

## Original Article

# Panax notoginseng saponins attenuates hypertrophic scar formation by inhibiting collagen synthesis in a rabbit ear model

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**Abstract:** Hypertrophic scarring is a pathological condition that results from an overproduction of collagen and excessive deposition of extracellular matrix. The purpose of the present study was to determine the therapeutic effects of panax notoginseng saponins (PNS) on hypertrophic scarring in a rabbit ear model and find the potential mechanisms. Fifteen healthy rabbits were randomized into three groups, namely, Sham group, Model group and PNS group. Acid fuchsin staining and ELISA assay were performed to detect the collagen synthesis and levels of collagen I and collagen III. The mRNA levels of collagen I A2, collagen III A1, matrix metalloproteinase-1 (MMP1) and connective tissue growth factor (CTGF) were examined through quantitative RT-PCR analysis. The average scar area was also determined. The results showed that hypertrophic scarring was significantly inhibited in the rabbit ears on Day 30 after PNS treatment. Collagen synthesis was markedly suppressed in the scar tissues following PNS administration. Furthermore, after PNS treatment, mRNA levels of collagen I A2, collagen III A1, CTGF were notably decreased, whereas MMP1 was evidently elevated in PNS group. These findings suggested that PNS could inhibit the accumulation of collagen and then inhibit hypertrophic scarring through reducing CTGF expression and increasing MMP1 expression.

**Keywords:** Panax notoginseng saponins, hypertrophic scar, collagen I, collagen III, CTGF, MMP1

## Introduction

As a complex dermal fibro-proliferative disorder, hypertrophic scarring occurs after deep trauma, surgical incision, or burns. Clinically, they are featured by an overabundant deposition of collagen in the dermis and subcutaneous tissues [1]. Patients with hypertrophic scarring often experience serious cosmetic and functional impairment caused by various complications, including compression, sensation of stiffness, loss of joint mobility, and anatomical deformities [2, 3]. Up to now, there are many treatment choices for hypertrophic scarring, including topical silicone gel, laser, and steroid injection [4]. Nevertheless, there remains dismal about the best therapeutic method to completely and permanently erase scars with few side effects. Therefore, it is crucial for us to identify a novel and effective therapeutic strategy for the patients with hypertrophic scars.

Up to now, at least 28 types of collagen have been defined [5-7]. Among them, collagen I is

the major component of the extracellular matrix (ECM), whereas collagen III is the predominant collagen type in the healing wound tissues. Excessive collagen deposition has been implicated in the pathogenesis of hypertrophic scar. Compared with normal skin, collagen synthesis is as much as three times higher in hypertrophic scar and 20 times higher in keloids [8]. Studies showed that the regulation of collagen synthesis is impaired in hypertrophic scars, leading to increased expression of collagen I and collagen III [9, 10].

Connective tissue growth factor (CTGF) is a matricellular protein that regulates cell proliferation, migration, differentiation, angiogenesis and extracellular matrix (ECM) production [11]. Overexpression of CTGF has been observed in chronic fibrotic disorders affecting skin as well as internal organs [12]. Although CTGF shows minimal basal expression in normal skin, it displays transient elevation for several days following dermal injury [13-15]. Besides, persistent overexpression of CTGF has been observed in

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biopsies of keloids and localized sclerosis [16], and fibroblasts isolated from human hypertrophic scars and scleroderma lesions express higher levels of baseline CTGF [17, 18].

As a highly valued traditional Chinese medicine, panax notoginseng saponins (PNS) has attracted more and more attention due to its beneficial biological effects [19]. The effects of PNS have been widely studied and its anti-oxidative, anti-allergic, anti-carcinogenic, and anti-bacterial functions have been well documented [20]. In addition, emerging evidence indicated that PNS exerted a protective role in hepatic fibrosis [21]. However, little is understood about the effect of PNS on hypertrophic scars.

Therefore, in the present study, we aimed to determine whether administration of PNS could attenuate hypertrophic scarring. Furthermore, we explored the potential biochemical mechanism that might be implicated in the anti-scar effect of PNS on hypertrophic scar formation.

### Materials and methods

#### *Animal and grouping*

Fifteen adult female New Zealand white rabbits (each weighing 2.3 to 2.6 kg) were obtained from Shanghai Laboratory Animal Center Co. Ltd. (Shanghai, China). They were fed with standard rabbit feed and water *ad libitum*, and acclimatized for at least two weeks before the commencement of experiments. All animal treatments were strictly in accordance with the international ethical guidelines and the National Institutes of Health Guide concerning the Care and Use of Laboratory Animals, and the experiments were performed with the approval of the Animal Experimentation Ethics Committee of The Second Affiliated Hospital of Kunming Medical University.

#### *Hypertrophic scar rabbit model and drug administration*

The above fifteen rabbits were randomized into three groups (n=5 per group), including Sham group, Model group, PNS group. The rabbits in Model group and PNS group were anesthetized with ketamine (40 mg/kg) and xylazine (5 mg/kg) and prepared for wounding. The rabbit ear model of hypertrophic scars was established as previously described [22]. A full-thickness

circular wound was created down to bare cartilage on the ventral side of each ear using a 1 cm dermal punch biopsy. Six punch wounds were made on each ear of the rabbits. The epidermis, dermis and perichondrium in the wound were then removed using a surgical blade. A total of 120 wounds were made in 20 rabbit ears. At day 14 post wounding, PNS was applied to the wounds of the experimental group (50  $\mu$ L to each wound) every day in concentrations of 2 mg/ml for 7 days, while Model group received equal amounts of 0.9% w/v saline treatment. The average scar area was measured with calipers. At 7, 30 days after PNS treatment, ear hypertrophic scar tissues were obtained from two rabbits of each group, respectively. The scar tissues were diced and rapidly frozen in liquid nitrogen until further processing.

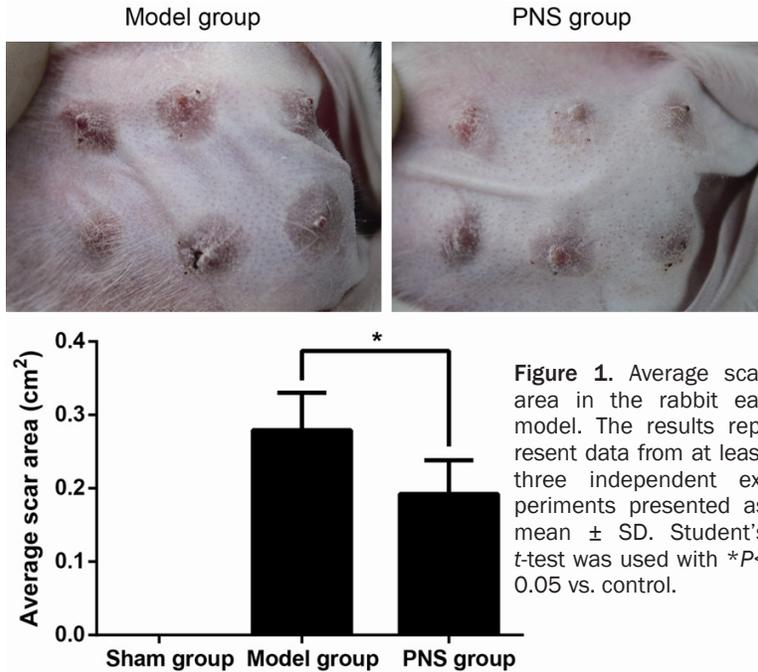
#### *RNA isolation and quantitative RT-PCR*

Total RNA from the scar tissues was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). RNA quantity and quality was assessed using the Nanodrop 2000. To quantify mRNA expression, total RNA was reverse-transcribed into cDNA using oligo dT and an M-MLV kit (Invitrogen, CA, USA). Next, quantitative RT-PCR was performed with the resulting RT product, SYBR Green Dye (Invitrogen) and specific primers on a Roche Light Cycler 480 Real-Time PCR System (Roche, Switzerland). The sequences of the primers were as follows: *ocu-collagen I A2* (Forward 5'-ACGAAGACATCCCACCAATC-3', Reverse 5'-AGATCACGTCATCGCACAAC-3'); *ocu-collagen III A1* (Forward 5'-CCGAACCGTGCCAAATATGC-3', Reverse 5'-CAACAGTGC GGGA-GTAGTT-3'); *ocu-CTGF* (Forward 5'-GAAGCCAGATGCTGAAAC-3', Reverse 5'-TGACCCTTGGA-GACTTTG-3'); *ocu-MMP1* (Forward 5'-GAAGCAGATGCTGAAAC-3', Reverse 5'-TGACCCTTG-GAGACTTTG-3') and *ocu-GAPDH* (Forward 5'-GGCAAAGTGGATGTTGTGCGC-3', Reverse 5'-GCCGTGGGTGGAATCATACT-3'). After the reactions were completed, the CT values were determined by setting a fixed threshold. The gene expression was analyzed by the  $2^{-\Delta\Delta Ct}$  method [23], and normalized to GAPDH, which was the endogenous reference in the corresponding samples.

#### *Histological analysis*

Scar tissues were fixed overnight in 4% formalin solution and embedded in paraffin. Tis-

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**Figure 1.** Average scar area in the rabbit ear model. The results represent data from at least three independent experiments presented as mean  $\pm$  SD. Student's *t*-test was used with  $*P < 0.05$  vs. control.

sue sections were stained with hematoxylin and eosin (H&E) for morphological assessment. The collagen analysis of skin scars were performed using acid fuchsin staining. After staining, the sections were observed under a light microscope.

### *ELISA assay for collagen I and collagen III quantification*

The quantification of collagen I and III was performed using an ELISA Kit (R&D Systems Inc., Minneapolis, MN, USA). In brief, 100  $\mu$ L of standards or samples were added to the appropriate well of the antibody pre-coated microtiter plate, followed by 50  $\mu$ L of conjugate; each well was then covered and incubated for 1 h at 37°C. Next, 50  $\mu$ L of substrates A and B were added to each well and incubated for 10 min at room temperature. Finally, added 50  $\mu$ L of the stop solution was added, and the optical density was read at 450 nm immediately using a Benchmark Plus microplate reader (Bio-Rad, Hercules, CA, USA).

### *Immunohistochemistry*

The paraffin-embedded sections were deparaffinized with xylenes and rehydrated. After treatment with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity, the sections were submerged into citrate buffer

and high-pressure boiled for antigenic retrieval, followed by incubation with 1% bovine serum albumin to block the non-specific binding. Rabbit anti-CTGF (1:200; Millipore Corp., Temecula, CA, USA) or rabbit anti-Collagen I (1:200; Cell Signaling, Danvers, MA, USA) was incubated with the sections overnight at 4°C. For negative controls, the rabbit anti-CTGF or anti-Collagen I antibody was replaced with normal goat serum. After washing, the tissue sections were treated with biotinylated anti-rabbit secondary antibody (Zymed, San Francisco, CA, USA), followed by further incubation with streptavidin-horseradish peroxidase complex (Zymed). The tissue sections

were incubated with 3,3-diaminobenzidine (DAB) and counterstained with hematoxylin, dehydrated and mounted. The sections were reviewed and scored independently by two observers blind to experimental assignments, based on both the proportion of positively stained cells and the intensity of staining.

### *Statistical analysis*

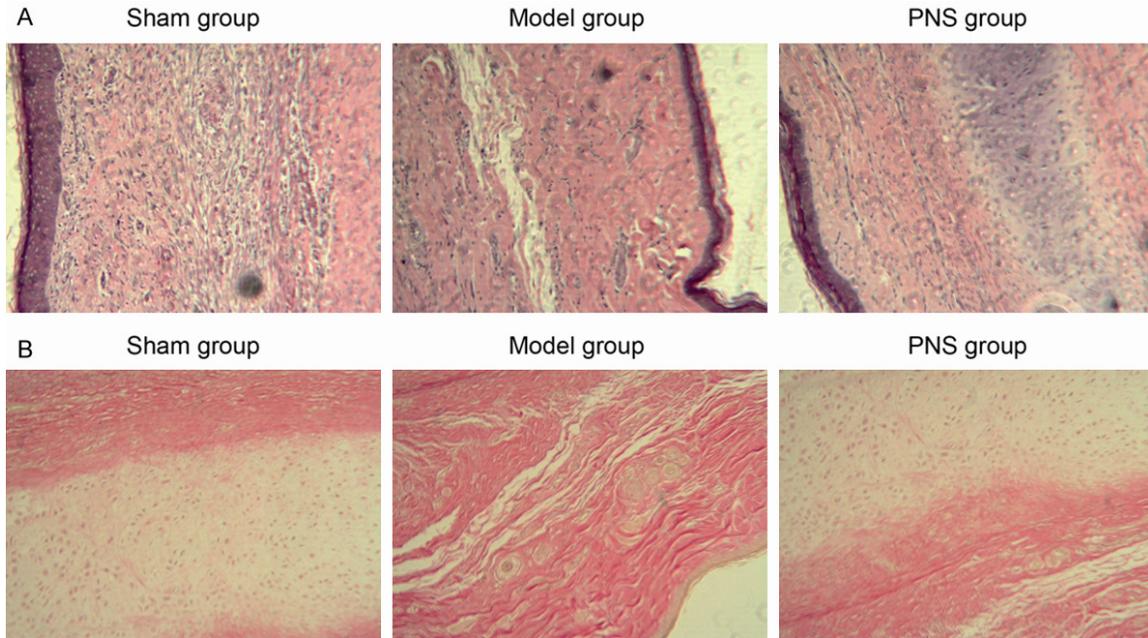
Statistical analysis was performed using GraphPad Prism version 5 software (GraphPad Software, Inc., La Jolla, CA, USA). All results were presented as the mean  $\pm$  SD of at least three independent experiments. A two-tailed Student's *t*-test was used for comparisons of independent groups. *P* values less than 0.05 were considered to indicate a significant result between groups.

## **Results**

### *PNS alleviates the scar formation in the rabbit ear model*

As shown in **Figure 1**, the mean scar area (length\*width/2, cm) in Model group was  $0.287 \pm 0.051$  cm<sup>2</sup>, which was significantly higher than that of PNS group with  $0.192 \pm 0.046$  cm<sup>2</sup> on day 30 after PNS treatment ( $P < 0.05$ ), indicating that PNS obviously suppresses scar formation in the rabbit ear.

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**Figure 2.** Formation of collagen in hypertrophic scar tissue. A: HE staining. B: Acid fuchsin staining.

### *PNS inhibits collagen formation in hypertrophic scar tissue*

Acid fuchsin staining was performed for observation of collagen deposition. As shown in **Figure 2**, compared to Sham group, collagen fibers were dense and disorderly arranged in Model group. By contrast, the deposition was eased and collagen fibers were well arranged following 7 days of PNS administration.

### *PNS suppresses collagen I and collagen III synthesis*

In order to evaluate the molecular effects of PNS on matrix production, ELISA assay was performed to determine the protein levels of collagen I and III, which constitute the bulk of the scar ECM. As expected, the concentration of both collagen I and collagen III in skin tissues was remarkably increased after wounding. Collagen I concentration was significantly decreased on day 7 in PNS group, compared with that in Model group ( $P < 0.05$ ); however, on day 30, the difference of collagen I concentration between Model group and PNS group were not statistically significant (**Figure 3B**). Collagen III concentration was evidently reduced on day 7 and day 30 (all  $P < 0.05$ ) in PNS group, compared with that in Model group (**Figure 3A**). In addition, the collagen density was significantly

reduced in scar tissues treated with PNS compared with the untreated wounds by immunohistochemical staining of collagen I on day 30 ( $P < 0.05$ ; **Figure 3C**).

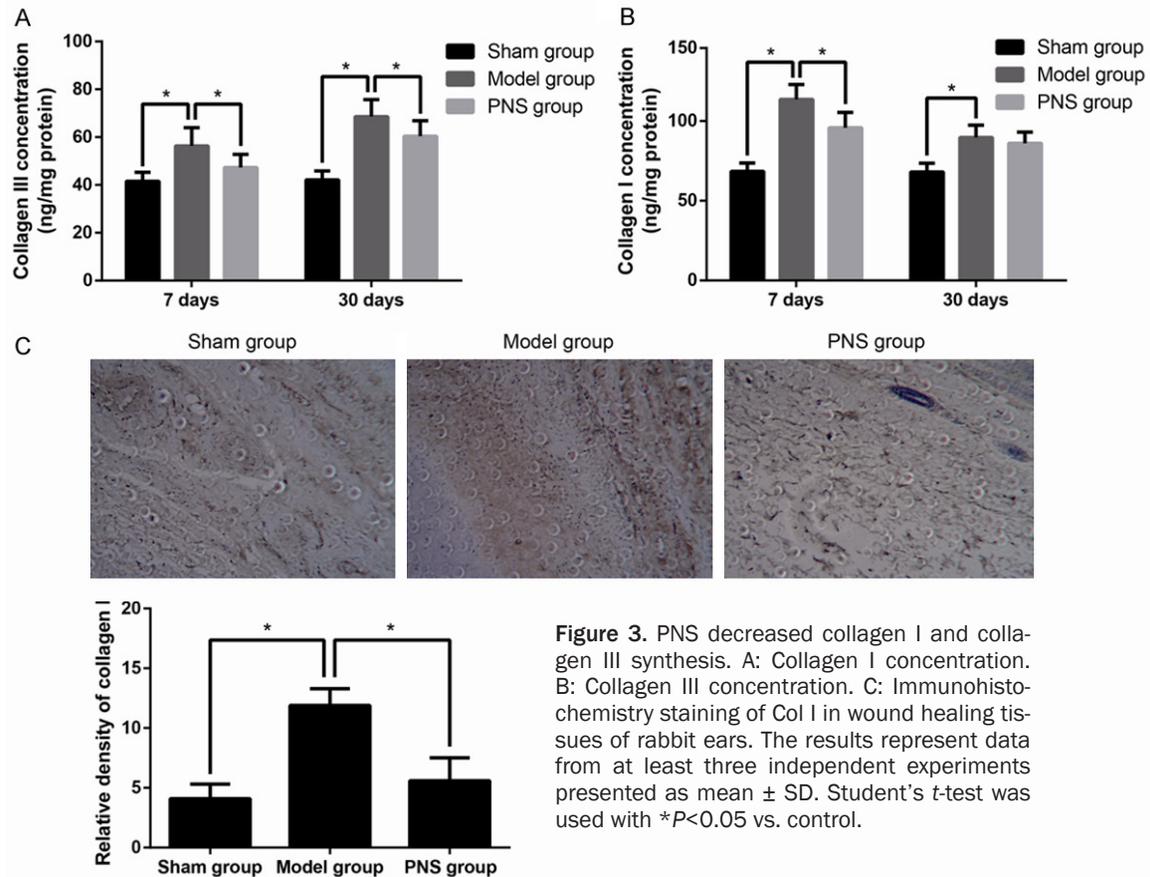
### *PNS suppresses CTGF protein expression*

Compared to normal skin tissues, immunohistochemical detection of CTGF in the untreated scar tissues showed significantly high staining intensity for CTGF. Further, the CTGF-positive cell number in the scar tissues treated with PNS was evidently decreased compared with that in Model group on day 7 (**Figure 4**).

### *Effects of PNS on mRNA expression of collagen I A2, collagen III A1, CTGF and MMP1*

The results of quantitative RT-PCR showed that the mRNA levels of collagen I A2, collagen III A1, CTGF and MMP1 were all noticeably elevated in scar tissues than in normal skin tissues. 7 days of PNS treatment markedly down-regulated the mRNA expression of collagen I A2, collagen III A1, CTGF in scar tissues; however, on day 30, the differences of collagen I A2 and CTGF mRNA expression between Model group and PNS group were not statistically significant (**Figure 5A-C**). The MMP1 mRNA expression increased in scar fibroblasts following 7 days of PNS administration (**Figure 5D**).

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**Figure 3.** PNS decreased collagen I and collagen III synthesis. A: Collagen I concentration. B: Collagen III concentration. C: Immunohistochemistry staining of Col I in wound healing tissues of rabbit ears. The results represent data from at least three independent experiments presented as mean  $\pm$  SD. Student's *t*-test was used with \* $P < 0.05$  vs. control.

### Discussion

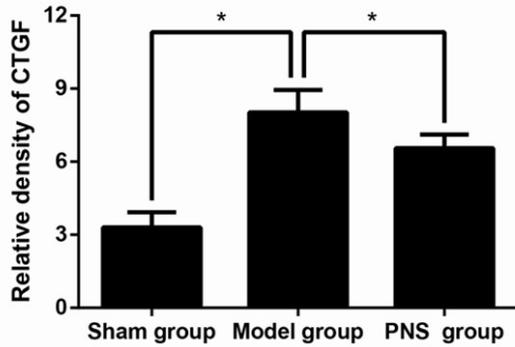
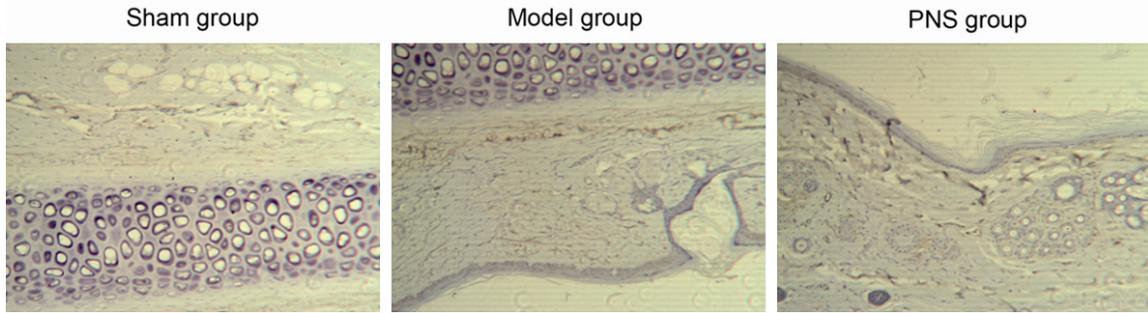
With the rapid development of molecular biology, more and more natural products have been identified as drug targets to treat hypertrophic scarring. In this study, a rabbit ear model mimicking the scarring process was established, and we found that PNS administration could inhibit excessive collagen synthesis and then inhibit the conformation of hypertrophic scars through decreasing the expression of CTGF and collagen-related mRNAs, including collagen I A2 and collagen III A1, and up-regulating the expression of MMP1.

During wound healing, the production of collagen can be a double-edged sword: on the one hand, it is necessary for the healing process; on the other hand, overproduction and excess deposition of collagen may lead to overhealing outcomes, such as scar formation [24, 25]. Hence, the balance between collagen synthesis and degradation is required for healing of wounds. Our findings indicated that PNS can accelerate wound healing with increasing col-

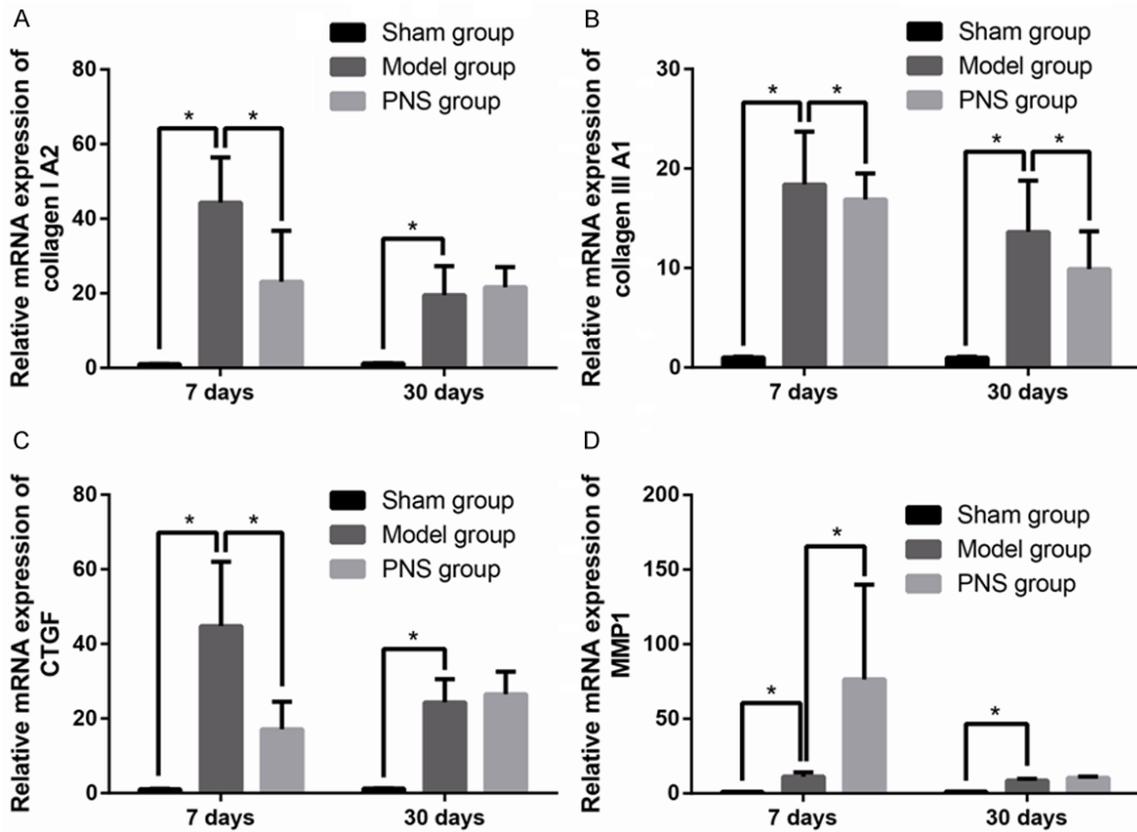
lagen production and subsequent collagen deposition. In the rabbit hypertrophic scarring model, PNS resulted in a sparse arrangement of the collagen, similar to normal skin collagen distribution, and reduced the production of collagen I and collagen III. These results support the alternative effect of PNS on collagen expression in the different stages of wound healing, which is beneficial for wound closure and scar diminution. Similarly, a study of Yao *et al.* [26] revealed the potential effects of PNS to accelerate wound healing and inhibit scar formation through regulating the balance of collagen synthesis and degradation.

Various moleculars are important components in the process of collagen synthesis. For example, CTGF plays an important role in promoting fibrosis and scarring in numerous tissues [27, 28]. Overexpression of CTGF has been shown to promote fibrosis and scar formation in skin, kidney, liver, brain, lung, vasculature and pancreas [28-30]. Because TGF- $\beta$  induces CTGF potentially through several pathways, CTGF has been considered to mediate many of its fibrotic

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**Figure 4.** PNS decreased CTGF expression in scar tissues by immunohistochemistry. The results represent data from at least three independent experiments presented as mean  $\pm$  SD. Student's *t*-test was used with  $*P < 0.05$  vs. control.



**Figure 5.** Effects of PNS on mRNA levels of (A) collagen I A2, (B) collagen III A1, (C) CTGF and (D) MMP1. GAPDH was used as loading control. The results represent data from at least three independent experiments presented as mean  $\pm$  SD. Student's *t*-test was used with  $*P < 0.05$  vs. control.

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effects. Indeed, previous studies have demonstrated roles of CTGF in the TGF- $\beta$ -dependent induction of fibronectin, collagen, and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) [11, 17, 31]. Hu *et al.* [32] reported that CTGF could induce hypertrophic scar fibroblast differentiation and collagen synthesis.

MMP1 is the main enzyme that degrades collagen I and collagen III in scar tissues and serves a critical role in physiological processes of tissue remodeling [33, 34]. In our study, following PNS administration, MMP1 mRNA expression was remarkably elevated in PNS group than in Model group. In other studies, such as the one using topical oleanolic acid to suppress hypertrophic scarring in the rabbit ear model, the MMP1 levels were higher in the control group than in the experimental group. Although this is difficult to explain, it may be due to the variable effects of the agents used on MMP1 and the biofeedback regulating mechanisms in wounds in vivo since a higher production of MMP1 is induced in wounds with higher collagen content [35].

In summary, our studies have demonstrated that PNS could inhibit the formation of collagen I and collagen III, and then repress the conformation of hypertrophic scarring through down-regulating the expression of CTGF and collagen-related gene, including collagen I A2 and collagen III A1, and up-regulating the expression of MMP1. Thus, our findings suggest that PNS might be a novel and effective agent for the patients with hypertrophic scarring.

### Disclosure of conflict of interest

None.

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