Hydroxysafflor Yellow A inhibits the viability and migration of vascular smooth muscle cells induced by serum from rats with chronic renal failure via inactivation of the PI3K/Akt signaling pathway

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Abstract. It has been reported that the viability and migration of vascular smooth muscle cells contributes to arteriovenous fistula stenosis. Hydroxysafflor Yellow A (HSYA) has been demonstrated to inhibit the viability and migration of VSMCs by regulating Akt signaling. The present study aimed to investigate the role of HSYA on the viability and migration of human umbilical vein smooth muscle cells (HUVSMCs) following stimulation using serum from rats with chronic renal failure (CRF), and to determine the effects of HSYA on PI3K/Akt signaling. Wistar rats were randomly divided into two groups, control and CRF groups. Serum from each group was collected to stimulate the HUVSMCs. Cell Counting Kit-8 and wound healing assays were performed to assess cell viability and migration, respectively. Flow cytometry analysis was performed to assess apoptosis, and western blot analysis was performed to detect protein expression levels of PI3K and Akt. Nitric oxide (NO) production was measured using the Nitrate/Nitrite assay kit. The results demonstrated that serum from CRF rats significantly enhanced cell viability, migration and apoptosis, the effects of which were reversed following treatment with HSYA. In addition, CRF serum decreased NO and endothelial NO synthase expression, whilst increasing the protein expression levels of PI3K and phosphorylated-Akt in HUVSMCs. Notably, treatment with HSYA markedly restored NO production and inactivated the PI3K/Akt signaling pathway. Furthermore, the PI3K/Akt inhibitor, AMG511, exerted similar effects to HSYA. Taken together, the results of the present study suggest that HSYA suppresses cell viability and migration in the presence of CRF serum by inactivating the PI3K/Akt signaling pathway.

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Introduction

Hemodialysis is the primary renal replacement therapy for patients with end-stage renal disease (ESRD), and is designed to perform some of the functions lost as a result of chronic renal failure (CRF), such as clearing metabolic wastes and regulating the balance of water, electrolytes and the acid-base balance, which increases the survival of patients and improves the quality of life (1). Arteriovenous fistula (AVF) is the preferred mode of access for hemodialysis. The artery near the wrist of the forearm and the adjacent vein are sutured to allow arterial blood flow in the vein following anastomosis, which forms the AVF (2), the 'lifeline' for patients with hemodialysis. However, the patency rate of AVF after 1 year is 70%, and 48% after 4 years (3). The failure of AVF can be attributed to venous stenosis, intimal hyperplasia, technical problems and inflow issues (4-6).

The primary cause of AVF stenosis is venous stenosis caused by intimal hyperplasia at the anastomosis site of AVF (7). Previous studies have reported that intimal hyperplasia is predominantly caused by the viability and migration of vascular smooth muscle cells (VSMCs) (8,9). Extensive neointimal hyperplasia composed of VSMCs has been observed at the anastomosis site of AVF (10). Thus, inhibiting the viability and migration of VSMCs may be an effective intervention of AVF stenosis. It has been confirmed that activation of the PI3K/Akt signaling pathway promotes aberrant viability and migration of VSMCs (11,12). Park *et al* (13) demonstrated that inhibiting the PI3K/Akt signaling pathway disrupts the viability of rat aortic VSMCs. Conversely, activation of the PI3K/Akt signaling pathway induces the viability and migration of VSMCs (14). Therefore, therapies targeting the PI3K/Akt signaling pathway may be promising in inhibiting AVF stenosis.

Hydroxysafflor Yellow A (HSYA) is a water-soluble chalcone glycoside extracted from Carthami Flos, the flower of safflower (*Carthamus tinctorius* L.), which is the primary active ingredient in the pharmacological action of Carthami Flos (15). HSYA exerts several pharmacological effects, such as cardiovascular effects (16), neuroprotective effects (17),

antitumor effects (18) and endothelium cell protection (19). Jiang *et al* (20) reported that HSYA suppresses the viability, migration and invasion of lipopolysaccharide-induced non-small cell lung cancer cells by suppressing the PI3K/AKT/mTOR signaling pathway. Previous studies have demonstrated that HSYA inhibits the viability and migration of VSMCs by regulating Akt signaling (21,22). However, whether HSYA can modulate AVF stenosis in patients with CRF via inhibiting VSMC viability and migration remains largely unknown.

In the present study, VSMCs were induced using serum from CRF rats, and the aim was to assess the effects of HSYA on the viability and migration of VSMCs, as well as uncover the potential mechanisms.

Materials and methods

Animal model of CRF. Animal experiments were performed as previously described (23). A total of 40 male Wistar rats (age, 6-7 weeks; weight, 160-180 g) were obtained from Nanjing Jiancheng Bioengineering Institute, and maintained in a 12 h light/dark cycle, with 50-60% humidity at 22-26˚C. All rats were provided *ad libitum* access to a standard diet and water. Rats were randomly divided into two groups; a control (n=20) and CRF (n=20) groups after 1 week of adaptive feeding. Adenine (2.5 g; Sigma-Aldrich; Merck KGaA) was added to 100 ml normal saline to prepare a 2.5% adenine suspension. Rats in the CRF group received 250 mg/kg adenine once a day via oral gavage for a total of 14 days, and adenine was administrated every other day for the next 14 days. Rats in the control group received the same amount of normal saline. All rats were fasted for 12 h prior to the last administration. Rats were anesthetized with 2% sodium pentobarbital (50 mg/kg) 1 h after the final administration, and cervical dislocation of the spine was immediately performed following collection of 4-6 ml blood from the abdominal aorta. All animal experiments were approved by the Experimental Animal Center of Lianyungang Hospital of Traditional Chinese Medicine (Lianyungang, China; approval. no. IACUC-20200312-07).

Serum parameters. All experiments were performed as previously described (23-26). Blood samples were centrifuged at 1,000 x g for 10 min to collect serum. The concentrations of serum creatinine (SCr) and blood urea nitrogen (BUN) were measured using commercial kits (cat. nos. C011-2-1 and C013-2-1, respectively; Nanjing Jiancheng Bioengineering Institute), and measured using a biochemical autoanalyzer (ROCHE Modular P800; Roche Diagnostics GmbH).

Cell culture and treatment. Human umbilical vein smooth muscle cells (HUVSMCs; cat. no. CP-H084) were purchased from Procell Life Science & Technology Co., Ltd., with the approval of Ethics Committee of Lianyungang Hospital of Traditional Chinese Medicine (Lianyungang, China. Approval no. IACUC-20200611-03), and maintained in DMEM supplemented with 10% FBS (both purchased from Gibco; Thermo Fisher Scientific, Inc.), at 37˚C with 5% CO_2.

The rat whole blood was collected from the control (control serum) and CRF (CRF serum) groups into coagulation tubes and allowed to clot on ice for 50 min. Subsequently, centrifugation at 1,000 x g for 15 min was used for separating serum. Rat serum was maintained in DMEM at 37˚C for 48 h at dilutions of 2.5:100, 5:100 or 10:100. HUVSMCs were pretreated with 1, 5 or 25 μM HSYA (27-30) (purity >98%; Beijing Solarbio Science & Technology Co., Ltd.) prior to stimulation of rat serum. AMG 511 (5 nM; MedChemExpress) was used to inhibit PI3K.

Cell viability assay. A Cell Counting Kit-8 (CCK-8) assay was performed to assess the effect of rat serum and/or HSYA on cell viability. HUVSMCs were seeded in 96-well plates at a density of $5x10^3$ cells/well and treated with rat serum and/or HSYA at 37˚C for 48 h. Cells were subsequently incubated with 10 μl CCK-8 reagent (cat. no. C0037; Beyotime Institute of Biotechnology) for 1 h at 37˚C. Cell viability was analyzed at a wavelength of 450 nm, using a microplate spectrophotometer (BioTek Instruments, Inc.).

Wound healing assay. HUVSMCs were seeded into 6-well plates at a density of $1x10^6$ cells/well. Sterile 200 μl pipette tips were used to scratch the cell monolayers. Cell medium was replaced with fresh serum-free DMEM. The migratory ability of cells characterized by wound width was observed at 0 and 48 h under a light microscope (Olympus Corporation; magnification, x100).

Apoptosis analysis. HUVSMCs ($5x10^5$) were collected by centrifugation at 37˚C and 300 x g for 3 min and re-suspended in 200 μl Annexin V binding buffer (cat. no. C1062M; Beyotime Institute of Biotechnology). After resuspension, cells were incubated with 10 μl PI (cat. no. C1062M; Beyotime Institute of Biotechnology) for 15 min at room temperature in the dark. Apoptotic cells were subsequently analyzed using a flow cytometer (Beckman Coulter, Inc.).

Measurement of nitric oxide (NO). HUVSMCs were seeded in 12-well plates at a density of $2x10^5$ cells/well and treated with rat serum and/or HSYA. HUVSMCs were subsequently lysed using Lysis Buffer (cat. no. S3090; Beyotime Institute of Biotechnology). The Nitrate/Nitrite assay kit (cat. no. S0023; Beyotime Institute of Biotechnology) was used to determine NO concentration in HUVSMCs, according to the manufacturer's protocol. NO concentration was measured at a wavelength of 540 nm, using a microplate spectrophotometer (BioTek Instruments, Inc.).

Western blotting. Total protein was extracted from HUVSMCs using RIPA lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology). Cell supernatants were collected by centrifugation at 12,000 x g for 15 min at 4˚C. Total protein was quantified using the BCA protein assay kit (cat. no. P0012s; Beyotime Institute of Biotechnology) and 20 μg protein/lane was separated by 12% SDS-PAGE. The separated proteins were subsequently transferred to PVDF membranes (EMD Millipore) and blocked with 5% skimmed milk for 2 h at room temperature. The membranes were incubated with primary antibodies against PI3K (1:1,000), Akt (1:1,000), phosphorylated (p)-Akt (cat. no. Ser473, 1:1,000), endothelial NO synthase (eNOS; 1:1,000), p-eNOS (1:1,000) and GAPDH (1:10,000) overnight at 4˚C (all purchased from Affinity Biosciences).

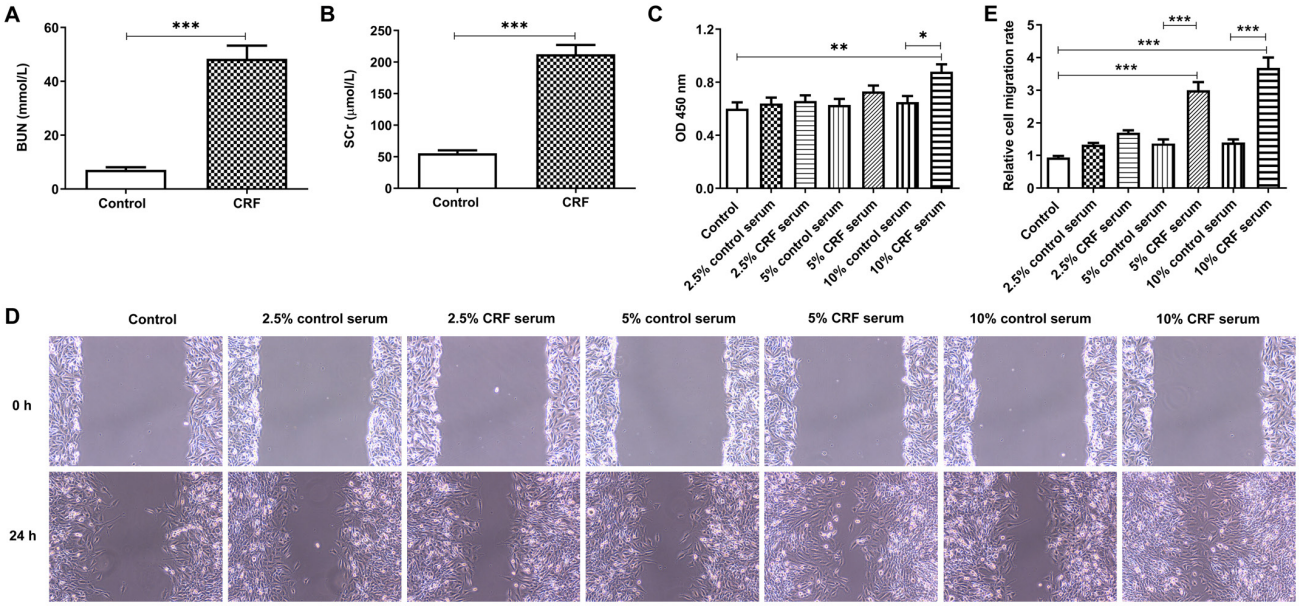

Figure 1. Serum from CRF rats promotes viability and migration of HUVSMCs. (A and B) Concentrations of BUN and SCr in the rat serum of the control or CRF group. (C) HUVSMCs were stimulated with different concentrations of serum from the control or CRF group. The viability of HUVSMCs was evaluated via examining the absorbance at 450 nm. (D and E) Migratory capability of HUVSMCs was determined using a wound healing assay and the relative migratory rate was quantified. *P<0.05, **P<0.01 and ***P<0.001. SCr, serum creatinine; BUN, blood urea nitrogen; CRF, chronic renal failure; HUVSMCs, human umbilical vein smooth muscle cells; OD, optical density.

Following the primary incubation, membranes were incubated with goat anti-rabbit/mouse IgG (H+L) HRP-conjugated secondary antibodies (1:10,000; Affinity Biosciences) for 2 h at room temperature. Protein bands were semi-quantified using Image Lab version 4.1 software (Bio-Rad Laboratories, Inc.).

Statistical analysis. All experiments were performed in triplicate and data are presented as the mean ± standard deviation. A Student's t-test was used to compare differences between two groups, and a one-way ANOVA followed by a Tukey's post hoc test was used to compare differences between multiple groups in GraphPad Prism version 5.0 (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Serum from CRF rats promotes the viability and migration of HUVSMCs. Rat serum from the control and CRF groups was collected to assess changes in the parameters of renal function. As presented in Fig. 1A, the BUN concentration was significantly higher in the CRF group compared with the control group. Similarly, SCr concentration was higher in the CRF group compared with the control group (Fig. 1B). These results suggest that the renal failure model was established successfully (24-26).

HUVSMCs were treated with rat serum to assess the effect of CRF serum on HUVSMCs. The results demonstrated that 10% CRF serum significantly promoted cell viability compared with 10% control serum, whereas low concentrations of CRF serum (2.5 and 5.0%) slightly enhanced cell viability (Fig. 1C). In addition, the migratory ability of HUVSMCs was inhibited following stimulation of CRF serum compared with the same concentration of control serum. Furthermore, the results of the wound healing assay demonstrated that CRF serum promoted cell migration in a concentration-dependent manner, whereas control serum had little effect on cell migration (Fig. 1D and E). Collectively, these results suggest that serum from CRF rats promotes the viability and migration of HUVSMCs.

Serum from CRF rats promotes the apoptosis of HUVSMCs. The results demonstrated that the apoptotic rate increased following treatment of HUVSMCs with rat serum. Notably, 10% CRF serum markedly promoted cell apoptosis compared with 10% control serum (Fig. 2A). This may be explained by the presence of toxic substances in the serum of rats with CRF, which affects cell survival and promotes apoptosis.

HSYA inhibits cell viability, migration and apoptosis of HUVSMCs. HUVSMCs were treated with HSYA or AMG 511 in the presence of 10% CRF serum. As presented in Fig. 3A, HSYA inhibited cell viability induced by 10% CRF serum in a concentration-dependent manner. Similarly, AMG 511 suppressed the viability of HUVSMCs. Notably, pretreatment with HSYA or AMG 511 alleviated 10% CRF serum-induced cell migration (Fig. 3B), suggesting that HSYA reverses the effect of CRF serum on cell migration. In addition, HSYA partially alleviated 10% CRF serum-induced apoptosis, the effects of which were reversed following pretreatment with AMG 511 (Fig. 3C). Taken together, these results suggest that HSYA counteracts the effects of CRF serum on the viability, migration and apoptosis of HUVSMCs.

HSYA inactivates the PI3K/Akt signaling pathway and promotes NO production. NO inhibits neointimal hyperplasia (31); thus, the NO concentration in HUVSMCs was detected. The NO concentration was notably decreased in

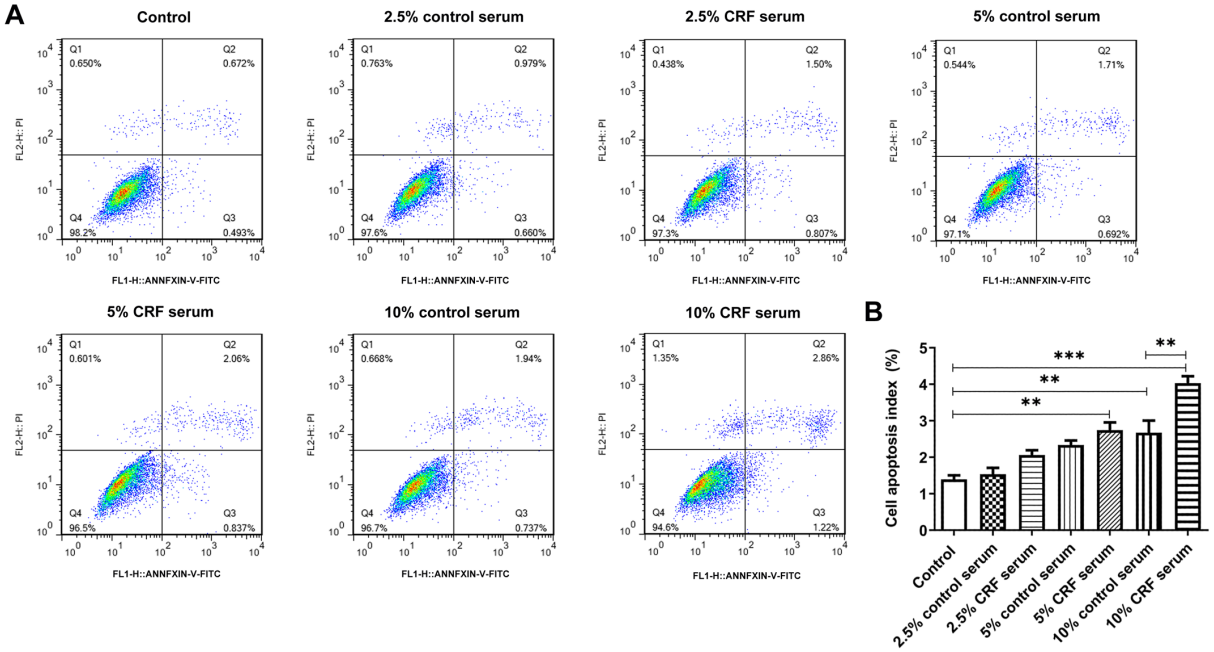

Figure 2. Serum from CRF rats enhances apoptosis of HUVSMCs. HUVSMCs were stimulated with 2.5, 5 or 10% serum from the control or CRF group. (A) Apoptotic ratio of each group was detected via flow cytometry. (B) Quantitative analysis of flow cytometry. **P<0.01 and ***P<0.001. CRF, chronic renal failure; HUVSMCs, human umbilical vein smooth muscle cells.

the presence of 10% CRF serum compared with the control group; however, treatment with HSYA elevated NO levels in a concentration-dependent manner. Similarly, treatment with AMG 511 promoted NO production (Fig. 4A).

Given that HSYA exhibited similar inhibitory effects to AMG 511 on the viability and migration of HUVSMCs, it was next investigated whether HSYA can exert these effects by inhibiting the PI3K/Akt pathway. As presented in Fig. 4B, 10% CRF serum upregulated PI3K expression and phosphorylation of Akt, suggesting that serum from CRF rats can activate PI3K/Akt signaling in HUVSMCs. Notably, the expression levels of PI3K and p-Akt decreased following treatment with AMG 511. In addition, treatment with HSYA markedly decreased the protein expression levels of PI3K and p-Akt. Treatment with both HSYA and AMG 511 upregulated p-eNOS expression, respectively, which explains the increase in NO production (Fig. 4A).

Discussion

AVF stenosis caused by neointimal hyperplasia is frequently observed in patients (32), and can lead to the morbidity of patients with ESRD (33,34). However, the molecular mechanism underlying neointima formation in AVF remains unclear. It is well-known that injury induced pathological viability and migration of VSMCs is a major cause of neointima formation (35,36). In the present study, CRF rats were established and the serum from rats were prepared to stimulate HUVSMCs to determine whether HSYA could inhibit neointimal hyperplasia *in vitro*.

High concentrations of CRF serum significantly promoted the viability and migration of HUVSMCs, which is consistent with the pathological condition of AVF stenosis in patients with ESRD (37,38). However, high concentrations of CRF serum also increase cell apoptosis, which may be due to toxicants in the serum that disrupt cell survival. Activation of the PI3K/Akt signaling pathway is closely associated with aberrant viability and migration of VSMCs (11,12,39). In addition, activation of the PI3K/Akt signaling pathway has been observed in mice with renal dysfunction caused by ischemia/reperfusion-injury (40,41). Previous studies have reported that the PI3K/Akt signaling pathway is activated in kidney injury induced by cisplatin (42) and kidneys of rats with unilateral ureteral obstruction (43).

HSYA is an active ingredient isolated from *Carthami Flos* (15). It has been reported that HSYA inhibits platelet derived growth factor BB-induced activation of Akt signaling, which in-turn disrupts the viability and migration of VSMCs (21). In addition, Yang *et al* (22) demonstrated that HSYA suppresses the viability and migration of lipopolysaccharide-induced VSMCs by inhibiting the Toll-like receptor 4/Rac1/Akt pathway. Thus, it was hypothesized that HSYA exerts antiproliferative and anti-migratory effects on HUVSMCs via the PI3K/Akt signaling pathway.

The results of the present study demonstrated that HSYA suppressed CRF serum-induced cell viability and migration, suggesting its role in HUVSMCs-mediated intimal hyperplasia. AMG 511, which is a potent and selective PI3K inhibitor that decreases the phosphorylation of Akt, exhibited similar effects to HSYA on the viability and migration of HUVSMCs. Notably, HSYA decreased cell apoptosis induced by CRF serum, whereas AMG 511 had little effect on the apoptosis of HUVSMCs. This may be explained by the hypothesis that HSYA ameliorates the toxic effects of toxicants in CRF serum on HUVSMCs.

In the present study, NO production and p-eNOS expression decreased in CRF serum-induced HUVSMCs, whereas treatment with HSYA and AMG 511 enhanced the levels of

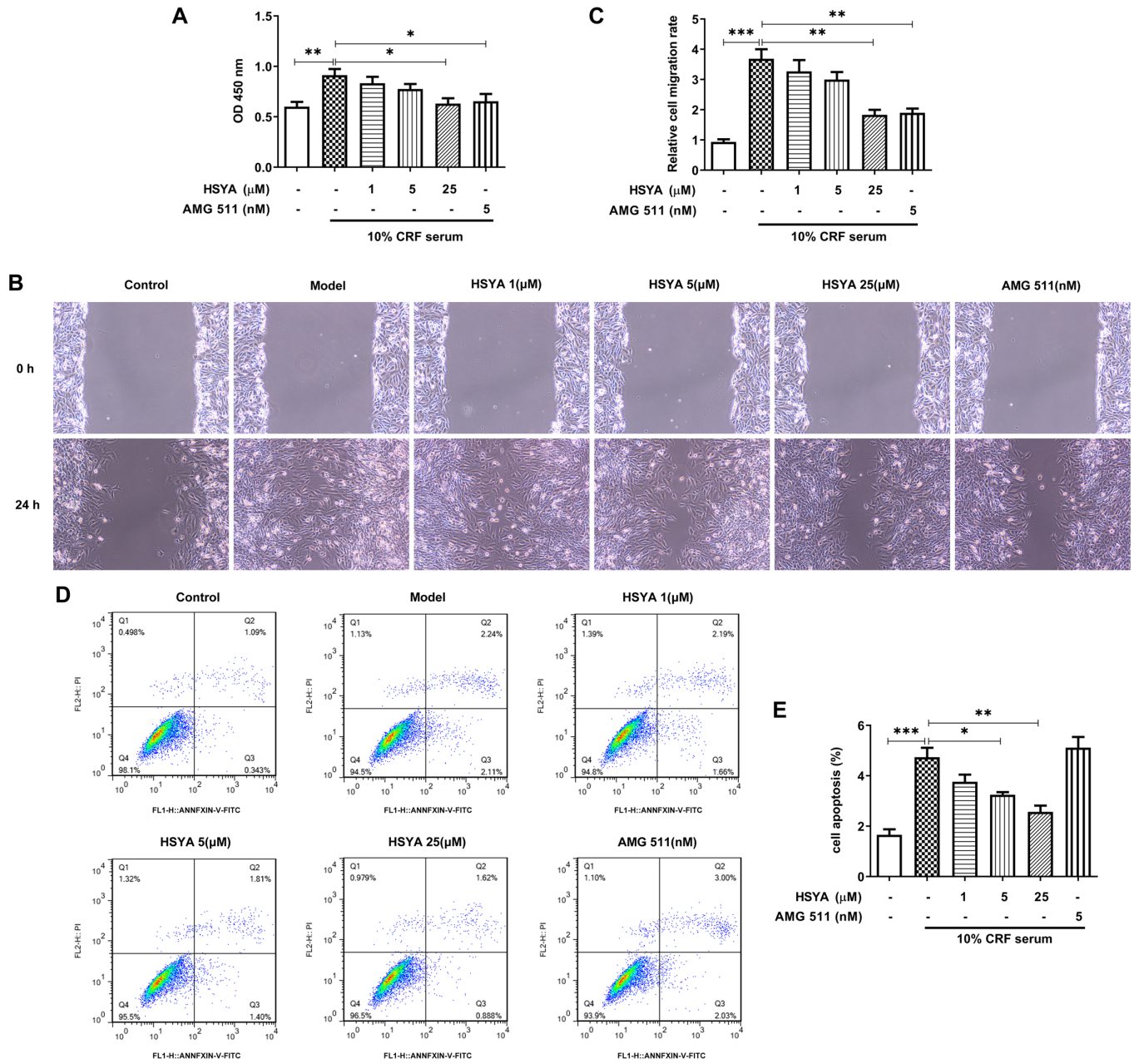

Figure 3. HSYA inhibits cell viability, migration and apoptosis. Solutions of 1, 5, 25 μM HSYA or 5 nM AMG 511 were prepared to treat HUVSMCs 30 min prior to stimulation with 10% CRF serum. (A) Viability of HUVSMCs was evaluated via examining the absorbance at 450 nm. (B and C) Migratory capability of HUVSMCs was determined using a wound healing assay and the relative migratory rate was quantified. (D and E) Apoptotic ratio of each group was detected via flow cytometry. *P<0.05, **P<0.01 and ***P<0.001. HSYA, Hydroxysafflor yellow A; CRF, chronic renal failure; HUVSMCs, human umbilical vein smooth muscle cells; OD, optical density.

NO and p-eNOS, respectively. Previous studies have demonstrated that NO-based therapies can decrease neointimal hyperplasia (44,45). NO production is regulated by NO synthases (NOSs), including eNOS. eNOS activity is mainly regulated through phosphorylation, which is primarily regulated by the PI3K/Akt/eNOS pathway. It has been reported that activation of PI3K/AKT/eNOS pathways serves an important role in regulating cell migration, migration, angiogenesis and apoptosis (46). The results of the present study showed that HSYA may rescue NO production in CRF serum-treated cells via inhibiting PI3K/Akt activation.

To further investigate whether HSYA can regulate the PI3K/Akt signaling pathway, the protein expression levels of PI3K and p-Akt were detected. The results demonstrated that HSYA affected PI3K expression and Akt phosphorylation. In addition, HSYA inhibited the viability and migration of HUVSMCs by regulating PI3K/Akt signaling, which suggests that HSYA may mediate intimal hyperplasia-induced AVF stenosis. However, given that the present study only focused on HSYA-mediated viability and migration of HUVSMCs, further studies are required to confirm the effects of HSYA in AVF stenosis *in vivo*. In addition, other alternative assays will be utilized to enrich the experimental content and further validate these findings. Finally, prospective studies should focus on investigating the involvement of other pathways on the effects of HSYA on AVF stenosis.

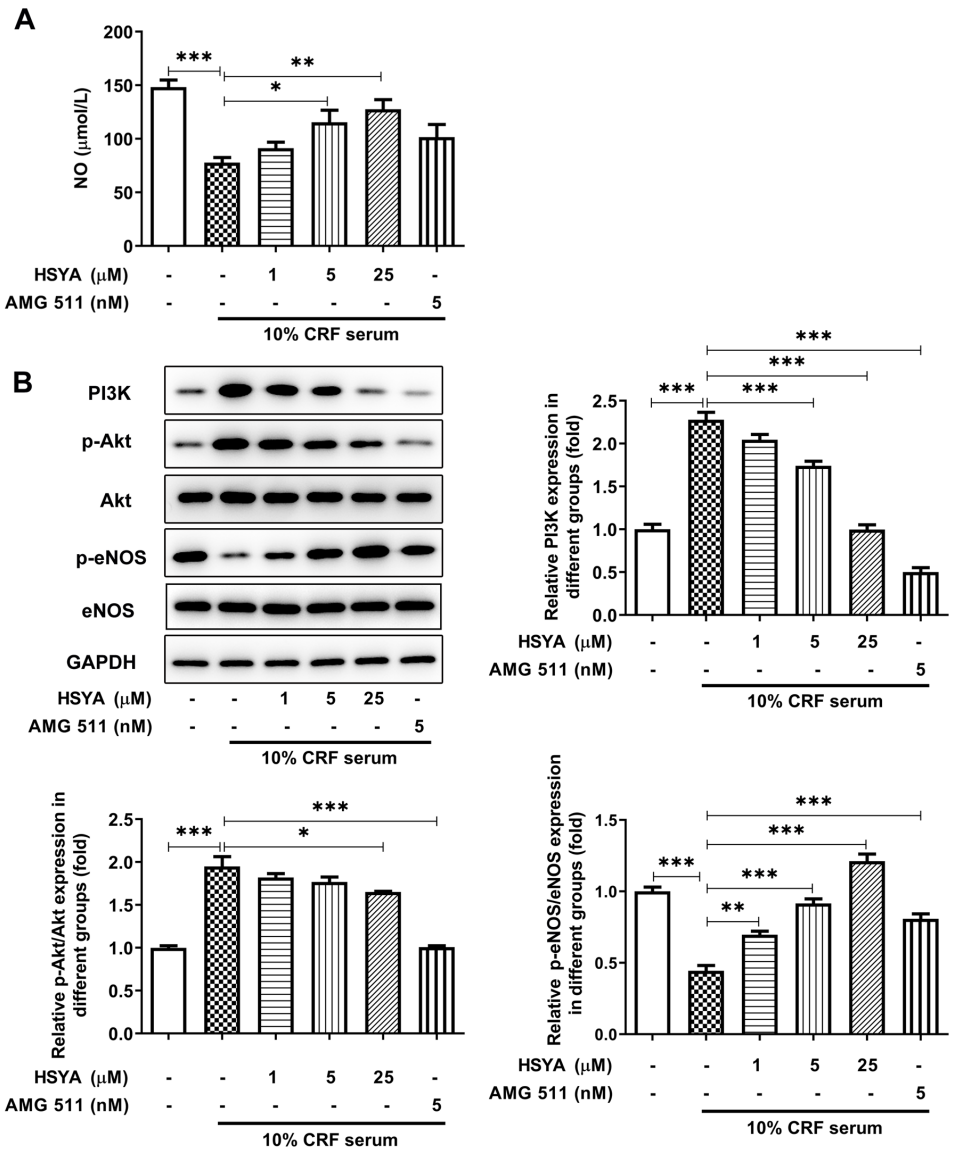

Figure 4. HSYA inactivates PI3K/Akt signaling and enhances NO production. Solutions of 1, 5, 25 μM HSYA or 5 nM AMG 511 were prepared to treat HUVSMCs 30 min prior to stimulation with 10% CRF serum. (A) Production of NO in each group was examined using a Nitrate/Nitrite assay kit. (B) Protein levels of PI3K, Akt, p-Akt, eNOS and p-eNOS were estimated via western blotting. *P<0.05, **P<0.01 and ***P<0.001. HSYA, Hydroxysafflor yellow A; CRF, chronic renal failure; HUVSMCs, human umbilical vein smooth muscle cells; p-, phospho-; NO, nitric oxide; eNOS, endothelial NO synthase.

Taken together, the current study for the first time demonstrated the inhibitory effects of HSYA on HUVSMC viability and migration, as well as showing the underlying mechanism involved regulation of the PI3K/Akt signaling pathway. The results provide primary evidence for the therapeutic application of HSYA in intimal hyperplasia-induced AVF stenosis.

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Availability of data and materials

All data generated or analysed during the present study are included in this published article.

Authors' contributions

BC, CH, LW and QW conceived and designed the present study. CH and LW, performed the experiments and acquired the data. QW and YY analyzed the data. BC drafted the initial manuscript, including the figures. All authors have read and approved the final manuscript. BC and CH confirm the authenticity of all the raw data.

Ethics approval and consent to participate

All animal experiments were approved by the Experimental Animal Center of Lianyungang Hospital of Traditional Chinese

Medicine (Lianyungang, China. Approval. no. IACUC-20200312-07). The use of Human umbilical vein smooth muscle cells (cat. no. CP-H084; Procell Life Science & Technology Co., Ltd.) was approved by the Ethics Committee of Lianyungang Hospital of Traditional Chinese Medicine (Lianyungang, China. Approval. no. IACUC-20200611-03).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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