

Artesunate Ameliorates Cigarette Smoke-Induced Airway Remodeling via PPAR- γ /TGF- β 1/Smad2/3 Signaling Pathway

Kunming Pan

Zhongshan Hospital Fudan University

Juanjuan Lu

Department of Pharmacology, Shanghai Jiao Tong University School of Medicine

Yun Song (✉ songwany@126.com)

Huashan Hospital Fudan University <https://orcid.org/0000-0002-7168-8274>

Research

Keywords: Artesunate, COPD, airway remodeling, cigarette smoke, PPAR- γ

Posted Date: January 7th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-139978/v1>

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Version of Record: A version of this preprint was published on March 23rd, 2021. See the published version at <https://doi.org/10.1186/s12931-021-01687-y>.

Abstract

Background: Airway remodeling, the major pathological feature of chronic obstructive pulmonary disease (COPD), leads to poorly reversible airway obstructions. Current pharmacological interventions are ineffective in controlling airway remodeling. Herein, in this study, we investigated the potential role of artesunate in preventing and treating airway remodeling and the underlying molecular mechanisms *in vitro* and *in vivo*.

Methods: The COPD rat model was established by cigarette smoke (CS) exposure. After 12 weeks' treatment of artesunate for COPD rats, the pathological changes of lung tissues were observed by ELISA test, histochemical and immunohistochemical staining. Lung functional experiment was also carried out to elucidate artesunates' effects. The human bronchial smooth muscle (HBSM) cells were used to clarify the underlying molecular mechanisms.

Results: Artesunate treatment inhibited CS-induced airway inflammation and oxidative stress in a dose-dependent manner, simultaneously significantly reduced airway remodeling through inhibiting α -SMA and cyclin D1 expressions. Mechanistically, upregulation of PPAR- γ expression and inactivation of TGF- β 1/Smad2/3 signaling by artesunate treatment were found *in vivo* and *in vitro*. Furthermore, PPAR- γ knockdown by siRNA transfection abolished the ability of artesunate to inhibiting HBSM cell proliferation, via triggering the activation of TGF- β 1/Smad2/3 signaling pathway and downregulating the expression of α -SMA and cyclin D1 in HBSM cells.

Conclusions: These findings suggest that artesunate could be valuable for treating airway remodeling by targeting PPAR- γ /TGF- β 1/Smad signaling in COPD.

1. Introduction

Airway remodeling is major pathological abnormality in chronic obstructive pulmonary disease (COPD) [1]. The oxidative stress by environmental factors including exposure to cigarette smoke (CS) has been shown to induce airway hyperreactivity and airway remodeling, effects that involve airway smooth muscle [2, 3, 4]. Airway remodeling is also caused by the upregulation of α -smooth muscle actin (α -SMA) and Cyclin D1, which are critical regulators in the cell proliferation and cell cycle progression [5].

The ligand-activated transcription factor peroxisome proliferator-activated receptor γ (PPAR- γ), a member of the nuclear hormone receptor superfamily, is frequently expressed in human airway smooth muscle cells [6]. Increasing evidence revealed that PPAR- γ agonists have the ability to inhibit proliferation of human airway smooth muscle, and reduces inflammatory cell infiltration and airway remodeling by the activation of PPAR- γ in COPD [7–9]. In addition, PPAR- γ was also reported to inhibit TGF- β 1-Smad2/3 pathway activation, which plays a vital role in the progression of epithelial-mesenchymal transition (EMT) and airway remodeling [10, 11]. Therefore, targeting PPAR- γ signaling might represent an effective strategy for the preventing and treating airway remodeling in COPD.

Current clinical pharmacologic therapies, including corticosteroids and bronchodilators, are able to reduce exacerbations and improve symptoms but not to suppress the development and progression of COPD [12]. Drug repurposing is one such strategy. The repurposing of 'old' drugs is gradually becoming an attractive proposition because it involves the use of less risky compounds with potentially lower overall development costs and shorter development timelines [13]. Artesunate is a semi-synthetic derivative of a Chinese herb named by *Artemisia annua* L, which is commonly used as an antimalarial agent [14]. In addition, it has been shown to possess anti-inflammatory and antioxidant activity [15, 16]. A recent report demonstrated that artesunate ameliorated oxidative lung damage in experimental allergic asthma and attenuated CS-induced lung damage and emphysema in mice [17, 18]. Artesunate could also inhibit the proliferation of primary human cultured airway smooth muscle cell [19, 20] and improves bleomycin-induced pulmonary fibrosis pathology in rats via the inhibition of by inhibiting TGF- β 1-Smad3 activation [21]. However, the effects of artesunate on airway remodeling and the underlying mechanism(s) remains to be further explored .

In the present study, we hypothesized that airway inflammation and airway remodeling, already accompanied by clearly abnormal lung function ameliorated by artesunate, is closely related to PPAR- γ /TGF- β 1/Smad signaling pathway. Using human bronchial smooth muscle cells (HBSM) and an experimental rat model of COPD, the present study mainly described the protective effects of artesunate, and underlying mechanism(s). The present study provided new uses for old drugs - artesunate and its potential mechanisms of action responsible for airway inflammation and remodeling in COPD.

2. Materials And Methods

2.1 Rat model of COPD

Thirty Sprague-Dawley (SD) rats (8 weeks, 230 ± 25 g, half male and half female) were purchased from Shanghai Laboratory Animal Company (Shanghai, China). All rats were housed in a room temperature of 25 °C and in a light-dark cycle of 12:12 h with free access to diet and water ad libitum. All animal experiments were approved by the Experimental Animal Ethics Committee of Fudan University and performed in accordance with the guidelines for the care and use of laboratory animals set by Fudan University (Shanghai, China).

The CS-induced COPD rat model was established as our previous report [22]. Rats were exposed to cigarette smoke from four cigarettes (Double Happiness, Shanghai) burning simultaneously and each exposure lasted 75 minutes consuming 48 cigarettes totally. The smoke exposure was performed twice per day and 5 days per week for 12 weeks by using a custom-designed and purpose-built nose-only, directed flow inhalation and smoke-exposure system (handmade) housed in a fume and laminar flow hood. Artesunate (25, 50, 100 mg/kg) was administered via intraperitoneal injection 1 h before the first exposure of a day. Control group was treated with normal saline. The rats were anesthetized with 40 mg/kg intraperitoneal sodium pentobarbital for sample collection and further analysis after 12 weeks of smoke exposure.

2.2 Lung function test

Lung function was measured as we previously described [22]. After anaesthesia, trachea cannula was inserted at the throat to examine lung function by PowerLab 8sp Life Analysis System (AD Instrument, Australia), including peak inspiratory flow (PIF), peak expiratory flow (PEF), airway inside pressure (IP) and airway pressure maximum rising slope (IP-slope).

2.3 Bronchoalveolar lavage fluid (BALF)

After rats were anesthetized by injecting sodium pentobarbital, tracheotomy was performed and a cannula was inserted into the trachea. BALF was collected from the right lungs through three lavages of 1 ml phosphate-buffered saline (PBS). Extracted BALF was immediately centrifuged at 1000 rpm for 5 min at 4 °C, and then used for further assays.

2.4 Airway smooth muscle isometric tension assay

The airway isometric contraction assay was performed as described previously [5]. To measure airway smooth muscle isometric tension of rats, the main bronchus was rapidly separated from surrounding connective tissue after the extraction of BALF. The bronchial rings were fixed on the stainless-steel hooks in a 37 °C bath of modified Krebs's solution (composition in mM: NaCl 118; KCl 4.7; MgSO₄ 1.2; KH₂PO₄ 1.2; NaHCO₃ 25; CaCl₂ 2.5; glucose 11, EDTA·Na₂ 0.5) and then continuously bubbled with 95% O₂ and 5% CO₂. The bronchial rings were connected vertically to a force-displacement transducer under a resting tension of 500 mg. After the separated bronchial rings were washed every 15 min for 1 h, the values of isometric tension were recorded by PowerLab 8sp Life Analysis System (AD Instruments, Sydney, Australia) to generate the cumulative concentration-response curves for carbachol (CCh). The values of isometric tension were expressed as force.

2.5 Histology and Immunohistochemistry

After bronchoalveolar lavage, the right lungs were immersed in 4% paraformaldehyde for 24 h. After fixation, paraffin embedding and section, H&E and Masson stainig were performed for inflammation and fibrosis evaluation, respectively. Immunohistochemistry staining of α -SMA and cyclin D1 (CST, Cell Signaling Technology, Beverly, MA, USA) was performed for remodeling evaluation. Quantitative analysis was performed by Image-Pro plus 6.0.

2.6 Cell culture, cell transfection and cigarette smoke extract (CSE) preparation

The human bronchial smooth muscle (HBSM) cells were purchased from the ScienCell Research Laboratories (San Diego, California, USA). Cells were cultured in Smooth Muscle Cell Medium (ScienCell, San Diego, California, USA) and performed for experiments at passage 2–4 with no mycoplasma contamination. The small interfering RNAs (siRNAs) were synthesized by Genema (Shanghai, China) for the PPAR- γ knockdown assay. The sequences of si-PPAR- γ are as follows: 5'-

TGGAAATGTGATACGCAAAAT-3'. The siRNA transfection was conducted with Lipofectamine 3000 (Invitrogen, Waltham, MA, USA) according to the manufacturer's protocol.

CSE was prepared as our previous reports [22]. The CSE was prepared by combusting one cigarette (Double happiness, Chinese, the amount of tar was 12 mg), using a pump and passing the smoke through 10 ml of FBS-free culture medium at a rate of 5 min/cigarette. The resulting solution was adjusted to pH 7.4 with 1 mol/L of concentrated NaOH and filtered through a 0.22 µm filter. The obtained solution was referred to as 100% strength and diluted to the desired concentrations with culture medium.

2.7 Measurement of indicated cytokines and cell proliferation

After above indicated treatment, BALF or cell supernatants were collected to examine TGF-β1, IL-8, TNF-α, IL-6 and ICAM-1 level using an enzyme-linked immunosorbent assay (ELISA, Elabscience Biotechnology, China). Reactive oxygen species (ROS) production was measured by H2DCFDA reagent (Sigma, St. Louis, MO, USA) using flow cytometry. Cell lysate was used to measure intracellular GSH with a commercial assay kits (Beyotime, Jiangsu, China) as manufacturer's instructions. The cell proliferation was measured by Cell Counting Kit (CCK)-8 assay and BrdU cell proliferation assay kit (CST, Beverly, MA, USA) according to manufacturer's protocols.

2.8 Western blot analysis

After serum deprivation for 24 h, HBSM cells were cultured in DMEM with CSE stimulation in the presence or absence of artesunate. α-SMA, Cyclin D1 and GAPDH expression were measured after 24 h. Frozen lung tissues were homogenized in RIPA lysate using ultra-sonic oscillation, total protein extracts were separated by 10% SDS-PAGE. Immunoblots were probed with anti-α-SMA, anti-cyclin D1, anti-PPAR-γ, anti-p-Smad2, anti-p-Smad3, anti-Smad2, anti-Smad3, anti-GAPDH and anti-β-actin (CST), followed by horseradish peroxidase-conjugated secondary antibody (Abcam). Protein bands were visualized with Biorad System (USA), β-actin and GAPDH were used as internal controls for total protein extracts, respectively. Band intensity was quantitated using Image J software.

2.9 Statistical analyses

All the experimental data are presented as the means ± SEM and analyzed by Prism version 6.0 (GraphPad Software, San Diego, USA). The *t*-test was performed to measure the differences between the two groups and one-way analysis of variance (ANOVA) followed by a Dunnett's test was performed to compare the differences among three or more groups. *p*-values < 0.05 were considered statistical significance.

3. Results

3.1 Role of artesunate in reducing airway inflammation and oxidative stress in lung of CS-exposed rats

The proinflammatory cytokines IL-6, IL-8, TNF- α , and ICAM-1, which amplify the inflammatory process and induce airway structural changes were found in BALF after 12 weeks of CS exposure. The levels of IL-6, IL-8, TNF- α , and ICAM-1 in the BALF were significantly higher in the CS-exposed group than in control group (Fig. 1A-1D). The increasing of IL-6, IL-8, TNF- α , and ICAM-1 were attenuated by artesunate treatment in a dose-dependent manner (Fig. 1A-1D). Consistently, artesunate treatment also markedly attenuated inflammatory infiltration, which significantly increased in lung of CS-exposed rats compared to the control rats (Fig. 1E and 1F).

Oxidative stress induced by CS exposure is also largely correlated with the process of airway remodeling [23]. As shown in Fig. 4C, CS significantly increased ROS levels in the BALF, which was reversed by artesunate treatment. Furthermore, the GSH level decreasing induced by CS exposure was also significantly reversed by artesunate treatment (Fig. 1G and 1H). Taken together, these findings demonstrated that artesunate could be valuable for protecting against airway inflammation and oxidative stress in CS exposure rat model and suggest the potential role in the prevention and therapy of airway remodeling.

3.2 Role of artesunate in attenuating CS-induced airway remodeling in rats

Airway remodeling, which is generally accepted, is closely related to persistent chronic inflammation and oxidative stress [24]. An obvious airway remodeling phenotype, showing fibrosis, epithelial and smooth muscle thickness, was found after 12 weeks of CS exposure, and these CS-induced events could be significantly reversed by artesunate in a dose-dependent manner (Fig. 2A-2D). α -smooth muscle actin (α -SMA) and cyclin D1 are identified as the key mediators in airway remodeling [5]. In the CS exposure groups, the expressions of α -SMA and cyclin D1 in lung dramatically up-regulated and were reversed by artesunate treatment (50 mg/kg and 100 mg/kg) as measured by IHC (Fig. 2E-2H) and western blot (Fig. 2I and 2J). These results suggest that airway remodeling following CS-exposure in rats could be reversed by artesunate treatment.

3.3 Role of artesunate in ameliorating lung function and airway hyperresponsiveness from CS-exposed rats

Airway remodeling contributes to the progressive loss of lung function, declining lung function caused by persistent airflow obstructions is an important feature of COPD [25]. We next assess the effects of artesunate in lung function from CS-exposed rat. PIF and PEF significantly decreased after CS exposure for 12 weeks, while IP, IP-sloPe and isometric force significantly increased compared with rats in control group ($P < 0.01$). Importantly, artesunate treatment improved CS-induced pathological alterations in lung functions, including PIF, PEF, IP and IP-slope, as well as isometric force compared to the CS-exposed rats (Fig. 2A-2D). These results demonstrated that declining lung function caused by airway remodeling could be ameliorate by artesunate treatment in the condition of oxidative stress induced by CS.

3.4 Role of artesunate in inhibiting HBSM cells proliferation triggered by CSE

To further validate the potential role of artesunate on airway remodeling, CCK-8 and BrdU incorporation assay was used to assess the proliferation of HBSM cells. We found that significantly increased proliferation of HBSM cells triggered by low concentration of CSE (2.5%) were inhibited by treatment with artesunate in a dose concentration-dependent manner indicated by both CCK-8 (Fig. 4A) and BrdU incorporation assay (Fig. 4B). As expected, treatment with artesunate also significantly attenuated CSE-induced cyclin D1 (Fig. 4C) and α -SMA (Fig. 4D) expressions as compared to CSE alone. These results indicated that artesunate has an inhibitory effect on proliferation of HBSM cells by attenuating the expression cyclin D1 and α -SMA.

3.5 Role of artesunate in the expression of PPAR- γ and TGF- β 1/Smad2/3 activation in both HBSM cells and rat lung exposed to CS

Next, we validated whether PPAR- γ pathway is involved in the protective effects of artesunate in HBSM cells and rat lungs. Western blot analysis showed that CS significantly decreased PPAR- γ expression in both HBSM cells and rat lungs, which were reversed by artesunate treatment in a dose-dependent manner (Fig. 5A and 5B). CS can trigger TGF- β 1 release, activating its downstream signaling pathway Smad2/3 cascade to promote airway remodeling. We subsequently validate the effects of artesunate on CS-induced activation of TGF- β 1 signaling, and we found that TGF- β 1 was significantly upregulated in CS-exposed HBSM cells and rat lungs, which were reversed by artesunate treatment (Fig. 5C and 5F). Additionally, CSE-activated Smad2/3 phosphorylation was also inhibited by artesunate treatment (Fig. 5D and 5E). Altogether, these results demonstrated that the attenuating effects of artesunate involving both PPAR- γ up-regulation and TGF- β 1/Smad2/3 dephosphorylation in the CS-induced airway inflammation and remodeling process.

3.6 Role of artesunate in suppressing cell proliferation by targeting PPAR- γ /TGF- β 1/Smad2/3 signaling

Given that the involvement of PPAR- γ and TGF- β 1/Smad2/3 signaling in CSE-induced airway remodeling and abnormal proliferation of airway smooth muscle cells directly contributes to airway remodeling [26], we therefore investigated whether the suppressive effects of artesunate on HBSM cell proliferation was related with PPAR- γ activation and TGF- β 1 signaling pathway. Western blot analysis showed that siRNA-PPAR- γ transfection significantly abolished PPAR- γ expression in HBSM cells (Fig. 6A). We also showed that PPAR- γ knockdown abolished artesunate's effect by enhancing TGF- β 1 level in supernatant of HBSM cells (Fig. 6B) and Smad2/3 dephosphorylation (Fig. 6E) in HBSM cells stimulated by CSE. As expected, PPAR- γ knockdown also enhanced the expression of α -SMA and cyclin D1 (Fig. 6C), along with significant increases in CSE-exposed HBSM cells proliferation, which was inhibited by artesunate treatment, as

measured by CCK-8 and BrdU assay (Fig. 6D). These results suggest that artesunate suppresses cell proliferation through TGF- β 1/Smad2/3 signaling pathway by targeting PPAR- γ .

4. Discussion

The main findings of the present study are that artesunate significantly suppressed CS-induced airway inflammation as well as airway remodeling *in vivo and in vitro*. The effect of artesunate was associated with the CS-induced airway remodeling by targeting PPAR- γ /TGF- β 1/Smad2/3 signaling.

Experimental studies have already confirmed that CS exposure directly contributed to the changes in structural cells seen in the lung tissue and small airways caused by airway inflammation and oxidative stress [27]. Thus, to some extent, inhibiting the inflammatory response, oxidative stress, as well as airway remodeling may offer viable choices for the therapy of COPD. First, we established a 12-week CS-exposure rat model to evaluate the therapeutic effects of artesunate, and we found that artesunate treatment reduced the levels of IL-6, IL-8, TNF- α , ICAM-1, ROS and GSH in the BALF of CS-exposed rats in a dose-dependent manner. These results are consistent with another study showing that artesunate could modulate multiple inflammatory and oxidative stress mediators in CS or ovalbumin exposure mice model [28].

Previous studies on both animals and humans have shown that CS induces the production of pro-inflammatory cytokines such as IL-6, IL-8, TNF- α , and ICAM-1 as well as ROS and GSH, which amplify the inflammatory process and play an integral role in the coordination and persistence of inflammatory process that occur in airway remodeling of COPD patients [29, 30]. This pathological changes in the lungs exposed to CS were significantly ameliorated by artesunate treatment. Importantly, isometric force increase and lung function decline, including PIF, PEF, IP and IP-sloPe, as representatives of airway hyperresponsiveness and small airway resistance, respectively, were also ameliorated. Besides its anti-malaria property, artesunate has also been reported to show a variety of pharmacological activities. For example, artesunate was reported to reduce lung damage in CS-induced mice [31], which is consistent with our results. Artesunate ameliorated CS-induced airway inflammation, inhibited the PI3K δ /Akt pathway, restored HDAC2 activity, consequently reversing CSE-induced glucocorticoid insensitivity [32]. All these findings have prompted us to further characterize the underlying mechanisms in detail. Therefore, artesunate, as a multi-target drug, provides a novel use for the repurposing of 'old' drugs in airway remodeling, a key feature of COPD and asthma [33]. To further validate the animal experiment results, cellular experiments were performed in HBSM cells. It was widely accepted that CSE has a significant effect on airway abnormal contractility and proliferation in HBSM cells [34]. Consistently, our results were consistent with these findings and further demonstrated that artesunate inhibited HBSM cells proliferation and reduced levels of the proliferation marker cyclin D1 as well as α -SMA, a marker of myofibroblast *in vivo and in vitro*. However, previous studies have reported that artesunate suppressed the proliferation of human leukemic cells proliferation by regulating c-Myb and cyclin D2 expression [35]. Moreover, artesunate significantly inhibited the proliferation of hepatoma cell line via STAT3 inhibition and DR4 augmentation [36]. In our study, we observed an increase in HBSM cells proliferation caused by

CSE can be inhibited by artesunate, accompanied with the recoveries of cyclin D1 and α -SMA expressions, revealing the different mechanisms of artesunate in inhibiting cell proliferation in various cell systems.

It has been reported that PPAR- γ activation reduces lipopolysaccharide-induced inflammation in mice model, suggesting that an activator of PPAR- γ may have a beneficial effect the inflammatory response of COPD [37]. In fact, several evidences have suggested the reduced level and activity of PPAR- γ in the lungs of both CS-exposed mice lungs as well as in the lung of smokers and COPD patients [38, 39].

Consistently, our data demonstrated that PPAR- γ expression in both rat lungs and HBSM cells were significantly inhibited in response to CS, which is in agreement with previous reports that CS can inhibit PPAR- γ activation [40]. Whereas treatment with artesunate significantly enhanced PPAR- γ activation *in vivo* and *in vitro*, which is in agreement with previous reports that PPAR- γ agonists reversed CS-induced airway injury in bronchial epithelial cells [41]. Thus, we have reason to speculate that the activation of PPAR- γ may involve the protection of artesunate for CS-induced airway inflammation and remodeling.

It has been reported that TGF- β 1-Smad signaling pathway could be activated by CS in bronchial rat explants, which is identified as the key signaling pathway in EMT and airway remodeling [11, 42]. It has been reported that TGF- β 1/Smad2 pathway was significantly activated in bronchial smooth muscle cells exposed to CS [43]. More important, the inhibition of the TGF- β 1 gene by PPAR- γ activation can be applied for treating TGF- β 1-induced pathophysiologic disorders such as fibrosis [44]. We further investigated in our this study whether artesunate's effect involve the TGF- β 1 signaling via activation of PPAR- γ . We found that PPAR- γ knockdown and the inactivation of TGF- β 1/Smad signaling pathway attenuated the effect of artesunate on CS-induced cell proliferation *in vitro*, which is consistent with previous study on the activation of PPAR- γ in various cells and diseases [45]. For instance, activation of PPAR- γ in myeloid cells could promote the progression of epithelial lung tumors through the regulation of TGF- β 1 signaling pathway. PPAR- γ expression was increased in NSCLC cell lines, and knockdown of PPAR- γ inhibited EMT [46]. Therefore, our results further confirmed that the effect of artesunate by ameliorating CS-induced bronchial remodeling involved in PPAR- γ /TGF- β 1/Smad2/3 signaling pathway.

5. Conclusion

In conclusion, our results revealed that artesunate treatment significantly protected against CS-induced airway inflammation, as well as airway remodeling via PPAR- γ /TGF- β 1/Smad2/3 signaling *in vivo* and *in vitro*, and provides a novel use for the repurposing of 'old' drugs in airway remodeling of COPD.

Abbreviations

ANOVA: one-way analysis of variance; α -SMA: alpha-smooth muscle actin; BALF: bronchoalveolar lavage fluid; CCh: carbachol; CCK-8: cell counting kit-8; COPD: chronic obstructive pulmonary disease; CS: cigarette smoke; CSE: cigarette smoke extract; ELISA enzyme-linked immunosorbent assay; EMT: epithelial-mesenchymal transition; GSH: reduced glutathione; HBSM: human bronchial smooth muscle;

HDAC2: histone deacetylase 2; ICAM-1: intercellular adhesion molecule 1; IL: interleukin; IP: airway inside pressure; IP-slope: airway pressure maximum rising slope; PPAR- γ : peroxisome proliferator-activated receptor γ ; ROS: reactive oxygen species; SD: Sprague-Dawley; TGF- β 1: transforming growth factor beta 1; TNF- α : tumor necrosis factor alpha; PIF: peak inspiratory flow; PEF: peak expiratory flow.

Declarations

Ethics approval and consent to participate

All animal experiments were approved by the Experimental Animal Ethics Committee of Fudan University (permission number: 2019 Huashan Hospital JS-112) and performed in accordance with the guidelines for the care and use of laboratory animals set by Fudan University (Shanghai, China).

Consent for publication

Not applicable.

Funding

This work was funded by the National Natural Science Foundation of China (No. 81900035).

Availability of data and material

The software and all relevant raw data are freely available to scientists.

Competing Interest

The authors declare that they have no competing Interests.

Author contributions

Juanjuan Lu and Yun Song conceived of the study. Kunming Pan and Juanjuan Lu participated in design of the study and performed the experiments. Kunming Pan carried out the statistical analysis. Yun Song helped in the interpretation of the data and coordination and drafted the manuscript. All authors read and approved the final version of the manuscript.

Acknowledgement

We thank professor Yongyao Cui for his guidance on the writing of this paper.

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Figures

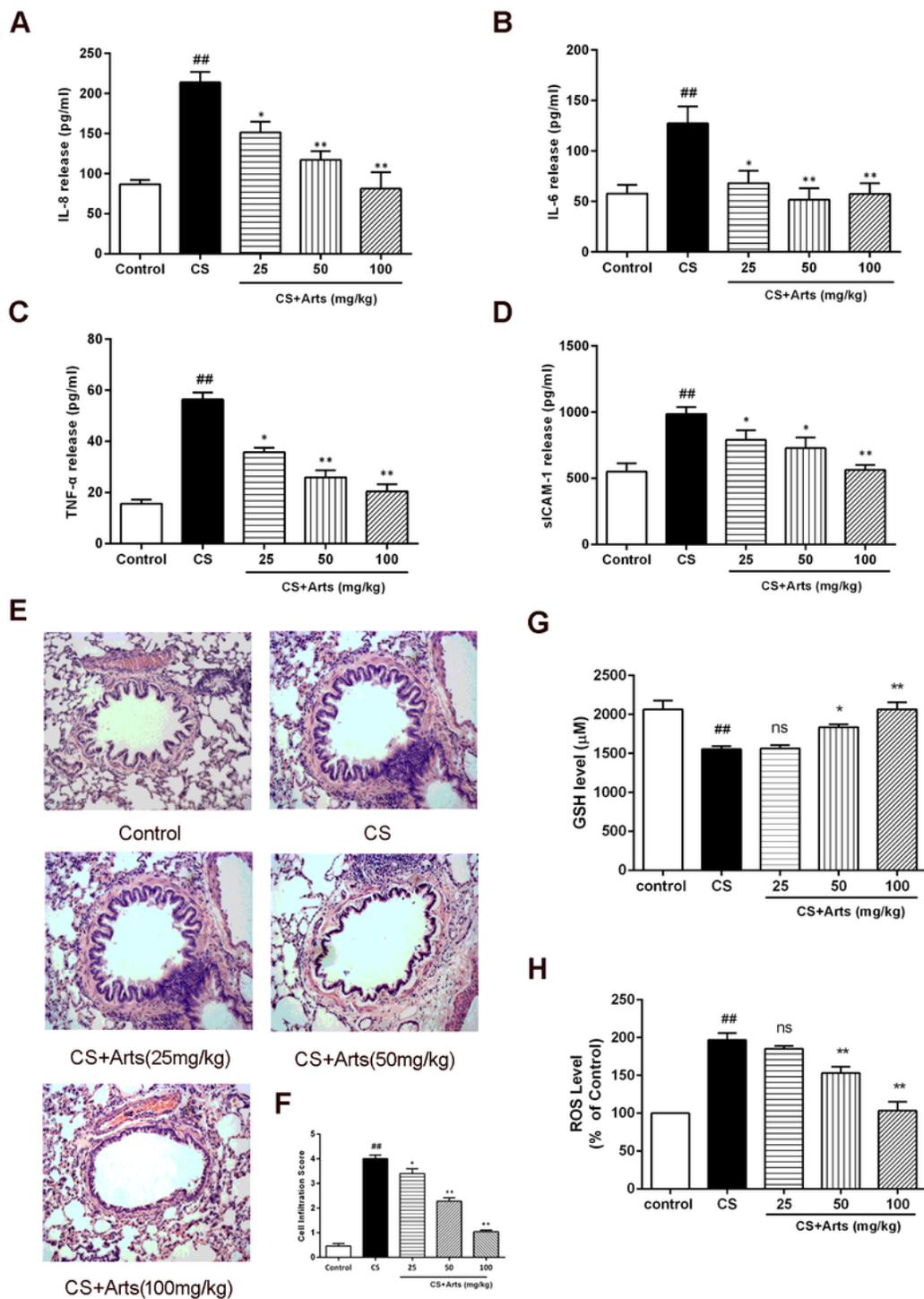


Figure 2

Artesunate attenuated CS-induced airway remodeling in rats. (A) Masson's trichrome staining was performed to determine airway histopathological changes. (B-D) The airway fibrosis, airway epithelial thickness and airway smooth muscle thickness were quantified by Image J. (E-H) Immunohistochemical staining were performed to determine α -SMA and cyclin D1 expressions in the lungs. (I, J) The proteins levels of α -SMA and cyclin D1 in the lungs were analyzed by Western blot. Data were expressed as mean \pm SEM of 6 rats/group, $##P < 0.01$ compared to control group; $*P < 0.05$ and $**P < 0.01$, compared to CS group. Arts: Articulate.

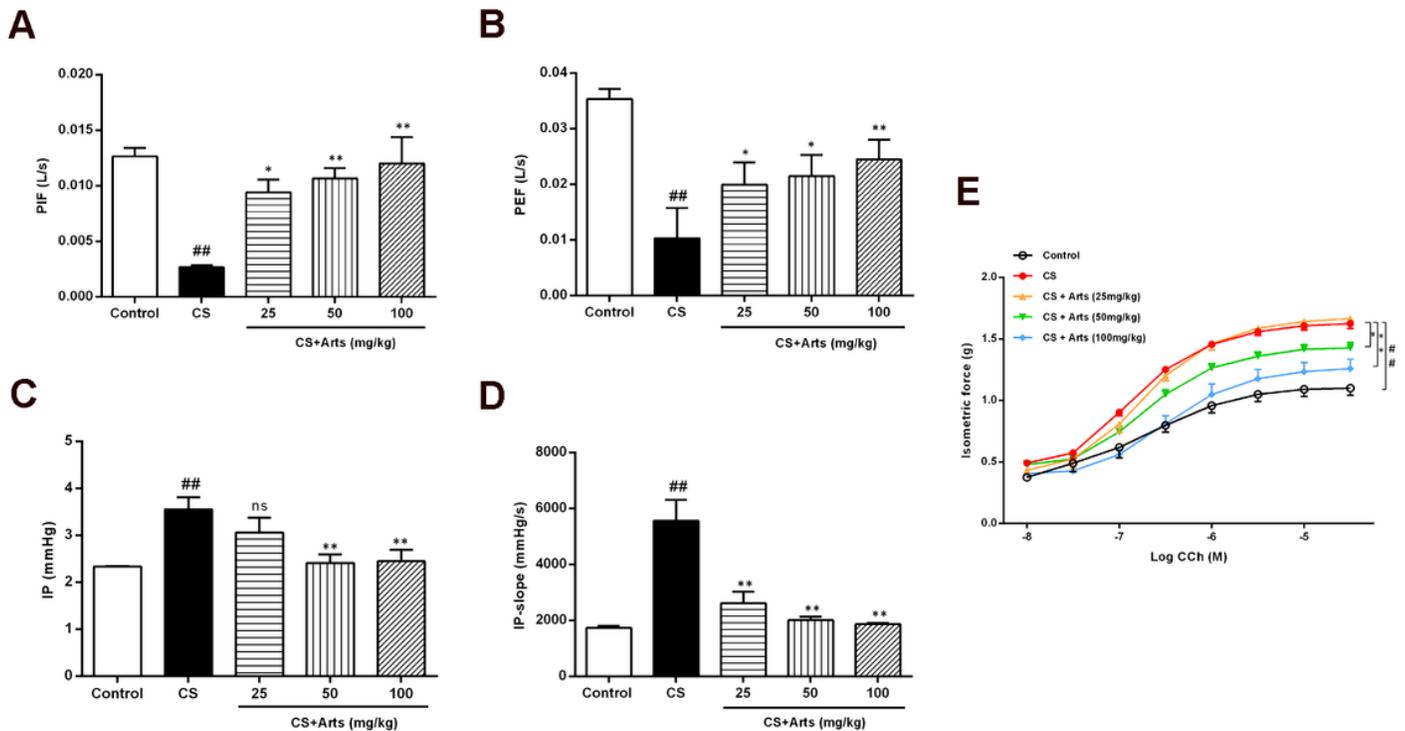


Figure 3

Artesunate restored lung function declining and airway hyperresponsiveness caused by CS. After CS exposure for 12 weeks, the lung function of rats was assessed by using a PowerLab 8sp Life Analysis System, including (A) peak inspiratory flow (PIF), (B) peak expiratory flow (PEF), (C) inspiratory pressure (IP), (D) airway pressure maximum rising slope (IP-slope). (E) isometric force. Data were expressed as mean \pm SEM of 6 rats/group, $##P < 0.01$ compared to control group; $*P < 0.05$ and $**P < 0.01$, compared to CS group. Arts: Articulate.

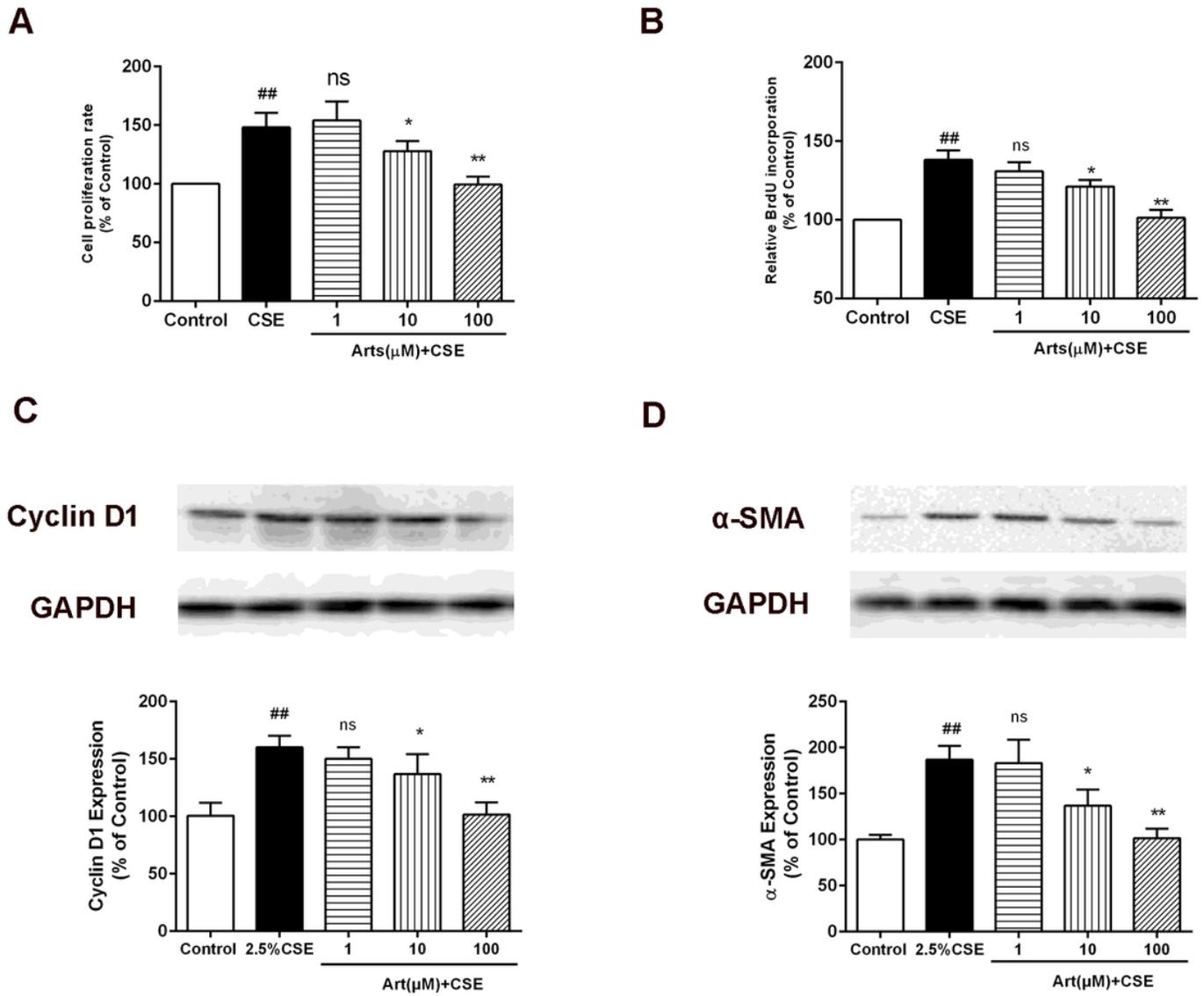


Figure 4

Artesunate inhibited the CS-induced proliferation in HBSM cells. (A) The cell viability was determined by CCK-8 assay. (B) The cell proliferation was determined by BrdU incorporation assay. The proteins levels of cyclin D1(C) and α-SMA (D) in HBSM cells were analyzed by Western blot. Data were expressed as mean ± SEM, ^{##}P < 0.01 compared to control group; ^{*}P < 0.05 and ^{**}P < 0.01, compared to CSE group. Arts: Articulate.

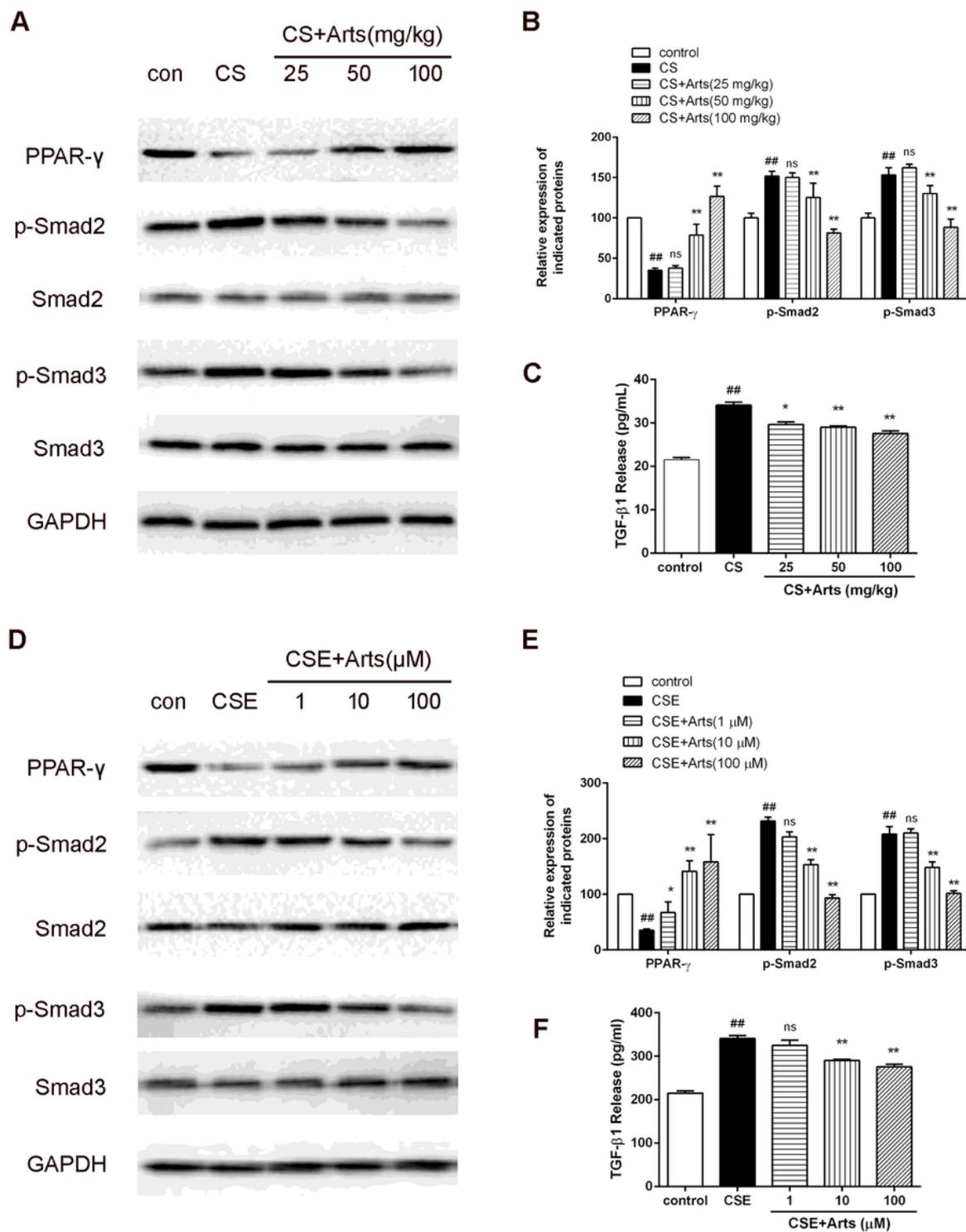


Figure 5

Effects of artesunate on expression of PPAR- γ and activation of TGF- β 1/Smad2/3 signaling in HBSM cells and rat lungs. The expressions of PPAR- γ , p-Smad2/3, Smad2/3 and GAPDH in HBSM cells (A, B) and rat lungs (D, E) were determined by using Western blot. The levels of TGF- β 1 in supernatant of HBSM cells (C) and BALF of rats (F) were determined by using ELISA method. Data were expressed as mean \pm

SEM, ##P < 0.01 compared to control group; *P < 0.05 and **P < 0.01, compared to CS group. Arts: Articulate.

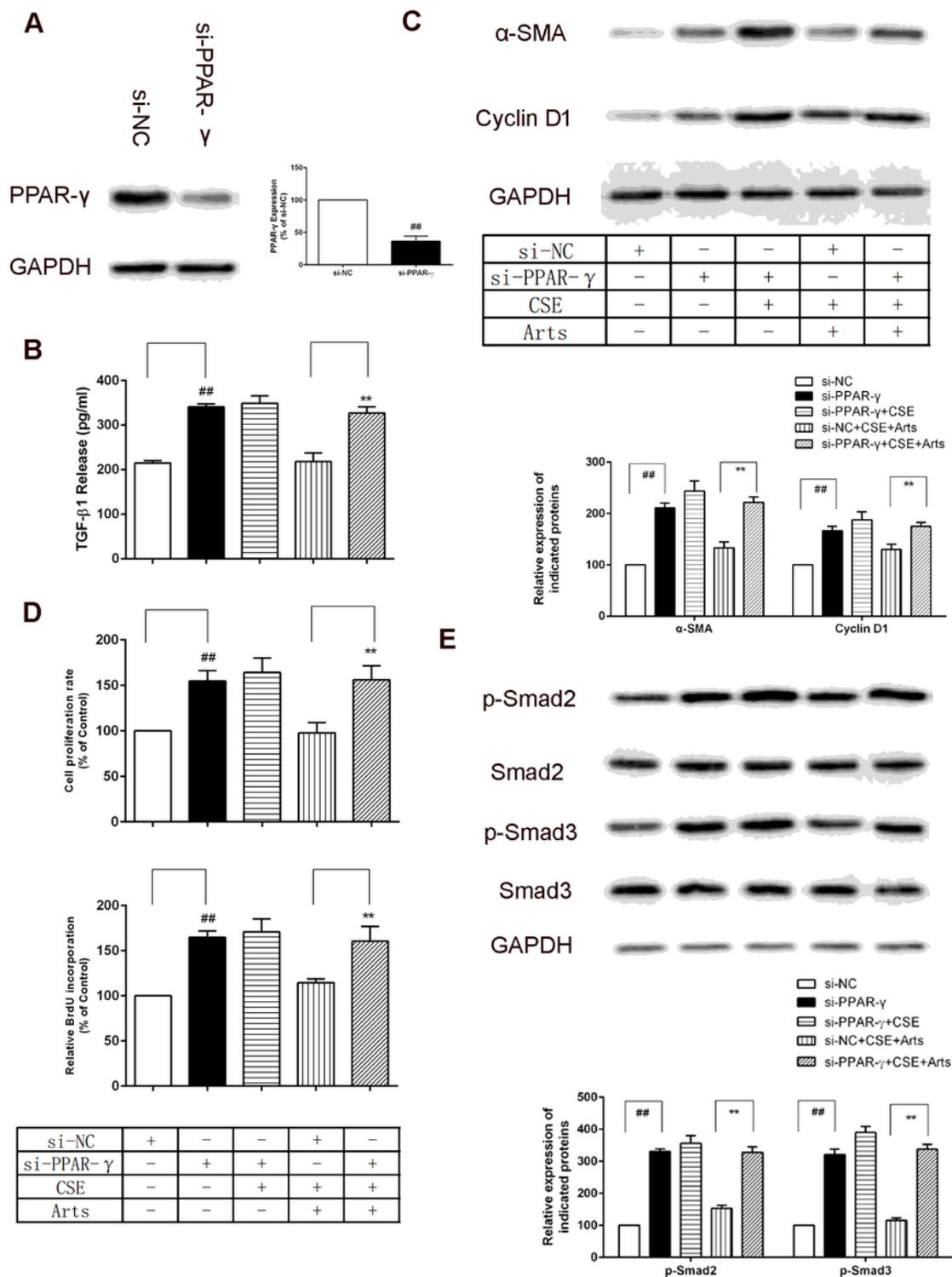


Figure 6

Suppressing cell proliferation by artesunate through PPAR- γ /TGF- β 1/Smad2/3 signaling pathway. HBSM cells were transfected with siRNA-PPAR- γ in the presence or absence of CSE/artesunate. (A) The interference efficiency was determined by Western blot. (B) The level of TGF- β 1 in supernatant of HBSM

cells was determined by using ELISA method. (C) The expressions of α -SMA, cyclin D1 and GAPDH were determined by using Western blot. (D) The cell viability was measured by CCK-8 assay and the cell proliferation was measured by BrdU incorporation assay. (E) The expressions of p-Smad2/3, Smad2/3 and GAPDH were determined by using Western blot. Data were expressed as mean \pm SEM, ##P < 0.01 compared to control group; *P < 0.05 and **P < 0.01, compared to CSE group. Arts: Articulate.