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Material and methods
Methods: This trial enrolled 14 men with a history of myocardial infarction (MI) and systemic inflammation. The patients were randomized in a 2:1 ratio to trehalose (15g/week, IV administration) (N=10) or placebo (equal volume 0.9% normal saline) (N=4) for a period of 12-weeks. The relative serum expression levels of miRNA-126, miRNA-24, miRNA-181b, miRNA-10a and miRNA-92a were assessed.

Results
Results: IV trehalose administration significantly increased the serum level of miRNA-24 (2.473±0.72; P=0.037) compared to the baseline, but did not alter the other miRNA serum levels. However, at the end of the study, miRNA-24 (4.58±0.99; P=0.002), miRNA-181b (4.08±1.75; P=0.009) and miRNA-10a (3.68±0.63; P=0.013) showed notably higher serum levels in the trehalose relative to the placebo group. Furthermore, the reduction (normalized to baseline) in serum levels of miRNA-126 (P=0.042) and miRNA-92a (P=0.001) were reduced in the trehalose versus placebo group, while the serum level of miRNA-24 (P=0.007) was notably higher than that in the placebo group.

Conclusions
Conclusion: Serum levels of miRNAs associated with vascular inflammation were altered following IV trehalose administration. The alterations in serum miRNAs, especially miRNA-126 and miRNA-24, could be considered as helpful biomarkers for the evaluation of trehalose potency in reducing arterial wall inflammation in patients with CAD.
Trehalose-induced alterations in serum expression levels of microRNAs associated with vascular inflammation in patients with coronary artery disease – the pilot results from the randomized controlled trial

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Conclusion: Serum levels of miRNAs associated with vascular inflammation were altered following IV trehalose administration. The alterations in serum miRNAs, especially miRNA-126 and miRNA-24, could be considered as helpful biomarkers for the evaluation of trehalose potency in reducing arterial wall inflammation in patients with CAD.

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Introduction

Coronary artery disease (CAD) and myocardial infarction (MI) are the most common cardiovascular diseases (CVDs) and remain the leading causes of mortality worldwide (1-3). CAD occurs as a consequence of atherosclerosis, a chronic inflammatory response in the arterial wall. Various cells, proteins, and inflammatory mediators play important roles in the pathogenesis of atherosclerosis (4-6). The assessment of circulatory lipid levels, especially low-density lipoprotein cholesterol (LDL-C), and the adjustment of its levels is the first step in the management of atherosclerotic cardiovascular disease (ASCVD) (3, 7). However, the reduction in mortality following lipid-lowering therapies such as statins is not only due to their cholesterol-lowering effects but also to their anti-inflammatory effects, one key mechanism of action of statins (8-10). As inflammation plays a principal role in the development of atherosclerosis, there is a need for anti-inflammatory drug therapy to effectively reduce the risk of atherogenesis-related complications.

Trehalose (C\textsubscript{12}H\textsubscript{22}O\textsubscript{11}) is a natural non-reducing sugar with an α,α-1,1-glycosidic linkage between two glucose units that prevents the destruction of biological molecules against environmental stresses (11, 12). The positive anti-oxidant and anti-inflammatory effects of trehalose have been shown in cellular and preclinical studies, suggesting the therapeutic capacity of this natural disaccharide against a variety of diseases (13-22). Trehalose can exhibit anti-inflammatory responses through NF-κB pathway inhibition (23) as well as induction of autophagy via lysosomal-mediated TFEB activation and an mTOR-independent pathway (24-27). Although alterations in sensitive indicators of inflammation, such as serum concentrations of cytokines and acute-phase proteins (APPs) are common to virtually all inflammatory responses, these traditional markers generally lack the specificity to identify the exact pathologic events occurring in the arterial wall during development of ASCVD (28).

MicroRNAs (miRNAs) are conserved endogenous short (~18-22 nucleotides) single-stranded non-coding RNA molecules found in a wide variety of organs, cells and body fluids and can regulate the gene expression at the post-transcriptional level (29). Dysregulation of miRNA expression has been linked to different pathophysiological conditions such as inflammation (30, 31). Therefore, circulating miRNAs have been considered as potential biomarkers for the prognosis and diagnosis of certain diseases, as well as a mechanism by which to track the efficacy of treatment strategies (29, 32-39). MiRNAs have been reported to affect vascular smooth muscle cell (VSMC) and inflammatory cell function, disrupting endothelial integrity and cholesterol homeostasis, factors involved in the onset of vascular inflammation and subsequent development of atherosclerotic plaque (40). For instance, miRNA-92a (41-44) and miRNA-126 (45) are highly expressed in endothelial cells (ECs) (46) and, through the regulation of adhesion molecule expression and pro-inflammatory cytokine production, play a role in vascular inflammation and the progression of atherosclerosis. It has also been demonstrated that miRNA-181b (47, 48) and miRNA-10a (49), by affecting the nuclear factor kappa B (NF-κB) pathway and the inflammatory process, may be involved in the pathogenesis of atherosclerosis.
Therefore, this study was designed to investigate trehalose-induced alterations in the serum levels of miRNAs known to be associated with vascular inflammation in patients with CAD in order to evaluate the effectiveness of IV trehalose administration in reducing arterial wall inflammation.

Material and Methods

Study population
This randomized, placebo-controlled, double-blind clinical trial enrolled 15 men (aged 18-80) with a history of MI and percutaneous coronary intervention (PCI)>90 days before study, as well as having evidence of inflammation, defined as a highly sensitive C-reactive protein (hs-CRP) >2 mg/l. The inclusion criteria were ST deviation, raised troponin and cardiac catheterization. Patients with impaired renal function (creatinine >3.0 mg/dL), diabetes, active hepatitis or severe hepatic dysfunction, active cancer, on immunosuppressive therapy, with active infectious or febrile disease and recipients of transplantation were excluded from the trial. Using computer-generated random numbers, patients were randomized in a 2:1 ratio to either trehalose (15 g/week, intravenous (IV) administration) or placebo (equal volume 0.9% normal saline) for a period of 12 weeks. All infusions were conducted by a trained nurse in the presence of a specialist physician over a 90-minute period. All participants provided written informed consent and the ethics committee of Mashhad University of Medical Sciences approved the study protocol and the study was conducted in Ghaem Educational, Research and Treatment Center, Mashhad, Iran. The trial was registered on ClinicalTrials.gov (NCT03700424) on October 9, 2018. Fasting blood samples were drawn from all participants. Serum was separated by centrifugation for 20 min at a relative centrifugal force (RCF) of 1000 and then stored at –80°C prior to analysis. Routine biochemical factors were also measured in the samples using commercial kits.

Serum miRNA extraction and cDNA synthesis
For evaluation of miRNA expression, total RNA was extracted from 300 μl of serum samples using BIOzol RNA lysis buffer (BN-0011.33, Bonyakhteh, Tehran, Iran) according to the manufacturer’s protocol with some modifications, such that, the time of centrifugation as well as incubation was increased to obtain the highest content of miRNAs in the samples. The quantity and quality of the extracted RNAs were evaluated by NanoDrop 2000 (Thermo, Wilmington, DE, USA).

Complementary DNA (cDNA) was synthesized by BONmiR High Sensitivity MicroRNA 1st Strand cDNA Synthesis kit (BN-0011.17.2, Bonyakhteh, Tehran, Iran) according to the manufacturer’s instructions. About 5 μg of total RNA with absorbance of 1.8-2 at 260/280 nm was used for the initial polyadenylation step and followed by using RT Stem-loop primer designed by Bonyakhteh company which was available in the kit; the universal cDNA synthesis was completed via the thermocycler device for 10min at 25°C, 60min at 42°C, and 10min at 70°C. Synthetized cDNA was stored at −20°C for future quantitative real-time PCR (qRT-PCR) analysis.

qRT-PCR
In order to measure the relative serum expression of miRNA-126, miRNA-24, miRNA-181b, miRNA-10a and miRNA-92a, the SYBR Green qPCR method was run in a Light Cycler 96
instrument (Roche Diagnostics, Mannheim, Germany) using a specific forward primer for each miRNA (Designed by Bonyakhteh company, Tehran, Iran) (Table 1) and BON microRNA 2x QPCR Master mix (BN-0011.17.4, Tehran, Iran) according to the following program: 2min at 95°C followed by 45 cycles at 95°C for 5s and at 60°C for 30s. All reactions were performed in duplicate. The expression levels of the miRNAs of interest were calculated using the Ct (Cycle threshold) value and quantified by the comparative (2\(^{-\Delta\Delta Ct}\)) method and normalized to U6 small nuclear RNA (U6snRNA) expression as an internal control.

**Statistical analysis**

All analyses were performed using SPSS software, version 11.5 (Chicago, IL, USA). P values less than 0.05 were considered statistically significant. Variables had normal distribution and were presented as mean ± standard error (SE). Within-group comparisons were performed using a paired samples t test. Between-group comparisons were performed using an independent samples t test. The relative expression software tool (REST) was used to analyze the miRNA-126, miRNA-24, miRNA-181b, miRNA-10a and miRNA-92a expression level changes for comparing relative ‘after treatment’ levels to ‘before treatment’ as well as before and after trehalose treatment relative to before and after placebo group, respectively. In addition, an independent samples t-test was applied for expression change comparison between groups.

*Table 1*: Sequence of forward primers used to evaluate the expression of miRNA-126, miRNA-24, miRNA-181b, miRNA-10a and miRNA-92a.

| miRNAs     | Sequences                        |
|------------|----------------------------------|
| miRNA-126  | 5´-GCGTCGTACCGTGAGT-3´           |
| miRNA-24   | 5´-ACATGGGCTCAGTTCAG-3´          |
| miRNA-181b | 5´-GGGCAACATTCAACGCTG-3´         |
| miRNA-10a  | 5´-ACCCTGTAGATACGAATTTG-3´       |
| miRNA-92a  | 5´-GGTTGGGATGGGTTG-3´            |
| U6 snRNA   | 5´-AAGGATGACACGCAAAT-3´          |

**Results**

Of the 15 patients, 14 were considered in final analysis and one patient did not due to the missing data. patients were categorized into trehalose (N=10) and placebo (N=4) groups. Figure 1 presents the flowchart of study.
Baseline comparison of biochemical factors in the studied groups

As are detailed in Table 2, the biochemical parameters analyzed included lipid profile, liver enzymes (alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT)), renal function tests (urea, creatinine, bilirubin total and direct) and hs-CRP, none of which were statistically different between trehalose and placebo groups at baseline. Following IV trehalose administration, increases in both HDL-C (mg/dL) (35.7±1.9 vs 32.4±1.6; final vs baseline, P=0.005) and LDL-C (mg/dL) (77.6±11.3 vs 60.2±8.9; final vs baseline, P=0.042) were found, as well as a decrease in aspartate transaminase (AST) level (23.3±2.8 vs 29.0±2.7; final vs baseline, P=0.039) at the end of the study compared to baseline. Other
biochemical parameters showed no significant changes in either the trehalose or placebo group. In addition, except for alanine aminotransferase (ALT) level (U/L) which changes showed a significant difference between the trehalose and placebo group (3.80±3.14 vs -10.25±5.10; P=0.035), there were no significant differences in the changes of other biochemical parameters between the groups.

Table 2. Baseline comparison of biochemical factors in the studied groups.

|                  | Trehalose (N=10) | Placebo (N=4) | P-value |
|------------------|------------------|---------------|---------|
| TG (mg/dL)       | 112.1±21.9       | 101.3±39.1    | 0.802   |
| Cholesterol (mg/dL) | 106.7±10.0       | 118.5±24.5    | 0.597   |
| HDL-C (mg/dL)    | 32.4±1.6         | 33.5±3.0      | 0.733   |
| LDL-C (mg/dL)    | 60.2±8.9         | 70.3±17.7     | 0.582   |
| Urea (mg/dL)     | 30.4±2.0         | 30.8±7.2      | 0.949   |
| Cr (mg/dL)       | 1.1±0.1          | 1.3±0.1       | 0.273   |
| AST (U/L)        | 29.0±2.7         | 34.0±1.9      | 0.296   |
| ALT (U/L)        | 19.4±2.3         | 27.5±3.0      | 0.074   |
| ALP (U/L)        | 228.5±22.3       | 182.0±11.3    | 0.088   |
| Bill T (mg/dL)   | 0.5±0.1          | 0.9±0.3       | 0.251   |
| Bill D (mg/dL)   | 0.2±0.1          | 0.5±0.2       | 0.267   |
| hs_CRP (mg/dL)   | 7.7±1.2          | 10.4±2.2      | 0.272   |

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

Changes in serum miRNA expression related to the studied groups

The results demonstrated that, with the exception of miRNA-10a (0.22±0.20; P=0.042) (Figure 2D) that showed a lower expression level in the trehalose relative to the placebo group, the serum expression levels of the other miRNAs, namely miRNA-126 (Figure 2A), miRNA-24 (Figure 2B), miRNA-181b (Figure 2C) and miRNA-92a (Figure 2E), were not different between groups at baseline. However, at the conclusion of the study, miRNA-24 (4.58±0.99; P=0.002) (Figure 2B), miRNA-181b (4.08±1.75; P=0.009) (Figure 2C) and miRNA-10a (3.68±0.63; P=0.013)
(Figure 2D) showed notably higher serum expression levels in the trehalose relative to the placebo group. In addition, the results demonstrated that IV trehalose administration significantly increased serum levels of miRNA-24 (2.47±0.72; P=0.037) (Figure 3B) relative to baseline, though none of the other miRNA levels were altered. A significant reduction in the serum level of miRNA-10a (0.12±0.15; P=0.028) (Figure 3D) was observed in the placebo group versus baseline.

Furthermore, the reduction relative to baseline of serum levels of miRNA-126 (P=0.042) (Figure 3A) and miRNA-92a (P=0.001) (Figure 3E) was significantly less in the trehalose than in the placebo group, while the alteration in serum levels of miRNA-24 (P=0.007) (Figure 3B) was notably higher in the trehalose relative to the placebo group. There were no significant changes in levels of miRNA-10a and miRNA-181b between the groups.

Figure 2: Serum miRNA-126 (A), miRNA-24 (B), miRNA-181b (C), miRNA-10a (D) and miRNA-92a (E) fold changes in the trehalose group relative to the placebo group, at baseline and at the conclusion of the study. Data are expressed as mean±SEM. *P<0.05, **P<0.01.
**Figure 3:** Serum miRNA-126 (A), miRNA-24 (B), miRNA-181b (C), miRNA-10a (D) and miRNA-92a (E) fold changes in the trehalose and placebo group at the conclusion of the study relative to baseline. Data are expressed as mean±SE. *: P<0.05, **: P<0.01, ***: P<0.001
Discussion

Several studies have indicated that trehalose exerts anti-atherosclerotic actions through its anti-inflammatory and anti-oxidant properties (50-53). IV trehalose administration has also been shown to alter serum levels of miRNAs associated with vascular inflammation and may effect reduction in inflammation in the arterial wall in patients with CAD. However, hs-CRP, as a nonspecific marker of inflammation, showed no significant changes in either the trehalose or the placebo groups indicating that this traditional biomarker is less effective in the evaluation of trehalose potency for reducing arterial wall inflammation in these patients. By contrast, miRNA-24 showed notably higher levels after trehalose treatment. In addition, the change in miRNA-24 level was interestingly higher than that found in placebo group. MiRNA-24 is highly expressed in ECs and, by targeting genes involved in the proliferation, apoptosis and inflammation pathways, plays a key role in the regulation of endothelial function (54-56). It has been suggested that miRNA-24, by targeting YKL-40 (an inflammatory glycoprotein involved in endothelial dysfunction) (57), may serve as a biomarker for predicting CHD patients, and reduction in the serum levels of miRNA-24 has been reported in these patients (58). Therefore, the higher level of miRNA-24 in the trehalose group in our study is indicative of the efficacy of treatment in CAD patients.

MiRNA-126 is another miRNA that is prominent in cardiac muscle and is significantly reduced in CAD patients (59). Although in this study the serum expression level of miRNA-126 was unchanged in the trehalose group, its reduction relative to baseline was significantly less and levels remained almost constant relative to the placebo group; this again is indicative of the efficacy of trehalose treatment in CAD patients.

Trehalose was also potent in maintaining the serum level of miRNA-92a, another endothelial miRNA, in these patients, so that its reduction relative to baseline was significantly less in the trehalose versus the placebo group. MiRNA-92a has been shown to contribute to the development of CVD through NF-κB and downstream inflammatory pathways (60) and its increased serum level has been reported in both stable CAD and acute coronary syndrome (60, 61), though this study failed to show a reduction in miRNA-92a levels in the trehalose group.

The serum levels of miRNA-181b were not different between groups at the baseline. While, miRNA-10a showed significant lower level in trehalose group than in placebo groups at the baseline. Nevertheless, at the conclusion of this study, serum levels of these miRNA were notably higher in the trehalose versus the placebo group. One study reported that miRNA-181 is upregulated in human atherosclerosis plaques and suggested an essential role for miRNA-181 in the development of atherosclerosis through regulation of endothelial dysfunction (62). However, another study reported lower levels of miRNA-181 in CAD patients (63). In addition, low levels of miRNA-10a were associated with the development of atherosclerosis (64). Thus, higher levels of miRNA-181b and miRNA-10a in the trehalose versus the placebo group are indicative of the efficacy of trehalose treatment in this study (65-69).

In conclusion, the serum levels of some miRNAs associated with vascular inflammatory processes were effectively changed after 12 weeks of IV trehalose administration. Therefore, these miRNAs,
especially miRNA-126 and miRNA-24, could be considered to be useful biomarkers for the evaluation of trehalose potency in reducing arterial wall inflammation in patients with CAD.
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