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HYDROXY-METHYL-FURFURAL INHIBITS SECRETION OF SELECTED INFLAMMATORY FACTOS IN A COCULTURE SYSTEM OF HUVECS WITH HUMAN MONOCYTIC CELL LINES

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ABSTRACT

Objectives. There is sufficient evidence that bioactive compounds in foods may reduce level of persistent inflammation, a key contributor to development of atherosclerosis. This study was sought to determine whether exposure to Hydroxymethylfurfural (5-HMF) diminishes levels of ICAM-1 and MMP-9 from coculture of Human umbilical vein endothelial cells(HUVECs) with human monocytic cell. ***Design.*** A co-culture system was established between HUVECs and human monocytic cell line (THP-1). After treatments of the cocltivated cells with different concentrations of HMF, the viability of cells in the system was investigated using MTT assay. Also, the anti-inflammatory responses of the system was studied by measuring the levels of ICAM-1 and MMP-9 in supernatants by ELISA. ***Results.*** It was found that treatment with 5-HMF significantly decreased MMP-9 level ($P<0.05$) with no significant effect on secretion of ICAM-1. Furthermore, HUVECs cell 's viability viability showed equal or decreasing tendency, but coculture cell viability increased with increasing the dose of 5-HMF ($p<0.05$). ***Conclusion***. Our data confirms the anti-inflammatory properties of 5-HMF. The finding of this study could be applicable in prevention and management of atherosclerosis.

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Introduction

Atherosclerosis is considered as a chronic inflammation of arterial wall which endothelial dysfunction, vascular inflammation and the atheromatous plaque are of its key markers [1, 2]. Dysfunction of vascular endothelium and subsequent inflammation, triggers leukocyte adhesion, platelet aggregation, cell proliferation and migration of vascular smooth muscle cells which leads to arterial thickness [3]. The key event in early pathogenesis of atherosclerosis is adherence of monocytes to injured endothelium that results in their migration to the sub-endothelial space and accumulation of monocyte-derived macrophages [4]. Aggregation of macrophages within the atherosclerotic lesions plays as an adhesive pole for locally concentrated oxidized low density lipoprotein and participates in generation of lipid-laden foamy macrophages which induce production of pro-inflammatory cytokines [4, 5].

It has been suggested that inflammation and inparticular inflammatory mediators are among the most influential contributors to the development of atherosclerosis. Accumulating evidence insisted that cytokines cause endothelial activation, and lead to overexpression of cell adhesion molecules (CAMs) especially vascular cell adhesion molecule-1 (VCAM-1) and intercellular

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adhesion molecule 1 (ICAM-1),) that are considered to play a key role when circulating monocytes attach endothelium and subsequently transmigrate into intima [6, 7].

It has been evident that VCAM-1–stimulated endothelial cells, rapidly activate endothelial cell–associated matrix metalloproteinases (MMPs) which are considered to be critical proteins in propagation of inflammation process [8]. The MMPs are a group of zinc-dependent neutral endoproteases which are hydrolysis components of extracellular matrix in atherosclerotic plaque [9]. It is reported that expression and activation of these compounds is essential for facilitation of monocytes migration into the intima [10-13]. Further, studies have shown an especial expression for MMP-9 in symptomatic coronary artery lesions [14].

5-hydroxymethylfurfural (5-HMF) is a bioactive compound which is produced in food environment in presence of acidic condition and high temperature via a non-enzymatic phenomenon called Maillard reaction [15, 16]. The compound exists in variety of foods and beverages such as coffee, fruits, cereals and dairy products. However, processing conditions such as duration, temperature, and water activity affects the amount of it in these foods [17]. Recently, some studies discovered health promoting effects of 5-HMF and defined it as a natural antioxidant [18]. Also, some other physiologically beneficial influences such as, neuro-protective [19], anti-allergic [20] and anti-inflammatory effects are attributed to 5-HMF [21].

As mentioned, recent reports illustrated that endothelium–monocyte interaction is the key role of the development of atherosclerosis [22, 23]. We have we conducted a preclinical study using a coculture system of human umbilical vein endothelial cells (HUVECs) with human monocytic cell lines (THP-1) By enableing direct cell to cell contact. The study was designed to evaluate wether the 5-HMF, a naturally occurring bioactive compound of many heat treated foods inhibits secretion of ICAM-1 and MMP-9.

Methods

Cell culture

HUVECs were purchased from the Pasteur Institute of Iran and were cultured in DMEM/F12 medium enriched with 10 % fetal bovine serum (FBS), 100 IU/mL penicillin, 80µg/mL streptomycin. The cells were subjected to 2–4 passages and incubated in a 5% CO2 humidified atmosphere at 37 °C. The THP-cells (Pasteur institute of Iran) were also grown, separately in a suspension culture in RPMI-1640 medium containing 10% FBS, 100 IU/mL penicillin, 80µg/mL streptomycin and supplemented with 0.05 mM 2-mercaptoethanol in a humidified 5% CO2 atmosphere at 37 °C.

Coculture system

HUVECs were grown to confluence on 24-well plates (2 × 105 cells/ well). After 48 h, THP-1 (1 × 105 cells/ well) seeded onto confluent HUVECs. Cocultures were then stimulated with lipopolysaccharide (LPS) (1µg/mL) and treated with 5-HMF (Matrix Scientific, Pontiac Business Center Drive, Elgin, USA) (at doses of 10, 30 and 50µg/mL). After 24 and 48 hour incubations, the supernatants were collected immediately and centrifuged to remove the cells. Then they were frozen at −80°C until the analysis.

MTT assay

Cell viability was assessed by MTT assay. HUVECs cells and Coculture cells were separately seeded on to 96-well plates. After 24h incubation, the cells were exposed to various concentrations of 5-HMF (10, 30, 50 µg/mL) andincubated for 24 and 48 hours, On the next step all of the medium was removed and 50 µL of MTT [3-(4,5-Dimethylthiazol-2-yl] with 150 µL of medium was added and incubation for 4 hours in a 5% CO2 humidified atmosphere at 37 °C. Then, 200 µL of dimethylsulfoxide was added to help dissolving of the formazan crystals produced by the cells. Finally, the plates were then agitated on a plate shaker for 5 minutes and the absorbance was determined at 540nm using a microplate reader.

Assessment of ICAM-1 and MMP-9 in coculture supernatants

Cocultures were incubated for 24 or 48 h. At each time point, culture medium was collected and centrifuged at 400 g for 5 min to eliminate nonadherent cells. The resultant supernatants were subjected to enzyme-linked immunosorbent assay (ELISA) analysis of ICAM-1 and MMP-9 levels. Levels of ICAM-1 and MMP-9 in coculture supernatants were measured following manufacturer's instructions using a commercially available kit (eBioscience).

In brief, monoclonal antibody coated to the microwells were binded with human sICAM-1 and MMP-9 in the coculture supernatants. Then, an incubation was performed with the HRP-conjugated anti-human sICAM-1 or biotin-conjugated anti-human MMP-9 antibodies. Finally, TMB substrate solutions were added to the plates and after 10 min incubation in the dark, a stop solution was added. Absorbance was measured at 450 nm in a microplate reader (Model 680; Bio-Rad Laboratories,Inc., USA).

Statistical analysis

Data were presented as mean ± SD. All data were tested for normal distribution and equal variance by the Kolmogorov-Smirnov test. Comparisons among the treatment groups were performed using one-way ANOVA followed by the Tueky's post hoc comparison.

Results

Effects of 5-HMF exposure on HUVECs and coculture of HUVECs and THP-1 cell viability

To determine the effects of 5-HMF on HUVECs and coculture of HUVECs and THP-1 cell viability the MTT assay test was applied after culturing with serial concentrations (10µg/mL, 30µg/mL, 50µg/mL) of 5-HMF for 24 and 48 hours. As shown in figure 1, viability of HUVECs was decreased after exposure with 30µg/mL of 5-HMF (by 80%). However, the viability in the other doses was comparable with control cells after 24 hours. On the other hand, it was found that viability was increased following exposure with different concentrations of 5-HMF in the coculture system cell (fig.2). In addition, treatment of the cells with 30 µg/ml of 5-HMF enhanced the coculture viability as compared to negative control group ($p<0.05$).

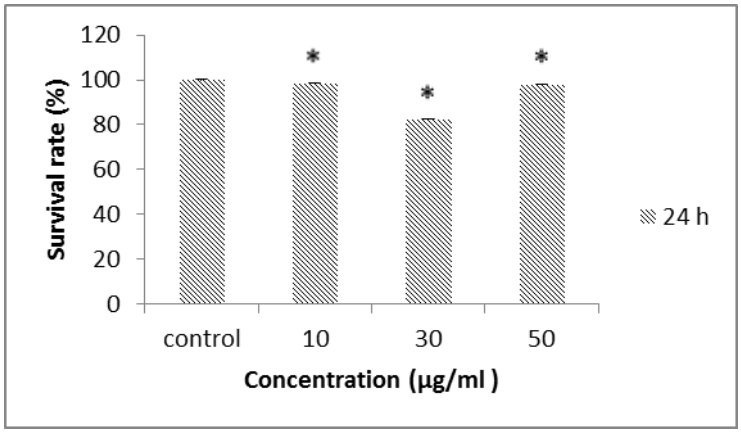

Fig.1. The effects of 5-HMF on HUVECs cell viability at different concentrations. HUVEC cells were pretreated with LPS and followed by treatment with various concentrations of 5-HMF for 24h. Cell viability was measured by MTT assay. * represent significant difference compared to the control group ($p < 0.01$).

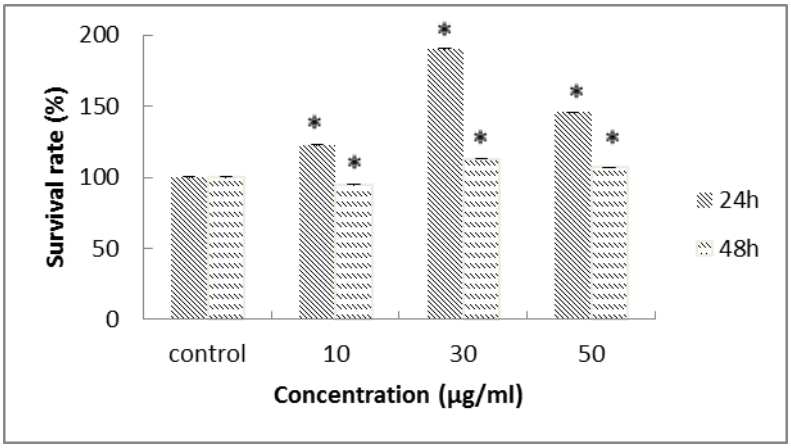

Fig.2. The effects of 5-HMF on coculture of HUVECs and THP-1 cell viability at different concentrations. Coculture cells were pretreated with LPS and various concentrations of 5-HMF for 24h and 48h. Cell viability was measured by MTT assay. *The data represent significant difference compared to control group ($p < 0.01$)

Effect of 5-HMF on MMP-9 and ICAM-1 secretion in coculture system:

To observe the effect of 5-HMF on some inflammatory markers involved in induction of atherogenesis, we determined levels of MMP-9 and ICAM-1 secretion in a system of LPS-stimulated coculture of HUVECs and THP-1. There were a significant differences in mean value of MMP-9 between the groups with various doses of 5-HMF (Table 1). The MMP-9 concentration of LPS stimulated coculture system after 24h treatment with 5-HMF at the dose of 10 µg/mL was 3.2 times more than that of dose of 30. In addition, the secretion of MMP-9 was slightly decreased at the dose of 50 µg/mL compared with the lowest exposure dose ($P < 0.01$). However, there was no significant difference between treatment with 30 µg/mL and 50 µg/mL. After 48h coculture, 5-HMF incorporation resulted in reduction of MMP-9 concentration at the dose of 10 µg/mL by 1.2 and by 1.1 times at dose of 30 µg/mL and 50 µg/mL. Also, level of MMP-9 in the culture supernatents in dose of 50 was significantly lower than 30 ($P < 0.001$) .

As shown in Table 2, analysis of ICAM-1 secretion in LPS-stimulated coculture of HUVECs and THP-1 system revealed any significant difference between different groups.

Table 1. The concentration of MMP-9 (ng/ml) in the coculture system

Groups (µg/mL)	24h	48h
10	0.513±0.237	0.119±0.048
30	0.158±0.054a	0.146±0.037b
50	0.133±0.109a	0.133±0.089bc

Comparisons were performed between different doses of 5-HMF. The data are expressed as mean±SD. Different letters represents statistical difference within each time point using ANOVA**.**

Table 2. The concentration (ng/ml) in the of ICAM system coculture

Groups (µg/mL)	24h	48h
10	29.31±19.39	32.28±7.65
30	30.91±4.77	33.70±3.89
50	33.53±5.34	28.86±5.64

Comparisons were performed between different doses of 5-HMF, $P < 0.05$ was considered as significant difference. No statistical significance was noted.

Discussion

The mechanism underlying atherosclerosis has been examined during last decades, and there is an agreement that endothelium–monocyte interaction has a key role in the development of atherosclerosis [22, 23]. It has become increasingly obvious that 5 - HMF has beneficial effects, including antitumor, cytoprotective, and antioxidant potentials rather than adverse properties. Additionally, it has been represented that 5-HMF inhibited atherosclerosis and has the similar protection potential to atrovastatin in terms of anti-oxidatory, anti-inflammatory and lipid-lowering properties in apoE-deficient mouse model [24]. It has been indicated that 5-HMF has antioxidant properties, and can protect human hepatocytes against cytotoxicity stimulated by H_2O_2 and HUVECs against cytotoxicity stimulated by glucose or H_2O_2 [25]. In another study, it was demonstrated that the 5-HMF purified from the extract of aged black garlic represents anti-inflammatory effects on HUVECs via inhibition of VCAM-1 expression, ROS production, NF- κB activation and monocyte adhesiveness [21]. So, the present study was done to examine the effects of 5-HMF on secretion of the ICAM and MMP-9 in LPS-stimulated coculture of HUVECs and THP-1.

Among various adhesion molecules, ICAM-1 is considered to play a key role when circulating monocytes attach to the endothelium and then transmigrate into the intima. In order to confirm role of ICAM-1 molecules on atherosclerosis in animal models, previous experiment using ICAM-1 knockout mice has indicated decreased atherosclerosis under ICAM-1 deficiency [26]. Another reports supported the notion that the amount of soluble intercellular adhesion molecule-1 (sICAM-1) is closely associated to the severity of atherosclerosis and cardiovascular incidents, and also offered that inhibition of ICAM-1 can postpone the development of atherosclerosis [27, 28].

On the other hand, MMP-9, is considered to be a critical protein in inflammation process. This protein cleaves components of ECM in the atherosclerotic plaque, such as elastin, collagen, and proteoglycans, and as such is a key modulator of plaque stability [29, 30]. High level of MMP-9 was observed in progressive atherosclerotic lesions in the mouse model [31]. Gough et al. demonstrated that macrophage expression of active form, but not pro-MMP-9, stimulated plaque disruption [32]. MMP-

9 is overexpressed in advanced atherosclerotic plaques obtained from humans undergoing endarterectomy. The recent investigation provides support by indicating an elevated danger of advanced atherosclerosis and unstable plaques in patients with higher levels of MMP-9 [33]. At the present, there is no report about effect of 5-HMF on MMP-9 expression.
The HUVEC-related inflammatory response is stimulated by a range of inflammatory mediators, including oxidized-low density lipoprotein, tumor necrosis factor-α and LPS. LPS is a major part of the outer membrane of gram-negative bacteria. LPS induces monocytes/macrophages, causing the activation of a set of signaling events that potentiate the generation of inflammatory mediators. LPS elicits the production of reactive oxygen species (ROS) and upregulates adhesion molecules, chemokines and cytokines in HUVECs [34]. These mechanisms have key role in endothelial dysfunction and may cause endothelial cell-associated illnesses. A number of reports have attempted to recognize methods to protect against the inflammatory response and death of endothelial cell [35, 36]. We applied LPS as inflammatory factor in present study. For more investigation of role of 5-HMF on inflammation, we surveyed the effect of different doses of 5-HMF on expression of ICAM-1 and MMP-9 on HUVEC and monocytes in presence of LPS.

Our results indicated that co-culture of HUVECs and monocytes along with different doses of 5-HMF decreases the secretion of MMP-9 whereas no meaningful statistical difference was observed in secretion of ICAM-1. During inflammatory process, the expression and secretion of MMP-9 is stimulated by proinflammatory mediators. MMP-9 mediates leukocyte migration during inflammation process [37]. Furthermore, it has been indicated 5-HMF has anti-inflammation potential during atherosclerosis. Thus, reduction in secretion of MMP-9 may be one of the reasons behind anti-inflammatory potential of 5-HMF.
Kim et al. determined 5-HMF purified from the extract of black garlic has anti-inflammatory potential in tumor necrosis factor-α (TNF-α)-induced HUVECs. Treatment of HUVECs with 5-HMF significantly suppressed TNF-α-stimulated cell surface and total protein and mRNA expression of ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1). Their results suggest novel evidence of the anti-inflammatory potential of 5-HMF in verification of its potential therapeutic application for the inhibition and control of vascular diseases such as atherosclerosis through mechanisms involving the prevention of NF-κB activation andVCAM-1 expression in vascular endothelial cells [21]. So, our results are in contrast with the Kim et al. study which suggests 5-HMF significantly suppressed expression of ICAM-1.

As depicted in fig1, HUVECs cell viability decreased by 5-HMF treatment at concentrations of 10, 30 or 50 µg/ml. Thus, 5-HMF has no anti-apoptotic effect on HUVECs. On the other hand, cell viability of coculture system was increased with different concentration of 5-HMF. Therefore, 5-HMF remarkably blocked LPS-stimulated apoptosis of coculture cells. Since 5-HMF block ROS production due to LPS, 5-HMF may prevent migration of inflammatory cell into tissue. Since hyperproduction of ROS stimulates cell death mediated by cytotoxicity, the anti-apoptotic effect of 5-HMF was hypothesized in coculture. Our results are in accordance with the Gang Cao et al. study which confirm protective effects of 5-HMF on HUVECs from oxidative stress at different concentrations by increased the viability of HUVECs [38]. Additionally, in another study, Wang et al. offered anti-apoptosis and protective effect of 5-HMF on hepatocytes [39]. Taken together, inflammation plays a critical role in the initiation and progression of atherosclerosis, and Tan et al. have recognized MMP-9 as key mediator [40]. It has been well established that the treatment of endothelial cells with TNF-α will significantly enhance the expression of adhensive proteins such as ICAM-1 and VCAM-1. So, it is suggested to stimulate TNF-α HUVECs by TNF-α in additionto LPS. Furthermore, more studies needs to examine accurate mechanism of low expression of MMP-9 after treatment with 5-HMF and possible complications.

Conclusion

In conclusion, our data suggests that 5-HMF may inhibit damage from inflammation. Although the underlying mechanisms are not completely elucidated, the protective effect of 5-HMF is likely related to low secretion of MMP-9 protein. The detailed mechanisms need to be more surveyed. Furthermore, this results confirms that 5-HMF has anti-inflammatory properties in HUVECs that include blockage of cell apoptosis and secretion reduction of MMP-9. So, 5-HMF may be a beneficial agent to prevention and management endothelial dysfunction -related illnesses such as atherosclerosis and cardiovascular diseases.

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Disclosure statement

The authors have declared that there is no conflict of interest.

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