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Regulation of c-Jun N-Terminal Protein Kinase (JNK) Pathway in Apoptosis of Endothelial Outgrowth Cells Induced by Asymmetric Dimethylarginine

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Endothelial outgrowth cells (EOCs) are terminal endothelial progenitor cells (EPCs). Asymmetric dimethylarginine (ADMA) has been identified as a novel risk factor for cardiovascular diseases. Our aim in the present study was to investigate the effect of regulation of asymmetric dimethylarginine (ADMA) on EOCs apoptosis and to explore the underlining mechanisms of c-Jun N-terminal protein kinase (JNK) pathway in the process.

Material/Methods: EOCs were harvested from umbilical cord blood and obtained by using density gradient centrifugation and adhesive culture methods. Endothelial characteristics were identified by immunohistochemistry and fluorescence staining. EOCs were treated with different concentrations of ADMA and detected by flow cytometry. After JNK specific inhibitor (SP600125) was added, EOCs apoptosis protein expressions were measured by Western blot analysis. Proliferation, migration, and vascularization were detected by CCK-8 assay, wound healing assay, and tube-like formation assay, respectively.

Results: EOCs were successfully extracted from umbilical cord blood and different concentrations of ADMA aggravated EOCs apoptosis. ADMA distinctly activates the phosphorylation activity of JNK. Supplementation of JNK-specific inhibitor (SP600125) decreased expression of Bax and cleaved caspase 3/9, and alleviated ADMA-induced apoptosis. SP600125 also promoted angiogenesis viability.

Conclusion: The JNK pathway participates in the apoptosis-promoting process of EOCs, and targeted inhibition of the JNK pathway can alleviate ADMA-induced injury, which I s the potential underlying mechanism of vascular endothelium injury in ischemic stroke.

MeSH Keywords: **Apoptosis • Endothelial Cells • MAP Kinase Kinase 7**

Abbreviations: **EOCs** – endothelial outgrowth cells; **ADMA** – asymmetric dimethylarginine; **JNK** – c-Jun N-terminal protein kinase; **EPCs** – endothelial progenitor cells

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Background

Endothelial progenitor cells (EPCs) are precursor cells that can differentiate into vascular endothelial cells. The present study shows that there are at least 2 types of endothelial precursor cell in the human body: primitive EPCs and terminal EPCs. Terminal EPCs are also known as endothelial outgrowth cells (EOCs). EOCs not only participate in the embryonic angiogenesis, but also regulate the process of angiogenesis after birth and promote angiogenesis of ischemic tissues [1,2]. The monoclonal number of EOCs is significantly higher than in EPCs, with a high level of telomerase activity [3]. Studies show that EOCs participate in angiogenesis after ischemic stroke [4]. In addition, EPCs can prevent in-stent restenosis [5] and thrombogenesis [6], and predict the occurrence and prognosis of cerebral ischemia [7]. In general, EOCs, the terminal EPCs, have important clinical application in the treatment of ischemic stroke.

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthases (NOS), which inhibits bioavailability of nitric oxide and can increase production of NOS-derived reactive oxidative species [8,9]. Under pathological conditions, ADMA concentrations significantly rise (3–5 μmol/l) and inhibit synthesis of NOS and NO, which leads to diastolic function depression of peripheral vascular and decreased cerebral blood perfusion [10,11]. Research shows that L-arginine-NO synthetic obstacles have a close relationship with hypertension and atherosclerosis [12,13]. *In vivo*, ADMA expression levels have obvious correlation with the occurrence and prognosis of acute cardiovascular and cerebrovascular diseases [14]. High-level expression of ADMA may be a significant risk factor for vascular endothelial dysfunction [15].

In vitro, ADMA has been confirmed to induce the apoptosis of vascular smooth muscle cells through the JNK signaling pathway [16]. However, the variation after JNK-specific inhibitors intervention has rarely been reported. Our study investigated the specific function of JNK-specific inhibitor in the apoptosis of EOCs induced by ADMA.

Material and Methods

Cell culture

Samples (30 ml) of fresh umbilical cord blood were collected from 30 healthy parturient patients at the Obstetrical Department, Second Hospital of Tianjin Medical University. Mononuclear cells were extracted from umbilical cord blood by density gradient centrifugation and then resuspended in 2 ml EGM-2 medium (containing 10% FBS). Cells were inoculated in culture plates coated with HFN for 2 h and then cultured in an incubator (37°C, 5% CO_2). After growing to confluence, primary cells were sub-cultured for the next experiments.

Immunohistochemistry

The 2nd passage cells were inoculated into 24-pore plates and cultured at attached state. Then, cells were fixed with 4% paraformaldehyde for 20 min. Endogenous peroxidase was blocked with 0.3% H_2O_2-formalin for 10 min. After washing with PBS, antibodies (all from Santa Cruz, Dallas, TX, USA), including FLK1 antibody (1: 100, mouse, sc-29318), VIII factor antibody (1: 100, rabbit, sc-400373), and CD34 antibody (1: 100, rabbit, sc-400197), were added and incubated at 4°C overnight. The combination of secondary antibody was according to the instructions of the ABC immunohistochemical detection kit (VECTASTAIN, Vector, USA, PK-6100). Cells were stained by AEC agent and re-dyed by hematoxylin. Staining results were observed under an inverted phase-contrast microscope (100×, Leica, Heidelberg, Germany).

Fluorescence staining

The cells were the same as in the previous section. We added DiI-acLDL (10 mg/l) to these cells and incubated them at 37°C for 4 h, followed by fixing with 4% paraformaldehyde solution for 10 min. After washing with PBS, we added FITC-UEA-I (10 mg/l) to the samples and incubated them at 37°C for 1 h. Staining results were observed under a fluorescence microscope (Olympus, Japan, CX31-32RFL). DiI-acLDL and FITC-UEA-I double-staining positive cells were EOCs in differentiation phase.

Annexin-V/PI staining and flow cytometry

Samples were centrifuged at low speed (100 g) for 10 min. The supernatant was discarded and the precipitate was washed with PBS, trypsinized, and resuspended in 100 μl Annexin binding buffer. Afterwards, we added 5 μl Annexin-V and 1 μl PI (100 μg/ml) to each tube, and incubated them at room temperature in the dark for 15 min. Finally, the apoptosis was detected by use of a flow cytometer.

Western blotting analysis

EOCs were disposed by RIPA lysis buffer (Santa Cruz, Dallas, USA). The liquid was transferred into sterile EP tubes and placed on ice for 20 min. Afterwards, tubes were centrifuged at 12 000 rpm for 10 min. Protein concentrations were measured and compared with BCA. A mixture of equal parts of protein and sample buffer was subjected to SDS-PAGE. The extracted protein was transferred onto PVDF membranes. The PVDF membrane was blocked in blocking buffer (TBST, 5% fat-free milk) for 1 h at room temperature. The following primary antibodies were used for immunoblotting: p-JNK (Cell Signaling, 1: 1000, no.4668),

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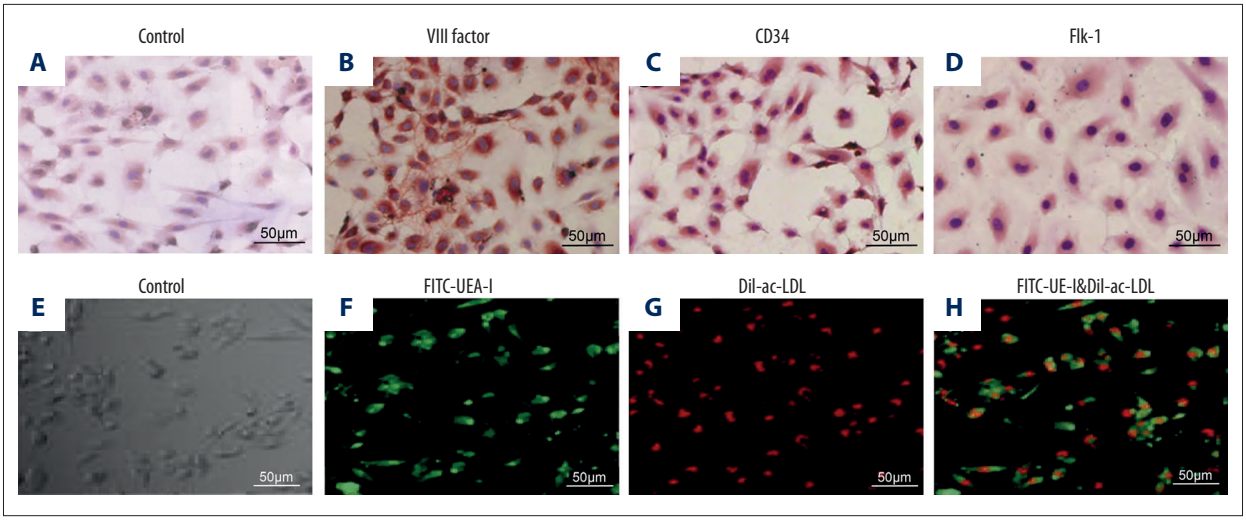

Figure 1. EOCs identification by immunohistochemistry and fluorescence staining. (**A**) The negative control group for IHC. (**B**) VIII factor-related antigen expression was positive on the surface of EOCs. (**C**) CD34 positive. (**D**) Flk-1 positive. (**E**) The negative control group. (**F**) Combination of FITC-UEA-I on surface of EOCs. (**G**) Combination of DiI-ac-LDL. (**H**) EOCs were double-staining positive. All graphs are exhibited with the magnification of 200×. The scale is in the corner.

JNK (Cell Signaling, 1: 1000, no.4672), Bcl-2 (Abcam, 1: 2000, ab32124), Bax (Abcam, 1: 2000, ab32503), cleaved caspase 3 (Abcam, 1: 2000, ab13585), and cleaved caspase 9 (Abcam, 1: 2000, ab32539). GAPDH was used as the internal reference. The PVDF membrane was incubated in the diluted primary antibodies at 4°C overnight and washed with TBST 3 times for 10 min. Then, the PVDF membrane was diluted in secondary antibody (Santa Cruz, Dallas, TX, USA, sc-33732, 1: 1000) at 4°C overnight. The PVDF membrane was demonstrated by Image Pro-Plus system (Media Cybernetics, Silver Spring, MD, USA).

Cell proliferation assay

Proliferation of EOCs was detected by use of the CCK-8 assay kit (Dojindo, Japan, CK04-500). Briefly, EOCs were seeded in 96-well culture plates at the density of 2×10^4 cells/well with 100 mL FBS-free culture medium. After 24 h, ADMA (10 μmol/l) and SP600125 (0, 1, 5, 10 μmol/l) were added to EOCs. After 12 h of incubation, 10 μl of CCK-8 reagent was added to each well. Then, the optical density (OD) values were read at 450 nm using a microplate reader (Tecan Sunrise, Männedorf, Switzerland). The final data collection was repeated at least 3 times.

Wound healing assay

Briefly, EOCs were seeded onto gelatin-coated 6-well plates (Nunc, Roskilde, Denmark). Confluent cell monolayers of EOCs were scratched with a sterile pipette tip. The monolayers were washed twice and then cultured for 12 h in basal medium. Images of the same areas were taken immediately after scratching (0 h) and at 24 h under an Axiovert 25 microscope (Zeiss, Jena, Germany). The width of the wounding scratches at different time-points was measured and is expressed as relative percentage compared with the initial distance. Experiments were performed in triplicate.

Tube-like formation assays

Matrigel (BD Biosciences, NJ, USA) (10 mg/mL) was coated on 24-well culture plates at 37°C (300 μl per well). After 30 min, EOCs (5×10^4) were seeded onto the gel. Varying concentrations of SP600125 (0, 1, 5, 10 μmol/l) and ADMA (10 μmol/l) were added to the cell culture medium. After 24 h of incubation at 37°C, tube-like structures were observed with a phase-contrast microscope (Axiovert 25, Zeiss). The semi-quantitative evaluation of the tube-like length from 4 randomly selected fields was performed by ImageJ software. Assessment was performed in triplicate.

Statistical analysis

Data are expressed as a mean ± standard deviation and analyzed with SPSS software (SPSS, Chicago, IL, USA). The data were analyzed by one-way ANOVA or Student's t test. $P<0.05$ was considered to be statistically significant.

Results

Immunohistochemistry and fluorescence staining

After being collected from umbilical cord blood, EOCs were identified by immunohistochemistry and fluorescence staining (Figure 1). Immunohistochemistry showed that CD34, FLK 1, and

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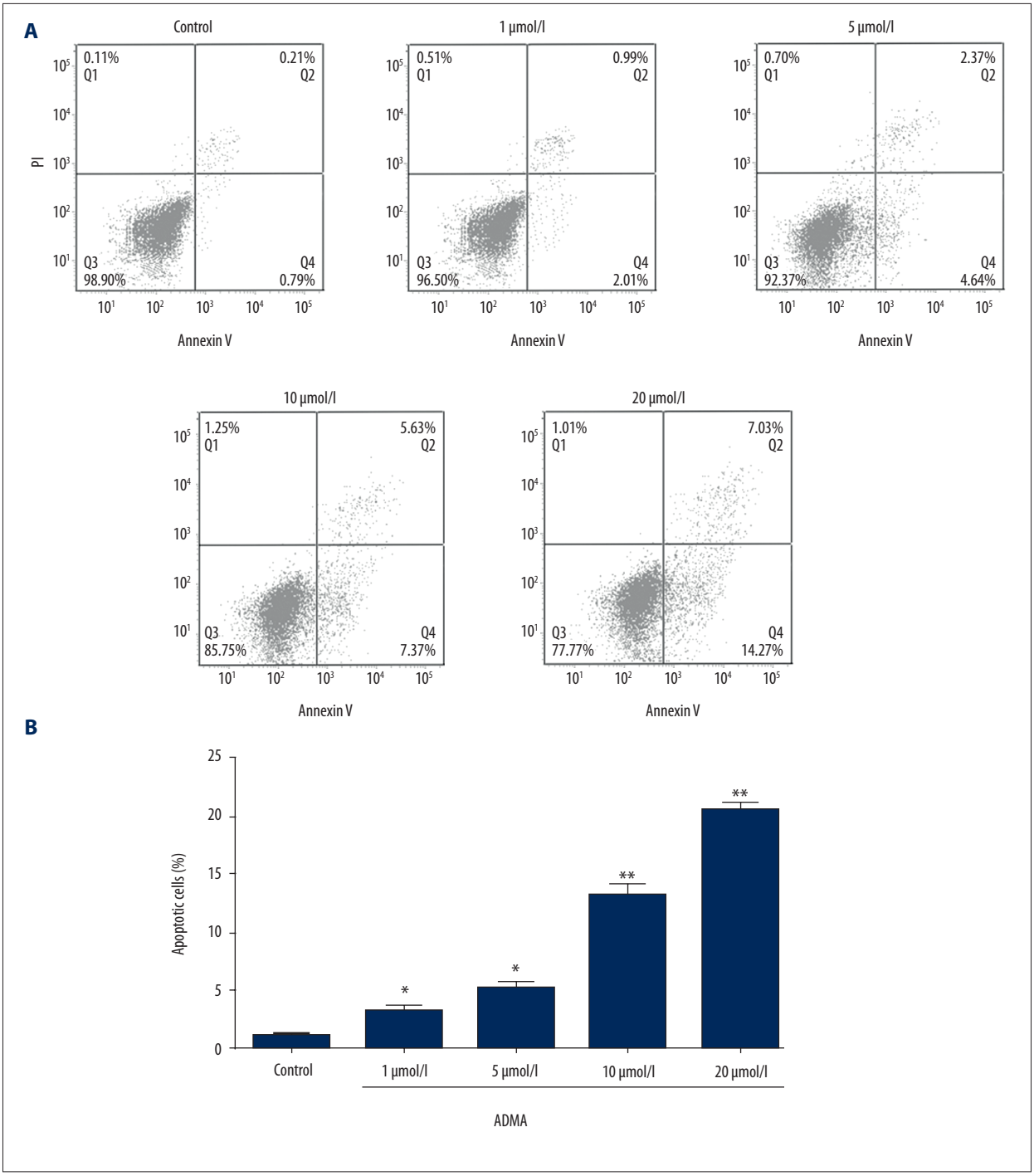

Figure 2. Flow cytometry showing the apoptosis-promoting effect of ADMA on EOCs. (**A**) EOCs were stained with Annexin-V/PI for flow cytometry analysis. Apoptotic cells in every group contained 2 stages (early apoptotic and late apoptotic cells). (**B**) The total apoptosis rates of EOCs were determined by flow cytometry. * P<0.05, ** P<0.01 compared with control group.

VIII factor-related antigen expressions were all positive on the surface of EOCs (Figure 1A–1D). DiI-ac-LDL and FITC-UEA-I combined with EOCs (Figure 1F, 1G). Fluorescence staining showed that double-staining positive cells were EOCs (Figure 1H).

AMDA aggravates EOCs apoptosis as detected by flow cytometry

AMDA has been reported to affect the apoptosis of EOCs and our study detected the regulation by flow cytometry (Figure 2).

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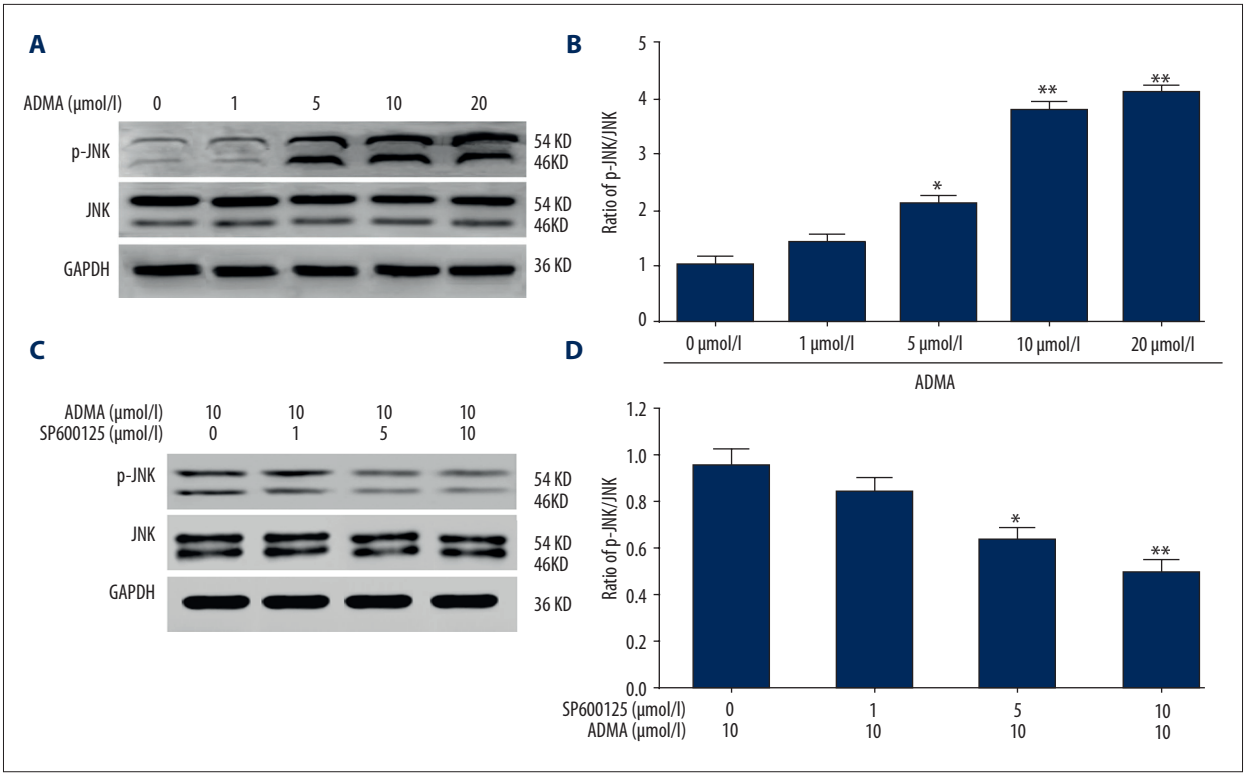

Figure 3. Phosphorylation activity of JNK was detected by Western blot. (**A**) Typical bands of p-JNK, JNK at different concentration (0, 1, 5, 10, and 20 μmol/l) of ADMA. (**B**) Relative quantified expression p-JNK/JNK. (**C**) Typical bands of p-JNK and JNK at different concentrations (0, 1, 5, and 10 μmol/l) of JNK-specific inhibitor (SP600125) and ADMA (10 μmol/l). (**D**) Relative quantified expression p-JNK/JNK. * P<0.05, ** P<0.01 compared with the first group.

Different concentrations of ADMA were applied to EOCs. After 48 h of co-incubation, results revealed that AMDA aggravated EOCs apoptosis, and higher concentration of ADMA had more obvious apoptosis-promoting effect.

JNK pathway was activated by different concentrations of ADMA

The effect of regulation of ADMA on the JNK pathway was detected by Western blot (Figure 3). Different concentrations (0, 1, 5, 10, 20 μmol/l) of ADMA activated the phosphorylation activity of JNK. In addition, the activation process exhibited distinct dose-dependent phenomenon, which means the higher concentration (20 μmol/l) of ADMA obviously promoted the phosphorylation activity (Figure 3A, 3B). JNK-specific inhibitor (SP600125) markedly suppressed the phosphorylation activity (Figure 3C, 3D). Combined with previous results, the apoptosis-promoting mechanism might be that ADMA promotes the phosphorylation activity of JNK and then induces the apoptosis.

JNK-specific inhibitor (SP600125) suppresses the apoptosis of EOCs

After treatment with varying concentrations of SP600125 (0, 1, 5, and 10 μmol/l), the apoptosis of EOCs was detected by flow cytometry and Western blot (Figure 4). Flow cytometry showed that higher concentrations of SP600125 decreased the number of apoptotic cells (Figure 4A, 4B). Western blot analysis showed that higher concentrations of SP600125 reduced the Bax and cleaved caspase 3/9 expression. The above results indicated that SP600125 specifically inhibited JNK expression and decreased apoptosis of EOCs.

EOCs proliferation viability

After treatment with varying concentrations of SP600125 (0, 1, 5, and 10 μmol/l) and ADMA (10 μmol/l), the proliferation vitality, migration, and tube formation were detected to evaluate biological effects of SP600125 (Figure 5). CCK-8 assay showed that EOCs proliferation viability was promoted by increasing concentrations of SP600125 (Figure 5A). Moreover, wound healing assay showed that migration of EOCs was significantly increased compared to the control group (Figure 5B, 5C). Tube-like structure formation and semi-quantitative evaluation of

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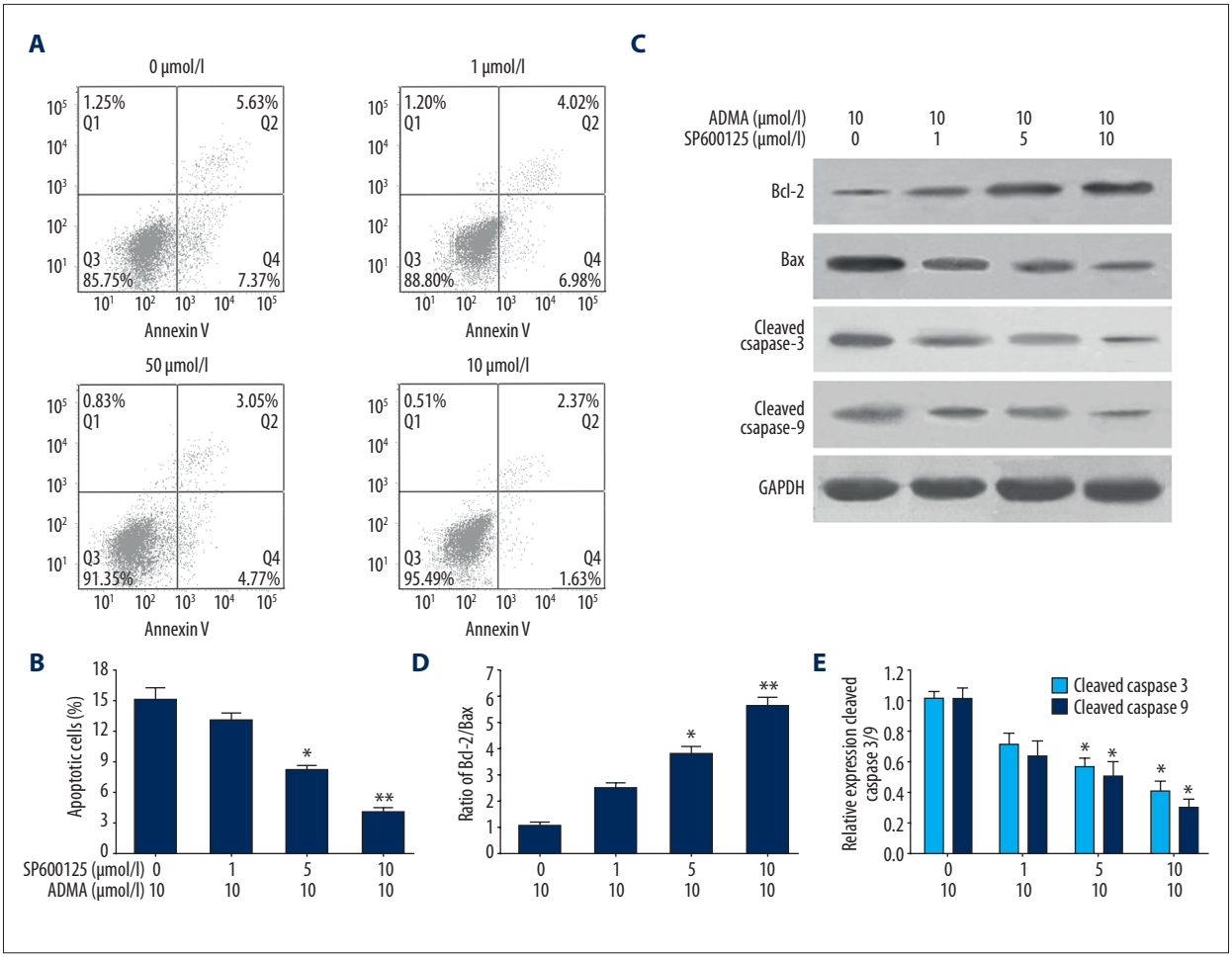

Figure 4. Apoptosis inhibition of JNK-specific inhibitor (SP600125) on EOCs. (**A**) Apoptosis was detected by flow cytometry (Annexin-V/PI) added with different concentration of JNK specific inhibitor (SP600125). (**B**) Apoptotic cells of EOCs. (**C**) Representative bands detected by Western blot. (**D**) Ratio of Bcl-2/Bax. (**E**) Relative expression of cleaved caspase 3/9. * P<0.05, ** P<0.01 compared with the first group.

the branch network showed that addition of different concentrations of SP600125 to the EOCs culture medium impaired the tube-like structure network (Figure 5D, 5E).

Discussion

The destruction of integrity and activity of the endothelial layer is a key factor in the pathogenesis and progression of atherosclerosis [17]. Endothelial progenitor cells (EPCs) are a group of precursor cells that differentiate into mature endothelial cells [18]. Our study investigated the apoptosis-promoting effect of ADMA on EOCs, a sub-type of terminal EPCs, and then explored the underlining mechanism of the JNK pathway in endothelial regeneration.

In our study, EOCs were extracted from umbilical cord blood and identified by immunohistochemistry and fluorescence staining (Figure 1). Dying results showed that EOCs were stained positive on CD34, FLK1, and VIII factor, as well as DiI-ac-LDL and FITC-UEA-I double-staining. In the apoptosis process, AMDA aggravated EOCs apoptosis and in a dose-dependent manner (Figure 2). Generally, higher concentrations of ADMA had more obvious apoptosis-promoting effect. ADMA is an endogenous inhibitor of nitric oxide synthase (NOS), and plasma ADMA level is closely associated with the morbidity of atherosclerosis [14]. Research shows that ADMA is closely related to the apoptosis of endothelial progenitor cells [19]. The possible mechanisms include decreasing of NO generation [20] and apoptosis induced by MAPKs pathways [21]. eNOS regulates the generation of NO and acts as a bio-marker of EOCs for cell identification [22]. On the other hand, ADMA inhibits the synthesis of NO and O_2^- and then hinders the cellular damage caused by NMDA [23].

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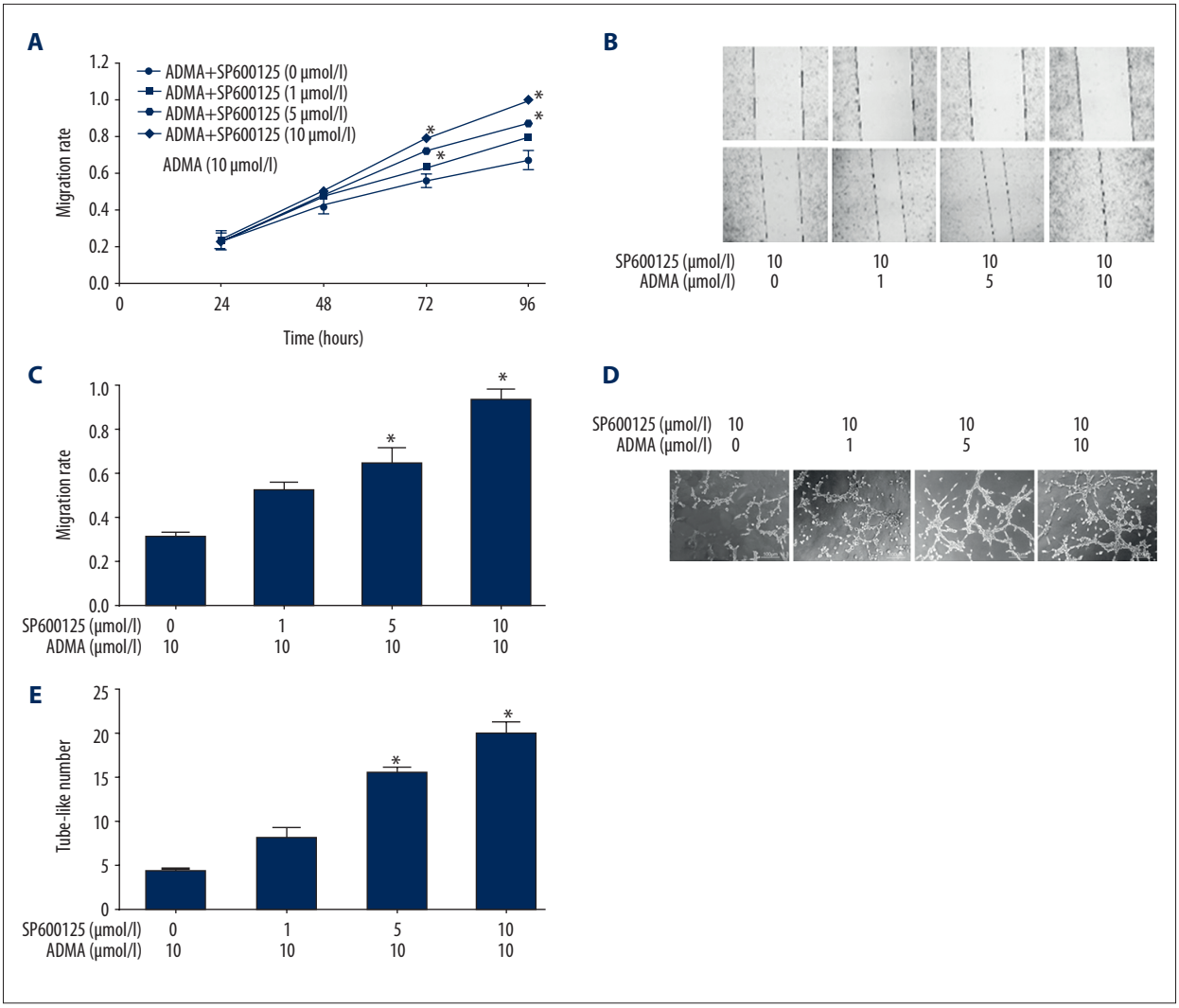

Figure 5. Proliferation vitality, migration, and tube formation were detected. (**A**) Proliferation vitality was estimated by CCK-8. (**B**) Representative pictures of the scratch assay acquired at time-points 0 and 24 h. (**C**) The migration rate. (**D**) Representative photographs of tube-like formation under optical microscope. (**E**) The branch network formation at ×200 magnification. Experiments were performed in triplicate. Scale bar is marked on the graphs. * P<0.05 compared with the first group.

Our study confirmed that different concentrations (0, 1, 5, 10, and 20 μmol/l) of ADMA activate the phosphorylation activity of JNK (Figure 3A, 3B). In addition, JNK-specific inhibitor (SP600125) markedly suppressed the phosphorylation activity (Figure 3C, 3D). Therefore, the apoptosis-promoting mechanism might be that ADMA promotes the phosphorylation activity of JNK and then induces the apoptosis. *In vitro*, cell research has confirmed that ADMA induces vascular smooth muscle apoptosis through the JNK signaling pathway [16]. In the next experiments, we used JNK-specific inhibitor (SP600125) to verify the hypothesis. Because ADMA regulates the NO-ROS balance and participates in the development of atherosclerosis, interventions aimed at NO-ROS balance could be protective in developmentally programmed cardiovascular and cerebrovascular diseases [24]. Apoptotic cells and apoptosis-related proteins (Bax and cleaved caspase 3/9) are decreased with the addition of SP600125 (Figure 4). Combined with previous results, this indicates that SP600125 specifically inhibits JNK expression and decreases apoptosis of EOCs. The JNK pathway has been verified to participate in the metabolic regulation of ADMA. Principally, dimethylarginine dimethylaminohydrolase-1 (DDAH1) is responsible for ADMA degradation, which plays an important role in maintaining NO bioavailability and preserving cardiovascular function in cerebral apoplexy [25]. SP600125 might up-regulate DDAH1 activity to accelerate ADMA degradation [26]. Proliferation, migration, and tube formation assays demonstrate that addition of SP600125 obviously accelerated the vitality and vascularization of EOCs (Figure 5). It has been

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frequently reported that cardiovascular drugs effectively cure ADMA-induced vascular injury via the JNK pathway [27]; for instance, probucol protects against ADMA-induced HBMEC injury and suppresses oxidative stress through the JNK/p38 MAPK pathway.

Conclusios

Multiple-factors analysis shows that ADMA is an independent risk factor for cerebral infarction [28]. Early-intervention treatment for ADMA is expected to reduce the incidence and mortality of ischemic cerebrovascular disease [29]. Our study reveals an effective function channel to suppress apoptosis induced by ADMA and promote vascular endothelial generation. However, details of the mechanism and more practical therapeutic targets need to be explored in our subsequent research.

Acknowledgements

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