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Astragaloside IV protects human trophoblast HTR8/SVneo cells from H_2O_2-Induced oxidative stress via Nrf2-Keap1-p62 feedback loop

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ABSTRACT

Preeclampsia is one of the serious complications of pregnancy and is the main cause of maternal death. Accumulation of trophoblastic reactive oxygen species (ROS) and placental dysfunction are the potential pathogenic bases of early-onset preeclampsia. Astragaloside IV (AS IV), one of the major and active components of *Astragalus membranaceus* (Fisch.) Bunge, is known for its antioxidant effects in many diseases. This study aimed to investigate the protective effects and potential mechanisms of AS IV against oxidative stress-induced damage in trophoblasts. We established an oxidative stress in vitro model using an extravillous trophoblast cell line (HTR8/SVneo). The results demonstrated that pretreatment of HTR8/SVneo cells with AS IV can potently suppress the increase in ROS level and the cell apoptosis rate and rescue the H_2O_2-induced decrease in cell viability. Mechanistically, the antioxidant effects of AS IV were achieved by activating nuclear factor erythroid 2-related factor 2 (Nrf2), along with an increase in autophagy makers and a decrease in Keap1 levels. Silencing Nrf2 and p62 with siRNA dramatically abolished the prosurvival effects of AS IV on treated HTR8/SVneo cells. Further study results on the relationship among Nrf2, Keap1, and p62 by siRNAs showed that the Keap1/Nrf2 pathway and p62 form a positive feedback loop. Our present study demonstrated that AS IV protects human trophoblasts against H_2O_2-induced oxidative stress via the activation of the Nrf2-Keap1-p62 feedback loop. These findings provide a cue for future studies on the use of AS IV as a protective and curative agent against preeclampsia.

1. Introduction

Preeclampsia (PE) is a common pregnancy complication, which is characterized by new-onset hypertension and proteinuria at ≥ 20 weeks of gestation (Shennan, Green, & Chappell, 2017). PE is a major cause of maternal, fetal, and neonatal mortality and affects an estimated 3%–5% of all pregnancies (Ananth, Keyes, & Wapner, 2013). In addition, PE has severe complications, including maternal organ dysfunction, such as uteroplacental dysfunction, fetal growth restriction, liver involvement, renal insufficiency, and hematological or neurological complications (Ananth et al., 2013; von Dadelszen et al., 2004; von Dadelszen et al., 2007). However, to date, the available therapeutic strategy for PE is the termination of pregnancy, which increases the risk of premature neonates. Hence, non-invasive and safe therapeutic strategies for PE must be sought.

Oxidative stress of trophoblast cells plays an important role in the pathophysiology of PE (Ferguson, Meeker, McElrath, Mukherjee, & Cantonwine, 2017). Oxidative stress may be one of the major triggers that lead to placental and endothelial dysfunction in PE (Barneo-Caragol et al., 2019; Ferguson et al., 2017). Hence, alleviating the oxidative stress of the placenta is a potential preventive and treatment strategy for PE. Astragaloside IV (AS IV), one of the major and active components of the *Astragalus membranaceus* (Fisch) Bunge, has a strong anti-oxidative effect as it can remove free radicals and decrease lipid peroxidation (Wang et al., 2019; W. J. Zhang, Hufnagl, Binder, & Wojta, 2003). The anti-oxidative effects of AS IV have been reported in both in vitro and in vivo studies (X. Li et al., 2013; Yu et al., 2006).

Autophagy is an important intracellular process responsible for resisting oxidative stress and maintaining cellular homeostasis (Kanninen, de Andrade Ramos, & Witkin, 2013). The impaired autophagy of

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the maternal–fetal interface weakens the capacity of trophoblast cells to clear excess reactive oxygen species (ROS), leading to oxidative stress, which contributes to the pathophysiology of some pregnancy-related diseases (Prokesch et al., 2017). In addition, autophagy is involved in the pathophysiology of PE (Melland-Smith et al., 2015; Prokesch et al., 2017).

AS IV plays a protective role by regulating autophagy and oxidative stress in some types of cells (Huang et al., 2017). However, the protective effects of AS IV on trophoblasts induced by H_2O_2 remain unclear. In addition, the Nrf2-Keap1 system and autophagy are both involved in the oxidative-stress response, metabolic pathways, and innate immunity, and dysregulation of these processes is associated with pathogenic processes (Jiang et al., 2015). It has been reported that phosphorylation of the autophagy-adaptor protein p62 markedly increases p62′s binding affinity for Keap1, an adaptor of the Cul3-ubiquitin E3 ligase complex responsible for degrading Nrf2(Ichimura et al., 2013; Jiang et al., 2015). The activation of the Nrf2-Keap1-p62 feedback loop were involved in the oxidative-stress response (Lau et al., 2013). Thus, whether its protective effects on trophoblasts are mediated by a feedback loop of autophagy and oxidative stress is not understood. In the present study, we used a vitro model to investigate the protective role and mechanisms of AS IV against H_2O_2-induced oxidative stress of trophoblasts.

2. Materials and methods

2.1. Cell culture and treatment

The human first-trimester extravillous trophoblast cell line (HTR-8/SVneo) (Graham, Fitzpatrick, & McCrae, 1998; Ulrich et al., 2016) was purchased from Fuheng Biology (Shanghai, China) and maintained in Roswell Park Memorial Institute (Hyclone, South Logan, UT, USA) medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) at 37 °C with 5% CO_2. A stock solution of 1 mM H_2O_2 (Sigma, St. Louis, MO, USA) was prepared before use. A new stock solution of 20 mM AS IV (Yuanye Biomart, Shanghai, China) was stored at room temperature. Astragaloside IV was dissolved in dimethyl sulfoxide (Sigma, St. Louis, MO, USA). The cells were pretreated for 4 h with AS IV (at the concentrations of 0 or 20 μM) and cultured for 24 h in a medium supplemented with 500 μM H_2O_2according to the cell counting kit-8 (CCK8) assay.

2.2. Cell viability assay

The cell viability of the HTR-8/SVneo cells was measured using a CCK8 kit (Dojindo, Kumamoto, Japan). The cells were seeded in 96-well plates (10^4 cells per well) for 4 h and treated with AS IV or H_2O_2 at different levels for 24 h. Then 10 μL of cck8 was added to each well of 96-well plates. After 2 h of incubation, the cells were counted using a universal microplate reader microplate reader (TECAN, Austria), and absorbance was recorded at a wavelength of 450 nm (OD450). The reading performed in triplicate. Each experiment was performed in triplicate.

2.3. Quantitative measurement of apoptosis by AnnexinV-FITC/PI staining

The apoptotic rate of HTR-8/SVneo cells was detected using FCM with fluorescein isothiocyanate (FITC)-labeled annexin V (annexin V-FITC) and propidium iodide (PI) double labeling, (Annexin V-FITC Apoptosis Detection kit, Beyotime, Shanghai, China). Cells were seeded in 12-well plates and treated with 5 mM ammonium chloride for 24 h. The cells were digested and washed by cold phosphate-buffered saline (PBS, Hyclone, South Logan, UT, USA) twice and then fixed with 70% pre-cooled ethanol for 24 h. After centrifugation at 1000 *g* for 5 min, the supernatant was discarded, and the cell pellets were washed twice. Staining was performed in 200 μL of bonding buffer containing 5 μL of Annexin V-FITC in the dark at room temperature for 20 min, followed by another 15 min in the dark at 4 °C. PI (10 μL) was then added. Samples were measured with a BD LSR FCM (BD Biosciences, Franklin Lakes, USA).

2.4. Detection of intracellular ROS

The levels of intracellular ROS were detected using an ROS assay kit (Beyotime, Shanghai, China). In brief, different treatments of HTR-8/SVneo cells were washed with PBS and incubated with dichlorodihydro-fluorescein diacetate at 37 °C for 30 min, followed by washing three times with PBS (pH = 7.2). The DCF fluorescence distribution of 20,000 cells was detected with a BD LSR FCM (BD Biosciences, Franklin Lakes, USA) at an excitation wavelength of 488 nm and an emission wavelength of 535 nm respectively.

2.5. RNA isolation and quantitative Real-Time PCR (qPCR)

The total RNA of HTR-8/SVneo cells for different treatments were isolated from cells using a TRIZOL Reagent kit (Takara, Tokyo, Japan) according to the manufacturer's instruction. Approximately 1 μg of total RNA was used for cDNA syntheses using a standard reverse transcription kit (Takara, Tokyo, Japan). The inverse transcription process was 25 °C for 10 min, 42 °C for 15 min, and 85 °C for 5 min (Ding et al., 2020; C. C. Wang et al., 2020). The primers (designed by AlleleID 6) are shown in Table 1 (synthesized in Comate Bioscience Co. Ltd, Changchun, China). qPCR was performed in a 7500c real-time PCR detection system (T. T. Zhang, Yang, & Jiang, 2015) (Applied Biosystems, Carlsbad, CA, USA) with the SYBR premix EX Taq (TaKaRa). GAPDH was regarded as the control.

2.6. Western blot

Total proteins from the HTR-8/SVneo cells for different treatments were extracted using a mammalian protein extraction reagent (M−PER, Thermo, Rockford, IL, USA). The nuclear proteins of cells were prepared using a CelLytic NuCLEAR Extraction kit (Sigma-Aldrich, St. Louis, MO, USA). Protein concentration was detected using a BCA protein assay kit (Beyotime, Shanghai, China). Equal amounts of protein were loaded into each well, separated by 10% SDS-PAGE, blocked with 5% bovine serum albumin dissolved in TBST for 1 h, and then incubated with primary antibodies (LC3 ab192890; GAPDH ab8245; p62 ab207305; Nrf2 ab89443; HO-1 ab68477; Histone H3 ab1791; Beclin 1 ab210498; Abcam, Cambridge, MA, USA; 1:1000) at 4 °C for 12 h. The membranes were washed four times for 6 min each and incubated with the appropriate second antibody conjugates (Abcam, Cambridge, MA, USA) or horseradish-peroxidase-conjugated protein antibody for 1 h at room temperature. Membranes were washed four times and then stained using DAB Horseradish Peroxidase (Color Development kit Beyotime, Shanghai, China). The proteins were detected by visualizing the gel

Table 1
Primers for real-time quantitative PCR.

Primer name	Primer sequence(5′-3′)	Product length (bp)
Nrf2	Sense Primer: GTATGCAACAGGACATTGAGCAAG Anti-sense Primer: TGGAACCATGGTAGTCTCAACCAG	107
p62	Sense Primer: CCAGCACCAAGAGCACGGACAGCG Anti-sense Primer: TGGGGAGAAGAAGGGGACCACGAA	446
Keap1	Sense Primer: CATCGGCATCGCCAACTTC Anti-sense Primer: ACCAGTTGGCAGTGGGAC	135
GAPDH	Sense Primer: CCATGGAGAAGGCTGGG Anti-sense Primer: CAAAGTTGTCATGGATGACC	195

Alpha Innotech and analyzing it using a Tanon gel imaging system (Tanon, Shanghai, China). GAPDH and Histone H3 were regarded as the controls (total and nuclear protein, respectively).

2.7. Transfection and p62 and Nrf2 small RNA interference

Three candidate siRNAs of p62 and Nrf2 and one negative control siRNA were synthesized by RiboBio (Guangzhou, China). The p62 siRNA sequences were si-001: GGAGTCGGATAACTGTTCA 5′- −3′, si-002: TGAGGAAGATCGCCTTGGA 5′- −3′, and si-003: GGACCCATCTGTCTTCAAA 5′- −3′. The Nrf2 siRNA sequences were si-001: GAGAAAGAATTGCCTGTAA5′- −3′, si-002: GCTACGTGATGAAGATGGA 5′- −3′, and si-003: GCCCTCACCTGCTACTTTA 5′- −3′. The specificity and effectiveness of the three candidate siRNAs of p62 and Nrf2 were detected by quantitative analysis of the mRNA level of p62 and Nrf2 36 h after siRNA transfection. The second candidate siRNA of p62 (Supplementary Fig. 1) and the third candidate siRNA of Nrf2 (Supplementary Fig. 2) were the most effective. The HTR-8/SVneo cells were transfected with the second siRNAs of p62 or the third siRNA of Nrf2 in subsequent experiments. The transfection experiments used the FUGENE® HD (Roche, Basel, Switzerland) transfection reagent following the manufacturer's instructions. In brief, HTR-8/SVneo cells were cultured in 12 wells, and the transfection reagent was used to transfect p62 siRNA or Nrf2 siRNA at a concentration of 50 nM following the manufacturer's instructions. The cells were treated with si-p62 or si-Nrf2 for 36 h in a serum-free medium.

2.8. Statistical analysis

All data were analyzed with SPSS 22.0 software (Version X, IBM, Armonk, NY, USA). One-way or two-way analysis of variance (ANOVA) with multiple comparison post test (Dunnett or Bonferroni) were used to compare the means between experimental groups as indicated. Data have been given as mean ± standard deviation (SD). *P*-values of 0.05 or

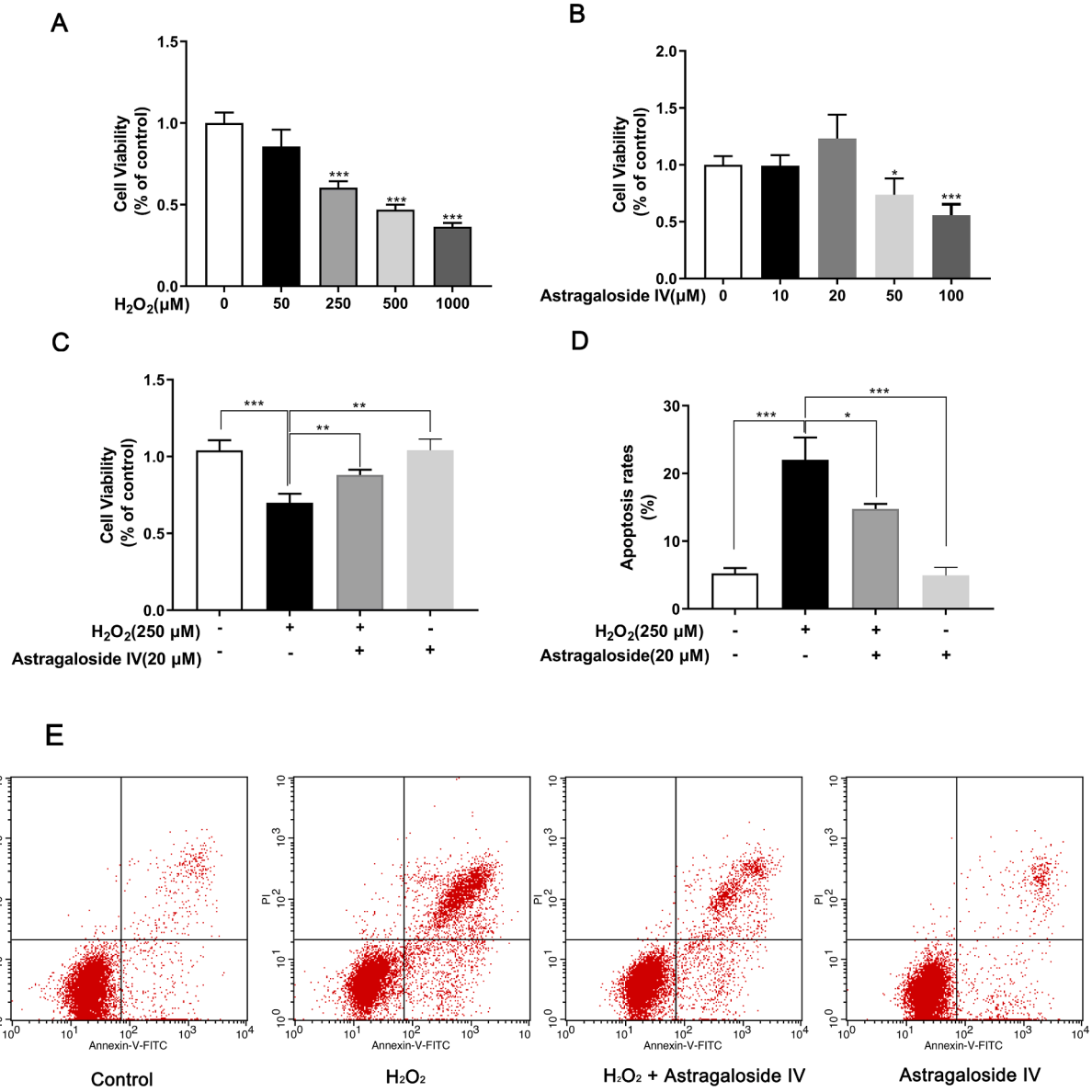

Fig. 1. The protective effects of astragaloside IV against H_2O_2-induced cytotoxicity in HTR8/SVneo cells. (A) The effects of different concentrations of H_2O_2 (0, 50, 250, 500, and 1000 μM) for 24 h on the cell viability of HTR8/SVneo cells. The cell viability was measured by CCK-8 assay. The data are shown as mean ± SD. n = 6. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (B) The effects of different concentrations of astragaloside IV (0, 5, 10, 20, and 50 μM) for 24 h on the viability of HTR8/SVneo cells. The cell viability was measured by CCK-8 assay. The data are shown as mean ± SD. n = 6. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The cell viability (C), the percentage of cell apoptosis (D and E) were measured. The data are shown as mean ± SD., n = 4, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

less were considered to be significant.

3. Results

3.1. AS IV protects against H_2O_2-induced cytotoxicity in HTR8/SVneo cells

HTR8/SVneo cells were treated with different concentrations of H_2O_2 (0, 50, 250, 500, and 1000 μM). Fig. 1 A show that H_2O_2 at various concentrations significantly decreased cell viability in a dose-dependent manner compared with the untreated control. At a concentration of 250 μM, H_2O_2 significantly decreased cell viability by approximately 40% compared with the control group ($P < 0.05$). Thus, we chose 250 μM as the appropriate concentration to establish the oxidative stress in vitro model for the following experiments. The suitable dose of AS IV on HTR8/SVneo cells was determined using the CCK8 assay. HTR8/SVneo cells treated with AS IV at various concentrations (0, 10, 20, and 50 μM) did not have an effect on HTR8/SVneo cell growth (Fig. 1B). Thus, we chose 20 μM as the appropriate concentration of AS IV for the following experiments. However, 50 μM AS IV significantly decreased cell viability (Fig. 1B). We predicted that a high concentration of AS IV might have a toxic effect on HTR8/SVneo cells. Then, pretreatment of cells with 20 μM AS IV for 4 h before exposure to H_2O_2 significantly increased cell viability (Fig. 1C). The effects of AS IV or H_2O_2 on the percentage of apoptotic rate were determined using annexin V-FITC/PI staining. After the cells were treated with AS IV or H_2O_2, the results of annexin V-FITC and PI double staining indicated that AS IV alleviated the H_2O_2-induced increase in apoptosis rate in the HTR8/SVneo cells (Fig. 1D and Fig. 1E).

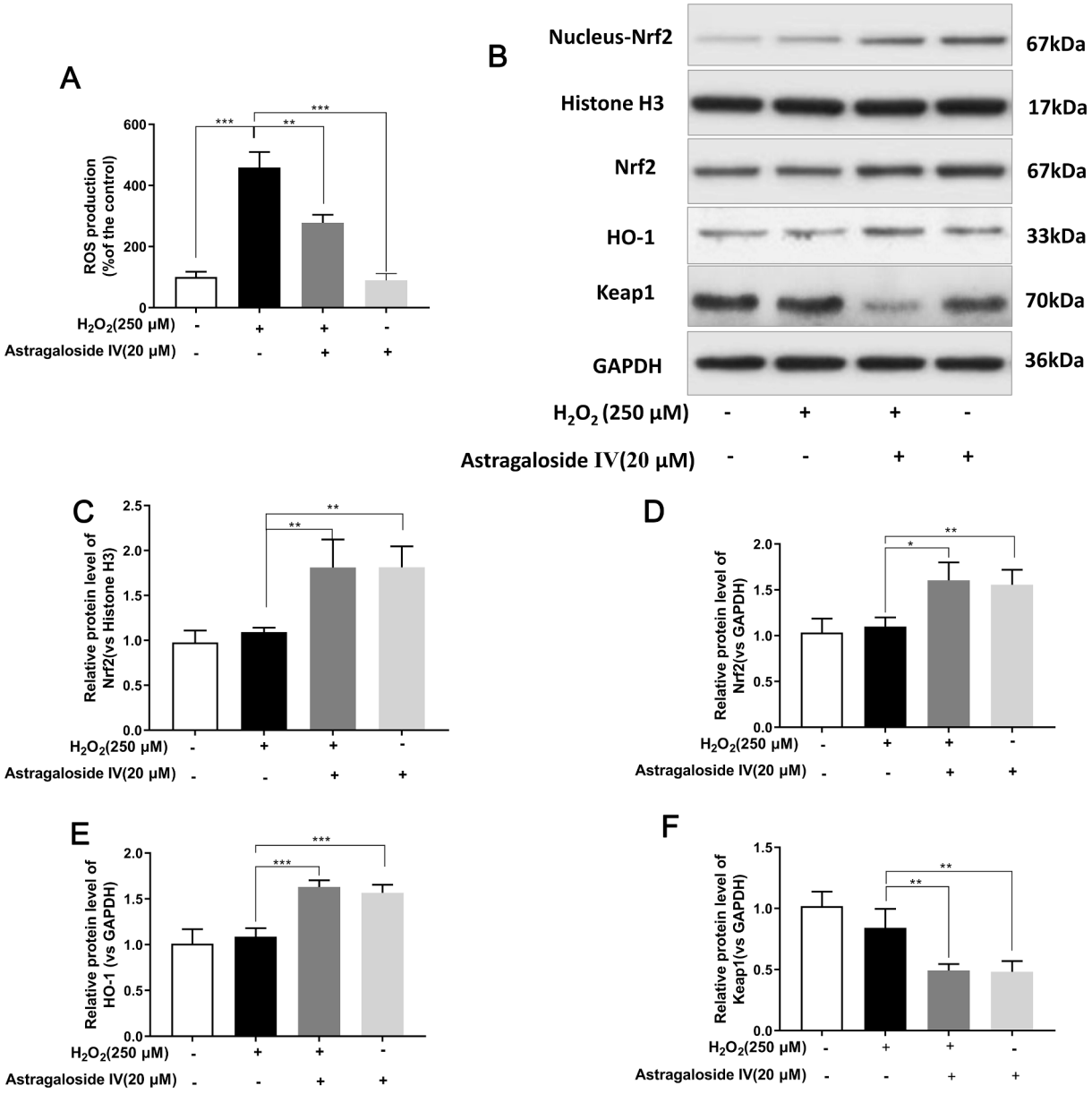

Fig. 2. Astragaloside IV protects HTR8/SVneo cells against H_2O_2-induced oxidative stress by the activation of Nrf2/Keap1 pathways. (A) ROS concentration in HTR8/SVneo cells pretreated with or without astragaloside IV for 4 h and then treated with or without H_2O_2 for 24 h. The data are shown as means ± SD, n = 4, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$;. (B) The nuclear protein level of Nrf2, Nrf2, HO-1, and the protein expression of Keap1 were analyzed by Western blotting. Relative nuclear protein levels of Nrf2 (C), relative protein levels of Nrf2(D), HO-1(E), and Keap1(F) were analyzed by grey scanning. The data are shown as means ± SD, n = 4, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$;. n.s. indicates no significance. Histone H3and GAPDH were regarded as the controls (nuclear protein and total, respectively).

3.2. Cytoprotective effects of AS IV against oxidative stress are dependent on the induction of Keap1-Nrf2 in HTR8/SVneo cells

ROS production increased significantly after 24 h of exposure to H_2O_2 compared with the control group (Fig. 2A). In addition, ROS production in the AS IV plus H_2O_2 group was significantly decreased compared with that in the H_2O_2 group. As shown in Fig. 2B, C, D, and E, AS IV strongly up-regulated nuclear protein levels of Nrf2, total Nrf2, and HO-1 under the H_2O_2-induced oxidative condition. In addition, AS IV down-regulated the protein level of Keap1 under the H_2O_2-induced oxidative condition (Fig. 2B, F).

3.3. AS IV enhanced autophagy on HTR8/SVneo cells under H_2O_2-induced oxidative stress

The effects of AS IV on the autophagy biomarkers induced by H_2O_2 in HTR8/SVneo cells were explored using Western blot analysis. The results showed significant changes in the ratio of LC3II/LC3I and the expression of Beclin 1 and p62. After pretreatment with AS IV, the ratio of LC3II/LC3I and Beclin 1 significantly increased, and p62 expression significantly decreased compared with the H_2O_2-treated group (Fig. 3).

3.4. Role of Keap1-Nrf2-p62 in the protective effects of AS IV on HTR8/SVneo cells under H_2O_2-induced oxidative stress

HTR8/SVneo cells of Nrf2-knockdown and control-siRNA groups were incubated in AS IV or H_2O_2 to assess the effects of Nrf2 signaling and oxidative stress on autophagy. The level of Nrf2 mRNA was significantly decreased after transfection into HTR8/SVneo cells with or without H_2O_2 treatment compared with the cells transfected with a control siRNA. In addition, the Nrf2 mRNA level was significantly decreased after transfection into HTR8/SVneo cells with or without pretreatment with AS IV compared with the cells transfected with a control siRNA (Fig. 4A). Furthermore, knockdown of Nrf2 abolished the protective effect of AS IV against an H_2O_2 -induced decrease in cell viability (Fig. 4B) and increased ROS level (Fig. 4C). Knockdown of Nrf2 restraint the increase of the ratio of LC3II/LC3I of the HTR8/SVneo cells AS IV induced (Fig. 4E and Fig. 4G). In addition, the decrease in p62 induced by AS IV was attenuated by Nrf2 siRNA (Fig. 4D, E, and F). These results confirmed that Nrf2 is upstream of this AS IV-induced Nrf2-Keap1-p62 feedback loop.

HTR8/SVneo cells of p62-knockdown groups and control-siRNA groups were incubated in AS IV or H_2O_2 to determine whether autophagy can modulate H_2O_2-induced oxidative stress. The expression of p62 was significantly decreased after transfection into HTR8/SVneo cells with or without H_2O_2 treatment compared with the cells transfected with a control siRNA. In addition, the level of p62 was significantly decreased after transfection into HTR8/SVneo cells with or without pretreatment with AS IV compared with the cells transfected with a control siRNA (Fig. 5A). Furthermore, knockdown of p62 decreased the protective effect of AS IV against an H_2O_2-induced decrease in cell viability (Fig. 5B) and increased ROS (Fig. 5C). Keap1 levels increased, while Nrf2 decreased in p62 knockdown group compared with those in the cells transfected with a control siRNA and challenged with H_2O_2 (Fig. 5D–H). As such, Keap1 degradation induced by AS IV might have been mediated by p62, which blocked the proteasomal degradation of Nrf2. Knockdown of p62 inhibit the increase of the ratio of LC3II/LC3I of the HTR8/SVneo cells AS IV induced (Fig. 4F and I).

4. Discussion

PE is one of the serious complications of pregnancy that threatens maternal, fetal, and neonatal health and life (J. Li et al., 2018).

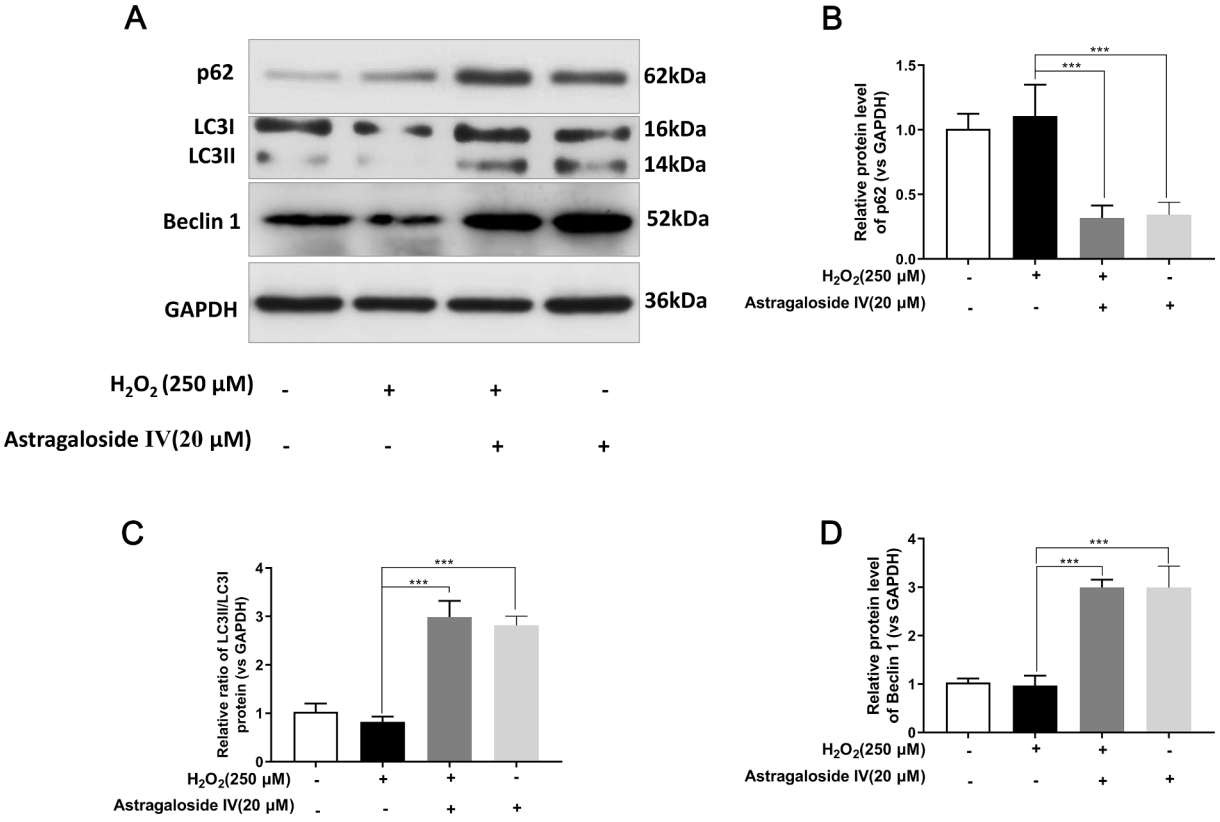

Fig. 3. Astragaloside IV increases autophagy in H_2O_2-induced HTR8/SVneo cells. (A) The protein level of p62, LC3II/LC3I, and Beclin-1were analyzed by Western blotting. Relative protein levels of p62(B), LC3II/LC3I (C), Beclin-1 (D), and were analyzed by grey scanning. The data are shown as means ± SD, n = 4, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$;. n.s. indicates no significance. GAPDH was regarded as the controls.

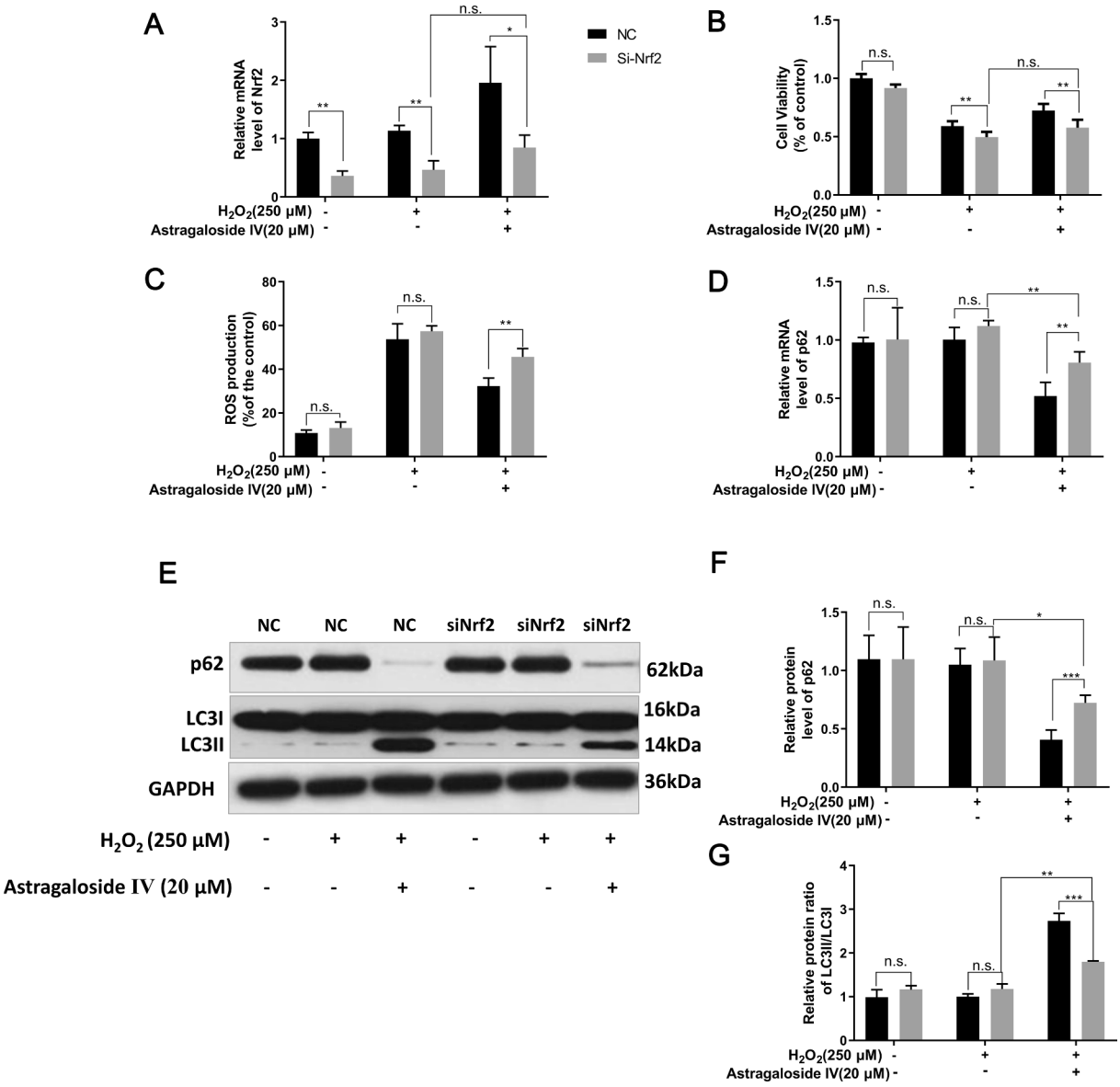

Fig. 4. Nrf2 is upstream of p62 in the H_2O_2-induced Nrf2-Keap1-p62 feedback loop. The mRNA expressions of Nrf2(A) and p62(D) in HTR8/SVneo cells transfected with either an Nrf2 siRNA (Si-Nrf2-2) or a control siRNA (NC) for 16 h and pretreated with astragaloside IV for 4 h followed by H_2O_2 treatment for an additional 24 h. The protein expressions of p62 (E and F) and LC3II/LC3I ratio (E and G) in HTR8/SVneo cells transfected with either an Nrf2 siRNA (Si-Nrf2-2) or a control siRNA (NC) for 16 h and pretreated with astragaloside IV for 4 h followed by H_2O_2 treatment for an additional 24 h. The data are shown as means ± SD, n = 4; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (B) Cell viability assay and (C) ROS level in HTR8/SVneo cells transfected with either an Nrf2 siRNA or a control siRNA (NC) for 16 h and with or without astragaloside IV pretreatment for 4 h followed by H_2O_2 treatment for additional 24 h. The data are shown as mean ± SD. n = 6. *, $p < 0.05$; **, $p < 0.01$. n.s. indicates no significance.

Therefore, finding a safe and effective solution to alleviate the symptoms of PE is imperative. In this study, we investigated whether AS IV could ameliorate the H_2O_2-induced trophoblast oxidative stress. Further mechanistic exploration indicated that the protective effect of AS IV in trophoblasts against oxidative stress is dependent on the Nrf2-Keap1-p62 pathways (Fig. 6).

Oxidative stress in trophoblast cells is well known as one of the pivotal causative factors in the pathophysiology of PE (Aouache, Biquard, Vaiman, & Miralles, 2018). Increased oxidative stress and placental dysfunction are the potential pathogenic bases of early-onset preeclampsia (Burton, Yung, Cindrova-Davies, & Charnock-Jones, 2009). Overproduction of ROS is widely recognized as an important index of oxidative stress (Hua et al., 2019). We established an in vitro model of oxidative stress by exposing the human trophoblast cell line (HTR8/SVneo) to H_2O_2 (Graham et al., 1998; Msheik, Azar, El Sabeh, Abou-Kheir, & Daoud, 2020; P. Wang et al., 2020). Inhibiting oxidative stress is an effective and rational treatment strategy for PE. ROS production and cell apoptosis are closely related. The present study showed that the percentage of apoptotic cells increased significantly with H_2O_2 treatment and may be closely related to ROS overproduction. Meanwhile, both the percentage of cell apoptosis and the level of ROS decreased under pretreatment with AS IV compared with under treatment with H_2O_2 alone, suggesting that AS IV may suppress H_2O_2-induced apoptosis in HTR8/SVneo cells.

Nrf2 is a vital transcription factor that protects cells against oxidative stress, harmful chemicals, and environmental toxicants (Lee & Surh, 2005; Tao, Justiniano, Zhang, & Wondrak, 2013). Keap1 is a critical repressor of Nrf2 activity (Lau et al., 2010). In case of oxidative stress, Nrf2 detaches from Keap1 and translocates to the nucleus, thereby playing an important biological function (Bellezza, Giambanco, Minelli,

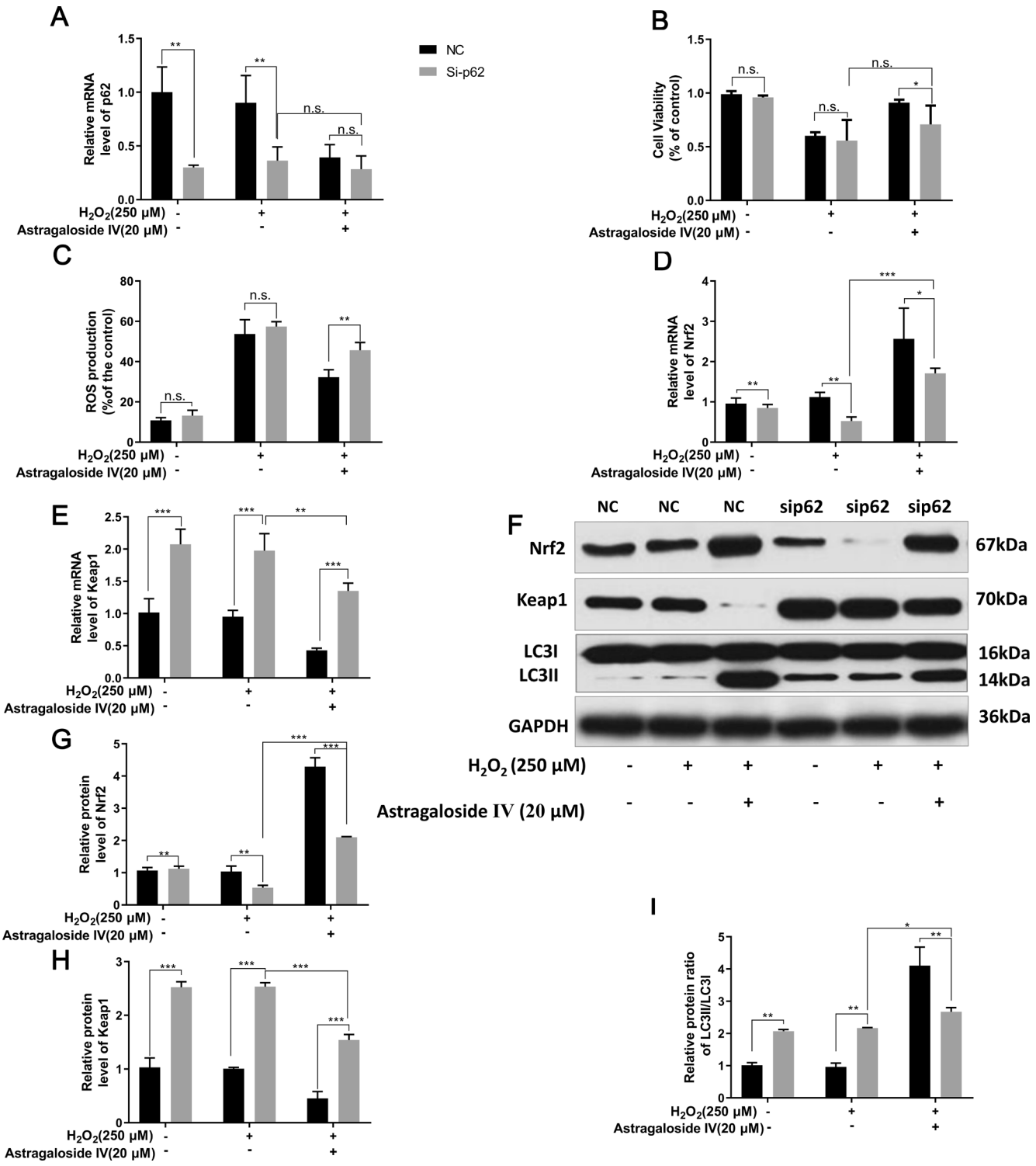

Fig. 5. p62 blocked the proteasomal degradation of Nrf2 and induced Keap1 degradation. The mRNA expressions of p62(A), Nrf2(D), and Keap1(E) in HTR8/SVneo cells transfected with either an p62siRNA (si-p62 −3) or a control siRNA (NC) for 16 h and pretreated with astragaloside IV for 4 h followed by H_2O_2 treatment for an additional 24 h. The protein expressions of Nrf2(F and G), Keap1(F and H), and LC3II/LC3I ratio (F and I) in HTR8/SVneo cells transfected with either an p62siRNA (si-p62 −3) or a control siRNA (NC) for 16 h and pretreated with astragaloside IV for 4 h followed by H_2O_2 treatment for an additional 24 h. The data are shown as means ± SD, n = 4; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. (B) Cell viability assay in HTR8/SVneo cells transfected with either an Nrf2 siRNA or a control siRNA (NC) for 16 h and with or without astragaloside IV pretreatment for 4 h followed by H_2O_2 treatment for additional 24 h. The data are shown as mean ± SD. n = 6. *, $p < 0.05$; **, $p < 0.01$. n.s. indicates no significance.

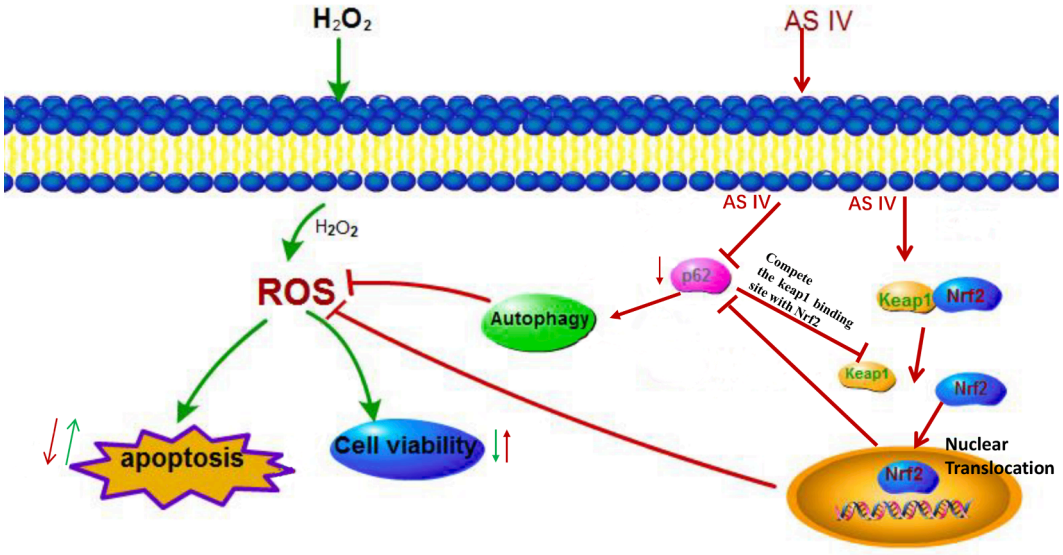

Fig. 6. A putative mechanism of astragaloside IV alleviating H_2O_2-induced apoptosis and oxidative stress in HTR8/SVneo cells. The green arrows indicate the effects of H_2O_2 on HTR8/SVneo cells, and the red arrows indicate the effects of astragaloside IV on H_2O_2-induced apoptosis and oxidative stress in HTR8/SVneo cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

& Donato, 2018). In the present study, AS IV increased the protein level of Nrf2 in HTR8/SVneo cells and decreased the level of Keap1. Furthermore, knockdown of Nrf2 abolished the protective effect of AS IV against an H_2O_2-induced decrease in cell viability, suggesting that AS IV protects HTR8/SVneo cells from H_2O_2-induced oxidative stress by activating the Nrf2/Keap1 pathway. Recent studies indicated that p62 is a target gene of the transcription factor Nrf2 (Copple et al., 2010; Komatsu et al., 2010).

Activation of autophagy can be regarded as a therapeutic target for oxidative stress-related disease. Autophagy is a cellular degradation process responsible for resisting oxidative stress and maintaining cellular homeostasis (Liu et al., 2017). p62 and LC3 are important autophagy biomarkers, and LC3II/LC3I ratio increases when autophagy occurs in cells (Holt et al., 2011; Rusten & Stenmark, 2010). p62, an autophagy adaptor protein, works as an adaptor that binds ubiquitylated protein aggregates and delivers them to the autophagosomes (Jiang et al., 2015). Expression of p62 decreases when autophagy occurs in cells. In the present study, AS IV increased the ratio of LC3II/LC3I and Beclin 1 expression and decreased the p62 expression induced by H_2O_2, suggesting that AS IV induced HTR8/SVneo cell autophagy.

A recent study suggests that oxidative stress is involved in the fine-tuning of the autophagic process in response to the level of oxidative stress and functions in a feedback loop (Tang et al., 2019). We performed siRNA-mediated silencing of p62 and Nrf2 to further investigate the relationship between the Nrf2-Keap1-p62 pathway and the protective effects of AS IV. Accumulating evidence suggests that when autophagy is impaired, p62 accumulates in the cytosol and tends to competitively bind with Keap1, a negative regulator of Nrf2, resulting in prolonged Nrf2 activation. In the present study, we found that knockdown of Nrf2 significantly attenuated AS IV-induced autophagy. Thus, Nrf2 can act as an upstream regulator of autophagy. Keap1 has been identified as a significant repressor of Nrf2 activity. Importantly, p62 not only plays crucial roles in removing ubiquitinated proteins but also in regulating the Nrf2/Keap1 signaling pathway (Jiang et al., 2015; Park, Kang, Lee, & Bae, 2015; Shen et al., 2018). p62 directly interacts with Keap1 and disrupts the interaction between Nrf2 and Keap1, resulting in increased Nrf2 activity (Tang et al., 2019). Nrf2 accumulation induces the transcription of numerous cytoprotective genes, whereas the p62 gene is an Nrf2 target. Therefore, Nrf2 induces p62 transcription, which in turn increases Nrf2 activity by inactivating Keap1 to produce a positive feedback loop. In the present study, knockdown of p62 significantly increased Keap1 levels. Nrf2 decreased in p62 knockdown that significantly increased the Keap1 expression level, suggesting the possible interaction between p62 and Keap1. This result is consistent with that of a previous report, which showed that RNAi depletion of p62 resulted in an increase in the Keap1 protein level by slowing down its rate of degradation and concomitantly decreasing the Nrf2 protein level (Bhide et al., 2018). In addition, p62 silencing reduced Nrf2 expression, which confirms that p62 is a target of Nrf2, and a feedback loop exists between them.

5. Conclusion

Taken together, our study found that AS IV protects human trophoblasts against H_2O_2-induced oxidative stress through the activation of the Nrf2-Keap1-p62 feeding loop. Our findings may serve as a reference for future studies investigating the role of AS IV as a protective and curative agent against PE. Given the complexity of the interactions between the Nrf2-Keap1-p62 pathway, oxidative stress, and vascular calcification, further studies are needed to provide additional information about the underlying mechanisms.

Credit authorship contribution statement

Fengge Wang, Dongmei Man, and Shuxiong Chen designed the work. Peng Lin, Miaomiao Qu, Jishui Wang, Haiyan Zhang, Lihua Zhang, and Miao Liu executed the experiments. Fengge Wang wrote the manuscript. Shuxiong Chen and Dongmei Man corrected the manuscript. Fengge Wang and Shuxiong Chen revised the manuscript.

Ethical statement

The Authors confirm that no animal/human studies have been carried out in the present.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2021.104621.

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