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Endosulfan inhibits proliferation through the Notch signaling pathway in human umbilical vein endothelial cells☆

Jialiu Wei [a, b], Lianshuang Zhang [a, b], Lihua Ren [a, b], Jin Zhang [a, b], Yang Yu [a, b], Ji Wang [a, b], Junchao Duan [a, b], Cheng Peng [c], Zhiwei Sun [a, b], Xianqing Zhou [a, b, *]

[a] Department of Toxicology and Hygienic Chemistry, School of Public Health, Capital Medical University, 100069, Beijing, China
[b] Beijing Key Laboratory of Environmental Toxicology, Capital Medical University, 100069, Beijing China
[c] National Research Centre for Environmental Toxicology (Entox), Member of Queensland Alliance for Environmental Health Science (QAEHS), The University of Queensland, Coopers Plains, 4108, Brisbane, QLD, Australia

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ABSTRACT

Our previous research showed that endosulfan triggers the extrinsic coagulation pathway by damaging endothelial cells and causes hypercoagulation of blood. To identify the mechanism of endosulfan-impaired endothelial cells, we treated human umbilical vein endothelial cells (HUVECs) with different concentrations of endosulfan, with and without an inhibitor for Notch, *N*-[*N*-(3, 5-difluorophenacetyl)-1-alanyl]−S-Phenylglycinet-butylester (DAPT, 20 μM), or a reactive oxygen species (ROS) scavenger, *N*-Acetyl-L-cysteine (NAC, 3 mM), for 24 h. The results showed that endosulfan could inhibit cell viability/proliferation by increasing the release of lactate dehydrogenase (LDH), arresting the cell cycle in both S and G2/M phases, and inducing apoptosis in HUVECs. We also found that endosulfan can damage microfilaments, microtubules, and nuclei; arrest mitosis; remarkably increase the expressions of Dll4, Notch1, Cleaved-Notch1, Jagged1, Notch4, Hes1, and p21; and significantly induce ROS and malondialdehyde production in HUVECs. The presence of DAPT antagonized the above changes of cycle arrest, proliferation inhibition, and expressions of Dll4, Notch1, Cleaved-Notch1, Hes1, and p21 caused by endosulfan; however, NAC could attenuate LDH release; ROS and malondialdehyde production; apoptosis; and the expression levels of Dll4, Notch1, Cleaved-Notch1, Notch4, and Hes1 induced by endosulfan. These results demonstrated that endosulfan inhibited proliferation through the Notch signaling pathway as a result of oxidative stress. In addition, endosulfan can damage the cytoskeleton and block mitosis, which may add another layer of toxic effects on endothelial cells.

1. Introduction

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a, 6,9,9ahexahydro-6,9-methano-2,4,3-benzodioxathiepine-3-oxide) is an organochlorine pesticide listed as a kind of persistent organic pollutant (POP) by Stockholm Convention in 2011 (Becker et al., 2011). However, it has been used in agriculture and viticulture worldwide in the past 50 years (Gandhi et al., 2015). Importantly, endosulfan has been a ubiquitous contaminant, and it was detected in a wide variety of environmental media because of its potential transport (Kafilzadeh et al., 2015). Environmental endosulfan residues are mainly absorbed into the body through the gastrointestinal tract, respiratory tract, and skin (Abdul Majeed et al., 2014). Studies have demonstrated that exposure to endosulfan could affect different organ systems and physiological functions in mammals, including reproductive (Du et al., 2015), nervous (Enhui et al., 2016), endocrine (Senthilkumaran, 2015), immune (Zhao et al., 2014), hepatic (Moses and Peter, 2010), and cardiovascular systems (Ozmen, 2013).

Cardiovascular diseases (CVD) have been the primary cause of mortality worldwide (Balfour et al., 2016; Bundy and He, 2016; Cruz et al., 2016). Studies have proved that POPs are associated with changes in LDL-cholesterol, which suggests that exposure to POPs is related to atherosclerosis and CVD (Kim et al., 2015a; Penell et al., 2014). In an attempted suicide, endosulfan ingestion overdose caused abnormal heart rate and blood pressure (Moon and Lee,

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* Corresponding author. Department of Toxicology and Hygienic Chemistry, School of Public Health, Capital Medical University, 100069, Beijing, China.
E-mail addresses: xqzhou@ccmu.edu.cn, xianqingzhou@aliyun.com (X. Zhou).

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2013). Moreover, endosulfan can disrupt cellular homeostasis and lead to toxic changes in the hearts of rabbits, which indicates that exposure to endosulfan may be associated with CVD (Ozmen, 2013).

Endothelial dysfunction has been recognized in CVD as the primum movens in the pathogenesis of multiple cardiovascular events that damage the vascular wall, form atherosclerotic plaque, and consequently promote vascular injury (Cimellaro et al., 2016). A study showed that endosulfan could infuse toxicity to endothelial cells leading to endothelial dysfunction (Li et al., 2015). Our previous study has also found that endosulfan induces hypercoagulation of blood by triggering the extrinsic coagulation pathway resulting from the damaging of endothelial cells (Wei et al., 2015; Zhang et al., 2015); however, the mechanism of endosulfan-induced endothelial dysfunction remains unclear.

Furthermore, endosulfan could damage endothelial cells through proliferation inhibition and apoptosis induced by reactive oxygen species (ROS) in human HaCaT keratinocytes (Antherieu et al., 2007). The Notch signaling pathway has also been proved to regulate adhesion, proliferation, and migration in endothelial cells (Kofler et al., 2011), which includes five transmembrane ligands [Jagged1, Jagged2, Delta-like (Dll) 1, Dll3, and Dll4] and four receptors (Notch1–4), and all of them have been recognized in mammalian cells. Two receptors (Notch1 and 4) and three ligands (Jagged1, Dll1, and Dll4) were identified in vascular endothelial cells (Kume, 2012). However, the functions of Jagged1 and Dll4 are different. Dll4-dependent Notch activation could prevent endothelial tip cell formation and inhibit vessel branching (Sainson and Harris, 2007), and Dll4 inhibition could lead to unrestricted proliferation in endothelial cells (Gu et al., 2009). Moreover, cardiac defects and developmental abnormality of vasculature were found in Jagged-null mouse, suggesting that Jagged1 plays a vital role in the development (Pedrosa et al., 2015). However, whether the two opposing Notch signaling pathways are involved in endosulfan-inhibited proliferation and apoptosis remains unknown. *N*-[*N*-(3, 5-difluorophenacetyl)-1-alanyl]—S-phenylglycinet-butylester (DAPT) has been shown to be a specific inhibitor of Notch signaling (Ma et al., 2007) and *N*-acetyl-L-cysteine (NAC) has been used as a ROS scavenger (Ma et al., 2016). Therefore, the present study was designed to further clarify the mechanism of endosulfan-induced cytotoxicity by investigating the effect of the Notch signaling pathway on it using NAC and DAPT as the regulators.

2. Materials and methods

2.1. Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) were obtained from Shanghai Institutes for Biological Sciences, China. Cells were cultured in DMEM (HyClone, USA) complemented with 10% fetal bovine serum (Gibco, USA) and incubated at 37 °C in a humid atmosphere with 5% CO_2. Endosulfan containing α and β isomers (7:3) was kindly donated by Jiangsu Kuaida Agrochemical Co. Ltd. (Nantong, China). Endosulfan was dissolved in DMSO and used for the experiment after dilution with DMEM. A control group of cells was added to DMEM containing an equal volume of 0.1% DMSO. NAC was purchased from KeyGen Biotech (China) and DAPT was obtained from Selleck Chemicals (USA).

2.2. Cell viability assay

The viability of HUVECs was detected by a cell counting kit (CCK)-8 (KeyGen). Briefly, cells (1×10^4 cells per well) were adhered to the bottom of 96-well plates for 16–24 h, followed by endosulfan treatment at various concentrations (0.125, 0.25, 0.5, 1, 6, 12, 16, and 32 μg/mL). After 24-h incubation, the CCK-8 reagent was added to each well at equal volumes, and the viability of the cells was measured by a microplate reader at 492 nm (Thermo Multiskan MK3, USA).

2.3. Assessment of lactate dehydrogenase release

In addition to analyzing the integrity of the cell membrane, lactate dehydrogenase (LDH) release was assessed by a commercial detection kit (Jiancheng, China) according to manufacturer's instructions. After HUVECs were treated with various doses of endosulfan and 12 μg/mL endosulfan + NAC (3 mM) for 24 h, the supernatants were harvested for the assessment. LDH activity was analyzed using 100 μL of cell medium by the microplate reader at 450 nm (Thermo MultiskanMK3, USA).

2.4. Assessment of oxidative damage

Briefiy, after treated with various concentrations of endosulfan and 12 μg/mL endosulfan + NAC (3 mM) for 24 h, HUVECs were lysed with cold RIPA lysis buffer containing 1 mM phenylmethylsulphonyl fiuoride (DingGuo, China). After being centrifuged at 12,000 rpm for 10 min at 4 °C, the collected supernatants were prepared for measurement by a malondialdehyde (MDA) kit (Jiancheng, China).

For the assessment of intracellular ROS level, flow cytometry was used with an oxidation-sensitive probe, 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (JianCheng, China). After treatment with various concentrations of endosulfan for 24 h, the cells were washed and coincubated with 10 μM DCFH-DA diluted in serum-free DMEM at 37 °C for 30 min. After incubation, the cells were rinsed with cold PBS and resuspended for measurement. Fluorescence intensity was quantified using a flow cytometer (Becton-Dickinson, USA).

2.5. Observation of mitosis

Briefly, HUVECs treated with 0 and 12 μg/mL endosulfan were observed under a real-time inverted phase contrast microscope (UltraVIEW VoX, USA) for 24 h. Cells at different stages were examined, and images were captured every 10 min in nine randomly selected visual fields. The percentage of normal mitosis of HUVECs was calculated by counting HUVECs from six random visual fields per group.

2.6. Measurement of cell apoptosis

Apoptosis in HUVECs was detected using annexin V and a propidium iodide (PI) assay kit (KeyGen). After being treated with various dosages of endosulfan and 12 μg/mL endosulfan + NAC (3 mM) for 24 h, HUVECs were suspended in a binding buffer and stained with 5 μL Annexin V-FITC for 15 min, followed by treatment with 5 μL PI at room temperature. The cells were then loaded on a flow cytometer (Millipore, USA), and the data from 10,000 cells/sample were collected. The apoptosis rates of early and late stages were together regarded as the total apoptosis rate.

2.7. Cell cycle assays

Distributions of cell cycle were measured using a detection kit for cell cycle (KeyGen). HUVECs (1.0×10^6/well) were plated and treated in six-well plates (three wells per group). After being treated with different concentrations of endosulfan and 12 μg/mL endosulfan + DAPT (20 μM) for 24 h, the cells were fixed in ice-cold 70% ethanol at 4 °C overnight. Then the cells were incubated at

37 °C for 30 min with 100 μL RNase A and 400 μL PI. Finally, the samples were analyzed using a flow cytometer (FC500, Beckman Coulter, USA).

2.8. Measurement of cell proliferation

Briefly, HUVECs were stained with 5 μM of fluorescent probe CFDASE (KeyGen) at 37 °C for 15 min, and then washed with PBS twice and cultured in six-well plates for 24 h. Then the cells were incubated with different concentrations of endosulfan and 12 μg/mL endosulfan + DAPT (20 μM) for 24 h, collected, and resuspended in 500 μL of PBS for analysis. The average fluorescence intensity of HUVECs was analyzed using a flow cytometer (BD FACS Aria, USA).

2.9. Damage assessment of microfilaments, microtubules, and cell nuclei

Cells were harvested and fixed for 10 min with 3.7% formaldehyde at room temperature. After washing with PBS containing 0.1% Triton X-100 thrice, the cells were divided into three parts: some were incubated with Hoechst 33258 (5 μg mL^{-1}) (KeyGen) for 20 min for staining nuclei, some were stained with Actin-Tracker Green (200 nM) (KeyGen) for microfilaments, and the microtubules of some cells were treated with Tubulin-Tracker Red (250 nM) (KeyGen). Laser confocal microscopy (Leica TCS SP5, Germany) was used to monitor the distribution of fluorescence. Twenty cells from four randomly selected visual fields per group were examined. The average fluorescence intensity of microfilaments, microtubules, and nuclei and diameters of nuclei were analyzed by Volocity Demo 6.0.

2.10. Determination of the Notch signaling pathway activation

To analyze whether endosulfan influences the expressions of cellular factors involved in the Notch signaling pathway, expressions of Jagged1 [1:1000, Cell Signaling Technology (CST), USA], Dll4 (1:1000, Abcam, USA), Notch1 (1:1000, CST, USA), Cleaved-Notch1 (1:1000, CST, USA), Notch4 (1:1000, Abcam, USA), Hes1 (1:1000, CST, USA), and p21 (1:200, rabbit antibodies, Boster, China) in HUVECs were assessed by Western blot. As an internal control, β-actin (1:1000, CST, USA) was also detected. The densitometric analysis of the protein bands was performed by Image Lab™ Software (Bio-Rad, USA).

2.11. Statistical analysis

The data were expressed as mean ± standard deviation. Statistical analyses were performed using SPSS 17.0 software. Differences among the groups were analyzed by one-way analysis of variance (ANOVA) followed by comparing the differences between various groups. Differences were considered statistically significant at $P<0.05$.

3. Results

3.1. Cytotoxicity of HUVECs induced by endosulfan

The viability of HUVECs gradually decreased with increasing endosulfan levels compared to that of the cells in the control group in a dose-dependent manner (Fig. 1A). In the middle- (6 μg/mL) and high- (12 μg/mL) dose groups, LDH release increased compared to that in the control group, whereas it significantly reduced in the 12 μg/mL endosulfan + NAC (3 mM) group compared to that in the 12 μg/mL group ($P < 0.05$) (Fig. 1B). Doses of endosulfan were selected at 1, 6, and 12 μg/mL for further experiments on the basis of the CCK-8 assay.

3.2. Apoptosis of HUVECs induced by endosulfan

As shown in Fig. 2, the apoptotic rate significantly increased in the middle- (6 μg/mL) and high- (12 μg/mL) dose endosulfan groups compared to that in the control group, whereas it obviously decreased in the 12 μg/mL endosulfan + NAC (3 mM) group compared to that in the 12 μg/mL endosulfan group ($P < 0.05$) (Fig. 2A and B).

3.3. Production of oxidative stress by exposure to endosulfan

To obtain a clear insight into endosulfan-induced cytotoxicity, we assessed the intracellular generation of ROS and MDA levels. After HUVECs were treated with endosulfan for 24 h, both intracellular ROS and MDA levels significantly increased in 6 and 12 μg/mL dose groups compared to those in the control group. ROS and MDA levels in the 12 μg/mL endosulfan + NAC (3 mM) group were significantly reduced compared to those in the 12 μg/mL endosulfan group ($P < 0.05$) (Fig. 2C and E).

3.4. Cell cycle and proliferation after endosulfan treatment

As presented in Fig. 3A and B, the percentage of cells in the S and G2/M phases increased and that in the G0/G1 phase decreased in the treatment group compared to that in the control group. The percentage of HUVECs in the S phase increased in a dose-dependent manner, that in the G2/M phase increased only in the high-dose (12 μg/mL) endosulfan group, and that in the G0/G1 phase decreased in the 6 and 12 μg/mL endosulfan groups compared to the control group ($P < 0.05$). Furthermore, the percentage of cells in the G2/M phase decreased, whereas that in the G0/G1 phase progressively increased in the 12 μg/mL endosulfan + DAPT (20 μM) group compared with the 12 μg/mL endosulfan group ($P < 0.05$). The results of proliferation test also showed that the level of average fluorescence intensity significantly increased in the 6 and 12 μg/mL endosulfan groups compared to that in the control group, whereas it decreased in the 12 μg/mL endosulfan + DAPT (20 μM) group compared to that in the 12 μg/mL endosulfan group ($P < 0.05$) (Fig. 3C and D).

3.5. Changes in the cytoskeleton and mitosis induced by endosulfan

Results showed that microfilaments and microtubules had maldistribution when endosulfan concentration increased (Fig. 4A and B). Endosulfan could decrease the fluorescence intensity of microfilaments and microtubules significantly in a dose-dependent manner, which demonstrated that the microfilaments and microtubules were damaged by endosulfan (Table 1) *($P<0.05$)*. Cells were round and nuclei were dyed homogeneously without endosulfan treatment, whereas karyopyknosis was clearly observed with middle (6 μg/mL) and high (12 μg/mL) doses of endosulfan. The average diameters of nuclei significantly decreased in the 12 μg/mL endosulfan group (9.53 ± 1.02 μm) compared to that in the control group (13.68 ± 0.70 μm) although there was no significant variation in the fluorescence intensity in the endosulfan-treated groups ($P < 0.05$) (Fig. 4C and Table 1). Furthermore, there were no obvious changes in the 12 μg/mL endosulfan + NAC (3 mM) and 12 μg/mL endosulfan + DAPT (20 μM) groups compared with the 12 μg/mL endosulfan group. The results also suggested that failure of complete mitosis dramatically increased and cells ultimately died after they were treated with 12 μg/mL endosulfan (Fig. 4E and G).

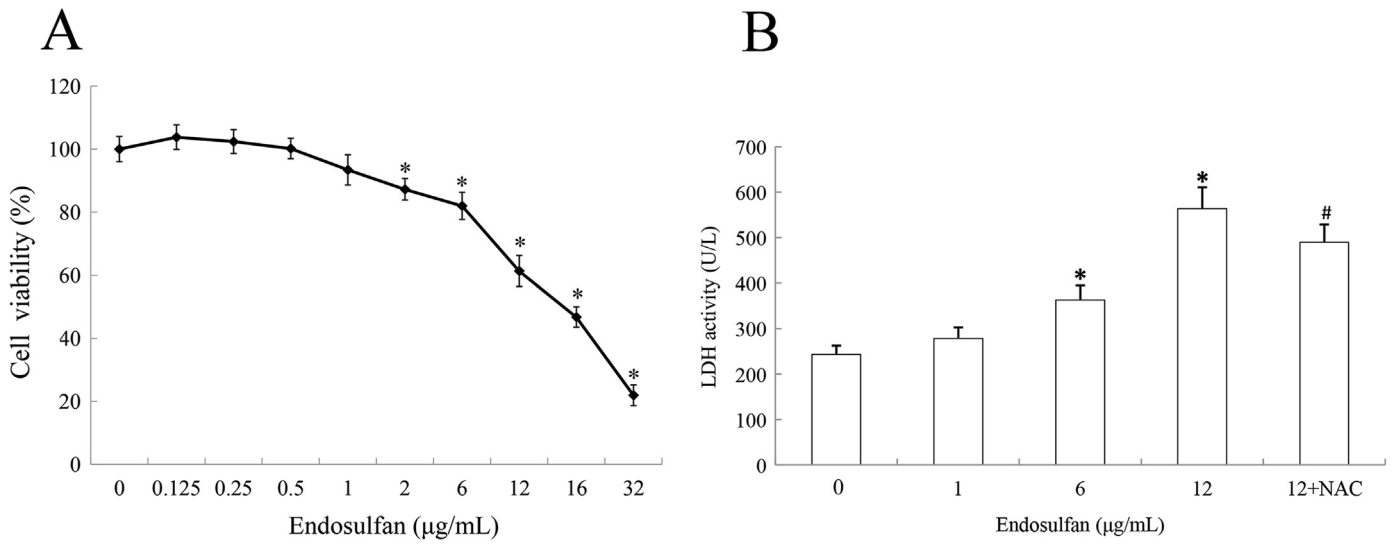


Fig. 1. Effects of endosulfan on the cytotoxicity of HUVECs. (A) Cell viability was assessed by the CCK-8 assay. (B) LDH release in cells treated with different dosages of endosulfan for 24 h * indicates significant difference compared with the 0 μg/mL endosulfan group, # indicates significant difference compared with the 12 μg/mL endosulfan group. The data are expressed as mean ± S.D. ($P<0.05$).

3.6. Notch signaling pathway activated by endosulfan

The results showed that the expressions of Dll4, Cleaved-Notch1, Hes1, and p21 obviously increased after treatment with endosulfan ($P<0.05$). The expression level of Notch1 was the highest in the 6 μg/mL dosage group and significantly decreased in DAPT and NAC-treated groups. The expressions of Jagged1 and Notch4 were upregulated at all three dosages (1, 6, and 12 μg/mL) of endosulfan compared to that in the control group and significantly decreased in the high-dose (12 μg/mL) endosulfan group compared with the 1 μg/mL endosulfan group ($P < 0.05$). DAPT and NAC could attenuate them in HUVECs (Fig. 5A and B).

4. Discussion

Our previous study showed that endosulfan could lead to blood hypercoagulation resulting from damage and apoptosis of endothelial cells (Wei et al., 2015). Apoptosis of endothelial cells was regarded as the key issue of research in revealing the molecular mechanisms of atherosclerotic vascular diseases (Lai and Kan, 2015).

The present results showed that endosulfan treatment caused significant decrease in cell viability; however, the apoptotic rates and LDH release were increased in HUVECs. This further explains the injury of vascular endothelial tissue and cells observed in rats in our previous study (Wei et al., 2015; Zhang et al., 2015). The present results were also similar to the results of de Lavor et al. who showed that cell viability reduction could result in LDH release and apoptosis (de Lavor et al., 2015). To acquire closer mechanistic insight into endosulfan-induced endothelial cytotoxicity, the cell cycle and proliferation in HUVECs were studied. The present study showed that endosulfan arrested the HUVECs in both S and G2/M phases and inhibited the proliferation of HUVECs. However, Li et al. found that HUVECs were arrested in the G1 phase but not in the G2/M phase when treated with 60-μM endosulfan for 48 h, which was not in accordance with our present results and could have been due to different dosages and exposure time of endosulfan (Li et al., 2015). Cell cycle block may lead to the inhibition of cell proliferation. Apoptosis may arise from the occurrence of depletion of ATP, DNA damage and cell cycle arrest (Kanno and Nishizaki, 2011). Cell cycle arrest can be caused by oxidative stress-induced DNA breakage. Our previous study showed that endosulfan could induce oxidative DNA damage in vessel endothelial cells (Wei et al., 2015). Cells have a resumable cell cycle arrest in response to instant or modest DNA damage, whereas extended or serious DNA damage may result in case of continuous cell cycle block (Lukin et al., 2015). In addition, the activation of the DNA damage response pathway could mediate cell cycle arrest (Abraham, 2001; Puente et al., 2014). Cells can activate genome surveillance in response to DNA damage to sense and repair damaged or abnormally structured DNA and maintain the genome stability (Abraham, 2001; Wang et al., 2011). Cell cycle arrest allows cells time to repair DNA damages and resultant gene mutations. However, when the DNA damages are too serious and exceeded the self-repairing capacity of the cells, apoptosis would take place (White, 1993). The present study illustrated that endosulfan could cause cell cycle arrest and apoptosis, which may be because of the DNA damage caused by endosulfan in HUVECs. In addition, cytoskeleton plays a pivotal role in cell proliferation and mitosis (Kim et al., 2015b; Ritchey and Chakrabarti, 2014). Cytoskeleton consists of microfilaments and microtubules. Our study suggested that endosulfan can significantly induce damages in the microfilaments, reduce microtubules, and form karyopyknosis. A similar study also showed that endosulfan can modulate cytoskeletal architecture in HepG2 cells (Peyre et al., 2012), and cytoskeleton may play a pivotal role in the execution phase of apoptosis organization (Oropesa Avila et al., 2015). It was reported that abnormal actin-based cytoskeleton could influence the structure and function of spindle, which led to a delayed mitosis or even cell death (Sandquist et al., 2016). For example, vinblastine, a microtubule-targeting agents, was proved to arrest mitosis and induce apoptosis subsequently (Bates et al., 2011). The present results indicate that endosulfan may impair microfilaments, microtubules, and cell nucleis and block mitosis.

We further investigated the cell signaling pathway of endosulfan-induced cell cycle arrest. The Notch signaling pathway regulates proliferation in endothelial cells (Kofler et al., 2011) and plays critical roles in cell cycle and proliferation. After Notch–ligand binding and a series of proteolytic cuts, the Notch intracellular domain (NICD) is translocated into the nuclei, leading to the expressions of downstream target genes (Guo et al., 2011;

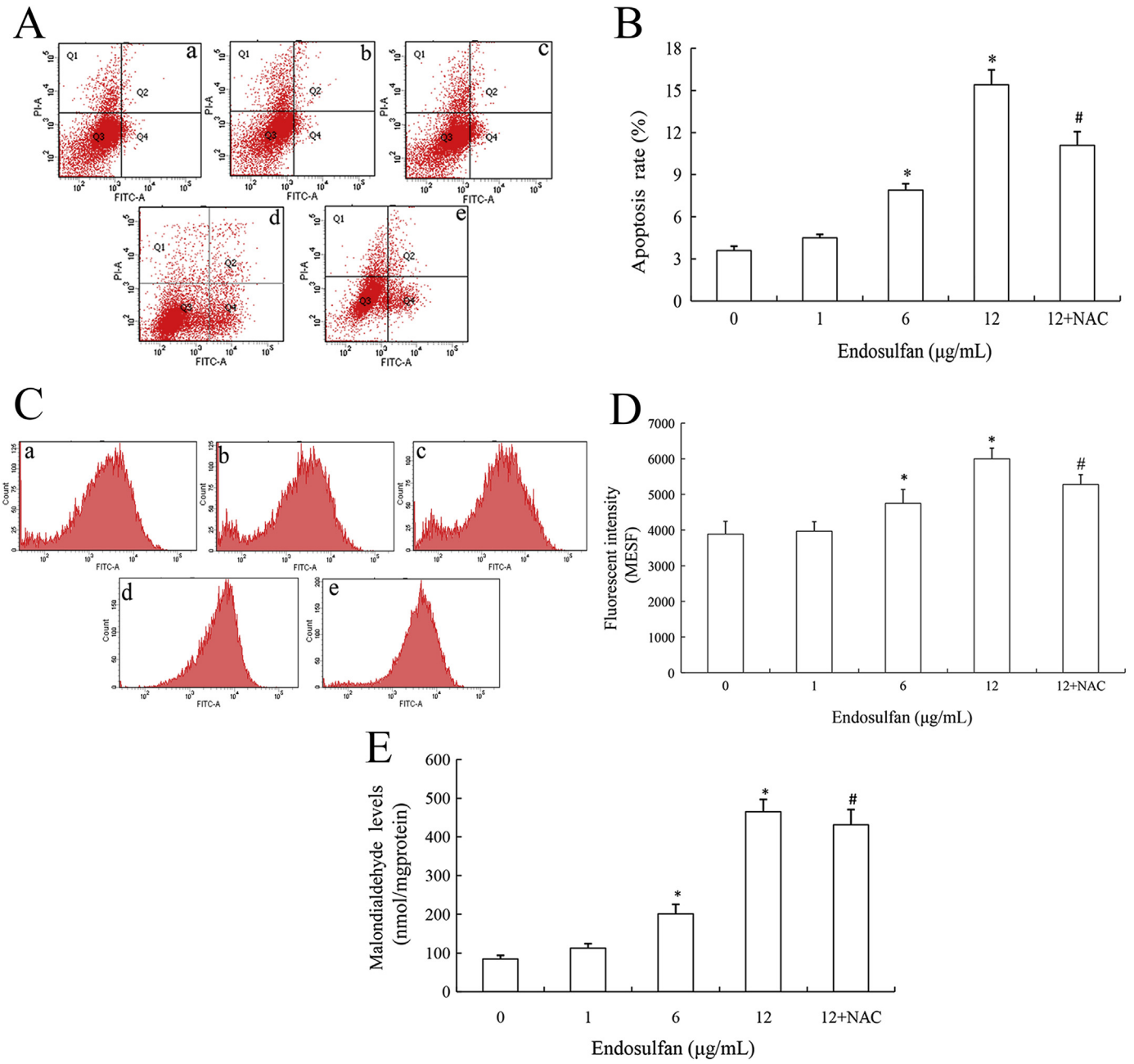


Fig. 2. Effects of endosulfan on oxidative stress, damage, and apoptosis of HUVECs. (A) a–e: 0 μg/mL endosulfan group (a), 1 μg/mL endosulfan group (b), 6 μg/mL endosulfan group (c), 12 μg/mL endosulfan group (d), 12 μg/mL endosulfan + NAC (3 mM) group (e). (B) HUVECs treated with endosulfan showed an increase in the apoptosis rate. (C, D) Fluorescence intensity of ROS was measured using flow cytometry. a–e: 0 μg/mL endosulfan group (a), 1 μg/mL endosulfan group (b), 6 μg/mL endosulfan group (c), 12 μg/mL endosulfan group (d), 12 μg/mL endosulfan + NAC (3 mM) group (e). (E) MDA level in HUVECs treated with various dosages of endosulfan for 24 h * indicates significant difference compared with the 0 μg/mL endosulfan group, # indicates significant difference compared with the 12 μg/mL endosulfan group. The data are expressed as mean ± S.D. ($P<0.05$).

Tan et al., 2014). NICD1 and NICD4 are the active intracellular domains of Notch1 and Notch4, respectively. Endothelial Jagged1 can activate Notch4 and regulate vascular maturation by modulating downstream of Dll4/Notch1 signaling (Pedrosa et al., 2015). The present study showed that endosulfan gradually induces the upregulation of Dll4, cleaved-Notch1, Hes1, and p21 with increasing dosages. We found that cleaved-Notch1, compared to Notch1, was significantly increased in the HUVECs treated with endosulfan, suggesting that Notch1 activation plays a vital role in endosulfan-induced adverse events of cellular biology. Although endosulfan also increased the expressions of Jagged1 and Notch4, the upregulated expressions gradually decreased with increasing endosulfan levels. The results also suggested that endosulfan could activate both the Dll4/cleaved-Notch1/Hes1/p21 pathway and Jagged1/Notch4 pathway, with the latter being more sensitive to endosulfan exposure than the former. Benedito et al. also reported that Jagged1 could inhibit Dll4-induced Notch activation to promote angiogenesis in endothelial cells (Benedito et al., 2009). Hes1 is the downstream effector of the Notch pathway involved in cell cycles (Dahlberg et al., 2011). The significantly increased protein levels of cleaved Notch1 and Notch target genes proved the activation of the Notch signaling pathway in endothelial cells (El Hindy et al., 2013). P21, the target gene of Hes1, is an inhibitor for cyclin-dependent kinase that negatively mediated cell cycle and induced

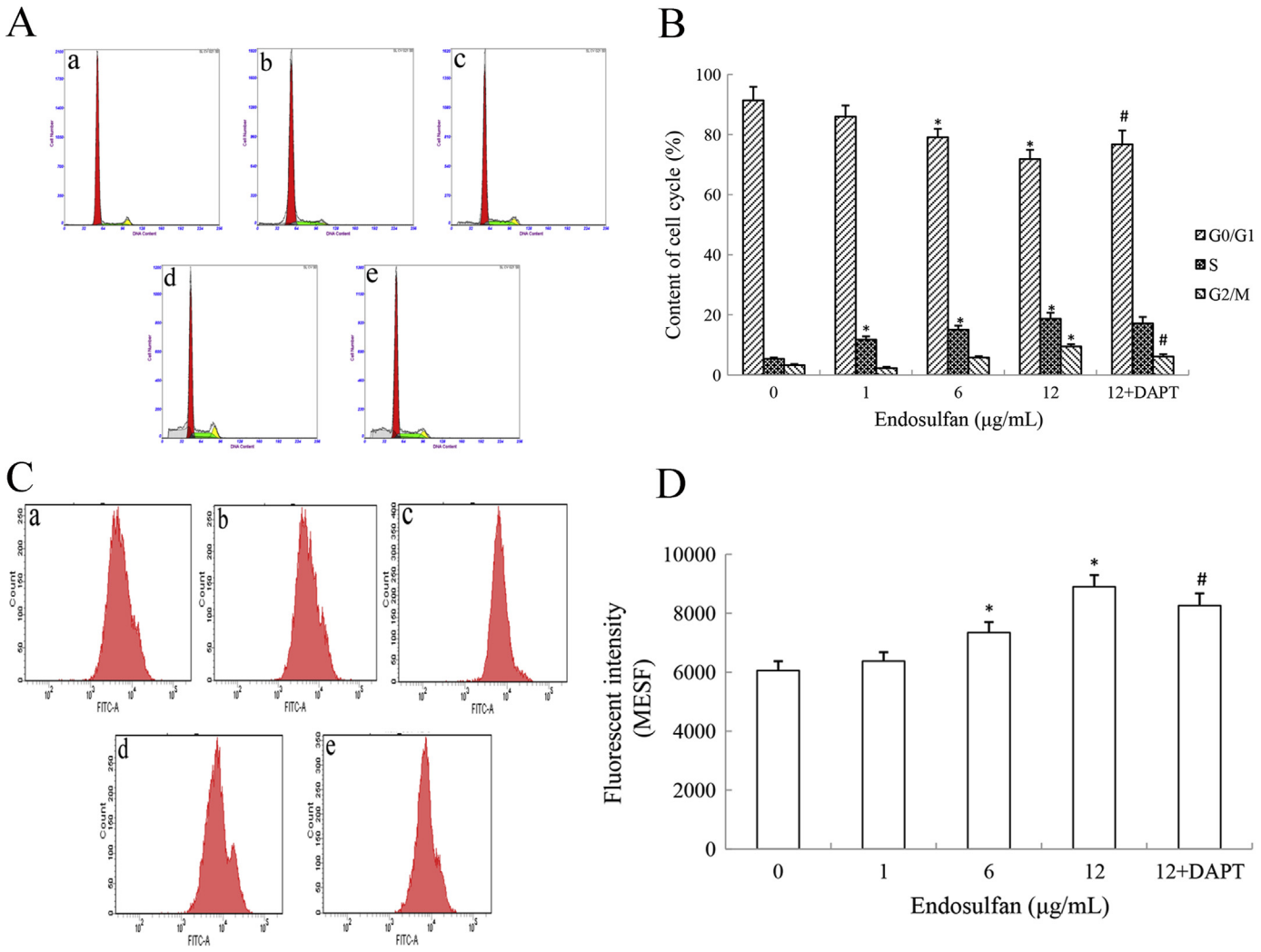


Fig. 3. Effects of endosulfan on the cell cycle and proliferation of HUVECs. (A, B) Distribution of cell cycle after endosulfan treatment was determined by flow cytometry for 24 h a–e: 0 μg/mL endosulfan group (a), 1 μg/mL endosulfan group (b), 6 μg/mL endosulfan group (c), 12 μg/mL endosulfan group (d), 12 μg/mL endosulfan + DAPT (20 μM) group (e). (C, D) Proliferation of HUVECs after endosulfan treatment was determined by flow cytometry. a–e: 0 μg/mL endosulfan group (a), 1 μg/mL endosulfan group (b), 6 μg/mL endosulfan group (c), 12 μg/mL endosulfan group (d), 12 μg/mL endosulfan + DAPT (20 μM) group (e). * indicates significant difference compared with the 0 μg/mL endosulfan group, #indicates significant difference compared with the 12 μg/mL endosulfan group. The data are expressed as mean ± S.D. ($P<0.05$).

G1/S and G2/M arrest (Atashrazm et al., 2016; Tian et al., 2015). Our present study suggested that endosulfan increases the p21 level through Hes1 activated by Notch signaling. Similar results proved that Hes1 expression inhibited cell cycle through the increase in the cell cycle inhibitor p21 in CD34 $^{+}$ cells and cell expansion in vivo (Yu et al., 2006). The blockade of Notch signaling may also partially explain the decreased protein levels of p21, which is also a target gene of Notch signaling (Rangarajan et al., 2001). Our present study investigated that proliferation inhibition and cell cycle arrest could be attenuated in DAPT-treated HUVECs, suggested that Notch signaling could be an important regulator in endosulfan-induced cell cycle arrest. This finding is consistent with previous results from Shyu et al. who showed that Notch signaling may arrest polar cells in the G2 phase by suppressing Stg expression (Shyu et al., 2009). These results demonstrated that endosulfan-induced cell cycle arrest and proliferation inhibition may occur through the activated Notch signaling pathway. If oxidative damage or cell cycle arrest is excessive, apoptosis may be activated (White, 1993). A simliar study also found that the activation of Notch1 signaling could lead to cell cycle arrest and the apoptosis of trophoblast stem cells in rabbit (Tan et al., 2014). What is interesting is that cell exposure to diallyl trisulfide, an organic chemical, led to proliferation inhibition, cell cycle arrest, and apoptosis through the down-regulation of Notch1 (Li et al., 2013). Structurally, diallyl trisulfide is completely different from endosulfan, which implies that diallyl trisulfide may exert the above effects through different mechanisms or Notch1 may have a bilateral effect in determining cell fate.

We postulated that oxidative stress has a significant effect on endosulfan-induced cytotoxicity. ROS, as an upstream stimulating signal, can activate protein kinases and related transcriptional factors; regulate the expressions of downstream genes; act in cell proliferation, differentiation, and apoptosis regulation; and affect cell structure, function, and even cell fate (Liu and Sun, 2010; Park and Park, 2009). Our results showed that NAC, an antioxidant, could antagonize the increases in the ROS and MDA levels, attenuate apoptosis, and inhibit the activation of Notch signaling pathway caused by endosulfan. The results suggested that oxidative stress could be a crucial aspect of cell proliferation inhibition and apoptosis induced by endosulfan. Rastogi et al. found that endosulfan significantly increased ROS generation and MDA levels in Sertoli-germ cells and that NAC can attenuate the apoptosis of human peripheral blood mononuclear cells induced by endosulfan

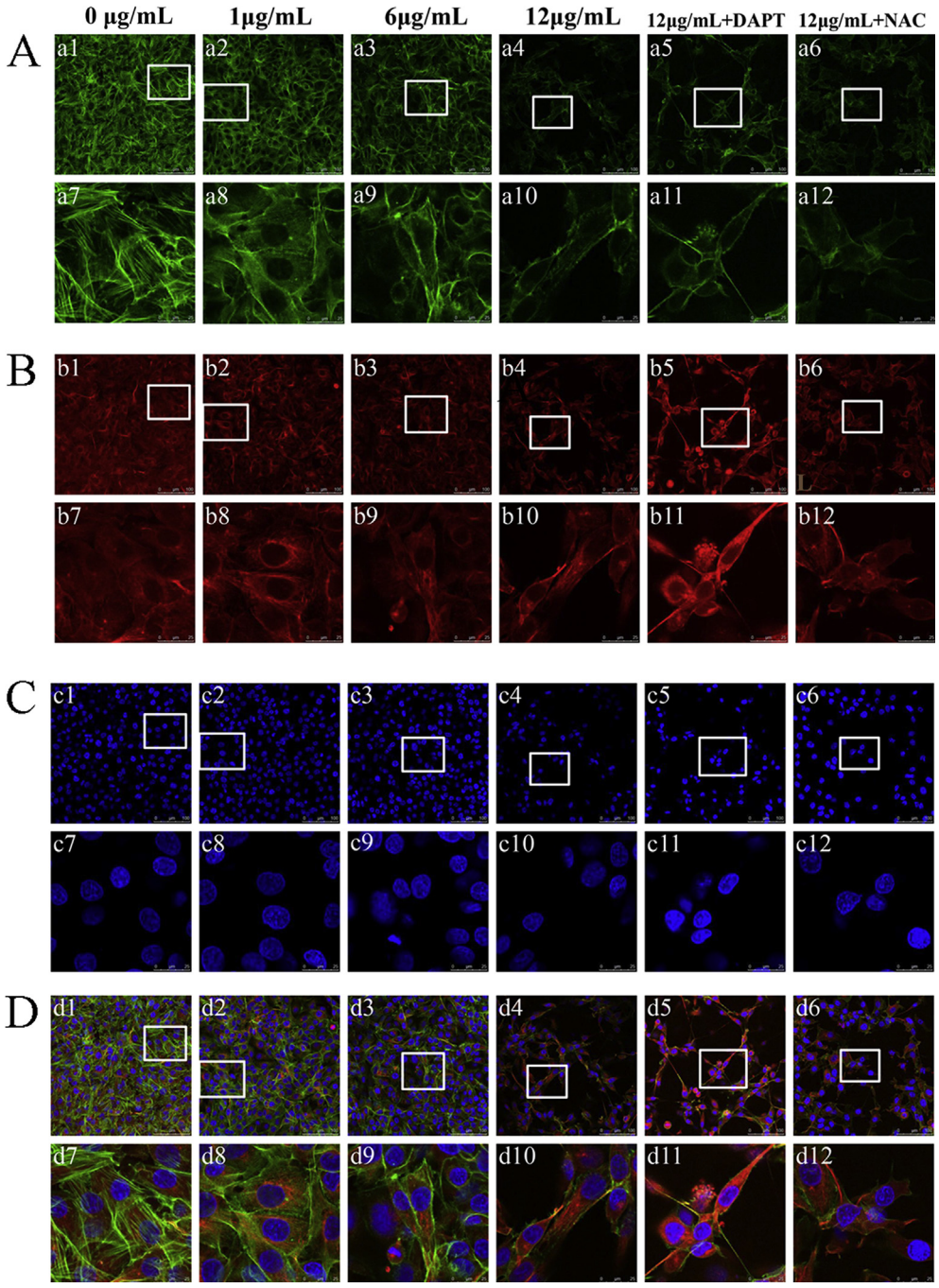
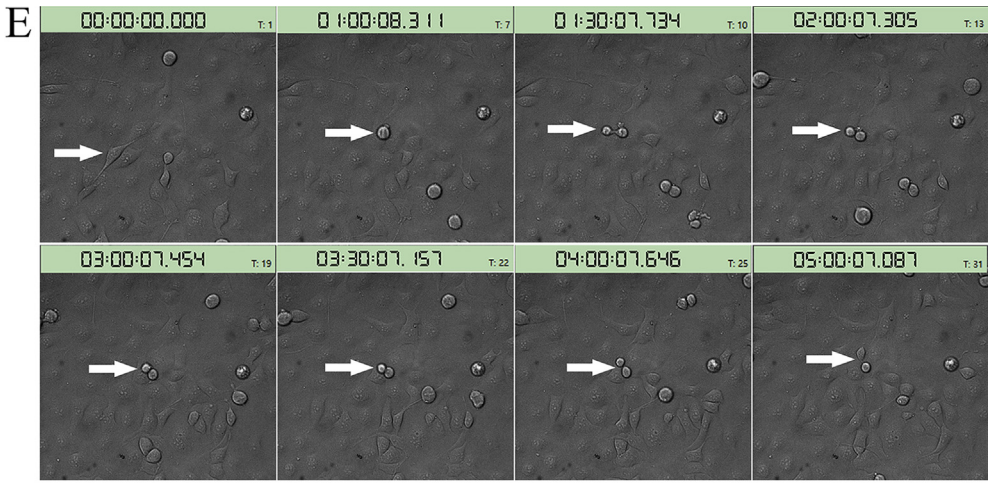
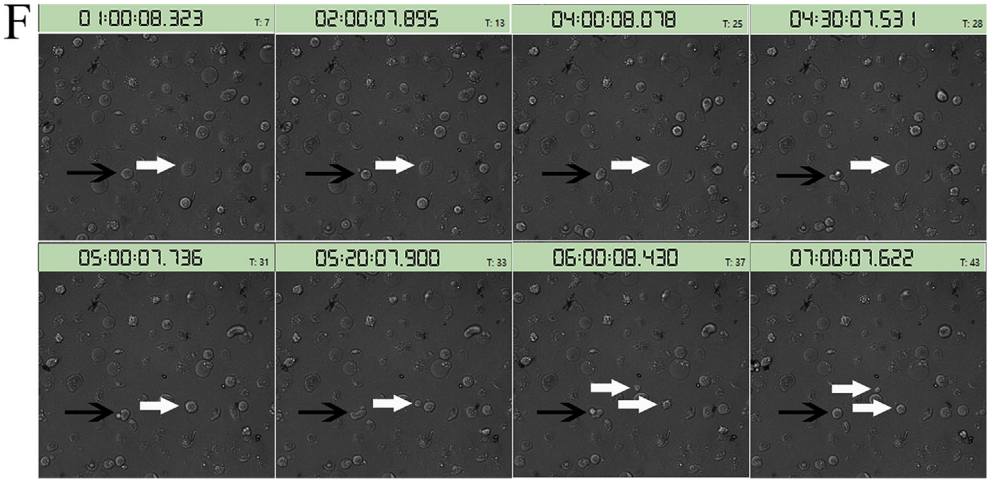


Fig. 4. Effects of endosulfan on cytoskeleton and mitosis in HUVECs. Images 7–12 are the 4 × magnified versions of 1–6, respectively. Microfilament (A), microtubule (B), and cell nucleus (C) were incubated with Actin-Tracker Green, Tubulin-Tracker Red, and Hoechst 33258 solution, respectively. (D) Merged graphs of A, B, and C. Cells were observed using a real-time inverted phase contrast microscope (200 ×) after treatment with 0 (E) and 12 μg/mL (F) endosulfan for 24 h. (G) The percentage of normal mitosis of HUVECs was calculated after treatment with 0 and 12 μg/mL endosulfan for 24 h. White arrow indicates regular mitosis, whereas the black indicates the failure of mitosis. *indicates significant difference between the two groups. The data are expressed as mean ± S.D. ($P<0.05$).

(Rastogi et al., 2014). Excessive ROS can cause oxidative damage in DNA, resulting in DNA modifications, such as single- and double-strand breaks. Endosulfan has been reported to increase the DNA damage resulting from excessive ROS in zebrafish (Shao et al., 2012). Kamarehei found that ROS could activate Notch signaling in SK-N-MC cells and induce apoptosis (Kamarehei and Yazdanparast, 2014). Because the Notch pathway is a highly conservative signaling system, the above Notch-medicated cell proliferation inhibition and apoptosis by endosulfan may be similar in HUVECs. We found that endosulfan can cause apoptosis, induce cell cycle arrest and proliferation inhibition, damage cytoskeleton and cell nuclei, and activate the Notch signaling pathway in HUVECs. Proliferation inhibition by endosulfan is possibly induced by the Notch signaling pathway as a result of oxidative stress. Therefore, the present findings determine the mechanism of endosulfan-induced cardiovascular toxicity and demonstrate that exposure to endosulfan may be an underlying risk factor for the cardiovascular system.





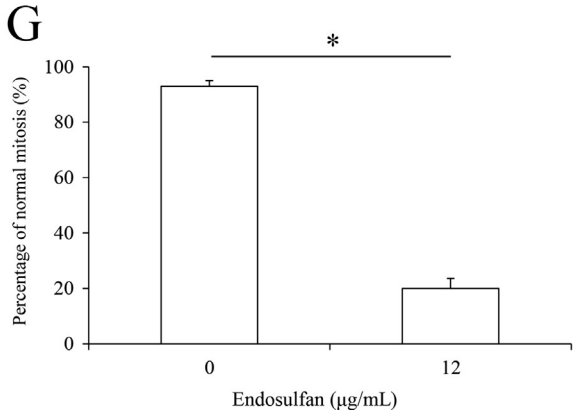


Fig. 4. (*continued*).

Table 1
Damage to microfilaments, microtubules, and nuclei in HUVECs induced by endosulfan.

Concentration (μg mL^{-1})	Fluorescence intensity ofmicrofilaments	Fluorescence intensity ofmicrotubules	Fluorescence intensity of nuclei	Diameters of nuclei (μm)
0	90.89 ± 6.22	70.18 ± 4.89	25.05 ± 2.07	13.68 ± 0.70
1	60.24 ± 2.09*	66.18 ± 3.65	20.68 ± 2.27	13.79 ± 1.25
6	53.95 ± 6.78*	53.02 ± 9.97	29.88 ± 5.07	11.98 ± 0.55
12	32.74 ± 4.74*	51.70 ± 4.59*	27.91 ± 4.02	9.53 ± 1.02*
12 + NAC (3 mM)	27.29 ± 4.37	67.23 ± 8.31	31.07 ± 4.01	11.92 ± 1.69
12 + DAPT (20 μM)	29.55 ± 1.73	51.56 ± 12.44	29.96 ± 4.83	10.68 ± 2.43

*indicates significant difference compared with the 0 μg/mL endosulfan group. The data are expressed as mean ± S.D. ($P<0.05$).

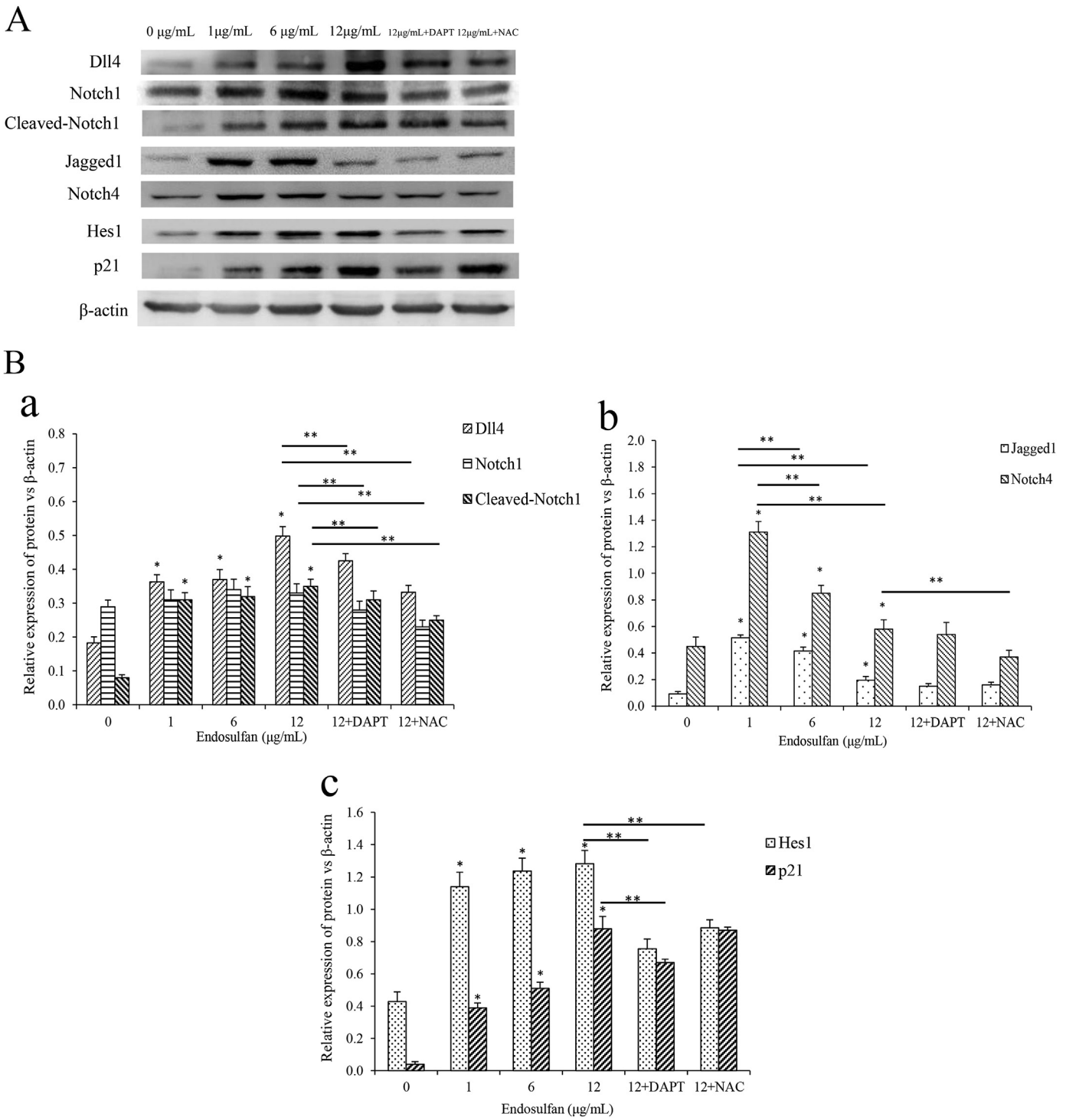


Fig. 5. Effects of endosulfan on the Notch signaling pathway in HUVECs. (A) Expressions of Dll4, Notch1, Cleaved-Notch1, Jagged1, Notch4, Hes1, and p21. (B) The relative densitometric analysis of Dll4, Notch1, Cleaved-Notch1 (a), Jagged1, Notch4 (b), Hes1, and p21 (c) expressions after treatment with different concentrations of endosulfan for 24 h *indicates significant difference compared with the 0 μg/mL endosulfan group, **indicates significant difference between two different groups. The data are expressed as mean ± S.D. ($P<0.05$).

5. Conclusions

The present study revealed a vital molecular mechanism of endosulfan-induced toxicity in endothelial cells through the Notch signaling pathway. Given that the Notch signaling pathway is central to determine cell fate and cellular processes, the current findings have biological implications in cellular organisms. Therefore, blocking the Notch signaling pathway may be a promising way to attenuate the toxicity of endosulfan on endothelial cells. In addition, this finding may have key implications in the prevention of CVD where the Notch signaling pathway can be explored as a preclinical evaluation.

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