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Original Article

Theaflavins ameliorate palmitic acid-induced human umbilical vein endothelial cells injury and mitochondrial dysfunction

Guang-Xue Fu[1], Ning Pan[2], Xiao-Feng Qin[2], Qing-Hui Li[1], Yu-Dong Chen[1]

[1]*Department of Cardiovascular Medicine, Central Hospital of Shengli Oil Field, Dongying 257000, China;*
[2]*Department of Emergency Medicine, Central Hospital of Shengli Oil Field, Dongying 257000, China*

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Abstract: The study was performed to investigate the influence of theaflavins on cell apoptosis, mitochondrial dysfunction and DNA damage in PA-induced atherosclerotic HUVECs injury model. The results indicated that treatment of HUVEC with PA induced cell death in a time-dependent manner, and the number of HUVECs was markedly decreased in PA treatment group. However, co-incubated with PA and theaflavins in HUVECs, the cell viability was significantly increased, and apoptotic cell proportion was decreased. In addition, the levels of NO and p-eNOS were significantly suppressed when the cells were exposed to PA, in contrast to that co-incubation with theaflavins reversed the decreased level of NO and p-eNOS in HUVECs. Furthermore, PA combination with theaflavins could significantly improve mitochondrial membrane potential and inhibit DNA damage in HUVECs. These results suggested that theaflavins could alleviate the mitochondrial dysfunction and DNA damage that were induced by palmitic acid. In conclusion, theaflavins had a potential protective effect against PA-induced mitochondrial dysfunction and DNA damage in HUVECs, and the underlying mechanism was mediated, at least partially, through the activation of eNOS/NO signaling pathway.

Keywords: Mitochondrial dysfunction, DNA damage, human umbilical vein endothelial cells, theaflavins, atherosclerosis

Introduction

Palmitic acid (PA) is the most common saturated free fatty acid and is known to induce endothelial dysfunction that leads to atherosclerosis [1, 2]. Endothelial cells are exposed to PA can induce apoptosis, oxidative stress and inflammation, all factors that aggravate the vascular damage [3, 4]. In human umbilical vein endothelial cells (HUVECs), PA-induced increase in the generation of reactive oxygen species, the activation of NADPH oxidase, the up-regulation of inducible nitric oxide synthase (iNOS) and down-regulation of endothelial nitric oxide synthase (eNOS) [5]. Moreover, PA can inhibit the attachment, migration, and tube formation of endothelial progenitor cells (EPCs) through suppression of the Akt/endothelial nitric oxide (NO) synthase (eNOS) signaling pathway [6]. In vivo, PA induces apoptosis in mouse aortic endothelial cells and endothelial dysfunction, and enhances oxidative and ER stress [7]. Thus, endothelial cell exposure to PA is a suitable cell model for the exploration of the molecular mechanisms by which free fatty acids induce vascular damage.

Theaflavins have been identified as the major active ingredient in black tea and are mixture of theaflavin 3-gallate (TF3G), theaflavin 3'-gallate (TF3'G), and theaflavin 3, 3'-digallate (TFDG) [8]. Theaflavins are characterized by the benzotropolone ring structure and a bright red or orange color, and they contribute to the unique taste of black tea [9]. Pharmacological and epidemiological studies demonstrate that theaflavins possess many health beneficial properties including antioxidant [10, 11], anti-inflammatory [12], anti-cancer [13, 14] and cardioprotective effects [15, 16]. It has been reported that theaflavins may attenuate H_2O_2-induced erythrocytes, homocysteine-induced HUVEC and lipopolysaccharide-induced bone marrow-derived macrophages injury [8, 9, 17]. These obser-

vations document that theaflavins possess the protective effect on injuring-induced cell injury in vitro. However, the protective effect of theaflavins on PA-induced injury in HUVEC and mitochondrial has not been clearly delineated.

Mitochondria are important organelles with diverse functions, not only in ATP production and calcium homeostasis, but also in reactive oxygen species (ROS) generation [18]. Mitochondrial damage is closely related to the development of cardiovascular diseases [19]. In endothelial cells, mitochondria are essential for maintaining cell homeostasis and functions, and mitochondrial damage can induce cell apoptosis [20]. The present study was undertaken to determine the protective potential of theaflavin, a major polyphenol derived from black tea, against mitochondrial injury in HUVECs.

Materials and methods

Cell culture

The human umbilical vein endothelial cells (HUVECs) was obtained from the Cell Resource Center, Shanghai Institutes for Biological Sciences (SIBS, China), and maintained in RPMI-1640 (Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA) at 37°C in a humidified incubator (Thermo, USA), 5% CO_2, 95% air atmosphere.

Cell viability detection by CCK8

HUVECs (5.0×10^3/well) were plated and treated in 96-well plates (three wells per group) with palmitic acid (50 μM) for 1 day, 2 days or 3 days respectively, and different concentrations (0, 5, 10, or 20 mg/L) of theaflavins were added. After the combination treatment, 10 μL of Cell Counting Kit-8 (CCK-8) was added to the cells, and the OD value of the cells was measured at 450 nm using an ELISA reader (BioTek, USA) according to the manufacturer's instructions.

Caspase-3 activity and cell apoptosis assay

HUVECs lysates were prepared and incubated with anti-caspase 3. Immunocomplexes were incubated with peptide substrate in assay buffer for 2 h at 37°C. Release of p-nitroaniline was measured at 405 nm using an ELISA reader (MD SpectraMax M5, USA) according to the manufacturer's instructions. Results signify percent change in activity compared to untreated control.

Cell apoptosis was measured using an Annexin-V and Propidium Iodide (PI) Apoptosis Detection Kit (Beyotime, China) by a flow cytometer (Becton Dickinson, USA) according to the guidelines.

Nitric oxide quantification

HUVECs were plated and treated in 96-well plates and were stimulated with palmitic acid and theaflavins. Forty-eight hours later centrifugate to obtain the supernatant, and the level of nitric oxide was measured by nitrite production using the Griess reagent (Invitrogen, USA) at 540 nm using an ELISA reader (BioTek, USA) according to the manufacturer's instructions.

Detection of Ca^{2+} concentrations

HUVECs were plated and treated in 12-well plates and were incubated with palmitic acid and theaflavins to detect changes in Ca^{2+} levels. Cells were harvested and washed twice, and re-suspension in Indo 1/AM (3 μg/ml) at 37°C for 30 min and analyzed by flow cytometry.

Determination of the mitochondrial membrane potential

The mitochondrial membrane potential was assessed using a fluorometric probe, DiOC6 (Molecular Probes). Briefly, cells were plated in 6-well culture dishes. After reaching confluence, cells were treated with palmitic acid and theaflavins. After incubation, cells were stained with DiOC6 (40 nM) for 15 min at 37°C. Cells were collected, washed twice in PBS, and analyzed by FACScan flow cytometry.

The comet assay

Briefly, fully frosted slides were precoated on each end with 100 mL of 0.8% agarose in phosphate-buffered saline (pH = 7.4) and covered with a 22 mm × 22 mm glass coverslip and left at room temperature for 20 minutes. Then, 30 mL of the cell culture was mixed with 70 mL of 1% low-melting point agarose in phosphate-buffered saline and maintained at 42°C on a dry-bath incubator. The mixture was immediately spread onto each end of a precoated slide and covered with a fresh glass coverslip, and the comets were captured with an Olympus

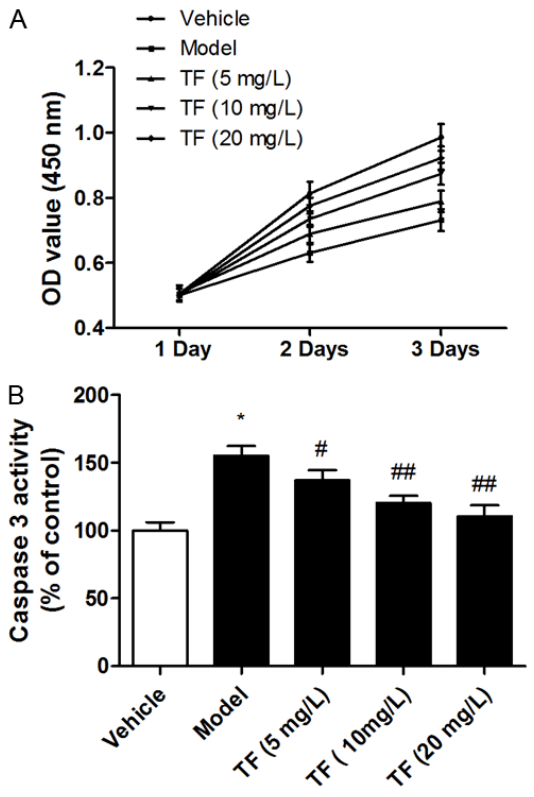

Figure 1. HUVECs were incubated with palmitic acid and theaflavins, and the cell viability was examined by CCK8 assay (A). HUVECs were incubated with palmitic acid and theaflavins for 48 h, and caspase 3 activity was examined by caspase 3 ELISA assay (B). Values were expressed as mean ± SEM, n = 3 in each group. $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus model group.

microscope equipped with a CCD camera connected to the fluorescent microscope.

Western blotting

HUVECs were homogenized and extracted in NP-40 buffer, followed by 5-10 min boiling and centrifugation to obtain the supernatant. Samples containing 30 μg of protein were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). After saturation with 5% (w/v) non-fat dry milk in TBS and 0.1% (w/v) Tween 20 (TBST), the membranes were incubated with the following antibodies: Bax, Bcl-2, caspase-3, caspase-9 and p-eNOS (Santa Cruz Biotechnoogy, CA, USA). After three washes with TBST, The membranes were next incubated with the appropriate HRP (horseradish peroxidase)-conjugated antibody visualized with chemiluminescence (Thermo, USA).

Statistical analysis

The data from these experiments were reported as mean ± standard errors of mean (SEM) for each group. All statistical analyses were performed by using PRISM version 4.0 (GraphPad). Inter-group differences were analyzed by one-way ANOVA, and followed by Tukey's multiple comparison test as a post test to compare the group means if overall $P < 0.05$. Differences with P value of < 0.05 were considered statistically significant.

Results

Effect of theaflavins on PA-induced apoptosis in HUVECs

To investigate the potential cell death of PA in HUVECs, we first examined the effect of PA on cell survival by CCK-8 assay. The results indicated that treatment of HUVEC with PA induced cell death in a time-dependent manner, and the number of HUVECs was markedly decreased in PA treatment group. The inhibition rate was about 30% with PA treatment at day 3 (**Figure 1A**). However, co-incubated with PA and theaflavins (10 mg/L and 20 mg/L) in HUVECs, the cell viability was significantly increased in day 2 and day 3 (**Figure 1A**). Moreover, we examined whether PA induced apoptosis in HUVECs through an apoptotic mechanism. Caspase-3 activity assay and Annexin V-PI double-labeling were measured after HUVECs exposure to PA for 48 h. The results indicated that HUVECs with PA treatment showed significant cell apoptosis as compared to that of the vehicle-treated group, and the caspase-3 activity in co-incubated with PA and theaflavins (10 mg/L and 20 mg/L) was significantly lower than that of the PA-treated group (**Figures 1B**, **2A** and **2B**). These results demonstrated that theaflavins could increase the cell viability and reverse PA-induced apoptosis in HUVECs. Furthermore, the apoptotic response was further investigated by measuring apoptosis-related proteins with western blot. Treatment with 50 μM PA led to increase the pro-apoptotic BAX level and decrease the anti-apoptotic Bcl-2 level in HUVECs. Simultaneously, the protein expression of caspase-3 and caspase-9 was significantly up-regulated in PA group as compared to vehicle-treated group (**Figure 2C** and **2D**). However, treatment with theaflavins dose-dependent reversed the PA-induced down-regulation of anti-apoptotic pro-

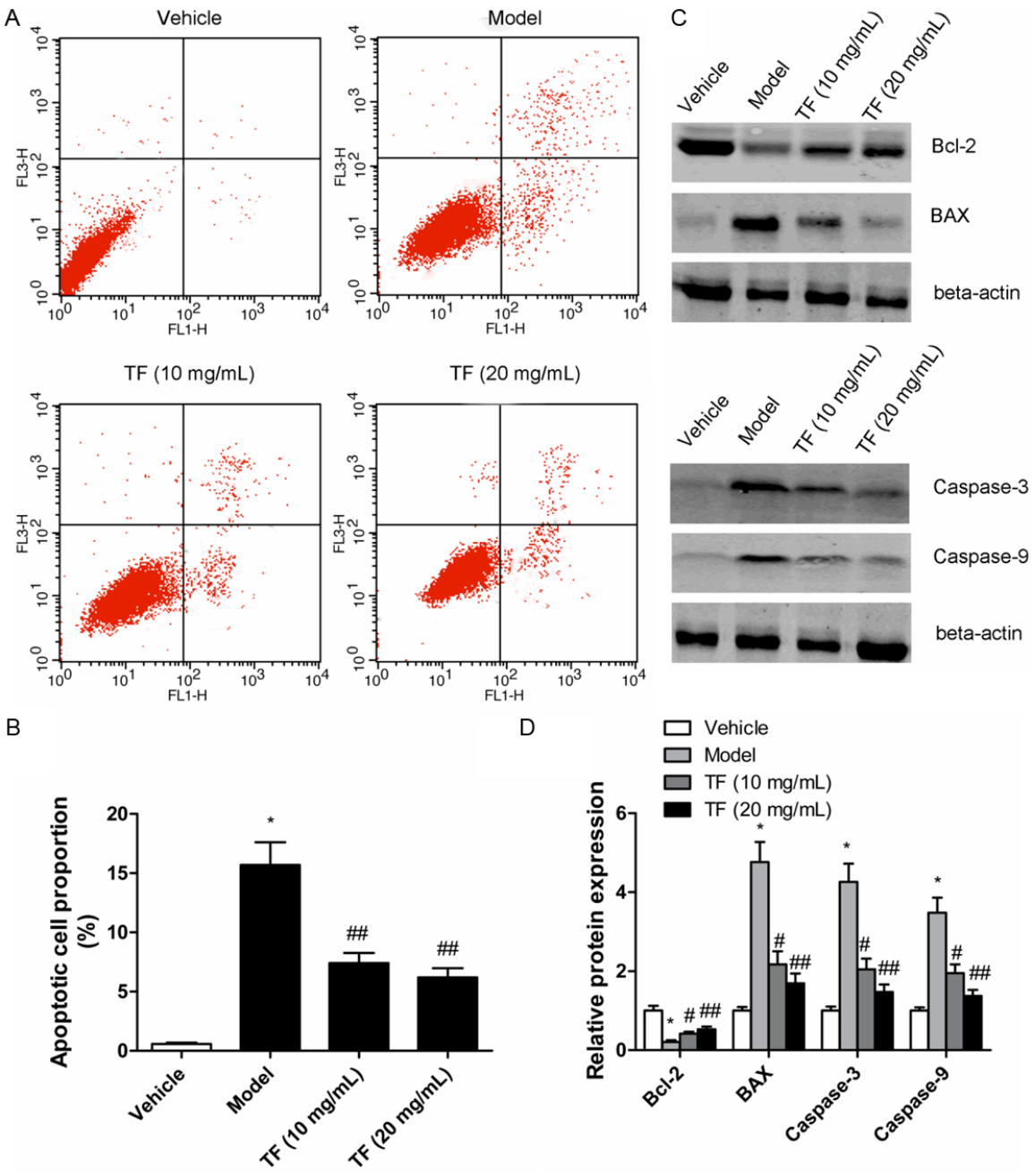

Figure 2. HUVECs were incubated with palmitic acid and theaflavins for 48 h, the percentage of apoptotic cells was also analyzed by flow cytometric analysis of annexin V/PI double staining (A) and bar graphs represent the percentage of apoptotic cells (B). The protein expression of BAX, Bcl-2, caspase-3 and caspase-9 was measured by western blotting (C) and densitometric quantification normalized to β-actin protein bands (D). Values were expressed as mean ± SEM, n = 3 in each group. $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus model group.

tein and up-regulation of pro-apoptotic proteins expression in HUVECs (**Figure 2C** and **2D**).

Theaflavins reverse PA-induced HUVECs injury by regulating NO and iNOS

The ability to generate NO has served as a marker for healthy endothelia, and nanomolar concentrations of NO have anti-inflammatory and protective effects on endothelial cell [1, 21]. To assess PA-induced secretory dysfunction of HUVECs and the modulation effect of theaflavins, we measured the levels of NO and p-eNOS in HUVECs. As shown in **Figure 3**, the levels of NO and p-eNOS were significantly suppressed when the cells were exposed to PA at

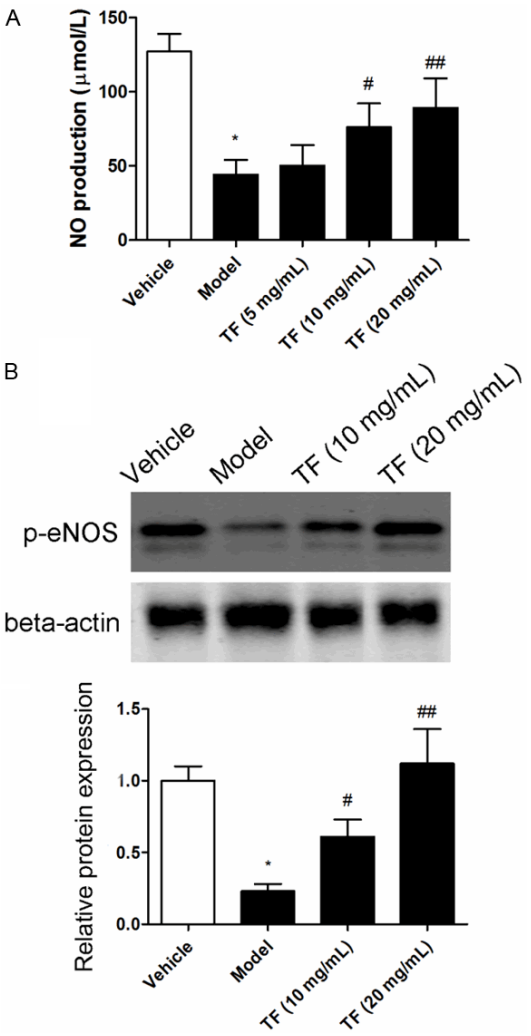

Figure 3. HUVECs were incubated with palmitic acid and theaflavins for 48 h, and the NO concentration was detected by ELISA assay (A). The protein expression of p-eNOS was measured by western blotting (B). Values were expressed as mean ± SEM, n = 3 in each group. $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus model group.

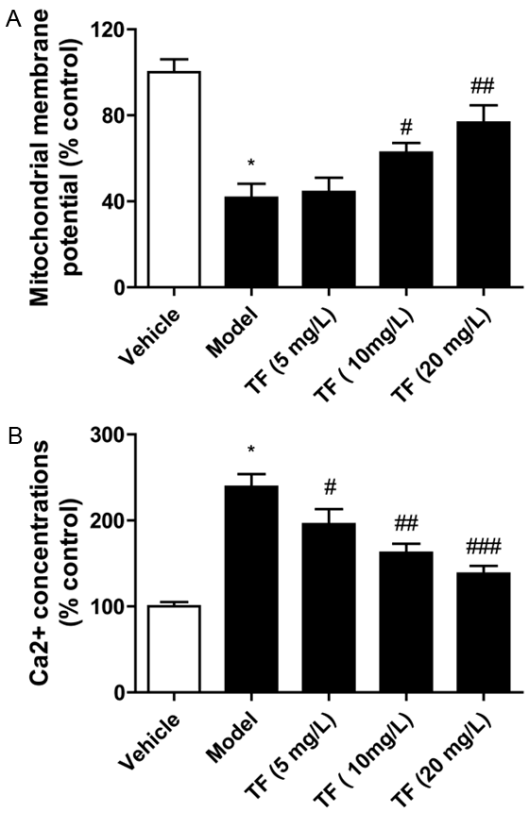

Figure 4. HUVECs were incubated with palmitic acid and theaflavins for 48 h, the mitochondrial membrane potential (A) and the release of Ca^{2+} (B) were examined by flow cytometry. Values were expressed as mean ± SEM, n = 3 in each group. $^{*}P < 0.05$ versus control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ versus model group.

the concentration of 50 μM (**Figure 3A** and **3B**). However, co-incubation with theaflavins (10 mg/L and 20 mg/L) for 48 hours reversed the decreased level of NO and p-eNOS in HUVECs (**Figure 3A** and **3B**). These results suggested that theaflavins could modulate the PA-induced secretory dysfunction of endothelial cells.

Theaflavins inhibit PA-induced mitochondrial dysfunction and DNA damage

Mitochondrial dysfunction and DNA damage are involved in injuring-induced cell proliferation inhibition and apoptosis in HUVECs [22, 23]. To further explore whether PA-induced cell apoptosis was mediated through mitochondrial dysfunction and DNA damage and the potential protective effects of theaflavins was investigated.

We determined the mitochondrial membrane potential with the mitochondria-sensitive dye, DiOC6, using flow cytometry. As shown in **Figure 4**, treatment of HUVECs with PA induced the loss of the mitochondrial membrane potential as compared to vehicle-treated group (**Figure 4A**). PA combination with theaflavins could significantly improve mitochondrial membrane potential in HUVECs when the concentration of theaflavins was more than 10 mg/L (**Figure 4A**). Next, we assessed the effect of PA on the mobilization of Ca^{2+}. When HUVECs were treated with PA, Ca^{2+} levels were significantly increased as compared with the vehicle-treated group (**Figure 4B**). The results demonstrated that PA promoted the secretory dysfunction of Ca^{2+} in HUVECs. Intriguingly, treatment of HU-

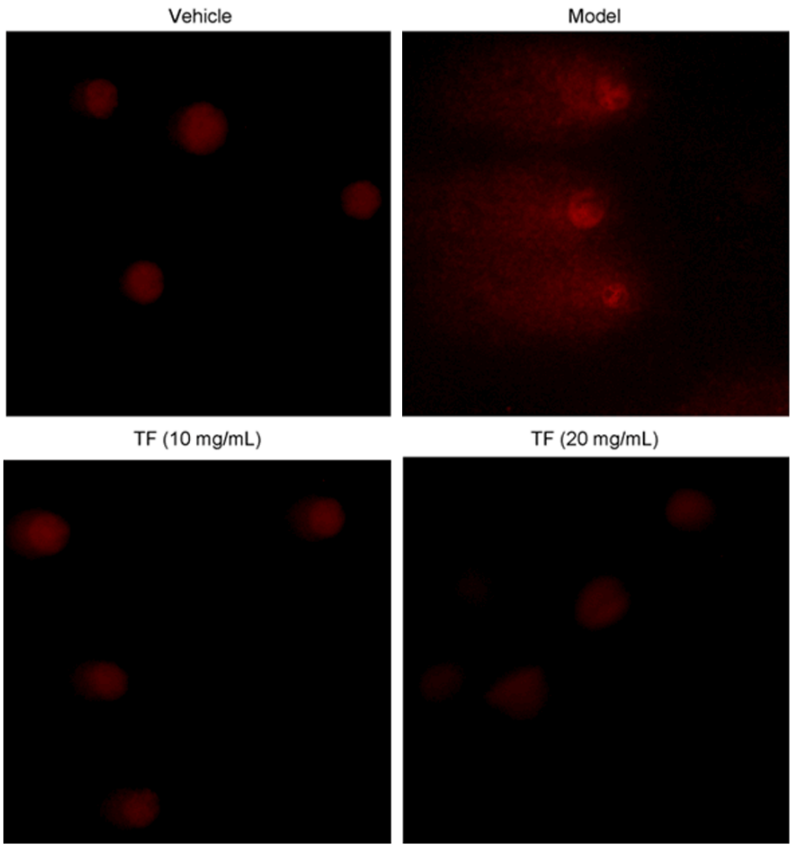

Figure 5. HUVECs were incubated with palmitic acid and theaflavins for 48 h, and the cell DNA damage was measured by the comet assay.

VECs with theaflavins inhibited PA-induced calcium releasing. DNA damage has been found in HUVECs with injuring-induced. In the present study, the tail length in the PA-treated group was markedly higher than in the control group. However, tail length was significantly suppressed by theaflavins co-incubated HUVECs (**Figure 5**). These results suggested that theaflavins could alleviate the mitochondrial dysfunction and DNA damage that were induced by palmitic acid.

Discussion

Endothelial dysfunction is a driving force in the initiation and development of atherosclerosis [22]. Although atherosclerosis development appears to be the result of multiple factors, a particularly important risk factor in the pathogenesis of atherosclerosis is saturated free fatty acid, which contributes to endothelial dysfunction [3, 24]. As a major component of dietary saturated fat and 20% of the total serum free fatty acids, PA is often used to induce endothelial dysfunction [25]. Inflammatory response and oxidant are induced in HUVECs stimulated with PA, and the reduction of insulin-mediated eNOS activity and production of NO are related to endothelial dysfunction [5]. In the present study, HUVECs were considered to approximately represent the endothelial monolayer in blood vessels. The exposure of PA to HUVECs inhibited cell viability and induced cell apoptosis that the proportion of the apoptosis cells was increased; however, theaflavins could increase the cell viability and reverse PA-induced apoptosis in HUVECs. Further studies conclusively showed that treatment with theaflavins dose-dependent reversed the PA-induced down-regulation of anti-apoptotic protein and up-regulation of pro-apoptotic proteins expression in HUVECs. Endothelial cells apoptosis plays an important role in the development of atherosclerosis, and palmitate induces apoptosis in mouse aortic endothelial cells through the promotion of oxidative and ER stress [7]. These observations document that theaflavins could inhibit PA-induced endothelial cells apoptosis via regulating apoptosis-related protein expression.

In vitro and in vivo studies show that exposure to free fatty acids for prolonged periods causes endothelial dysfunction, including a reduction of endothelial cell NO levels, which has served as a marker for healthy endothelia and protects against injuring-induced endothelial dysfunction [5, 7]. Endothelial-derived NO is produced by eNOS and regulates vascular tone, and NO has anti-inflammatory and protective effects through the inhibition of the activation of NF-κB [26, 27]. Our results indicated that PA inhibited NO production and eNOS phosphorylation. However, co-incubation with theaflavins (10 mg/L and 20 mg/L) for 48 hours reversed the decreased level of NO and p-eNOS in HUVECs.

Recent in vitro studies have demonstrated that PA inhibits AKT and eNOS phosphorylation and increased iNOS expression, and the AMPK/AKT/eNOS/NO signaling pathway plays a protective role in endothelial dysfunction [1]. Moreover, Akt/eNOS signaling pathway mediates inhibition of endothelial progenitor cells (EPC) by palmitate-induced ceramide, and ceramide-induced reduction of NO may be the molecular mechanism for PA-mediated EPC inhibition [6]. Therefore, theaflavins-targeted NO synthesis might be an effective means for improvement of endothelial cell functions.

Mitochondrial dysfunction has been implicated in cardiovascular diseases. In view of the utilization of most oxygen in the respiratory chain, mitochondria have been presumed as the major source of cellular ROS generation and a portion of electron leakage during oxygen consumption [22, 28]. Previous studies show that lipid oxidation plays an important role in the initial mitochondrial dysfunction and cell death caused by hemi in endothelial cells [29]. However, for all we know, no literature has been reported that theaflavins possesses the efficacy of improving mitochondrial dysfunction in vitro. In our work, we found that theaflavins could significantly improve mitochondrial membrane potential in HUVECs when the cells were exposed to PA, and PA-induced dysfunction of Ca^{2+} release in HUVECs was reversed by theaflavins treatment. Moreover, our study showed that PA could induce considerable DNA damage in HUVECs by using the method of the comet assay. Some studies have shown that the release of mitochondrial cytochrome c to the cytosol can lead to caspase-3 and caspase-9 activation and endothelial cell apoptosis, which may also cause DNA damage [30]. Our results suggested that theaflavins could alleviate the mitochondrial dysfunction and DNA damage that were induced by palmitic acid, and the underlying mechanism was mediated, at least partially, through the activation of eNOS/NO signaling pathway.

This result further confirmed the important role of theaflavins in the PA-induced injury of HUVECs. In conclusion, we found that theaflavins could ameliorate palmitic acid-induced HUVECs injury, mitochondrial dysfunction and DNA damage through the activation of eNOS/NO signaling pathway.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yu-Dong Chen, Department of Cardiovascular Medicine, Central Hospital of Shengli Oil Field, Dongying 257000, China. Tel: (86) 546-8552224; Fax: (86) 546-8552224; E-mail: yd_chenmed@hotmail.com

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