The effect of trehalose administration on the serum expression levels of microRNAs associated with lipid metabolism and the autophagy process in patients with myocardial infarction – post-hoc analysis of the IR-TREAT trial

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Abstract

Introduction: This study aimed to evaluate the trehalose (TRE)-induced changes in the serum expression levels of microRNAs (miRNAs) associated with lipid metabolism and the autophagy process in myocardial infarction (MI) patients to assess the potential protective effects of TRE in these patients.
Material and methods: This post hoc investigation was performed on serum samples obtained from a pilot randomized, double-blind, placebo-controlled clinical trial (IR-TREAT) that recruited 14 men (aged 18–80 years) with MI and systemic inflammation. The patients were randomized in a 2 : 1 ratio to either TRE (15 g/week, intravenous (IV) administration) (n = 10) or placebo groups (equal volume of saline 0.9%) (n = 4) for a period of 12 weeks. To measure the relative serum expression levels of miRNA-155 (macrophage function regulator), miRNA-221 (autophagy regulator), and miRNA33a (regulator of macrophage autophagy and cholesterol efflux pathway), the SYBR Green quantitative polymerase chain reaction (qPCR) method was used.
Results: miRNA-155 showed significantly higher serum expression levels in the TRE group (2.772 ±0.73; p = 0.009) when compared to the placebo group. Also, significant reductions in miRNA-155 (0.171 ±0.03; p = 0.016), miRNA-221

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(0.116 ±0.07; $p = 0.013$), and miRNA-33a (0.076 ±0.07; $p = 0.025$) were observed in the placebo group at the end of the study. Nevertheless, the reduction (normalized to the baseline) of the serum expression levels of miRNA-221 (fold change (FC): 0.87 ±0.20 vs. FC: 0.18 ±0.08; $p = 0.009$) and miRNA-33a (FC: 0.73 ±0.22 vs. FC: 0.13 ±0.08; $p = 0.025$) were significantly lower in TRE group than in the placebo group.

Conclusions: Intravenous trehalose administration did not reduce the expression of miRNA-221 and miRNA-33a as much as placebo. Keeping a steady state of the serum expression levels of these miRNAs associated with lipoproteins metabolism and autophagy in the TRE group might have protective effects in patients with MI.

Key words: trehalose, microRNA, myocardial infarction, autophagy, lipid metabolism.

Introduction

Myocardial infarction (MI) is the most severe consequence of atherosclerotic coronary artery disease (ACAD) and the cause of more than 7 million deaths every year [1, 2]. There is a close relationship between impaired lipid metabolism, autophagy [3], and inflammatory pathways that make all of them important targets for therapy with the aim of preventing the development of coronary atherosclerotic plaques and the formation of thrombus on ruptured plaques causing adverse clinical events [4]. For instance, therapeutic approaches could be directed to stabilize vulnerable, rupture-prone plaques by selective induction of macrophage autophagic death [5].

A growing body of evidence suggests that non-coding RNAs, particularly microRNAs (miRNAs), are the key regulators of atherosclerotic plaque progression [6–8]. miRNAs regulate post-transcriptionally the expression of genes involved in plaque initiation, progression, and rupture [9]. For instance, miRNA-155 regulates the inflammatory response of macrophages during atherogenesis [10]. miRNA-33a also plays a beneficial role in the development of atherosclerotic plaques by regulating ATP binding cassette transporter A1 (ABCA1) and ABCG1 genes which participate in the process of cholesterol efflux to high-density lipoprotein (HDL) particles from macrophages overloaded with cholesterol [9]. It seems that miRNA-221, by modulating the p27/CDK2/mTOR axis, might be an important regulator of autophagy balance and cardiac remodeling, and it was suggested that it could also be a therapeutic target in heart failure [11]. Because microRNAs have stable expression in circulation, they might be biomarkers in the prognosis of atherosclerotic cardiovascular disease (ACVD) and diagnosis of some other heart diseases and could be useful in the follow-up of the efficacy of treatment strategies [12].

Recently, there has been a trend toward the use of natural products for the management of dyslipidaemia [13–15] as adjuncts to standard treatments [16]. Trehalose (TRE, $C_{12}H_{22}O_{11}$) is a natural, non-reducing disaccharide with an α,α-1,1-glycosidic link between 2 glucose units that inhibits the destruction of biological molecules against environmental stresses [17]. Evidence suggests that TRE protects cells against a wide range of stresses. TRE also accumulates rapidly in lower organisms like yeasts and tardigrades, making them resistant to dehydration, oxidative stress, heat shock, and protein aggregation [18]. Mammalian cells can also benefit from TRE as a stress-reducing mechanism. As a result of exposure to TRE, cells can retain more water during stress, protecting intracellular organelles from disruption by hydration/dehydration cycles [19]. Additionally, TRE reduces the accumulation of misfolded proteins and intracellular protein aggregates [20]. It has been shown that TRE reduces the accumulation of ubiquitinated proteins, can reduce skeletal muscle denervation, protect mitochondria, and (taken in general) it can be neuroprotective in the amyotrophic lateral sclerosis (ALS) mouse model, suggesting that it might be a therapeutic target for the treatment of ALS and several other types of neurodegenerative disease [21]. TRE also reduces hepatic steatosis by removing intracellular lipid droplets from hepatocytes [22]. However, it is not known what effect TRE administration might have during MI. Several preclinical studies have shown that TRE could be a promising therapeutic agent with pleiotropic effects including anti-inflammation and autophagy-regulating activities [23–25]. TRE can achieve an anti-inflammatory response by induction of autophagy in the lysosomal-mediated transcription factor EB (TFEB) activation and mTOR-independent pathway [26, 27]. The promising anti-atherogenic benefits of TRE have consistently been shown in various pro-atherogenic animal models [23, 25], substantiating its potential to improve MI-induced complications in patients with MI.

Therefore, this *post hoc* pilot study, for the first time, aimed to explore the potential protective effects of TRE in MI patients by tracking the miRNA expression levels involved in lipid metabolism and autophagy pathways.

Material and methods

Study population

This study was performed as a *post hoc* investigation of our previously published trial [28]. The

study population and selection criteria were defined previously [28]. Briefly, 15 men (aged 18–80 years) with a history of MI and percutaneous coronary intervention (PCI) > 90 days before study, as well as having evidence of inflammation, defined as an hs-CRP > 2 mg/l, were enrolled. Patients were randomized in a 2 : 1 ratio to either trehalose (15 g/week, intravenous (IV) administration) or placebo groups (equal volume normal saline 0.9%) for a period of 12 weeks. Fasting blood samples were drawn from all participants at baseline and at the end of the trial. The serum was separated and then stored at –80°C before analysis. Biochemical parameters were also measured using commercial kits. All participants gave written informed consent, and the Institutional Ethics Committee of Mashhad University of Medical Sciences approved the study protocol. The trial – Inflammation Reduction by TREhalose AdminisTration (IR-TREAT) – is registered on ClinicalTrials.gov (NCT03700424).

Serum miRNA extraction and cDNA synthesis

The method of serum miRNA extraction, cDNA synthesis, and qRT-PCR were explained in our previously published article [29]. Briefly, 300 µl of serum samples were used for total RNA extraction using BIOzol RNA lysis buffer (BN-0011.33, Bonyakhteh, Tehran, Iran) according to the manufacturer's protocol, with some modifications including increasing the time of centrifugation, as well as incubation, to the efficiency of extraction. After measuring the quality and quantity of the extracted RNAs, about 5 µg of total RNA with an absorbance of 1.8–2 at 260/280 nm was used for the initial polyadenylation step of complementary DNA (cDNA) synthesis, using a BONmiR High Sensitivity MicroRNA 1st Strand cDNA Synthesis kit (BN-0011.17.2, Bonyakhteh, Tehran, Iran). The universal cDNA synthesis was completed with 10 min at 25°C, 60 min at 42°C, and 10 min at 70°C. At the final step, the relative serum expression levels of miRNA-155, miRNA-221, and miRNA-33 were measured by the SYBR Green qPCR method using a specific forward primer for each miRNA (Bonyakhteh company, Tehran, Iran) (Table I) and BON microRNA 2× QPCR Master mix (BN-0011.17.4, Tehran, Iran). The program was as follows: 2 min at 95°C followed by 45 cycles at 95°C for 5 s and at 60°C for 30 s, and all reactions were performed in duplicate. Finally, the comparative ($2^{-\Delta\Delta CT}$) method was performed for the expression levels of the miRNAs of interest (Table I).

Statistical analysis

SPSS software, version 11.5 (Chicago, IL, USA) was used for statistical analysis. All variables were normally distributed and presented as mean ± standard error (SE). The paired-samples *t*-test and independent samples *t*-test were used for within-group and between-group comparisons, respectively.

Relative expression software tool (REST) was used to analyse the miRNA-155, miRNA-221, and miRNA-33-fold changes in expression levels to compare after-treatment expression levels in relation to those before treatment as well as those before and after TRE treatment in relation to those before and after in the placebo group, respectively.

Table I. Forward primer sequences of miRNA-155, miRNA-221, and miRNA-33

miRNAs	Sequences
miRNA-155	5´-CCGTTAATGCTAATCG- 3´
miRNA-221	5´-AGCCGAGCTACATTGTC- 3´
miRNA-33a	5´-GCATTGTAGTCGCATT- 3´
U6 snRNA	5´-AAGGATGACACGCAAAT- 3´

Results

Fourteen patients were considered in the final analysis and were classified into TRE (n = 10) and placebo (n = 4) groups. Figure 1 presents the flowchart of the study.

Baseline comparison of biochemical factors in the studied groups

According to Table II, lipid profile, renal function biomarkers, liver enzymes, and high-sensitivity C-reactive protein (hs-CRP) were not statistically different between the 2 studied groups at baseline.

Changes in the serum miRNAs expression levels related to the studied groups

The results showed that the serum expression levels of miRNA-155 (Figure 2 A), miRNA-221 (Figure 2 B), and miRNA-33a (Figure 2 C) were not statistically different between the studied groups at baseline, although, at the end of the study, miRNA-155 showed significantly higher serum expression levels in the TRE group (2.772 ±0.73; p = 0.009) when compared with the placebo group (Figure 2 A). In addition, the results showed that IV TRE administration could not significantly change serum miRNAs expression levels. However, the significant reduction in miRNA-155 (0.171 ±0.03; p = 0.016) (Figure 3 A), miRNA-221 (0.116 ±0.07; p = 0.013) (Figure 3 B), and miRNA-33a (0.076 ±0.07; p = 0.025) (Figure 3 C) were observed in the placebo group at the end of the study. Nevertheless, the reduction (normalized to the baseline) of the serum expression levels of miRNA-221 (FC: 0.87 ±0.20 vs. FC: 0.18 ±0.08; p = 0.009) and

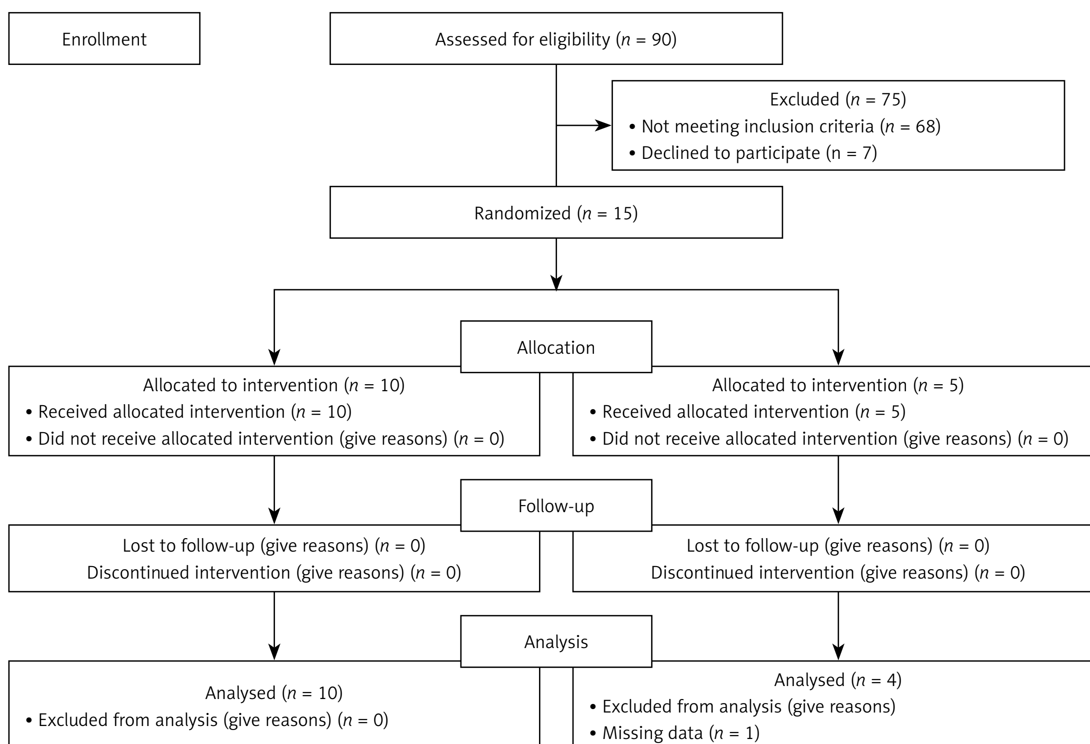

Figure 1. The flowchart of the study

miRNA-33a (FC: 0.73 ±0.22 vs. FC: 0.13 ±0.08; p = 0.025) were significantly lower in the TRE group than in the placebo group. The expression level of miRNA-155 also showed borderline lower changes in the TRE group than in the placebo group although they were not statistically significant (1.35 ±0.34 vs. 0.19 ±0.04; p = 0.056).

Discussion

Previous studies have shown that miRNA levels are up- or down-regulated according to the disease status. They are also used as biomarkers to track whether certain drug changes the course of the disease [30]. Furthermore, there are data suggesting the existence of the crosstalk between lipid metabolism and autophagy pathway in CVD [3]. Since some studies indicated that the beneficial effects of TRE could be mediated by the regulation of lipid metabolism and activation of autophagy [31, 32] as two important pathophysiological pathways involved in MI, this *post hoc* pilot study for the first time explored the effects of TRE

Table II. Biochemical factors at the baseline of the study

Parameter	TRE (n = 10)	Placebo (n = 4)	P-value
TG [mg/dl]	112.10 ±21.93	101.25 ±39.14	0.802
Cholesterol [mg/dl]	106.70 ±10.00	118.50 ±24.54	0.597
HDL-C [mg/dl]	32.40 ±1.61	33.50 ±3.00	0.733
LDL-C [mg/dl]	60.20 ±8.90	70.25 ±17.73	0.582
Urea [mg/dl]	30.40 ±2.00	30.75 ±7.20	0.949
Cr [mg/dl]	1.12 ±0.10	1.28 ±0.13	0.273
AST [U/l]	29.00 ±2.74	34.00 ±1.91	0.296
ALT [U/l]	19.4 ±2.32	27.50 ±3.00	0.074
ALP [U/l]	228.50 ±22.34	182.00 ±11.25	0.088
Bill T [mg/dl]	0.50 ±0.10	0.95 ±0.32	0.251
Bill D [mg/dl]	0.22 ±0.10	0.50 ±0.17	0.267
Hs-CRP [mg/l]	7.73 ±1.20	10.40 ±2.20	0.272

Data are expressed as mean ± SE. AST – aspartate aminotransferase, ALT – alanine aminotransferase, ALP – alkaline aminotransferase, Bill T – bilirubin total, Bill D – bilirubin direct, Cr – creatinine, HDL-C – high-density lipoprotein cholesterol, Hs-CRP – high-sensitivity C-reactive protein, LDL-C – low-density lipoprotein cholesterol, TG – triglycerides.

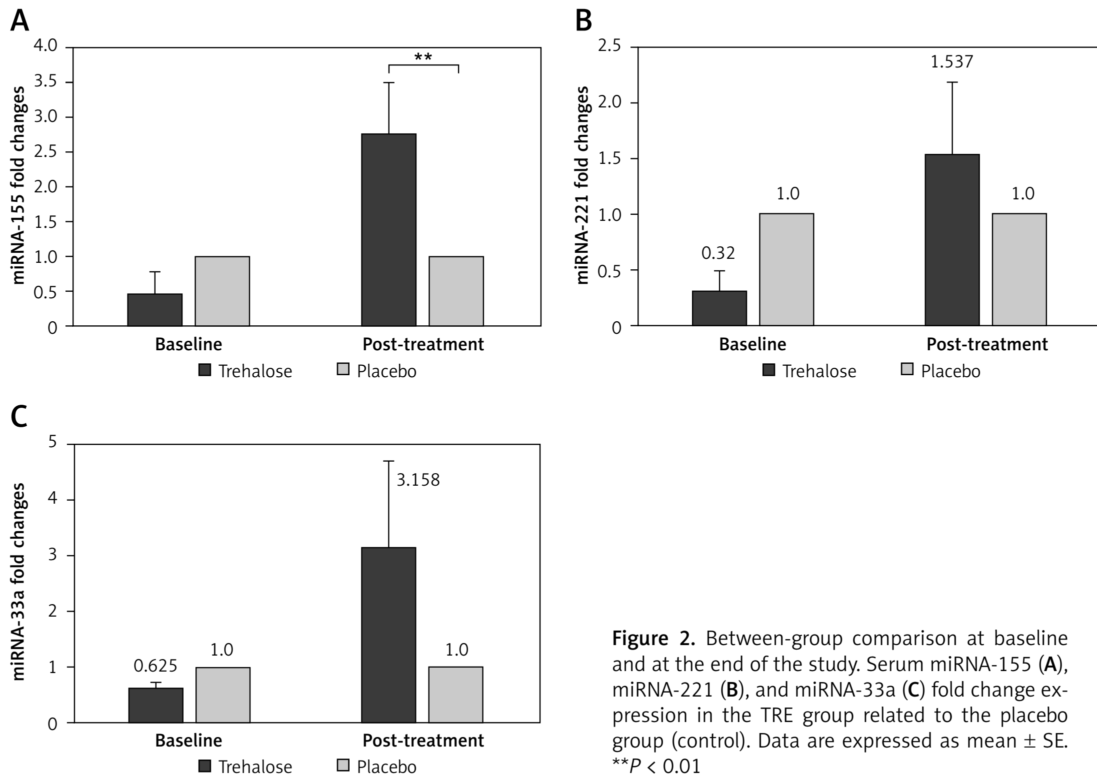

Figure 2. Between-group comparison at baseline and at the end of the study. Serum miRNA-155 (**A**), miRNA-221 (**B**), and miRNA-33a (**C**) fold change expression in the TRE group related to the placebo group (control). Data are expressed as mean ± SE. $^{**}P < 0.01$

in improving MI-induced complications by tracking the miRNAs expression levels involved in these pathways. It has been previously shown that TRE had a beneficial effect on miRNA expression levels involved in inflammatory pathways [29]. However, to the best of our knowledge, this was the first study to investigate the effect of TRE on the expression levels of miRNAs involved in the lipid metabolism and autophagy pathways (like miRNA-155, miRNA-221, and miRNA-33a) in patients with MI. The results showed that IV TRE administration could not significantly change the expression lev-

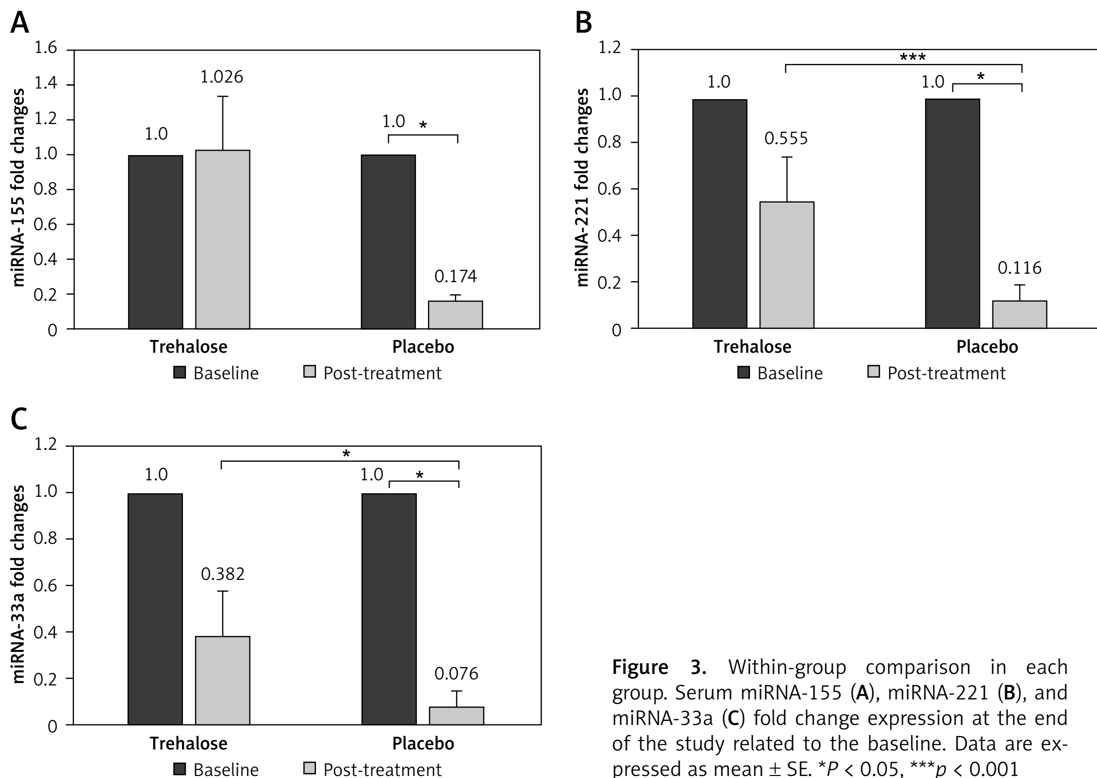

Figure 3. Within-group comparison in each group. Serum miRNA-155 (**A**), miRNA-221 (**B**), and miRNA-33a (**C**) fold change expression at the end of the study related to the baseline. Data are expressed as mean ± SE. $^{*}P < 0.05$, $^{***}p < 0.001$

els of serum miRNAs. However, the changes in the serum expression levels of miRNA-221 and miRNA-33a indicated significant differences between the TRE and placebo groups so that the reduction (normalized to the baseline) was significantly lower in the TRE group than in the placebo group although the expression level of miRNA-155 also showed less change in TRE group than in the placebo group. However, this was statistically not significant [33].

miRNA-155 is an oncogene or a tumour suppressor in different types of cancer [34]. Several studies have shown its pleiotropic effects in the regulation of cell homeostasis, and miRNA-155 is considered as a multifunctional molecule that modulates many pathophysiological pathways in immune reactions and inflammation, which all play an important role in atherosclerotic CVD. Conflictingly it can be either upregulated or downregulated in atherosclerosis, more precisely in patients with ACAD [35, 36]. It seems that miRNA-155 was specifically expressed in atherosclerotic plaques and proinflammatory macrophages [37]. Increased serum expression level of miRNA-155 was also shown in MI patients at high risk of cardiac death [38]. It was demonstrated that rosuvastatin therapy could suppress miRNA-155 serum expression levels in patients with acute coronary syndrome (ACS), who underwent PCI. This indicated an association with lower inflammatory cytokine expression in these patients. However, at the end of this study, the serum expression level of miRNA-155 was considerably higher in the TRE group than in the placebo group. This might be due to a stronger reduction of miRNA-155 serum expression level in the placebo group. Nevertheless, the changes in the serum expression level of miRNA-155 were not statistically different between the studied groups. It must be mentioned that downregulation of miRNA-155 was shown in some studies. For instance, it was shown in patients with ACS [39], in infarcted heart tissue from MI patients with ventricular rupture [40], in coronary arteries of patients who died because of ACS [41], and in patients with ACAD when compared with controls [42]. One study suggested that increased miRNA-155 via targeting of calcium-regulated heat stable protein 1 (CARHSP1) played a protective role during atherosclerosis-associated foam cell formation [35]. miRNA-155 was also reported to promote cholesterol efflux from macrophages via the KEGG pathway, and it played an important role in the inhibition of foam cell formation [43]. It seems that increased miRNA-155 by targeting mitogen-activated protein kinase 10 (MAP3K10) could contribute to the prevention of atherogenesis [44]. Also, it has been reported that overexpression of miR-155 improved autophagic activity in HUVECs [45]. Therefore, a significant reduction of the serum level of miRNA-155 in the placebo group at the end of the study related to the baseline levels might indicate a worse prognosis in this group when compared with the TRE group.

This study also showed a significantly decreased serum level of miRNA-221 in the placebo group at the end of the study when compared with the baseline, and this reduction was significantly stronger than the one found in the TRE group. Previously it was reported that miRNA-221 could induce cardiomyocyte hypertrophy *in vitro* [46] and that *in vivo* overexpression resulted in cardiac dysfunction and heart failure due to inhibition of cardiomyocyte autophagy accompanied by mTOR activation [11]. In the present study, the serum expression level of miRNA-221 was reduced by TRE treatment, but this was not statistically significant. On the other hand, it was reported that miRNA-221 by inhibition of NLRP3/ASC/pro-caspase-1 inflammasome pathway has an anti-inflammatory effect in ACAD [47]. In this study, higher serum levels of miRNA-221 in the TRE group when compared to the placebo group might show that the protective effect of TRE in MI patients can be mediated by pathways other than autophagy regulation.

miRNA-33a is another key miRNA that is involved in lipid phagocytosis and metabolism. miRNA-33a potentially affects the growth of atherosclerotic plaques by reducing macrophage autophagy via downregulation of crucial transcriptional activators such as forkhead box protein O3 (FOXO3) and TFEB, as well as ABCA1, which result in reduced macrophage lysosomal activity and cholesterol efflux, respectively [48–51]. The results of this study show that IV TRE administration cannot significantly reduce miRNA-33a serum expression levels. A greater decrease in miRNA-33a serum levels was observed in the placebo group than in the TRE group. Although it suggests that the beneficial effects of TRE might be mediated by autophagy activation [51], the results of this study not only fail to show TRE-induced reduction of miRNA-33a in the TRE group but also that increased miRNA-33a levels in TRE group might induce macrophage autophagy leading to the protective effect of TRE in patients with MI.

One of the explanations of why TRE did not change the expression levels of target miRNAs might be the dosing schedule chosen for the administration of trehalose. In fact, the optimal dosage for trehalose administration in humans is not yet known, and based on a previous animal study [25] an intravenous dose of 350 mg/kg 3 times a week, equivalent to a total weekly dose of approximately 75 g in an average-sized adult, should have been used, while due to the burden of a parenteral infusion on multiple days every week, we adopted the

dosage schedule of 15 g once a week, which may have decreased the potential effects of TRE.

The main limitation of this pilot study is that it was performed as a *post hoc* investigation and the small sample size (similarly to many first-in-human studies) was not *a priori* estimated for the present analysis on miRNAs. However, we were still able to find significant changes in the levels of miRNAs between the study groups, which opens possibilities for future studies to explore primarily the regulatory role of TRE intervention in modulating selected biologically important miRNAs.

In conclusion, the results of this study showed that although TRE did not reduce the serum expression of miRNA-221 and miRNA33a as much as placebo in patients with MI, maintaining a steady state of the serum expression levels of these miRNAs associated with lipid metabolism and autophagy pathway following TRE administration might reflect its beneficial effects in these patients. This clinical finding can open windows as to the potential value of TRE in interfering with autophagy and lipid metabolism pathways in humans. However, additional studies on more patients and simultaneous assessment of additional inflammatory and autophagy biomarkers are necessary to validate these findings and to confirm the potential clinical implications of using these miRNAs as biomarkers for monitoring the efficacy of TRE treatment in patients with MI.

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Conflict of interest

The authors declare no conflict of interest.

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