Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.1590/fst.117121

Hydroxysafflor yellow A (HSYA) improve scars by vivo and vitro study

Yan LI[1], Jingping GE[1], Yuanyuan YIN[1], Xu HE[1]*, Jianping GU[1]*

Abstract

The purpose of this research was to evaluate HSYA' effects and mechanisms to improve Scars Induced by Anticoagulant Injection by vivo and vitro study. New Zealand rabbits were divided into Normal control (NC), Anticoagulant, Anticoagulant+HSYA-Low, Anticoagulant+HSYA-Middle and Anticoagulant+HSYA-High. Measuring TGF-β1 and IL-1β concentration by Elisa assay; evaluating pathology and fibrosis level by HE and Masson staining, measuring collage I, collage III, TLR4 and NF-κB(p65) protein expression by IHC assay. Relative gene expression (Collage I, Collage III, TLR4 and NF-κB(p65)) were evaluated by RT-qPCR assay. Relative proteins expression (Collage I, Collage III, TLR4 and NF-κB(p65)) were evaluated by WB assay. And using TGF-β1 to stimulate cell to make cell model. Compared with NC group, TGF-β1 and IL-1β concentration were significantly increased ($P < 0.001$); The pathology and fibrosis level were significantly deteriorated, meanwhile, Collage I, Collage III, TLR4 and NF-κB(p65) proteins and gene expression were significantly up-regulation in Anticoagulant group ($P < 0.001$). With HSYA supplement, TGF-β1 and IL-1β concentration were significantly depressed, Pathology and fibrosis levels were significantly improved, Collage I, Collage III, TLR4 and NF-κB(p65) proteins and gene expressions were significantly improved with dose-dependent ($P < 0.05$). HSYA could improve anticoagulant injury induced subcutaneous scar via regulation TLR4/NF-κB(p65).

Keywords: HSYA; Collage I; Collage III; TLR4; NF-κB(p65).

Practical Application: HSYA could improve scars via TLR4 pathway in clinical in future.

1 Introduction

Hypertrophic scar (HS) is a refractory skin disease caused by severe physical injury or other inflammations, mostly accompanied by appearance damage, redness, swelling, itching and pain, which always affect the physical and mental health of patients. At present, the pathogenesis of HS is not yet completely clear, but its pathological process is mainly manifested by the overexpression of multiple fibrosis-related factors and increased synthesis or decreased degradation of extracellular matrices (ECMs) such as collagen (Berman et al., 2017). Therefore, the treatment of HS is difficult and lacks effective means. Although some surgical procedures, in vitro physiotherapy or glucocorticoid and anti-tumor drug therapy can inhibit HS, the efficacy is not very definite (Lee et al., 2001; Gauglitz et al., 2011; Wu et al., 2019). Clinical practice has found that the subcutaneous scars of patients after anticoagulant injection are severe, which seriously affects subsequent treatment and infusion of the patients. Natural drug extracts have unique advantages in the treatment of HS. The current studies have shown that natural drug extracts can inhibit HS, and the mechanism is mainly related to increasing antioxidant capacity, inhibiting the expressions of transforming growth factor-β1 (TGF-β1) and Smad and reducing the content of collagen fibers at HS (Si et al., 2018; Mathangi Ramakrishnan et al., 2015; Park et al., 2008; Hsu et al., 2010).

Safflower is the dry tubular flower of Carthamus tinctorius L., which is a notated plant medicine for improving blood circulation and dispersing stasis, and commonly used in the treatment of cardio-cerebrovascular and thrombotic diseases (Yuan et al., 2014; Nie et al., 2012). Hydroxysafflor yellow A (HSYA) is the most important active component in safflower. Pharmacological studies have shown that HSYA has significant effects of cardioprotection and cerebrovascular protection, which are related to vasodilation (Liu et al., 2008), antioxidant damage (Fan et al., 2011) and anti-inflammation (Wu et al., 2012). HSYA can inhibit the expressions of lipopolysaccharide and hypoxia-induced inflammatory factors, and suppress the activity of NF-κB, thus playing an anti-inflammatory role (Li et al., 2013; Liu et al., 2014; Jiang et al., 2014). Whether HSYA can improve the subcutaneous ecchymoses caused by anticoagulant injection has not been studied. On this basis, in this study, the effect of HSYA on the subcutaneous ecchymoses of New Zealand rabbits after anticoagulant injection was observed, and the expressions of related proteins and genes were detected, so as to explore the mechanism of HSYA.

2 Materials and methods

2.1 Materials

Male New Zealand rabbits were provided by LAIFU farm, Pukou District, Nanjing [Laboratory animal production license: SCXK (Su) 2019-0005]. TNF-α kit (Sino-American Biotechnology Co., Ltd., CSB-E06998Rb), IL-1β kit (Thermo, ER5RB), NF-κB (p65), TLR4, α-SMA and Collage III antibodies (Abcam, USA), first-strand cDNA synthesis kit (TaKaRa, Japan, RR036B), and One Step TB Green™ PrimeScript™ RT-PCR Kit II (SYBR Green) (TaKaRa, Japan, RR086B) were used in this study. HSYA standards

Received 14 Nov, 2021
Accepted 04 Jan, 2022
[1]*Department of Vascular and Interventional Radiology, Nanjing First Hospital, Nanjing Medical University, Nanjing, China*
*Corresponding author: hexunj@163.com; gujianping@tom.com

were purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China.

2.2 Animal grouping and administration

Male New Zealand rabbits weighing 2.0-2.5 kg were randomly divided into normal control group (NC), anticoagulant group, anticoagulant + HSYA-L group, anticoagulant + HSYA-M group and anticoagulant + HSYA-H group, with 4 rabbits per group. In the injection groups, the periumbilicus of experimental animals were divided into 6 areas, with an interval of 1 cm. Among them, 3 areas were injected with anticoagulant using imported needles, while the other 3 areas using domestic needles, once every 12 h, a total of 18 injections. All administration groups were intragastrically administrated with HSYA when anticoagulant injection, once a d for 18 d. The anticoagulant + HSYA-L group, anticoagulant + HSYA-M group and anticoagulant + HSYA-H group were intragastrically administrated with 10 mg/kg, 20 mg/kg and 40 mg/kg HSYA, respectively. The anticoagulant group was intragastrically administrated with a corresponding volume of normal saline. The animals in each group were treated with corresponding treatments for 7 d, and then sacrificed for sampling. Our animal experiments were approved by Ethics committee of Nanjing First Hospital. The work described followed by carried out in accordance with the ARRIVE Guidelines for reporting in vivo animal experiments

2.3 Scar cell model and treatment of each group

Using 5 ng/ml TGF-β1(Sigma, USA) to stimulate HKE cell line (ATCC, USA) for 48 h as scar cell model (Shook et al., 2018). NC:HKF cell were maintained in DMEM containing 10% fetal bovine serum and 1% streptomycin/penicillin (all from Thermo Fisher Scientific, Inc.) at 37˚C in a humidified 5% CO_2 incubator. Model: HKF cell were stimulated by 5ng/ml TGF-β1 for 48 h before following experiment; DMSO: HKF cell were stimulated by 5ng/mL TGF-β1 and DMSO for 48 h before following experiment; HSYA-L: HKF cell were stimulated by 5ng/mL TGF-β1 and 40mg/L HSYA which were dissolution DMSO for 48 h before following experiment; HSYA-M: HKF cell were stimulated by 5ng/ml TGF-β1 and 80 mg/L HSYA which were dissolution DMSO for 48 h before following experiment; HSYA-H: HKF cell were stimulated by 5ng/ml TGF-β1 and 160 mg/L HSYA which were dissolution DMSO for 48 h before following experiment; Model+si-NC: HKF were transfected with si-NC(F: 5'- UUCUCCGAACGUGUCACGUTT-3'; R: 5'-ACGUGACACGUUCGGAGAATT-3') and stimulated by 5ng/ml TGF-β1 and 160mg/L HSYA which were dissolution DMSO for 48 h before following experiment; HSYA: HKF were stimulated by 5ng/ml TGF-β1 and 160mg/L HSYA which were dissolution DMSO for 48 h before following experiment; si-TLR4: HKF were transfected with si-TLR4(F: 5'- CUUCAUAAGCUGACUUUAATT-3'; R: 5'- UUAAAGUCAGCUUAUGAAGTT-3') and stimulated by 5ng/ml TGF-β1 for 48 h before following experiment; si-TLR4+HSYA: HKF were transfected with si-TLR4(F: 5'- CUUCAUAAGCUGACUUUAATT-3'; R: 5'-UUAAAGUCAGCUUAUGAAGTT-3') and stimulated by 5ng/ml TGF-β1 and 160mg/L HSYA which were dissolution DMSO for 48 h before following experiment.

2.4 Elisa assay

The concentrations of TGF-β1 and IL-1β in tissue samples were detected according to the instructions of the kit.

The cells of difference groups were treated by difference methods for 48 h, Collecting culture medium, Centrifuge the supernatant (8000 × g, 3 min, 4 °C), measuring hydroxyproline (Hyp) concentrations by Hyp kit (cat no. KGT562, KeyGen, Nanjing, China) depending on supplier instructions.

2.5 Histopathological examination

After the experiment, the animals were sacrificed by injecting air into the heart. Sampling was carried out from the central and surrounding tissues of rabbit scars, with the size of about 1.5 cm × 1.5 cm × 0.3 cm. After fixation with 4% paraformaldehyde for 24 h, the sections were embedded in paraffin, with the thickness of 5 μm, for HE staining and Masson staining, respectively. Under an orthoplan microscope, the sections were observed and photographed. The main observation indexes included: 1) the morphology of scar tissues; and 2) collagen morphology and arrangement. Three fields of vision (200 times magnification) were randomly selected from each section, and the thickness of the dermis was measured using Olympus airborne digital microscopic image analysis software.

2.6 Detection of related proteins in scar tissues using immunohistochemistry (IHC)

Using the two-step SP method, after conventional dehydration and sectioning, the tissues were placed in an oven at 60 °C for 15 min for tight adhesion. Then, the sections were dripped with retrieval buffer for antigen retrieval twice in a high-pressure cooker, with 15 min/time. After cooling naturally to room temperature, the sections were washed with phosphate buffer solution (PBS) 3 times for 5 min each time. With 3% H_2O_2 dripped, the sections were incubated at 37 °C for 20 min, and then washed with PBS 3 times for 5 min each time. Subsequently, cell membrane perforator (3% TritonX-100 solution) was added, followed by washing with PBS, sealing with goat serum for 30 min, incubation with primary antibody in a wet box overnight at 4 °C, and then washing with PBS. Afterwards, secondary antibody (1: 200) was added for incubation at 37 °C for 1 h, followed by washing with 3 times. Under a microscope, development was performed using the DAB method, and staining was stopped by PBS rinsing. Then, the sections were dehydrated in gradient ethanol, cleared in xylene and sealed with neutral gum. Sections were randomly selected in each group and photographed under a microscope (100×). The results were quantitatively analyzed using Image J, and the average absorbance (AA) was determined for statistical analysis.

2.7 Real-time qPCR

mRNA was extracted from tissues using the Trizol method, followed by cDNA transcription and PCR detection. The sequences of primers for RT-PCR are shown in Table 1. The amplification conditions of PCR were as follows: pre-denaturation at 94 °C for 13 min, then 32 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, and finally elongation at 72 °C for 8 min. With

Table 1. The primer sequence.

Gene Name	F:(5'-3')	R:(5'-3')
Collage I	CTGGCCCTAATGGATTCGCT	TCACCCTTAGGTCCCTTGGT
Collage III	TTCAAATGGCTCCCCTGGAC	CCGCCAGGACTACCATTGTT
TLR4	TCCAGAGCCGTTGGTGTATCTT	ACAATTCGACCTGCTGCCTCA
NF-κB(p65)	TCATCTTCCCGGCAGAGCCAG	GTGGGTCTTGGTGGTAGCTGT
GAPDH	TCTGACTTCAACAGGGACACC	CTGTTGGTGTAGCCAAATTCGT

GAPDH as internal reference, the expressions of related genes were calculated using the $2^{-\Delta\Delta CT}$ method.

2.8 Western blot (WB)

The scar tissues of the rabbits were washed with normal saline, weighed, crushed with a mortar, mixed with RIBA tissue lysate and PMSF, homogenized and then incubated on ice for 30 min. After centrifugation at 10,000 r·min-1 under 4 °C for 20 min, the supernatant was collected. The protein concentration was quantified using a BCA protein determination kit. Total protein (30 μg) was collected from each sample, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the separating gel concentration of 12%. Subsequently, the proteins were transmembraned to methanol-treated PVDF for 90 min. The transferred PVDF membranes were sealed in T-BST sealing solution containing 5% bovine serum albumin with shaking for 1 h at room temperature, and then added with primary antibody for incubation at 4°C overnight. Afterwards, the PVDF membranes were collected, washed with TBST 3 times for 15 min each time, added with secondary antibody (1: 2,000) for incubation at room temperature for 1 h, and then washed with TBST 3 times for 5 min each time. Finally, electrogenerated chemiluminescence (ECL) reaction solution was added for development in a gel imaging system. The gray value of the target protein was determined using Image J, and the relative expression of the protein was expressed with the obtained ratio of GAPDH to the target protein.

2.9 Cellular immunofluorescence

The samples were cells from difference groups cultured on thin glass sheets. Washing every samples by 0.01M PBS for 5 min every times at 4 °C before incubation by antibodies. Fixing samples by 4% paraformaldehyde 15 min at 4 °C. Blocking was performed by immersing samples in 3% BSA (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature. Incubating samples by primary antibodies overnight at 4 °C, using PBS to wash and incubated by secondary antibody (1 h, room temperature). Every image was captured at a single confocal plane. In each group of samples, three fields of view were chosen randomly, all cells in each field were observed and quantified. The primary antibody Collage I (cat no. ab260043), Collage III (cat no. ab224532), TLR4 (cat no. ab30667) and p-NF-κB(p65) (cat no. 32536) were from Abcam.

2.10 Statistical analysis

The statistical analysis of the data was conducted using SPSS 19.0. All measurement data were expressed as mean ± standard deviation (SD). Inter-group comparison was carried out using one-way analysis of variance (ANOVA), and pairwise comparison between the treatment group and the model group was performed using the LSD-t test. $P < 0.05$ was considered as statistically significant.

3 Results

3.1 TGF-β1 and IL-1β concentrations and expressions

Compared with the NC group, the concentrations of TGF-β1 and IL-1β in the skin tissue in the anticoagulant group were significantly higher ($P < 0.001$, respectively, Figure 1). After intervention with HYSA, the concentrations of TGF-β1 and IL-1β reduced significantly compared with the anticoagulant group ($P < 0.001$, respectively, Figure 1), presenting an obvious dose-effect relationship.

3.2 HE staining results

In the NC group, the structure of the skin tissue was integral and wavy, collagens arranged tightly and evenly, and the fibers were fine. In the anticoagulant group, not only the epidermis was obviously thickened, but also the thickness of the dermis was increased. Under the microscope, a large number of collagen fibers with different thickness and irregular arrangement were observed. Compared with the anticoagulant group, the thickness of the epidermis and dermis of New Zealand rabbits treated with HSYA reduced, and the arrangement of collagens was more regular, showing a certain dose-effect relationship (Figure 2).

3.3 Masson staining results

Curvilinear fiber (CVFs) in the anticoagulant group increased significantly compared with the NC group ($P < 0.001$, Figure 3), arranged irregularly in swirls or nodules. Compared with the anticoagulant group, CVFs after intragastric administration with HSYA decreased significantly and presented a significant dose-effect relationship ($P < 0.05$, respectively, Figure 3). The arrangement was regular, gradually presenting a cord-like shape.

3.4 Detection of collage I and collage III protein expressions by IHC

IHC staining showed that compared with the NC group, the protein expressions of collagen I and collagen III in the anticoagulant group increased significantly ($P < 0.001$, respectively, Figure 4A & 4B). After intervention with HYSA, the protein expressions of collagen I and collagen III were significantly inhibited compared with the anticoagulant group ($P < 0.05$, respectively, Figure 4A & 4B), and presented a certain dose-effect relationship.

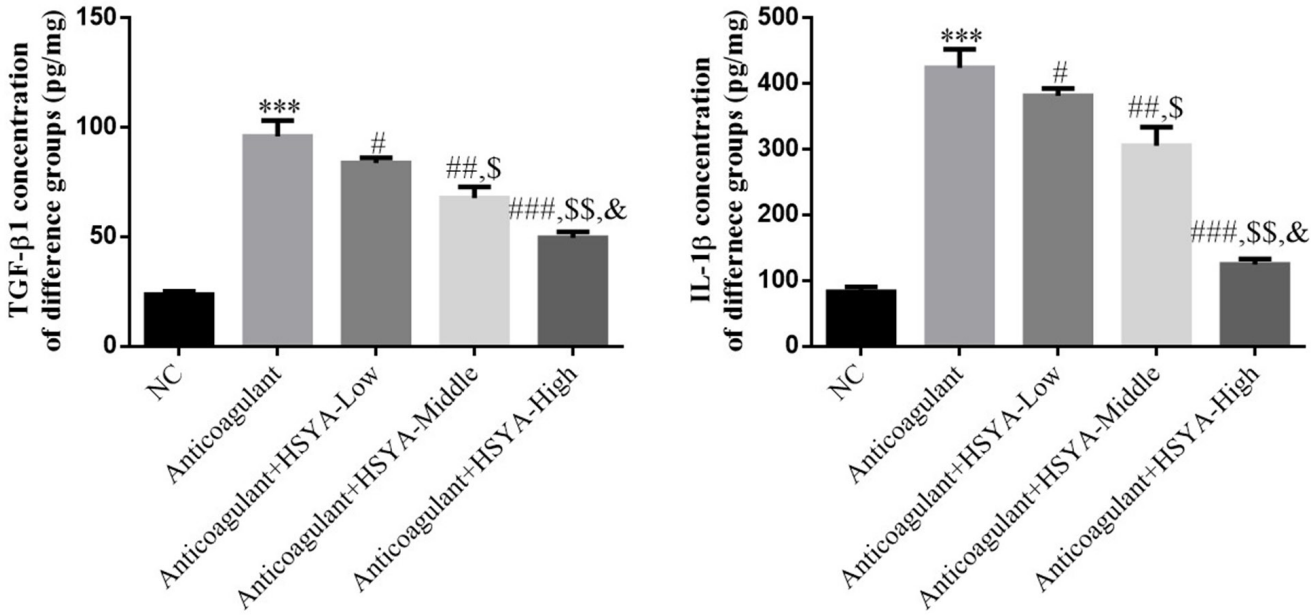

Figure 1. TGF-β1 and IL-1β concentration of difference groups (pg/mg). ***: P < 0.001, compared with NC group; #: P < 0.05, ##: P < 0.01, ###: P < 0.001, compared with Anticoagulant; $: P < 0.05, $$: P < 0.01, compared with Anticoagulant+HSYA-Low; &: P < 0.05, compared with Anticoagulant+HSYA-Middle.

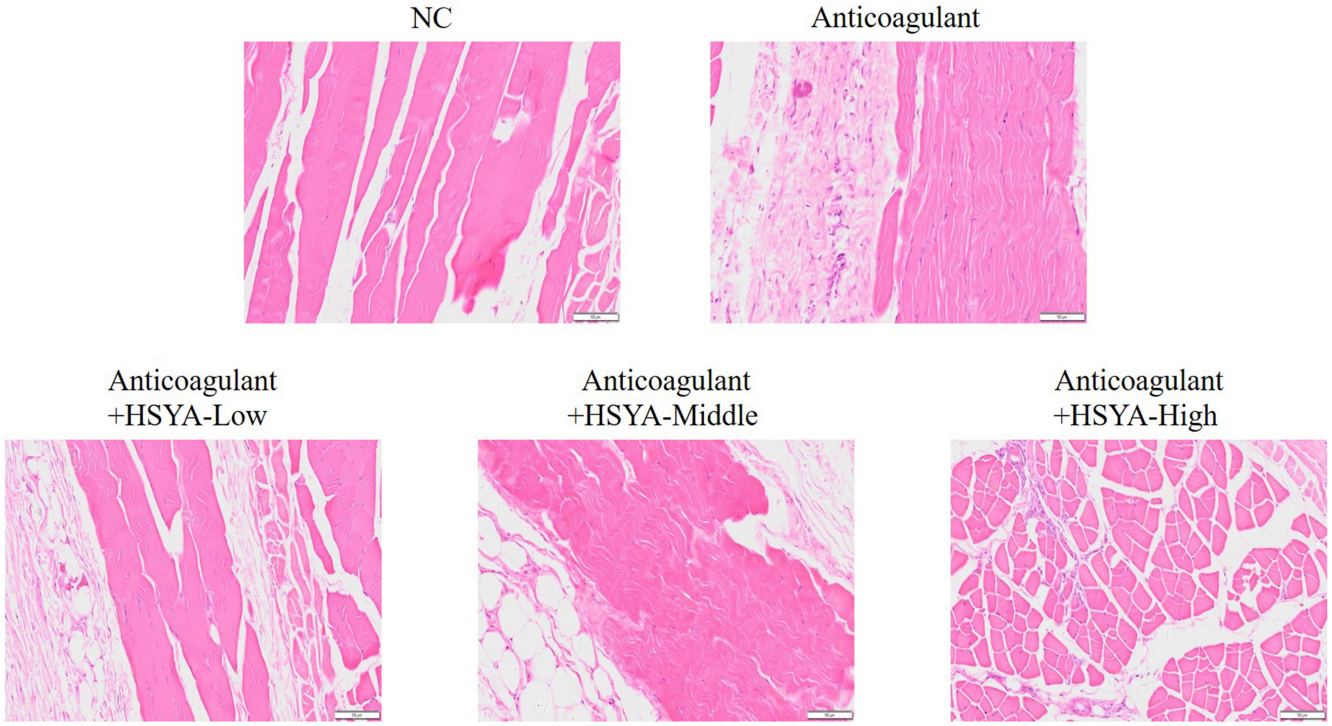

Figure 2. Pathology by HE staining (100×).

3.5 Detection of TLR4 and NF-κB (p65) protein expressions by IHC

IHC staining demonstrated that compared with the NC group, the protein expressions of TLR4 and NF-κB (p65) in the anticoagulant group increased significantly ($P < 0.001$, respectively, Figure 5A & 5B). After intervention with HYSA, the protein expressions of TLR4 and NF-κB (p65) were significantly inhibited compared with the anticoagulant group ($P < 0.05$, respectively, Figure 5A & 5B), and presented a certain dose-effect relationship.

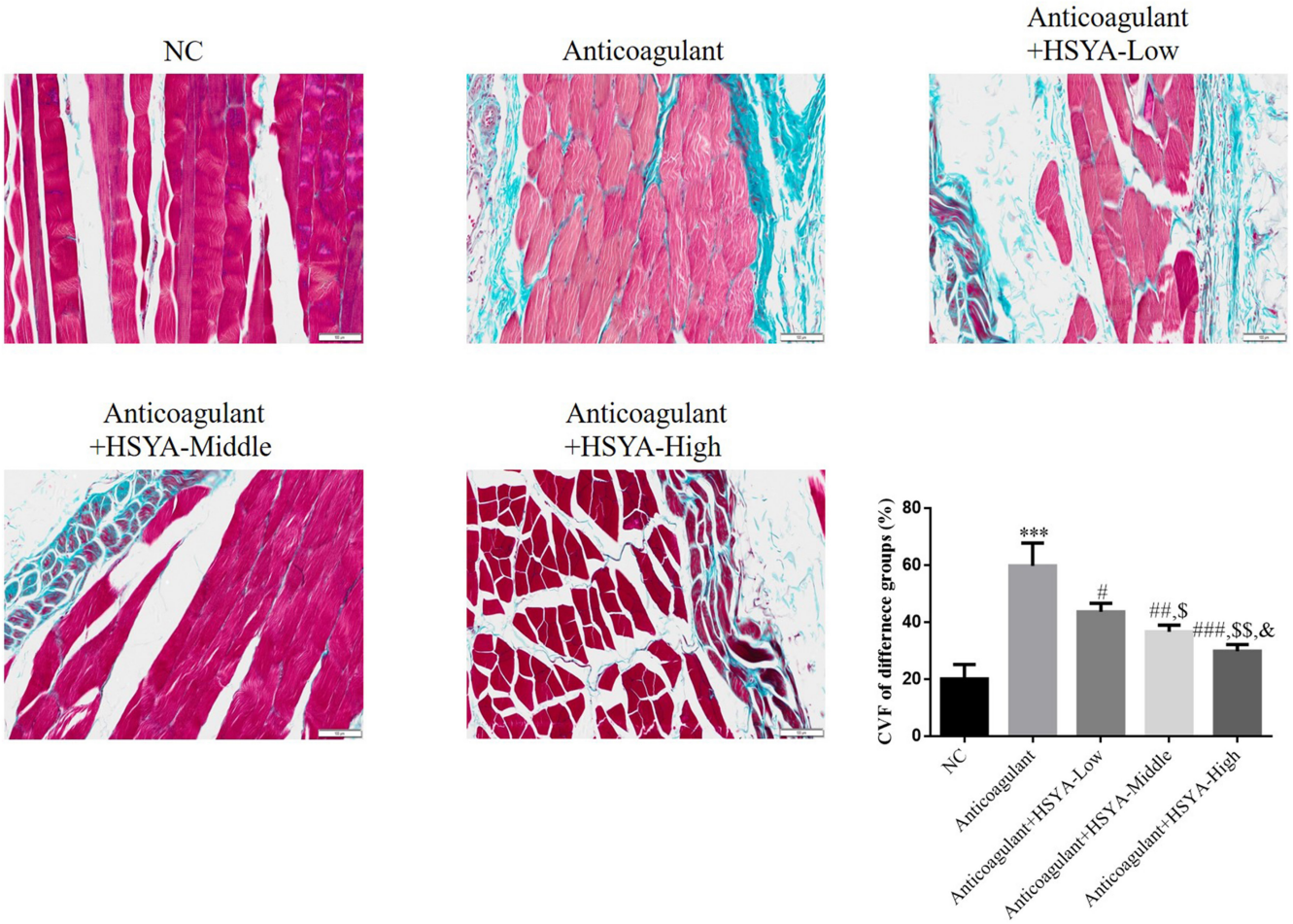

Figure 3. CVF of difference groups by Masson staining (100×). ***: P < 0.001, compared with NC group; #: P < 0.05, ##: P < 0.01, ###: P < 0.001, compared with Anticoagulant; $: P < 0.05, $$: P < 0.01, compared with Anticoagulant+HSYA-Low; &: P < 0.05, compared with Anticoagulant+HSYA-Middle.

3.6 Detection of related gene expression

RT-qPCR results revealed that compared with the NC group, the expressions of collage I, collage III, TLR4 and NF-κB (p65) genes in the anticoagulant group increased significantly (P < 0.001, respectively, Figure 6A & 6B). After intervention with HYSA, the expressions of collage I, collage III, TLR4 and NF-κB (p65) genes were significantly inhibited compared with the anticoagulant group (P < 0.05, respectively, Figure 6A & 6B), and presented a certain dose-effect relationship.

3.7 Detection of related protein expression by WB

WB results suggested that compared with the NC group, the expressions of collage I, collage III, TLR4 and NF-κB (p65) proteins increased significantly in the anticoagulant group (P < 0.001, respectively, Figure 7A & 7B). After intervening with HYSA, the expressions of collage I, collage III, TLR4 and NF-κB (p65) proteins were inhibited significantly compared with the anticoagulant group (P < 0.05, respectively, Figure 7A & 7B), and presented a certain dose-effect relationship.

3.8 HSYA affected Hyp concentration in scars cell model

Compared with NC group, Hyp level of Model and DMSO groups were significantly up-regulated (P<0.001, respectively, Figure 8); based on scars cell model treatment, with difference HSYA concentrations supplement, HSYA treated groups were significantly depressed in Hyp levels (P < 0.05, respectively, Figure 8).

3.9 HSYA affected Collage I, Collage III, TLR4 and NF-κB(p65) gene expressions in scars cell model

Compared with NC group, Collage I, Collage III, TLR4 and NF-κB(p65) mRNA levels of Model and DMSO groups were significantly up-regulated (P<0.001, respectively, Figure 9); based on scars cell model treatment, with difference HSYA concentrations supplement, HSYA treated groups were significantly depressed in Collage I, Collage III, TLR4 and NF-κB(p65) mRNA levels (P<0.05, respectively, Figure 9).

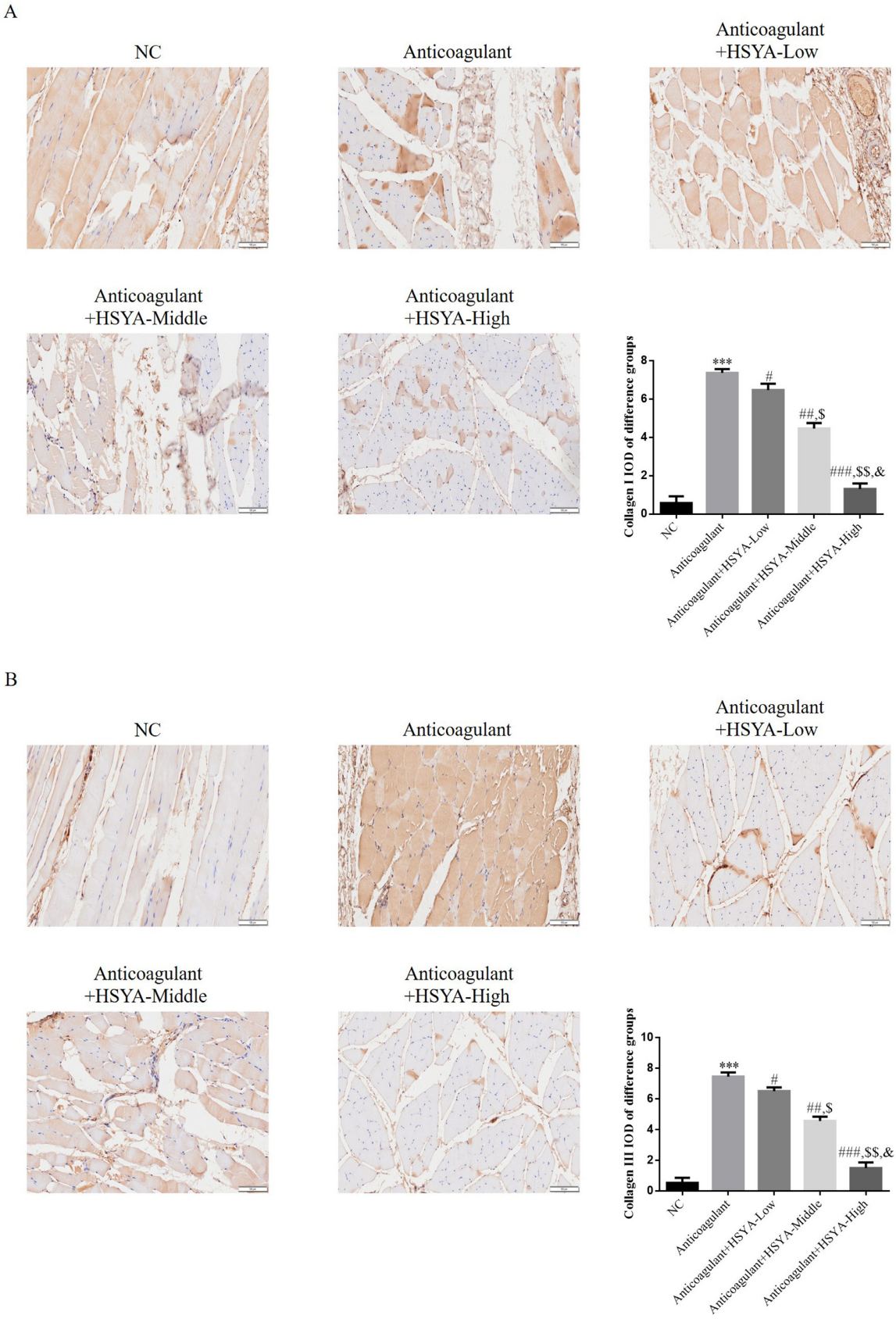

Figure 4. Collage I and Collage III proteins expression by IHC assay (100×). (A) Collage I protein expression by IHC staining; (B) Collage III protein expression by IHC staining ***: $P < 0.001$, compared with NC group; #: $P < 0.05$, ##: $P < 0.01$, ###: $P < 0.001$, compared with Anticoagulant; $: $P < 0.05$, $$: $P < 0.01$, compared with Anticoagulant+HSYA-Low; &: $P < 0.05$, compared with Anticoagulant+HSYA-Middle.

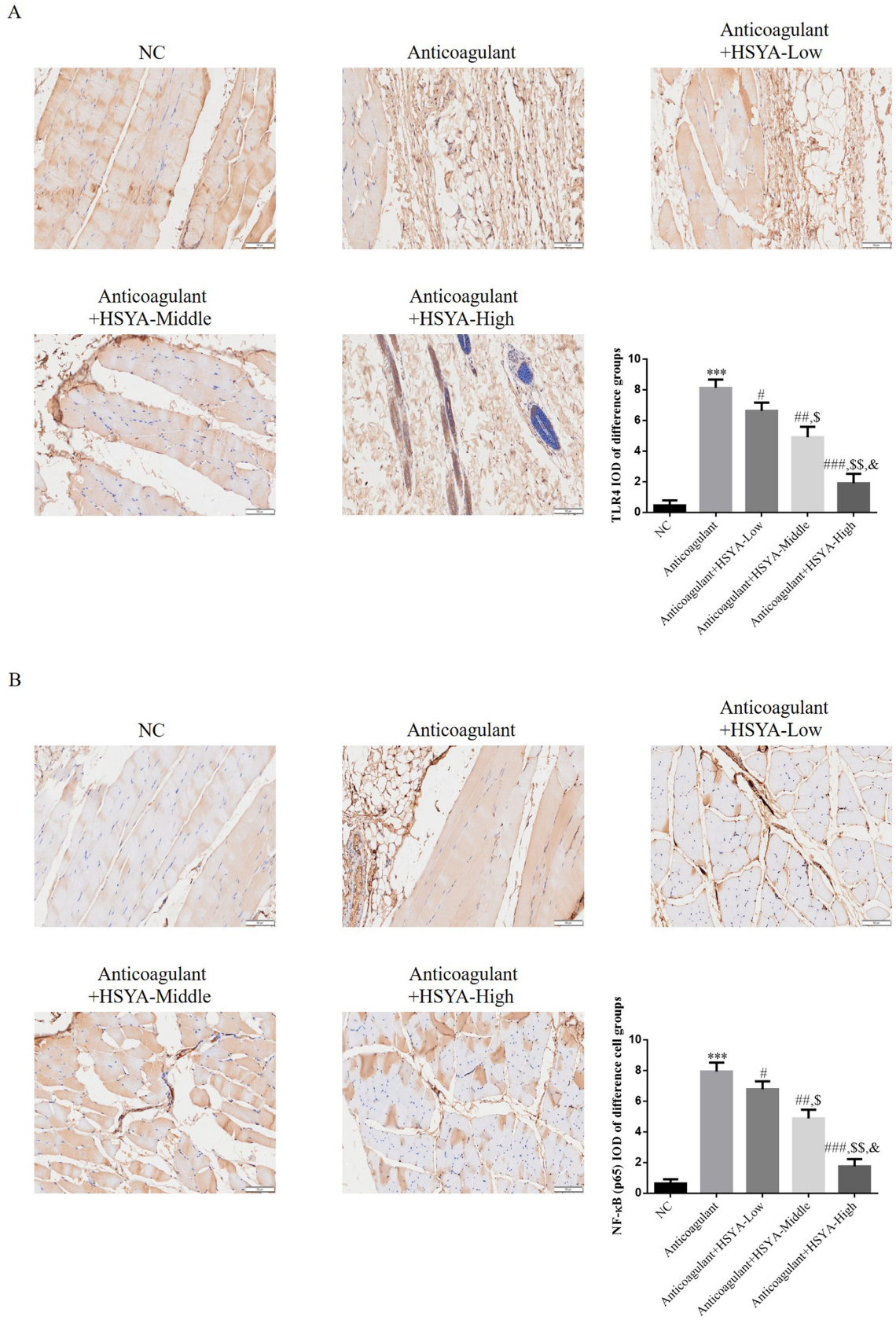

Figure 5. TLR4 and NF-κB(p65) protein expression by IHC assay. A. TLR4 protein expression by IHC staining; B. NF-κB(p65) protein express by IHC staining ***: $P < 0.001$, compared with NC group; #: $P < 0.05$, ##: $P < 0.01$, ###: $P < 0.001$, compared with Anticoagulant; $: $P < 0.05$, $$: $P < 0.01$, compared with Anticoagulant+HSYA-Low; &: $P < 0.05$, compared with Anticoagulant+HSYA-Middle.

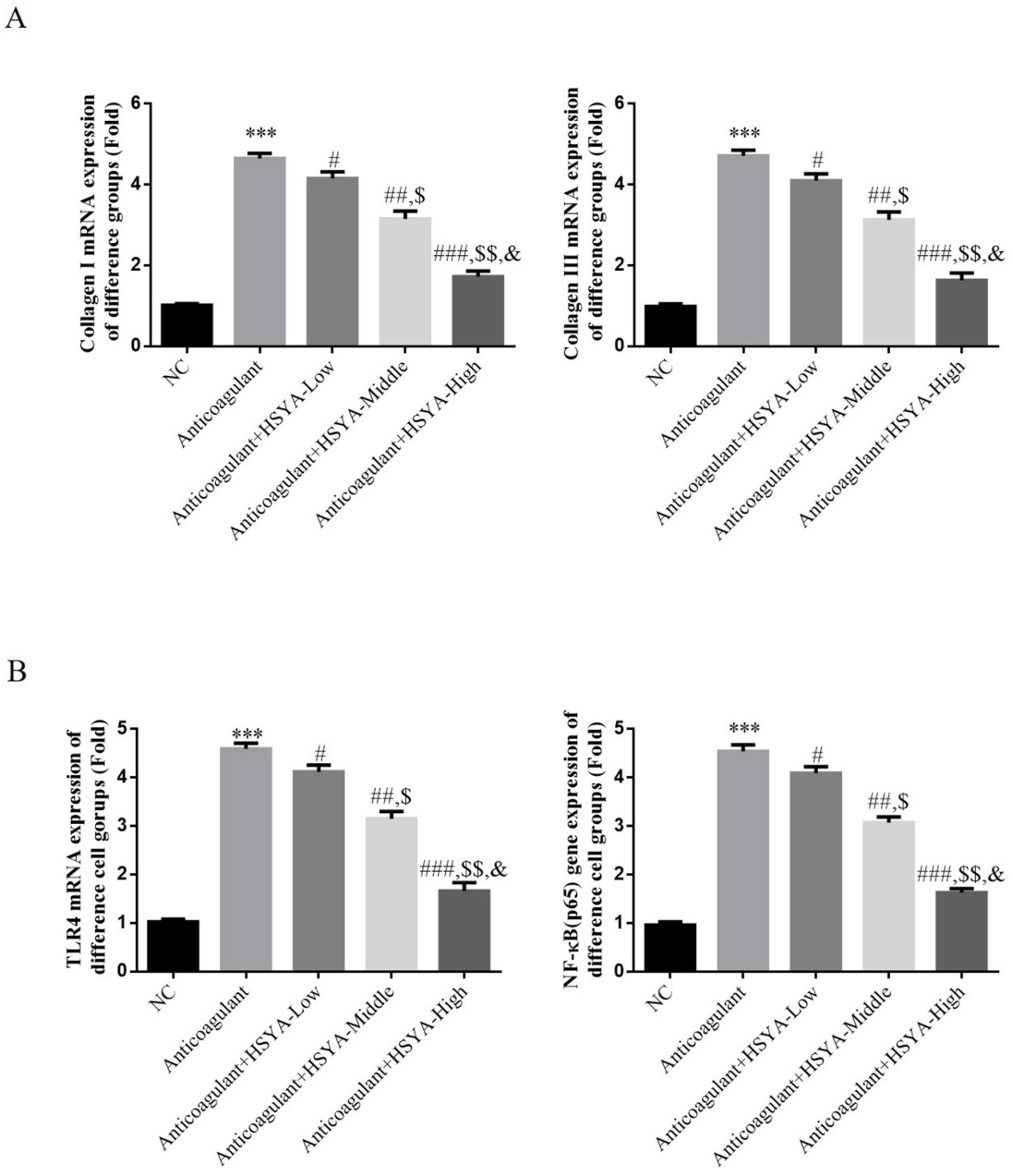

Figure 6. Relative mRNA expression by RT-qPCR assay. A. Collage I and Collage III gene expression by RT-qPCR assay ***: P < 0.001, compared with NC group; #: P < 0.05, ##: P < 0.01, ###: P < 0.001, compared with Anticoagulant; $: P < 0.05, $$: P < 0.01, compared with Anticoagulant+HSYA-Low; &: P < 0.05, compared with Anticoagulant+HSYA-Middle; B. TLR4 and NF-κB(p65) gene expression by RT-qPCR assay ***: P < 0.001, compared with NC group; #: P < 0.05, ##: P < 0.01, ###: P < 0.001, compared with Anticoagulant; $: P < 0.05, $$: P < 0.01, compared with Anticoagulant+HSYA-Low; &: P < 0.05, compared with Anticoagulant+HSYA-Middle.

3.10 HSYA affected Collage I, Collage III, TLR4, NF-κB(p65) and p- NF-κB(p65) proteins expressions in scars cell model by WB assay

By WB assay, Compared with NC group, Collage I, Collage III, TLR4 and p-NF-κB(p65) proteins levels of Model and DMSO groups were significantly up-regulated (P<0.001, respectively, Figure 10); based on scars cell model treatment, with difference HSYA concentrations supplement, HSYA treated groups were significantly depressed in Collage I, Collage III, TLR4 and p-NF-κB(p65) proteins levels (P<0.05, respectively, Figure 10).

3.11 HSYA affected Collage I, Collage III and TLR4 proteins expressions in scars cell model by cellular immunofluorescence

By cellular immunofluorescence, Compared with NC group, Collage I, Collage III and TLR4 proteins levels of Model

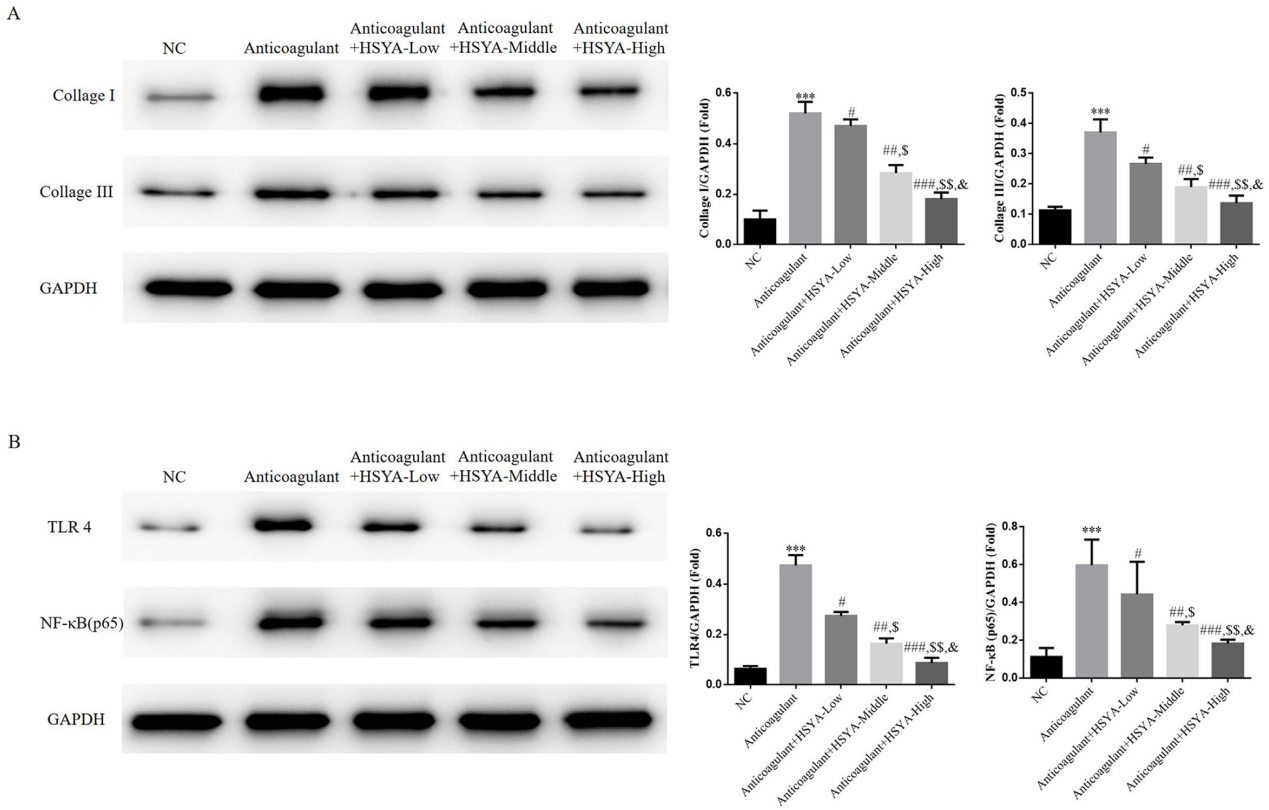

Figure 7. Relative proteins expression by WB assay. (A) Collage I and Collage III proteins expression by WB assay ***: P < 0.001, compared with NC group; #: P < 0.05, ##: P < 0.01, ###: P < 0.001, compared with Anticoagulant; $: P < 0.05, $$: P < 0.01, compared with Anticoagulant+HSYA-Low; &: P < 0.05, compared with Anticoagulant+HSYA-Middle; (B) TLR4 and NF-κB(p65) protein expression by WB assay ***: P < 0.001, compared with NC group; #: P < 0.05, ##: P < 0.01, ###: P < 0.001, compared with Anticoagulant; $: P < 0.05, $$: P < 0.01, compared with Anticoagulant+HSYA-Low; &: P < 0.05, compared with Anticoagulant+HSYA-Middle.

and DMSO groups were significantly up-regulated (P < 0.001, respectively, Figures 11, 12, 13); based on scars cell model treatment, with difference HSYA concentrations supplement, HSYA treated groups were significantly depressed in Collage I, Collage III and TLR4 proteins levels (P < 0.05, respectively, Figures 11, 12, 13).

3.12 HSYA affected p-NF-κB(p65) protein nuclear volume in scars cell model by cellular immunofluorescence

By cellular immunofluorescence, Compared with NC group, p-NF-κB(p65) proteins nuclear volume of Model and DMSO groups were significantly up-regulated (P < 0.001, respectively, Figure 14); based on scars cell model treatment, with difference HSYA concentrations supplement, HSYA treated groups were significantly depressed in p-NF-κB(p65) proteins nuclear volume (P < 0.05, respectively, Figure 14).

3.13 TLR4's effect in HSYA's treatment to Hyp concentration in scars cell model

Compared with NC group, Hyp level of Model and Model+si-NC groups were significantly up-regulated (P<0.001, respectively, Figure 15); based on scars cell model treatment, with HSYA

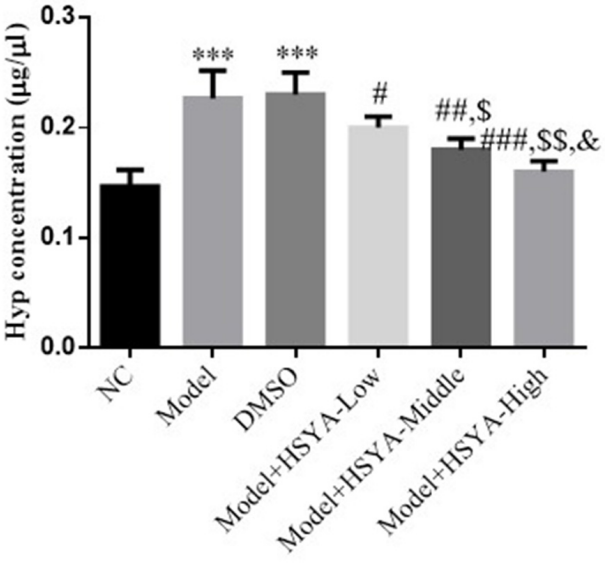

Figure 8. Hyp concentration in difference HYSA treated groups. ***: P < 0.001, compared with NC group; #: P < 0.05; ##: P < 0.01, ###: P < 0.001, compared with Model; $: P < 0.05; $$: P < 0.01, compared with Model+HSYA-L; &: P < 0.05, compared with Model+HSYA-M.

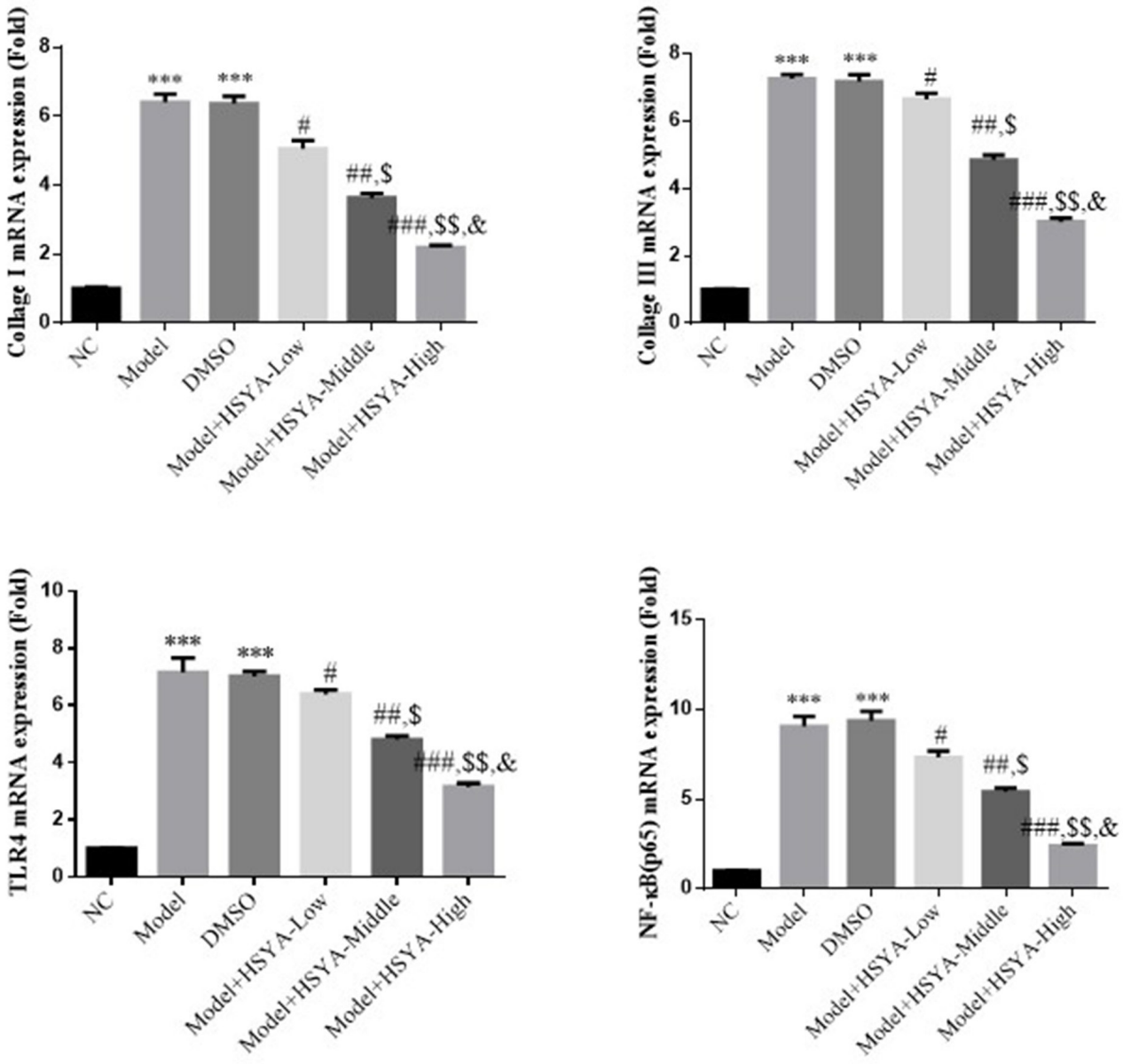

Figure 9. Relative mRNA expressions in difference groups by RT-qPCR assay. ***: P < 0.001, compared with NC group; #: P < 0.05;##: P < 0.01, ###: P < 0.001, compared with Model; $: P < 0.05; $$: P < 0.01, compared with Model+HSYA-L; &: P < 0.05, compared with Model+HSYA-M.

supplement or/ and si-TLR4 which knockdown TLR4 expression transfection, HSYA, Model+si-TLR4 and Model+si-TLR4+HSYA groups were significantly depressed in Hyp levels (P < 0.001, respectively, Figure 15).

3.14 TLR4's effect in HSYA's treatment to Collage I, Collage III, TLR4 and NF-κB(p65) mRNA expression in scars cell model

By RT-qPCR assay, Compared with NC group, Collage I, Collage III, TLR4 and NF-κB(p65) mRNA levels of Model and Model+si-NC groups were significantly up-regulated (P < 0.001, respectively, Figure 16); based on scars cell model treatment, with HSYA supplement or/ and si-TLR4 which knockdown TLR4 expression transfection, HSYA, Model+si-TLR4 and Model+si-TLR4+HSYA groups were significantly depressed in Collage I, Collage III, TLR4 and NF-κB(p65) mRNA levels (P < 0.001, respectively, Figure 16).

3.15 TLR4's effect in HSYA's treatment to Collage I, Collage III, TLR4, NF-κB(p65) and p- NF-κB(p65) proteins expression in scars cell model by WB assay

By WB assay, Compared with NC group, Collage I, Collage III, TLR4 and p-NF-κB(p65) proteins levels of Model and Model+si-NC groups were significantly up-regulated (P < 0.001, respectively, Figure 17); based on scars cell model treatment, with HSYA supplement or/ and si-TLR4 which knockdown TLR4 expression transfection, HSYA, Model+si-TLR4 and Model+si-TLR4+HSYA groups were significantly depressed in Collage I, Collage III, TLR4 and p-NF-κB(p65) proteins levels (P < 0.001, respectively, Figure 17).

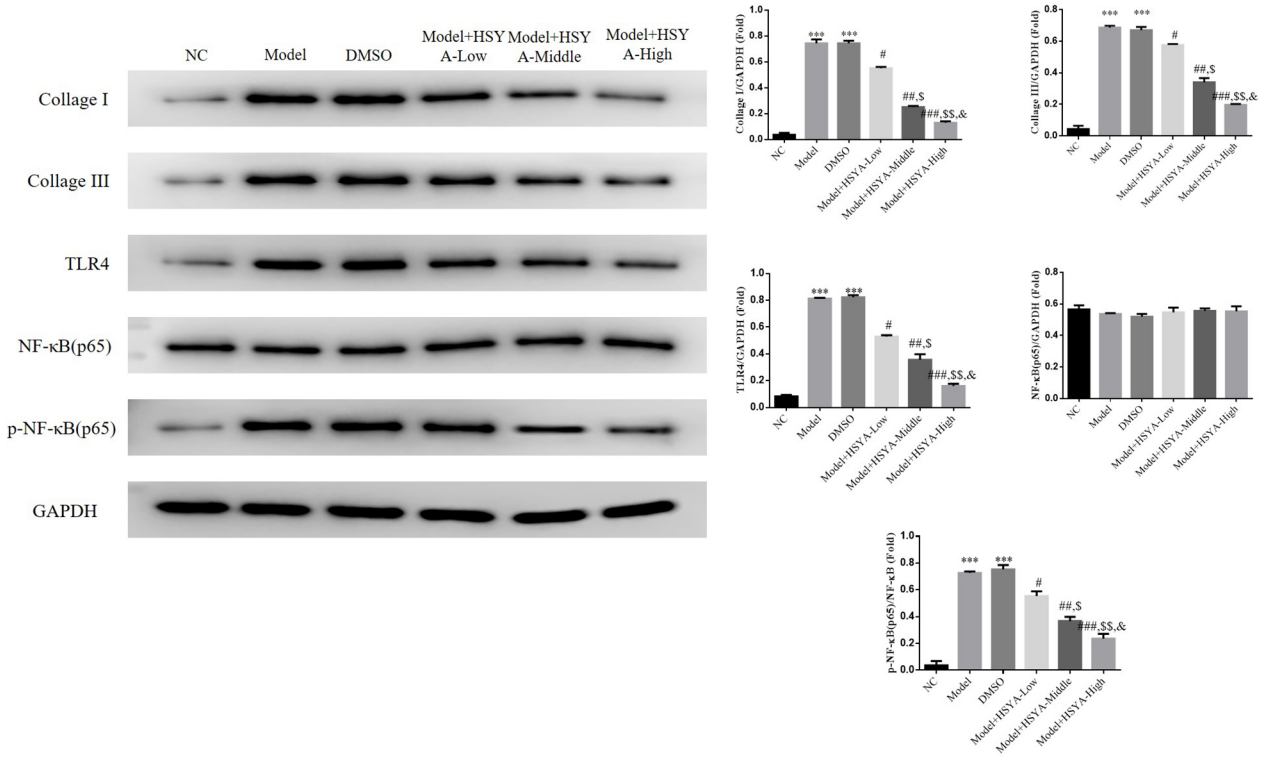

Figure 10. Relative proteins expressions in difference groups by WB assay. ***: P < 0.001, compared with NC group; #: P < 0.05; ##: P < 0.01, ###: P < 0.001, compared with Model; $: P < 0.05; $$: P < 0.01, compared with Model+HSYA-L; &: P < 0.05, compared with Model+HSYA-M.

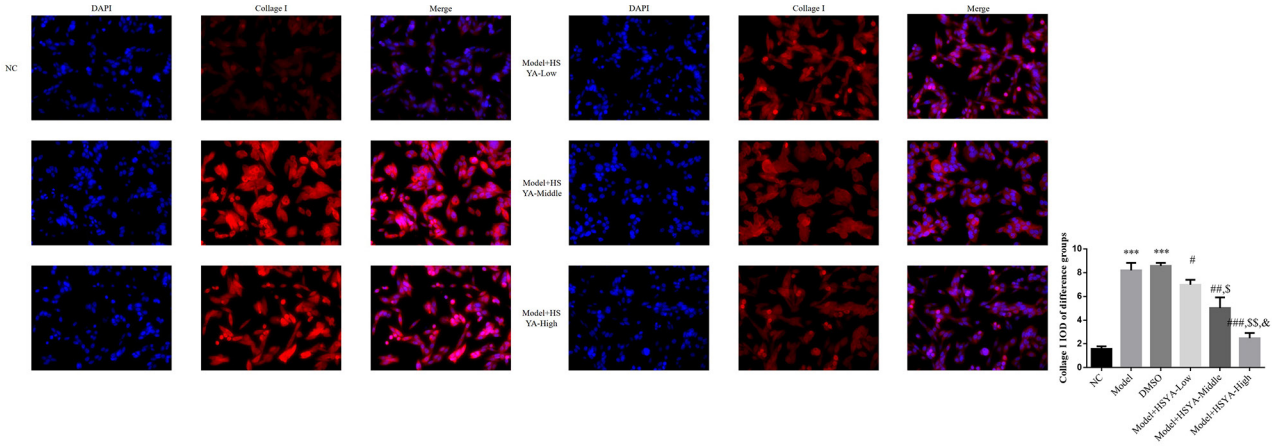

Figure 11. Collage I protein expression (×200). ***: P < 0.001, compared with NC group; #: P < 0.05;##: P < 0.01, ###: P < 0.001, compared with Model; $: P < 0.05; $$: P < 0.01, compared with Model+HSYA-L; &: P < 0.05, compared with Model+HSYA-M.

3.16 TLR4's effect in HSYA's treatment to Collage I, Collage III and TLR4 proteins expression in scars cell model by cellular immunofluorescence

By cellular immunofluorescence, Compared with NC group, Collage I, Collage III and TLR4 proteins levels of Model and Model+si-NC groups were significantly up-regulated (P < 0.001, respectively, Figures 18, 19, 20); based on scars cell model treatment, with HSYA supplement or/ and si-TLR4 which knockdown TLR4 expression transfection, HSYA, Model+si-TLR4 and Model+si-TLR4+HSYA groups were significantly depressed in Collage I, Collage III and TLR4 proteins levels (P < 0.001, respectively, Figures 18, 19, 20).

3.17 TLR4's effect in HSYA's treatment to p-NF-κB(p65) proteins nuclear volume in scars cell model by cellular immunofluorescence

By cellular immunofluorescence, Compared with NC group, p-NF-κB(p65) proteins nuclear volume of Model and Model+si-

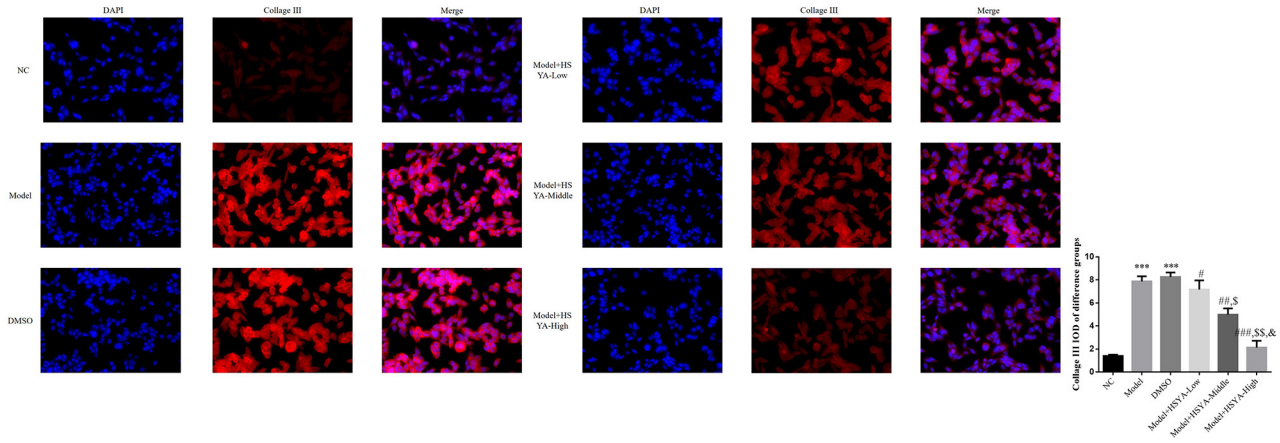

Figure 12. Collage III protein expression (×200). ***: P < 0.001, compared with NC group; #: P < 0.05;##: P < 0.01, ###: P < 0.001, compared with Model; $: P < 0.05; $$: P < 0.01, compared with Model+HSYA-L; &: P < 0.05, compared with Model+HSYA-M.

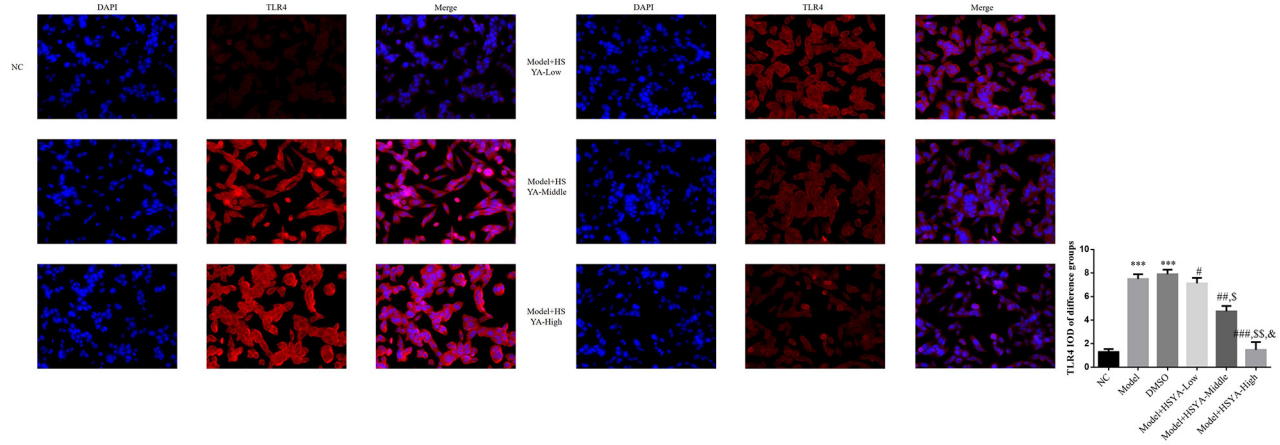

Figure 13. TLR4 protein expression (×200). ***: P < 0.001, compared with NC group; #: P < 0.05;##: P < 0.01, ###: P < 0.001, compared with Model; $: P < 0.05; $$: P < 0.01, compared with Model+HSYA-L; &: P < 0.05, compared with Model+HSYA-M

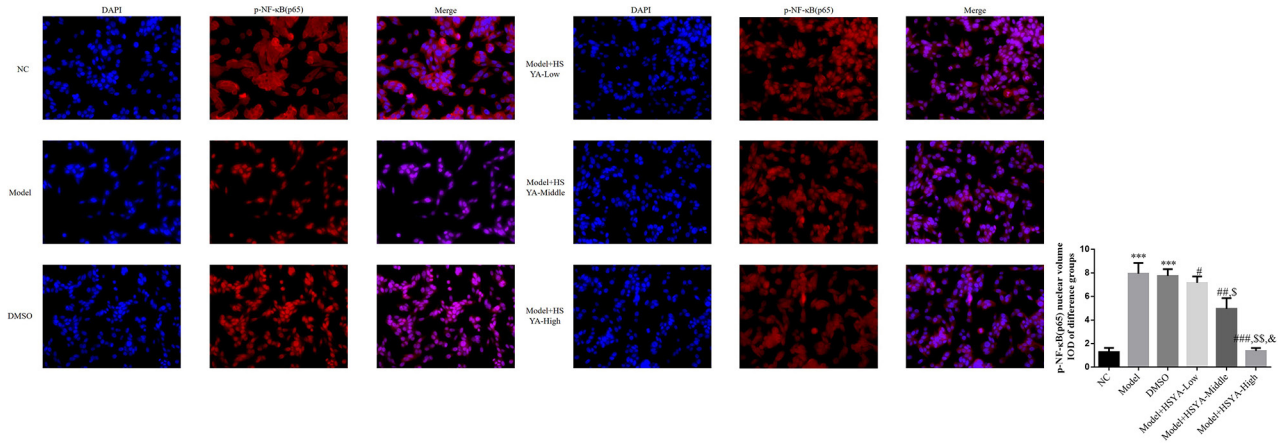

Figure 14. p-NF-κB(p65) protein nuclear volume (×200). ***: P < 0.001, compared with NC group; #: P < 0.05;##: P < 0.01, ###: P < 0.001, compared with Model; $: P < 0.05; $$: P < 0.01, compared with Model+HSYA-L; &: P < 0.05, compared with Model+HSYA-M.

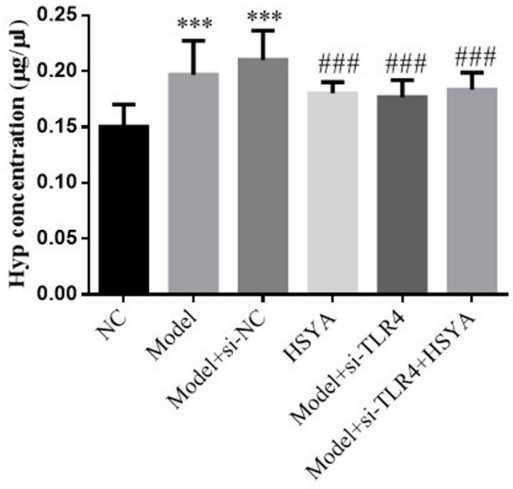

Figure 15. Hyp concentration. ***: P < 0.001, compared with NC group; ###: P < 0.001, compared with Model.

NC groups were significantly up-regulated (P < 0.001, respectively, Figure 21); based on scars cell model treatment, with HSYA supplement or/ and si-TLR4 which knockdown TLR4 expression transfection, HSYA, Model+si-TLR4 and Model+si-TLR4+HSYA groups were significantly depressed in p-NF-κB(p65) proteins nuclear volume (P < 0.001, respectively, Figure 21).

4 Discussion

Skin scar is the fibrosis and hyperplasia of the skin tissue after trauma or surgery, mainly characterized by excessive proliferation of fibroblasts, excessive ECM deposition and abnormal activation of various cytokines and growth factors (Xu et al., 2018; Nong et al., 2019). When the skin is seriously injured or infected, excessive repair always occurs, resulting in HSs or keloids. Scars not only affect the appearance of patients and cause dysfunction of joints and local organs, but also may lead to serious psychological problems. Therefore, it is of great significance to study the treatment of scars (Xu et al., 2018; Nong et al., 2019). At present, it is believed that inflammatory

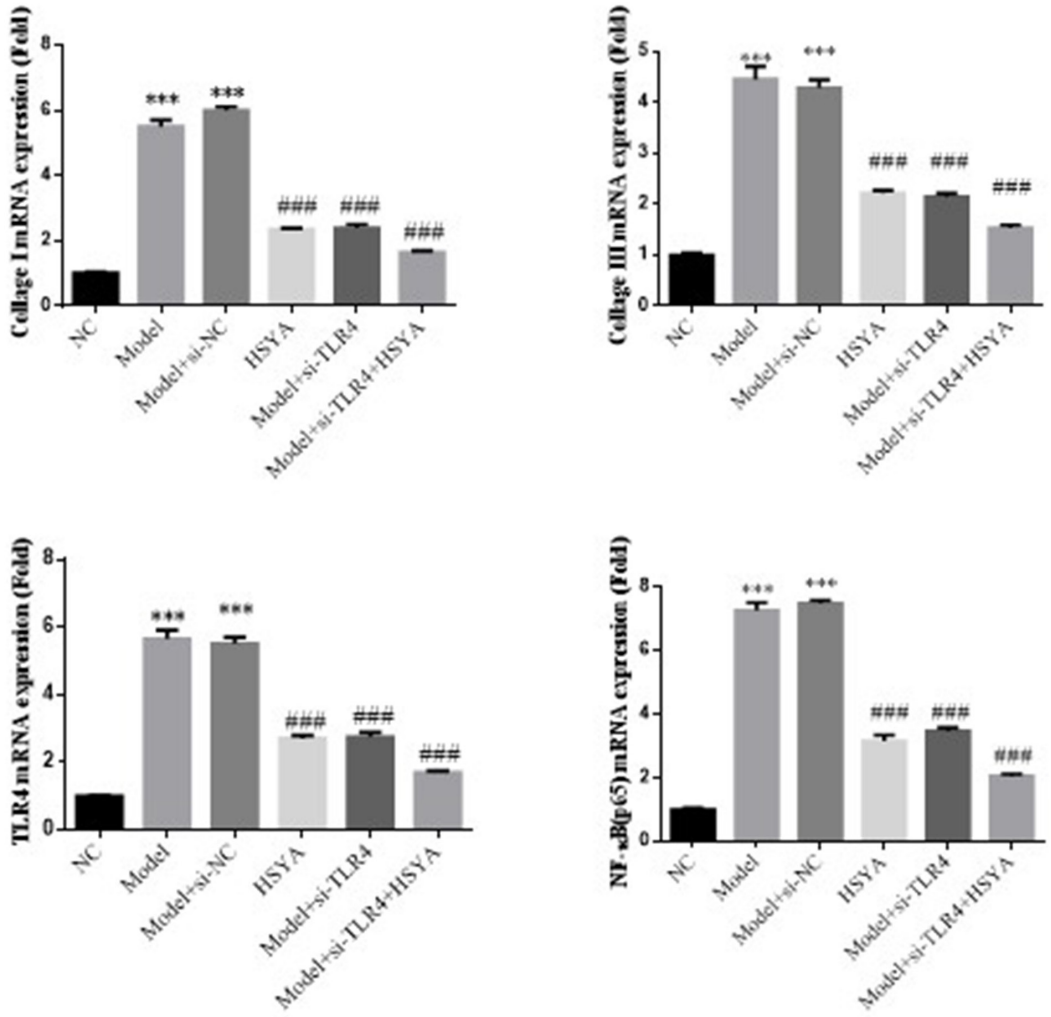

Figure 16. Relative mRNA expression in difference groups by RT-qPCR assay. ***: P < 0.001, compared with NC group; ###: P < 0.001, compared with Model.

factors are closely related to wound healing and HS formation (Lichtman et al., 2016; Klinge et al., 2000), and they are key factors during skin scar formation. The research results have confirmed that the increased gene and protein expressions of collagen I and collagen III are the markers of scarring and fibrosis (Klinge et al., 2001; Sun et al., 2013). In clinical practice, we found that anticoagulant injection would cause damage to the subcutaneous tissue, and meanwhile lead to subcutaneous scars in patients, which not only causes damage to the patients' body, but also hinders subsequent treatment. The results of this experiment showed that after anticoagulant injection, obvious subcutaneous scars appeared in New Zealand rabbits, and a large number of collagen fibers deposited in the tissues. These results suggest that anticoagulant injection can induce the massive secretion of inflammatory factors in the subcutaneous tissue, and thus resulting in collagen fiber deposition.

In traditional medicine, safflower has the effects of promoting blood circulation, dredging meridians, removing blood stasis and relieving pain, and is widely used in the treatment of cardio-cerebrovascular diseases such as coronary heart disease and hypertension (Bai et al., 2012; Yang et al., 2011). HSYA is the main effective component in safflower, which is used for quality control of safflower in the laboratory (Lei et al., 2019). In this study, we found that intragastric administration with HSYA at different doses on New Zealand rabbits could effectively improve the occurrence of HS caused by anticoagulant injection, and effectively reduce the concentrations of inflammatory factors TGF-β and IL-1β. In order to further explore the mechanism of HSYA in improving subcutaneous scars, we detected genes and proteins of the related signaling pathways.

Toll-like receptor 4 (TLR4) is the natural receptor of LPS, a common gram-negative bacterium in skin trauma, and increasingly expressed in keloids and various human fibrotic tissues. Some scholars stimulated Fb in scar and normal skin tissue using LPS, and revealed that both Fb proliferated significantly, intracellular TLR4 content increased, and MyD88 and TGF-β1-indcuced procollagen expression increased, especially in scar (Lei et al., 2019). In this study, we found that after intragastric administration with HSYA, the activation of the TLR4/NF-κB (p65) signaling pathway in the subcutaneous tissue caused by anticoagulant injection was significantly inhibited, and the improvement showed a dose-effect relationship with HSYA.

In conclusion, HSYA can improve subcutaneous scars caused by anticoagulant injection through inhibiting inflammation and the activity of related TLR4/NF-κB (p65) signaling pathway in vivo and vitro study.

Funding

This study was supported by National Natural Science Foundation of China (No.81871463), Project of Science and Technology of Nanjing (No.201803006) and Medical Science and Technology Development Foundation of Nanjing Department of Health (YKK19086).

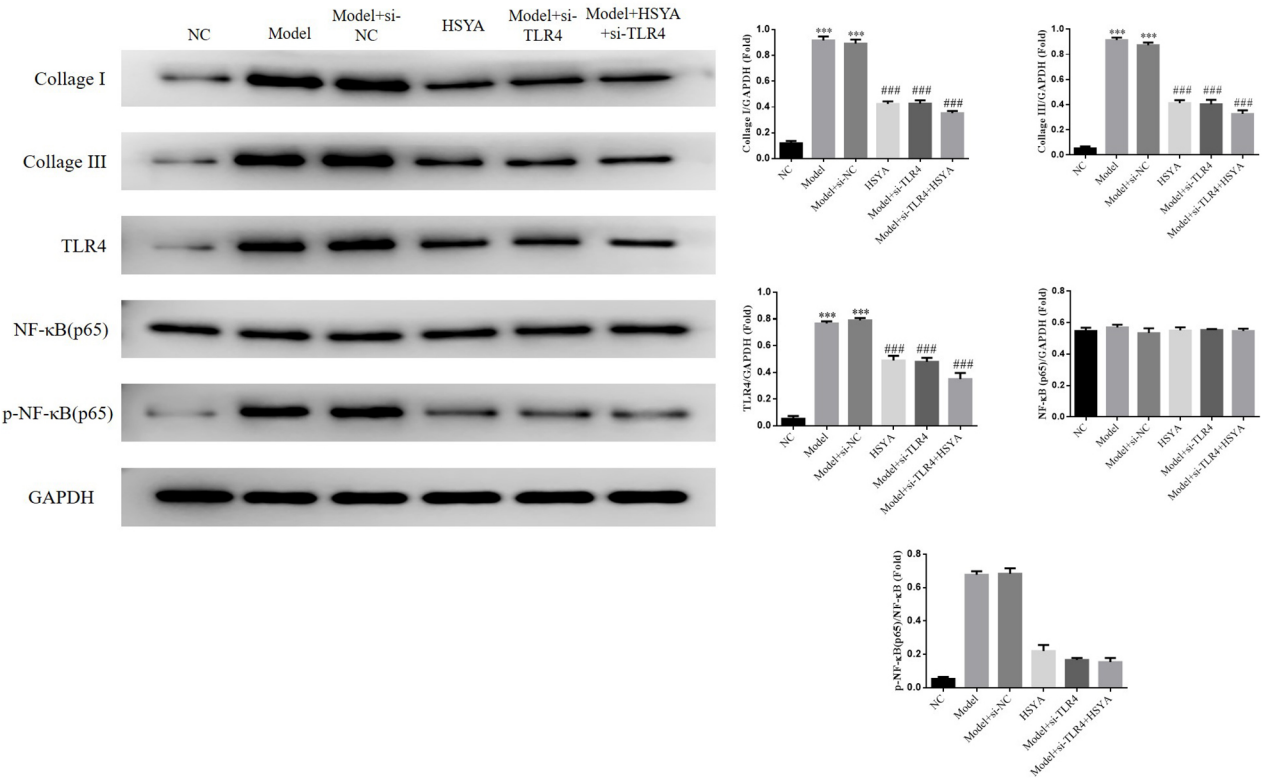

Figure 17. Relative proteins expression in difference groups by RT-qPCR assay. ***: P < 0.001, compared with NC group; ###: P < 0.001, compared with Model.

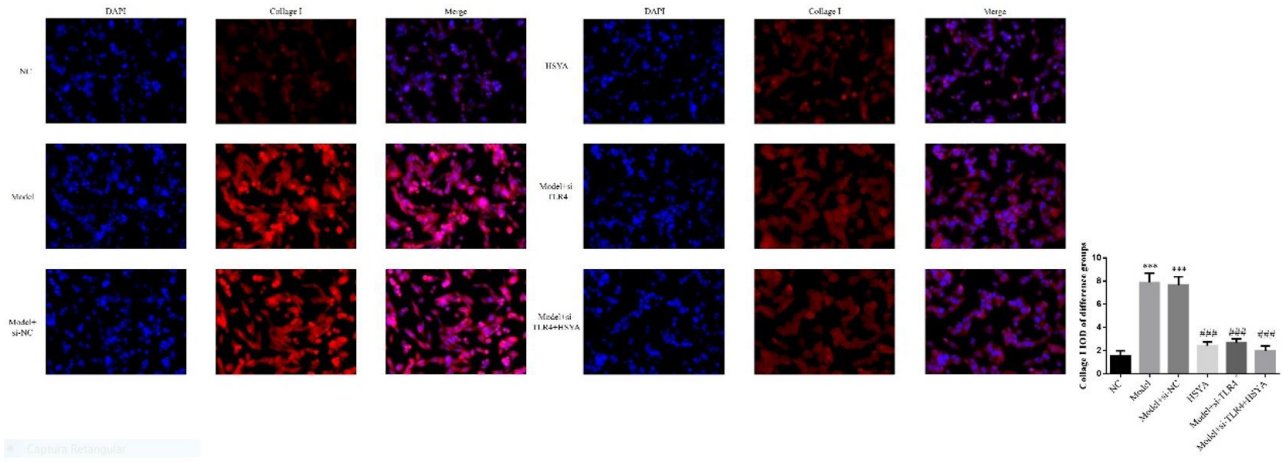

Figure 18. Collage I protein expression (×200). ***: P < 0.001, compared with NC group; ###: P < 0.001, compared with Model.

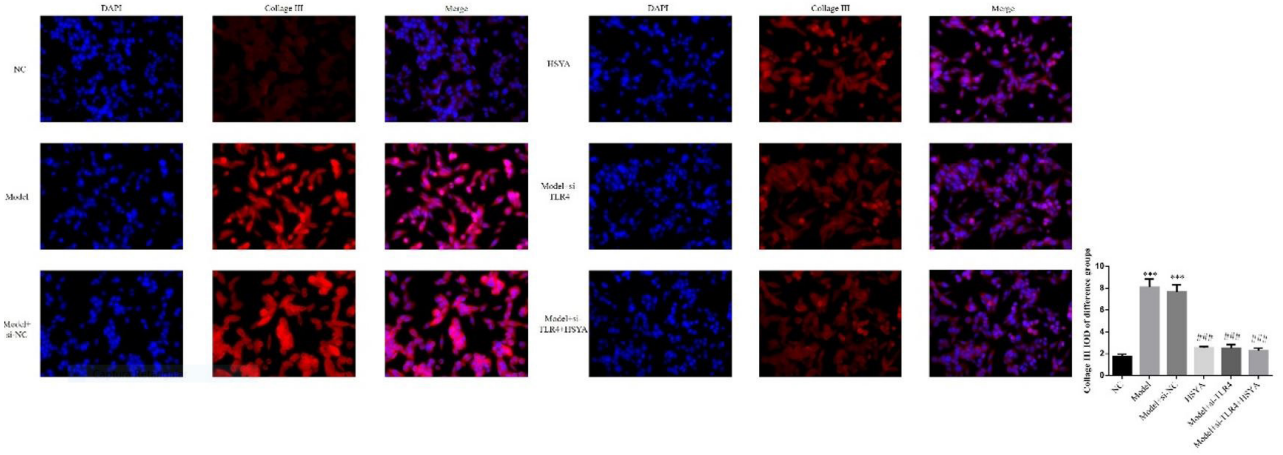

Figure 19. Collage III protein expression (×200). ***: P < 0.001, compared with NC group; ###: P < 0.001, compared with Model.

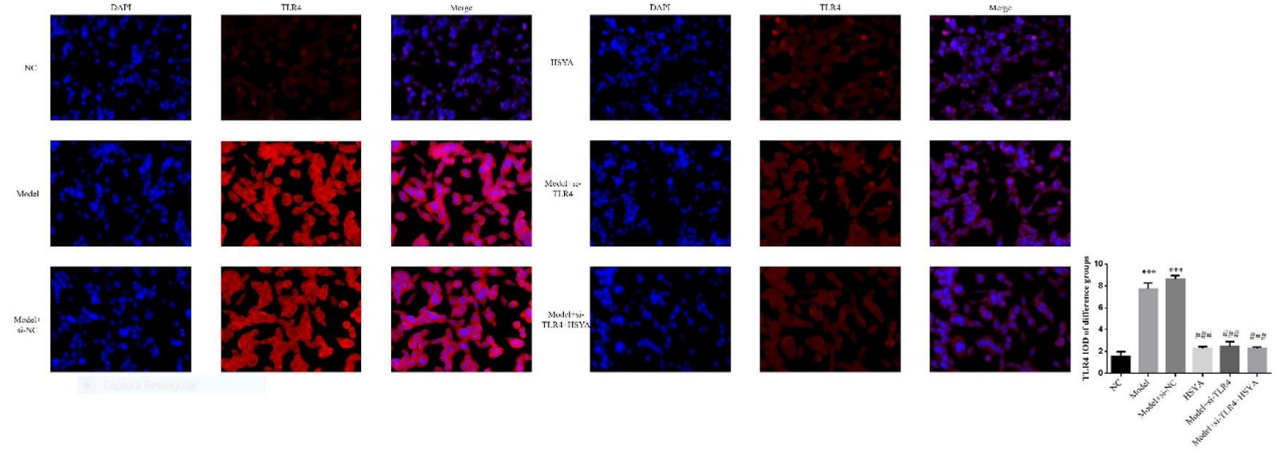

Figure 20. TLR4 protein expression (×200). ***: P < 0.001, compared with NC group; ###: P < 0.001, compared with Model.

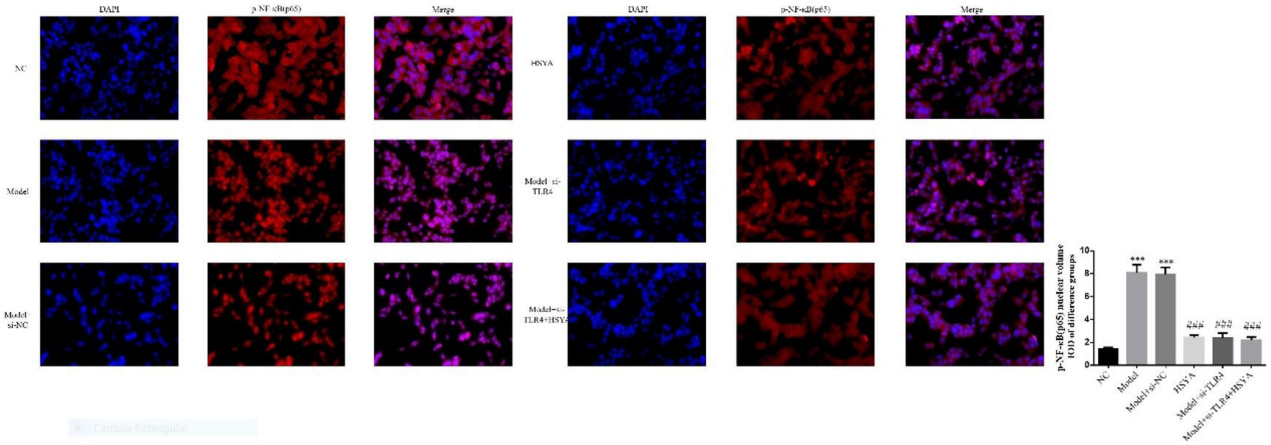

Figure 21. p-NF-κB(p65) protein nuclear volume (×200). ***: P < 0.001, compared with NC group; ###: P < 0.001, compared with Model.

References

Bai, Y., Lu, P., Han, C., Yu, C., Chen, M., He, F., Yi, D., & Wu, L. (2012). Hydroxysafflor yellow A (HSYA) from flowers of Carthamus linctorius L. and its vasodilatation effects on pulmonary artery. *Molecules (Basel, Switzerland)*, 17(12), 14918-14927. http://dx.doi.org/10.3390/molecules171214918. PMid:23519263.

Berman, B., Maderal, A., & Raphael, B. (2017). Keloids and hypertrophic scars: pathophysiology, classification, and treatment. *Dermatologic Surgery*, 43(Suppl 1), S3-S18. http://dx.doi.org/10.1097/DSS.0000000000000819. PMid:27347634.

Fan, L., Dang, X., Shi, Z., Zhang, C., & Wang, K. (2011). Hydroxysafflior yellow A protects PC12 cell against the apoptosis induced by oxygen and glucose deprivation. *Cellular and Molecular Neurobiology*, 31(8), 1187-1194. http://dx.doi.org/10.1007/s10571-011-9720-3. PMid:21688125.

Gauglitz, G. G., Korting, H. C., Pavicic, T., Ruzicka, T., & Jeschke, M. G. (2011). Hypertrophic scarring and keloids: pathomechanisms and current and emerging treatment strategies. *Molecular Medicine (Cambridge, Mass.)*, 17(1-2), 113-125. http://dx.doi.org/10.2119/molmed.2009.00153. PMid:20927486.

Hsu, Y.-C., Chen, M.-J., Yu, Y.-M., Ko, S.-Y., & Chang, C.-C. (2010). Suppression of TGF-beta1/SMAD pathway and extracellular matrix production in primary keloid fibroblasts by curcuminoids: its potential therapeutic use in the chemoprevention of keloid. *Archives of Dermatological Research*, 302(10), 717-724. http://dx.doi.org/10.1007/s00403-010-1075-y. PMid:20717830.

Jiang, S., Shi, Z., Li, C., Ma, C., Bai, X., & Wang, C. (2014). Hydroxysafflor yellow A attenuates ischemia/reperfusion-induced liver injury by suppressing macrophage activation. *International Journal of Clinical and Experimental Pathology*, 7(5), 2595-2608. PMid:24966974.

Klinge, U., Si, Z. Y., Zheng, H., Schumpelick, V., Bhardwaj, R. S., & Klosterhalfen, B. (2000). Abnormal collagen I to III distribution in the skin of patients with incisional hernia. *European Surgical Research*, 32(1), 43-48. http://dx.doi.org/10.1159/000008740. PMid:10720845.

Klinge, U., Si, Z. Y., Zheng, H., Schumpelick, V., Bhardwaj, R. S., & Klosterhalfen, B. (2001). Collagen I/III and matrix metalloproteinases (MMP) 1 and 13 in the fascia of patients with incisional hernias. *Journal of Investigative Surgery*, 14(1), 47-54. http://dx.doi.org/10.1080/089419301750072202. PMid:11297060.

Lee, Y., Minn, K. W., Baek, R. M., & Hong, J. J. (2001). A new surgical treatment of keloid: keloid core excision. *Annals of Plastic Surgery*, 46(2), 135-140. http://dx.doi.org/10.1097/00000637-200102000-00008. PMid:11216607.

Lei, R., Li, J., Liu, F., Li, W., Zhang, S., Wang, Y., Chu, X., & Xu, J. (2019). HIF-1alpha promotes the keloid development through the activation of TGF-beta/Smad and TLR4/MyD88/NF-kappaB pathways. *Cell Cycle (Georgetown, Tex.)*, 18(23), 3239-3250. http://dx.doi.org/10.1080/15384101.2019.1670508. PMid:31645185.

Li, J., Zhang, S., Lu, M., Chen, Z., Chen, C., Han, L., Zhang, M., & Xu, Y. (2013). Hydroxysafflor yellow A suppresses inflammatory responses of BV2 microglia after oxygen-glucose deprivation. *Neuroscience Letters*, 535, 51-56. http://dx.doi.org/10.1016/j.neulet.2012.12.056. PMid:23333598.

Lichtman, M. K., Otero-Vinas, M., & Falanga, V. (2016). Transforming growth factor beta (TGF-β) isoforms in wound healing and fibrosis. *Wound Repair and Regeneration*, 24(2), 215-222. http://dx.doi.org/10.1111/wrr.12398. PMid:26704519.

Liu, Y.-L., Liu, Y.-J., Liu, Y., Li, X.-S., Liu, S.-H., Pan, Y.-G., Zhang, J., Liu, Q., & Hao, Y.-Y. (2014). Hydroxysafflor yellow A ameliorates lipopolysaccharide-induced acute lung injury in mice via modulating toll-like receptor 4 signaling pathways. *International Immunopharmacology*, 23(2), 649-657. http://dx.doi.org/10.1016/j.intimp.2014.10.018. PMid:25466274.

Liu, Y.-N., Zhou, Z.-M., & Chen, P. (2008). Evidence that hydroxysafflor yellow A protects the heart against ischaemia-reperfusion injury by inhibiting mitochondrial permeability transition pore opening. *Clinical and Experimental Pharmacology & Physiology*, 35(2), 211-216. PMid:17941891.

Mathangi Ramakrishnan, K., Babu, M., & Lakshmi Madhavi, M. S. (2015). Response of keloid fibroblasts to Vitamin D3 and quercetin treatment - in vitro study. *Annals of Burns and Fire Disasters*, 28(3), 187-191. PMid:27279805.

Nie, P. H., Zhang, L., Zhang, W. H., Rong, W. F., & Zhi, J. M. (2012). The effects of hydroxysafflor yellow A on blood pressure and cardiac function. *Journal of Ethnopharmacology*, 139(3), 746-750. http://dx.doi.org/10.1016/j.jep.2011.11.054. PMid:22197825.

Nong, X., Rajbanshi, G., Chen, L., Li, J., Li, Z., Liu, T., Chen, S., Wei, G., & Li, J. (2019). Effect of artesunate and relation with TGF-β1 and SMAD3 signaling on experimental hypertrophic scar model in rabbit ear. *Archives of Dermatological Research*, 311(10), 761-772. http://dx.doi.org/10.1007/s00403-019-01960-7. PMid:31396694.

Park, G., Yoon, B. S., Moon, J.-H., Kim, B., Jun, E. K., Oh, S., Kim, H., Song, H. J., Noh, J. Y., Oh, C., & You, S. (2008). Green tea polyphenol epigallocatechin-3-gallate suppresses collagen production and proliferation in keloid fibroblasts via inhibition of the STAT3-signaling pathway. *The Journal of Investigative Dermatology*, 128(10), 2429-2441. http://dx.doi.org/10.1038/jid.2008.103. PMid:18463684.

Shook, B. A., Wasko, R. R., Rivera-Gonzalez, G. C., Salazar-Gatzimas, E., López-Giráldez, F., Dash, B. C., Muñoz-Rojas, A. R., Aultman, K. D., Zwick, R. K., Lei, V., Arbiser, J. L., Miller-Jensen, K., Clark, D. A., Hsia, H. C., & Horsley, V. (2018). Myofibroblast proliferation and heterogeneity are supported by macrophages during skin repair. *Science*, 362(6417), eaar2971. http://dx.doi.org/10.1126/science.aar2971. PMid:30467144.

Si, L.-B., Zhang, M.-Z., Han, Q., Huang, J.-N., Long, X., Long, F., Zhao, R. C.-H., Huang, J.-Z., Liu, Z.-F., Zhao, R., Zhang, H.-L., & Wang, X.-J. (2018). Sensitization of keloid fibroblasts by quercetin through the PI3K/Akt pathway is dependent on regulation of HIF-1alpha. *American Journal of Translational Research*, 10(12), 4223-4234. PMid:30662665.

Sun, L., Yang, L., Fu, Y., Han, J., Xu, Y., Liang, H., & Cheng, Y. (2013). Capacity of HSYA to inhibit nitrotyrosine formation induced by focal ischemic brain injury. *Nitric Oxide*, 35, 144-151. http://dx.doi.org/10.1016/j.niox.2013.10.002. PMid:24126016.

Wu, J., Fang, L., Cen, Y., Qing, Y., Chen, J., & Li, Z. (2019). MiR-21 regulates keloid formation by downregulating Smad7 via the TGF-beta/smad signaling pathway. *Journal of Burn Care & Research; Official Publication of the American Burn Association*, 40(6), 809-817. http://dx.doi.org/10.1093/jbcr/irz089. PMid:31184708.

Wu, Y., Wang, L., Jin, M., & Zang, B. (2012). Hydroxysafflor yellow A alleviates early inflammatory response of beomycin-induced mice lung injury. *Biological & Pharmaceutical Bulletin*, 35(4), 515-522. http://dx.doi.org/10.1248/bpb.35.515. PMid:22466555.

Xu, H.-L., Chen, P.-P., Wang, L.-F., Tong, M.-Q., Ou, Z.-H., Zhao, Y.-Z., Xiao, J., Fu, T.-L., & Wei-Xue, (2018). Skin-permeable liposome improved stability and permeability of bFGF against skin of mice with deep second degree scald to promote hair follicle neogenesis through inhibition of scar formation. *Colloids and Surfaces. B, Biointerfaces*, 172, 573-585. http://dx.doi.org/10.1016/j.colsurfb.2018.09.006. PMid:30218983.

Yang, J., Wang, Y., & Guo, M.-L. (2011). Identification and mapping of a novel hydroxysafflor yellow A (HSYA) biosynthetic gene in Carthamus tinctorius. *Biochemical Genetics*, 49(5-6), 410-415. http://dx.doi.org/10.1007/s10528-011-9417-9. PMid:21253824.

Yuan, W., Yang, D., Sun, X., Liu, W., Wang, L., Li, X., Man, X., & Fu, Q. (2014). Effects of hydroxysafflor yellow A on proliferation and collagen synthesis of rat vascular adventitial fibroblasts induced. *International Journal of Clinical and Experimental Pathology*, 7(9), 5772-5781. PMid:25337218.