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Preparation of Reproducible and Responsive Scar Model and Histology Analysis

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ABSTRACT – Unlike human, with some exceptions, animals do not heal with excessive scar. The lack of suitable animal model has hindered the development of effective scar therapy. We previously reported that partial thickness rabbit ear wound model resembles human wound heal process. This study was designed to prepare a hypertropic scar wound model which can be employed for testing anti-scar therapy. Four wounds were created down to the bare cartilage on the anterior side of each rabbit ear using 8-mm dermal biopsy punch and histology analysis at post operation day (POD) 5, 28 and 48 were performed. As the outcome of scar formation is largely determined by the early inflammatory response to the wounding and the degree and the duration of occlusion, cephalodin(50 mg/kg) was injected daily and medical occlusive dressings were applied. Five micro wound and scar sections were stained with hematoxylin and eosin for quantification of epidermal regeneration and scar hypertrophy. Sections were also stained using Masson's trichrome and Sirius red to evaluate collagen organization and rete ridge formation. Wound closure process was assessed to 7wks post wounding. Complete removal of the epidermis, dermis and perichondrial layer caused delayed epithelialization, which results in hypertropic scarring. The inability of the wounds to contract and the delay in epithelialization in rabbit ear was likely due to cartilage and it created scar elevation. The results suggest that full thickness surgical punch wound model in rabbit ear could be employed as a reliable and reproducible scar wound model for testing anti-scar therapy.

Key words – Animal model, Scar, Rabbit ear, Histology

Each year in the developed world, 100 million patients acquire scars, aside from psychological and social difficulties associated with prominent scars, some of which cause considerable problems with excessive scar tissue, including joint motility, impaired growth, and loss of organ function.[1)]

Unfortunately, unlike in other areas of medicine, there have been few advances in the management and treatment of abnormal scars over the past 20 years.[2-4)]

Main reason for this delayed advancement in scar treatment is due to a lack of suitable scar model.

Different animal models including pigs, mouse, rats and rabbit have been investigated.[5,6)] Animals, with some exceptions, do not heal with excessive scar. Animals have a very short resolution phase with a very rapid resolution in inflammation. And most animals are loose skinned, in response to open wound, healing occurs mainly by contracture with rapid epithelial closure. And also the loose skin means that there is an absence of wound tension.[7)]

In dermal wound healing, the initial stage of healing is cell migration and inflammation followed by cell proliferation, matrix synthesis, followed by resolution. In resolution phase of wound healing, inflammatory cells undergo apoptosis with a resolution of inflammation and a drop in collagen synthesis so that collagen production is balanced by collagen breakdown. As this balance between collagen production and breakdown disrupted, proliferative scarring occurred. Scarring is a very complicated process involving many different factors with activation through multiple pathways and much of the outcome of the scar formation is determined by the early inflammatory response to wounding. Fetal wounds do not heal with scar while adult skin could lead to scar formation; an understanding of the difference between scarless fetal wounds heal and adult scarring process could partly explain the pathophysiology of scarring.[8)]

Fetal wound healing differs from adult wound healing by a number of parameters including altered and downregulated inflammatory response, rapid reepithelialization, decreased angiogenesis, altered growth factor response, different rates of extracellular matrix(ECM) deposition and restoration of the architecture of the involved tissue. By contrast, the wound healing process in the adult appears to be optimized for rapid restoration of tissue integrity and the prevention of infection by ensuring rapid closure of the defect. Thus, adult wound healing has a robust inflammatory, increased neovascularization, excessive ECM deposition, and scar formation.[9)]

Although the etiology and mechanism of hypertrophic scarring in adult human are not fully understood, it has been long speculated that important factor in humans that lead to scar for-

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mation might be delayed epithelialization and persistent tension on wound closure.

We previously reported that epidermal regeneration was the primary wound closure process in rabbit ear wound model.[10] Due to the presence of cartilage, rabbit ear could not close the wound rapidly by contracture. Because the wounds do not heal by contracture, epithelialization is delayed and a raised scar could be formed. In this study, we prepared scar model and assessed the hypertropic scar formation by histology analysis.

Experimental

Preparation of hypertropic scar wound model

Wound creation

New Zealand white rabbit (average body weight, 2.0±0.2 kg) were housed in individual cages and acclimated for 1 week prior to the study. The rabbits were fed and watered ad libitum. Rabbits were anesthesized by ethyl ether inhalation (10 mL) for 60 sec and rabbits became sedated.

Four sites of full-thickness excisional circular wound (1cm diameter) were created down to the cartilage on the anterior side of each ear using a 8-mm dermal biopsy punch as shown in Figure. 1. Complete removal of the perichondrial layer is necessary as it delays epithelialization for 8-14 days in 8-mm punch biopsy wounds. Total 24 wound sites were created. And the punched area was cooled and disinfected with 5 mL 0.5% acrinol aqueous solution which was chilled in ice-bucket, for 30 seconds. Wound animals were placed in fixant for the tested periods.

Location of wound lesions

The biopsy punch was inflicted on the anterior part of the rabbit ear with a diameter of 1 cm; two horizontal inflicted sites were prepared as shown in Fig. 1.

Application of occlusive dressing and wound treatment

After wounding, the inflicted area was treated with 5 mL of ice cold 0.5% acrinol solution for 30 seconds to prevent bacterial contamination. Exudates and blood from the wound were absorbed by pressing sterile cotton swabs on the wound. As wound healing process was affected by the occlusion condition and the exudate absorption capacity of the dressing, all the treated area were covered with an occlusive dressing except open, untreated wound as a comparison. As an occlusive dressing, Duoderm® (Convatec, NJ, USA) was employed. Cephalodin (50 mg/kg) was injected daily to prevent the inflammation. Wound closure rate and the state of healing in terms of re-epithelialization, wound contraction and scar formation were continuously evaluated until complete wound closure.

Histological analysis

On days 5, 28 and 48 post wounding, cross-sectional, full-thickness terminal biopsies were taken from the wounds. Samples were fixed in 10 % neutral buffered formaldehyde solution, and paraffin embedded. Samples were sectioned (5 μm) perpendicular to the surface and stained with hematoxylin and Eosin, Masson' trichrome, Sirius red. Presence of hypertropic scar and disorganized collagen matrix were investigated in the specimens. The degree of rete ridges in the neo-epidermis of each wound histological cross-section was investigated. Scar tissue was defined in each Masson's trichrome slide as the area of disorganized collagen deposition aberrant from the surrounding tissue. Olympus microscope (model BX41, Japan) and image recording equipment (models DP11 and PM10SP, Japan) were employed

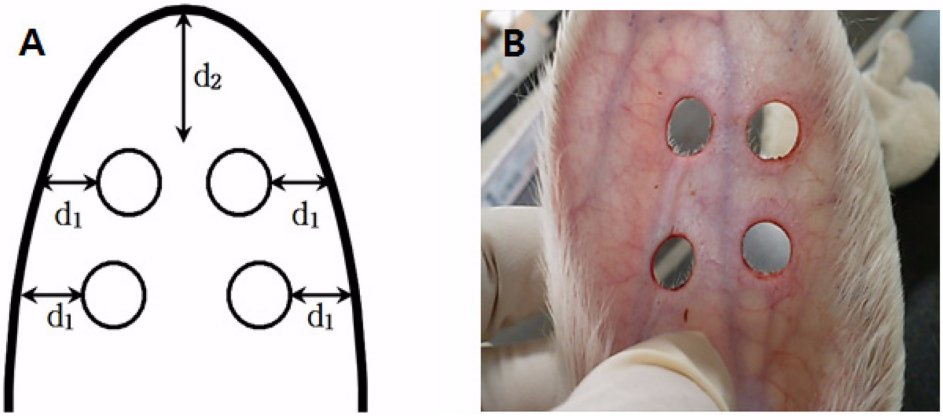

Figure 1—A: Four circular wounds (diameter 8 mm) were created on the ventral side of each ear using a 8-mm surgical punch. For a reliable comparative wound healing study, the inflicted wound position should be well controlled to have same distance from the top and the side. B: Macroscopic image shows complete removal of epidermis, dermis and cartilage.

Assessment of collagen arrangements

At least three histological sections were cut out of each tested region and stained with hematoxylin-eosin, Masson's trichrome and Sirius red to differentiate vital and damaged skin cells. The healthy dermis has a characteristic collagen structure. As hype tropic scarring causes a characteristic change in the collagen structure of the skin, the presence of weave like collagen fibers and irregular collagen bundles were investigated.

Results and Discussion

Figure 1-A shows the preparation of hypertropic scar wound model on rabbit ear. Four circular wounds (diameter: 8 mm) were created on the ventral side of each ear of rabbit. For a reliable comparative wound healing study, the wound position should be well controlled to have same distance from the top (d2) and the side (d1). Figure 1-B shows macroscopic image of complete removal of epidermis, dermis and other auxillary parts including cartilage.

Figure 2 shows photographic image of gross wound closure at post wounding days at 1day(A), 5day(B), 7day(C), 11day (D), 13day (E) and 48day (F). 8mm surgical punch wound was created and occlusive dressings were applied. Inflammation was controlled by injecting cephalodin (50 mg/kg) daily. No signs of contraction observed (A through E). Wound healing in this model is carried out exclusively through granulation tissue formation and re-epithelialization. The inability of the wounds to contract, which exposes fibroblast within the wound to ten-

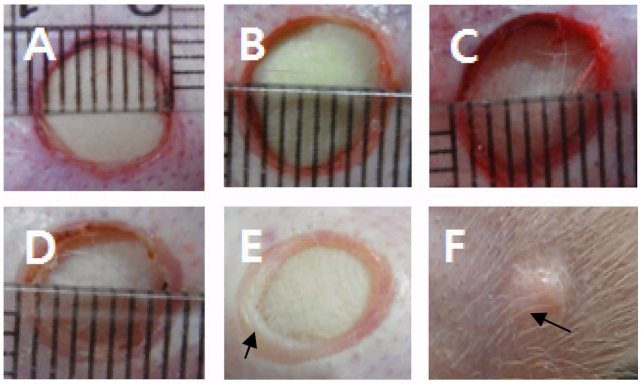

Figure 2–Photographs of gross wound closure at post wounding days at 1day(A), 5day(B), 7day(C), 11day (D), 13day (E) and 48day (F): 8 mm surgical punch wound was created and occlusive dressings were applied. Inflammation was controlled by injecting cephalodin (50 mg/kg) daily. No signs of contraction observed (A through E). Wound healing in this model is carried out exclusively through granulation tissue formation and re-epithelialization. The inability of the wounds to contract, which exposes fibroblast within the wound to tension, and the delayed epidermal regeneration of 2 weeks (E), results in scar elevation as shown in photo F. Scar elevation is depicted as an arrow in photo F.

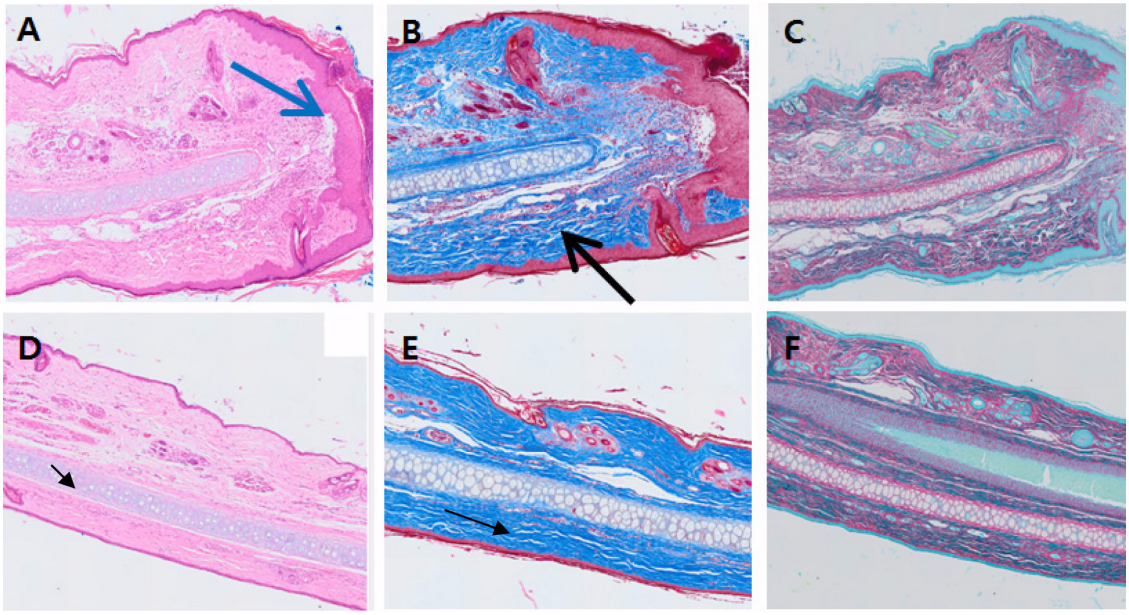

Figure 3–Histology of 8 mm full thickness surgical wound at five days post wounding (A,B,C) as compared with normal skin (D,E,F) stained with hematoxylin-eosin (A,D) and Masson's trichrome stained (B,E) and Sirius red (C,F), respectively. Normal skin shows well organized dermal collagen structure, blood vessels and other auxillary parts; cartilage (depicted as an arrow in D) locates between ventral side and posterior side and occupies more than 20% of total skin area. Well arranged weave-like collagen structure, depicted as an arrow in E, also more clearly shown in Masson's trichrome stained sections of normal skin (E). Sirius red stained sections of wounded skin (C) shows disrupted dermal collagen architecture as compared normal skin (F).

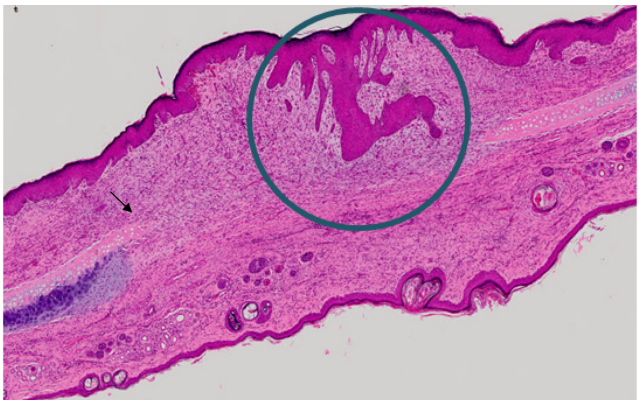

Figure 4–Histologic cross-section of 48day post wounding (hematoxylin & eosin stain, ×100): Significant epithelial hypertropic scar formation with greater infiltration of scar tissue and disruption of collagen dermal arrangements observed (depicted as an circle); damaged cartilage (arrow) has not been restored yet and no macrophases and neutrophils observed.

sion, and the delayed epidermal regeneration of 2 weeks (E), resulted in scar elevation as shown in photo F. Scar elevation is depicted as an arrow in photo F. Like human wounds, rabbit wounds delayed epithelialization result in more scar formation.

Figure 3 shows histology of 8mm full thickness surgical wound at five days post wounding (A,B,C) as compared with normal skin (D,E,F) stained with hematoxylin-eosin (A,D) and Masson's trichrome stained (B,E) and Sirius red (C,F), respectively. Normal skin shows well organized dermal collagen structure, blood vessels and other auxillary parts; cartilage (depicted as an arrow in D) locates between ventral side and posterior side and occupies more than 20% of total skin area. Well arranged weave-like collagen structure, depicted as an arrow in E, also more clearly shown in Masson's trichrome stained sections of normal skin (E). Sirius red stained sections of wounded skin (C) shows disrupted dermal collagen architecture as compared normal skin (F).

Figure 4 shows the histologic cross section of forty-eight days of post-wounding (hematoxylin & eosin stain, ×100). The scar tissue has significantly greater infiltration through epidermis and dermis. Significant epithelial hypertropic scar formation and disruption of collagen dermal arrangements were observed (depicted as a circle); damaged cartilage (arrow) has not been restored yet and no macrophases and neutrophils observed. Hypertrophic scars represent an exaggerated fibroproliferative response of the dermis, which creates an imbalance of matrix degradation and collagen synthesis, resulting in excess accumulation of dermal collagen. Complete removal of the perichondrium induced delayed epithelialization for 8-14 days in 8-mm punch biopsy wounds. Delayed epithelialization increased persistence of scar elevation beyond 48 day post wounding. The inability of the wound to contract, which exposes fibroblasts within the wound to tension, and the delay in epithelialization of approximately 2 weeks, results in predictable scar elevation and a histological appearance of hypertropic scar.

In recent years, it has become clear that the wound inflammatory response may be responsible for fibrosis at sites of tissue repair. Inflammatory cells such as leucocytes secrete factors that stimulate fibroblast growth which might be beneficial to the repair process. But, uncontrolled, persistent inflammation causes over-proliferation of damaged tissue and fibrosis.[11] Controlling inflammation is very critical to prepare a reliable wound model. And inflammation could be controlled by daily injection of cephalodin. Based on this study, we suggest rabbit ear wound model with inflammation control as a reproducible and reliable human-resembled scar wound model.

Conclusion

The results suggest that rabbit ear model could be employed as a reliable and human-resembled hypertropic scar model.

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