

## LETTER TO EDITOR

# Diethylstilbestrol mediates vascular endothelial inflammatory injury in vitro and in vivo

Dear Editor,

Diethylstilbestrol (DES) is a common environmental contaminant that may cause adverse human health effects via endocrine disruption. Recently, DES has been implicated in the impairment of the cardiovascular system.<sup>1</sup> Accumulating data from human and animal studies indicate that DES exposure contributes to carcinogenesis, reproductive system injury, and impairment of the immune system.<sup>2,3</sup> Nevertheless, the effects of DES on the circulatory system remains largely unknown. In this study, evidence for adverse impacts of DES in vascular ECs, both in vitro and in vivo, is presented that highlights the need for additional research focused on DES toxicity and underlying molecular mechanism. Human umbilical vein endothelial cells (HUVECs), chick embryo chorioallantoic membrane (CAM), and matrigel plug were treated with VEGF and DES. We found that DES prevented VEGF mediated action on viability, motility, and capillary network formation of HUVECs by CCK-8, migration, and capillary tubule formation assay, respectively. Also, DES prevented VEGF-mediated angiogenesis and vasculogenesis by CAM and subcutaneous matrigel. DES facilitated anti-angiogenic effects on HUVECs by inducing inflammatory cytokines via YAP/Mst1–FOXO3A signaling. This study demonstrates for the first time that DES promotes a proinflammatory response in vascular endothelial cells (ECs) by inducing inflammatory cytokines both in vitro and in vivo. Exposure to DES might lead to cellular inflammation events and ECs injury, and consequently, cause vascular dysfunction increased risk of cardiovascular disease.

DES significantly suppress the growth of testis cells, stem cells, NK cell, and others. However, the effect of DES on EC angiogenesis role is still unknown. In the present study, HUVECs viability reduced blocked by treatment with DES. DES exposure significantly reduced HUVECs viability at concentrations of 25  $\mu$ M or more (Figure 1A). DES also repressed viability in a time dependent manner (Figure 1B).

DES also effectively prevented chemotactic migration and capillary-like tube formation. ECs migration contributes to

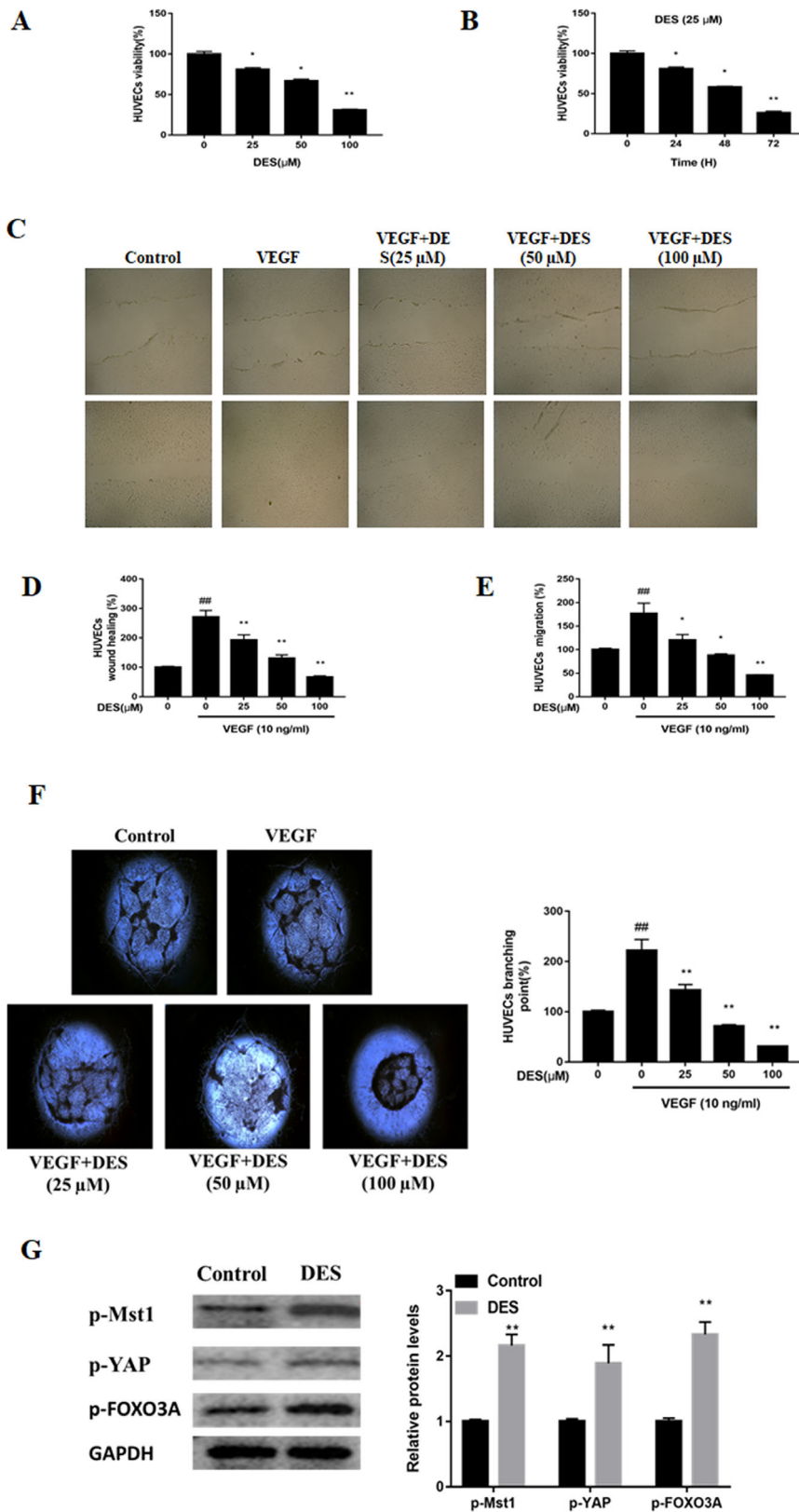
angiogenesis. Wound sizes in HUVEC cultures were substantially narrowed by incubation in the presence of VEGF (10 ng/mL) for 24 h (Figure 1C,D). DES reversed VEGF-mediated wound closure of HUVECs between edges of the scratch. Moreover, at a concentration of 100  $\mu$ M, the wound area of ECs was almost reversed, from  $271.76 \pm 22.29\%$  of control (VEGF treatment) to  $67.21 \pm 4.31\%$  (with DES treatment) (Figure 1C,D). Transwell assays also revealed that ECs migration rate was suppressed in by DES treatment (Figure 1E). Formation of capillary tubes was assessed after incubation for 12 h in the presence of VEGF and DES. VEGF (10 ng/mL) significantly stimulated capillary lumen formation to  $225 \pm 22\%$  of the control cells (Figure 1F). DES at concentrations of 25, 50, or 100  $\mu$ M dramatically diminished network formation (Figure 1F). DES induces an inflammatory response ECs, and current findings indicate that this response is due induction of vascular inflammation via inflammatory mediators and proinflammatory cytokines.

Both CAM and matrigel plug assays are frequently used for assessing angiogenesis. These methods are low-cost, simple, and highly reproducible<sup>4-6</sup> In this study, CAM assay showed that DES significantly inhibits VEGF-mediated vessel formation (Figure 2A,B). Using Matrigel plugs, DES also notably suppressed VEGF mediated microvessel integrity. Compared to the control mice, the capillary density was significantly higher in the VEGF treated plugs, and DES reversed these effects (Figure 2C,D). VEGF-induced more than a fourfold increase in hemoglobin levels compared to the control mice, and this increase was absent in DES-treated plugs (Figure 2E). These data suggest that DES might inhibit angiogenesis in vivo.

Increasing evidence demonstrates that FOXO3A expression inhibits cell proliferation, migration, and tube formation. FOXO3A is a key downstream target of the Hippo–YAP pathway.<sup>7</sup> As an essential factor of the Hippo/YAP pathway, Mst1 is functionally upstream of FOXO3A. Mst1 elevates phosphorylation expression of FOXO3A and triggers expression of downstream genes.<sup>8</sup> Activation of the Hippo–YAP pathway can induce FOXO3A, and subsequently affect

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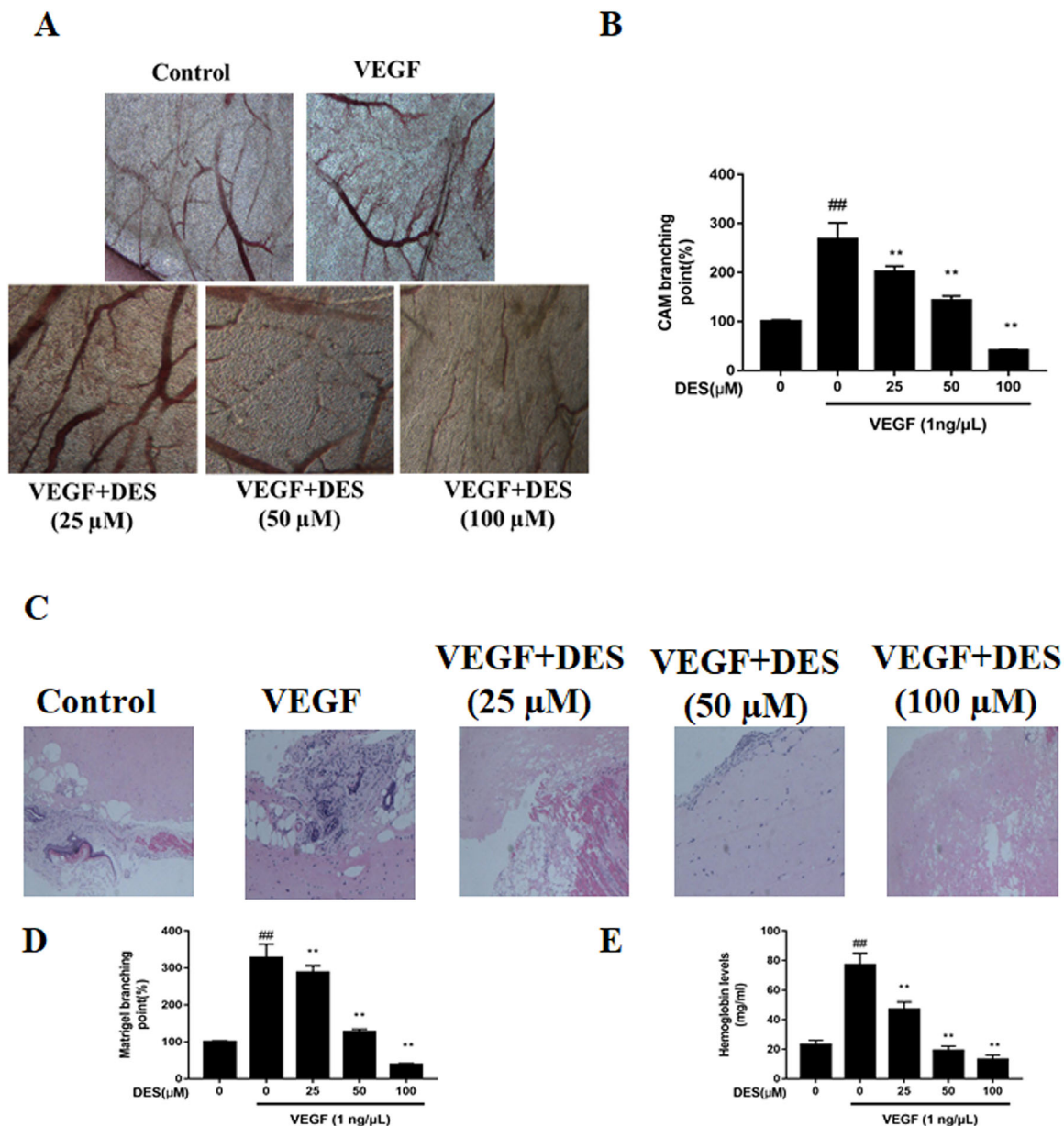
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**FIGURE 1** A, DES reduces the viability of HUVECs. HUVECs were treated with the 0-100  $\mu\text{M}$  of DES. After indicated times, viability was assessed using CCK-8 assays. B, Time-course study of viability. \* $P < .05$ , \*\* $P < .001$ ; DES prevents VEGF-induced chemotactic migration in HUVECs. C,D, HUVECs were exposed to 10 ng/mL of VEGF and 0-100  $\mu\text{M}$  of DES; representative images of cells from in vitro wound healing assays. E, HUVECs were treated with 10 ng/mL of VEGF and 0-100  $\mu\text{M}$  of DES for 24 h, the number of cells in in vitro transwell assays. \* $P < .05$ , \*\* $P < .001$ ; F, VEGF induces capillary-like lumen formation in HUVECs. HUVECs were treated with 10 ng/mL of VEGF and 0-100  $\mu\text{M}$  of DES. Lumen formation of HUVECs was stopped and measured by counting lumen formations. # $P < .05$ , ## $P < .001$ ; G, Effect of DES on the activation of YAP/Mst1-FOXO3A in HUVECs. Total cell lysates were used to evaluate the levels of proteins. \* $P < .05$ , \*\* $P < .001$

EC function. Phosphorylation of Mst1, YAP, and FOXO3A were higher in the DES-treated cells (Figure 1G), suggesting that DES exerts its impairment effect on ECs via inducing the YAP-FOXO3A signaling pathway.

In conclusion, DES impaired angiogenic function in HUVECs, including overall viability, migration, and capillary-like lumen formation. Additionally, the anti-angiogenic activity of DES was found both in CAM and



**FIGURE 2** DES limited VEGF-induced number of capillary formation in the CAM model. A,B, The fertilized eggs were treated with 0-100  $\mu$ M of DES and 1 ng/ $\mu$ L of VEGF for 7 days. The left panel shows magnified blood vessels. The right panel shows relative blood vessel density in the CAM model. \* $P$  < .05, \*\* $P$  < .001, ### $P$  < .01; C,D, DES limited VEGF-induced angiogenesis in vivo. C57BL/6 mice were injected with 0.8 mL of matrigel containing 0-100  $\mu$ M of DES and 1 ng/ $\mu$ L of VEGF. Matrigel plugs were obtained from mice and stained with H&E assay. E, Hemoglobin levels of the Matrigel plugs were estimated with a Drabkin's reagent kit. \* $P$  < .05, \*\* $P$  < .001, ### $P$  < .01

matrigel plug in vivo assays. Impaired angiogenic function caused by DES is associated with the upregulation of YAP/Mst1-FOXO3A signaling, providing new insight into DES cytotoxicity.

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#### CONFLICT OF INTEREST

The authors have declared no conflict of interest.

#### AUTHOR CONTRIBUTIONS

M.Z. and J.S. were associated with conception and design of the study. M.Z. and J.S. performed the experiments. M.Z. and J.S. drafted the manuscript. M.Z. and J.S. reviewed and edited the manuscript. All authors approved the manuscript.

## DATA AVAILABILITY STATEMENT

Data are available upon reasonable request.

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## SUPPORTING INFORMATION

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