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EXPERIMENTAL STUDY

Can atypical response in endothelial dysfunction-related genes and microRNAs arise from low hydrogen peroxide exposure?

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ABSTRACT

OBJECTIVE: Vascular endothelium is a tissue in which several vasoactive substances are produced and secreted. Reactive oxygen species can cause endothelial dysfunction (ED). miRNAs can be implicated in the oxidative stress-related ED during vascular disease pathogeneses. Our aim is to investigate effect of H_2O_2-induced oxidative stress on expression levels of genes and miRNAs that are key players in ED.

METHODS: H_2O_2 effect on cell viability of human umbilical-vein endothelial cells (HUVEC) at 24-hour was measured with MTT. Low sub-cytotoxic H_2O_2 concentrations (25, 50 μM) were selected to analyze their oxidative stress-inducing capacities with MDA assay and their effects on EDN1, NOS3, VCAM1, SERPINE1, miR21, miR22, miR126, and miR146a levels with RT-qPCR.

RESULTS: Each tested H_2O_2 concentration reduced HUVEC cell viability. Fifty μM H_2O_2 augmented cellular MDA levels. Intriguingly, EDN1, VCAM1, and SERPINE1 and all analyzed miRNAs' levels attenuated upon H_2O_2 treatment whereas there was no change in NOS3 levels compared to control. There was a positive correlation between miR-21 and VCAM1.

CONCLUSION: Rather than individual alterations in analyzed parameters, consistent changes in our findings *i.e.*, parallel decreases in EDN1, VCAM1, SERPINE1 mRNA levels as well as miRNAs, suggests that H_2O_2 concentration-dependent modulation of expression patterns can bring about various impacts on ED *(Tab. 1, Fig. 5, Ref. 63). Text in PDF www.elis.sk*

KEY WORDS: oxidative stress, endothelial dysfunction, hydrogen peroxide (H_2O_2), microRNA, gene expression.

Introduction

Endothelial cells (ECs) cover the inner surface of blood vessels and act as a barrier between blood and tissue (1). ECs are not single type building blocks, and their function depends on the organ where they reside (2, 3). Vascular endothelium is not solely a barrier but also an important endocrine organ which secrets many vasoactive substances including vasodilatation factors e.g., nitric oxide (NO) and prostacyclin (PGI2) together with vasoconstrictors e.g., platelet activating factor (PAF) and endothelin-1 (EDN1) (3–5). By means of its endocrine activity, vascular endothelium regulates vascular wall tension, blood flow, inflammatory antagonism of vascular wall, and sustainment of angiogenesis (6-8). Endothelial dysfunction (ED) results in an imbalance between the synthesis of vasodilators like NO and vasoconstrictors like EDN1 (3, 9, 10). Furthermore, ED causes deregulation of the hemostasis between pro- and anti-oxidative, pro- and anti-inflammatory, and pro- and anti-thrombotic signals which can lead to upregulation of vascular cell adhesion molecule-1 (VCAM1) and intercellular adhesion molecule-1 (ICAM1), a plasminogen activator inhibitor-1 (PAI1) (11, 12). Vascular ED characterized with impaired endothelium-related vasodilatation, high levels of oxidative stress, chronic inflammation, leukocyte-endothelial cell adhesion, severe vascular leakage, and aged endothelial cell is considered as the distinctive feature of several cardiovascular diseases (CVD) (1, 3, 7, 8, 10) as well as atherosclerosis (13), hypertension (9), and diabetes (14). In aerobic organisms, the levels of cellular reactive oxygen species (ROS) and anti-oxidant defense system against ROS, are in a dynamic balance. The breakdown of the oxidative balance against anti-oxidant capacity is defined as oxidative stress (15). It was demonstrated in previous studies that the increment or insufficient clearance of ROS results in functional impairments of endothelium (13, 16, 17). It is the evident based on previous studies that oxidative stress has a major role in mediating cardiovascular pathogeneses, via direct or indirect signaling mechanisms, covering atherosclerosis, hypertension, hypercholesterolemia, cardiac insufficiency, and stroke (3).

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Tab. 1. Primers used in qPCR analyses of mRNA and miRNA coding genes.

Gene symbol	Primer sequence	Amplicon (bp)	Anneal, Cycle	Ref. Seq.
EDN1	F:5′-GACATCATTTGGGTCAACACTC-3′ R:5′-GGCATCTATTTTCACGGTCTGT-3′	121	60 °C, 40X	NM_001955.5
NOS3	F:5’-GTTTGTCTGCGGCGATGT-3’ R:5’-GTGCGTATGCGGCTTGTC-3’	192	60 °C, 40X	NM_000603.5
VCAM1	F:5′-TCTGGAAATGCAACTCTCACC-3′ R:5′-CAAAACTCACAGGGCTCAGG-3′	316	60 °C, 40X	NM_001078.4
SERPINE1	F:5′-GGCCATTACTACGACATCCTG-3′ R:5′-GGTCATGTTGCCTTTCCAGT-3′	150	60 °C, 40X	NM_001386460.1
GAPDH	F:5′-CTGGTAAAGTGGATATTGTTGCCAT-3′ R:5′-TGGAATCATATTGGAACATGTAAACC-3′	81	60 °C, 40X	NM_002046.7
	Gene Globe ID*			
hsa-miR21-5p	MS00009079		55 °C,40X	
hsa-miR22-5p	MS00009142		55 °C,40X	
hsa-miR126-5p	MS00006636		55 °C,40X	
hsa-miR146a-5p	MS00003535		55 °C,40X	

EDN1 – Endothelin 1, *NOS3* – Nitric oxide synthase 3, *VCAM1* – Vascular cell adhesion molecule 1, *SERPINE1* – Serpin family E member 1, *GAPDH* – Glyceraldehyde-3-phosphate dehydrogenase, *hsa-miR* – Human microRNAs, bp – base pair, F – forward primer sequence, R – reverse primer sequence, Anneal – Annealing temperature, X – times of cycle, Ref. Seq. – NCBI reference sequence. *Qiagen miScript qPCR Primer Assays (Maryland, USA)

MicroRNAs (miRNAs) are ~19–24 nucleotide-long small non-coding RNA molecules (18–20). miRNAs are involved in the post-transcriptional regulation of gene expression and play role in several cellular events such as proliferation, differentiation, aging, and death (21). Previous studies asserted that miRNAs can be implicated in the modulation of oxidative stress during the pathogenesis of vascular disease, and serve pivotal roles in oxidative stress-induced ED and dysregulation of mitochondrial metabolism (20, 22, 23). As a response to the intra-cellular stress, many miRNAs are upregulated, which underlines the potential significance of miRNAs during the modulation of pathological processes (18). Recent studies have revealed the fundamental roles of miRNAs in the physiopathology of CVD and thus emphasized the idea that miRNAs can bear potential as therapeutic targets in treatment of patients with vascular disorders (24–27). The evidence emerged from the previous in vitro and in vivo studies points out that alteration of blood flow conditions regulates the expression of flow-sensitive miRNAs in endothelial cells (18). Moreover, several flow-sensitive miRNAs also called as “athero-miRNAs”, *e.g.*, miR-10a, miR-19a, miR-23b, miR-21, miR22, miR-126, miR-146, miR-155, *etc.*, have been shown to play critical roles in the modulation of ED- and atherosclerosis-related genes’ expression (18, 28–31).

The aim of the present study is to investigate whether hydrogen peroxide (H_2O_2)-induced oxidative stress in endothelial cells is involved in the modulation of the expression levels of target protein encoding genes *i.e.,* EDN1, nitric oxide synthase-3 (NOS3), VCAM1, and serpin family E member-1 (SERPINE1, also known as PAI1) and non-coding miRNAs, *i.e.*, miR-21-5p, miR-22-5p, miR-126-5p, and miR-146a-5p, involved in the ED. Understanding the underlying roles of these miRNAs in endothelial cells after induction with various stimuli can yield novel therapeutic targets regarding management of cardiovascular and related chronic diseases.

Materials and methods

Cell culture and H_2O_2 treatment

For the experimental analyses, human umbilical vein cell line (HUVEC), purchased from American Type Culture Collection previously, was utilized. HUVEC cells were cultured in the complete medium formulized as 1 % penicillin / streptomycin (Gibco), 20 % fetal bovine serum (FBS) (Gibco), 0.3 ng/mL epidermal growth factor (EGF, Sigma) and 10 mg/mL heparin (VEM) in DMEM/F-12 basic medium (Gibco) and proliferated in 5 % CO_2, 95 % air supplying incubator (Thermo Scientific, HeraCell 150i) at 37 °C. The adherent cells were passaged after reaching 70–80 % confluence by using 0.05 % trypsin (Gibco) in order to seed the cells for further experiments.

Assessment of cell viability in H_2O_2-induced oxidative stress model

Cell viability analysis was assayed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) spectrophotometrically (Thermo Scientific, Multiskan GO) as described previously (32). Shortly, 1×10^5 cells/mL was seeded into multi-well plates and after 24 hours (h) of incubation, the adhered cells were treated with various concentrations of H_2O_2 (0, 25, 50, 100, 200, 250, 400, 500 μM) for another 24 h. Then the cells were incubated with 1 mg/mL MTT solution for 3–4 hours. The insoluble formazan crystals were solved with dimethyl sulfoxide after gentle shaking for 2–5 minutes (min) and the colorimetric change was read under 570 nm spectrum. The cell viability was calculated and expressed as the percentage of untreated control cells. The half maximal inhibitory concentration (IC_{50}) value was calculated by using “[Inhibitor] vs normalized response – variable slope – least squares fit analysis function” of GraphPad Prism software (version 8.0.2). The sub-cytotoxic 25 and 50 μM H_2O_2 concentrations were picked for further experiments.

Malondialdehyde (MDA) assay

MDA is one of the final products and a common analytic parameter of lipid peroxidation (13). After H_2O_2 exposure, the MDA analysis was performed following a previous protocol modified for cell culture models (33). To summarize, the cells were seeded into 75 cm^2 flasks with 1×10^5 cells/mL density and after 70–80 % confluence was reached, were treated with 25 or 50 μM H_2O_2 for 24 h. The cells were lysed with proteinase inhibitor cocktail-containing RIPA buffer (Thermo Scientific). The cell lysates were mixed with 8.1 % sodium dodecyl sulfate (Sigma, CAS number: 151-21-3), 20 % acetic acid (Merck), and 0.8 % thiobarbituric acid solutions at 95 °C for 30 min and then they were let to cool down at room temperature (RT). Thereafter, the samples were mixed with n-butanol (Merck)/pyridine (Merck) solution and spun down at 4000

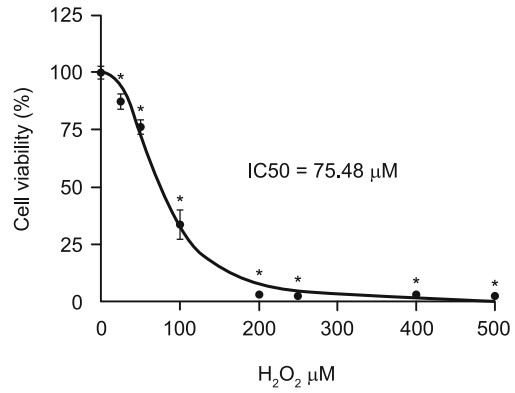

Fig. 1. The effect of H_2O_2 (0-500 μM) on HUVEC cell viability after 24 h treatment. Data were expressed as the mean ± standard deviation (n = 3), *p < 0.05 vs control. The curve-fit was plotted and IC_{50} was calculated by using non-linear regression analysis in GraphPad. H_2O_2: Hydrogen peroxide, IC_{50}: Half maximal inhibitory concentration.

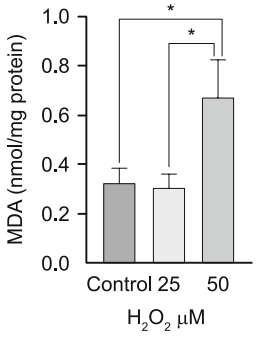

Fig. 2. The effect of 25 and 50 μM H_2O_2 on MDA level (nmol/mg protein) in HUVEC cells after 24 h treatment. Data were expressed as the mean ± standard deviation (n = 3), *p < 0.05. H_2O_2: Hydrogen peroxide, MDA: Malondialdehyde.

rpm (Sigma) for 10 min at RT. The supernatants were analyzed spectrophotometrically under 532 nm wavelength. The samples were measured in duplicates and the analyses were repeated for thrice. For normalization, the total protein concentrations in each sample were measured by using Pierce BCA (Bicinchoninic acid) Protein Assay Kit (Thermo Scientific). The MDA levels were expressed as nmol per mg total protein.

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

The expression levels of the target genes were analyzed with RT-qPCR method as described previously (34). First, HUVEC cells were seeded into 6-well plates with the final cell density of 5×10^4 cells/mL and after confluence was achieved, they were treated with 25 or 50 μM H_2O_2 for 24 h. After trypsinization, the cells were harvested for total RNA isolation (Qiagen; RNeasy Mini Kit). The total RNA amounts of the samples were measured spectrophotometrically with μDrop device (Thermo Scientific) and cDNA conversions for mRNA (1 μg) and miRNA (500 ng) samples were performed with RevertAid (Thermo Scientific) and miScript RT II cDNA synthesis kits (Qiagen), respectively. After appropriate dilutions, cDNA samples were amplified for qPCR analysis (Qiagen, Rotor Gene Q) by using either Real-Q Plus SYBR green (Amplicon) for mRNA or miScript qPCR sybr green kit (Qiagen) for miRNA samples. The following qPCR conditions were applied; for mRNA: 15 min at 95 °C, 40 cycles of; 15 s at 95 °C and 60 s at 60 °C, melting curve analysis between 62–95 °C rising by 1 °C, for miRNA: 15 min at 95 °C, 40 cycles of; 15 s at 94 °C, 30 s at 55 °C, and 30 s at 70 °C, melting curve analysis between 60–95 °C rising by 1 °C. The qPCR reactions were run in triplicates and for normalization, GAPDH or U6 genes were used. $2^{-\Delta\Delta Ct}$ method was utilized to calculate fold changes. The primers used for mRNA and miRNA qPCR analyses are listed in Table 1.

Statistical analysis

All statistical analyses and graphical presentations were performed with GraphPad Prism software (version 8.0.2). The normal distribution of the data was analyzed by using Shapiro-Wilk test.

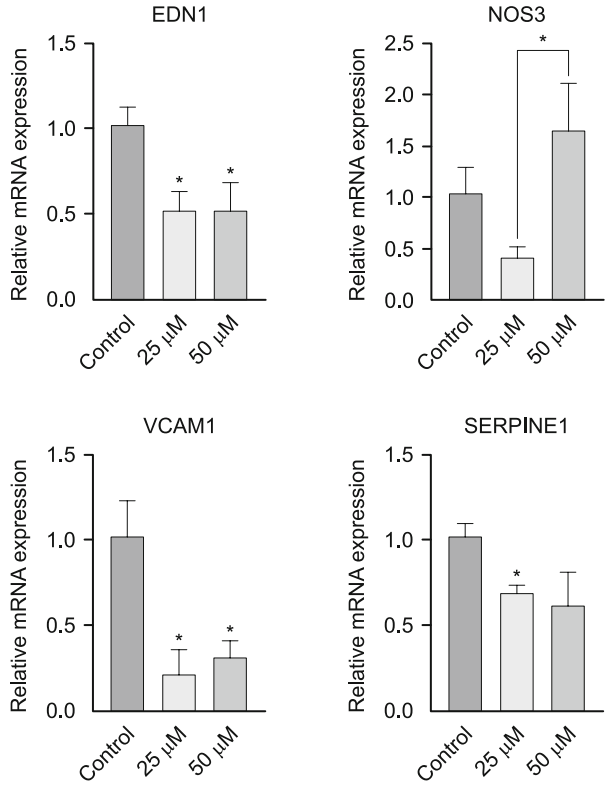

Fig. 3. Relative mRNA levels of EDN1, NOS3, VCAM1, and SERPINE1 after 25 and 50 μM H_2O_2 treatment for 24 h in HUVEC cells. qPCR data were expressed as the mean ± standard deviation of fold changes ($2^{-\Delta\Delta Ct}$) (n = 3), *p < 0.05. EDN1: Endothelin-1, NOS3: Nitric oxide synthase-3, VCAM1: Vascular cell adhesion molecule-1, and SERPINE1: Serpin family E member-1.

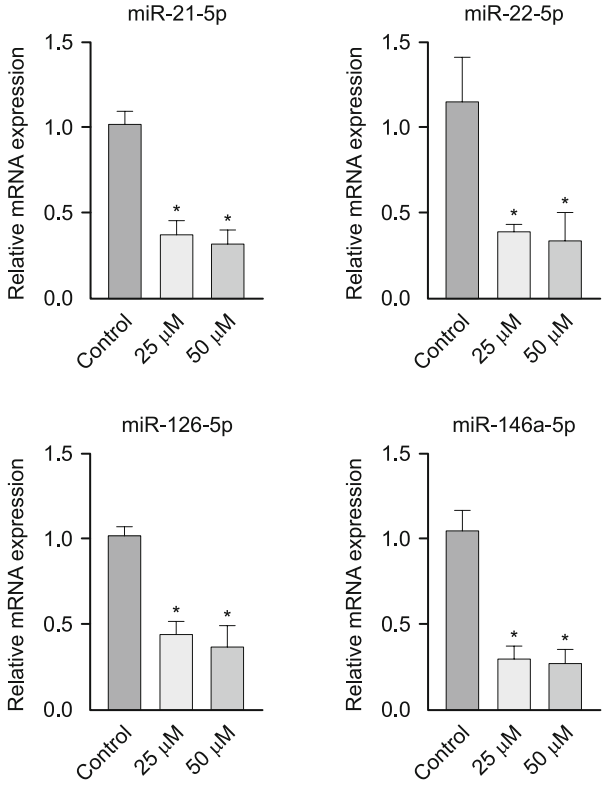

Fig. 4. Relative expression levels of miR-21-5p, miR-22-5p, miR-126-5p, and miR-146a-5p after 25 and 50 µM H_2O_2 treatment for 24 h in HUVEC cells. qPCR data were expressed as the mean ± standard deviation of fold changes ($2^{-\Delta\Delta Ct}$) (n = 3), *p < 0.05.

The differences among the groups were analyzed with one-way ANOVA test and two individual groups were compared with Holm-Sidak's or Tukey's multiple comparisons tests. Less than 0.05 for the p values was set as statistically significant.

Results

Cell viability levels after H_2O_2 treatment in HUVEC cells

In the present study, HUVEC cells were treated with H_2O_2 to obtain an *in vitro* oxidative stress cell culture model. MTT analysis was performed to test several concentrations of H_2O_2 (0–500 µM). We found that, each tested concentration caused a significant cell viability reduction ($p < 0.05$) and the IC_{50} value was calculated as 75.48 µM by using non-linear regression curve fit analysis. For further experiments, the lowest two H_2O_2 concentrations *i.e.*, 25 and 50 µM were picked (Fig. 1).

Elevated MDA levels in H_2O_2-induced HUVEC cells

Sub-cytotoxic H_2O_2 concentrations were tested in HUVEC cells to observe whether oxidative stress was induced post-24 h treatment by measuring MDA levels. 50 µM H_2O_2 elevated MDA levels significantly ($p < 0.05$) whereas there was no significant change in 25 µM H_2O_2-treated HUVEC cells ($p > 0.05$) (Fig. 2).

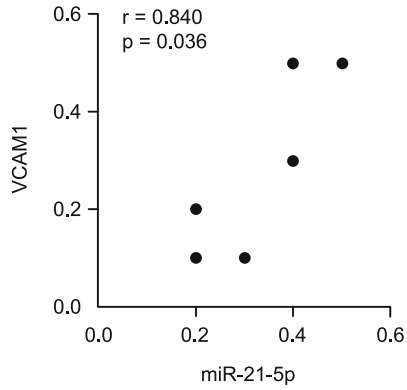

Fig. 5. Correlation between miR-21-5p and VCAM1. r = 0.84, p < 0.05. VCAM1: Vascular cell adhesion molecule-1.

Downregulated mRNA and miRNA levels upon H_2O_2 exposure and correlation between VCAM1 and miR-21-5p

In order to observe the effect of relatively low oxidative stress status in HUVEC cells on the mRNA and miRNA levels of ED-related parameters, we performed RT-qPCR analyses depicted in Figures 3 and 4. We found that, there was almost 2-fold and 4-fold decreases in EDN1 and VCAM1 relative mRNA levels respectively after treatment with both H_2O_2 concentrations ($p < 0.05$, Fig. 3). Besides, SERPINE1 mRNA level post-25 µM H_2O_2 treatment also decreased significantly despite nonsignificant change at 50 µM. However, there was no significant effect of H_2O_2 exposure on NOS3 mRNA levels at both concentrations in HUVEC cells ($p > 0.05$) (Fig. 3) together with a significant increase after 50 µM H_2O_2 treatment compared to 25 µM concentration ($p < 0.05$) (Fig. 3). As to miRNA levels, H_2O_2 exposure led to more than 2-fold decrease in miR-21, -22, -126, and -146a at both concentrations compared to the control group ($p < 0.05$) (Fig. 4). After the correlation analyses between miRNAs and the target genes, we found a positive correlation between VCAM1 and miR-21 ($r = 0.84$, $p < 0.05$) (Fig. 5).

Discussion

In the current study, we investigated the effect of H_2O_2-induced oxidative stress on the cell viability, MDA levels and the expression levels of ED-related mRNAs *i.e.*, NOS3, EDN1, VCAM 1, and SERPINE1 and miRNAs *i.e.*, miR21, miR22, miR126, miR146a in HUVEC cells. We found significant alterations in the levels of oxidative stress and the target genes including miRNAs after 24-h H_2O_2 exposure.

H_2O_2, which is one the main forms of ROS synthesized in the human body, can cause vascular endothelial cell damage by inducing oxidative stress in various ways (35–38). Hence, H_2O_2 is commonly used to produce oxidative stress-induced apoptotic in vitro cell culture model (37, 39, 40). On this basis, we created oxidative stress cell culture model by using H_2O_2 in HUVEC cells. H_2O_2 has the potential of acting like an endothelium-derived hyperpolarizing

factor. Despite the accumulating evidence that it can contribute to ED and CVD by causing oxidative processes and inflammation (41), relatively less is known regarding the effect of pathophysiological concentrations of H_2O_2 on the ED (1).

Based on our findings, 25 and 50 μM H_2O_2 sub-cytotoxic concentrations were used due to significant reduction of cell viability in HUVEC cells. The higher H_2O_2 concentrations (100-500 μM) were out of focus due to their cytotoxic effects with more than 70 % reduction in cell viability. In previous studies, unlike the present study, higher H_2O_2 concentrations (100–700 μM) were used in order to create oxidative stress model in HUVEC cells (20, 42, 43). The oxidative stress induced with relatively lower H_2O_2 in our study gave rise to different responses by the cells than expected.

We measured the MDA levels to determine the effect of H_2O_2 on oxidative stress in HUVEC cells. Wang et al reported a dose-dependent increase in MDA levels after 200, 500, and 700 μM H_2O_2 exposure in HUVEC cells (20). In parallel with these findings, we also observed a significant increase in MDA levels even after treating with lower H_2O_2 concentration (50 μM). ROS can act as a signaling molecule beside a cell-damaging agent. Until now plenty of studies have been conducted regarding the implication of ROS in oxidative stress responses and modulation of gene expression (44). However, still less is known about the exact mechanisms with respect to involvement of H_2O_2, as one the most abundant forms of ROS in cells, in how the gene expression patterns related to the activation of defense mechanisms were modulated (45). In the current study, we evaluated the effect of H_2O_2 on the mRNA levels of ED-related genes *i.e.*, EDN1, NOS3, VCAM1, and SERPINE1 (46) in HUVEC cells and we observed significant alterations.

NOS3 induces the production of NO which is an important vasodilator secreted by endothelial cells to maintain the vascular tonus (47). The mRNA level of NOS3 post- H_2O_2 treatment did not show a significant alteration compared to control group whereas it was significantly higher in 50 μM H_2O_2-induced HUVEC cells compared to the one in 25 μM group. We observed significant reductions in EDN1 and VCAM1 at both H_2O_2 concentrations while SERPINE1 mRNA level decreased at 25 μM group and did not show a significant change at 50 μM group. It was often reported in the literature that the level of NO, which is the main product of NOS3, decreases in ED pathogenesis (48, 49). Reactive oxygen products reduce the bioavailability of NO by reacting with it and trigger the cellular damage (49). In addition to NO and NOS3, EDN1 is also considered as the major regulatory molecule in vascular endothelial tonus. There are several reports asserting that NO has an inhibitory role in the transcriptional regulation of EDN1 expression in endothelial cells. Of which Nakamura-Utsunomiya et al claimed that NOS3 and EDN1 have opposite functions during the collaborative regulation of vascular tonus. EDN1 is a strong vasoconstrictor supporting the vascular homeostasis. The high levels of EDN1 expression in endothelial cells can lead to ED. It was demonstrated in a previous study that, upon ED progression, while the secretion rate of NO, which is produced as a response to hemodynamic stress and can cause vasodilation, decreased, the production of angiotensin converting enzyme and EDN1 increased (50). In another study conducted with HUVEC cells, Duerrschmid et al showed that EDN1 augmented the susceptibility to ED-triggered atherosclerosis by elevating oxidative stress levels (51). Moreover, a previous study sought to reveal any association between NOS3 and EDN1 levels in HUVEC cells treated with low and high H_2O_2 concentrations (47). The researchers found a proportional increase in mRNA expression levels of NOS3 based on increased H_2O_2 concentration and exposure time. These findings are partially in line with the present study suggesting the elevated NOS3 mRNA levels after higher H_2O_2 concentration compared to lower one. Contradictory to these findings, Han et al reported a significant reduction in NOS3 levels in HUVEC cells after 24 h-long 400 μM H_2O_2 treatment (7). Previous studies demonstrated that the endothelial cells could respond to H_2O_2 exposure in various ways depending on their reside in arteries or veins (1, 52). The difference in oxidative stress responses was claimed to be raised from the shear stress of blood flow (18). Hence further studies are needed to understand the molecular mechanisms involved in the gene expression regulation during endothelial cells' responses against oxidative stress. In a study conducted with a similar model with ours, after treatment of endothelial cells derived from pig aorta with low H_2O_2 concentrations, the NO accumulation was found to be decreased in the cells despite a ~3-fold increase in NO synthase activity after 50 μM H_2O_2 exposure (1). Furthermore, in bovine aorta-derived endothelial cells, H_2O_2 treatment (100, 150 μmol/L) was reported to cause concentration depended significant upregulation of NOS3 mRNAs (~3–5 folds) via transcriptional and post-transcriptional mechanisms (52). Nicholson et al found that in line with our findings, 50 μM H_2O_2 treatment increased NOS3 levels compared to 25 μM concentration, however, it showed an opposite effect by upregulating also EDN1 mRNA levels contradicting with decreased EDN1 levels in our study (53). In a similar study, relatively higher concentration of H_2O_2 (100 μM) caused a reduction in NOS3 mRNA levels in HUVEC cells together with increased levels of SERPINE1 (42). As aforementioned, the imbalances between the pro- and anti-active factors regulating oxidation, inflammation, and thrombosis play role in H_2O_2-induced ED. The upregulation of SERPINE1, as one of these factors, delays the fibrinolysis by inhibiting the effect of plasminogen activators and hence leads to thrombus accumulation in vascular wall (54). In contrary to previous findings reporting increased VCAM1 and SERPINE1 mRNA levels after H_2O_2 treatment in HUVEC cells (11, 12, 55–57), we found attenuated mRNA expressions for both factors upon H_2O_2 exposure. Despite some unexpected findings, the results in the present study are compatible with each other, meaning that NOS3 and EDN1 showed opposite expression patterns (47) and EDN1, VCAM1, and SERPINE1 showed a parallel alteration pattern. It can be inferred from the contradicting findings of previous studies that ED induced CVD can emerge due to the general imbalance between NO and ROS producing systems' alterations rather than unidirectional alterations of single factors (7).

The dysregulation at gene and miRNA expression patterns plays vital role in the fate of cellular pathways which eventually affects an organism's physiology. miRNAs serve as gene expression regulators with respect to functioning as key molecules in both crucial cellular pathways pertaining to vascular biology and modulation of various diseases' onsets like CVD (29, 58). Previous studies unveiled that a significant number of miRNAs have pivotal roles in the cells residing heart tissue such as cardiomyocytes, fibroblasts and endothelial cells (29) and also in the pathogenesis of ED which is the major cause of CVD (59). In the present study, we analyzed the expression of miR-21, miR-22, miR-126, and miR-146a and observed significant reductions in the levels of all miRNAs at both H_2O_2 concentrations post-24 h treatment. Wang et al demonstrated the upregulation of miR-146a and miR-146b in high glucose-induced endothelial cells and their endothelium-protective effects by inhibiting oxidative stress formation (30). miR-126, which is highly expressed in endothelial cells, is an endothelium-specific miRNA (31). The amount of miR-126 is increased in endothelial cells in order to build up the vascular walls as well as to support and maintain angiogenesis together with other miRNAs (29). Moreover, Harris et al showed that the overexpression of miR-126 and VCAM1 expression could regulate the vascular inflammation (60). It can be considered from these findings that miRNAs can regulate the expression of molecular adhesion markers together with controlling the vascular inflammation (61). VCAM1 and miR-126 that is targeting proinflammatory mediators are considered as the major regulators of endothelial homeostasis and vascular integrity. miR-126 exhibits indirect antithrombotic features and reduces the vascular inflammatory responses, e.g., the expressions of VCAM1 and fibrinogen, leukocyte numbers, etc., via targeting the tissue factors in monocytes (60, 62). It was shown in a previous study that miR-126 can inhibit VCAM1 expression (18). Contrary to this finding, in the present study, we showed a reduced expression of both factors after H2O2 exposure and there was no significant correlation between VCAM1 and miR-126 levels. Another study found a reduction in basal mRNA levels of VCAM1 upon treatment with miR-146 inhibitor (63). In the same study, the decreased NOS3 mRNA levels after IL-1β treatment could be reversed by augmenting its levels after miR-146 inhibitor treatment. In a previous study it was reported that several miRNAs, such as miR-21, miR-22, miR-146a, miR-155, miR-221, miR-222, and miR-34a, were related to the angiogenesis in persons with CVD (58). Among plenty of miRNAs, the miR-21-5p expression is deregulated, mostly upregulated, during the pathogeneses of various CVD such as cardiac hypertrophy, myocardial infarction, and aortic aneurysm (29). miR-22-3p was shown to modulate the expression of certain genes that are associated with hypertension in human microvascular endothelial cells (28).

In the present study, we also analyzed to check whether there is any correlation between the downregulated miRNA levels with the ED-related target genes and found only a significant positive correlation between VCAM1 and miR-21-5p expression levels. When their crucial roles in the posttranscriptional repressive regulation are considered, miRNAs are of pivotal importance as potential targets in the adaptation of cells for survival against stress during pathophysiology of several diseases (18). In this context, we suggest that the downregulated miRNAs in the current study can possess direct or indirect regulatory roles in the ED induced by cellular oxidative stress.

Conclusion

Overall, our findings demonstrated that relatively lower H_2O_2 concentration could produce oxidative stress model in HUVEC cells by augmenting cellular MDA levels. Besides, H_2O_2-concentration dependent dysregulation in ED-related factors *i.e.*, NOS3, EDN1, VCAM1, and SERPINE1, and in CVD-related miRNAs, *i.e.*, miR-21, miR-22, miR-26, and miR-146a, underlines their significance in maintaining the cellular oxidative balance. For better understanding the specific targets and action mechanisms of these miRNA, in vitro and in vivo mechanistic models are warranted.

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