

Inhibition of hypertrophic scar formation with oral asiaticoside treatment in a rabbit ear scar model

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Abstract

Hypertrophic scar (HS) is a fibrotic skin disease characterised by over-productive collagen and excessive inflammatory reaction, which can be functionally and cosmetically problematic. A scar-prone constitute will accelerate HS formation and functional disorder, which deserves systemic therapy with oral medicine. To examine the oral therapeutic effectiveness on HS with convincing evidence of gross view and histological improvement, a rabbit ear HS model was employed with oral administration of asiaticoside (AS) at the doses of 12 and 24 mg kg⁻¹ d⁻¹ daily for 60 consecutive days. Gross observation and histological findings showed that oral AS treatment could significantly inhibit HS formation in a dose dependent manner. Semi-quantification of scar elevation index at days 7, 15, 30, and 60, and quantitative polymerase chain reaction at days 30 and 60 also provided the evidences of reduced scar thickness and inhibited fibrotic gene expressions of *collagens I, III, TGF-β1, interleukins 1β, 6 and 8*, and enhanced gene expression of *SMAD 7* and *PPAR-γ* with a dose-dependent manner. These results indicated that AS is likely to serve as a systemic therapeutic agent of HS treatment for those who may have scar-prone constitute via anti-inflammation, inhibiting fibrotic process, and enhancing matrix degradation.

KEYWORDS

asiaticoside, histological and gene evidences, hypertrophic scar, oral administration, rabbit ear model

1 | INTRODUCTION

Hypertrophic scar (HS) is a common fibro-proliferative disorder causing pain, itch, and contractures with limited joint mobility, and disfigured deformities, which

significantly affects psychological health of the patients.¹ HS is characterised by hyperproliferation of dermal fibroblasts, overproduction of collagen, and excess deposition of extracellular matrix (ECM) elicited by deep burn and traumatic injuries, and even acne.² Normal inflammatory response can stimulate collagen synthesis and promote wound healing. Whereas, excessive inflammatory reaction,

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a process with excessive secretion of inflammatory cytokines released from wound fibroblasts and inflammatory cells such as interleukin (IL)-1 β , IL-6, and IL-8, can promote fibroblast proliferation, boost collagen overproduction, and aggravate other ECM synthesis, which ultimately result in pathological scar formation, including HS and keloid.^{3,4}

Currently, extensively used therapies for HS are limited in surgical excision, radiation, cryotherapy, intralesional corticosteroid injections, lasers, and topical silicone gel sheets. However, an efficient, convenient, and sustainable therapeutic agent with low cost and minimum side effects is in great need. It has been commonly recognised that natural herbs and their extracts play a distinctly beneficial role in the prevention and treatment of various diseases. Asiaticoside (AS) is a saponin component extracted from the medicinal plant *Centella asiatica*, which is recommended long ago in the treatment of many dermatoses and skin injuries such as HSs, keloids, and burns as well as in non-dermatological diseases such as anxiety,⁵ gastric mucosal lesions,⁶ and neurodegenerative disorders.⁷

AS has been reported to have a variety of biological effects, including anti-inflammatory,⁸ anti-oxidant,⁹ and anti-ulcer properties.¹⁰ AS was also found to prevent scar formation with reduced inflammatory reaction and collagen deposition, transforming growth factor (TGF)- β 1 secretion and enhanced activity of SMAD 7 that is a negative regulator of TGF- β signalling.¹¹

These effects suggested that AS had a potent anti-scarring effect. Actually, it has been used clinically for many years in the treatment of HS. Two to four tablets (6 mg/tablet, Shanghai Modern Pharmaceutical Company), three times a day for adults are recommended by the State Food and Drug Administration of China for the treatment of non-healing wounds, HSs, or keloids in the active phase.¹² Nevertheless, there are few reports proving that oral administration of AS alone could grossly reduce scar formation, possibly due to the native disadvantage of its poor bioavailability. In addition, literature also showed that the side effect AS could be barely detected with no obvious LC50 (lethal concentrations required to kill 50%).¹³ Therefore, non-apparent therapeutic effect of AS on gross improvement of treated HS is likely due to the insufficient drug dose.

This study aimed to test the therapeutic effect of AS on HS using a rabbit ear HS model by daily oral-taken AS with high dose (12 mg kg⁻¹ d⁻¹, 10-folds higher than that of clinical recommendation) and double-high dose (24 mg kg⁻¹ d⁻¹, 20-folds higher than that of clinical recommendation) to observe its possible HS improvement from the aspects of gross view, histological feature, and gene expression of fibrotic factors.

Key Messages

hypertrophic scar is a fibro-proliferative disorder causing pain, itch, limited joint mobility, and disfigured deformities this study presented that asiaticoside oral therapy can significantly improve the gross view and histological structure of formed hypertrophic scar in a rabbit ear scar model when given a sufficient dose, which overcomes the shortcoming of asiaticoside low bioavailability

2 | METHOD AND MATERIALS

2.1 | Creation of rabbit ear hypertrophic scarring model

Young adult New Zealand white rabbits (3-4 months old, 2.5-3.0 kg weight, no sex restriction) were obtained from the Shanghai Jiagan Biotechnology Co., Ltd. The animals were individually housed under standard conditions and the experimental protocol was approved by the Ethics Committee of Shanghai Ninth People's Hospital. As previously described,¹⁴ the rabbits were anaesthetised with sodium pentobarbital (30 mg kg⁻¹), then four full-thickness circular wound, with the diameter of 10 mm for each, were made on the ventral surface of each ear down to the perichondrium (perichondrium was not contained) using a punch in asepsis condition. Haemostasis was obtained by applying pressure after creating the wounds. Then, to observe the scar forming process, the wounds were exposed to air and the secretions were removed every day.

2.2 | Experimental design and drug administration

A total of 48 rabbits were enrolled in the study; after removing the rabbits that had wound infection or necrosis, the remaining rabbits were divided into three groups: (a) control group: 13 rabbits (3 rabbits examined at day 7, 4 rabbits at day 15, 3 rabbits at day 30, 3 rabbits at day 60) were treated with double-distilled water (DDW); (b) high dose group: 13 rabbits (3 rabbits examined at day 7, 3 rabbits at day 15, 4 rabbits at day 30, 3 rabbits at day 60) were treated with AS at a dose of 12 mg kg⁻¹ d⁻¹; (c) double-high dose group: 14 rabbits (4 rabbits detected at day 7, 3 rabbits at day 15, 4 rabbits at day 30, 3 rabbits at day 60) were treated with AS at a dose of 24 mg kg⁻¹ d⁻¹. The drug was kindly provided by Shanghai Modern Pharmaceutical Company and was dissolved

in DDW with the help of an ultrasonic extractor. The AS solutions were administered three times daily through transoral gavage. The rabbits of each group were, respectively, sacrificed at postoperative days of 7, 15, 30, and 60 ($n = 3$ for each time point) with euthanasia of anaesthesia overdose, and wound gross view were recorded by photography via a digital camera followed by wound tissue harvesting for histological evaluation and real-time quantitative polymerase chain reaction (RT-qPCR).

2.3 | Histologic examination

The scar was cut open along the long axis and fixed in 4% paraformaldehyde followed by paraffin embedding and tissue section at 5 μm thickness. Afterward, the sections were stained with haematoxylin and eosin (H&E) and Masson's trichrome and observed under a light microscope (Nikon, Tokyo, Japan) as previously described.¹⁵

2.4 | Evaluation of scar elevation index

A scar elevation index (SEI) was calculated to quantify the degree of scar proliferation using H&E stained slides of the wounds. The SEI is defined as the ratio of the length of a line of the scar to that of normal skin, which is perpendicular to the ear surface and is measured from the top point of the scar/skin epithelium to the surface of the ear cartilage as previously described.² The SEI of each wound was measured using an Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, Maryland) by a blinded examiner.

2.5 | RNA extraction and RT-qPCR

RNA was isolated from the scar tissue on postoperative day 30 and day 60 using a Trizol Reagent (Invitrogen, Carlsbad, California) with the help of a rotor-stator homogeniser. Amount of 2 μg total RNA, 4 mL 5 \times buffer, 2 mL dNTP, 1 mL oligo-(dT), 0.5 mL AMV reverse transcriptase, 0.5 mL RNase inhibitor, and DDW was filled up to the total volume of 20 mL mixture. The cDNA was reversely transcribed by cultivating at 30°C for 10 minutes, 45°C for 60 minutes, 95°C for 5 minutes, and 5°C for 5 minutes. Then cDNA was amplified using a Strata Gene Mx3000p (Applied Biosystems). qPCR conditions were set as follows: initial denaturation at 95°C for 10 minutes, followed by 40 cycles at 95°C for 30 seconds, 58°C for 30 s, and 72°C for 45 seconds. Each interested gene was normalised to the housekeeping gene *GAPDH* and the fold change was compared relative to the control

sample. The designed primers were listed in Table 1. qPCR assay was repeated at last three times.

2.6 | Statistical analysis

Statistical analysis was performed by SPSS software (version 19.0, SPSS, Inc., Chicago, Illinois) using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. All results were presented as the mean \pm standard derivation. $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | AS reduced HS formation

By gross observation, all wounds began to close up after postoperative day 7 and AS treatment especially in double-high dose could enhance the wound healing process (Figure 1). Wounds almost completely healed after postoperative day 15 and AS treated group especially the double-high dose group healed the best (Figure 1). As shown in Figure 1, wounds were completely re-epithelialised at postoperative day 30. An apparently raised and tangibly local HS towered above the circumambient unscathed skin was observed in the control group with hard texture and pale red colour. However, AS-treated groups showed softer and less visible scars than the control group (Figure 1). Scar regression was found at postoperative day 60 in all groups. By contrast, palpation and visualisation of the wounding sites treated with AS revealed softer and flatter tissue, with the colour gradually closer to that of normal skin (Figure 1). Moreover, the scars in the double-high dose AS group revealed a fading pink colour and lighter coloration comparable with the surrounding tissue, suggesting that AS inhibited HS formation at a dose-dependent manner.

3.2 | The effect of AS on histologic characteristics of HS

Histologically, the lesions were nearly flat with the wound level similar to that of the epidermis in all groups at postoperative day 7, but the dermal layer in the control group was a bit thickened and the collagen fibres were relatively disarranged comparing with the scars of AS-treated groups (Figures 2A and 3). At postoperative day 15, the dermal layer of the untreated control scars became significantly thickened, and collagen fibres were much denser, with disorganised collagen bundles in the dermis when observed in double-high power field (Figures 2A and 3). However, AS treated groups showed relatively thinner dermis and

TABLE 1 Primers used in quantitative polymerase chain reaction analysis

Gene	Primer Sequence (5'-3')	Annealing Temperature, °C	Product Size, bp
Collagen I	Sense: AACTTGCTTCATGCGTCTG	58	153
	Antisense: CCTCGGCAACAAGTTCAACA		
Collagen III	Sense: TGACAAGGGAGACACAGGAC	58	217
	Antisense: ACCAGCACCACTCTAAGAC		
TGF-β1	Sense: CACCCAGTGACACAGATGAG	58	248
	Antisense: ATGTTGAGCCCGTTCCAG		
SMAD7	Sense: AGCAGAAATCCAAGCACC	58	433
	Antisense: CTGGCAGGAAGGGAATAAG		
PPAR-γ	Sense: TTGCTGTGGGGATGTCTCAT	58	208
	Antisense: TTTCTGTCAAGATCGCCCT		
IL-1β	Sense: CCAGTGAGATGATGGCTTACC	58	152
	Antisense: GTTGTAGGGTTGGCAGGAGA		
IL-6	Sense: TAATGAGACCTGCCTGCTGA	58	191
	Antisense: GCTTGAGGGTGGCTTCTTC		
IL-8	Sense: TACAGAGCTTCGATGCCAGT	58	152
	Antisense: GGTCCAGGCAGAGTTCTCTT		
GAPDH	Sense: CTCCTGCGACTTCAACAGTG	58	72
	Antisense: TGAGGGCTCTTACTCCTTGG		

the collagen fibres were relatively less dense and irregular than that of the control group (Figures 2A and 3). At day 30 post-operation, both H&E and Masson staining exhibited that scars in the control group were obviously elevated, raised above the level of uninjured adjacent skin (at low power), and presenting abundantly thicker, denser, and more tangled collagen fibres (at high power). In contrast, with the AS treatment, the dermal layer was thinner at the scar site, the deposition of collagens was significantly reduced, and collagen fibres were thinner with a relatively less disorganised pattern. The reduced scar formation was particularly pronounced in the double-high dose group (Figure 2A and 3). At postoperative day 60, the scars obviously subsided in all groups when comparing with those of day 30. Even so, significant differences in scar thickness and histological structure remained at day 60 with fine collagen fibres and reduced density in the AS-treated group in a dose-dependent manner, which was consistent with gross presentations (Figures 2A and 3).

3.3 | AS alleviated SEI of HS

To investigate the effect of oral AS on the degree of scar hyperplasia, the SEI was measured by using H&E stained slices. SEI was significantly lower in AS groups in a dose-dependent manner than that of the control group at all timepoints (Figure 2B-E). In detail, there was a significantly

reduced SEI in the double-high dose AS group (1.573 ± 0.1320) than that of the control group (1.952 ± 0.3157) and high dose group (1.908 ± 0.1413) on day 7 post wounding (Figure 2B, $P < 0.05$). SEI gradually increased with the value 1.952 ± 0.3157 , 1.908 ± 0.1413 , and 1.573 ± 0.1320 at day 15 post-operation, respectively, in the control, high dose, and double-high dose AS group, and the SEI value in the double-high dose AS was lowest among all three groups (Figure 2C, $P < 0.05$). Scar hyperplasia reached a peak at day 30 post-wounding, with the average SEI value of 4.846 ± 0.4111 , 3.951 ± 0.2389 , and 3.008 ± 0.3672 , respectively, in control, high dose, and double dose groups. However, consecutive administration of AS markedly and dose-dependently inhibited scar hyperplasia (Figure 2D, $P < 0.05$). In addition, scars began to regress and soften at day 60, which was also reflected by reduced SEI in all groups. Still, the SEIs in double-high dose AS group (2.279 ± 0.2272) and in high dose AS group (2.115 ± 0.1269) was much lower than that of the control group (2.626 ± 0.1886) (Figure 2E, $P < 0.05$), although no significant difference between two AS treated groups (Figure 2E, $P > 0.05$).

3.4 | The effect of AS on gene expression of HS

At postoperative day 30, the qPCR showed that the expression levels of collagen (COL) were significantly

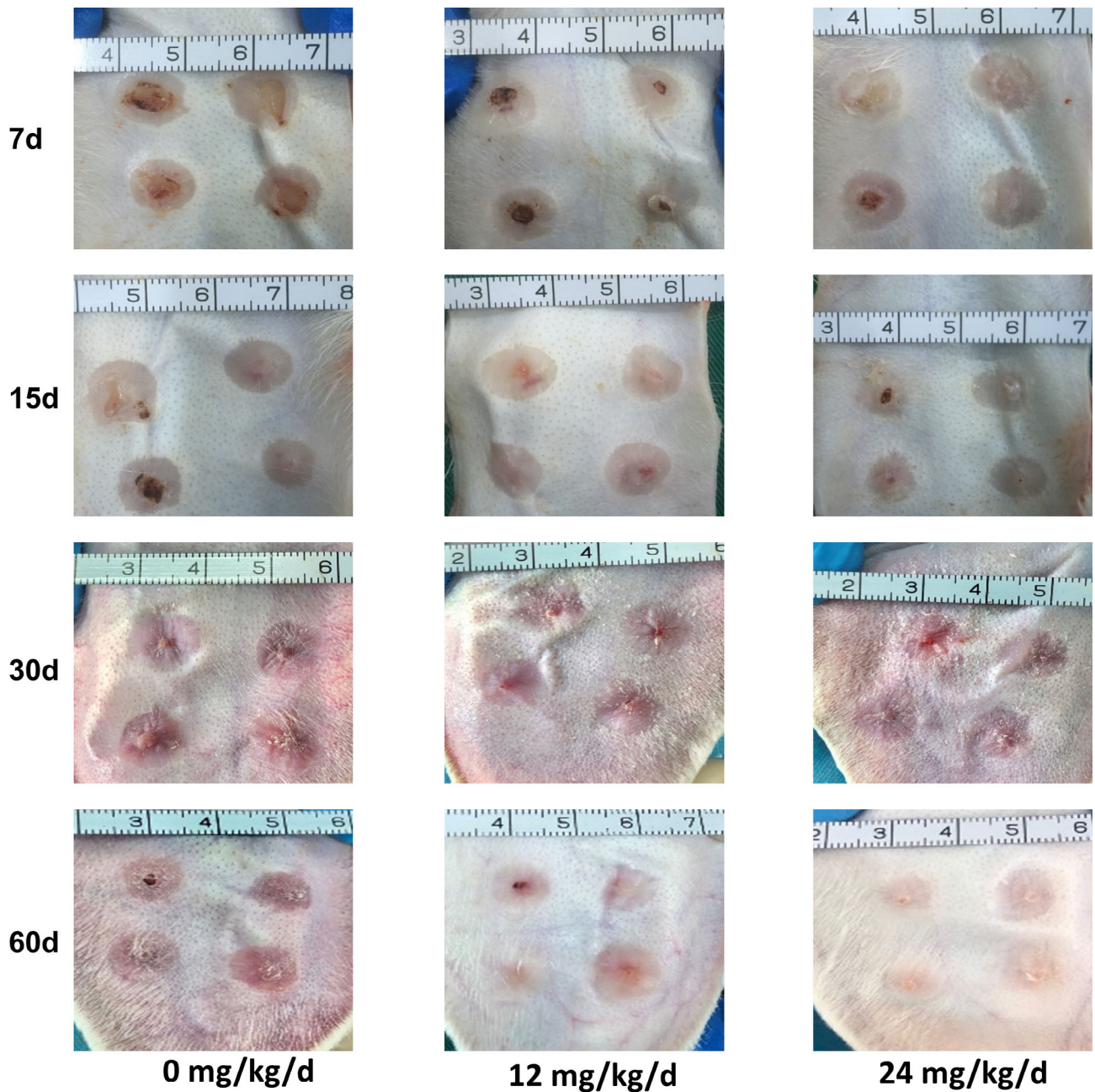


FIGURE 1 Gross view of healed wounds and formed hypertrophic scar (HS) tissue at postoperative days 7, 15, 30, and 60. A dose-dependent effect on inhibiting HS formation can be observed grossly with significantly inhibited angiogenesis and flattened tissues

lower in the high dose group (0.6500 ± 0.2004 -folds of *COL I*, 0.6768 ± 0.2039 -folds of *COL III*) and in the double-high dose group (0.5573 ± 0.1799 -folds of *COL I*, 0.4483 ± 0.2360 -folds of *COL III*) compared with those of control group (Figure 4A, $P < .05$). TGF- $\beta 1$ is a pivotal mediator in scar formation, resulting in excessive cumulation of COL and ECM in the process of HS formation. As shown, AS could obviously reduce TGF- $\beta 1$ gene expression in a dose-dependent manner (0.8168 ± 0.09574 -folds in high dose group and 0.6440 ± 0.1377 -folds in double-high dose group) when

compared with that of the control group (Figure 4A, $P < .05$). SMAD 7, a definite inhibitory factor in TGF- β signalling pathway, was found at an increased gene expression level with 1.130 ± 0.01000 -folds and 1.453 ± 0.1069 -folds, respectively, in high and double-high dose groups when compared with that of the control group (Figure 4A, $P < .05$). Peroxisome proliferator-activated receptors (PPARs) are widely expressed in cutaneous tissues, among which PPAR- γ was closely negatively related to skin fibrosis.¹⁶ Double-high dose AS stimulation led to the upregulation of PPAR- γ mRNA (2.770 ± 0.4392 -folds)

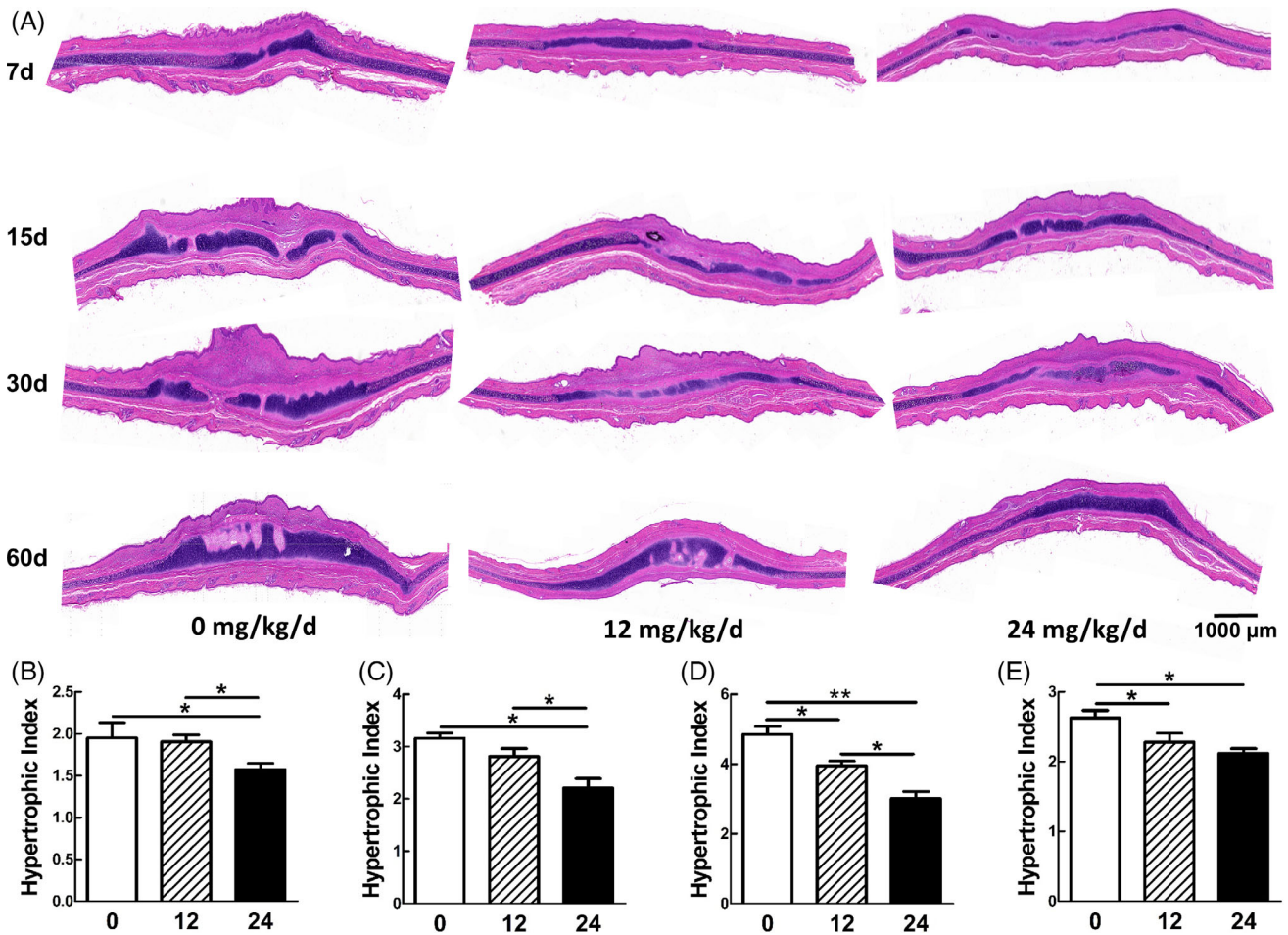


FIGURE 2 Histological analysis of formed hypertrophic scar (HS) tissue with semi-quantitative analysis. A, histology overview of HSs treated with double-distilled water (the left panel), 12 mg kg⁻¹ asiaticoside (AS) (the middle panel), and 24 mg kg⁻¹ AS (the right panel), respectively. The scars and their collagen fibers with AS treatment were flatter and thinner than those of the control group in a dose-dependent manner. Prominently elevated scar relative to uninjured adjacent skin was noted in control group, moderate elevations were noted in 12 mg kg⁻¹ AS groups, less conspicuous scar was presented in 24 mg kg⁻¹ AS group. Magnification×20, Bar = 1000 μm. Semi-quantitative analysis of scar elevation index (SEI) at postoperative days 7, B; 15, C; 30, D; and 60, E showed that AS treatment decreased SEI in a dose-dependent manner with significant differences among the groups. 0, 12, and 24, respectively, represents AS dose of 0, 12, and 24 mg kg⁻¹. (**P* < 0.05, ***P* < 0.01)

compared with the control (1-fold) and high dose treated groups (1.620 ± 0.5522 -folds) (Figure 4A, *P* < 0.05). Moreover, the expression levels of inflammation-related genes of *IL-1β*, *IL-6*, *IL-8* were markedly decreased in AS treated group in a dose-dependent manner compared with those of the control group (Figure 4A, *P* < 0.05).

At day 60 post-wounding, the mRNA expression levels of *COL I* and *COL III* remained significantly reduced in the AS groups compared with the control group in a dose-dependent manner (Figure 4B, *P* < 0.05). And AS treatment could also significantly bring down the expression level of *TGF-β1* mRNA to 0.8390 ± 0.06321 -folds in high dose group and 0.7050 ± 0.1184 -folds in double-high dose group compared with that of the control group (Figure 4B, *P* < 0.05). In addition, double-high dose AS treatment could significantly promote *SMAD 7* mRNA expression (1.510

± 0.07550 -folds) as opposed to the level of the control group (one-fold) and high dose AS group (1.177 ± 0.1350 -folds) (*P* < .05). *PPAR-γ* showed an increased tendency in the AS groups especially in double-high dose group, but no statistical difference was found. As shown, AS dose-dependently suppressed *IL-1β*, *IL-6* expression levels compared with the levels of control group, and there was a lower gene expression level of *IL-8* in the AS-treated scar tissue than in the control scar (Figure 4B, *P* < 0.05).

4 | DISCUSSION

HS is often caused by trauma and burn injury deep to the dermis, followed by excessive cutaneous wound healing. HS is painful, itchy, and rigid with disfiguring scars

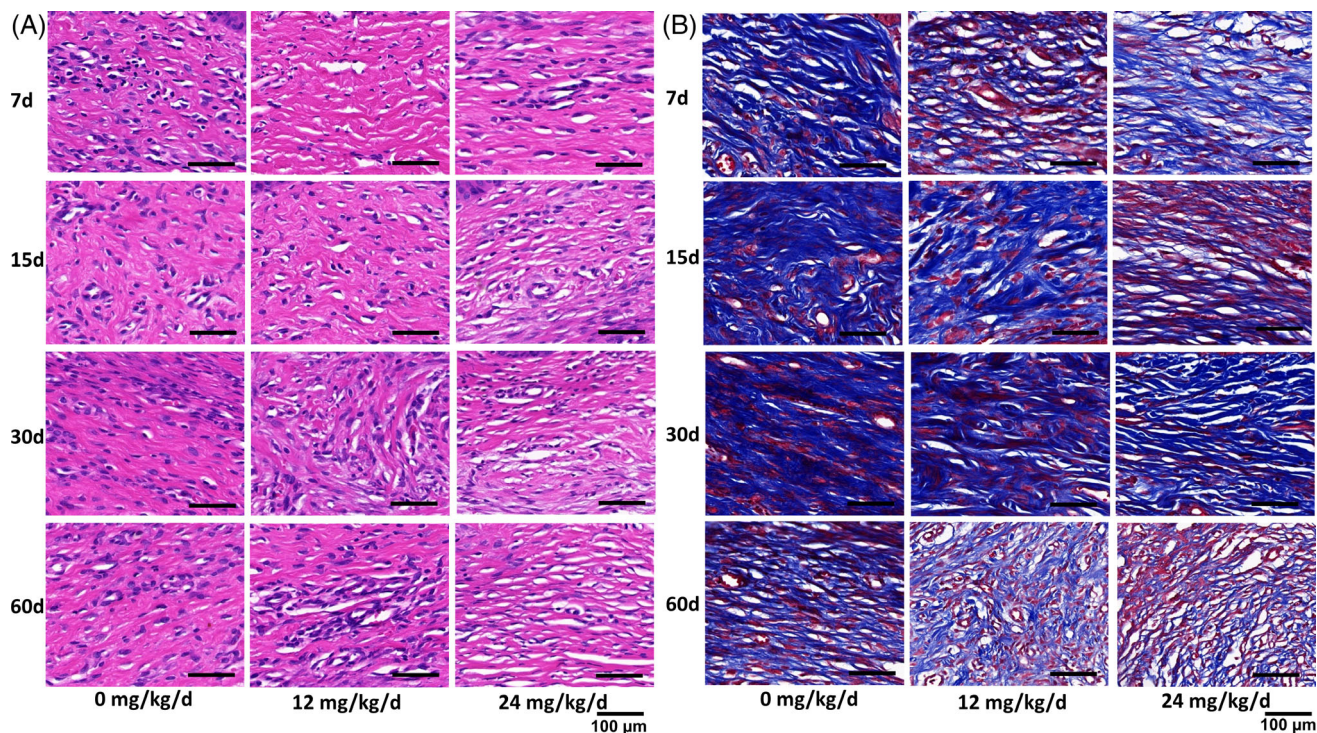


FIGURE 3 High power examination of haematoxylin and eosin (H&E) and Masson staining. A, H&E staining in the dermal region immediately below the epidermis showed decreased and relatively organised collagen fibres in asiaticoside (AS) treated scar tissue at postoperative days 7, 15, 30, and 60 comparing to those of the control group. B, Masson's trichrome staining in the dermal region immediately below the epidermis found regularly arranged, thinner, sparser collagen fibres in AS treated scar tissue as opposed to the control scars at postoperative days 7, 15, 30, and 60. Magnification $\times 40$, Bar = 100 μm

characterised by an excess multiplication of dermal fibroblasts, overproduction and deposition of collagens, and exaggerated inflammation reaction.

Patients suffering from HS usually exhibit a scar constitution mainly characterised by systemic inflammation. Their scars are persistently enlarging during the wound healing, in which process excessive immune cells are recruited rapidly to the injury site through the regulation of prolonged inflammatory chemokines once the trauma occurred.

Oral medication might be an optimal solution for spread and severe HS patients prone to systemic inflammation. However, there was no clearly relevant data or literature to support that oral drug therapy could improve the gross appearance and histological structure of HS.

AS is a white needle-like crystal extracted from the traditional herbal medicine, *Centella asiatica*, a medicinal plant that was already used as a "panacea" 3000 years ago, which has been demonstrated to be effective for the treatment of dermatoses and skin lesions such as excoriations, burns, HSs as well as in non-dermatological diseases like gastric ulcers, gastric mucosal lesions, lupus, leprosy, and cancer.¹⁷⁻¹⁹ However, almost all published studies focused on topical medications of animal wounds or just cytological studies.¹⁹⁻²² And the effect of oral AS

treatment on HS has rarely been explored experimentally.

Although many reports showing that AS could be used for HS treatment.^{20,22,23} It seemed to work invalidly or only show little benefit on improving gross and histological appearance of HS.^{24,25} The poor response to AS treatment is probably associated with the fact that AS tablet for oral intake is a glycoside herbal medicines with low bioavailability and the adjuvant usually occupies 92% of volume with no pharmacological effects.¹³ Thus, an appropriate treatment efficacy cannot be expected from the dose regimen of clinical recommendations. As a supportive evidence of low bioavailability, LC50 and side effects were difficult to be detected.²⁶

Therefore, this study used AS drug manufactured by Shanghai Modern Pharmaceutical Company to explore the oral therapeutic effect of AS on the improvement of HS gross view, histological structure, and fibrotic gene expression using a rabbit ear HS model. Because of the disadvantage of low bioavailability, the drug dose with 10-folds (12 mg kg⁻¹ d⁻¹) and 20-folds (24 mg kg⁻¹ d⁻¹) higher than that of clinical recommendation were applied to conduct the oral drug efficacy on HS. In addition, the transoral gavage was employed to make sure proper intake of administered drugs.

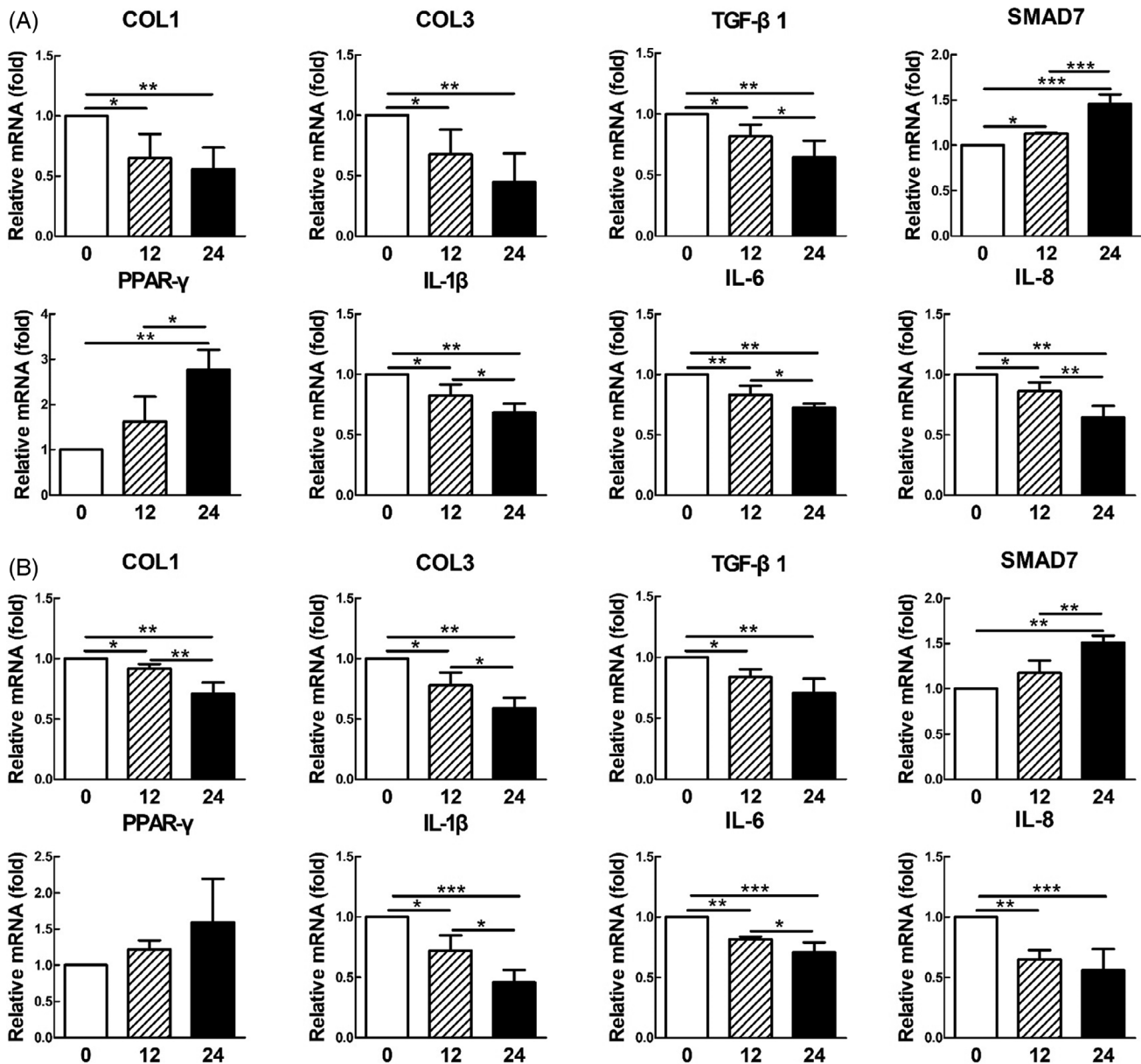


FIGURE 4 Asiaticoside (AS) suppressed pro-fibrogenic and promoted anti-fibrogenic gene expression in treated scar tissues. A, The gene expression levels of *COL I*, *COL III*, *TGF-β1*, *IL-1β*, *IL-6*, and *IL-8* were significantly decreased but *SMAD 7*, *PPAR-γ* was evidently increased in the scar tissues after 30 consecutive days of AS oral treatment with a dose-dependent change and significant differences among the groups. B, The gene expression levels of *COL I*, *COL III*, *TGF-β1*, *IL-1β*, *IL-6*, and *IL-8* were significantly decreased but *SMAD 7*, *PPAR-γ* was evidently increased in a dose-dependent manner following 60 consecutive days of oral administration of AS with significant differences among the groups. Each assay was repeated in at least three independent tissue samples. 0, 12, and 24, respectively, represents AS dose of 0, 12, and 24 mg kg⁻¹. (**P* < 0.05, ***P* < 0.01, ****P* < 0.001)

The results showed that oral AS therapy at an adequate dose could significantly assist the wound healing process and improve the gross view of HS with more flattened appearance (Figure 1). When quantitatively evaluating the thickness of formed HS tissue, SEI analysis clearly demonstrated AS effect on reducing scar thickness with significant differences among groups (Figure 2). The histochemical experiment also revealed that COL fibre

alignment was improved, and COL content was reduced after AS treatment (Figure 3).

Interestingly, this study demonstrated a clear dose-dependent effect of AS not only for gross appearance (Figure 1) and histological structure (Figures 2 and 3) improvement, but also the reduction of fibrotic gene expression (Figure 4), suggesting that the therapeutic effect of AS on HS is truly dependent on the dose that is

sufficient to overcome the drug shortcoming of low bioavailability, this also explained why previous clinical studies were not able to show significant improvement of gross view and histology of HS treated by oral AS therapy alone.

In this study, none of the treated rabbits displayed obvious side effects or died after oral AS treatment at these two doses, which suggested that AS indeed had low bioavailability and non-detectable side effects. Future research should further explore how to purify or improve the structure of AS in order to enhance the bioavailability. Based on our findings, ascending the clinically recommended dose of AS could be a safe and straightforward approach to treat HS.

5 | CONCLUSION

This study presents a supportive evidence that AS oral therapy alone can significantly improve the gross view and histological structure of formed HS tissues given a sufficient dose, which can overcome the shortcoming of AS low bioavailability. The underlying mechanism is likely to exert anti-scarring effect via down-regulating TGF- β 1 signalling¹¹ and inhibiting the inflammatory process,⁸ resulting in decreased collagen production and deposition as well as improved tissue structure. Due to the limited bioavailability of this drug and non-detectable side effect, the increase of clinical recommended dose will be a simple and efficient way to exert its anti-scarring therapeutic effect of current format of AS tablet.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available online of International Wound Journal.

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