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Original research article

miR-214 and miR-126 were associated with restoration of endothelial function in obesity after exercise and dietary intervention

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ABSTRACT

Obesity would result in increased cardiovascular morbidity including endothelial destruction, and miRNAs are recognized as potent regulators on endothelial function. We therefore explored pivotal miRNAs before and after exercise and dietary intervention in obese adults and examined their potential relationships with selected endothelial function and biomarkers. Obese adults were included in an exercise and dietary intervention training program for 2 months. At the beginning and the end, measurements of anthropometric and metabolic parameters were performed. Flow-mediated dilation, endothelial related biochemicals and circulating miR-214 and miR-126 levels were also determined. Results showed that circulating miR-214 and miR-126 levels were significantly enhanced ($P < 0.05$) by exercise and dietary intervention along with improved endothelial function. The relationship between relative changes of miR-214 and that of endothelial progenitor cells was significant ($r = 0.589$, $P < 0.05$); relative expression of miR-126 was also significantly ($r = 0.433$, $P < 0.05$) correlated with endothelial nitric oxide synthase. The intervention lead to upregulation of circulating miR-214 and miR-126 in obesity, and these molecular adaptations are associated with improved endothelial function during the restoring process.

Introduction

Obesity impairs vascular homeostasis and induces cardiovascular pathologies, among which endothelium alteration and dysfunction usually reflect the initial stages at various vessel levels (Mahmud et al., 2009; Tounian et al., 2001). Exercise combined with dietary intervention could achieve great improvement of endothelial function in the obese (Meyer et al., 2006; Woo et al., 2004). The basic underlying mechanism of these lifestyle modifications on reversing endothelial dysfunction might be involved transcriptional and translational regulations of the essential genes. Up to one-third of the mammalin transcriptome were regulated by microRNAs (miRNA, miR) (Fukushima et al., 2011; Lewis et al., 2005); these post-transcriptional regulators emerged as key molecules in obesity-associated vasculopathy process. miRNAs are a class of endogenous non-coding short nucleotides (18–25 bp in length) and can bind to the 3′ untranslated region of the target mRNA completely to degrade it or incompletely to suppress the translation (Forstemann et al., 2007; Grimson et al., 2007).

The role of miRNAs in endothelium has been increasing recognized. Among the miRNAs involved in cardiovascular pathology, miR-214 and miR-126 play vital roles in controlling endothelial related vascular function and were associated with reduced cell proliferation, enhanced apoptosis and inflammation (Rippe et al., 2012). These two miRNAs were also chosen as biomarkers for malignant endothelial proliferative diseases; the data suggested their diagnostic roles on the non-invasive human researches (Heishima et al., 2015).

Therefore, the current study was designed to examine the effects of exercise combined with dietary intervention on miR-

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214 and miR-126 in obese subjects and to investigate their relationship with obesity induced endothelial dysfunction. The elucidation of novel miRNAs in recovering endothelial vascular physiology of obesity by exercise and dietary intervention is of high value that could extend insight into the basic mechanisms of the beneficial effects of lifestyle modification on endothelial biology.

Materials and methods

Subjects and ethics statement

A total of 22 obese adults without known severe cardiovascular or malignant diseases were recruited in the study. Body mass index (BMI) was calculated as weight divided by squared height; subjects were considered obesity for BMI $\geq 30\,kg/m^2$. All subjects gave written informed consent before participating in the study. 17 subjects have completed all of the intervention and testing program.

Exercise and dietary intervention

The study was part of a larger investigation of the effects of exercise and dietary intervention on the obesity in a closed boot camp for 2 months. Diet was nutritionally complete (20% protein, 20% fat, and 60% carbohydrate) and calorie intake ranged from 1300 to 2200 kcal/d, which were monitored at the beginning and after this program by nutritionists. Exercise physiologists planed a series of physical exertion (1500–2500 kcal/d) that involved both endurance exercise (70–85% of maximum heart rate; aerobic treadmill running, sports games, bicycling, and dancing) and resistance training (12–15 repetition maximum); the interventions were performed 5 h/d and 6 d/wk., supervised by qualified trainers throughout the program. Measurements were performed at baseline and endline of the intervention.

Biochemical analysis

Blood were taken after overnight fast to determine glucose, insulin, and other standard parameters. Enzyme-linked immunosorbent assay (ELISA) kits (Cusabio, Biotech. Co., LTD, Wuhan, China) were used to determined serum endothelial nitric oxide synthase (eNOS), C-reaction protein (CRP), and vascular endothelial growth factor (VEGF) concentration according to the manufacturer's recommended procedure. Homeostatic model of assessment of insulin resistance (HOMA-IR) was calculated as [fasting insulin ($\mu U/ml$) $\times$ fasting glucose (mmol/l)]/22.5.

Endothelial related vascular reactivity test

Flow-mediated dilation (FMD) of the brachial artery was measured using echography (UNEX-EF, UNEX Co. Ltd., Nagoya, Japan). Details of the procedure have described in previous study (Thijssen et al., 2011).

Quantification of circulating endothelial progenitor cells (EPCs)

CD34+/KDR+ cells were analyzed with flow cytometry following the previous study (Van Craenenbroeck et al., 2008). Data were analyzed using Cytomics FC500 flow cytometer (Beckman Coulter, USA). Each sample was analyzed for a minimum of 40,000 events; fluorescent isotype-matched antibodies were used as controls. Data were analyzed using CXP software 2.0.

miRNA analysis

Serum preparation and RNA extraction

Blood from the subjects was collected avoiding skin contamination and then clotted at room temperature for 1 h. Serum was prepared by centrifugation at 3000 rpm for 10 min and removed carefully from the supernatant into a plastic sterile polycarbonate tube. Serum samples were kept on ice before use or frozen at $-80\,^{\circ}C$ for long time storage. miRcute Serum/plasms miRNA isolation kit (DP503, TIANGEN, Beijing, China) was used to isolate total RNA (including miRNAs) from serum according to manufacturer's instructions. For each serum sample, 25 fmol of *C. elegans* cel-miR-39 (5′-UCACCGGGUGUAAAUCAGCUUG-3′) was added as spike in control immediately after 200 μl serum and 900 μl lysis buffer were fully vortexed avoiding degradation. The RNA was quantified using a spectrophotometer (ND1000 Nanodrop, Massachusetts, USA) by measuring the absorbance at 260 nm (A260) and 280 nm (A280), with A260/A280 ratios above 1.8 indicating high-quality RNA.

Quantitative real-time PCR

Total RNA was reverse-transcribed into cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Massachusetts, USA). cDNA was then stored at $-20\,^{\circ}C$ until RT-PCR reactions was performed. cDNA was amplified in a reaction mixture (20 μl) contained of 10 μl of Power SYBR Green PCR Master Mix (2 ×) (Applied Biosystems, Massachusetts, USA), 0.8 μl of forward primer, 0.8 μl of reverse primers, 2 μl cDNA, and 6.4 μl DNase-free water. Monitoring the amplification was performed using a detection system (ABI 7500, Applied Biosystems, Massachusetts, USA), initiating at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 60 s. At the end of the PCR reaction, the samples were subjected to 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, and 60 °C for 15 s in order to draw the dissociation curve. The stem-loop primers and amplification primers were designed and purchased from Ribobio (Ribobio Co., Ltd, Guangzhou, China). Synthetic *C. elegans* cel-miR-39 was the reference gene used for normalization; relative expression of each gene was determined using the 2-△△ CT method.

Statistical analysis

Data were analysed using SPSS 16.0 and presented as mean ± standard error mean (SEM). Normality tests were used to determine the normal distribution of a data, and if the data were not normally distributed would be applied to log-transformation. Differences between the groups were analyzed by ANOVA, followed by paired sample *t*-test to compare the data before and after the intervention. Correlation between relative changes of miRNAs and FMD, eNOS, CRP, VEGF or EPC were determined by Pearson correlation analysis. Linear dependence between two variables was estimated through correlation coefficient (r); the value of r gives strength of linear relationship with 0.3, 0.5 and 0.8 being interpreted as poor, moderate and strong, respectively. $P < 0.05$ was considered significant. Figure was obtained using Sigma Plot 11.0.

Results

The effect of exercise and dietary intervention on weight losing and metabolic parameters

Both BMI and body fat decreased significantly by ∼11.4% ($P < 0.001$) and ∼10.5% ($P < 0.001$), respectively. Changes in metabolic parameter were summarized in Table 1; circulating

Table 1
The effect of exercise and dietary intervention on anthropometric characteristics and metabolic parameters.

	Baseline	Endline	P
Age (yr)	22 ± 4		
BMI (kg/m^2)	37.76 ± 1.20	33.53 ± 1.07	<0.001
Body fat (%)	40.94 ± 1.12	36.57 ± 1.43	<0.001
Cholesterol (mmol/l)	5.35 ± 0.22	4.74 ± 0.33	0.046
Triglycerides (mmol/l)	2.15 ± 0.26	1.32 ± 0.21	0.002
HDL-L (mmol/l)	0.99 ± 0.03	0.99 ± 0.05	0.981
LDL-L (mmol/l)	3.44 ± 0.18	2.99 ± 0.23	0.045
FPG (mmol/l)	5.61 ± 0.14	5.39 ± 0.14	0.196
FINS (pmol/l)	200.81 ± 28.08	116.49 ± 19.69	0.017
HOMA-IR	7.34 ± 1.19	4.08 ± 0.70	0.006

Note: Data are mean ± SEM. BMI, body mass index; HDL-L, high-density lipoprotein cholesterol; LDL-L, low-density lipoprotein cholesterol; FPG, fasting plasma glucose; FINS, fasting serum insulin; HOMA-IR, homeostasis model assessment estimated insulin resistance.

cholesterol, triglycerides, low-density lipoprotein cholesterol (LDL-L), fasting serum insulin (FINS), and HOMA-IR showed significant reduction ($P < 0.05$) after 2 months intervention.

The effect of exercise and dietary intervention on endothelial related function and biochemicals

FMD improved significantly ($P < 0.05$) by ~14.4% in brachial artery of the subjects after the intervention. Exercise combined with caloric restriction also enhanced circulating concentration of eNOS and VEGF and lowered CRP expression, all with significant differences ($P < 0.05$) (Table 2).

The effect of exercise and dietary intervention on circulating miR-214 and miR-126

miR-214 and miR-126 levels of endline were significantly enhanced ($P < 0.05$) compared with that of baseline as shown in Fig. 1.

The circulating miRNAs were normalized using spiked-in synthetic elegans miR-39 as control. Bars represent the mean ± SEM; * $P < 0.05$and ** $P < 0.01$ vs. baseline.

The correlation between relative changes of miRNAs and that of metabolic parameters

Table 3 summarized the correlation between changes of the miRNAs and metabolic parameters. The moderate correlation between the change of miR-214 and that of FPG was observed ($P < 0.05$); whereas there was no statistical correlation between miR-126 and those metabolic indicators.

The correlation between relative changes of miRNAs and that of endothelial parameters

The correlation between relative changes of the miRNAs and selected endothelial parameter were summarized in Table 3. There was no correlation between miRNAs and FMD, CRP or VEGF. The changes of miR-214 and EPC were moderately correlated with significance ($P < 0.05$); the alteration of miR-126 and eNOS were also observed significantly correlated ($P < 0.05$).

Table 2
The effect of exercise and dietary intervention on endothelial function.

	Baseline	Endline	P
FMD (%)	7.28 ± 0.46	8.33 ± 0.42	0.023
eNOS (IU/ml)	150.00 ± 33.65	530.81 ± 63.03	<0.001
CRP (μg/ml)	2.50 ± 0.41	1.36 ± 0.39	0.009
VEGF (pg/ml)	27.57 ± 3.91	42.96 ± 6.64	0.030
EPC (%)	0.0282 ± 0.0045	0.0647 ± 0.0116	0.011

Note: Data are mean ± SEM. FMD, brachial artery diameter; eNOS, endothelial nitric oxide synthase; CRP, C-reaction protein; VEGF, vascular endothelial growth factor; EPC, endothelial progenitor cells.

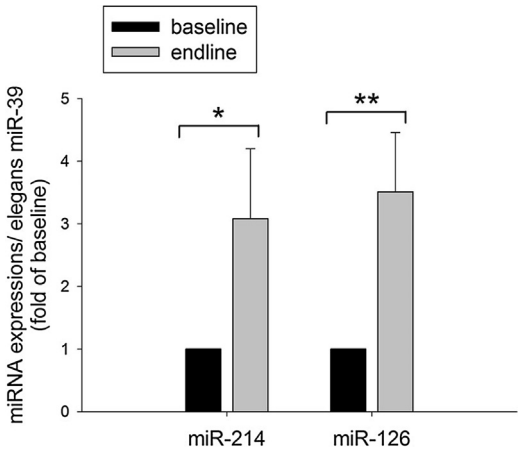

Fig. 1. The effect of exercise and dietary intervention on miR-214 and miR-126 expressions determined by Quantitative real-time PCR.

Table 3
Correlation with relative changes of miR-214 and miR-126.

	miR-214		miR-126	
	P	*r*	*P*	*r*
Cholesterol	0.823	0.061	0.621	0.129
Triglycerides	0.286	0.284	0.614	−0.132
HDL-L	0.986	−0.005	0.534	0.162
LDL-L	0.824	0.061	0.329	0.252
FPG	0.028	0.549	0.993	−0.002
FINS	0.169	0.361	0.396	−0.220
HOMA-IR	0.182	0.351	0.441	−0.200
FMD	0.929	0.024	0.912	0.029
eNOS	0.611	−0.138	0.038	0.433
CRP	0.520	0.174	0.336	0.248
VEGF	0.143	−0.383	0.798	0.067
EPC	0.025	0.589	0.634	−0.124

Note: HDL-L, high-density lipoprotein cholesterol; LDL-L, low-density lipoprotein cholesterol; FPG, fasting plasma glucose; FINS, fasting serum insulin; HOMA-IR, homeostasis model assessment estimated insulin resistance; FMD, brachial artery diameter; eNOS, endothelial nitric oxide synthase; CRP, C-reaction protein; VEGF, vascular endothelial growth factor; EPC, endothelial progenitor cells. Relative change was calculated as endline-baseline.

Discussion

In this study, for the first time we observed the changes of circulating miR-214 and miR-126 in obese adults after exercise combined with dietary interventions, which were associated with selected endothelial biochemicals. Human studies have showed that obesity is independently associated with endothelial dysfunction as reviewed by Avogaro and de Kreutzenberg (2005), and exercise combined with dietary intervention are important therapy for restoring endothelial physiology (Hamdy et al., 2003). Consistent with previous studies, 2 months of the camp program of the current research obviously attenuated the condition of obesity as seen in both anthropometric and metabolic parameters. Meanwhile, the selected endothelium-related function indicators: FMD, eNOS, CRP, VEGF, and EPC, were all significantly altered; the unpublished results from our research team have also showed details in altered endothelium function after the intervention. Taking together, endothelial functions of the obese subjects were improved by exercise and caloric restriction.

Even though great attention have been paid on lifestyle modification on endothelial dysfunction induced by obesity, little is known regarding how alterations of miRNAs affect or correlate with the biomarkers or molecules in animal or even human. miRNAs are functionally responsible for a variety of endothelium-dependent vascular functions in obesity and other metabolic diseases (Ortega et al., 2013; Shilo et al., 2008); those non-coding RNAs influence gene expression in a rapid means instead of being translated into protein (Hansen et al., 2013; Memczak et al., 2013). For the current research, miR-214 and miR-126 were chosen and assessed because of their importance in regulating endothelium related vascular function including cell proliferation, apoptosis, and inflammation. In addition, the secretion of these two miRNAs in circulating has been proved to be stable and accurate, which are effective candidates in revealing endothelial dysfunction in diseases (Heishima et al., 2015).

miR-214 is highly expressed in endothelial cells, and implicated in several regulation including angiogenesis through targeting Quaking (van Mil et al., 2012), cell proliferation through Phosphatase and Tensin Homolog (PTEN) (Zhang et al., 2010), and apoptosis through c-Jun NH2-terminal kinase (JNK1) (Yang et al., 2009). Most studies found increased expression of miR-214 in patients with vascular diseases, but the effects of miR-214 vary in different cell types. For example, in heart failure, miR-214 reduces endothelial cells angiogenesis; the expression level of miR-214 from both circulation and regional myocardium were enhanced (Duan et al., 2015; Melo et al., 2015). On the other hand, overexpression of miR-214 has also been reported protective in patients with myocardial ischemia and infarction (Liu et al., 2014; Wan et al., 2015; Wang et al., 2015). Given limited significant shift in FPG after intervention, the increase of miR-124 was moderately correlated with it, showing miR-214 might be sensitive to glycemic load during weight loss. Accordingly, under diabetic conditions, miR-214 responds to attenuate renal impairment through PTEN (Wang et al., 2016) and activating transcriptional factor 4 (ATF4) (Li et al., 2015).

In this research, circulating miR-214 was significantly enhanced by exercise and dietary intervention, and there was a moderate correlation existed between the relative changes of miR-214 and that of EPC. The number of EPCs indicated impaired EPC function, while the intervention acted to increase EPCs. The results imply that miR-214 might be implicated in the regulation of recovering angiogenesis from obesity, since EPCs are critical to endothelial maintenance and repair (Loomans et al., 2004). On the contrary, in the pathogenesis of coronary heart disease patients, miR-214 was able to suppress VEGF expression and EPC activities and predict severity of coronary lesions (Jin et al., 2015). These results collectively suggest that miR-214 may manifest variation in the same cell type with specific internal environment. Besides, miR-214 was closely related to eNOS in angiogenesis; through eNOS, miR-214 has roles on cell migration and tube formation in human vein endothelial cells (Chan et al., 2009). However, we did not observed a correlation between them in the circulation of obese subjects. This result may be influenced by different testing tissues, various physiological environments and also the limited sample size.

Furthermore, recent research (van Balkom et al., 2013) has identified that miR-214 in exosome controlled endothelial cell by mediating signalling between neighbouring target cells, thus preventing senescence and promoting blood vessel formation. Exosomal miR-214 is functional in stimulating vessel formation as proved *in vivo* and *in vitro* experiment (van Balkom et al., 2013); the current intervention increased endogenous miR-214 level, which indicates that exercise and caloric restriction could act to regulate miR-214 expression and positively influence cell signalling during the pathological process of obesity. On the other hand, exercise training reportedly normalized the upregulation of miR-214 in the myocardium of cardiac infarction mice (Melo et al., 2015). This could be explained by the contradictory effects of miR-214 for intracellular and extracellular functions on angiogenesis (Heishima et al., 2015); exercise combined with dietary constriction might serve as different modulator on circulating miR-214 in obese pathology.

miR-126 also expresses high in endothelial cells (ECs) and has an EC specificity, with great importance in cells' fate and function. miR-126 is closely associated with several aspects of vasculogenesis: inhibits cell apoptosis through Akt signalling via phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2) (Chen et al., 2016), and also participates in inflammation through targeting vascular cell adhesion molecule-1 (VCAM-1) (Harris et al., 2008), activated leucocyte cell adhesion molecule (ALCAM), and SetD5 protein (Poissonnier et al., 2014). By directly targeting Sprouty-related protein, Spred1, and PIK3R2, negative regulators of the vascular endothelial growth factor (VEGF) signalling pathway, miR-126 has a protective effect on vascular integrity and participates several biological functions of endothelial cell including cell migration, reorganization of the cytoskeleton, capillary network stability, and cell survival (Fish et al., 2008). The genes and signalling network controlled by miR-126 have emphasized its essential regulatory roles in ECs.

Down-regulation of circulating miR-126 were observed in most subjects with metabolic disorders and cardiovascular diseases including diabetes (Zampetaki et al., 2010), congestive heart failure (Fukushima et al., 2011), and atherosclerosis (Wei et al., 2013), which indicate that miR-126 released by ECs into circulation may provide with useful biomarkers for related human research. miR-126 in plasma and circulating blood cells has great positive influences on vascular function; the study of Jansen et al. (2013) has showed that miR-126, carried and mediated by endothelial micro particles, served as messengers taking regenerative signals to promote endothelial healing and thus determined repair effects on the target cells. Although miR-126 would be usually blunted when ECs are exposed to high glucose levels (Olivieri et al., 2014), the current increase of miR-126 in obesity by exercise and calorie restriction was not correlated with related metabolic parameters. The results might be due to the young age of our research subjects, who are not involved in major cardiovascular diseases or diabetic complications. Endothelial dysfunction might develop prior to glycaemia in the obese, and weight loss resulted from exercise and diet was accompanied by improved insulin resistance and endothelial function as demonstrated in the current study. The relative expression of miR-126 was associated with eNOS in obese subjects after intervention and might be involved in processes including angiogenesis and wound healing. Compared to a healthy endothelium, lower expression of eNOS represents reduced nitric oxide bioavailability and creates a negative environment for ECs. Even though the expression of eNOS gene was reportedly not affected by miR-126 overexpression in ECs (Fish et al., 2008), miR-126 was involved in both Ras/ERK/VEGF and PI3 K/Akt/eNOS signalling pathways to modulate EPCs (Meng et al., 2012). There are multiple functions of miR-126 on EPCs, but in the current research we did not explore further on this aspect.

Of note, Wang et al. (2008) revealed that miR-126 content was reduced in adipose tissue stem cell-derived extracellular vesicles of obese subjects compared to the normal, which might result in impaired angiogenic potential. Our study demonstrates that circulating miR-126 of obese subjects was enhanced by exercise and dietary intervention, suggesting the training program restored the protective role of miR-126 for endothelium-related angiogenesis. In accord with the current results, the protective mechanism of miR-126 has been confirmed by other researches; for instance, Jakob et al. (2012) reported that reduced miR-126 was responsible

for the impaired capacity of cardiac neovascularization and function; artificial increased miR-126 restored endothelium function of angiogenic ability to healthy level (Potus et al., 2014). For short term exercise training, one bout of endurance exercise would induced an increased concentration of plasma miR-126 while resistance exercise has no such effect, as examined by the study of Uhlemann et al. (2014) Along with our research, these data indicate that exercise alone seems to be sufficient to induce augmentation of miR-126, and that long-term exercise could have an accumulating effect. Indeed, in skeletal muscle of hypertension, Fernandes et al. (2012) found that long-term exercise restored miR-126 level and promoted peripheral revascularization.

Conclusion

The endothelial related miR-214 and miR-126 could be enhanced by exercise combined with dietary intervention in obesity, and these molecular adaptations are associated with improved endothelial function during the process. These findings increase our understanding of the molecular mechanisms underlying lifestyle modifications on endothelial dysfunction during obesity.

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Conflict of interests

The authors declare no conflict of interest.

Ethical approval

All procedures in this study involving human participants were in accordance with the Declaration of Helsinki and were approved by the ethics committee of Guangzhou Sport University.

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