ET and its combination with MitoQ increased serum miR-133a significantly compared to baselines (Figure 4). ET, MitoQ, and their combination caused a significant increase in serum miR-145 (Figure 5). Furthermore, the effect of the combination of ET and MitoQ on miR-145 expression was more than the effect of treatment with either MitoQ or ET alone ($P < 0.001$).

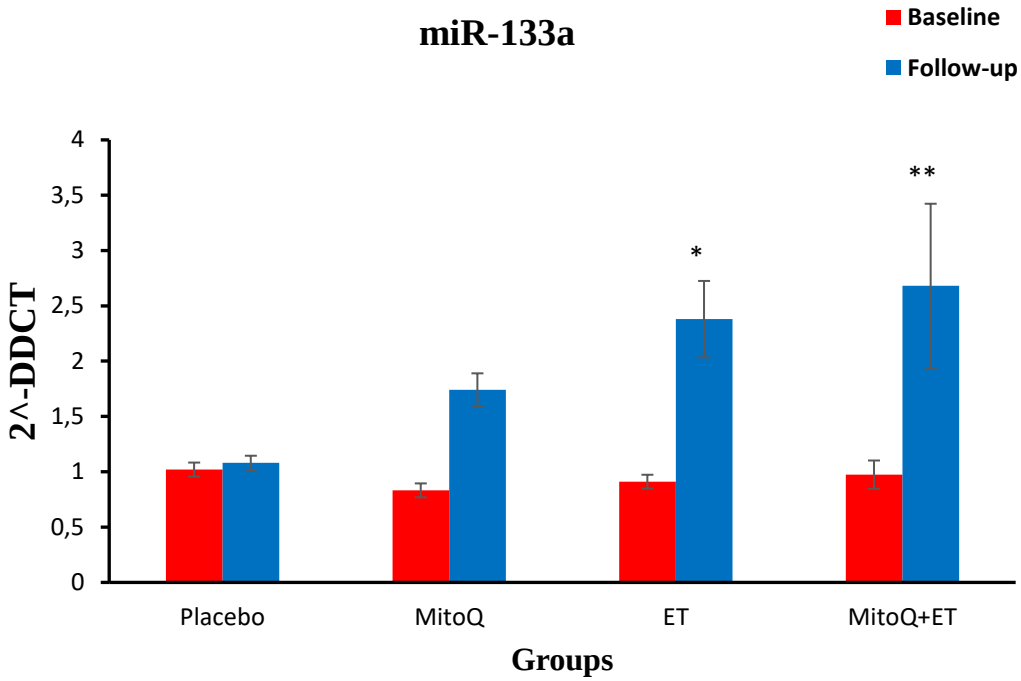

Figure 4. Circulating miR-133a expression (Mean ± SEM) in HTN patients, and the effect of ET, MitoQ, and MitoQ+ET (n = 13 in each group). * $P < 0.05$, ** $P < 0.01$ *vs* baseline. ET: endurance training.

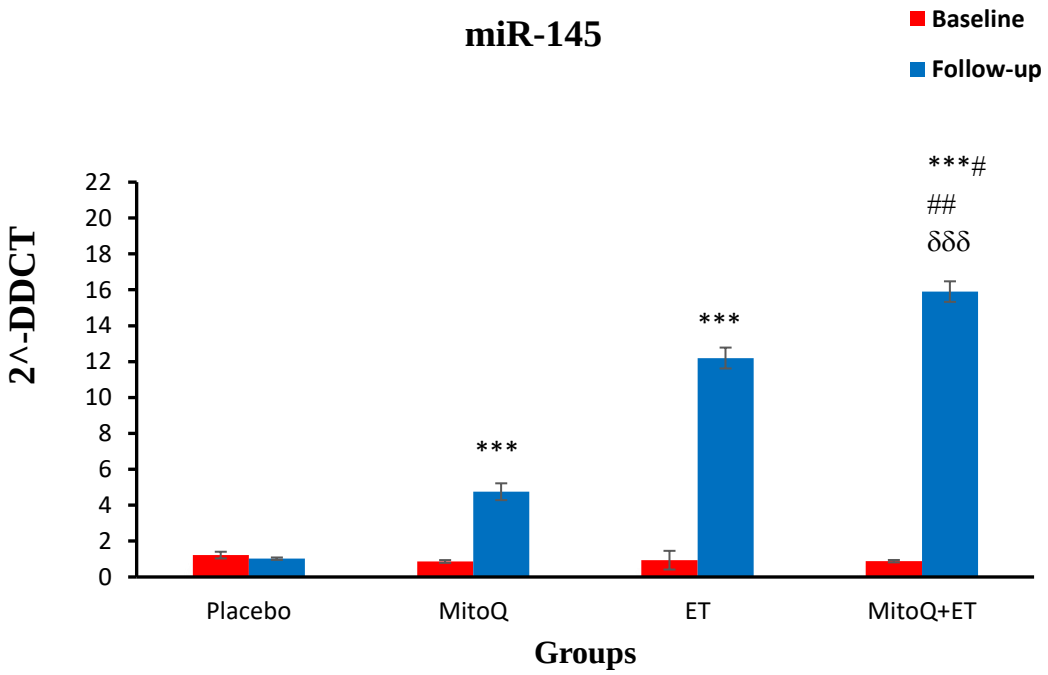

Figure 5. Circulating miR-145 expression (Mean ± SEM) in HTN patients with, and the effect of intervention with ET (endurance training), MitoQ, and MitoQ+ET (n = 13 in each group). * Significant *vs* related baseline, # significant vs MitoQ follow-up, δ significant *vs* ET follow-up. All differences are with $P < 0.001$.

DISCUSSION

In the present study, we found that TOS, hs-CRP, and BP decreased and serum miR-133a and miR-145 levels increased after 6 weeks of treatment of moderately hypertensive patients with MitoQ, ET, and their combination. These alterations were associated with improved cardiac function, confirmed by reduction in left atrial pressure and left ventricular hypertrophy. Combination therapy was more effective than single treatment in most cases.

It has been documented that HTN is related to increased generation of ROS and a reduced antioxidant defense system resulting in oxidative stress [33]. Also, the levels of inflammatory factors are higher in the blood of these patients [34-36]. Accordingly, exercise is recommended for individuals with moderate hypertension as it attenuates blood pressure by decreasing ROS levels, improving redox status [36], and by attenuating the level of inflammatory factors in the blood [38, 39]. Mitochondria contribute to HTN pathophysiology as it has been shown that these organelles are the primary origin of ROS production [20]. Therefore, it is anticipated that MitoQ, as a mitochondria-targeted antioxidant agent, may have beneficial effects on the blood pressure of patients with HTN, especially when it is co-administered with ET, another redox improver. In agreement with this anticipation, we showed that ET and MitoQ co-administration effectively reduced the blood pressure of hypertensive patients, which was associated with reduction of TOS and inflammatory factor hs-CRP. Antioxidants such as vitamin E and ascorbic acid have not shown remarkable effects on hypertension, and it was proposed that these agents cannot reach the ROS generation sites such as mitochondria [33]. Similarly, another study has reported that aerobic training is an effective way to reduce blood pressure. Swati and coauthros in their study have compared the effect of 6 weeks aerobic (at 60-70% of maximum HR) and resistance exercise trainings on blood pressure of patients with hypertension. They found that both aerobic and resistance trainings were effective in reducing systolic and diastolic blood pressures and heart rate, while aerobic training was significantly more effective than resistance training [40]. Tsai and coauthors found that low to moderate intensity exercise is effective in lowering blood pressure, and in comparing the effect of different exercise intensities, low intensity exercise was more effective in lowering blood pressure than was high intensity exercise [41]. The reason is that endurance training has a greater impact on maximum oxygen uptake (VO2 max) and associated cardiopulmonary variables and it more effectively modifies cardiovascular disease risk factors associated with the development of coronary artery disease [40]. In the present study we used moderate intensity endurance training, which showed similar effects on blood pressure, and in addition this effect was augmented when combined with MitoQ administration.

miR-133a, as a myo-miRNA, is essential for the appropriate development and function of cardiac and skeletal muscles [42]. It has been shown that the level of miR-133 is lower in patients with HTN compared to healthy controls [17], and this miRNA has antihypertrophic properties [43]. Our results showed that after 6 weeks, ET and its combination with MitoQ caused a significant increase in miR-133a levels. Mechanistically, it has been reported that angiotensin II increases blood pressure and induces myocardial fibrosis when miR-133a is downregulated, which confirms that miR-133a has cardio-protective properties [44]. It seems that in the present study ET and the combination of ET and MitoQ improved cardiac function and BP in hypertensive patients, probably by modulating miR-133a levels. In line with these findings, it has been shown that fourteen weeks of ET increases miR-133 expression in the left ventricle [19], and circulating miR-133 level increases in athletic and marathon runners [45].

It has been reported that miR-145 is also reduced in patients with HTN [46], and this miRNA has a vital role in the pathogenesis of HTN [18, 46]. The finding that ET, MitoQ, and MitoQ+ET caused a significant increase in miR-145 levels in hypertensive individuals and this effect was associated with reduced blood pressure may confirm the antihypertensive effect of this miRNA. Accordingly, the combination therapy that reduced blood pressure more effectively caused a more remarkable increase in the level of miR-145. The miR-143/miR-145 gene cluster inhibits ACE gene expression and therefore suppresses ACE/Ang II/AT1R action [18, 46]. It has been reported that in healthy young subjects, training increases miR-145 levels [47] and in the spontaneously hypertensive rat model, exercise restores miR-145 expression [48].

Overall, although ET+MitoQ decreased BP more than MitoQ or ET alone, the maximum reduction of 9 mm Hg may not seem a remarkable effect. This may be due to the moderate HTN of the patients who participated in the study, because we were cautious about exposing severe hypertensive subjects to ET. Probably, more beneficial effects may be achieved in combination therapy in patients with more severe hypertension.

CONCLUSION

Overall, this study demonstrated that moderate ET, MitoQ, and, especially, their co-administration improved cardiac function and reduced BP significantly in patients with hypertension via improvement in oxidative status, hs-CRP serum level, and increasing the circulating miR-133a and miR-145 levels. Based on these findings, we propose MitoQ and its combination with ET to be considered possible HTN treatment strategies.

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Conflicts of Interest: The authors declare no conflict of interest.

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