

## Original Research Article

# Suppressive effect of bacoside A on hypertrophic scar formation by downregulation of TGF- $\beta$ 1

Zimiao Chen<sup>1</sup>, Lina Lu<sup>2\*</sup>

<sup>1</sup>Department of Burn Surgery, Yuncheng Central Hospital, Yuncheng, <sup>2</sup>Institute of Hematological Diseases, The Third Hospital of Yuncheng, Shanxi 044000, China

\*For correspondence: **Email:** LAngelukit@yahoo.com; **Tel:** 0086-1860359024

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### Abstract

**Purpose:** To determine the anti-scarring property of bacoside A (BA) in a rabbit model of post-burns hypertrophic scar (HS).

**Methods:** A total of 16 healthy male rabbits were divided into four groups (4 rabbits/group). The rabbits were subjected to full-thickness burn wound by placing a 10-mm brass rod at 90 °C on their ears for 20 s, and were untreated (HS group). The remaining rabbits also underwent HS process but were topically treated daily with 0.2, 0.4 or 0.6 % BA gel (HS + 0.2, 0.4, and 0.6 % BA groups) from day 8 to day 34.

**Results:** The mean values of collagen (types I & III), hydroxyproline (precursor of collagen) and hexosamine, as well as scar index significantly ( $p < 0.001$ ) decreased after 28 days of topical treatment with BA. Moreover, BA application resulted in improved histological changes with proper arrangement of collagen fibers. Significant ( $p < 0.001$ ) upregulation or downregulation of transforming growth factor beta-1 (TGF- $\beta$ 1) and matrix metalloproteinase-1 (MMP-1) were observed in the BA-treated rabbits.

**Conclusion:** Treatment with BA significantly lowers the scarring area of burn wounds by downregulation of TGF- $\beta$ 1. Thus, BA can potentially be developed into a drug for managing injury scars.

**Keywords:** Hypertrophic scar, Bacoside A, Scar elevation index, Transforming growth factor beta-1, Collagen

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## INTRODUCTION

Hypertrophic scar (HS) reflects the dysfunctional response from the skin during injury especially burns and surgery, which leads to excessive deposition of fibroblast and collagen and thick elevated scar/fibrosis [1]. Post-burn HS presents an emotional and physical burden on the patient owing to excessive pain, pruritus, discomfort, disability (joint contracture) and disfigurements [2]. Epidemiological studies have indicated that

about 40 – 70 % of thermal injury (burn wound) result in HS. Thus, there is need for its treatment as soon as possible [3]. To date, there are no effective treatment procedures or protocols for managing post-burn HS. However, a few treatment strategies (sub-optimal treatments) are employed efficiently to manage HS. These include silicone gel sheeting, massage therapy, laser surgery, and peptide therapy with natural products (combination therapy) [4].

The pathophysiology of HS is poorly understood. However, an ample amount of evidence implicate transforming growth factor beta-1 (TGF- $\beta$ 1) and matrix metalloproteinase-1 (MMP-1) in HS formation because they are directly involved in collagen formation and deposition [5]. Therefore, any natural drug that can modulate these proteins may be beneficial for suppressing HS formation or for scar management. Bacoside A (BA) is a triterpenoid saponin isolated from *Bacopa monniera* L. (syn. *Herpestis monniera*). Several studies have reported that BA possesses various biological properties [6,7]. It exerts many pharmacological properties such as anti-inflammatory, anti-stress, anti-oxidant, anti-microbial, neuroprotective, hepatoprotective and renoprotective effects [8-10]. It co-exists with bacoside B (BB). Being isomers, the two saponins differ only in their optical rotation (spatial configuration) [11].

Previously, Sharath *et al* [12] demonstrated that topical treatment with BA exhibited better wound healing property than standard dermal ointment (nitrofurazone). Moreover, saponins influence balance in the secretion of protease inhibitor in various models, and so might contribute to anti-scarring property [13,14]. Therefore, the present animal study was designed to investigate the anti-scarring effect of BA in the rabbit ear scarring model by assessing various biochemical indices such epidermal thickness index (ETI) and scar elevation index (SEI), and collagen levels (Types I and III). In addition, glycosaminoglycans (hexosamine), protein levels of MMP-1 and TGF- $\beta$ 1 and histopathological alterations were studied.

## EXPERIMENTAL

### Chemicals and BA gel preparation

Lysis buffer, xylene, formaldehyde, toluene, hematoxylin and eosin (H & E), and acetone were bought from Sigma-Aldrich (MO, USA). Other chemicals and reagents used in this study were of HPLC or analytical grade. The different concentrations of BA (for topical application) were prepared as reported by Sharath *et al* [12]. Different concentrations of BA gel (0.2, 0.4 and 0.6 % w/w gel) were prepared by adding 100, 200 and 300 mg of BA (each) to 50 g of sodium alginate.

### Experimental animals

A total of sixteen male healthy adult New Zealand Albino rabbits weighing  $2.4 \pm 0.4$  kg were procured from the animal center at Shanxi University. The rabbits were housed in a

polycarbonate cage, and were maintained under standard laboratory conditions (22 - 25 °C; 55 % humidity) with 12-h light/dark cycle, and unlimited access to water and food. This study was approved by the ethical committee of The Third Hospital of Yuncheng (approval no. THY-15-0052). All the animal procedures/protocols were performed based on NIH guidelines.

### Procedure for HS production

Thermal injury was used for inducing HS in line with the method of Friedrich and his colleagues [15], but with slight modifications. The rabbits were anesthetized with meloxicam (0.2 mg/kg) and xylazine (5 mg/kg) under sterile conditions. On the contralateral ear, four 10-mm deep, full-thickness thermal wounds (burn wound) were made by placement of 10-mm brass rod at 90 °C for 20 s using Fisher dry bath incubator (Fisher Scientific, PA, USA). Each ear received 4 burn wounds with full removal of epidermis, dermis and perichondrium. All the thermal wounds were covered with a sterile Tegaderm occlusive dressing for the first three days to prevent cartilage desiccation. After the HS procedure, the rabbits were returned to their respective cages (after recovery from anesthesia) and placed with an Elizabethan collar to avoid wound infection between rabbits. They were inspected daily for topical application and also to confirm any infection or any discomfort.

### Experimental design

Sixteen rabbits were divided into four groups (4 in each group) with 32 scars in each group. Group I rabbits were inflicted 10-mm deep full-thickness thermal wound as indicated above without any treatment, and they formed the HS group. Groups II, III and IV rabbits also underwent HS procedure but were topically treated daily with 0.2 or 0.4 or 0.6 % BA gel from the 8<sup>th</sup> day to the 34<sup>th</sup> day.

### Sample preparation

On the 35<sup>th</sup> day, overnight fasted rabbits were sacrificed by cervical decapitation. The scar samples were harvested immediately from each rabbit (8 scars in each rabbit) and stored at -80 °C until use. For histological analysis, a small portion of scar tissue was fixed in 10 % formaldehyde. The remaining scar tissues were homogenized with Tris-phosphate buffer (lytic buffer) and centrifuged at 3500 rpm for 15 min at 4 °C, and the resultant supernatant was used for biochemical and molecular assays. Protein levels in the scar tissue homogenate were determined

using a protein quantification kit (Bradford Assay) purchased from Abcam (Cambridge, UK).

### Determination of collagens and glycosaminoglycans

Collagen types I and III contents in the scar tissues were determined using commercial ELISA kit purchased from R & D Systems (MN, USA) based on the supplier's protocol, and expressed as mg/g scar tissue. Hexosamine and hydroxyproline levels in the scar tissues (homogenate) were estimated by the methods of Wagner [16] and Woessner [17], respectively. Both hexosamine and hydroxyproline were expressed as mg per 100 mg scar tissue.

### Histological evaluation

The formaldehyde-fixed scar tissues were dehydrated and processed with xylene and acetone, followed by embedding in paraffin wax. They were then sectioned into 4- $\mu$ m diameter slices using a microtome, fixed in microscope slides and stained with H & E stain for 8 h at 37°C. Finally, the scar tissue slides were viewed using light microscopy by a professional pathologist to assess any histological changes. Pictures of salient features were captured by the pathologist.

### Assessment of scar index

Scar elevation index (SEI) and epidermal thickness index (ETI) were determined based on hematoxylin and eosin (H & E) staining images captured by light microscope (Olympus Co., Tokyo, Japan) and analyzed with ImageJ software (ver 5.1; NIH, MD, USA). The images were graded by the method of Zhao *et al* [26]. Here, SEI corresponded to the ratio of total scar area (wound area) to the area of underlying dermis formed (normal tissue). An index >1 indicated HS formation, but an index of 1 meant no newly formed HS. On the other hand, ETI, represented by the degree of hypertrophy, was calculated as the ratio of mean epidermal height (cm) of scar tissue to mean epidermal height (cm) of normal tissue. An ETI > 1 indicated hypertrophic epidermal formation.

### Quantification of MMP-1 and TGF- $\beta$ 1 by western blot

An equal quantity (40  $\mu$ g) of protein extract of scar tissue homogenate was transferred onto polyacrylamide gel (10%) and separated by SDS-PAGE technique. Then the gel was electrotransferred onto polyvinylidene difluoride (PVDF) membrane. The membrane was blocked

using Tris-phosphate buffer solution (TPBS) with tween 20 and 5 % skimmed milk. It was then probed for 10 h at 37°C with primary antibodies: rabbit monoclonal anti-matrix metalloproteinase-1 (MMP-1) and anti-transforming growth factor beta-1 (TGF- $\beta$ 1) at dilutions of 1:1000 and 1:800, respectively (Zhongshan Biotechnology, Beijing, China) and housekeeping gene  $\beta$ -actin (1:800; Zhongshan Biotechnology, Beijing, China). This was followed by incubation with secondary antibody conjugated with polyclonal anti-rabbit horseradish peroxidase (HRP) antibody (1:10,000; Abcam, Cambridge, UK) for 1h at 37 °C. To remove unbound antibodies, the membrane was washed with TPBS solution. The protein bands were visualized with an enhanced ECL kit and photographed using chemiluminescent image analyzer (ChemiDoc; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The specific proteins were quantified using ImageJ software (5.1 ver) from NIH (MD, USA).

### Statistical analysis

Data are presented as mean  $\pm$  standard deviation (SD, n = 4)). Significant differences between HS and BA treatment groups at different BA concentrations were determined using one-way ANOVA, and by Dunnett's multiple comparison test. All statistical analyses were done using Statistical Package for the Social Sciences (Ver 21, IBM, NY, USA). Values of  $p < 0.05$  were considered to indicate statistically significant difference.

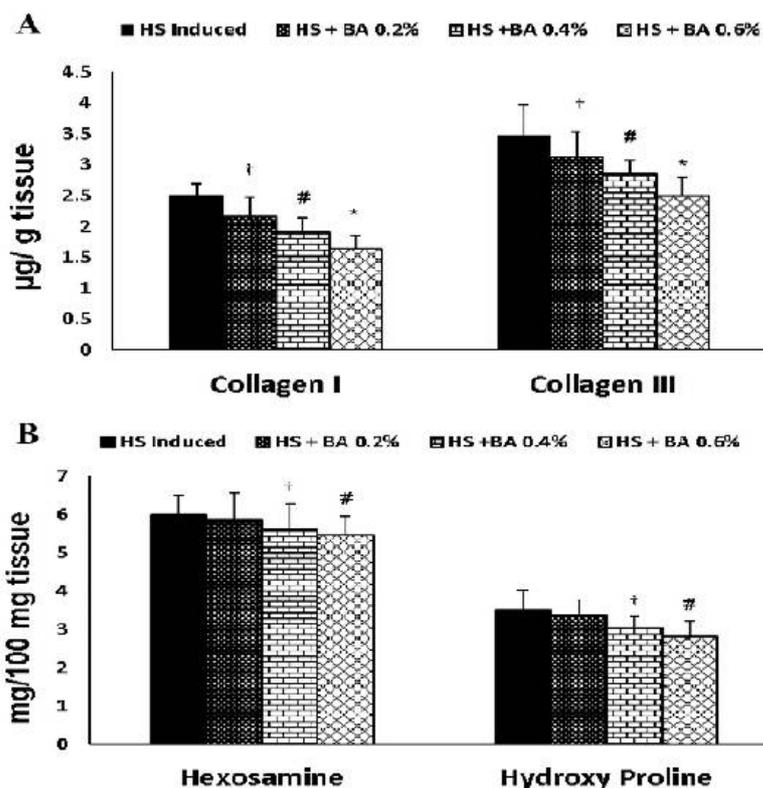
## RESULTS

### Collagens and glycosaminoglycans

The effect of BA on collagen types I and III, and glycosaminoglycans in the experimental rabbits are shown in Figure 1A and Figure 1B, respectively. The values of collagen (types I & III), hydroxyproline (precursor of collagen) and hexosamine were significantly decreased ( $p < 0.05$ ) by topical application of BA in a concentration-dependent manner, when compared to HS group.

### Histopathological changes

Figure 2 illustrates the effect of BA on scar tissue histo-morphology as revealed by H & E staining. The scar tissue section of HS rabbits (positive control) revealed the presence of excessively scattered fibroblasts with an irregular arrangement of collagen fibers leading to thickened dermal layers (projection). However, in the 0.2 % BA-treated rabbit scar tissue (Figure 2 B), there were moderate amounts of fibroblasts



**Figure 1:** Effect of BA on collagen types (I and III; A) and glycosaminoglycans (B) in the experimental rabbits. The values are presented as mean  $\pm$  SD (n = 4). † $p < 0.05$ , # $p < 0.01$ , \* $p < 0.001$ , compared with BA treatment group (HS vs. 0.2 or 0.4 or 0.6 % BA gel)

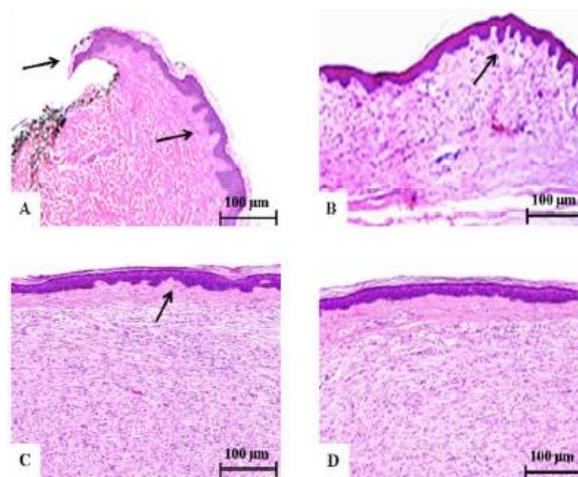
and slightly thicker dermal layers with few irregularly arranged collagen fibers. The scar tissue section of 0.4 % BA-treated rabbits (Figure 2 C) showed lesser amounts of fibroblasts with better collagen fiber arrangements, and much thinner dermal layers. Topical treatment with 0.6 % BA gel for 28 days resulted in regularly-arranged collagen fibers with thinner dermal layers devoid of any projections (Figure 2 D).

### Scar index

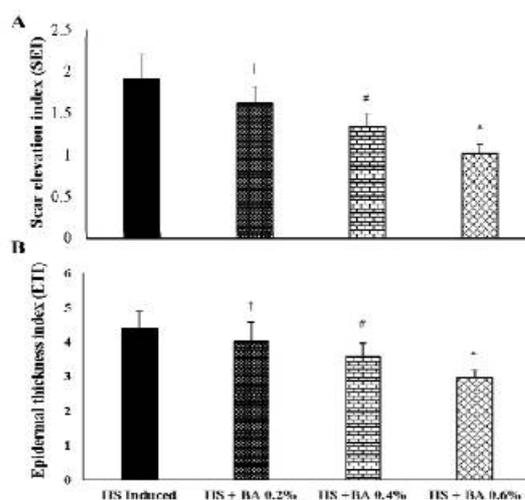
The effect of BA on the SCI and ETI in the experimental rabbits are shown in Figure 3A and Figure 3B, respectively. As the concentration of BA increased (0.2 to 0.6 %), the levels of SEI and ETI were significantly decreased, relative to the HS group (0.2 %,  $p < 0.05$ ; 0.4 %,  $p < 0.01$ ; 0.6 %,  $p < 0.001$ ).

### Protein expression of MMP-1 and TGF- $\beta$ 1

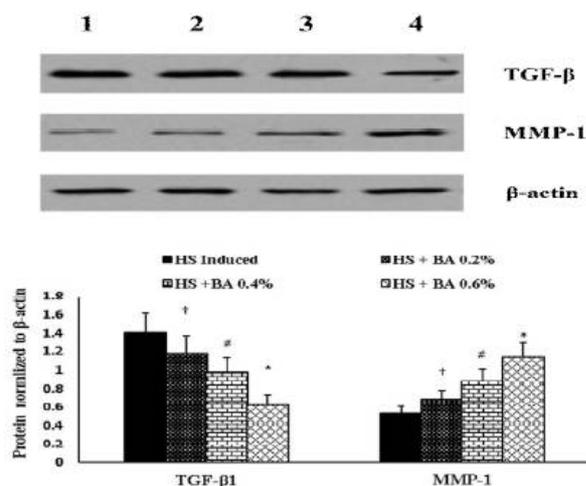
Figure 4 shows the effect of BA on the protein expressions of TGF- $\beta$  and MMP-1 in the scar tissue homogenate of the rabbits. The protein expressions of TGF- $\beta$  and MMP-1 were upregulated or downregulated in a dose-dependent fashion on treatment with BA, when compared with the HS group (0.2 %,  $p < 0.05$ ; 0.4 %,  $p < 0.01$ ; 0.6 %,  $p < 0.001$ ).



**Figure 2:** Effect BA on scar tissue histopathology (H & E staining;  $\times 40$ ); 2A: HS rabbit (positive control; scar tissue section revealed an irregular arrangement of collagen fibers with thickened dermal layers (projections denoted by arrow); 2B: 0.2 % BA-treated rabbit scar tissue showing lesser irregularly arranged collagen fibers with thicker dermal layers (arrowed); 2C: scar tissue section of 0.4 % BA-treated rabbits showing better collagen fiber arrangements with thinner dermal layers (arrowed); 2D: scar tissue section treated with 0.6 % BA for 28 days showing arranged collagen fibers with thinner dermal layers without any projections (arrowed)



**Figure 3:** Effect of BA on scar elevation index (SEI) and epidermal thickness index (ETI) in experimental rabbits. The values are presented as mean  $\pm$  SD ( $n = 4$ ).  $^{\dagger}p < 0.05$ ,  $^{\#}p < 0.01$ ,  $^*p < 0.001$ , compared with BA treatment group (HS vs. 0.2 or 0.4 or 0.6% BA gel)



**Figure 4:** Effect of BA on the protein expressions of MMP-1 and TGF-β1 in experimental rabbits. Lane 1 represents HS group; lanes 2 to 4 represent HS + 0.2 % BA, HS + 0.4 % BA and HS + 0.6 % BA groups, respectively. The values are presented as mean  $\pm$  SD ( $n = 4$ ).  $^{\dagger}p < 0.05$ ,  $^{\#}p < 0.01$ ,  $^*p < 0.001$ , compared with BA treatment groups (HS vs. 0.2, 0.4 and 0.6 % BA gel) (TGF-β1: transforming growth factor beta-1; MMP-1: matrix metalloproteinase-1)

## DISCUSSION

The present study was designed to investigate the anti-scarring property of BA in post-burn, hyper scarring rabbit ear model. The results showed that all concentrations of BA used (0.2, 0.4 or 0.6 %) lowered scar formation. However, the best anti-scarring effect was produced with 0.6 % BA. During wound healing process, the production/synthesis of collagen and GAGs act as a double-edged sword: they enhance the healing process, but excessive production or

deposition may result in HS formation. Hence, the balance between degradation and synthesis of collagen plays a vital role in scarring process [18, 19]. The contents of collagen (types I & III), hydroxyproline and hexosamine (GAG) were considerably decreased on topical treatment with increasing doses of BA gel (0.2 to 0.6 %). These results are in agreement with that of Fang *et al* [20].

To further demonstrate the anti-scarring effect of BA, histopathological changes in the scar tissues were evaluated. The positive control scar tissue (HS) showed increased presence of scattered fibroblasts with irregular arrangement of collagen fibers/bundles which resulted in thickened dermal layers. Likewise, Wei and his colleagues demonstrated that untreated scar tissue (HS model) showed thickened dermal layers with excessive disarrangement of collagen fibers [5]. Topical treatment with BA for 28 days reversed those abnormal histological changes, resulting in collagen fibers with thin dermal layers, with 0.6 % BA showing the best histology which was similar to normal scar with regularly arranged collagen fibers or bundles. Similarly, it has been reported that treatment of burn scar with 0.5 % of asiaticoside (saponin from *Centella asiatica*) resulted in collagen fiber arrangements similar to control scar tissue [21].

The scar index levels were substantially increased in HT rabbits, but on treatment with increasing concentrations of BA (0.2 to 0.6 %), the levels of SEI and ETI were significantly reduced, possibly due to decreased collagen deposition. Studies have shown that treatment with *Bacopa monniera* remarkably lowered burn scar area [8]. Matrix metalloproteinases (MMPs) are collectively called as matrixins. They are calcium-dependent zinc containing proteinases which play crucial roles in extracellular matrix (ECM) protein degradation and are thus involved in wound healing and scar/keloid formation [22]. However, MMP-1 (collagenase) is of one the pivotal proteolytic enzymes that is involved directly in the degradation of collagen types I and III in scarring tissue, and so serves as a major marker of tissue remodeling such as wound healing and scar/keloid formation [5]. Therefore, to evaluate the role of MMP-1 in scar formation, the protein expression of MMP-1 was assayed using western blot technique. The MMP-1 protein expression was significantly upregulated after 28 days of treatment with BA in a dose-dependent fashion. This is in agreement with a report showing that treatment with opuntia (rich in saponins) for 54 days significantly increased the expression of MMP-1 in a hypertrophic scar rabbit model [20]. The results obtained in this

study indicate that BA positively modulates MMP-1 protein expression and thereby reduces excessive collagen deposition in the wound/scar tissue.

Transforming growth factor- $\beta$  (TGF- $\beta$ ) exists in three isoforms (TGF- $\beta$  1 to 3). It plays a unique role in various pathological conditions especially wound healing and scarring [23]. Abundant evidence have demonstrated that hypertrophy-derived fibroblasts of HS tissue are highly active (for a long time) and so they significantly upregulate the protein expression of TGF- $\beta$ 1 more than the other isoforms [24]. The protein expression of TGF- $\beta$ 1 was markedly downregulated by treatment with increasing concentrations of BA (0.2 to 0.6%), relative to the HS group. Similarly, it has been reported that asiaticoside, a saponin isolated from *Centella asiatica* significantly downregulated the protein expression of TGF- $\beta$ 1 by modulating TGF- $\beta$ /Smad signaling pathway in hypertrophic scarring rabbit model [22].

## CONCLUSION

Topical treatment with BA significantly lowers the scarring area and scarring thickness of rabbit ear in a dose-dependent fashion following a thermal wound, by reducing collagen (types I and III), GAG contents, and SEI and ETI levels. These changes are due to upregulation or downregulation of MMP-1 or TGF- $\beta$ 1 proteins. Therefore, BA possesses a potential for use in the development of drugs for suppression of scar formation (HS).

## DECLARATIONS

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### Conflict of Interest

No conflict of interest associated with this work.

### Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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