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MitoQ Supplementation During Vigorous Training Improves Reactive Oxygen Species, Glutathione Peroxidase, and miRNAs Regulating Vascular Inflammation in Cyclists

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HIGHLIGHTS

- Mitochondrial antioxidant supplementation can reduce oxidative stress and improve miRNAs regulating vascular inflammation.
- This finding suggests that short-term mitochondrial antioxidant supplementation can be a principal strategy to enhance endurance performance in athletes.

Abstract: Vigorous training increases the production of reactive oxygen species (ROS) from skeletal muscle, which can contribute to impairing athletic performance and health. Mitochondrial antioxidant such as MitoQ may contribute to protecting against training-induced oxidative stress such as mitochondrial ROS, but the effects on athletic performance are unknown. The purpose of this study was to determine the effects of MitoQ to exercise training (EX) on VO_{2max}, miRNA expression involved in inflammation and oxidative stress. In this double-blind clinical trial, 32 professional cyclists (25.6±3.8yr) were randomly divided into 4 groups (n=8); 1) Placebo, 2) EX (cycle ergometer, 75% of VO_{2max}, 90 minutes, 3 sessions/week, and 4 weeks), 3) MitoQ (20 mg.day-1 for 4 weeks), and 4) EX+MitoQ (combined EX with MitoQ for 4 weeks). $\dot{V}O2_{max}$ and gene expression of MicroRNAs (miRNAs)-19b, -181b, -155, and -146a and Serum levels of ROS (peroxides, superoxide, hydroxyl radical, singlet oxygen, and alpha oxygen), glutathione peroxidase

(GPx), and superoxide dismutase (SOD) were measured by the gas analyzer, real-time polymerase chain reaction ($2^{-\Delta\Delta Ct}$) and ELISA, respectively. Both EX+MitoQ and MitoQ reduced serum levels of ROS ($P < 0.05$), but there was no change in EX group. Serum GPx levels were increased in EX, MitoQ, and EX+MitoQ ($P < 0.05$), but there was no difference between groups. Serum SOD remained unchanged in all groups. miRNA-155 and miRNA-19b expressions were decreased in EX+MitoQ compared to EX, whereas miRNA-146a was increased in EX+MitoQ compared to EX ($P < 0.05$). Placebo-controlled intervention (tapioca powder) had no effects on all outcomes ($P < 0.05$). MitoQ supplementation and EX reduced ROS levels and altered expression of key miRNAs mediating ROS generation and vascular endothelial inflammation could contribute to improving vascular function.

Keywords: Antioxidant; Athletic performance; Maximal Oxygen utilization; MitoQ.

INTRODUCTION

During vigorous exercise, metabolism and oxygen consumption are highly elevated, leading to excessive production of reactive oxygen species (ROS) even in trained cyclists [1, 2]. ROS-induced physiological effects result in contractile dysfunction in skeletal muscle and impair vascular function, which can limit appropriate blood flow to working muscles [3, 4]. A significant statistical inverse correlation was observed between ROS production and athletes' performance [5]. Considering professional cyclists ride 30,000-35,000 km each year, it is important to find a nutritional strategy for reducing their ROS levels to improve endurance sports performance.

Antioxidants are doubtless to cut back the impairment of performance that happens throughout tournaments or training by mostly reducing inflammation on skeletal muscles [1]. Mitochondrial antioxidant such as MitoQ, as a developed antioxidant that was ~800-fold more effective than the untargeted antioxidant [6], is designed to condense on the matrix surface of the inner mitochondrial membrane and exerts antioxidative effects by oxidizing ubiquinol to ubiquinone. In particular, MitoQ has both anti-inflammatory and antioxidant properties by decreasing nuclear factor-κb (NF-κb) and tumor necrosis factor (TNF)-α and increasing glutathione peroxidase 1 (GPx-1) levels [7], which could contribute to maintaining vascular homeostasis during vigorous exercise. Higher levels of inflammation factors are independently associated with lower $V_{O2\,max}$ [8]. Reactive oxygen species (ROS) are formed as natural by-products of normal cell activity and the increase in ROS levels has harmful effects on cell homeostasis, structures, and functions and results in oxidative stress [7]. The improvement of ROS and vascular endothelial inflammation could contribute to enhancing vascular endothelial function, which enhances oxygen delivery during exercise through increased blood flow by up-regulating endothelial nitric oxide synthase (eNOS), which increases bioavailability of NO, a potent vasodilator [9-11]. Thus, antioxidants could improve aerobic performance by improving ROS and endothelial inflammation.

There are key microRNAs (miRNA) involved in modulating ROS generation and inflammation. miRNA-155 leads to ROS generation by suppressing the antioxidant genes, such as Nfe2l2, Sod1, and Hmox1 [12]. Also, miRNA-155 induces inflammation by up-regulating the expression levels of interleukin (IL)-1β and TNF-α [12]. miRNA-155 also has a positive relationship with aging [13]. miRNA-19b also contributes to endothelial dysfunction by suppressing PGC-1α expression [14]. Overexpression of miR-19b was found in atherosclerotic blood vessels, which could be associated with suppressed PGC-1α expression, leading to endothelial injury [14]. miRNA-146b and miRNA-181b also play a role in anti-inflammatory action in endothelial cells by inhibiting pro-inflammatory transcription activation, including the NF-κB, AP-1, and MAPK/EGR pathways [15, 16]. For example, miRNA-146b attenuated IL-1β-induced pro-inflammatory response by the release of RANTES [17] and systemic administration of miR-181b reduced NF-κB signaling in the vascular endothelium, which would contributes to improving vascular function [18]. There is a controversial effect of exercise training (EX) on those miRNAs and to our knowledge, no study found the beneficial effect of antioxidants on those miRNAs.

Therefore, given the importance of miRNAs and mitochondrial ROS production in inflammatory effects in athletes 'performance, this double-blind randomized placebo-controlled trial aimed *to investigate* the effects of MitoQ supplementation, alone or in combination with EX on the maximal oxygen uptake ($\dot{V}O2max$), oxidative stress response, miRNAs regulating inflammation and ROS generation in professional cyclists. Serum levels of ROS, GPx, and superoxide dismutases (SOD) were measured to evaluate oxidative stress response. We hypothesized that MitoQ supplementation to EX may improve $\dot{V}O2$ max by reducing ROS levels and positively regulating the miRNAs expression in cyclists more than EX alone.

MATERIAL AND METHODS

Subjects

32 male professional cyclists (25.6±3.85yr) were selected from all professional cyclists (40 athletes) in Kerman province (four athletes have been excluded from the study because of sport injury and four athletes because of administrative work). Participant characteristics are given in Table 1. All participants were non-smokers, free from any cardiovascular or pulmonary disease, and were not taking any medications. All procedures, goals of the study, and the potential benefits and risks of MitoQ were described to the participants, and an informed consent form was signed by them. The study protocol was according to the standards set by the latest version of the Declaration of Helinski and was verified by the ethics committee of Kerman University of Medical Sciences (Ethics code: IR.KMU.REC.1397.526).

Experimental protocol

This is a parallel design, double-blind randomized placebo-controlled clinical trial. All subjects completed an incremental test to exhaustion (VO_{2max}), two familiarization rides and two performance trials on a stationary cycle ergometer (Ergomedic 839 E, Monark, Sweden). During all visits, subjects were instructed not to consume caffeine within 4 h or exercise intensely within 24 h before each session. At the beginning of each exercise session, participants performed a 5 min warm-up on a cycle ergometer at 30 W. Subjects performed cycling with an intensity of 75% of peak power for 90 minutes. During exercise, the intensity was maintained by monitoring heart rate with the smart-watch (Garmin, Forerunner 235). Participants were asked to remain seated and maintain a consistent cadence of 70 RPMs throughout all testing procedures [19].

Study design

All participants were randomly (based on random number tables) divided into 4 groups (Appropriate sample size per group was calculated by the G*Power software; effects size f: 0.25, α: err prob: 0.05, power (1-β err prob): 0.95; *n*=8); 1) Placebo, 2) EX (aerobic training, cycle ergometer, moderate-intensity (75% of peak power; matched ventilatory reserve, 3 sessions per week), 3) MitoQ (20 mg/day, oral), and 4) EX+MitoQ. The groups were matched according to VO_{2max}, BMI, sex, and age. All participants in exercise training groups completed a total of 12 exercise sessions for four weeks. The blood samples were taken after overnight fasting, before (on day 1) and at the end of the study (day 30). The samples were centrifuged at 5,000 rpm for 10 minutes, and the serum was aliquoted and stored at -80°C for future quantifications. Serum was used to determine miRNAs and also SOD and GPx by ELISA method (Figure 1).

MitoQ supplementation

MitoQ groups received MitoQ (mitoquinone mesylate 20 mg·day−1, MitoQ company, New Zeeland) and placebo (Tapioca powder, precipitated silica and microcrystalline cellulose). Tablets had been distributed into unmarked bottles with the aid of using the researcher who carried out the randomization, and those bottles had been given to the researcher engaging in the trial for distribution to participants. Participants were instructed to consume one tablet per day orally 30 min before breakfast for 4 weeks before completing the first performance trial. The selection of a 4-week supplementation period was informed by previous human research [20].

Materials

The materials used and their sources were: Mitoquinone mesylate (MitoQ) (MitoQ Ltd, New Zealand), kits of SOD (Randox, #RS504, UK), glutathione peroxidase (GPx) (Randox, #SD125, UK), ROS (MyBioSource, Cat No. MBS2603394, Canada), cDNA synthesis (Norgen Biotek, #54410, Canada), and cel-miRNA-39 (Norgen biotek, #59000, Canada), SYBR green (Ampliqon, #A325402, Denmark), universal primer (reverse) (Norgen biotek, #59000, Canada), and primers (metabion, Germany).

Body Composition Measurements

Bodyweight was measured using a medical beam balance (Allegro Medical, USA), and body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2) and used to differentiate between normal weight (BMI<25) and overweight (BMI≥25-29.9) (Table 1).

Measurements of VO2max

All athletes performed incremental cycling (matching ventilatory rates) (Metalayzer-3B, CORTEX, Germany).

Determination of SOD activity

The Randox ELISA kit was used to determine SOD levels in serum according to the manufacturer's instructions. SOD acts as a catalyst in the dismutation of O_2 radicals to hydrogen peroxide (H_2O_2) and in the conversion of NBT to NBT-diformazan, which absorbs light at 560 nm [21]].

Determination of GPx activity

The Randox ELISA kit was used to determine GPx activity using the method described by Paglia and Valentine. The assay Kit indirectly measures GPx activity through a reaction with glutathione reductase, the enzyme responsible for regenerating the reduced form of oxidized glutathione (GSSG). When NADPH is oxidized to NADP+, its absorbance at 340 nm decreases [22].

miRNAs measurement by RT-qPCR

Total RNA was isolated from the serum using the total RNA purification kit. Briefly, RNA was isolated from 150 µl of serum using the RL buffer washed and eluted in RNAse free water. RNA concentration and purity were quantified using nanodrop ND-2100 (Thermo Fisher Scientific, USA). To normalize between samples, 3.5µl Canorhabditis elegans miRNA-39 (cel-miRNA-39) was added to each sample. Immediately after RNA isolation, 5µl of RNA was reverse transcribed using the microscript miRNA cDNA synthesis kit. cDNA was PCR-amplified (step one plus instrument, Applied Biosystems, USA) using real q Plus Master Mix Green; high ROX and miRNA specific primers for miRNA-126 and miRNA-27a. All samples were assayed in duplicate. The relative expression level for a given miRNA was normalized to cel-mirna-39 as external control. The expression was calculated as fold change according to the formula: Fold change = $2^{-\Delta\Delta CT}$ in which $\Delta\Delta CT$ = [(CT gene - CT cel-miRNA-39) $_{treatment}$ – [CT gene - CT cel-miRNA-39)] $_{CTL}$ (23)]. The forward primer sequences of miRNAs were:

miRNA-155: 5′- TGGGGATAGTGCTAATCGTAATT-3′.

miRNA-146a: 5′- ATGATTCACTTTTCAGTTCTCA-3′.

miRNA-181b: 5′- AACATTCATTGCTGTCGGTGGGT-3′.

miRNA-19b: 5′- AGTCAAAACGTACCTAAACGTGT-3′.

Cel-miRNA-39: 5′-UCACCGGGUGUAAAUCAGCUUG-3′.

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

Statistical analysis

The data (means ± *SD*) were analyzed by the SPSS software (SPSS version 26, SPSS Inc., Chicago, IL, USA) and GraphPad Prism (GraphPad v.8.4.3., San Diego, LLC, USA). First, the data distribution was examined by the Kolmogrov-Smirnov test and if it was normal. Two-way repeated-measures ANOVA was used to compare variables followed by Tukey'post hoc test for between-group comparisons. Statistical significance was set at $P < 0.05$.

RESULTS

Anthropometric, Demographic, and general characteristics of the study population

In total, 32 professional cyclists (mean age 25.65±3.85yr) were recruited for this study. At baseline, no significant difference was found between groups regarding the participants' baseline characteristics including age, height, weight, body mass index, maximal oxygen uptake (VO_{2max}), and power output ($P > 0.05$) (Table 1).

Effects on reactive oxygen species and antioxidant enzymes

Both MitoQ consumption alone and MitoQ supplementation to EX significantly reduced serum levels of ROS compared to the pre-interventions. ($P < 0.0001$ and $P < 0.01$, respectively), but there was no change of ROS in EX ($P = 0.83$) (Figure 2A). GPx levels were increased follow all three interventions (MitoQ: $P = 0.013$, EX: $P < 0.0001$, and EX+MitoQ: $P = 0.003$) (Figure 2B). There was no change of SOD in all groups (MitoQ: $P = 0.70$, EX: $P > 0.99$, and EX+MitoQ: $P = 0.98$) (Figure 2C). Placebo-controlled intervention had no effects on the concentrations of ROS, GPx, and SOD ($P < 0.05$).

Effects on serum levels of miRNA-155, 181b, 19b, and 146a

MitoQ consumption decreased the serum levels of miRNA-155 compared to the pre-intervention ($P < 0.05$) whereas EX increased it ($P < 0.01$) and MitoQ supplementation to EX did not result in a significant change (Figure 3A). Both EX and MitoQ supplementation to EX increased miRNA-181b levels ($P < 0.05$ and $P < 0.001$, respectively) whereas MitoQ consumption had no change (Figure 3B). miRNA-19b levels were increased following MitoQ consumption, EX, and MitoQ supplementation to EX ($P < 0.01$, $P < 0.0001$, and $P < 0.0001$, respectively) (Figure 3C). miRNA-146a levels were increased following MitoQ consumption, EX, and MitoQ supplementation to EX ($P < 0.0001$, $P < 0.001$, and $P < 0.0001$, respectively) (Figure 3D). Placebo-controlled intervention had no effects on the serum levels of miRNA-155, 181b, 19b, and 146a ($P < 0.05$).

There was no difference in VO_{2max} between the groups before and after the interventions ($P > 0.05$).

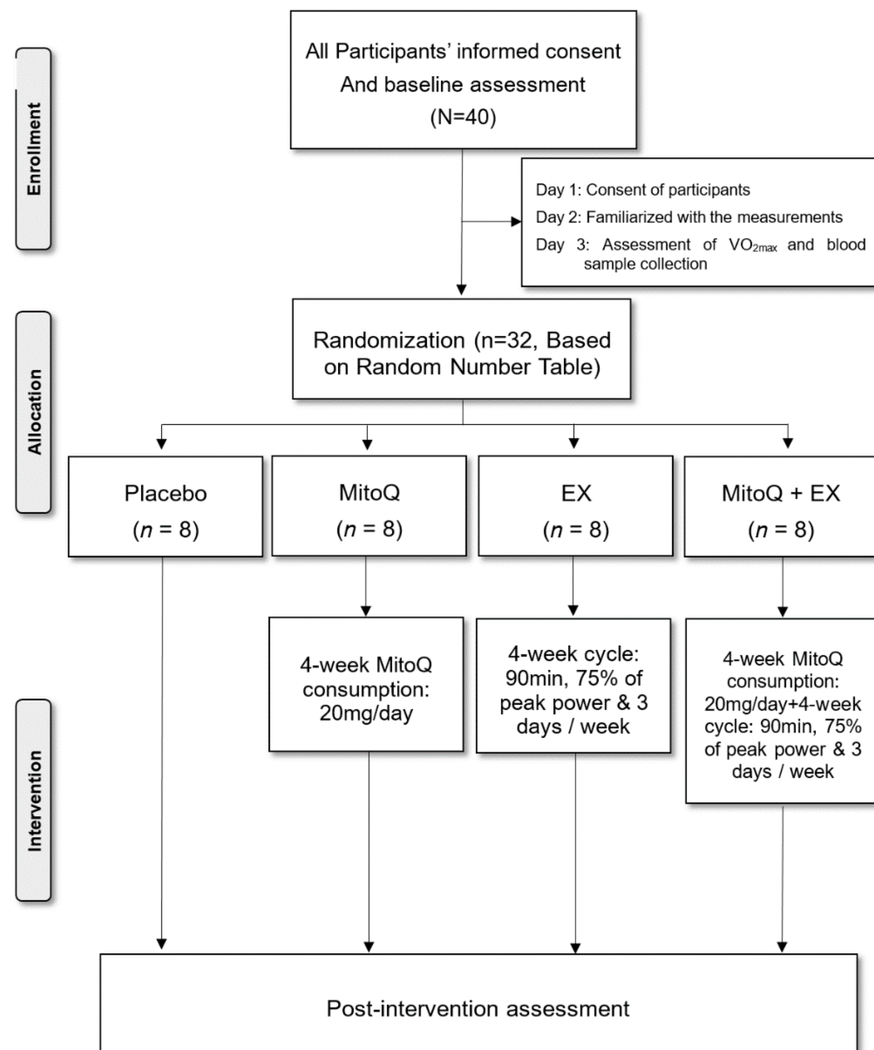

Figure 1. The protocol of the study

Table 1. Subject characteristics

Indexes	Placebo	MitoQ	EX	EX+MitoQ	*P*-value
Age (year)	23.25±1.25	25.43±1.35	24.40±0.74	25.40±1.17	0.1
Height (m)	1.78±0.08	1.80±0.05	1.85±0.08	1.82±0.09	0.09
Weight (kg)	73.9±9.7	77.6±4.67	78.6±5.67	82.6±3.67	0.11
BMI (kg/m^2)	23.38±2.1	23.95±2.52	22.90±2.52	24.95±2.52	0.09
VO_{2max} (ml.kg^{-1}.min^{-1})	59.56±2.34	61.66±2.61	61.66±3.42	60.84±2.83	0.08
Power output (W)	318±36.03	318±37.86	320±38.77	305±31.62	0.09

Values are means ± *SD*. *N* = 8/group. MitoQ, mitochondrial antioxidant; EX, exercise training; BMI, body mass index; VO_{2max}, maximal oxygen consumption. There is no significant change between groups.

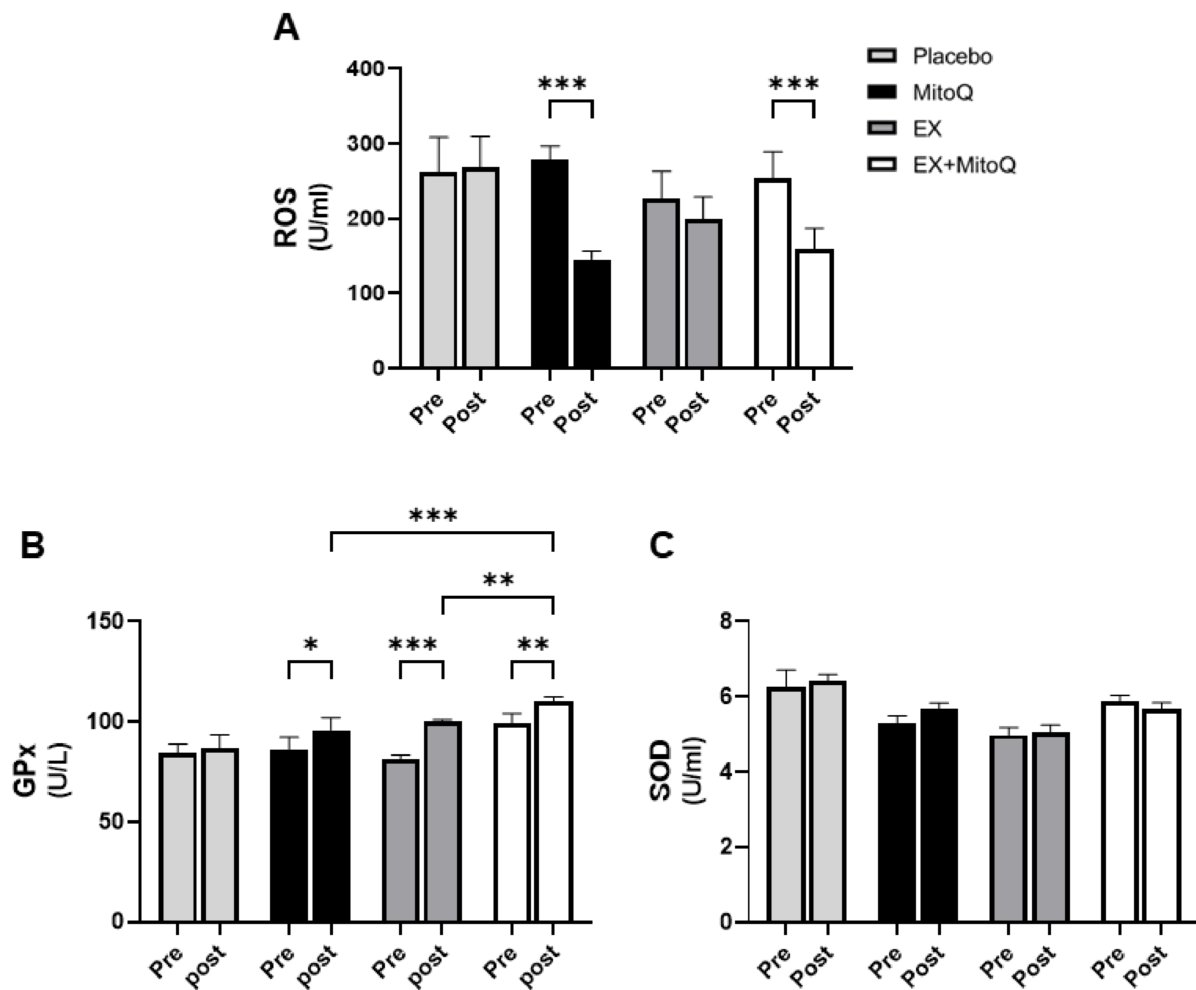

Figure 2. MitoQ supplementation alone and MitoQ supplementation to exercise training (EX) reduced serum levels of reactive oxygen species (ROS) compared to the pre-interventions (A). Glutathione peroxidase (GPx) was increased follow the interventions (B). There was no change of superoxide dismutase (SOD) in all groups (C). MitoQ, mitochondrial antioxidant; and EX, exercise training. **P* < 0.05; ***P* < 0.01; ****P* < 0.001. All data are presented as means ± *SD*.

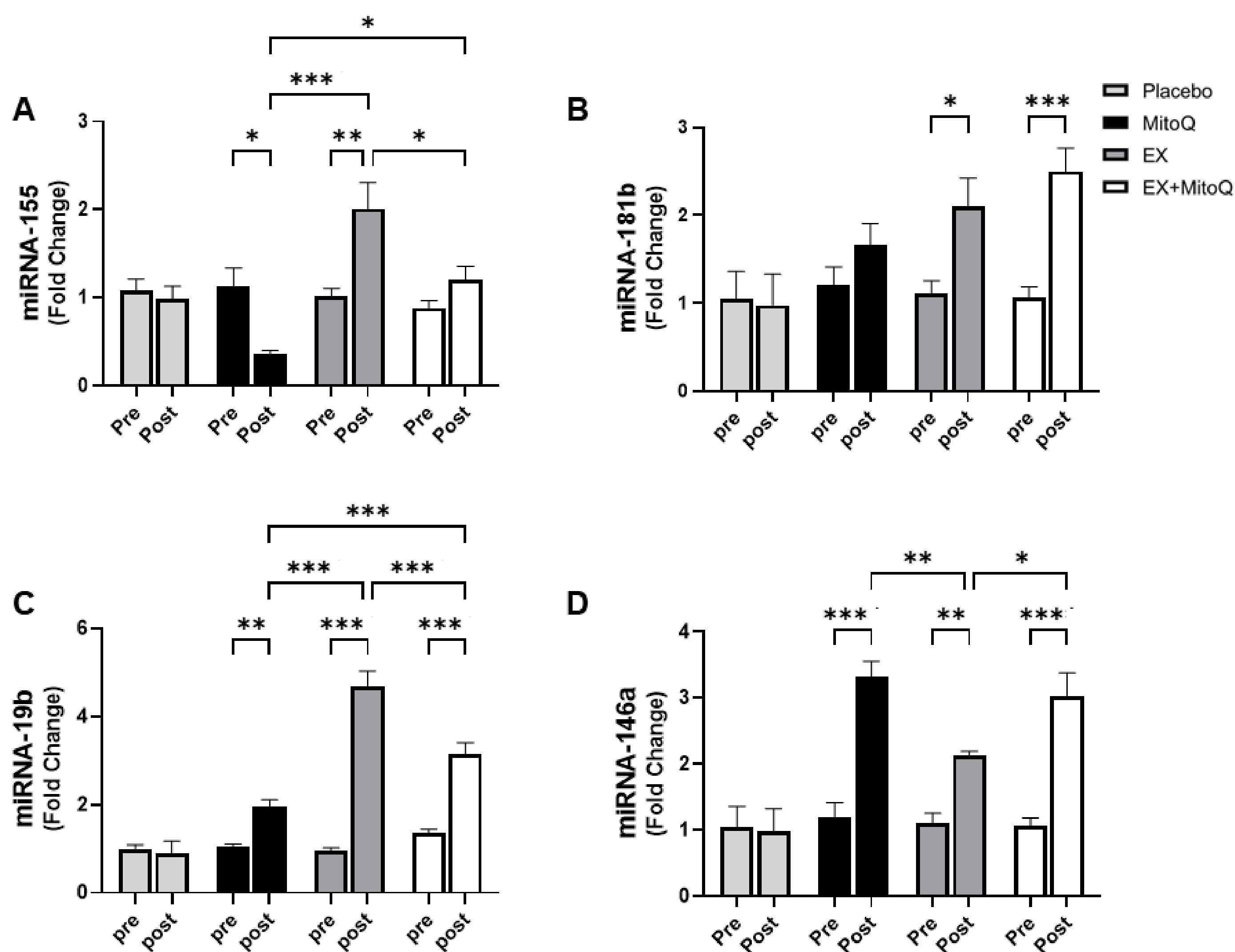

Figure 3. The relative gene expression of miRNA-155, 181b, 19b, and 146a in serum. MitoQ, mitochondrial antioxidant; and EX, exercise training. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. All data are presented as means ± *SD*.

DISCUSSION

This study demonstrates that MitoQ supplementation to aerobic training significantly reduced blood ROS levels and altered expression of miRNAs mediating ROS generation and vascular endothelial inflammation, which could contribute to increasing blood flow to working muscles by improving vascular function. Considering that vigorous training can lead to ROS overproduction in athletes [2], MitoQ supplementation can be a principal strategy to enhance their endurance performance by reducing exercise-induced oxidative stress and vascular endothelial inflammation.

Interestingly, four weeks of MitoQ supplementation to EX induced a significant decrease in ROS levels in professional cyclists (Figure 2A). The increases in ROS generation and inflammation uncouples eNOS, which reduces NO bioavailability and increases vascular tone [9-11]. Decreased NO levels lead to impaired endothelium-dependent vasorelaxation, which consequently limits oxygen delivery to skeletal muscles during exercise [9]. This study identified that MitoQ supplementation to EX significantly prevented the increase of miRNA-155 expression (Figure 3A) and miRNA-19b (Figure 3C). Increased expression of miRNA-19b can lead to endothelial dysfunction by damaged endothelial restoration resulting from suppressed PGC-1α expression [14]. Decreased miRNA-146b expression in monocytes is associated with dysregulated anti-inflammatory action [24]. We determined that MitoQ supplementation to EX significantly elevated miRNA-146a compared to EX (Figure 3D), which could contribute to promoting anti-inflammatory responses and improving vascular inflammation. The slightly elevated expression of miRNA-181b was found following MitoQ supplementation to EX compared to EX alone (Figure 3B). A previous animal study determined that systemic administration of miR-181b reduced pro-inflammatory transcription through inhibited NF-κB signaling in the vascular endothelium [18]. It is known that increased ROS and inflammation levels are associated with decreased NO bioavailability by dysregulating eNOS and up-regulating eNOS uncoupling [9, 10]. This study

found the favorable effects of MitoQ supplementation with EX on those miRNAs at first. Taken together, reduced ROS production and altered expression of key miRNAs regulating ROS and vascular inflammation contribute to enhancing vascular endothelial function following MitoQ supplementation, which could enhance oxygen delivery to working muscles during vigorous exercise by increased blood flow.

Serum GPx levels significantly increased following all three interventions (Figure 2B) but there was no change in SOD in all groups (Figure 2C). Previous studies presented similar results that antioxidant supplementation increased plasma levels of GPx but had no significant effect on SOD [26, 27]. A recent animal study found that MitoQ supplementation up regulated both expressions of GPx and SOD in brain tissue [28]. GPx and SOD serum levels as oxidative stress markers generally increase following vigorous exercise [29, 30], or long-term training [31], which suggests activated antioxidant defense. Similarly, this study observed that GPx serum levels increased following all three interventions, MitoQ, EX, and EX+MitoQ. On the other hand, since there was no change in SOD following all interventions and no difference between interventions, the positive effect of MitoQ on the prevention of ROS overproduction might be relatively less dependent on SOD activity although MitoQ is known as a mitochondria-targeted antioxidant. However, it seems that both serum concentrations of GPx and SOD are positively associated with increased aerobic performance in healthy young or elderly adults following moderate-intensity aerobic training [33, 34]. The change of antioxidant enzymes including GPs and SOD following EX could be different depending on the intensity of exercise [35, 36], and this study might have little effect on serum levels of SOD due to the vigorous intensity of EX. Future studies are warranted to examine those antioxidant enzymatic responses to MitoQ supplementation.

There are some limitations to this study. As we analyzed the miRNAs from serum it was not possible to identify cell-type or tissue-specific expression of the miRNAs. Accumulating evidence has shown the contrasting roles of those miRNAs, such as miRNA-19b and miRNA-146b, depending on the tissue location [14]. Performance at low intensity is not relatively influenced by ROS levels since ROS generation increases in proportion to exercise intensity.

Not only MitoQ supplementation to EX but also MitoQ consumption alone significantly reduced ROS levels whereas EX did not affect ROS levels. These results suggest that MitoQ can be recommended for athletes to control oxidative stress during training cessation as well as a training period, which could contribute to alleviating detraining effects or recovering from tough training. In this double-blind randomized placebo-controlled trial, the amount, frequency, and duration of MitoQ consumption were based on the previous study that selected healthy and active people as subjects [37]. In the present study, there were several limitations, including the small number of subjects, and the short period of supplementation (4 weeks), which these guidelines could be differently applied to athletes according to exercise type as well as gender and physical characteristics of athletes. Further study is warranted to investigate the long-term effect or dose-response effect of MitoQ consumption in different exercise performances.

CONCLUSION

In conclusion, the current study demonstrated that a short-term MitoQ supplementation to aerobic training reduced ROS levels and altered expression of key miRNAs mediating ROS generation and vascular endothelial inflammation could contribute to enhancing oxygen utilization by improving vascular function. Taken together, MitoQ can be a promising antioxidant to enhance performance in athletes. A future study is warranted to provide a more optimized strategy for MitoQ consumption.

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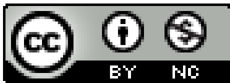

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