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Original Research

CagA-positive and *CagA*-negative *Helicobacter pylori* strains differentially affect the expression of micro RNAs 21, 92a, 155 and 663 in human umbilical vein endothelial cells

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Abstract: Given that the basic mechanism of the effect of *Helicobacter (H.) pylori* in the induction of atherosclerosis remains unknown and regarding the regulatory role of micro RNAs (miRNAs) in endothelial cell (EC) functions, we aimed to investigate the effect of *H. pylori* on the expression of miRNAs involved in atherosclerosis (atheromiRs) and their correlation with apoptosis in human umbilical vein EC (HUVEC). HUVECs were treated with different cytotoxin associated gene A (CagA) positive and negative *H. pylori* derived products, then the levels of apoptosis and miR-21, 92a, 155 and 663 were measured using flowcytometry and real time-PCR methods, respectively. Although, comparing induced apoptosis and necrosis in HUVECs revealed that water extract of $CagA^+$ *H. pylori* (HpWE) was more potent than $CagA^-$ one and *H. pylori* lipopolysacharide (Hp-LPS), no significant difference was observed between LPS extracted from $CagA^+$ and $CagA^-$ strains. Besides, $CagA^+$ HpWE significantly increased the levels of anti-apoptotic miR-21, and inflammatory miRNAs 155 and 663 but not miR-92a. A positive correlation was observed between apoptosis and necrosis and miR-155 as well as the expressions of miR-21 with miR-155 (P=0.024) and miR-663 (P=0.0001). As *H. pylori* products differentially influenced phenotypic and epigenetic changes in ECs pictured in apoptosis and in the expression of atheromiRs, we suggest that the presence of CagA molecule accompanied by these atheromiRs may act as beneficial biomarkers predicting ECs apoptosis as a sign of plaque rupture.

Key words: Endothelial cells; *H. pylori*; Apoptosis; Micro RNA.

Introduction

Atherosclerosis is a chronic inflammatory process in the large and medium sized arteries triggered by lipid deposition (1), endothelial cells (EC) dysfunction (2), innate and adaptive immune systems activation (3) which leads to development and progression of plaques. Apoptosis and necrosis in endothelial and smooth muscle cells (4) may cause plaque rupture, a process that can activate the coagulation pathway and clot formation in the blood vessels and consequently leads to stroke and myocardial infarction. Several predisposing factors have been considered to be related to atherosclerosis such as genetics (5), hypertension (6), hyperlipidemia (7), diabetes mellitus (8), and infections (9). Among the infectious agents, *Helicobacter (H.) pylori,* has been reported to be associated with atherosclerosis though the mechanisms through which it induces and promotes atherosclerosis remain unknown. The presence of *H. pylori* DNA in the atherosclerotic plaque (10), and seropositivity to *H. pylori* have been postulated as risk factors for cardio- and cerebro-vascular diseases (11) (12). Furthermore, it has been revealed that *H. pylori* can impel ECs to secrete neutrophil recruiting factors (13), and its eradication could improve the ECs dysfunction (14). The cytotoxin associated gene (*Cag*) *A* and vacuolating toxin (*Vac*) *A*, are described as the two most important virulent factors for the *H. pylori* strains (15). CagA is an immunodominant protein which is an activator of the immune system (16), and *CagA* positive *H. pylori* strains are considered to induce the development and instability of the atherosclerotic plaques (17). However, a definitive consensus is not achieved about the role of these virulence factors in the induction of apoptosis predisposed the plaques to the rupture. While it has been reported that *H. pylori* could induce apoptosis in human umbilical vein endothelial cells (HUVEC), independent of CagA and VacA (18), other studies showed that VacA induces apoptosis, and conversely CagA decreases apoptosis in the gastric epithelial cells by enhancing the degradation of p53 protein (19, 20).

Many efforts are made to understand the inflammopathology of atherosclerotic plaques and explore new biomarkers to determine the susceptibility of plaques to the rupture, of which, micro RNAs (miRNAs) are interesting candidates. MiRNAs are small 19-24 nucleotide RNAs encoded by their specific genes, for some of which a role in cardiovascular disease and atherosclerosis is shown (21, 22). Based on the miRNA array data, several miRNAs, namely atheromiRs, have been found to be involved in atherosclerosis. Among which, miR-21 acts as the inducer of nitric oxide (NO) synthe-

sis whereby inhibits apoptosis in ECs (23) and induces collagen synthesis leading to plaque stability (24). Two endothelial transcription factors including Kruppel like factor (KLF) 2 and 4 with reported athero-protective properties are inducers of anti-inflammatory or anti-oxidant phenotype in ECs (25, 26). MiR-92a, by targeting KLF-2 and 4, plays a pro-atherogenic role in ECs (27). Moreover, miR-92a confers resistance to apoptosis in vascular smooth muscle cells under oxidative stress condition by suppressing the mitogen-activated protein kinase kinase 4 (MKK4) (28). MiR-155 known as a modulator of inflammed endothelial cells which can down-regulate the expression of NO synthase (29), type I angiotensin-II receptor (30) and downstream inflammatory molecules (31). MiR-663 is expressed in HUVECs under shear stress condition and is shown to play a role in the ECs inflammatory responses and atherosclerosis (32).

Previous investigations on the miRNA expression in *H. pylori*-infected human gastric mucosa revealed that CagA+ strain decreased the expression of let-7 more effectively compared to CagA- one; implying that CagA might be involved in the regulatory processes of some miRNAs (33). It is also shown that infection with CagA+ *H. pylori* upregulates the expression of miR-1289 in gastric mucosa which results in the inhibition of $H^{+}/K^{+}\alpha$ and consequently facilitated the *H. pylori* colonization (34). Recognition of *H. pylori* by toll like receptor (TLR) expressed on the myeloid and lymphocyte cells is shown to induce the expression of miR-146a and miR-155 as negative regulators of pro-inflammatory immune responses independent of CagA molecule (35). Although the majority of findings support the association of *H. pylori* with miRNAs in different cell types and diseases especially in gastric cancer; there are a few studies to determine the effects of *H. pylori* and their components on the expression of atheromiRs in the ECs. EC dysfunction is the first step in a complex and multifaceted process leading to the initiation of the atherosclerotic lesions and their complications. Since deregulation of miRNAs is considered to be involved in the ECs dysfunction and it has been shown that *H. pylori* could alter the expression of miRNAs, it seems that investigating the impact of *H. pylori* on the expression of atheromirs in ECs may be useful to clarify the mechanisms of *H. pylori* pathogenesis in atherosclerosis. With respect to the various effects of *H. pylori* constituents such as CagA molecule and LPS on the EC dysfunction and atherosclerosis, this study aimed to investigate the effect of different *H. pylori* products in the induction of cell death in human umbilical vein endothelial cell (HUVEC) and evaluate whether these products can stimulate the expression of the signature atherosclerotic miRNAs such as miR-21, 92a, 155 and 663.

Materials and Methods

Cultivation of HUVECs

Endothelial cells were isolated from the vein of umbilical cord using collagenase digestion method described previously (36). The purity of the cells was confirmed by flow cytometer using FITC labeled antibody against CD31 (BD pharmingen, USA). HUVEC cells were serially sub-cultured and used for the next experiments at the fourth passages.

Isolation and identification of *H. pylori* strains

The strains of *H. pylori* were isolated from patients with gastritis. Gastric biopsies were homogenized and cultured on Columbia agar (Merck, Germany) supplemented with 10% horse lysed blood, 10 µg/ml vancomycin, 5 µg/ml trimethoprim, and 2.5 U/ml polymyxin B (Merck, Germany). *H. pylori* colonies were confirmed by gram staining and biochemical reagents such as positive urease, oxidase and catalase tests. DNAs were extracted and the presence of *UreC*, *CagA*, *VacA* and allelic types of *VacA*, were confirmed by polymerase chain reaction (PCR) using specific primers (Table

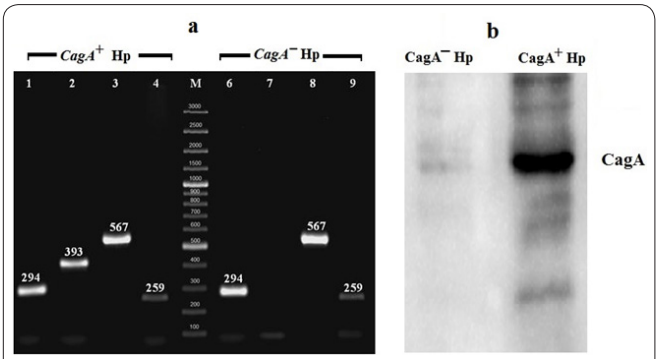

Figure 1. Agarose gel electrophoresis result on 3% gel showing two different strains of *H. pylori*, one positive (lanes 1 to 4) and the other negative (lane 6 to 9) for *CagA* gene (a). M represents the 100bp molecular weight marker. Immunoblotting with polyclonal specific antibody against CagA protein confirmed two strains of $CagA^{+}$ and $CagA^{-}$ *H. pylori* (b). Hp: *H. pylori.*

Table 1. The sequences of specific primers and their annealing temperature (AT) used for amplifying the *UreC*, *CagA* and *VacA* genes of the *H. pylori* strains and the produced amplicon size after PCR performance.

Gene	Sequence of Primers 5' to 3'	AT	Amplicon size (bp)
UreC	**F:** aag ctt tta ggg gtg tta ggg gtt t **R:** aag ctt act ttc taa cac taa cgc	55°C	294
CagA	**F:** gat aac agg caa gct ttt gag agg ga **R:** cca tga att ttt gat ccg ttc gg	55°C	393
VacAS	**F:** atg gaa ata caa caa aca cac **R:** ctg ctt gaa tgc gcc aaa c	52°C	259 (type s1)
VacAM	**F:** caa tct gtc caa tca agc gag **R:** gcg tca aaa taa ttc caa gg	56°C	567 (type m1)

[a] Standard deviation, [b] *H. pylori* water extract, [c] *H. pylori* lipopolysaccharide, [d] *H. pylori* conditioned media, [*] the level of significancy less than 0.05, [**]the level of significancy less than 0.01.

1). The strains with similar *UreC* and *VacA* (allelic types) and different *CagA* genotypes (Figure 1a) were selected for further experiments. Also, proteins from the bacterial cell lysates were extracted and separated using SDS PAGE and then transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA). To determine CagA protein, blots were developed by anti CagA antibody (Santa Cruz Biotechnology, Texas) using ECL Western blot detection kit (Millipore) according to the manufacturer's instructions (Figure 1b). The genitically similar strains were selected and pooled (3 strains) for the next experiments.

Extraction of lipopolysaccharide

Lipopolysaccharide from *H. pylori* (Hp-LPS) was extracted using the LPS extraction kit (Bulldog, USA) and extracted LPS was treated with proteinase K (75µg per 30µg LPS) to digest the contaminating proteins. LPS concentrations were determined using the Pierce limulus amebocyte lysate chromogenic endotoxin quantitation kit (Thermo Scientific, USA) according to the manufactures' instructions.

Preparation of *H. pylori* water extracts

To prepare CagA positive and negative *H. pylori* water extracts (HpWE), the colonies were re-suspended in sterile distilled water in a concentration of 10^9–10^{10} CFU/ml and after 1 h incubation at room temperature the cells were sonicated 8 times for 45 second at density 5. Then the suspensions were centrifuged for 10 min at 12000 x g and supernatants were passed through a 0.2 µm filter membrane. The protein concentration was measured using a Nano Drop spectrophotometer (ND 1000, USA).

Preparation of *H. pylori* conditioned media (HpCM)

The colonies of CagA positive *H. pylori* were harvested and re-suspended in 3 ml PBS. The optical density of the suspension was adjusted to a final OD of 1.0 at 600 nm. 2.5 ml of bacterial suspension was added to a T25 tissue culture flask containing 40 ml of M199 complete medium and incubated at 37°C under microaerophillic conditions for 24 h. Then, following the centrifugation at 12000 x g for 10 min, the supernatant was filtered using a 0.2 µM sterile filter to yield HPCM.

Treatment of HUVECs with Bacterial extracts

Obtained from the previous and our pilot study to set the suitable concentrations of LPS and *H. pylori* water extracts in stimulating HUVECs, 80% confluent HUVECs at passage 4 were separately treated in 6 well culture plates with 20 ng/ml LPS, 100 mg/ml of CagA positive and negative HpWEs and HpCM. After 48 h incubation at 37ºC, 5% CO2 and 95% humidity, the HUVECs were harvested using 0.05% trypsin and 0.1% EDTA for further experiments.

Detection of apoptosis using flowcytomety

Early and late apoptosis were measured in treated HUVECs using Phycoerythrin (PE) Annexin V apoptosis detection kit I (BD pharmingen, USA) according to the manufacturers' instructions. The stained cells were analyzed by flow cytometer instrument (BD FACS Calibur, USA) and the data were calculated using FlowJo software version 7.6.2.

Determination of the miRNAs levels in treated HUVECs by real time-PCR

Total RNAs from HUVECs were isolated using Trizol reagent (Invitrogen). cDNAs were synthesized in two steps. At first, a poly-A tail was added at the 3´ end of the RNAs by poly A polymerase enzyme (Biolabs, NEB). Thereafter, RNAs containing poly-A tail were converted to cDNA using cDNA synthesis kit (Fermentas, Lithuania). Eventually, PCR was performed by SYBR Green PCR Kit (Takara, Japan) and ROX reference dye in the presence of specific primers (Pars Genome, Iran). A pair of specific primers was also used for amplification of U6 small nuclear RNA as a housekeeping gene. All PCR reactions were performed using 7500 Real-time PCR Cycler (Applied Biosystems). The relative changes in miRNAs expression were analyzed using the $2^{-\Delta\Delta CT}$ algorithm.

Statistical analysis

All experiments were repeated five times and the relative expressions of miRNAs and the frequency of Annexin V and 7-AAD positive HUVECs were reported as mean ± standard deviation. Nonparametric Mann Whitney U test was used to compare the frequency of Annexin V and 7-AAD expressing HUVECs and also the relative miRNAs expression in treated and untreated HUVECs. Pearson correlation test was used to determine the association between miRNAs expression and the induction of apoptosis and necrosis. *P* values less than 0.05 were considered statistically significant.

Results

In all experiments, the purity of HUVECs was determined more than 95% using FITC conjugated anti-CD31 antibody.

Induction of apoptosis and necrosis in HUVECs by different *H. pylori* products

The flowcytometric analysis gated on HUVEC population revealed that all *H. pylori* products except HpCM were able to induce apoptosis in treated HUVECs. Amongst, $CagA^+$ HpWE induced the highest rate of apoptosis in HUVECs (Table 2 and Figure 2a-f). Comparing the extracted LPS from CagA positive and negative *H. pylori* revealed no significant differences between these groups (Figure 3a), therefore for further experiments, the effect of Hp-LPS (regardless of being CagA positive or negative) on HUVECs was cumulated and compared with other stimuli.

Assessment of 7-AAD positive HUVECs as a marker of necrosis showed that $CagA^+$ HpWE and Hp-LPS are potent inducers of necrosis in HUVECs. Interestingly, $CagA^+$ HpWE was found to be a more potent inducer of necrosis than the $CagA^-$ HpWE. However, no significant differences were observed between other study groups (Table 2 and Figure 2g-l).

Expression of miRNAs 21, 92a, 155 and 663 in HUVECs treated with different stimuli

There were no significant differences (P = 0.35) in the expression of the mentioned miRNAs between HU-

Table 2. Paired comparison analysis table of apoptosis and necrosis induced in HUVECs by different *H. pylori* products. The results of 5 independent experiments are shown.

		Mean ± SD[a]	HpWE[b]		Hp-LPS[c]	HpCM[d]
			CagA⁻	*CagA*⁺		
Annexin V						
HpWE	*CagA*⁺	22.1 ± 5.8	---			
	CagA⁻	12.7 ± 3.3	0.016*	---		
Hp-LPS		13 ± 3	0.005**	1	---	
HpCM		10.7 ± 2.1	0.009**	0.25	0.14	---
Untreated		8.6 ± 0.63	0.009***	0.014*	0.002**	0.12
7-AAD						
HpWE	*CagA*⁺	10.2 ± 2.3	---			
	CagA⁻	7.1 ± 1.8	0.047*	---		
Hp-LPS		9 ± 2.7	0.14	0.1	---	
HpCM		7.4 ± 3.1	0.17	0.92	0.46	---
Untreated		6.4 ± 1.6	0.047*	0.75	0.037*	0.75

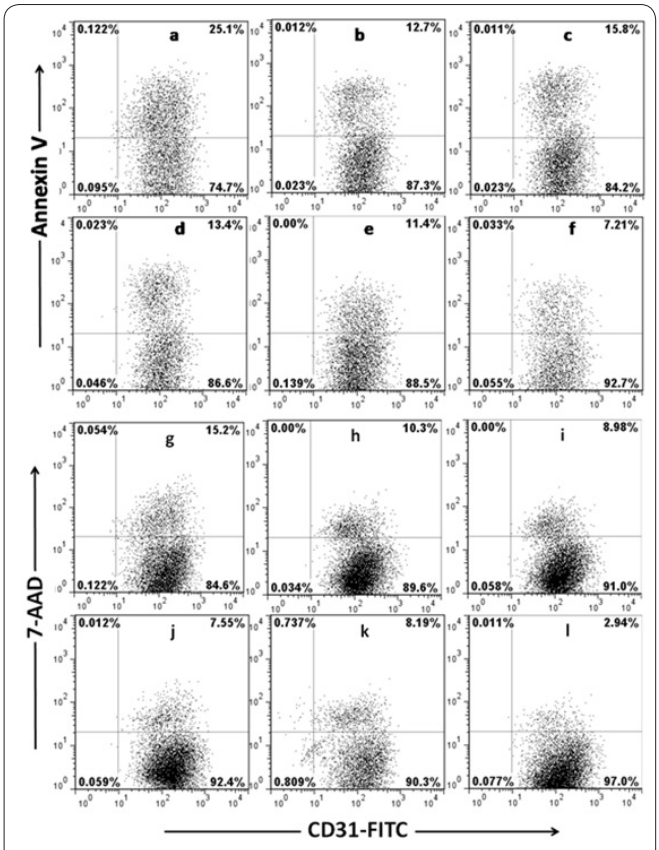

Figure 2. Flow cytometric dot plots represent induction of apoptosis (a-f) and necrosis (g-l) in HUVECs treated with CagA⁺ HpWE, CagA⁻ HpWE, Hp-LPS, *E. coli* LPS, HpCM and untreated control, respectively. HpWE: *H. pylori* water extract, Hp-LPS: *H. pylori* lipopolysaccharide, HpCM: *H. pylori* conditioned media.

VECs treated with LPS extracted from CagA positive and negative *H. pylori* strains (Figure 3b). As shown in Figure 4, in general, treatment of HUVECs with CagA⁺ HpWE, CagA⁻ HpWE, Hp-LPS and HpCM resulted in a change in the expression of the mentioned miRNAs. However, in a few instances there were no significant differences between treated and untreated HUVEC. When we compared the apoptosis and necrosis as well as the expression of the above mentioned miRNAs between HUVECs treated with Hp-LPS and LPS extracted from *E. coli*, no remarkable differences were observed (data are not shown).

Correlation between apoptosis, necrosis and miRNAs expression

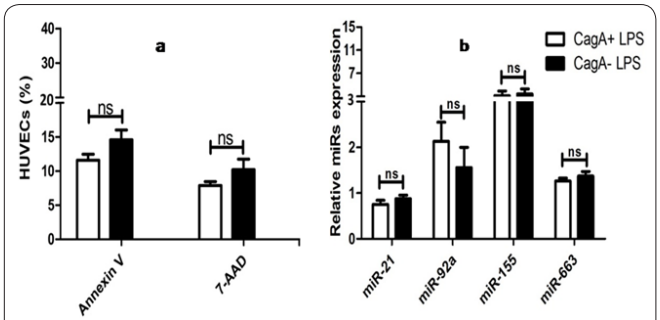

Figure 3. The graphs depict apoptosis and necrosis (a) as well as the expression of different miRNAs (b) in HUVECs treated with CagA⁺ and CagA⁻ *H. pylori* lipopolysaccharide (Hp-LPS).

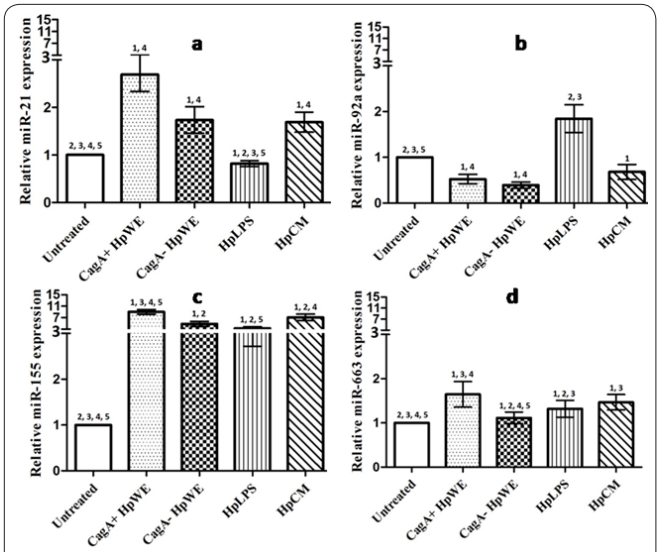

Figure 4. The graphs show the level of miR-21 (a), 92a (b), 155 (c) and 663 (d) in HUVECs treated with different *H. pylori* products. The numbers on the bars represent groups showing significant difference ($P < 0.05$) with the designated groups (1: Untreated, 2: CagA⁺ HpWE, 3: CagA⁻ HpWE, 4: HpLPS, 5: HpCM). HpWE: *H. pylori* water extract, Hp-LPS: *H. pylori* lipopolysaccharide, HpCM: *H. pylori* conditioned media.

Pearson correlation test revealed a positive correlation between miR-92a and miR-663 ($r = 0.517$ and $P = 0.0001$), as well as miR-155 and the rate of apoptosis ($r = 0.304$, $P = 0.024$) though, no significant associations were observed between the other parameters.

Discussion

While the extent of *H. pylori* involvement in the

formation and instability of atherosclerotic plaques and the mechanisms of such effects are still unknown, interplay between stress signals and gene expressions may provide a link between infection and atherosclerosis. Because the expression of 30% of the human genome is regulated by miRNAs, induction of different miRNAs by infectious agents points to the role of epigenetic in triggering intracellular changes based on the environmental cues (37). Recently, deregulation of miRNAs in cardiovascular diseases has received enormous attention. A delicate regulation is suggested to exist between miRNAs expression and predisposing factors of cardiac diseases, of which, bacterial pathogens with different virulence factors are still under scrutiny. We used different products of CagA positive and negative *H. pylori* strains to evaluate their impacts on EC apoptosis and necrosis as the main phenomena in the instability and rupture of plaques. The selected *H. pylori* strains were also positive for *VacA s1m1* gene and which are reported to induce higher levels of cytotoxicity (38). Therefore, simultaneous presence of *CagA* and *VacA s1m1* genes may induce higher levels of death in ECs which can act as a predisposing factor for plaque instability and rupture in the infected patients. Accordingly, previous studies revealed that $CagA^+$ but not $CagA^-$ strains were associated with the plaque instability (17) and the risk of recurrent atherosclerosis stroke (39). Purified Hp-LPS could also induce apoptosis in HUVECs to a lesser extent, and this effect was comparable to $CagA^-$ HpWE and lower than that of $CagA^+$ HpWE. Since one of the common components of $CagA^-$ HpWEs and purified Hp-LPS is LPS itself, it can be suggested that Hp-LPS is the main element in death induced by these extracts. LPS, by increasing the levels of caspase-1, caspase-3, pro-apoptotic Bax and tumor suppressor gene p53, is able to induce apoptosis in the ECs (40). Because we observed that the effect of Hp-LPS in ECs apoptosis was similar to *E. coli* LPS, it seems that the presence of CagA can potentially increase the pro-apoptotic properties of LPS.

Since HpCM was prepared from the $CagA^+$ *H. pylori,* we expected that its effect on HUVECs be somewhat similar to $CagA^+$ HpWE, however, this was not the case. Although it seems oversimplification, lower concentrations of CagA protein in HpCM compared to $CagA^+$ HpWE may be responsible for decreased apoptosis.

Morphological changes or alteration in the involved molecules can be used to evaluate apoptosis in individual cells. Since the study of morphological changes in the atherosclerotic plaques is not feasible, the levels of the apoptosis regulators such as miRNAs, cell-associated or soluble, could be markers of apoptosis in the plaques which in turn is regarded as an inducer of plaque rupture. Hence, the levels of apoptosis-related miRNAs (apoptomiRs) expressed in the ECs can act as predictive factors for ECs death and plaque instability. Therefore, we also analyzed the expressions of miRNAs involved in atherosclerosis such as miR-21, 92a, 155 and 663, and their association with apoptosis and necrosis in HUVECs treated with different *H. pylori* products. Among the *H. pylori* products, $CagA^-$ HpWE and HpCM showed similar effects to $CagA^+$ HpWE albeit to a lesser extent. Besides, while the effect of Hp-LPS in the upregulation of miR-155 and miR-663 was similar to other *H. pylori* products it showed opposite effects on the expression of miR-21 and miR-92a. MiR-21, which is highly expressed in the ECs and smooth muscle cells, is considered as an anti-apoptotic miRNA. By targeting the programmed cell death 4 and phosphatase and tensin homology molecules, this miRNA is able to protect ECs from apoptosis (41). Considering the higher rate of apoptosis observed in $CagA^+$ HpWE treated HUVECs, it is plausible to expect that the level of miR-21 be lower in these cells. Interestingly, our results showed that $CagA^+$ HpWE upregulated the level of this miRNA by 2.7 folds; implying a possible compensation mechanism induced by miR-21 to decrease apoptosis in the injured HUVECs. In addition, $CagA^+$ HpWE may induce apoptosis in HUVECs with another mechanism rather than downregaulation of miR-21. In agreement with this suggestion, we observed no significant association between the level of miR-21 and the frequency of apoptotic HUVECs. Furthermore, HpWE was able to downregulate the level of miR-92a in treated HUVECs. Since the lower level of miR-92 is associated with higher level of apoptosis, it is likely that *H. pylori* products (except Hp-LPS) induce apoptosis by decreasing miR-92. MiR-155 is an inflammatory miRNA that its role has been highlighted in a wide variety of physiological and pathological processes including cardiovascular diseases (42). Although our results revealed that all *H. pylori* products were potent stimulators of miR-155, $CagA^+$ HpWE and HpCM were more effective than the others. Since the CagA+ strains induced higher levels of miR-155, it is expected that CagA bearing *H. pylori* strains contribute to severe ECs dysfunction. Furthermore, we observed a significant positive correlation between the level of miR-155 and the rate of apoptosis. This finding is in agreement with a previous study reported the association between inflammatory markers and induction of apoptosis (43). Mir-663 is also known as a pro-inflammatory miRNA which has been shown to be able to induce inflammation in ECs (32). Resulted from our study, all *H. pylori* products except $CagA^-$ HpWE were stimulators of miR-663. Regarding the significant difference between $CagA^+$ and $CagA^-$ HpWE in the induction of miR-663, it can be proposed that CagA is a main contributor in the upregulation of this miRNA.

In conclusion, it can be suggested that each *H. pylori* products can differentially induce deregulation in ECs by influencing different molecules involved in EC function including atheromiRs. Among the *H. pylori* strains, CagA bearing ones were more potent in the induction of apoptosis and necrosis and also in the upregulation of miR-155 and miR-663 (inflammatory miRNA) thereby predisposing infected individuals to plaque rupture and consequent thrombotic events. In this regard, using molecular methods for determining microbial virulent factors in combination with the levels of micro RNA expressed exclusively in ECs could be valuable in the better understanding of inflammopathology of atherosclerosis and finding new biomarkers for predicting the situation of the atherosclerotic plaques in infected individuals.

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