Effect of electroacupuncture on haemodynamic changes during intubation for general anaesthesia is mediated by nitric oxide synthase-3 via the regulation of microRNA-155, microRNA-335 and microRNA-383

WEI WANG[1], KANG WANG[2] and XING ZHONG[3]

[1]Department of Anesthesiology, The First Affiliated Hospital of Jinan University, Guangzhou, Guangdong 510632; [2]Office of Construction, Sun Yat-Sen University, Guangzhou, Guangdong 510000; [3]Department of Ultrasound, The First Affiliated Hospital of Jinan University, Guangzhou, Guangdong 510632, P.R. China

Received April 13, 2021; Accepted November 25, 2021

DOI: 10.3892/mmr.2023.12970

Abstract. Intubation for general anaesthesia is a life-threatening risk because it can cause haemodynamic changes. Electroacupuncture (EA) has been reported to alleviate the risk of intubation. In the present study, haemodynamic changes were measured at different time points before and after EA. Reverse transcription-quantitative PCR was performed to measure the expression of micro (mi)RNAs and endothelial NO synthase (eNOS) mRNA. Western blotting was performed to evaluate the expression of eNOS protein. A luciferase assay was used to explore the inhibitory role of miRNAs in eNOS expression. The transfection of miRNA precursors and antagomirs was performed to assess their effect on eNOS expression. The systolic blood pressure, diastolic blood pressure and mean arterial pressure of patients were significantly decreased by EA, while the heart rate of patients was markedly increased. The expression of micro RNA (miR)-155, miR-335 and miR-383 was effectively inhibited by EA in the plasma and peripheral blood monocytes of patients, whereas eNOS expression and NOS production were markedly elevated by EA. The luciferase activity of the eNOS vector was significantly inhibited by miR-155, miR-335 and miR-383 mimics but activated by miR-155, miR-335 and miR-383 antagomirs. miR-155, miR-335 and miR-383 precursors suppressed the expression of eNOS, while miR-155, miR-335 and miR-383 antagomirs enhanced the expression of eNOS. The present study demonstrated that EA may exert a vasodilative effect during intubation for general anaesthesia by promoting NO production and upregulating eNOS expression. The effect of EA on upregulating eNOS expression may be mediated by its inhibitory effect on the expression of miRNA-155, miRNA-335 and miRNA-383.

Correspondence to: Dr Xing Zhong, Department of Ultrasound, The First Affiliated Hospital of Jinan University, 613 Huangpu Avenue (West), Guangzhou, Guangdong 510632, P.R. China
E-mail: tumorbio@163.com

Key words: electroacupuncture, microRNA, general anaesthesia, haemodynamic changes, nitric oxide, endothelial NO synthase

Introduction

Endotracheal intubation during general anaesthesia may cause haemodynamic changes, which can be life threatening in elderly patients with diseases (1). Throughout the operation, epipharyngeal and parapharyngeal areas can be stimulated, leading to sympathoadrenal incitement and, consequently, significant rises in serum levels of catecholamine, blood pressure (BP) and heart rate. These increases may result in myocardial infarctions and arrhythmias in patients (2,3). The therapeutic effects of medications on attenuating haemodynamic reactions during intubation of laryngoscopy and avoiding a significant increase in BP have been studied (4-6).

Electroacupuncture (EA) is based on traditional acupuncture and involves placing needles onto acupoints to generate stimulation as well as nervous system actions in the human body. Acupoints from the 'governor vessel' are used as a remedy method by acupuncture in human beings and in animals following the onset of spinal cord injury (SCI) (7,8). EA at the Zusanli point (ST-36) and Neiting point (ST-44) boosts microcirculation and cerebral integrity in mature rats (9).

MicroRNAs (miRNAs/miRs) are a category of noncoding RNAs that control posttranscriptional gene expression by repressing or enhancing RNA degradation. miRNAs are crucial regulators of different biological and behavioral procedures, such as cell regeneration, apoptosis, differentiation and carcinogenesis (10). Evidence indicates that ~30% of individual genes are modulated by miRNAs (11). A previous analysis detected miRNA levels after EA therapy and found that the expression of a group of miRNAs, including miR-214, was changed following the EA procedure; hence, miR-214 was further investigated by predicting the putative binding sites of miR-214 (12).

The endogenous generation of nitric oxide (NO), especially in cardiovascular disease, is mainly related to binding to its receptor, endothelial NO synthase (eNOS). Lower NO generation enhances cardiovascular ailments, which are responsible for a number of fatalities throughout the world (13). Under physiological states, eNOS is responsible for the generation of the majority of endothelium-derived NO. eNOS serves a critical role in cardiovascular homeostasis (14,15). Inhibition of eNOS causes important alterations as demonstrated by increased blood pressure in eNOS knockout mice (15). These findings suggest a protective role for eNOS against heart failure. In bronchial cells, the localization of both Arg1 and Arg2 compared with eNOS is not important compared with the severity of L-arginine depletion and inhibition of NO generation (16). The role of LPS-induced NOS3 has been verified in mouse bone marrow-derived macrophages *in vitro* and *in vivo* (17). The involvement of Fc receptors triggers NOS-1 (along with eNOS) activation with minimal NO generation, which suppresses autocrine and paracrine phagocytosis (18).

EA may exert an inhibitory effect on the expression of miR-155, miR-335 and miR-383 and eNOS may be a shared target gene of these three miRNAs (19-21). Furthermore, previous reports have shown that acupuncture may have a vasodilative effect during intubation for general anaesthesia (22). The present study enrolled subjects who received intubation for general anaesthesia and treated them with EA to study its effect on the expression of miR-155, miR-335, miR-383 and NO/eNOS.

Materials and methods

Human subject sample collection. This was a prospective study conducted between January 2019 and August 2020. Patients who had received elective abdominal operations with a physical status of class 1 or 2 based on the American Society of Anesthesiologists physical status classification system were enrolled in this study at the First Affiliated Hospital of Jinan University. The exclusion criteria were as follows: Coagulation ailments; present or past histories of drug misuse; alcoholism; and childbirth and pregnancy issues.

All subjects were randomly divided into two groups. The patients in the control group (n=36) received EA therapy at 1 cm below the acupoints and the patients in the treatment group (n=36) received EA at the exact acupoints. The acupoints were located as the Taichong (LR3) site at the distal end of the first metatarsal space on the dorsum of the foot and the Hegu (LI4) site at the radial side of the middle of the second metacarpal bone of the hand. To avoid over-stimulation, first the Hegu site and then the Taichong site were stimulated instead of stimulating them at the same time. The time lapse between the stimulation of the Hegu site and Taichong site was 15 min per session. The acupoints of both groups were ~6.4-12.8 mm deep and the acupuncture apparatus was an Acuhealth Guru 900 (Eastern Currents, Ltd.). For the acupuncture treatment, the acupuncture needles were inserted either at 1 cm below the acupuncture point or at the exact acupuncture point. Following needling, fentanyl (0.001 mg/kg) or midazolam (0.04 mg/kg) was administered orally to reduce pain. After acupuncture, an anesthesiologist intubated the individuals receiving the treatment and a monitoring device was inserted immediately to measure the indices for 5 min. The demographics and features of all subjects were collected and compared between the two groups. In addition, the systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP) and heart rate (HR) of patients in the two groups were measured before and after needling and the collected data was presented in Table I. Furthermore, peripheral blood monocytes (PBMCs) and peripheral blood samples were collected from all patients for later analyses. The Ethics Committee of the First Affiliated Hospital of Jinan University approved the protocol of the present study (approval no. JNDX007546228FSY). Written informed consent was obtained from all participants before the initiation of the present study.

RNA isolation and reverse transcription-quantitative (RT-q) PCR. Complete RNA was isolated from the human and cell samples ($1x10^5$ cells) with TRIzol® (Thermo Fisher Scientific, Inc.) according to the manufacturer's guidelines. RNA concentration and purity were tested by UV spectrophotometer. RNA (300 μl) extracted from each sample was precipitated with ethanol, washed, resuspended and converted to cDNA using reverse transcription following manufacturer's instructions. cDNA was then subjected to RT-qPCR using the SYBR-green method (MilliporeSigma) and a 7900HT real-time PCR cycler (Applied Biosystems; Thermo Fisher Scientific, Inc.). An annealing temperature of ~62˚C for 15 min was used. Subsequently, the relative expression of miR-155 (Forward: 5'-TGCTAATCGTGATAGGGG-3'; Reverse: 5'-GAACATGTCTGCGTATCTC-3'), miR-335 (Forward: 5'-AAGAGCAATAACGAAAAATG-3'; Reverse: 5'-GAACATGTCTGCGTATCTC-3'), miR-383 (Forward: 5'-TCAGAAGGTGATTGTGGC-3'; Reverse: 5'-GAACATGTCTGCGTATCTC-3') and eNOS mRNA (Forward: 5'-GAAGGCGACAATCCTGTATGGC-3'; Reverse: 5'-TGTTCGAGGGACACCACGTCAT-3') was determined through the $2^{-\Delta\Delta Cq}$ method (23). The thermocycling protocol for RT-PCR was 10 min at 95˚C, followed by 40 cycles of 30 sec at 95˚C, 30 sec at 60˚C and 30 sec at 72˚C. U6 (Forward: 5'-CTCGCTTCGGCAGCACA-3'; Reverse: 5'-AACGCTTCACGAATTTGCGT-3') and β-actin mRNA (Forward: 5'-CACCATTGGCAATGAGCGGTTC-3'; Reverse: 5'-AGGTCTTTGCGGATGTCCACGT-3') were used as the internal controls for miRNAs and mRNA respectively. Each experiment was repeated 3 times.

Cell culture and transfection. THP-1 cells and human umbilical vein endothelial cells (HUVECs) from ATCC were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) containing 100 μg/ml streptomycin, 100 U/ml penicillin (Thermo Fisher Scientific, Inc.) and 10% inactivated fetal bovine serum (Harlan Sera-Lab, Ltd.). Cells were cultured in 24-well plates at a density of $5x10^5$ cells/well for the establishment of two different sets of experiments. In one set, cells were divided into the following three groups: NC group in which cells were transfected with 50 nM negative controls (NC; 5'-UUCUCCGAACGUGUCACGUTT-3' for miR-155 precursors and 5'-CAGUACUUUUGUGUAGUACAA-3' for anti-miR-155), miR-155 precursors group in which cells were transfected with miR-155 precursors (5'-CTGTTAATGCTAATCGTGATAGGGGTTTTTGCCTCCAACTGACTCCTACATATTAGCATTAACAG-3')

Table I. Demographic and clinicopathological features of subjects.

Characteristics	Control group (n=36)	Treatment group (n=36)	P-value
Age, years	47.2±6.8	5.8±5.6	0.428
Sex			0.407
Male (%)	25 (69.4)	28 (77.8)	
Female (%)	11 (30.6)	8 (22.2)	

and anti-miR-155 group in which cells were transfected with anti-miR-155 (5'-ACCCCUAUCACGAUUAGCAUUAA-3'). In the other set, cells were divided into the following three groups: NC group in which cells were transfected with NC, miR-383 precursors group in which cells were transfected with miR-383 precursors and anti-miR-383 group in which cells were transfected with anti-miR-383. All cells were transfected with the corresponding miRNA precursors or antagomirs with TransIT-LT1 (Mirus Bio, LLC) 4°C overnight according to the manufacturer's instructions. The cells were collected via trypsinization and assayed for the expression of the corresponding genes of interest 24 h post-transfection.

Vector construction, mutagenesis and luciferase assay. The sequence analysis results by bioinformatic tool TargetScan (Relase 8.0, http://www.targetscan.org/vert_80/) indicated that eNOS may be a potential target gene shared by miR-155, miR-335 and miR-383. To further explore the inhibitory role of miR-155, miR-335 and miR-383 in the expression of eNOS, i.e., the regulatory relationship of miR-155/eNOS, miR-335/eNOS and miR-383/eNOS, luciferase vectors containing wild-type and mutant eNOS 3' UTRs were established. In brief, the eNOS 3' UTRs containing the binding sites for miR-155, miR-335 and miR-383 were amplified by PCR and cloned into pmirGLO dual-luciferase miRNA target expression vectors (cat. no. E1330; Promega Corporation) downstream of the reporter gene to generate wild-type eNOS vectors which contained according binding sites for miR-155, miR-335 and miR-383. In addition, site-directed mutagenesis was performed using a Stratagene Quick Change II mutagenesis assay kit (Stratagene; Agilent Sumitomo Dainippon Pharma Co., Ltd.) following the manufacturer's guidelines to generate mutant eNOS 3' UTR sequences which contained according binding sites for miR-155, miR-335 and miR-383. Finally, both the mutant and wild-type eNOS vectors were transfected into the THP-1 cells and HUVECs with along with the mimics and antagomirs of miR-155, miR-335 and miR-383 using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's guidelines. At 24 h after transfection, a Bright Glo luciferase assay kit (Promega Corporation) was used to analyze the luciferase activity of the transfected cells which were normalized to *Renilla* luciferase activity following the manufacturer's guidelines.

Western blot analysis. Western blots were used in conjunction with an Odyssey Infrared Imaging system (LI-COR Biosciences) using conventional western blotting procedures. In brief, collected PBMCs and cultured cells were subjected to RIPA lysis treatment to collect the proteins, the concentrations of which were determined using a BCA assay kit (Bio-Rad Laboratories, Inc.) following the manufacture's guidelines. Protein samples (50 μg) were then resolved by 12% SDS-PAGE and blotted onto NC membranes (Thermo Fisher Scientific, Inc.). Subsequently, the membranes were blocked with 5% non-fat milk for 60 min at room temperature, followed by overnight incubation at 4°C with primary anti-eNOS antibodies (1:1,000; cat. no. ab76198; Abcam). GAPDH (1:1,000; cat. no. ab8245; Abcam) was used as the loading control. Furthermore, the membranes were incubated with anti-rabbit IgG secondary antibodies conjugated to horseradish peroxidase (HRP) (1:1,000; cat. no. 7074; Cell Signaling Technology, Inc.) for 2 h at room temperature and then developed using an ECL reagent (Thermo Fisher Scientific, Inc.). The relative protein expression of eNOS was calculated using Quantity One software (v4.6.7; Bio-Rad Laboratories, Inc.).

ELISA. The supernatants of peripheral blood samples collected from different patient groups were retrieved by centrifugation (250 x g) at 4°C for 5 min, and the content of NO in each sample was assayed by a sandwich ELISA kit (cat. no. ab233628; Abcam) following the manufacturer's protocol.

Statistical analysis. All statistical analyses were conducted with SPSS 16.0 statistical computer software (SPSS, Inc.). Non-parametric parameters were compared using the χ^2 test. Wilcoxon sum rank test or Student's t test were used to analyze differences between two groups, while one-way analysis of variance (ANOVA) in conjunction with Tukey's post hoc test were used to analyze differences among multiple groups. Pearson correlations were used to determine the values of correlation coefficients. All statistical values are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

Demographic and clinicopathological characteristics of the participants recruited in the present study. The participants of this study were divided into two groups. One group of patients (n=36) received EA therapy 1 cm below the acupoints and the other group (n=36) received EA at the exact acupoints. The average age for the control group was 47.2±6.8 years old, while the average age for the treatment group was 51.8±5.6 years old. There were 25 male participants in the control group and 28 male participants in the treatment group. There were no obvious differences in these indicators between the two groups.

EA decreases the SBP, DBP and MAP but increases the HR of patients. The diagrams which indicated the locations of the acupuncture points, Taichong site and Hegu site, are shown in Fig. 1A. The SBP, DBP, MAP and HR of patients in the above two groups were measured before and after needling and 3 min of intubation. It was found that the SBP, DBP, MAP and HR were changed following needling in both groups, whereas

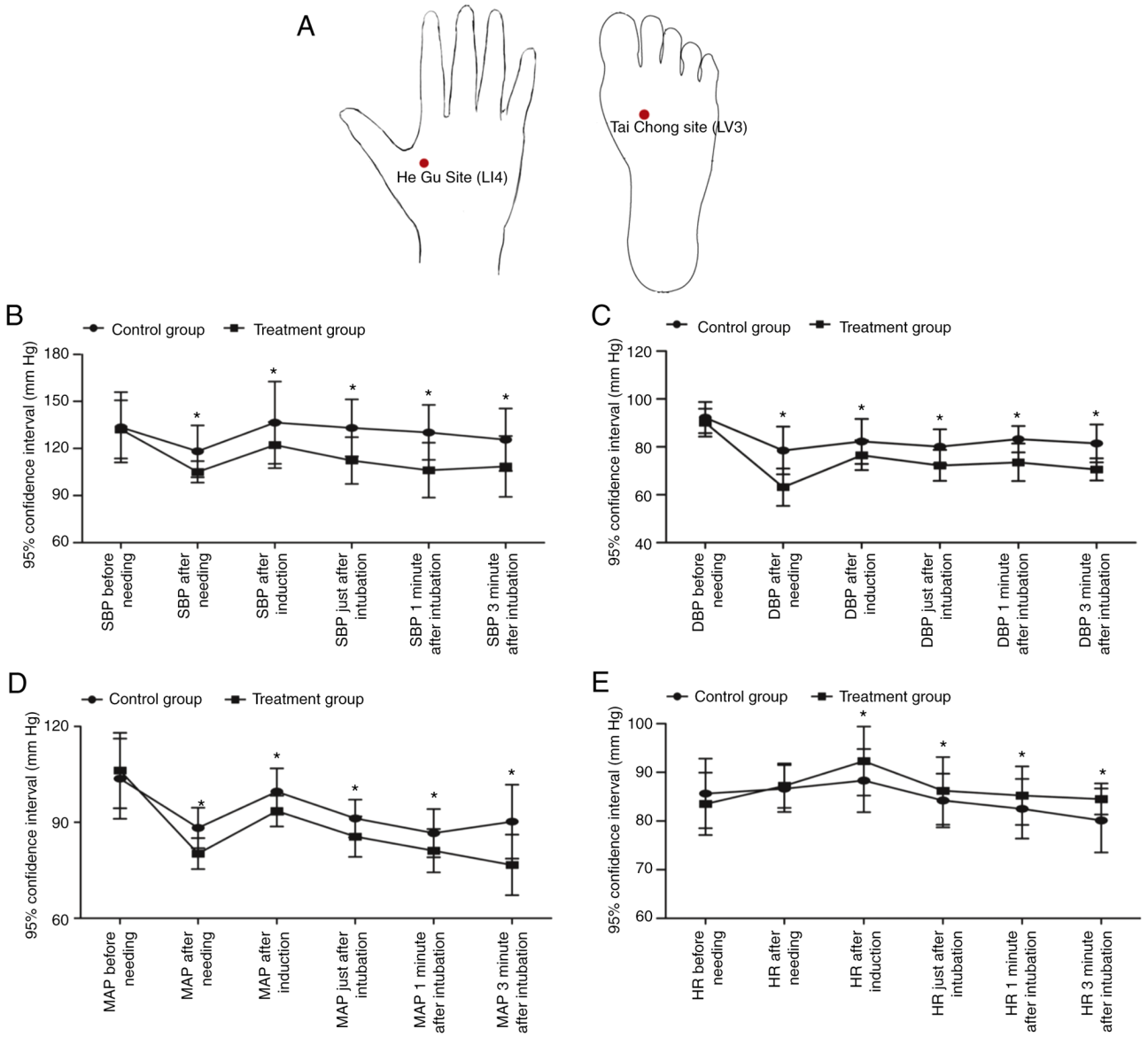

Figure 1. Electroacupuncture decreases the SBP, DBP and MAP but increased the HR of patients (*P<0.05 vs. control group; Student's t tests). (A) Diagrams of the locations of the Taichong and Hegu sites. (B) The SBP of patients receiving electroacupuncture on the exact acupoints was decreased than that in patients receiving electroacupuncture therapy at 1 cm below the acupoints following the time-point of needling. (C) The DBP of patients receiving electroacupuncture on the exact acupoints was decreased than that in patients receiving electroacupuncture therapy at 1 cm below the acupoints following the time-point of needling. (D) The MAP of patients receiving electroacupuncture on the exact acupoints was decreased than that in patients receiving electroacupuncture therapy at 1 cm below the acupoints following the time-point of needling. (E) The HR of patients receiving electroacupuncture on the exact acupoints was increased than that in patients receiving electroacupuncture therapy at 1 cm below the acupoints following the time-point induction. SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HR, heart rate.

no obvious difference was observed for the SBP, DBP, MAP and HR before needling. The SBP (Fig. 1B), DBP (Fig. 1C) and MAP (Fig. 1D) in patients receiving EA therapy at 1 cm below the acupoints were significantly elevated compared with those in patients receiving EA at the exact acupoints following the time-point of needling. However, the HR (Fig. 1E) in patients receiving EA therapy at 1 cm below the acupoints was markedly reduced compared with that in patients receiving EA at the exact acupoints following the time-point of induction.

EA inhibits the expression of miRNAs and increases the expression of NO in the peripheral blood and PBMCs of patients. The expression of miR-155, miR-335, miR-383 and NO was examined in the peripheral blood collected from patients receiving EA therapy at 1 cm below the acupoints or on the exact acupoints. The expression of miR-155 (Fig. 2A), miR-335 (Fig. 2B) and miR-383 (Fig. 2C) was significantly inhibited in peripheral blood samples from patients receiving EA therapy at the acupoints compared with that in patients receiving EA therapy at 1 cm below the acupoints. In addition, ELISA was performed to evaluate the production of NO in the peripheral blood of patients receiving EA therapy at 1 cm below the acupoints or on the exact acupoints. The production of NO (Fig. 2D) in the peripheral blood of patients receiving EA therapy at the acupoints was increased compared with those receiving EA therapy at 1 cm below the

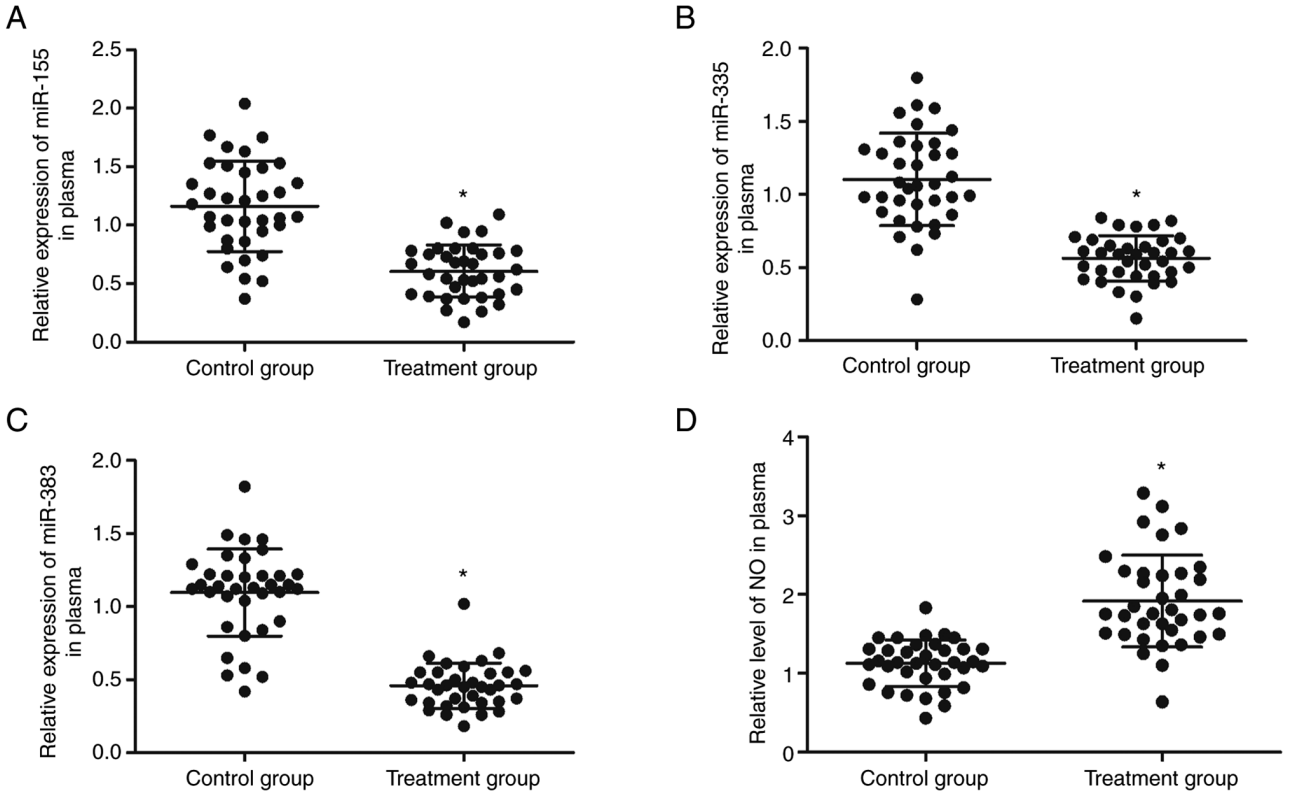

Figure 2. Electroacupuncture inhibits the expression of miRNAs and increased the production of NO in the peripheral blood of patients (*P<0.05 vs. control group; Student's t-tests). (A) The expression of miR-155 was inhibited in the plasma of patients receiving electroacupuncture on the exact acupoints than that in patients receiving electroacupuncture therapy at 1 cm below the acupoints. (B) The expression of miR-335 was inhibited in the plasma of patients receiving electroacupuncture on the exact acupoints than that in patients receiving electroacupuncture therapy at 1 cm below the acupoints. (C) The expression of miR-383 was inhibited in the plasma of patients receiving electroacupuncture on the exact acupoints than that in patients receiving electroacupuncture therapy at 1 cm below the acupoints. (D) The production of NO was elevated in the plasma of patients receiving electroacupuncture on the exact acupoints than that in patients receiving electroacupuncture therapy at 1 cm below the acupoints. miRNA/miR, microRNA.

acupoints. Furthermore, PBMCs were isolated from patients receiving EA therapy at 1 cm below the acupoints or on the exact acupoints and the expression of miR-155, miR-335, miR-383 and eNOS analyzed using qPCR. The expression of miR-155 (Fig. 3A), miR-335 (Fig. 3B) and miR-383 (Fig. 3C) was significantly reduced in the PBMCs from patients receiving EA therapy at the exact acupoints compared with those receiving EA therapy at 1 cm below the acupoints, while the expression of eNOS mRNA (Fig. 3D) was significantly enhanced in the PBMCs of patients receiving EA therapy at the exact acupoints.

miR-155, miR-335 and miR-383 inhibit the expression of eNOS by binding to the 3' UTR. Sequence analysis indicated that eNOS was a potential binding target of miR-155 (Fig. 4A), miR-335 (Fig. 4D) and miR-383 (Fig. 4G). Luciferase vectors containing wild-type and mutant eNOS 3' UTR were generated and cotransfected into THP-1 cells and HUVECs with miR-155, miR-335 and miR-383 mimics and antagomirs. The luciferase activity of wild-type eNOS was efficiently inhibited by miR-155 (Fig. 4B and C), miR-335 (Fig. 4E and F) and miR-383 (Fig. 4H and I) mimics in THP-1 cells and HUVECs, whereas miR-155, miR-335 and miR-383 antagomirs significantly enhanced the luciferase activity of wild-type eNOS vectors. No apparent changes were observed for the luciferase activity of mutant eNOS.

miR-155, miR-335 and miR-383 precursors suppress the expression of eNOS mRNA and protein, while miR-155, miR-335 and miR-383 antagomirs activate the expression of eNOS mRNA and protein in THP-1 cells and HUVECs. To further explore the inhibitory role of miR-155, miR-335 and miR-383 in the expression of eNOS, miR-155, miR-335 and miR-383 precursors and antagomirs were transfected into THP-1 cells and HUVECs. The successful transfection of miR-155, miR-335 and miR-383 precursors and antagomirs were confirmed by observing the changes of miR-155 (Supplementary Fig. S1A and B), miR-335 (Supplementary Fig. S1C and D) and miR-383 (Supplementary Fig. S1E and F). Subsequently, the expression of eNOS mRNA and protein was analyzed using qPCR and western blot analysis, respectively. The expression of eNOS mRNA and protein was significantly suppressed by miR-155 (Figs. 5A and B; 6A and B), miR-335 (Figs. 5C and D; 6C and D) and miR-383 (Figs. 5E and F; 6E and F) precursors in THP-1 cells and HUVECs, while the expression of eNOS mRNA and protein was activated by miR-155 (Figs. 5A and B; 6A and B), miR-335 and miR-383 antagomirs in THP-1 cells and HUVECs.

Discussion

The present study found that EA decreased the levels of SBP, DBP and MAP and increased the production of

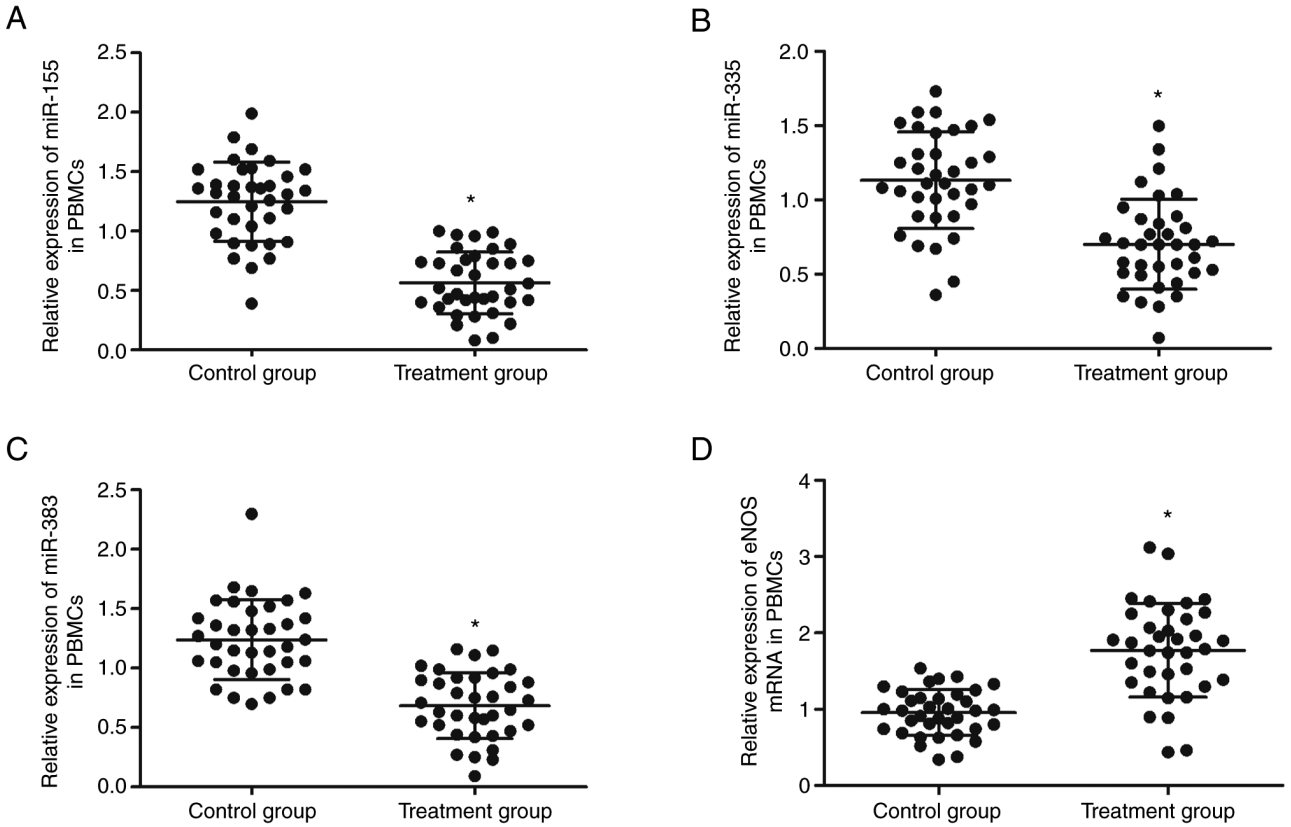

Figure 3. Electroacupuncture inhibits the expression of miRNAs and increases the production of NO in the PBMCs of patients (*P<0.05 vs. control group; Student's t tests). (A) The expression of miR-155 was inhibited in the PBMCs of patients receiving electroacupuncture on the exact acupoints than that in patients receiving electroacupuncture therapy at 1 cm below the acupoints. (B) The expression of miR-335 was inhibited in the PBMCs of patients receiving electroacupuncture on the exact acupoints than that in patients receiving electroacupuncture therapy at 1 cm below the acupoints. (C) The expression of miR-383 was inhibited in the PBMCs of patients receiving electroacupuncture on the exact acupoints than that in patients receiving electroacupuncture therapy at 1 cm below the acupoints. (D) The production of NO was elevated in the PBMCs of patients receiving electroacupuncture on the exact acupoints than that in patients receiving electroacupuncture therapy at 1 cm below the acupoints. miRNA/miR, microRNA; PBMCs, peripheral blood monocytes.

peripheral blood NO and the level of HR in the recruited patients. Luciferase assays confirmed that the overexpression of miR-155, miR-335 and miR-383 inhibited the luciferase activity of eNOS. Therefore, EA may exert a vasodilative effect during intubation for general anaesthesia by promoting NO production and upregulating eNOS expression. As reported by Dashti *et al* (24), haemodynamic responses to tracheal intubation are caused by laryngoscopy and stimulation of the structures of both the larynx and trachea (24), and the upward lifting force needed to expose the glottis is less with a GlideScope than the laryngoscope, resulting in less traction applied to soft tissues, which may be correlated with significantly less sympathetic stimulation. Nevertheless, fluctuations in the concentration of anesthetic agents in blood and effector sites occur with regard to the onset and offset stimulation. Moreover, cardiovascular symptoms leading to hypotension and bradycardia might be depressed by pharmacologic interventions, such as opioids. However, opioids can have deleterious respiratory effects on the fetus (24). Another strategy to reduce the cardiovascular response to tracheal intubation is to alter the method of tracheal intubation (25).

EA (EA) is an essential sensory stimulation that is accompanied by a range of responses from the nervous system. The responses to EA treatment have been demonstrated to be suppressed using anaesthesia and these modifications are manifested by somatic afferent and adrenal efferent nerves (26-28). The present study recruited patients to receive EA treatment. The SBP, DBP, MAP and HR of the patients was measured to show that EA effectively decreased the SBP, DBP and MAP but increased the HR. The interaction of certain methods with acupuncture was studied. In a crossover study, treatment with anesthetic at acupuncture site PC6 helps to avoid an antiemetic response (29). In a controlled study on the ramifications of acupuncture under anaesthesia, no differences were found in volunteers awarded acupuncture or placebo (30). Responses to EA have been shown in animals, demonstrating the consequences of reduced use of anaesthesia following EA (31). Saleh (32) conducted a randomized clinical analysis on more than 200 surgical samples to evaluate the consequence of EA at the Neiguan (P6) and Quchi (Li 11) sites in the prevention of endotracheal intubation-induced strain, reporting that the DP, SP and MAP in the control group is increased but that the increase in the experimental group is much higher.

Microarrays using total RNA have been used to measure the expression of miRNAs at medullas immediately following acupuncture remedy at the Taichong site to show the reaction of miRNA profiling to acupuncture treatment of SHRs. Preliminary evaluation of miRNA expression statistics shows that >200 miRNAs have considerable fluctuations with regard to their expression inside the body, including 23 miRNAs with

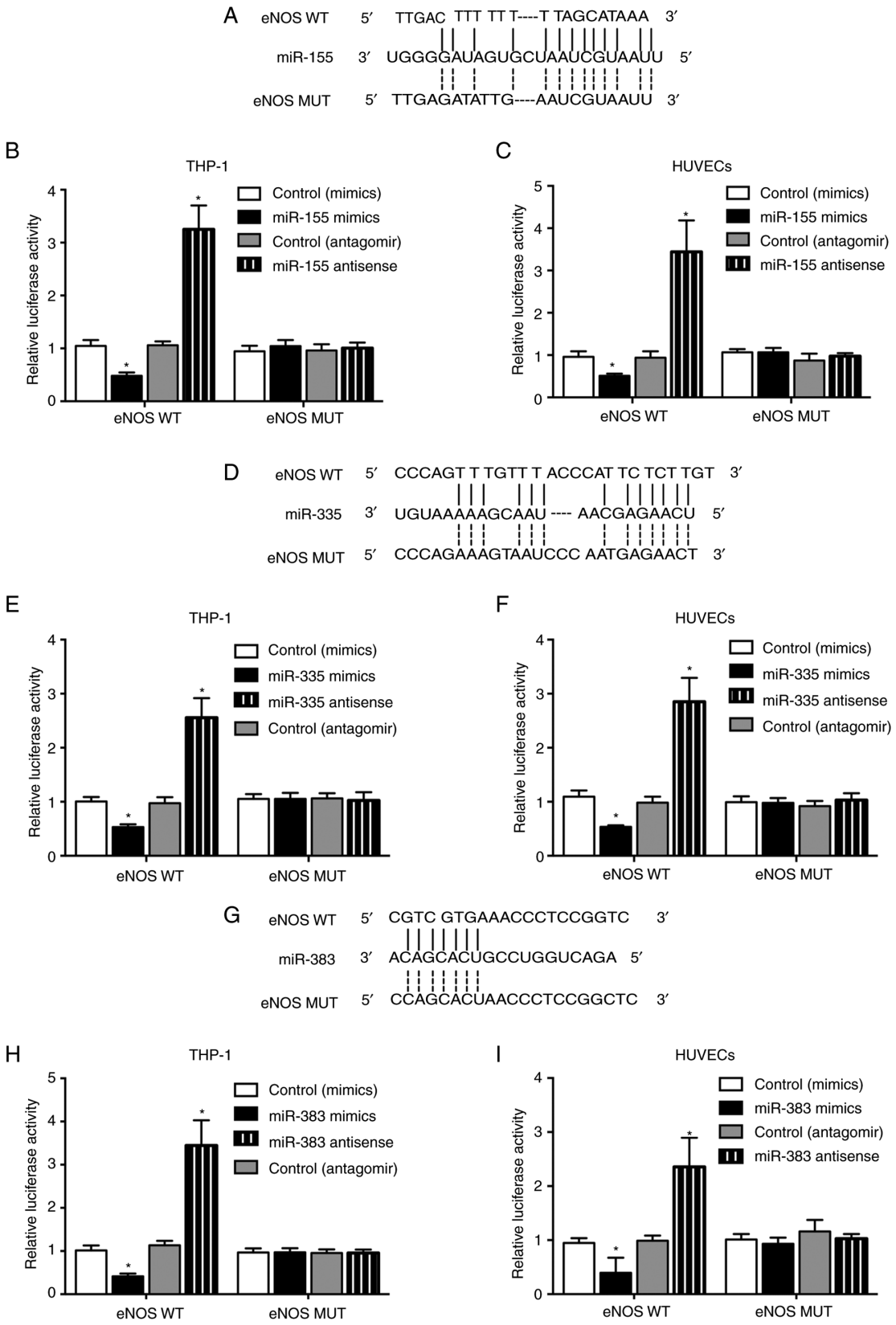

Figure 4. The luciferase activity of WT eNOS is suppressed by miR-155, miR-335 and miR-383 mimics and elevated by miR-155, miR-335 and miR-383 antagomirs (*P<0.05 vs. control group; one-way ANOVA). (A) Sequence analysis indicated potential binding of miR-155 to the 3' UTR of eNOS. (B) The luciferase activity of WT eNOS vector was inhibited by miR-155 mimics and enhanced by miR-155 antagomirs in THP-1 cells. (C) The luciferase activity of WT eNOS vector was inhibited by miR-155 mimics and enhanced by miR-155 antagomirs in HUVEC cells. (D) Sequence analysis indicated potential binding of miR-335 to the 3' UTR of eNOS. (E) The luciferase activity of WT eNOS vector was inhibited by miR-335 mimics and enhanced by miR-335 antagomirs in THP-1 cells. (F) The luciferase activity of WT eNOS vector was inhibited by miR-335 mimics and enhanced by miR-335 antagomirs in HUVEC cells. (G) Sequence analysis indicated potential binding of miR-383 to the 3' UTR of eNOS. (H) The luciferase activity of WT eNOS vector was inhibited by miR-383 mimics and enhanced by miR-383 antagomirs in THP-1 cells. (I) The luciferase activity of WT eNOS vector was inhibited by miR-383 mimics and enhanced by miR-383 antagomirs in HUVEC cells. WT, wild type; eNOS, endothelial NO synthase; HUVEC, human umbilical vein endothelial cell; miRNA/miR, microRNA; MUT, mutant.

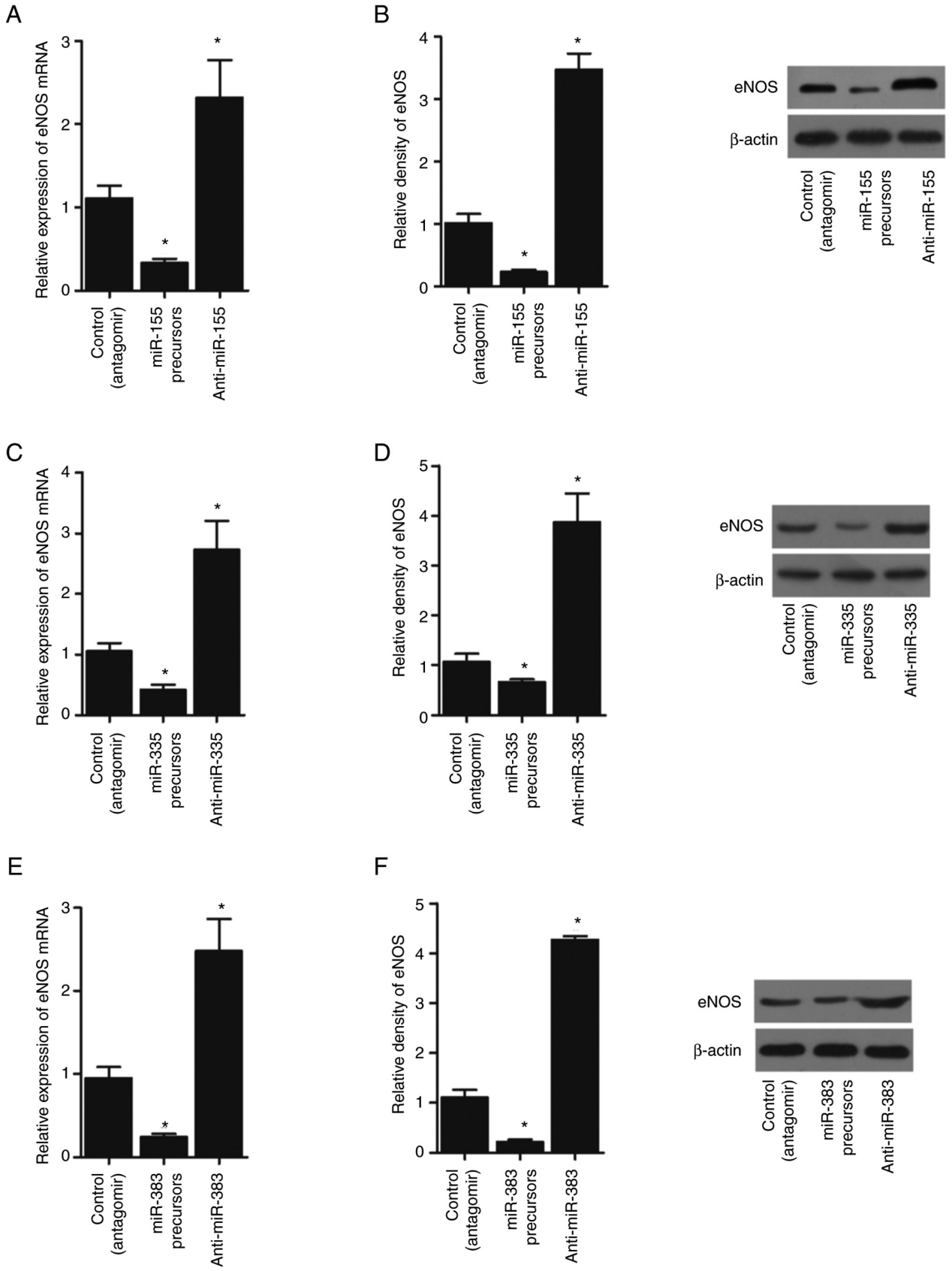

Figure 5. miR-155, miR-335 and miR-383 precursors suppress the expression of eNOS, while miR-155, miR-335 and miR-383 antagomirs activate the expression of eNOS in THP-1 cells (*P<0.05 vs. control (antagomir) group; one-way ANOVA). (A) miR-155 precursors suppressed the expression of eNOS mRNA, while miR-155 antagomirs enhanced the expression of eNOS mRNA in THP-1 cells. (B) miR-155 precursors suppressed the expression of eNOS protein, while miR-155 antagomirs enhanced the expression of eNOS protein in THP-1 cells. (C) miR-335 precursors suppressed the expression of eNOS mRNA, while miR-335 antagomirs enhanced the expression of eNOS mRNA in THP-1 cells. (D) miR-335 precursors suppressed the expression of eNOS protein, while miR-335 antagomirs enhanced the expression of eNOS protein in THP-1 cells. (E) miR-383 precursors suppressed the expression of eNOS mRNA, while miR-383 antagomirs enhanced the expression of eNOS mRNA in THP-1 cells. (F) miR-383 precursors suppressed the expression of eNOS protein, while miR-383 antagomirs enhanced the expression of eNOS protein in THP-1 cells. miRNA/miR, microRNA; eNOS, endothelial NO synthase.

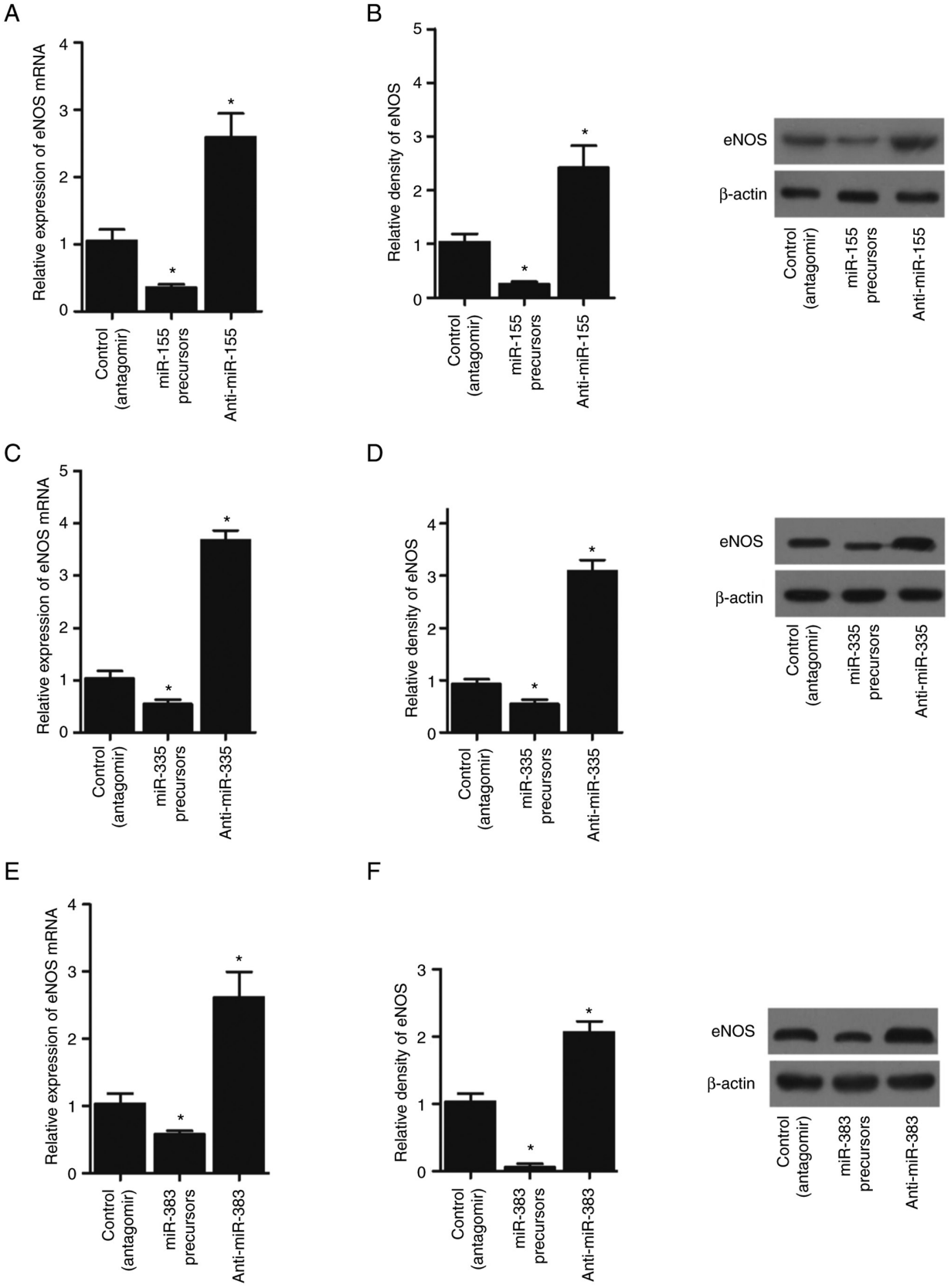

Figure 6. miR-155, miR-335 and miR-383 precursors suppressed the expression of eNOS, while miR-155, miR-335 and miR-383 antagomirs activated the expression of eNOS in HUVECs (*P<0.05 vs. control (antagomir) group; one-way ANOVA). (A) miR-155 precursors suppressed the expression of eNOS mRNA, while miR-155 antagomirs enhanced the expression of eNOS mRNA in HUVEC cells. (B) miR-155 precursors suppressed the expression of eNOS protein, while miR-155 antagomirs enhanced the expression of eNOS protein in HUVEC cells. (C) miR-335 precursors suppressed the expression of eNOS mRNA, while miR-335 antagomirs enhanced the expression of eNOS mRNA in HUVEC cells. (D) miR-335 precursors suppressed the expression of eNOS protein, while miR-335 antagomirs enhanced the expression of eNOS protein in HUVEC cells. (E) miR-383 precursors suppressed the expression of eNOS mRNA, while miR-383 antagomirs enhanced the expression of eNOS mRNA in HUVEC cells. (F) miR-383 precursors suppressed the expression of eNOS protein, while miR-383 antagomirs enhanced the expression of eNOS protein in HUVEC cells. miRNA/miR, microRNA; eNOS, endothelial NO synthase; HUVEC, human umbilical vein endothelial cell.

a ≥1.5-fold shift under acupuncture therapy. Compared with healthy SD rats, miR-155, miR-335 and miR-383 are significantly downregulated in the experimental group compared with the control group (21). The present study collected peripheral blood and PBMCs from patients receiving EA. The expression of miR-155, miR-335 and miR-383 was reduced the peripheral blood and PBMCs. Furthermore, luciferase assays were performed to explore the regulatory role of miRNAs in eNOS expression. miR-155, miR-335 and miR-383 inhibited the luciferase activity of eNOS by binding to its 3' UTR. Moreover, THP-1 cells and HUVECs were transfected with miR-155, miR-335 and miR-383 precursors and antagomirs. The expression of eNOS was significantly inhibited by miR-155, miR-335 and miR-383 precursors but enhanced by miR-155, miR-335 and miR-383 antagomirs.

NO is a soluble compound synthesized by endothelial cells using L-arginine amino acid via the constitutively active calcium calmodulin-dependent enzyme, nitric oxide synthase (NOS) (33). A five-electron oxidation reaction in L-arginine is catalyzed by NOS along with several cofactors (34). The first evidence that NO acts as a calming substance for the relaxation of vascular tissue was found by Devlin *et al* (35), who reveal that endothelial cells release NO if they are subjected to bradykinin treatment according to a chemiluminescence assay. As eNOS and neuronal NOS (nNOS) are constitutively expressed, they are termed calcium-dependent enzymes (although eNOS might be activated in a calcium-independent manner) (35). The mRNA level of the eNOS enhancer is reduced in a reaction to hypoxia in HUVECs (36). However, from the molecular point of view, eNOS stimulation and eNOS expression are more complex and rely on PI3K/Akt- or AC/PKA signaling at the posttranscriptional, transcriptional and posttranslational levels (37,38).

As a major part of traditional Chinese medicine, EA is widely used in China. Although some tragedies have been reported in the past years in western countries (39,40), from our perspective, this was mainly caused by the mis-location of the pressor points (although few details are known). Similar to other part of the traditional medicine, the factor that prevents widespread use of EA in clinical practice is the lack of comprehensive understanding of the underlying molecular mechanism and the risk of EA is primarily caused by unexperienced practitioner. Therefore, it will take a long time for the widely use of EA in clinical practice outside China.

In conclusion, the findings of the present study suggested that EA may exert a vasodilative effect during intubation for general anaesthesia, which may be due to the ability of EA to promote the production of NO by upregulating eNOS expression. Furthermore, the effect of EA on eNOS may be mediated by its inhibitory effect on the expression of miR-155, miR-335 and miR-383.

Acknowledgements

Not applicable.

Funding

The present study was supported by Shandong Traditional Chinese Medicine Science and Technology Development Project (grant no. 2017-469; 2019-0851; 2019-0861) and the Key Research Plan of Shandong Province (grant no. 2019GSF108253).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XZ and WW planned the study, WW and KW searched the literature, WW, KW and XZ collected and analyzed the data. WW and XZ confirm the authenticity of all raw data. XZ and WW wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Ethics Committee of the First Affiliated Hospital of Jinan University approved the protocol of the present study (Approval no. JNDX007546228FSY). Written informed consent was obtained from all participants before the initiation of the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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