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ORIGINAL ARTICLE

(-)-Epigallocatechin-3-Gallate Protects against Uric Acid-Induced Endothelial Dysfunction in Human Umbilical Vein Endothelial Cells

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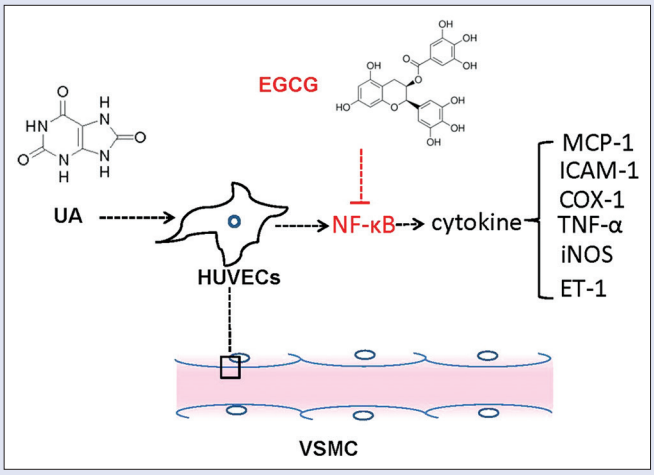
ABSTRACT

Background: Hyperuricemia is recently reported to be associated with hypertension, metabolic syndrome, and vascular damage. (-)-Epigallocatechin gallate (EGCG) is a major polyphenol component of green tea involving in potent anti-inflammatory and antioxidant effects. The study assessed the effect of EGCG on uric acid (UA)-induced vascular damage. **Materials and Methods:** The cell viability and angiogenic formation of human umbilical vein endothelial cells (HUVECs) treated with UA and EGCG were determined by methylthiazol tetrazolium and tube formation assays, respectively. The expression level of inflammatory cytokine and vascular factor, including nuclear factor-kappa B (NF-κB), monocyte chemotactic protein-1 (MCP-1), intercellular adhesion molecule 1 (ICAM-1), cycloxygenase-2, tumor necrosis factor (TNF-α), induced nitric oxide synthetase (iNOS), endothelin-1 (ET-1), and von Willebrand factor, were examined by real-time quantitative reverse transcription polymerase chain reaction. The expression of P65 in HUVECs treated with UA in different time points or different concentrations was detected by Western blotting. **Results:** EGCG suppressed UA-inducing HUVEC cells death. UA treatment of HUVEC significantly increases the expression of P65. EGCG significantly inhibited the UA-induced mRNA expression of NF-κB, MCP-1, ICAM-1, TNF-α, iNOS, and ET-1 in HUVECs. Functional analysis of angiogenic inhibition showed that pretreatment with EGCG improved the vascular tube formation. **Conclusion:** Our results suggested that UA-induced inflammatory cytokine production and impaired endothelial cell function in HUVECs were attenuated by EGCG. These data indicated that EGCG has a therapeutic potential for UA-mediated endothelial damage.

Key words: (-)-Epigallocatechin-3-gallate, angiogenic formation, endothelial cell, inflammation, uric acid

SUMMARY

- Hyperuricemia is recently reported to play a role in hypertension, metabolic syndrome, and vascular damage. (-)-Epigallocatechin gallate (EGCG) is a major polyphenol component of green tea with potent anti-inflammatory and antioxidant effects. The present study was undertaken to assess the effect of EGCG on uric acid (UA)-induced vascular damage. Our results suggest that UA-induced inflammatory cytokine production and impaired endothelial cell function in human umbilical vein endothelial cells can be attenuated by EGCG. Our study provides new insight for UA-related cardiovascular disorder.



Abbreviations used: EGCG: (-)-Epigallocatechin-3-gallate; HUVECs: Human umbilical vein endothelial cells; UA: Uric acid; NF-κB: Nuclear factor-kappa B; MCP-1: Monocyte chemotactic protein-1; ICAM-1: Intercellular adhesion molecule 1; COX-2: Cycloxygenase-2; TNF-α: Tumor necrosis factor; iNOS: Nitric oxide synthase; ET-1: Endothelin-1; VSMC: Vascular smooth muscle cell.

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INTRODUCTION

Hyperuricemia has been long recognized as the major etiologic factor in gout.[1] In recent years, a number of epidemiologic studies have shown that hyperuricemia may play an important role in the development and pathogenesis of many metabolic, hemodynamic, and systemic pathologic diseases, including metabolic syndrome, hypertension, stroke, and atherosclerosis.[2-5] In 2004, a prospective cohort study showed that hyperuricemia may be an independent risk factor for cardiovascular disease (CVD) in middle-aged men.[6] Recent studies have consistently supported that hyperuricemia may be an independent risk factor for hypertension. A systematic review with meta-analysis of 26 prospective cohort studies has shown a modest but significant association between hyperuricemia and coronary artery disease (CAD) events, independent of traditional risk factors.[7] The overall risk of CAD death increased

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by 12% for each increase of 1 mg/dl serum uric acid (SUA), although hyperuricemia appeared to significantly increase the risk of death only in women.[7] Neogi *et al.*[8] have assessed that SUA was-associated with carotid plaques in men, even in the absence of other cardiovascular risk factors in a large multicenter study.

The mechanism of hyperuricemia in CVD has not been well-known. Many experimental and epidemiological data have suggested that a possible role for hyperuricemia is endothelial dysfunction secondary to inflammation and oxidative stress.[9-11] In vascular smooth muscle cell (VSMC), uric acid (UA) may activate the transcription factors nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) as well as the mitogen-activated protein kinase (MAPK) signaling molecules, extracellular signal-regulated kinase p44/42 and p38, which stimulate monocyte chemotactic protein-1 (MCP-1), intercellular adhesion molecule 1 (ICAM-1), and cycloxygenase-2 (COX-2) expression.[12] UA also increases the upregulation of C-reactive protein in both VSMCs and endothelial cells, which directly causes the development of renal microvascular disease, possibly increasing blood pressure.[13] Endothelial cell nitric oxide synthetase (NOS) maladjustment plays an important role in initiating the vascular endothelial dysfunction associated with UA.[14,15] Studies in rat and cell culture models have shown that this endothelial dysfunction is due to the inhibition of endothelial generation of nitric oxide by UA.[9] Soluble UA has complex pro and con effects on vascular pathology depending on different cellular environments.[16] Compounds that enhance the excretion of UA or inhibit UA biosynthesis or have anti-inflammatory actions are generally used for the treatment of gout and hyperuricemia. Allopurinol, the most common and perhaps only inhibitor of xanthine oxidase (XO), is used in clinical practice. However, allopurinol has severe adverse effects on liver and renal function or allergic reactions.[17] Therefore, it is particularly important to find natural substances that can reduce UA and protect blood vessels.

Green tea is a popular beverage with beneficial health effects. (-)-Epigallocatechin-3-O-gallate (EGCG), the main catechin in green tea, has anti-inflammatory, antiatherosclerotic, and antioxidant properties in experimental studies conducted *in vivo* and *in vitro.*[18-20] Previous studies have also demonstrated that EGCG inhibited tumor necrosis factor (TNF-α)-induced production of MCP-1/CCL2 from bovine coronary artery endothelial cells, providing direct vascular benefits in inflammatory CVDs.[21] Further, EGCG may prevent MCP-1 expression by blocking p38 MAPK, AP-1, and NF-κB in endothelial cells. Yang *et al.* has shown that EGCG suppressed high glucose-induced vascular inflammatory process through the inhibition of protein kinase C and NF-κB activation in human umbilical vein endothelial cells (HUVECs).[22] Otherwise, EGCG was an inhibitor of XO.[23] However, the effect of EGCG on UA-induced endothelial dysfunction in endothelial cells has not been investigated.

In this study, EGCG is detected whether it decreases the UA-induced inflammation response in HUVECs and improves the tube formation of the vascular endothelium.

MATERIALS AND METHODS

Materials

UA, EGCG powder (≥95%), pyrrolidine dithiocarbamate, and other reagents were obtained from Sigma (St. Louis, MO, USA). EGCG stock solution was prepared in sterile double-distilled water at 20 mM. UA was dissolved in deionized water and filtered and was free of crystals (by polarizing microscopy), endotoxin (limulus amebocyte assay; BioWhittaker Inc, Walkersville, MD), and mycoplasma contamination (ImmuMark Myco-Test, ICN Biomedicals, Irvine, CA, USA).[24] NF-κB (p65) was purchased from Abcam Biotechnology (Abcam, British).

Cell culture

The HUVECs were purchased from American type culture collection (Rockville, MD, USA). HUVEC cells were grown in endothelial growth media-2 bullet kit (Lonza, Basel, Switzerland) containing penicillin G (100 units/ml; Sigma-Aldrich) and streptomycin sulfate (100 μg/ml; Sigma-Aldrich) at 37°C in a humidified atmosphere of 5% CO_2. HUVECs were treated with different concentrations of UA (2, 4, 8, and 16 mg/dl) at or different time (0, 24 h) for cell viability analysis. HUVEC cells were cultured with UA (8 mg/dl) for another 24 h after pretreating with or without EGCG (10, 20, and 50 μM) for real-time polymerase chain reaction (RT-PCR).

Cell viability analysis

To assess the effects of UA and EGCG on cell viability, the methylthiazol tetrazolium assay was used as previously described.[25] Briefly, cells (3×10^5 cells/well) in log phase were plated into 96 well plate and treated with UA in different concentrations or different periods. On the other hand, for the pretreatment, EGCG was added to the culture media (10, 20, and 50 μM) 12 h before UA (8 mg/dl) treatment and further incubated together for another 24 h. After treatment with UA at indicated concentrations of 24 h, 10 uL of WST-1 was added to each well and the plates were further incubated for 2 h at 37°C. The absorbance was measured using a spectrophotometer (molecular devices, Sunnyvale, CA, USA) at 450 nm.

Tube formation assay

Matrigel matrix 400 μl (BD, Bedford, MA, USA) using a chilled pipet tip was added into a 24 well plate and incubated at 37°C for 1 h to allow solidification to occur. HUVECs (8×10^4 cells/well) in 0.5 ml of growth medium were seeded on the matrigel in each well. The plate was then incubated at 37°C with 5% CO_2 for 6 h.[26] For the pretreatment, 50 μM EGCG was added to the culture media 12 h before UA (8 mg/dl) treatment and further incubated for another 24 h. The images of tube formation were captured under a light microscope (Leica DMLB [Leica, German]) from 5 random fields in 100 magnification.

Real-time quantitative polymerase chain reaction

Total RNA was extracted by using trizol (Invitrogen Corp., Carlsbad, CA, USA). The transcriptor first-strand cDNA synthesis kit (Roche, Mannhein, Germany) was used according to the manufacturer's protocol to synthesize cDNA from 500 ng total RNA. The resulting first-strand cDNA was amplified in a final volume of 20 μL containing 10 pmol of each primer and 1U of taq polymerase (TaKaRa, Shuzo, Japan). The primers that were used for the PCR amplifications was listed in Table 1 primer sequences for the quantitative RT-PCR. The oligonucleotide primers were synthesized by Sangon Technologies, Inc., (Shanghai, China). All values were calculated using $2^{-\Delta\Delta Ct}$ method and expressed as the change relative to the expression of glyceraldehyde phosphate dehydrogenase (GAPDH). The amplification conditions were 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 30 s.

Western blot analysis

The cells were rinsed with ice-cold phosphate-buffered saline (PBS) and lysed with lysis buffer (1% triton, 1% nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), and 1% deoxycholate in PBS). Supernatants were mixed with an equal volume of 2 × SDS sample buffer, boiled for 5 min, and separated on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred onto a polyvinylidene difluoride (Millipore, Bedford, MA, USA) membrane. The membranes were blocked for 2 h in 5% skim milk, rinsed, and incubated overnight at

Table 1: Primer sequences for the quantitative real-time polymerase chain reaction

Gene name	Forward	Reverse
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC
NF-kB	GAAGCACGAATGACAGAGGC	GCTTGGCGGATTAGCTCTTTT
COX-2	CAGCCAGATGCAATCAATGCC	TGGAATCCTGAACCCACTTCT
ICAM-1	TTGGGCATAGAGACCCCGTT	GCACATTGCTCAGTTCATACACC
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
ET-1	AAGGCAACAGACCGTGAAAAT	CGACCTGGTTTGTCTTAGGTG
vWF	AGCCTTGTGAAACTGAAGCAT	GCCCTGGTTGCCATTGTAATTC
iNOS	AGGGACAAGCCTACCCCTC	CTCATCTCCCGTCAGTTGGT

GAPDH: Glyceraldehyde phosphate dehydrogenase; NF-kB: Nuclear factor-kappaB; COX-2: Cycloxygenase-2; ICAM-1: Intercellular adhesion molecule 1; TNF-α: Tumor necrosis factor; ET-1: Endothelin-1; vWF: Von Willebrand factor; iNOS: Induced nitric oxide synthetase

4°C with primary anti-p65 antibodies. Membranes were then incubated for 1 h with horseradish peroxidase-conjugated secondary antibody at room temperature. GAPDH (Bioworld, Nanjing, China) on the same membrane was used as a loading control. After 3 time washes in PBST, protein bands were visualized using SuperSignal West Femto Substrate (Pierce, Rockford, IL, USA).

Statistical analysis

Data were expressed as the mean ± standard deviation of at least three independent experiments. Statistical analyses were performed by a two-tailed *t*-test between two groups; one-way ANOVA analysis was used to test the differences among three groups. Statistical significance was set at $P < 0.05$.

RESULTS

Protective effects of (-)-epigallocatechin gallate against uric acid-induced cell death in human umbilical vein endothelial cells

To evaluate the effects of EGCG on cell viability *in vitro*, UA affected cell viability in dose-dependent manner [Figure 1a]. Then, HUVECs were pretreated with 0–50 μM EGCG for 12 h and then exposed to 8 mg/dl UA for 24 h. As shown in Figure 1b, low doses of EGCG (10 μM) did not affect cell viability, but middle and high doses of EGCG (20 and 50 μM) improved cell viability. UA exposure also resulted in dose-dependent cell damage; however, moreover, we found that EGCG exerted a significant protective influence against UA-induced cell death [Figure 1b], thereby providing an insight into the inflammation mediating UA-induced endothelial damage in HUVECs for 24 h.

Uric acid activates nuclear factor-kappa B expression in human umbilical vein endothelial cells

Since UA could induce NF-κB expression in VSMC,[12] we speculated that NF-κB expression may be regulated by UA in HUVECs. We examined the expression of activated p65 NF-κB protein in HUVECs incubated with UA for various time periods and different dose. UA induced a significant decrease in p-NF-κB-p65 protein levels in a dose-dependent manner [Figure 2a]. In the presence of 8 mg/dl UA, the total p65 NF-κB protein levels decreased by nearly 50% ($P < 0.01$ vs. control). As shown in Figure 2b, the total p65 NF-κB was observed as early as 4 h after UA incubation. Meanwhile, the total p65 NF-κB abundance peaked at 24 h. Accordingly, 8 mg/dl UA was used to induce the HUVECs after various doses of EGCG pretreat in the next experiment.

Protective effect of (-)-epigallocatechin gallate on uric acid-induced mRNA expression of inflammatory cytokines and vascular factor in human umbilical vein endothelial cells

Inflammatory cytokine and vascular factor could be activated by NF-κB, which was important in endothelial damage.[12-14] Quantitative RT-PCR was performed to determine the expression of NF-κB and inflammatory cytokine and vascular factor gene transcription in HUVECs. RT-PCR analysis showed that EGCG decreased UA-mediated NF-κB mRNA expression in a dose-dependent fashion [Figure 3a]. We also examined the effect of EGCG on UA-induced MCP-1, ICAM-1, COX-2, and TNF-α mRNA expressions. Compared to control, MCP-1 mRNA expressions were decreased according to dose-dependent EGCG [Figure 3b]. ICAM-1, COX-2, and TNF-α mRNA expressions were markedly increased after induction of hyperuricemia, however, decreased after pretreated different dose EGCG, which were consistent with NF-κB mRNA expression [Figure 3c-e]. We further investigated the expression of induced NOS (iNOS), endothelin-1 (ET-1), and von Willebrand factor (vWF) produced by endothelial cells for regulating vascular function. As presented in Figure 3f-h, measured by RT-PCR assay, iNOS expression was also markedly decreased at all EGCG groups; the change was dose dependent. ET-1 expression reduction was observed in 40 μM EGCG. There was no significant difference in vWF between these groups.

Protective effect of (-)-epigallocatechin gallate on uric acid-induced angiogenic inhibition

To estimate the association of the HUVECs functions with EGCG on UA-induced, we used tube formation assay. In control, after 12 h, tuber formation could be observed in inverted microscope [Figure 4a]. As shown in Figure 4b, UA (8 mg/dl) with HUVECs was effective at tube formation. Based on these findings, we investigated the effect of EGCG on UA-induced angiogenic inhibition. Relative cell angiogenesis was shown in Figure 4c. Compared with UA, pretreat with EGCG promoted tuber formation in HUVECs.

DISCUSSION

In the present study, results show the protective role of EGCG against UA-induced endothelial cell *in vitro*. These data suggest that EGCG polyphenols attenuate inflammatory responses.

Many studies have documented that SUA is a risk factor for CVD.[27-29] Dysfunction of the endothelium is regarded as an important factor in the pathogenesis of vascular disease in CVD.[30] Inflammation is a critical factor in the pathogenesis and destabilization of endothelial dysfunction.[31,32] Recent studies have demonstrated that an elevated SUA

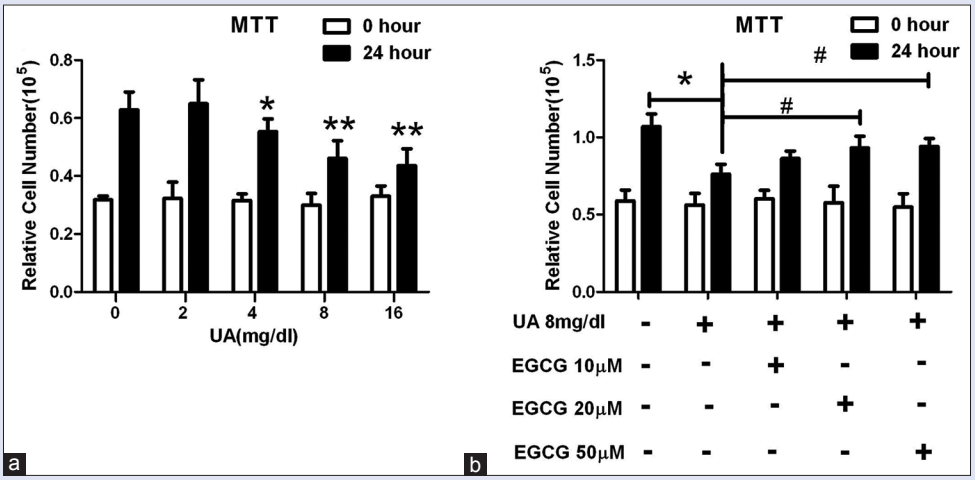

Figure 1: Effects of (-)-epigallocatechin gallate against uric acid-induced cell viability. (a) Uric acid. (b) (-)-epigallocatechin gallate (*$P < 0.05$ vs. control, **$P < 0.01$ vs. control, #$P < 0.05$ vs. uric acid alone)

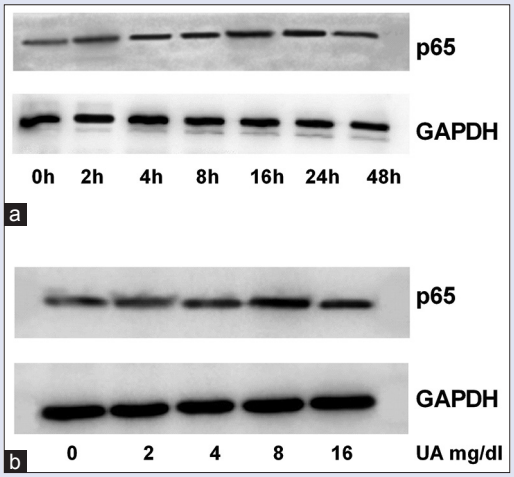

Figure 2: Nuclear factor-kappa B expression in human umbilical vein endothelial cell by uric acid induction. (a) Human umbilical vein endothelial cells were pretreated with 8 mg/dl uric acid in difference times. (b) Human umbilical vein endothelial cells were pretreated with 0–16 mg/dl uric acid in 24 h

level is also associated with circulating levels of systemic inflammatory mediators in a variety of conditions, including congestive heart failure.[33,34] In experimental animal models, elevated SUA levels are associated with vascular disease *in vivo*.[35] *In vitro*, UA can increase MCP-1 production in rat VSMCs. The activation of p38 MAPK and NF-κB and AP-1 are also increased.[12] There is accumulating evidence of the harmful effects of persistent hyperuricemia in the endothelium such as accelerating inflammatory response and inducing endothelial barrier dysfunction.[7] In the current studies, we confirmed that the UA-induced increase in NF-κB protein expression was in a time- and dose-dependent manner. Along with dose increased, the expression of NF-κB is increased. At 24-h incubation period, the expression of NF-κB is the top. UA not only induces inflammatory cytokine production but also impairs vascular endothelial cells integrity. This study found that UA significantly suppresses endothelial cell functions which could be extenuated if pretreated with EGCG by tube formation assay.

Until the past decade, the traditional conception that green tea consumption is beneficial to health has received great attention.[36-38] Epidemiological and experimental observations have confirmed that there is a positive relationship between green tea consumption and protection from CVD including atherosclerosis.[16] EGCG, a major component of tea polyphenol, improves atherosclerosis attributed to exhibit wide pharmacological activity, such as reducing inflammation and attenuating myocardial ischemia-reperfusion injury and repressing VSMC proliferation.[36] It is critical factor for EGCG anti-inflammatory activity to protect endothelium. In recent years, studies showed that EGCG attenuates the increased vascular inflammation induced by some factors. Pretreatment with EGCG inhibited the augmentation of VCAM-1 expression in vascular endothelial cells exposed to high glucose. Li *et al*. have shown that EGCG attenuated lipopolysaccharide-induced inflammatory cytokine production in human cerebral microvascular endothelial cells.[19] The results are consistent with other studies.[21,22] In the current study, varying concentrations of EGCG (10–50 μM) inhibited the UA-induced vascular inflammation in cultured HUVECs in a dose-dependent manner. It is observed that Pro inflammatory mediators, such as MCP 1, ICAM 1, COX 2, TNF-α decreased.

The molecular link between EGCG and pro-inflammatory mediators' expression under UA conditions may involve NF-κB activation. Normally, NF-κB, which is composed of heterogeneous dimer p65 and p50, are not activated in the cytoplasm.[18,22] It has been reported that NF-κB plays an important role in the upregulation of various cytokine and chemokine in endothelium, VSMC, and tumor.[18,39] EGCG has been implicated in the prevention of atherosclerosis. Many studies have shown that EGCG protected the endothelial cell through NF-κB pathway in different conditions of induction, such as high glucose,[22] NOS,[40] and the phorbol 12-myristate 13-acetate.[41] In the present study, EGCG attenuates vascular inflammatory response induced by UA, perhaps involving NF-κB pathways. However, in the nucleus, NF-κB activates genes that respond to inflammation and oxidative stress.[42] Oxidative stress is another key role in endothelium impaired by UA induction.[43] In addition, the antioxidant characteristics of EGCG have been well documented.[44] Further studies will be needed to clarify the precise relationship between NF-κB and ROS in UA-mediated ototoxicity.

Angiopoiesis test is mostly used in tumor research for the cell function. To evaluate the endothelial cells' integrity, tube formation assay was applied. EGCG significantly promotes HUVECs' tube formation, which suggests that EGCG can attenuate the inhibition of UA and protects the endothelium integrity.

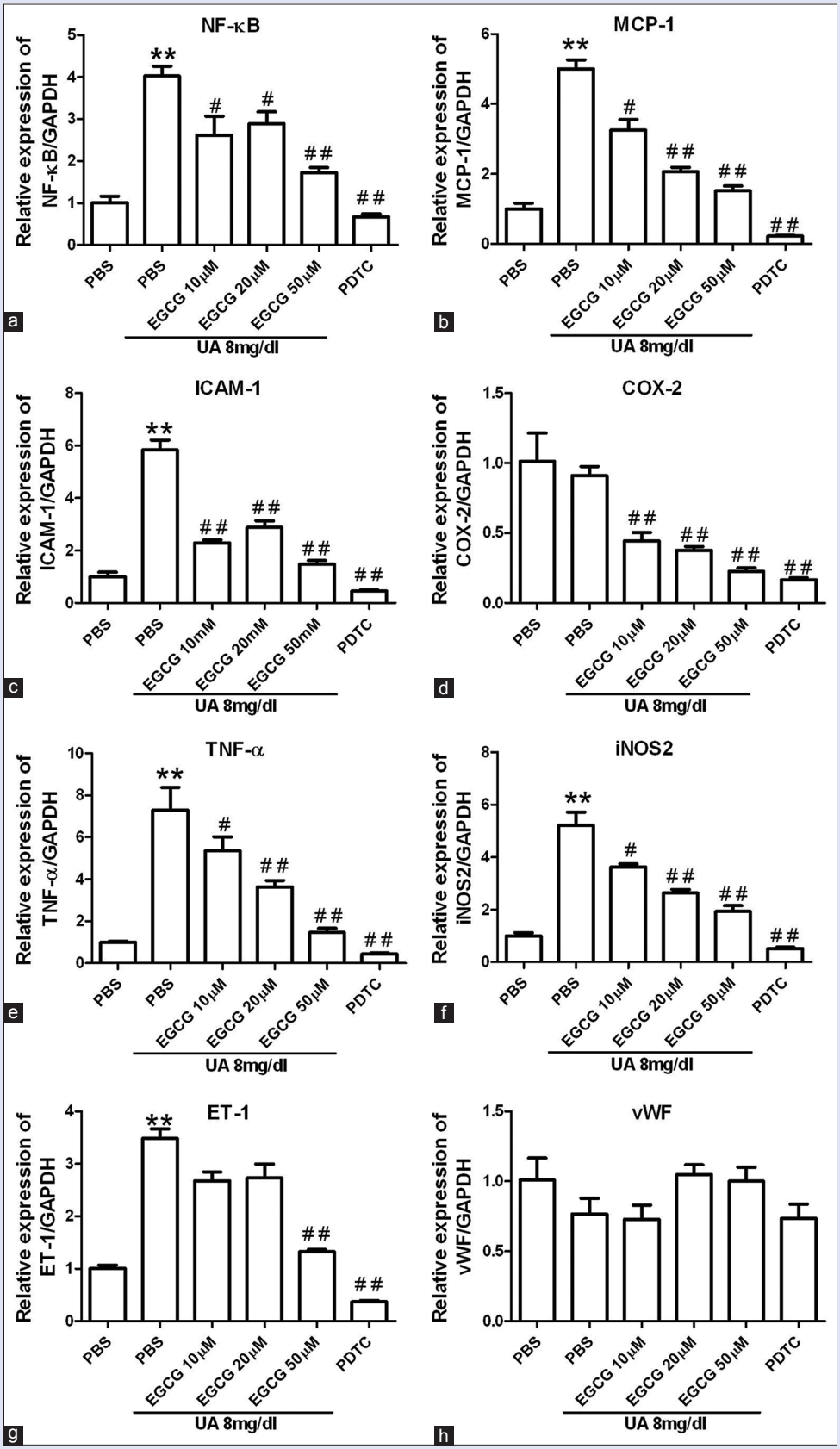

Figure 3: Effect of (-)-epigallocatechin gallate on uric acid-induced expression of inflammatory cytokines and chemokines in human umbilical vein endothelial cells. Human umbilical vein endothelial cells were pretreated with 0–50 μM (-)-epigallocatechin gallate for 12 h followed by treatment with 8mg mg/dl uric acid for 24 h. (a) Nuclear factor-kappa B. Glyceraldehyde phosphate dehydrogenase was used as an internal cytosolic marker control. (b) Monocyte chemotactic protein-1. (c) Intercellular adhesion molecule-1. (d) Relative levels of cycloxygenase-2 are shown. (e) Tumor necrosis factor-α. (f) Induced nitric oxide synthetase. (g) Endothelin-1. (h) The levels of von Willebrand factor (**$P < 0.01$ vs. control, #$P < 0.05$ vs. uric acid alone, ##$P < 0.01$ vs. uric acid alone)

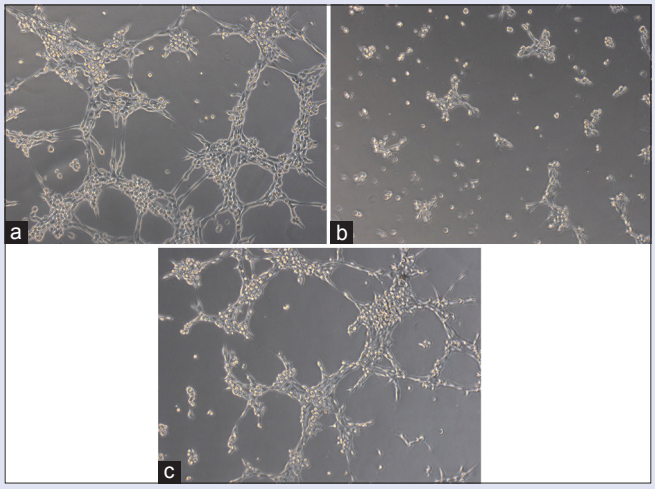

Figure 4: Protective effect of (-)-epigallocatechin gallate on uric acid-induced angiogenic inhibition × 100. (a) Control. (b) 8 mg/dl uric acid. (c) 50 μM (-)-epigallocatechin gallate + 8 mg/dl uric acid

CONCLUSION

This study demonstrates that UA-induced inflammatory cytokine production and impaired endothelial cell functions could be inhibited by EGCG in HUVECs. These results raise the possibility that EGCG is a possible candidate for the treatment and prevention of the vascular complication associated with hyperuricemia.

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Conflicts of interest

There are no conflicts of interest.

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