

Moderate Endurance Training and MitoQ Improve Cardiovascular Function, Oxidative Stress, and Inflammation in Hypertensive Individuals: The Role of miR-21 and miR-222: A Randomized, Double-Blind, Clinical Trial

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Abstract

Objective: Hypertension (HTN) is among the leading causes of myocardial infarction, stroke, and kidney disease. The MitoQ supplement is a mitochondrial-targeted antioxidant that attenuates the generation of reactive oxygen species (ROS). miRNAs play an essential role in the pathophysiology of HTN. Regular aerobic exercise is recommended to decrease the risk of cardiovascular disease. We aimed to evaluate the effects of MitoQ supplementation and moderate endurance training (ET), alone and in combination, on cardiac function, blood pressure, the circulatory levels of miRNA-21 and miRNA-222, and oxidative status in individuals with HTN.

Materials and Methods: In a double-blind, randomized clinical trial (except for ET group), 52 male hypertensive subjects (40-55 years old) were randomly divided into four groups (n=13): Placebo, MitoQ (20 mg/day, oral), ET (Cycle ergometer, moderate intensity, 40-60% VO₂ peak, three sessions/week for six weeks), and MitoQ+ET. Cardiac echocardiography indices, serum oxidative and inflammation status, and miRNAs 21 and 222 were assessed before and after interventions.

Results: Left ventricular mass [effect size (ES): -6.3, 95% confidence interval (CI): -11.2 to -1.4] and end-systolic/diastolic diameters significantly improved in the intervention groups (ES: -0.05, 95% CI: -0.11 to 0.00 and -0.09, 95% CI: -0.16 to -0.02). Total serum antioxidant capacity (TAC) increased (ES: 36.0, 95% CI: 26.1 to 45.8), and malondialdehyde (MDA) (ES: -0.43, 95% CI: -0.53 to -0.32), IL-6 (ES: -1.6, 95% CI: -1.98 to -1.25), miR-21 (ES: -0.48, 95% CI: -0.61 to -0.35), and miR-222 (ES: -0.31, 95% CI: -0.44 to -0.18) significantly decreased in response to ET, MitoQ, and their combination.

Conclusion: MitoQ and ET, individually and more pronouncedly in combination, can improve cardiovascular health in people with high blood pressure (BP) by reducing inflammation and increasing antioxidant defense, in association with reduction in circulatory miR-21 and miR-222 levels (registration number: IRCT20190228042870N1).

Keywords: Endurance Training, Hypertension, miR-21, miR-222, MitoQ

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Introduction

Hypertension (HTN) is a risk factor for other notable cardiovascular diseases (CVDs) such as myocardial infarction and stroke (1). HTN causes 9.4 million deaths annually worldwide and is estimated to affect about 1.56 billion people by 2025 (2). Primarily because of the unhealthy and sedentary lifestyle in the new generation, the prevalence and incidence of HTN are still increasing in many countries despite the numerous antihypertensive drugs available. Therefore, having physical activity is being increasingly advised as a non-pharmaceutical treatment strategy to prevent and treat HTN. Regular aerobic exercise reduces blood pressure (BP) and

attenuates the risk of CVDs (3).

Oxidative stress and inflammation have also been introduced as mediators for the initiation and progression of HTN (4). Exercise training has been shown to modify the production of cytokines involved in inflammatory processes, such as interleukin-1beta (IL-1 β), IL-6 and tumor necrosis factor-alpha (TNF- α) (5), and long-term endurance training (ET) has been demonstrated to reduce IL-6 and hs-CRP levels (6). Gaeini et al. (7) showed that the oxidant factor malondialdehyde (MDA) decreases even after one session of ET. Other investigators have also reported that strength training decreases MDA and increases total antioxidant capacity (TAC) and glutathione

peroxidase (GPx) activity in older men and women (8, 9).

Mitochondria are the sites of energy production in cells but are also involved in the production of free radicals (10), and their dysfunction plays an essential role in the pathophysiology of hypertensive injuries. In this regard, MitoQ, a mitochondrial-targeted antioxidant, has been reported to reduce free radicals, improve heart function, and attenuate BP (11-13).

MicroRNAs (miRNAs) are short single-stranded endogenous RNAs that target mRNAs and are involved in the development of HTN (4, 14). MiR-21 is expressed in the cardiovascular system and is associated with CVD and different types of HTN (15). MiR-222 suppresses mitochondrial and endothelial cell function by reducing PGC-1 α (16). This micro-RNA plays an essential role in cardiac physiology and pathophysiology and has been introduced as a cardiac function biomarker (17).

Due to the role of oxidative stress in HTN, the role of mitochondria in ROS production, the protective effect of MitoQ on mitochondrial free radical production, and the role of exercise training on reducing oxidative stress and amelioration of HTN, this study was aimed to evaluate the effects of MitoQ supplementation and ET, alone and in combination, on cardiac function and oxidative and inflammatory status in hypertensive individuals. In this regard, the effect of the described interventions was assessed on the circulating levels of miR-21 and miR-222 as probable mediators in the interaction between ET and MitoQ supplementation in hypertensive patients.

Materials and Methods

Materials

In this double-blind randomized clinical trial study, the materials used and their sources were MitoQ (MitoQ Ltd., New Zealand), TAC, MDA, IL-6 kit (Thermo Fisher Scientific, USA), RNA isolation kit (Norgen Biotek, Cat. No. 17200, Canada), cDNA synthesis kit (Norgen Biotek, Cat. No. 54410, Canada), cel-miR-39 (Norgen Biotek, Cat. No. 59000, Canada), SYBR green (Ampliqon, Cat. No. A325402, Denmark), universal primer (reverse) (Norgen Biotek, Cat. No. 59000, Canada), and forward primers (Metabion, Germany). MitoQ capsules contained MitoQuinol (as MitoQuinol Mesylate) 20 mg as active ingredient, and microcrystalline cellulose (MCC), tapioca, and silicon dioxide (SiO₂) as excipients. The role of these excipients compounds in capsule formulation are: SiO₂ as an anti-caking agent, adsorbent, or glidant to allow powder to flow freely; MCC as a segregation inhibitor to improve drug content uniformity; and tapioca starch, as diluent due to its good flow ability. These compounds are generally inactive ingredients especially when used in low amounts, and we did not find any evidence for them to have side effects on the cardiovascular system.

Subjects

We used G*Power:3.1.9.4 version software to calculate the study sample size. Based on the nature of

the study; repeated measure ANOVA, within-between interactions (see below: statistical analysis), number of groups=4, given the assumptions of $\alpha=0.05$, $1-\beta=0.8$, effect size (ES)=0.24, the total sample size was computed to be 52. In this double-blind, randomized clinical trial, middle-aged patients (40-55 years old) with moderately high BP (between SBP/DBP 140/90 to 150/100 mmHg) were entered into the study. Blinding was complete for the laboratory to measure inflammatory and oxidant factors; and for the cardiologist assessing cardiac hypertrophy and functional variables. It was not possible to make the protocol blinded in all aspects for those that exercise (ET group). Participants were selected mostly from participant in KERCADRS (Kerman Coronary Artery Disease Risk Factor Study with sample size of 10,000, aged 15- 80 years old) (45 subjects) and some from patients referred to the cardiovascular clinic of Shafa Hospital in Kerman, Iran (7 subjects) from May 2019 to March 2020. All protocols, goals, and potential benefits and risks of the study were explained to the participants, and they signed an informed consent form. All procedures followed the standards set by the latest revision of the Declaration of Helsinki and were reviewed and approved by the Ethics Committee of Kerman University of Medical Sciences (IR.KMU.REC.1397.595) and by the national RCT registry (IRCT20190228042870N1). Figure 1 shows the study flowchart.

HTN was defined according to the criteria from the European Heart Association as systolic BP (SBP) ≥ 140 mm Hg or diastolic BP (DBP) ≥ 90 mm Hg. Participants with kidney, liver, and lung diseases, diabetes, cancer, known CVDs other than HTN (e.g., valvular heart disease, coronary disease and heart failure), antihypertensive and diuretic medications, SBP>150 mmHg and/or DBP>100 mmHg, high body mass index (BMI ≥ 30 kg/m²), and orthopedic disabilities were excluded from the study. The participants' demographic information (age, sex, history of HTN, level of physical activity, alcohol consumption, and medications) was collected by face-to-face interviews using a validated questionnaire. In the KERCADR study the baseline level of physical activity is determined using the global physical activity questionnaire (GPAQ), which includes all kind of activities, working, playing, training, housekeeping, and recreational activities (18).

Fifty-two subjects were randomly divided on the basis of throwing dice into four groups of Placebo, MitoQ (20 mg/day, oral) (13), ET, and MitoQ+ET, with 13 individuals in each group (Fig.1). Moderate-intensity ET [40 to 60 % VO₂ peak, heart rate (HR) 120-140 b/minutes, duration 45 minutes, three sessions/week], was performed for six weeks. The blood samples were taken at baseline and at the end of the study (day 43). The clotted blood sample was centrifuged (3000 g for 10 minutes), and the serum was stored at -80°C for determining miRNAs 21 and 222, TAC, MDA, and IL-6.

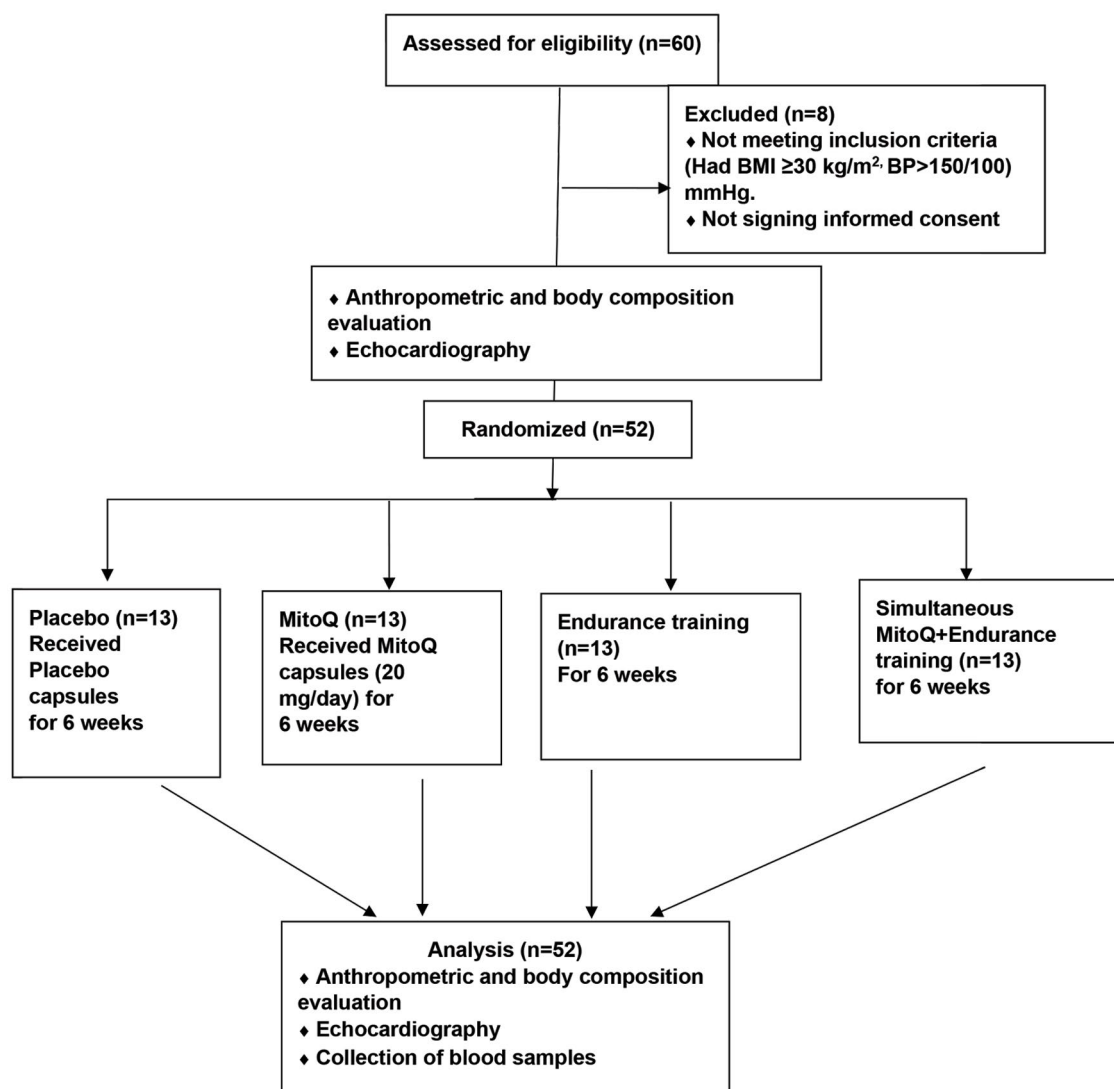


Fig.1: Study flow diagram. BMI; Body mass index.

Blood pressure measurement

BP (SBP and DBP) was measured with an automated device (Omron, M6 Comfort, Japan) to avoid the possibility of investigator bias in measurement. Measurement was performed twice after at least 10 minutes at rest (30 minutes apart) in a sitting position, and the values were averaged. The participants were asked to avoid consuming coffee, tea, soft drinks, supplements, and alcohol at least two hours before BP recording.

Body composition measurements

Bodyweight was measured by a medical beam balance (Allegro Medical, USA), and BMI was calculated [weight (in Kg)/height (in meter)²] and classified as normal (BMI<25), overweight (BMI between 25 and 29.9) and obese (BMI≥30). For assessing body fat, we used a caliper (Saehan skinfold caliper, South Korea) to measure skinfold thickness (at seven points). The Jackson and Pollock formula was used for calculating the percentage of body fat as follows (19):

$$\text{Body fat (\%)} = 495 / ((1.112 - (0.00043499 \text{ s}) + (0.00000055 \text{ s s}) - (0.00028826 \text{ a})) - 450$$

s: Sum of skinfolds at seven points, a: Age in years.

Modified Astrand-Ryhming Cycle Ergometer Test (Measurement of VO₂ Peak)

This test is a kind of ergometer test that is used for measuring VO₂ max, as a factor for determining aerobic capacity and physical fitness. The subjects were asked to avoid drinking alcohol or caffeine-containing products, smoking, and doing strenuous activity for at least 12 hours before the test. In groups with ET, a cardiopulmonary exercise test (CPET) was performed for estimating the peak power and VO₂ peak by the ergometer. The Astrand test consisted of having subjects pedal for six minutes against a constant load. This was conducted on a cycle ergometer (Monark, Ergomedic 839 E, Sweden) coupled with a gas analyzer (Cortex, Metalyzer 3B, Germany) while participants cycled in an upright position. The test consisted of a steady-state resting period, 2 minutes of warm-up without load, followed by a constant protocol in

which participants pedaled at a rate of 50 ± 5 rpm for six minutes while maintaining the heart rate (HR) between 120 and 140 bpm (20) (the HR range was required to predict VO_2 peak from the nomogram, Fig.2). Oxygen saturation (SpO_2) (pulse oximeter, Beurer, Germany), HR, oxygen uptake (VO_2), and respiratory exchange ratio (RER) were determined. Mean HR and output wattage was used to calculate the maximum oxygen consumption, and finally, the age coefficient was added to the values (21). Successful tests were defined as the completion of the 6-min test at a workload to induce HRs within the range of 120-140 bpm.

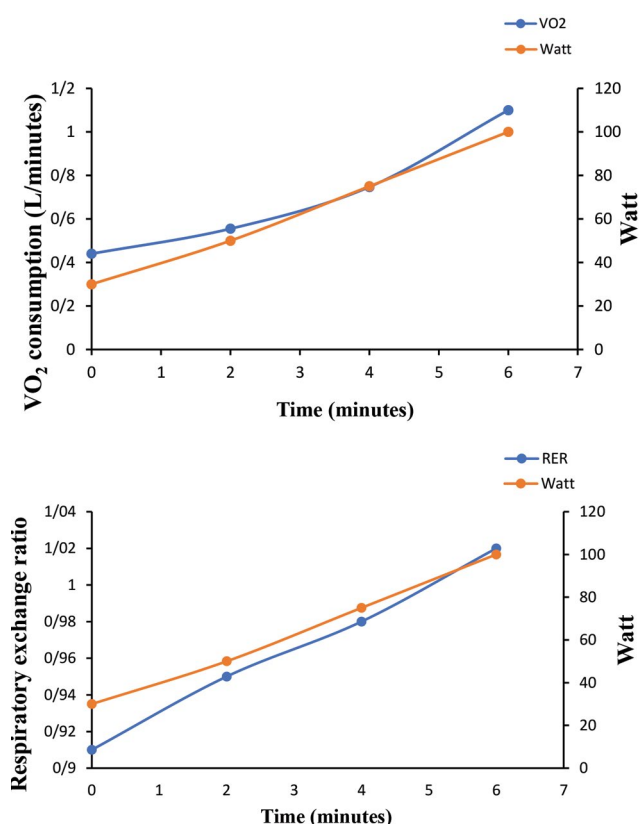


Fig.2: Oxygen uptake kinetics and changes in respiratory exchange ratio (RER) of participants in training groups in the Astrand test.

Endurance training protocol

Moderate intensity ET was performed on a cycle ergometer for six weeks, three sessions a week, in the Faculty of Sport Sciences of Shahid Bahonar University of Kerman under the guidance of an expert tutor. Based on the output wattage and the amount of oxygen consumption in the Astrand test, the first training session was performed for 15 minutes with 40% to 60% of the maximum output wattage. In subsequent sessions, an average of 2 minutes was added to the training time until the duration reached

~45 minutes. The ET duration and intensity were maintained constant at these levels in the last two weeks. Before and during training (at exercise peak), SBP, DBP, SpO_2 and HR were measured (22).

Cardiac function assessment by echocardiography

Cardiac function and hypertrophy parameters were assessed by a cardiologist. Parameters including left ventricle ejection fraction (EF), left ventricular end-systolic diameter (LVESD), left ventricular end-diastolic diameter (LVEDD), relative wall thickness (RWT), left ventricular mass (LV mass), LV mass index (LV mass per body surface area in m^2), and LV filling as measured by the early-to-late trans-mitral valve flow velocity ratio (E/A ratio) were obtained by a two-dimensional mode ultrasound machine (Philips, EPIQ, USA). Guided M-mode frames were scanned with simultaneous ECG for determination of HR. These parameters were assessed at baseline and the day after the end of the study.

Determination of total antioxidant capacity

A 5 mm fasting venous blood sample was taken from the participants at baseline and at the end of the study (day 43). The samples were centrifuged (3000 g for 10 minutes) after 20 minutes clotting time at room temperature. The ferric reducing ability of plasma (FRAP) method suggested by Benzie and Strain (1996) was used to quantify the serum TAC (23). At low pH, antioxidants present in the sample are able to reduce ferric (Fe III) tripyridyltriazine complex to an intense blue-colored ferrous (Fe II) form. This complex has a maximum absorbance at 593 nm and the blue color intensity is proportional to the antioxidant capacity of the sample. In brief, 5 μL of serum sample and 70 μL of FRAP reagent were incubated at 37°C for 5 minutes. Then the absorbance at 593 nm was measured. For providing standard curve, known concentrations of ferrous iron were incubated with FRAP reagent and their optimal density (OD) was recorded at 593 nm to provide a concentration-response curve. Then the sample ODs were fitted on the curve to find out each serum TAC value.

Determination of serum malondialdehyde

MDA is an organic compound considered an index of cell membrane lipid peroxidation. The thiobarbituric acid (TBA) assay method was used (24). In a mixture of trichloroacetic acid (TCA) and TBA-hydrochloric acid, MDA reacts with TBA and develops a pink color with maximum absorbance at 535 nm. We used 20 μL of serum sample with the mixture mentioned above to determine MDA concentrations.

Determination of serum interleukin-6

Serum IL-6 concentration was measured by a specific human IL-6 ELISA kit (EH2IL6, Thermo Fisher Scientific,

USA). In this method, 50 µl of serum is loaded into the wells containing IL-6 antibody. Then a washing step was performed to wash the other analytes. In the next step, a substrate was added, resulting in a blue color development proportional to the amount of IL-6 in the serum. Finally, the reaction was stopped by adding the stop solution, and the amount of yellow color was assessed at a wavelength of 450 nm by an ELISA reader (DRG instrument, Cat. No. ELM-2000, Germany).

miR-21 and miR-222 measurement by RT-qPCR

miRs were measured by reverse transcription quantitative polymerase chain reaction (RT-qPCR) method. The total serum RNA was extracted using a total RNA extraction kit. Briefly, 150 µl of serum was incubated with RL buffer and then loaded into the column that specifically captured the RNA. Finally, these captured RNAs were washed from the column by RNase-free water. The extracted RNA concentration and purity were determined by NanoDrop ND-2100 (Thermo Fisher Scientific, USA). To reduce sampling errors and normalize samples 3.5 µl *Canorhabditis elegans* miR-39 (*cel-miR-39*) was added to each sample as external control. Then, cDNA was synthesized from 5 µl extracted RNA using the microScript microRNA cDNA synthesis kit. To perform real-time PCR, we used synthesized cDNA, specific primers (for miR-21 and miR-222), and high ROX RealQ Plus Master Mix Green, and the mixture was amplified in the StepOnePlus instrument (Applied Biosystems, USA). The relative expressions of miR-21 and miR-222 were normalized to *cel-miR-39* as external control. The expression was calculated as fold change according to the formula, $\text{Fold change} = 2^{-\Delta\Delta\text{CT}}$, where $\Delta\Delta\text{CT} = [(\text{CT gene-CT } \textit{cel-miR-39})_{\text{treatment}} - (\text{CT gene-CT } \textit{cel-miR-39})_{\text{CTL}}]$ (25). The forward primer sequences of miRs were:

miR-21: 5'-TAGCTTATCAGACTGATGTTGA-3'

miR-222: 5'-AGCTACATCTGGCTACTGGGT-3'

cel-miR-39: 5'-UCACCGGGUGUAAAUCAGCUUG-3'

We used a universal primer that the company supplied as a reverse primer in the reactions.

Statistical analysis

Data analysis was performed by SPSS software (SPSS version 26, SPSS Inc., Chicago, IL, USA). The data distribution was determined by the Kolmogorov-Smirnov test, and if it was normal, two-way repeated measure ANOVA was used to assess the differences among the study groups, followed by Bonferroni post hoc test for pairwise comparisons. Nonparametric equivalent tests were used when the distribution of the data was not normal. Comparison of variables in each group between the baseline and its own follow up value (e.g., for HR, BMI, VO₂ peak values) was

performed by the paired t test. The Chi-Square test was used for descriptive statistics (history of HTN, smoking, and the level of physical activity). $P \leq 0.05$ was considered as the significance level.

Results

Anthropometric, demographic and clinical characteristics

The study groups were similar in demographic and general characteristics at baseline, and no significant differences were observed among them in these aspects (Table 1). The baseline parameters including: age ($P=0.16$), history of HTN ($P=0.39$), BMI ($P=0.18$), the basal level of physical activity ($P=0.15$), SBP ($P=0.29$), and DBP ($P=0.09$) were not significantly different among the groups. Body weight and BMI in the groups that performed ET significantly decreased ($P < 0.05$) compared to the baseline values. Resting heart rates (HR) were 76 ± 1.8 , 76 ± 1.9 , 72 ± 1.8 and 74 ± 2.0 beats/minutes in placebo, MitoQ, ET and MitoQ+ET groups, respectively ($P > 0.05$). Peak HR and baseline VO₂ in the ET group were 127 ± 2.5 beats/minutes and 3.1 ± 0.13 L/minutes, respectively; in MitoQ+ET group these values were 132 ± 2.5 beats/minutes and 3.0 ± 0.13 L/minutes, respectively. Both of these variables were in the range anticipated by executing moderate ET protocol.

Effects of MitoQ and ET on SBP and DBP

Exercise and MitoQ alone and in combination significantly decreased SBP compared to the baseline ($P < 0.001$, Table 1), DBP decreased only in the combined (MitoQ+ET) group compared to the baseline ($P < 0.01$).

Ventilatory parameters and body fat in hypertensive patients

Body fat percentage showed a significant decrease in the ET and the ET+MitoQ groups ($P < 0.05$) compared to their baseline (Table 1). Also resting SpO₂s were $95 \pm 0.1\%$, $93 \pm 0.2\%$, $95 \pm 0.2\%$ and $95 \pm 0.2\%$ in the placebo, MitoQ, ET and MitoQ+ET groups, respectively ($P > 0.05$). Peak SpO₂ in ET was $94 \pm 0.2\%$ and in MitoQ+ET was $93 \pm 0.2\%$, which were both in the normal range.

Effects of MitoQ and ET on cardiac function

Table 2 shows that LV mass (normal range: 96-200 g) and LVESD (normal range: 2.5-4.0 cm) significantly decreased in the combined group compared to their pre-intervention values ($P < 0.01$). However, EF (normal range: 52-72%), LV mass index (normal range: 50-102 g/m²), LVEDD (normal range: 4.2-5.8 cm), RWT (normal range: 0.24-0.42 cm), and E/A ratio (normal value: 1.35 ± 0.5) were not changed significantly by the interventions (Table 2).

Table 1: Anthropometric, demographic and clinical characteristics of the study groups

Groups	Placebo (n=13)		MitoQ (n=13)		ET (n=13)		MitoQ+ET (n=13)	
	Baseline	Follow-up	Baseline	Follow-up	Baseline	Follow-up	Baseline	Follow-up
Age (Y)	49 ± 0.7	NA	49 ± 0.7	NA	48 ± 0.9	NA	47 ± 1.1	NA
Weight (kg)	76 ± 1.5	76 ± 1.6	79 ± 2.0	79 ± 2.2	81 ± 2.4	80 ± 2.0*	83 ± 1.7	81 ± 1.7*
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*
SBP (mmHg)	140.5 ± 1.3	139.5 ± 1.3	142.4 ± 1.4	136.8 ± 1.7***	139.6 ± 0.7	126.5 ± 1.1***	142.7 ± 1.4	128.1 ± 1.3****##
DBP (mmHg)	92 ± 1.9	90.7 ± 2	93.3 ± 1.4	89.2 ± 1.1	89.6 ± 0.9	85.6 ± 0.6	94.5 ± 1.8	89.2 ± 1.2**
Body fat (%)	23.3 ± 0.7	23.4 ± 0.8	23.9 ± 0.8	23.2 ± 0.6	25.1 ± 0.7	23.7 ± 0.7*	26.4 ± 0.8	24.9 ± 0.7*

Values are expressed as mean ± SEM. *: Significant vs. baseline (*; P<0.05, **; P<0.01, ***; P<0.001), #: significant vs. MitoQ follow-up (###; P<0.001), BMI; Body mass index, SBP; Systolic blood pressure, DBP; Diastolic blood pressure, and NA; Not applicable data.

Table 2: General echocardiographic and cardiac function indices at the baseline (pre-intervention) and follow-up (6 weeks) in hypertensive patients (n=52)

Variable	Placebo (n=13)		MitoQ (n=13)		ET (n=13)		MitoQ+ET (n=13)	
	Pre-intervention	Follow-up	Pre-intervention	Follow-up	Pre-intervention	Follow-up	Pre-intervention	Follow-up
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
LV mass (g)	156 ± 3.8	154 ± 3.1	175 ± 6.5	168 ± 5.5	172 ± 4.5	169 ± 6.6	188 ± 10.1	174 ± 9.3**
LV mass index (g/m ²)	87.3 ± 2.2	86.8 ± 2.8	86.8 ± 3.2	89.7 ± 2.6	84.2 ± 2.8	83.3 ± 3.4	94.3 ± 4.8	88.3 ± 5.7
LVESD (cm)	2.8 ± 0.10	2.8 ± 0.11	3.0 ± 0.08	3.0 ± 0.08	3.0 ± 0.07	2.9 ± 0.05	3.1 ± 0.08	2.9 ± 0.09*
LVEDD (cm)	4.8 ± 0.10	4.8 ± 0.11	4.9 ± 0.06	4.9 ± 0.06	4.8 ± 0.04	4.6 ± 0.07	5.0 ± 0.10	4.9 ± 0.14
RWT (cm)	0.33 ± 0.01	0.31 ± 0.01	0.32 ± 0.01	0.33 ± 0.01	0.39 ± 0.02	0.33 ± 0.01	0.36 ± 0.01	0.34 ± 0.01
E/A ratio	0.7 ± 0.07	0.7 ± 0.06	0.8 ± 0.05	0.8 ± 0.06	0.9 ± 0.05	0.9 ± 0.08	0.8 ± 0.06	0.8 ± 0.05

Data are presented as mean ± SEM. *: Significantly vs. baseline (pre-intervention) (*; P<0.05, **; P<0.01), EF; Ejection fraction, LV mass; Left ventricular mass, LV mass index; Left ventricular mass index, LVESD; Left ventricular end-systolic diameter, LVEDD; Left ventricular end-diastolic diameter, RWT; Relative wall thickness, and E/A ratio; Early-to-late trans-mitral valve flow velocity ratio.

Effects of MitoQ and ET on serum TAC, MDA, and IL-6

After six weeks of ET and MitoQ intake, the serum TAC level significantly increased, and MDA and IL-6 significantly decreased in all intervention groups compared to their baseline values ($P < 0.001$, Fig.3). The effect of combined therapy on TAC and MDA was more than the effect of ET or MitoQ alone.

Effects of MitoQ and ET on miR-21 and miR-222 expression

MitoQ, ET, and MitoQ+ET interventions caused a significant reduction in serum miR-21. ET and MitoQ+ET reduced serum miR-222 significantly as well (Fig.4).

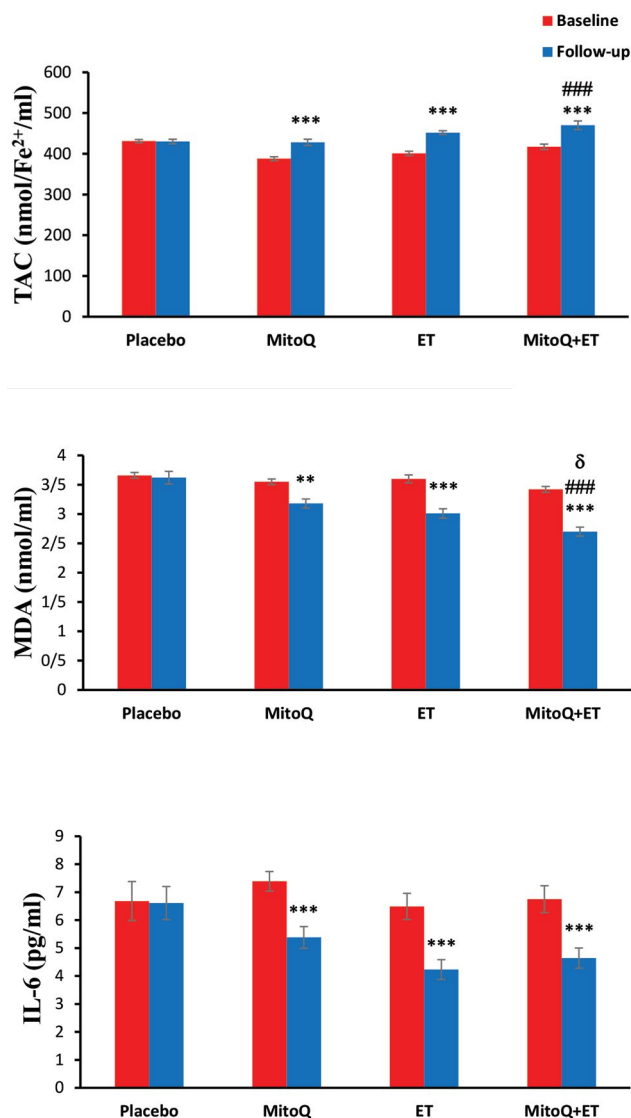


Fig.3: Serum TAC, MDA and IL-6 levels at baseline and follow-up in patients with HTN. Values are mean \pm SEM. $n=13$ in each group. ***, $P < 0.001$, significant difference vs. baseline, ###, $P < 0.001$ significant difference vs. MitoQ follow-up, δ ; Significant difference vs. ET follow-up, TAC; Total anti-oxidant capacity, MDA; Malondialdehyde, IL-6; Interlukine-6, HTN; Hypertension, and ET; Endurance training.

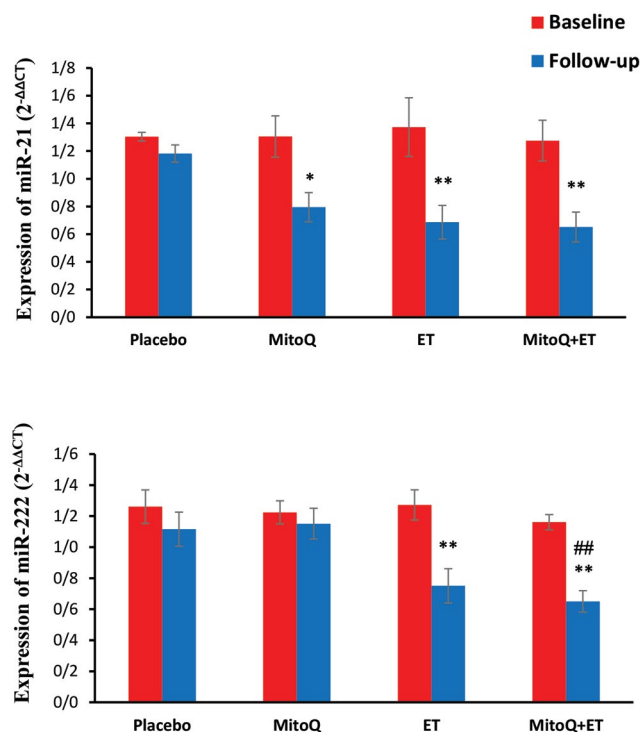


Fig.4: Relative expression (mean \pm SEM) of circulating miR-21 and miR-222 in patients with hypertension, and the effect of treatment with MitoQ, ET, and MitoQ+ET combination. $n=13$ in each group. *, Significant difference vs. baseline (*; $P < 0.05$, **; $P < 0.01$), ##; $P < 0.01$; Significant difference vs. MitoQ follow-up, and ET; Endurance training.

Discussion

The main results of the present study were that the combination of MitoQ and ET significantly decreased SBP and improved cardiac function indices, including LV mass and LVESD in hypertensive patients. Also, MitoQ, ET, and their combination reduced IL-6 levels and increased antioxidant defense capacity. Serum miR-21 levels decreased in all intervention groups, while ET and MitoQ+ET interventions reduced miR-222.

MiR-21 is related to vascular remodeling, ROS production, level of C-reactive protein (CRP), and arterial stiffness (15, 26, 27). MiR-21 was also related to left ventricular (LV) mass index in patients with HTN. Our results showed that MitoQ, ET, and their combination significantly lowered circulating miR-21 levels, which was associated with reducing LV mass and LVESD. This confirms the improving effect of ET, MitoQ, and their combined therapy on cardiac hypertrophy and on improving cardiac systolic function.

It has been well documented that oxidative stress, some miRNAs, and inflammation are involved in HTN development (4). Here, MitoQ and ET alone and in combination could reduce the expression of the two HTN-associated miRNAs, miR-21 and miR-222, caused a reduction in the inflammatory (IL-6) and oxidative (MDA) factors, and increased the antioxidant capacity (TAC) in our hypertensive participants. MitoQ and ET also reduced

SBP more efficiently when they were combined compared to their individual effects. The results are in line with our hypothesis that MitoQ, as a mitochondria-targeted antioxidant, may cause improvement in body redox state in association with reduction of miR-21 and miR-222. The more efficient effect of combination therapy in reducing HTN may be related to a more efficient reduction in MDA and a greater decrease in miR-222 expression.

On the other hand, it has been reported that exercise attenuates pro-inflammatory cytokines (IL-1, IL-6, and TNF- α) levels and increases anti-inflammatory cytokines (IL-10) (28). In this study, MitoQ and moderate ET, alone and in combination, reduced IL-6 and increased TAC serum levels in hypertensive individuals associated with lowering of miR-21 and miR-222 levels (especially miR-21). However, there are some studies whose findings are inconsistent with our results. For instance, in one study, two weeks of continuous ET (3 days/week) did not change CRP, IL-6, and IL-10 levels (29). In another study, after 16 weeks of ET, there was no change in IL-6 levels (30). Also, combined aerobic and resistance training (12 weeks) had no effects on IL-6 level (31). The discrepancies may be related to the differences in the intensity of training, which has been reported to be a determining factor affecting IL-6 release into the circulation (32). Also, the different periods and types of training may be factors that can affect the IL-6 production or release. In a study in young healthy men with MitoQ supplementation for 3 weeks during ET, it was observed that MitoQ did not affect skeletal muscle or whole-body aerobic adaptations to exercise training (33). These results verify that the responses to MitoQ may be dependent on healthy/diseased conditions, and/or the duration of supplementation. Meanwhile this study suggests that although MitoQ may not modulate the ET adaptations, it still potentiates the beneficial effects of exercise on HTN, being found in the present study.

Previous studies have proved that miR-21 exacerbates HTN by increasing IL-6 and TNF- α levels (15, 26). However, some studies have shown that miR-21 was not altered by exercise training (34). These discrepancies over miR-21 levels following exercise may be due to the different types of exercise or the sample used to measure miR-21 levels (blood or tissues). Similarly, there has been inconsistent data regarding miR-222 actions, including both its beneficial and detrimental effects. In one study on aortic endothelial cells, miR-222 caused mitochondrial dysfunction and ROS generation (16), and its inhibition reduced inflammation-induced ROS generation (35). Conversely, circulating miR-222 has been shown to be an athero-protective agent by affecting eNOS activity, thereby dilating blood vessels and lowering BP (36). It also protects cardiac tissue against ischemic injury (17). However, there is no data about the effect of MitoQ on miR-222 in human. This study showed that oral MitoQ supplementation did not change circulating miR-222 levels in hypertensive patients in spite of reducing their BP. At the same time, ET and the combination of MitoQ and ET significantly reduced miR-222 and BP levels. It

seems that, unlike ET, the mechanism by which MitoQ reduces BP is independent of changes in miR-222 levels. That is why the BP lowering effect of MitoQ is added to the BP lowering effect of ET without more reduction in the level of miR-222 when they are combined.

Regarding the effect of exercise on miR-222, a study showed that circulating miR-222 increased after acute exhaustive cycling exercise (37). In the present study, in which the participants performed moderate ET, a reduction in the circulating miR-222 was observed. It seems that the expression of this miRNA and its beneficial/detrimental effects depend on the type, intensity, and duration of exercise (38).

We acknowledge the limitations of our study. Due to ethical considerations, we could not include patients with more severe HTN in the study as otherwise we had to discontinue their medications and put them at a high risk when performing ET. MitoQ may be found more effective in lowering BP in patients with more severe HTN, especially when combined with ET. Also, in a study with a higher sample size the effect of MitoQ may be more pronounced and the results may strengthen. Moreover, higher doses of MitoQ and more extended periods of treatment may show better outcomes. These modalities need further investigation.

Conclusion

Overall, the data from this study showed that concurrent moderate ET and MitoQ significantly reduced BP, MDA, and IL-6 serum levels in HTN subjects. It also increased TAC and either improved the antioxidant status or reduced free radicals. Other outcomes were reduction in serum miR-21 and miR-222 levels in hypertensive subjects, which was associated with improvement in cardiac LV mass index and systolic function. More studies with higher doses of MitoQ and more extended periods of treatment, alone and in combination with ET, are needed to further clarify the effects of these interventions on BP of patients with different levels of HTN.

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Authors' Contributions

H.N., Y.M.-A.; Contributed to the conception and design of the study. H.N.; Supervised the project. Y.M.-A., S.A., Z.S.; Wrote the first draft of the manuscript. H.R.N., Sh.J.; Performed the clinical examinations and helped in the interpretation of the echocardiography results. Y.M.-A., S.J., S.A., Z.S.; Performed data collection. All authors

contributed in reviewing and finalizing the manuscript.

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