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Compound *Astragalus* and *Salvia miltiorrhiza* Extract Suppresses Rabbits' Hypertrophic Scar by Modulating the TGF-β/Smad Signal

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Key Words
CASE · Hypertrophic scar · TGF-β_1 · Smads · Animal experiment

Abstract
Background: Hypertrophic scar is a fibro-proliferative disease. Our previous studies demonstrate that compound *Astragalus* and *Salvia miltiorrhiza* extract (CASE) inhibits proliferation and invasion in keloid fibroblasts. ***Objective:*** To investigate the effects of CASE on hypertrophic scar. ***Methods:*** Rabbits were divided into the control, model and three dosage groups of CASE (0.94, 1.88, 3.76%). An animal model of hypertrophic scar was established and treated with CASE ointment or ointment base. The histopathological detection by hematoxylin & eosin and Masson's trichrome staining and protein expression of scars by Western blot were performed. ***Results:*** The hydroxyproline content was decreased under CASE treatment. Transforming growth factor beta 1 (TGF-β_1) protein expression increased in the model group while it decreased under CASE treatment. The elevated expression of Smad4 protein was decreased under CASE treatment. Additionally, CASE promoted Smad7 protein expression. ***Conclusion:*** CASE could inhibit formation of hypertrophic scar by modulating TGF-β/Smad signal and may be useful for the treatment of hyperplastic scars.

Introduction

Hypertrophic scarring is a common proliferative disorder of dermal fibroblasts characterized by collagen overproduction and excessive deposition of extracellular matrix and occurs in healing wounds elicited by trauma, inflammatory reactions, and deep burn [1, 2]. As a result of aberrant wound healing, hypertrophic scar is characterized by a raised, rigid and red appearance associated with pain and itch [3]. It will lead to cosmetic disturbance and affect the daily activities of a person if the scar is contracted causing joint stiffness and deformities [3]. The molecular mechanisms of hypertrophic scars are not accurately understood, though the ratio imbalance of matrix metalloproteinases (MMPs) degrading collagen and tissue inhibitors of metalloproteinases (TIMPs) mediated via the transforming growth factor beta (TGF-β)/Smad pathway is a widely accepted mechanism for fibrosis [4–6].

Compound *Astragalus* and *Salvia miltiorrhiza* extract (CASE) is made up of astragalosides, astragalus polysaccharide and salvianolic acids extracted from *Astragalus membranaceus* Bunge (Leguminosae) and *S. miltiorrhiza*

C. Wu and J. Jiang contributed equally to this work.

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Bunge (Lamiaceae) with a standard ratio [7]. Our previous in vitro study demonstrated that CASE significantly inhibited the proliferation, collagen synthesis and cell invasion in keloid fibroblasts by affecting TGF-β/Smad signal transduction [8, 9]. Characteristically, hypertrophic scar, a kind of fibro-proliferative disease with collagen overproduction and excessive deposition, shares pathological features with human keloids [10]. This study was designed to investigate whether CASE can reduce or eliminate formed hypertrophic scars in vivo and to elucidate the possible mechanism of action of CASE, especially its effects on the role of the TGF-β_1/Smad signaling pathway in hypertrophic scars pathogenesis.

Methods

Preparation of CASE

The herbs *A. membranaceus* Bunge (Leguminosae) and *S. miltiorrhiza* Bunge (Lamiaceae) were purchased from Bozhou Crude Drug Market (Anhui, China), and astragalosides, astragalus polysaccharide and salvianolic acids were made into powers with a standard ratio (70:1:1.85) in weight of crude herbs. CASE was prepared as described previously [7]. Three kinds of concentration CASE ointment (0.94, 1.88, 3.76%; w/w) were prepared by adding Vaseline, liquid paraffin, glycerinum and distilled water. The placebo ointment was identical to the CASE ointment but without three CASE components.

Animal Experiments and Treatment

Thirty female New Zealand white rabbits, obtained from Nanjing Jing Ling Zhong Tu Chang (Nanjing, China) and with an initial body weight of 2.5 ± 0.2 kg, were used. All animals were kept under constant conditions (temperature 25 ± 1°C) and had free access to a standard diet and drinking water. 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 carried out with the approval of the Animal Experimentation Ethics Committee of Anhui Medical University.

Rabbits were randomly distributed into five groups, the control group, the model group, and three CASE groups (0.94, 1.88, 3.76%); six rabbits in each group. Hypertrophic scars were induced on the ventral surface of rabbit ears as previously described [11, 12]. Briefly, the rabbits were anesthetized with ketamine (45 mg/kg), and then cycloid wounds 1 cm in diameter were created down to bare cartilage and dislodged the perichondrium on the rabbit's ear, four wounds in each ear. Following closely, each wound was fully covered by a Tegaderm dressing until the total wound appeared epithelized. Then the Tegaderm dressing was removed at the end of the third week. The scars of the model group were thinly daubed with placebo cream twice a day; the three CASE groups were applied corresponding ointment with CASE twice a day from the 4th week to the end of the 7th week. In the control group, the rabbits were given sham operation on their ears and administrated only with placebo cream treatment.

At the end of 7th week, all animals were anesthetized and the hypertrophic scars and the corresponding parts in the control group resected.

Determination of Hydroxyproline Content

The hydroxyproline (Hyp) content of the rabbit ear tissue was utilized to assess fibrosis, determined according to the method described previously [13]. Briefly, scar tissue was hydrolyzed, oxidized by H_2O_2 and colored by p-dimethylaminobenzaldehyde, and absorbance was tested at 540 nm. The amount of Hyp was expressed as μg/mg tissue.

H&E Staining and Determination of Scar Elevation Index (SEI)

Scar tissues were embedded in paraffin and cut in 4-μm sections for H&E and Masson's trichrome staining. H&E staining was performed as described previously [14]. Briefly, the tissue sections were stained in hematoxylin dye, washed in running water for blue, then stained using eosin and finally mounted under coverslips. The pathological features were observed and the degree of scar hyperplasia was expressed as SEI, defined as the ratio of the scar tissue height to the normal tissue below the hypertrophic scar [15, 16]. An SEI ratio of 1 meant that there was no difference between comparative groups.

Masson's Trichrome Staining

The degree of fibrosis on hypertrophic scars was estimated by Masson's trichrome staining using Masson's trichrome stain kit (MST-8003, MAIXIN-Bio, Fuzhou, China) according to the manufacturer's instructions. Briefly, the tissue was dewaxed, rehydrated, stained in Masson's compound staining fluid (including Weigert's iron hematoxylin and Biebrich's scarlet-acid fuchsin) for 5 min, washed in distilled water, placed in phosphomolybdic acid for 5 min and spun dry, stained in aniline blue solution for 5 min, rinsed in 1% acetic acid solution for 60 s and repeated. Finally the tissue was dehydrated, cleared and sealed. Collagen deposition was evaluated by the Image Pro Plus 4.5 software and the relative density of collagen in each group was counted by normalizing to the collagen density in the control group [17].

Western Blot Analyses

The scar tissue was homogenized in cell lysis buffer (P0013, Beyotime, China) for Western blot described in detail previously [8]. The primary antibodies used were goat polyclonal anti-Smad7 and mouse monoclonal anti-Smad4 obtained from Santa Cruz Biotechnology (Santa Cruz, Calif., USA) and mouse monoclonal glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody obtained from Cell Signaling Technology (Beverly, Mass., USA). GAPDH protein was as internal control. Results were analyzed by the Image J software (National Institutes of Health, Bethesda, Md., USA) [18].

Statistical Analyses

Data are presented as means ± standard deviation (SD), n = 6. Statistical analyses were performed by the SPSS software version 13.0 (SPSS Inc., Chicago, Ill., USA) for Windows. The experimental and control groups were compared by one-way ANOVA. $p < 0.05$ was deemed to be statistically significant.

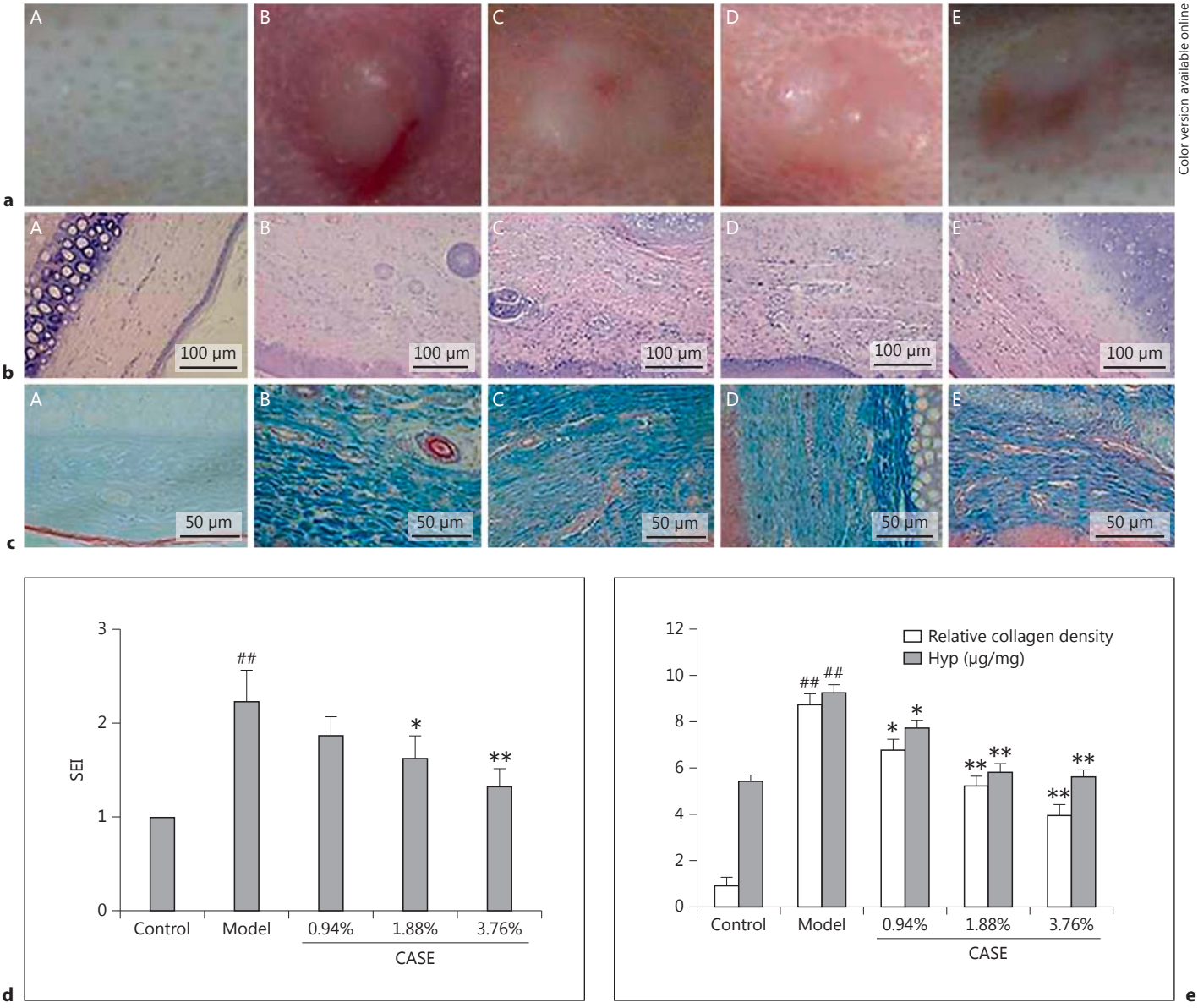

Fig. 1. Effects of CASE on hypertrophic scar in rabbits. **a** Typical scar hyperplasia was observed at the end of the 7th week. **b** H&E staining for histologic analysis of hypertrophic scars. **c** Masson's trichrome staining for collagen fibers. **d** The degree of scar hyperplasia was expressed as SEI. **e** The degree of fibrosis was assessed by using relative collagen density and Hyp content. A = Control group; B = model group; C–E = CASE treatment groups (0.94, 1.88, 3.76%). Error bars represent SD. Columns represent mean ± SD, n = 6. ## $p < 0.01$ compared with control group; * $p < 0.05$, ** $p < 0.01$ compared with model group.

Results

Effects of CASE on Hypertrophic Scars in Rabbits

A visibly raised and evident scar in a primary wound stage initiated gradually after 3 weeks post-operation. Appearance of representative wounds in each group was assessed at the end of the 7th week (fig. 1a), which showed that hypertrophic scars from the model group were highly elevated from surrounding tissue, while scars in the treatment groups were flatter than those in the model group. H&E staining of the rabbit ear scar sections revealed manifest incrassation of the dermic layer with ridges, angiogenesis and irregular fibroblasts in the model group, however this was evidently ameliorated in the CASE treatment groups in a dose-dependent manner (fig. 1b).

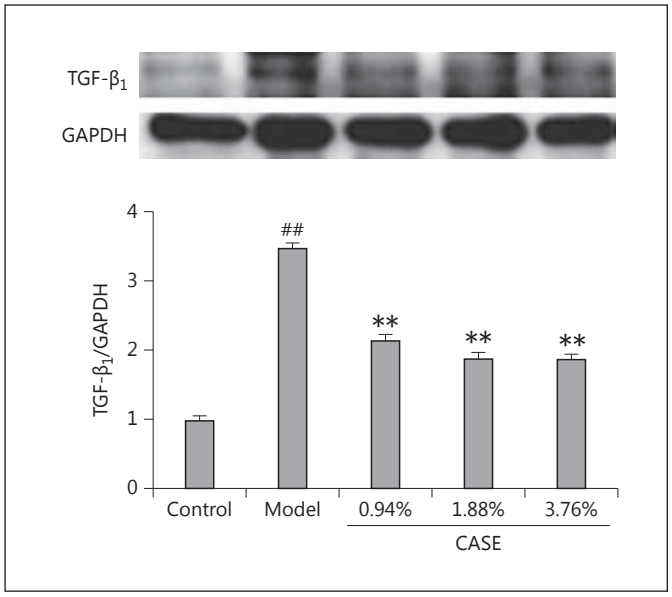

Fig. 2. Effects of CASE on TGF-β_1 protein expression. The total protein was extracted from rabbit ear tissue. TGF-β_1 was analyzed by immunoblotting using anti-TGF-β_1 and anti-GAPDH antibodies. The intensities of TGF-β_1 bands were normalized to GAPDH of the corresponding treatment groups. The ratio of TGF-β_1 to GAPDH in the control group is assigned a value of 1. Columns represent mean ± SD, n = 6. ## $p < 0.01$ compared with control group; ** $p < 0.01$ compared with model group.

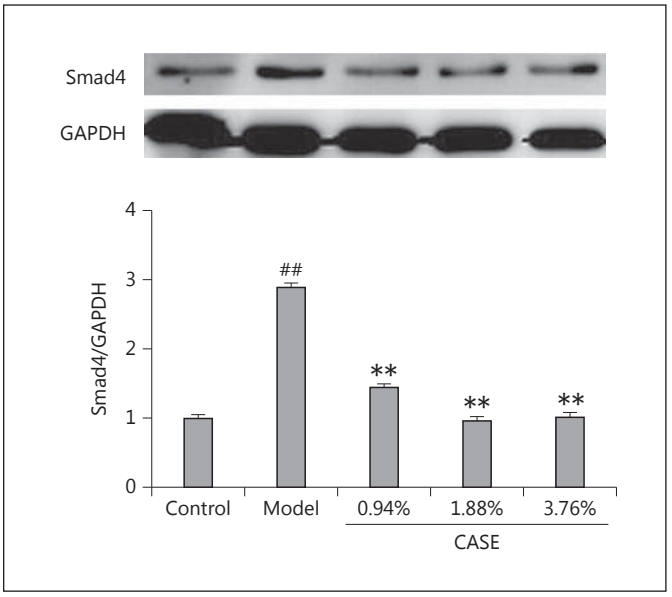

Fig. 3. Effects of CASE on Smad4 protein expression. The total protein was extracted from rabbit ear tissue. Smad4 was analyzed by immunoblotting using anti-Smad4 and anti-GAPDH antibodies. The intensities of Smad4 bands were normalized to GAPDH of the corresponding treatment groups. The ratio of total Smad4 to GAPDH in the control group is assigned a value of 1. Columns represent mean ± SD, n = 6. ## $p < 0.01$ compared with control group; ** $p < 0.01$ compared with model group.

Figure 1c shows typical rabbit ear scar sections stained with Masson's trichrome staining. The collagen fibers were denser, thicker and more disordered than those of the control group, and we observed typical bundle-shaped collagen fibers in the model group, while there was a marked improvement in treatment groups. In addition, collagen deposition areas, estimated by the Image Pro Plus 4.5 software (fig. 1e), showed that collagen deposition decreased quite significantly under CASE treatment in a dose-dependent manner. Figure 1 shows that both SEI and Hyp content obviously increased in the model group scars, while CASE treatment was able to decrease the elevated SEI and Hyp content in a dose-dependent manner, indicating that CASE can inhibit the synthesis or accelerate the degradation of collagen.

Effects of CASE on TGF-β_1 Protein Expression

Figure 2 shows the expression of TGF-β_1 protein extracted from frozen rabbit ear tissue. The expression of TGF-β_1 protein in the model group markedly increased, while CASE treatment dose-dependently decreased TGF-β_1 protein expression.

Effects of CASE on Smad4 Protein Expression

Figure 3 shows the expression of total Smad4 protein extracted from rabbit ear tissue. In the model group, the expression of Smad4 protein increased significantly compared with that of the control group, while the CASE treatment decreased the elevated Smad4 protein in the hypertrophic scarring model of rabbit ears.

Effects of CASE on Smad7 Protein Expression

As shown in figure 4, in the model group the expression of Smad7 protein extracted from rabbit ear tissue evidently decreased compared to the control group; however, following CASE treatment Smad7 protein expression increased, especially in the 3.76% CASE treatment group.

Discussion

Overproduction of fibroblasts commonly occurs in the process of wound repair, leading to the formation of scars, including hypertrophic scars and keloids [19].

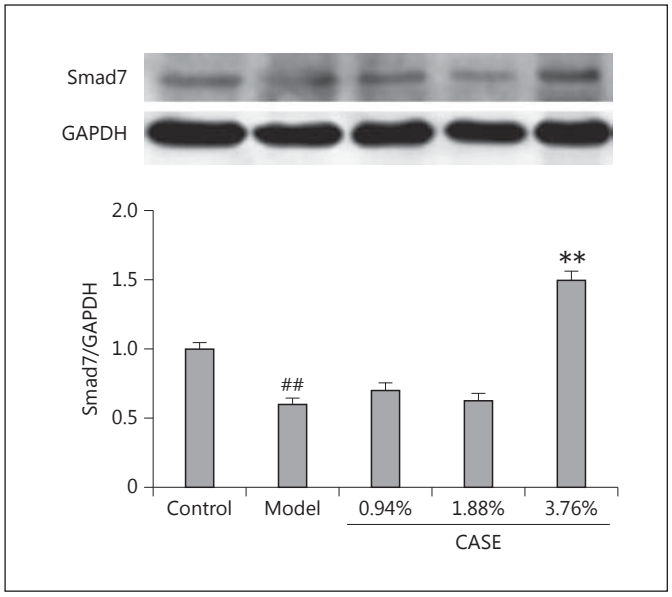

Fig. 4. Effects of CASE on Smad7 protein expression. The total protein was extracted from rabbit ear tissue. The expression of Smad7 was monitored by immunoblotting using anti-Smad7 antibody. The intensities of Smad7 bands were normalized to GAPDH of the corresponding treatment groups. The ratio of Smad7 to GAPDH in the control group is assigned a value of 1. Columns represent mean ± SD, n = 6. $^{\#\#}$ $p < 0.01$ compared with control group; ** $p < 0.01$ compared with model group.

Many reports have shown that hypertrophic scar fibroblasts have a very strong capacity to induce proliferation, production of cytokine and synthesis of collagen [11, 14, 20]. In our previous study CASE was shown to inhibit cell proliferation and invasion in human keloid fibroblasts [9]. In the present study we successfully established an animal model of hypertrophic scars on rabbit ears by incision wounding, in which each wound ensured removal of full-thickness skin and perichondrium [11]. Histopathological assessment showed that CASE significantly ameliorated fibroplasia and angiogenesis in the dermis. Additionally, CASE was able to decrease Hyp content in a dose-dependent manner. Hyp is one of the known derogative products of collagen protein [21], thus a decrease in Hyp content under CASE treatment is indicative of an inhibitory effect of CASE on collagen deposition and scar hyperplasia in general.

Further studies found that the inhibition by CASE of hypertrophic scar formation was associated with a cytokine termed TGF-β, especially TGF-β_1. Previous studies have shown that TGF-β_1 is one of the most important cytokines participating in wound healing and overproduced in hypertrophic scars [1, 24]. In addition, TGF-β_1 is also one of the most vital signal molecular to initiate TGF-β/Smad pathway, which regulates a variety of biological effects such as cell invasion, proliferation, differentiation and apoptosis [9, 24]. The activation of the TGF-β/Smad pathway promoted the production of fibroblasts, enhanced the synthesis and inhibited the degradation of collagen [5, 24]. Our current results indicate that the expression of TGF-β_1 protein notably increased in the model group compared to the control group, while it decreased under CASE treatment in a dose-dependent manner (fig. 2). Our results further demonstrate that CASE improved scar hyperplasia by inhibiting overexpression of TGF-β_1 protein.

TGF-β_1 transmits its signals mainly via the Smad family. The signaling cascade involves that TGF-β_1 combines with TGF-β receptor II, then activates TGF-β receptor I on the cell membrane, the activated TGF-β receptor I phosphorylates receptor-regulated Smads (Smad2, Smad3), then phosphorylated Smad2/3 form a complex with Smad4 and are transferred into the nucleus, resulting in target gene expression [5, 9]. The present study showed that the expression of Smad4 protein of the hypertrophic scar increased markedly in rabbits, which is different from the previous unchanged Smad4 protein in keloid fibroblasts induced by TGF-β_1 for a short time [8, 9]. The reason might be that Smad2 and Smad3 phosphorylation promoted the expression of Smad4 protein during the pathological progression of hypertrophic scar and then formed the complex of Smad2/3/4 [5, 9].

In summary, TGF-β signaling is regulated by multiple factors mainly including inhibitory Smads (containing Smad6 and Smad7) in cells [22, 23]. Our previous study also found that the expression of Smad7 protein, which negatively mediates TGF-β signal transduction, increased in human keloid fibroblasts under CASE treatment in the dose-dependent manner [9]. Our current results showed that CASE could up-regulate the expression of Smad7 in hypertrophic scar (in vivo), corroborating our previous observation in keloid fibroblasts (in vitro) [9]. All put together, CASE decreased collagen deposition and hyperplasia of hypertrophic scars partly by up-regulating inhibitory Smad (Smad7) expression.

In conclusion, CASE could inhibit hypertrophic scar by modulating the TGF-β/Smad pathway and must be considered as an investigational or candidate drug for further clinical trials, since it could be useful for the treatment of keloid and hyperplastic scar in humans in the near future.

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Disclosure Statement

The authors have no potential conflicts of interest.

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