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The effect of surgical denervation on prevention of excessive dermal scarring: A study on rabbit ear hypertrophic scar model

Caglayan Yagmur [a,*], Ethem Guneren [a], Mehmet Kefeli [b], Rei Ogawa [c]

[a] *Samsun Training and Research Hospital, Department of Plastic, Reconstructive and Aesthetic Surgery, 55020 Ilkadim, Samsun, Turkey*
[b] *Ondokuz Mayis University Medical School, Department of Pathology, Samsun, Turkey*
[c] *Nippon Medical School Hospital Department of Plastic, Reconstructive and Aesthetic Surgery, Tokyo, Japan*

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Substance P;
Free nerve ending;
Axon reflex

Summary *Background:* Previous reports have suggested that the extent of wound contraction, epithelisation and total healing time were influenced by denervation of tissues. In this article, we studied for the first time the effect of sensory denervation on prevention of excessive dermal scarring.
Materials and Methods: Sixteen New Zealand white rabbits were used. Denervation of the right ears was performed by surgical excision of two main sensory nerves. Dissections were also performed on left ears without any nerve excision for the control group. After 14 days of follow-up and confirmation of tissue denervation, an excessive dermal scarring model as defined by Morris et al. was made by surgery on both ears. Twenty-eight days after making the wounds, the tissues were extirpated for analyses. The scars were evaluated by the scar elevation index (SEI), epithelisation time and inflammatory cell count.
Results: The SEI of the denervated side scars was significantly lower than that of the non-denervated side. The rate and timing of total epithelisation and inflammatory cell count between groups yielded no difference.
Conclusions: In this study, the surgical denervation skin reduced scarring. It was suggested that understanding the exact role of sensory nerves and neural mediators in excessive dermal scarring is necessary for the prevention and treatment of scarring.

* Corresponding author. Tel.: +90 (0) 532 794 75 75.
E-mail address: caglayanyagmur@gmail.com (C. Yagmur).

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Complex wound healing events are categorised into four general processes: coagulation, inflammation, proliferation and maturation (remodelling). In the proliferation phase, epithelisation, angiogenesis, granulation tissue formation and collagen deposition are the main events.

In some instances, wound healing may end up with the formation of a proliferative scar rather than a self-limited mature scar. 'Proliferative scarring' may occur when the synthesis of extracellular matrix (ECM) is not sufficiently opposed by degradation in the maturation phase. The protruded erythematous and inelastic 'proliferative scar tissue' may be pruritic and painful, and may also cause important functional and aesthetic problems.

Apart from the well-identified cell types (fibroblasts, macrophages, neutrophils, etc.) in wound healing dynamics, there is limited knowledge about the role of nerve fibres and neural mediators. Sensory and autonomic nerves of skin serve in many physiologic and pathophysiologic conditions. Physical (trauma, thermal, ultraviolet light and electrical), chemical, microbiologic and allergic stimulation of skin sensory nerves end up in increased amounts of released neuropeptides, neurotrophins, neurotransmitters and oxygen derivatives.[1] As they are constant and physiologically active components of the skin anatomy, previous reports have suggested that the extent of wound contraction, epithelisation and total healing time were influenced by denervation of tissues.[2–6] In this article, we studied for the first time the effect of sensory denervation on the prevention of excessive dermal scarring.

Materials and methods

Preparation of denervated rabbit ear model

The Animal Care and Use Ethical Committee of Ondokuz Mayis University approved all procedures. Sixteen female New Zealand white rabbits weighing 2.9—4.4 kg (5—7 months of age) were kept under standard conditions and fed *ad libitum*, and a two-step surgery (denervation and making wounds) was performed. For obtaining sensory denervation on right ears ($n = 16$), animals were anaesthetised with ketamine (45 mg kg^{-1} intramuscularly) and xylazine (6 mg kg^{-1} intramuscularly). For prophylaxis, 10 mg kg^{-1} single-dose cefazoline was administered intramuscularly. Prior to surgery, the ear dorsum and auriculocranial sulcus region were shaved bilaterally. The skin was prepared with povidone—iodine.

Bilateral markings for 2-cm skin incisions were made on auriculocranial sulci (Figure 1a and Figure 2a). After making the skin incisions, meticulous dissection was carried out to expose the great auricular nerve (GAN) and lesser occipital nerve (LON), respectively, without causing any harm to the vascular structures (Figure 1b and Figure 2b). The GAN and LON, the two main sensory nerves in rabbit ear skin,[7] were exposed by considering their anatomic relationships with neighbouring structures. All dissections were made on both ears.

For the purpose of sensory denervation of ventral skin, 1 cm of nerve segment was excised from the GAN and LON on the right ear of each animal (Figure 1c and Figure 2c). Prior to nerve excision, a local anaesthetic (prilocaine hydrochloride 2%, Citanest, AstraZeneca) was applied to the region to prevent extreme stimuli. After excision, the distal and proximal nerve stumps were tied. On the left side, as described previously, dissection on both nerves was performed in a meticulous manner to prevent neuropraxy. No nerve excision was made on the left side; innervation of left ears remained intact.

Following nerve excision, a horizontal skin incision on proximal 1/3 border of ventral ear (Figure 1d and Figure 2c) was also performed on the right ear. A 0.5-cm-wide proximally based horizontal skin flap was harvested. Fine neurovascular bundles on the ground and flap were cauterised by low-adjusted electrocautery (Figure 1e). This additional procedure was performed depending on the results of our preliminary study. As rabbit ear is also innervated by minor sensory nerves,[7] nerve excision from the GAN and LON alone might be insufficient to ensure permanent sensory denervation of the region (middle 1/3 ventral skin region). Horizontal incision to 1/3 proximal border was also performed on the left ear (Figure 2c), but flap harvesting and cauterisation procedures were not performed on this side. At the end of surgery, skin incisions were sutured and animals were followed up for 14 days. While planning the study, this time period was thought to be essential to allow the clearance of any chemical mediator that may have been released by the nerve manipulation and the acute wound healing of the flaps and skin incisions. Thus, to minimise the influence of any possible interference with

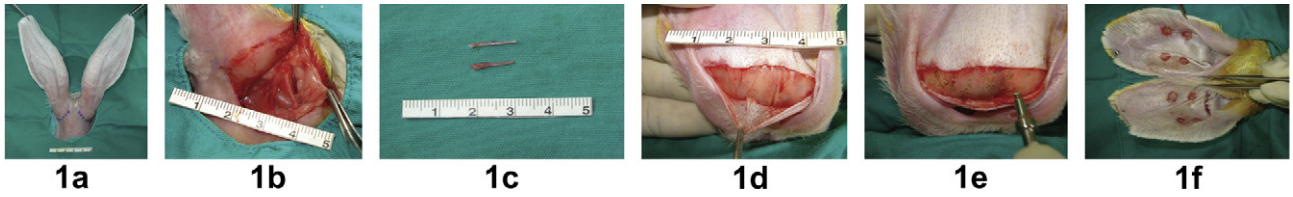

Figure 1 Two-step surgery was performed to all animals. At first step (1a to 1e), following bilateral 2 cm skin incisions to auriculocranial sulci (1a) GAN and LON was exposed by meticulous dissection without giving any harm to vascular structures (Figure 1b depicts GAN with neighbouring vasculature). As bilateral dissection of GAN and LON was performed, 1 cm segment from both nerves was excised on the right side for sensory denervation (1c). To increase the effect of denervation on the right side, 0.5 cm wide proximally based ventral horizontal skin flap was harvested (1d) and fine neurovascular structures were cauterized (1e). After the procedure, all wounds were sutured and animals were followed up for 14 days. On postoperative day 14, second step surgery was performed to create 4 full-thickness excisional wounds to bare cartilage on each ear (8 wounds per animal) (1f). GAN: Great Auricular Nerve, LON: Lesser Occipital Nerve.

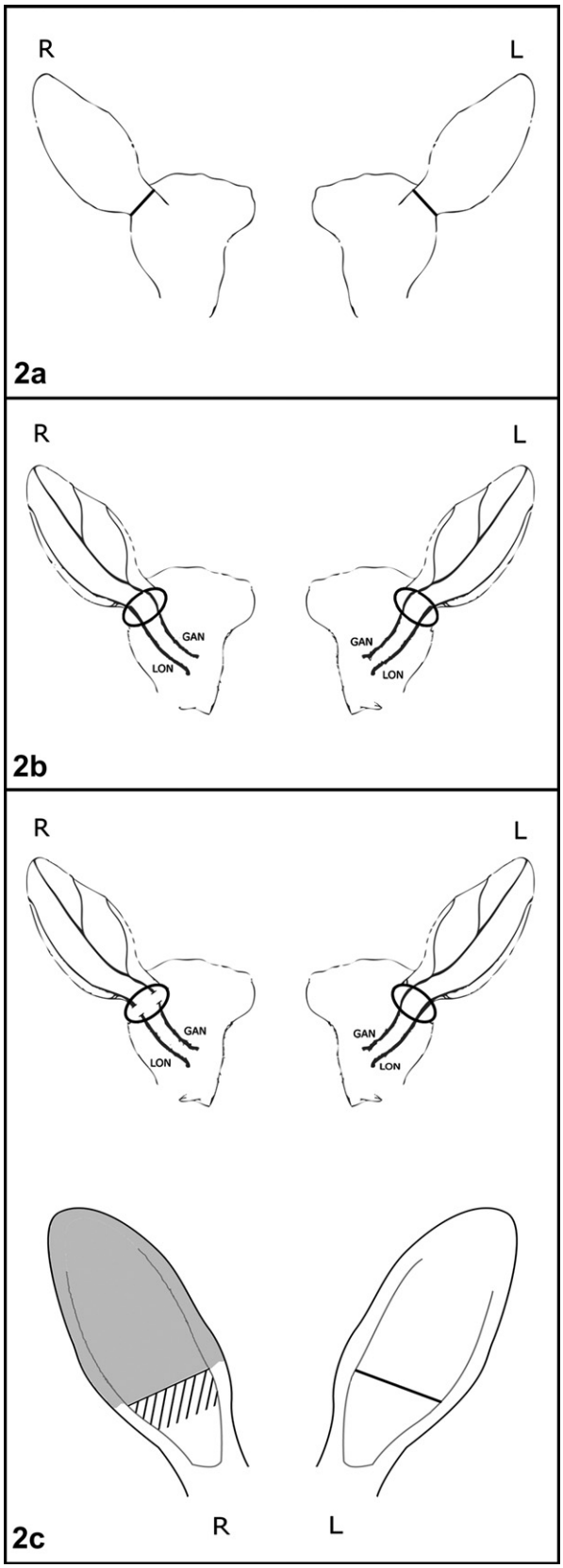

Figure 2 Diagram of surgery representing the steps of surgical denervation of right side. 2a: Bilateral 2 cm skin incisions to auriculocranial sulci. 2b: Bilateral dissection of GAN and LON. 2c: 1 cm segment from both nerves was excised on the right side, left side remained intact. Additionally, a proximally based ventral horizontal skin flap was harvested on the right side and fine neurovascular structures were cauterized. The denervated area on right ventral ear is represented in gray. GAN: Great Auricular Nerve, LON: Lesser Occipital Nerve.

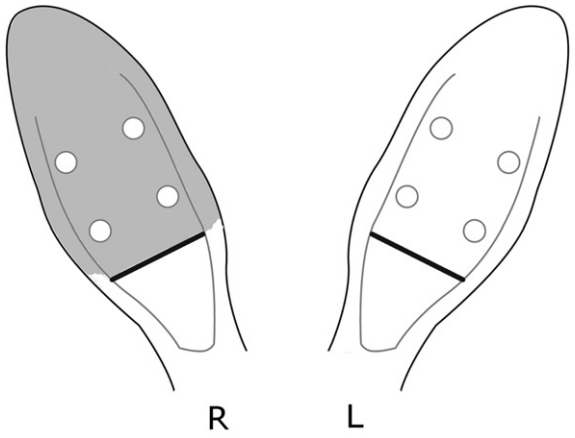

Figure 3 Application of excisional wound model to both denervated and non-denervated ventral ear skin regions (14 days after first step denervation surgery).

the next step, we waited for 14 days before preparing the rabbit ear wound model.

Assessment of denervated rabbit ear model

Two different tests were used to assess the sensory status of the middle 1/3 ventral ear skin region before the 'denervation surgery' and postoperative day 14: (1) pinching the ear region with the aid of forceps and (2) pinpricking with a 21-G needle. Both subjective tests depend on the principle of applying painful stimuli to unanaesthetised animals. Both tests were repeated for 5 times on the related skin region in both ears. The absence of any withdrawal subsequent to the painful stimuli in at least four trials for each test was considered as 'successful sensory denervation'. Conversely, the presence of any withdrawal in at least two trials was considered as 'inadequate sensory denervation' in operated animals. On preoperative examination, all intact animals were shown to have cutaneous sensation bilaterally. However, on postoperative day 14 — using both tests — all animals ($n = 16$) demonstrated 'sensory denervation' of the middle 1/3 ventral skin regions of the right ears, while all left ears continued to have cutaneous sensation.

Preparation of rabbit ear wound model

On postoperative day 14, an excisional wound model was applied to both denervated (right, $n = 16$) and non-denervated (left, $n = 16$) ventral ear skin regions, as described by Morris et al.[8]

Four full-thickness 7-mm wounds were created down to bare cartilage on the middle 1/3 ventral skin of each ear (eight wounds per animal) with a 7-mm biopsy punch

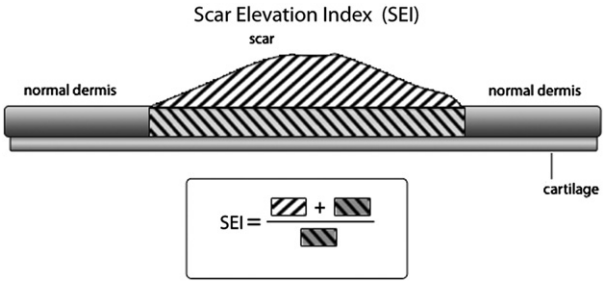

Figure 4 Scar Elevation Index (SEI) for evaluating rabbit ear excessive dermal scarring model.

(Figure 1f and Figure 3). To leave bare cartilage at the wound base, removal of skin and perichondrium was performed with a surgical operating microscope. The wounds on each ear were numerated and covered with Tegaderm dressing (3M Health Care). A total of 128 wounds ($n = 64$, left: $n = 64$, right) were created in 16 animals. Following this procedure, the ears were covered by adhesive tapes in a circular fashion. As dressings were on the concave ventral side, that procedure was an easy, reproducible and safe way to prevent dressings from scratching. It has also acted as a splint, decreasing the flexibility of ear. Thus, scratching becomes less possible. In addition, the distal opening has allowed inspection and dressing changes. The total epithelisation time for each individual wound was assessed by gross examination and recorded on a daily basis. The formation of stratified epithelium with complete healing was used to decide on complete epithelisation.[9–12]

Tissue harvesting and evaluation of scars

The scars were excised with a 3-mm unwounded margin, bisected at their highest point and processed for histological analysis using haematoxylin and eosin (HE) staining. Elevation of scars was quantified by measuring the scar elevation index (SEI). The SEI is the ratio of the total wound area tissue height to the area of normal tissue below the hypertrophic scar. An SEI of 1 indicates that the scar is of equal height to the surrounding unwounded dermis; an SEI >1 indicates a raised, hypertrophic scar[8,10] (Figure 4). To measure the SEI, digital images of scar sections were obtained by a blinded observer and processed by image analysis software (Image J)[13] (Figure 5). The inflammation scores of scar sections were calculated by counting the inflammatory cells in five different microscopic regions ($\times$40 HPF) for each scar section. The average number for each scar section was then calculated. An average between 0 and 10 corresponded to 1 point, 11 and 100 to 2 points, 101 and 1000 to 3 points, and any average more than 1000 cells to 4 points.[14]

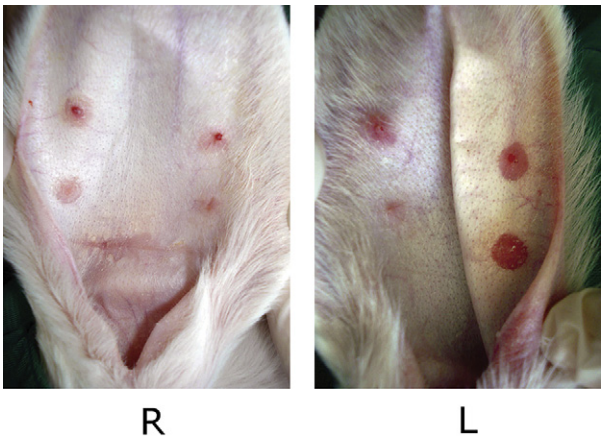

Figure 6 28 days after excisional wounding. Denervated right side scars (R) macroscopically appeared to be less hypertrophic than non-denervated left side scars (L) in most animals.

All wounds were created and harvested in a matched fashion. As for the SEI and epithelisation time, the collected data were 'paired observations' and followed a normal distribution; statistical analysis was made by using a paired t-test. The data on inflammation scores did not follow a normal distribution, and non-parametric Wilcoxon signed test was used. A value of $p > 0.05$ was considered insignificant.

Results

Among a total of 128 ($n = 64$, right; $n = 64$, left) wounds, eight ($n = 3$, right; $n = 5$ left) were lost to analysis because of secondary desiccation. The remaining wounds (93.75%) were healed completely by post-wounding day 15. On post-wounding day 28, the wounds were in the appearance of raised mature scars. The right ear (denervated) scars were visibly less hypertrophic than the left ear (non-denervated) scars (Figure 6). This finding was more evident when scar-bearing skin regions were taken extracorporeally. On histologic analysis, all wounds demonstrated features of scar tissue (an ECM with disorganised collagen fibres, increased number of fibroblasts and a well-organised vasculature).

The mean SEI for the right side (denervated) scars ($n = 61$) was 1.26 ($\pm$0.22) compared with 1.6 ($\pm$0.34) for the left side (non-denervated) scars ($p < 0.05$). This represents a surgical denervation-dependent decrease in scar hypertrophy in the right-side scars (Figure 7).

The mean epithelisation time of the right side (denervated) scars ($n = 61$) was 12.49 ($\pm$1.43). The left side (non-denervated) scars share the same mean, with a standard

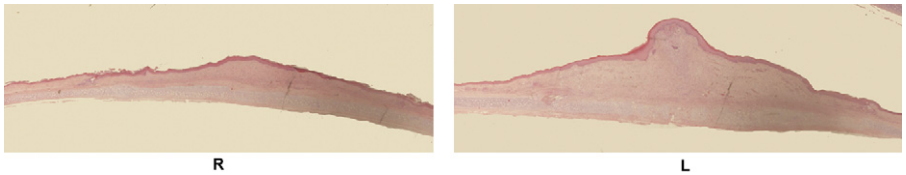

Figure 5 Digital images of scar sections were obtained from both denervated (R) and non-denervated (L) side scars.

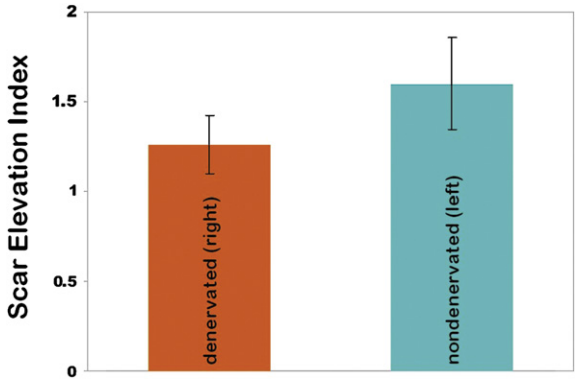

Figure 7 SEI of denervated (right) side scars ($n = 61$) was significantly lower when compared to non-denervated (left) side ($n = 59$) scars ($p < 0,05$).

deviation of 1.49, indicating no difference between the groups.

The median inflammation score was 3 for both groups. Between the minimum 1 and the maximum 4, no significant difference was observed.

Discussion

The role of sensory nerves in wound healing

The nerve system of the skin is involved in wound healing.[2–6,15] This may be attributed to the proliferative effect of neuropeptides (calcitonin gene-related peptide (CGRP), substance P and other mediators) on epithelial, vascular and connective tissue.[2,16–19] Impaired healing may occur in deficiently innervated tissues, as in quadriplegic or paraplegic patients.[20] Another example may be in diabetic wound healing. Peripheric neuropathy is a common finding in diabetic patients with chronic ulcers. Slower wound healing was observed in diabetic mice compared to heterozygous and control littermates.[6] A lower number of nerve fibres in normal and wounded skin were also demonstrated in diabetic mice.[21] Additionally, increased neutral endopeptidase (NEP) activity has been observed in tissues of diabetic mice and humans.[22] NEP is the main enzyme that degrades substance P, an important neuropeptide secreted by free nerve endings. Moreover, the topical administration of an NEP inhibitor to an excisional wound model in mice normalised wound-healing kinetics and increased wound bed inflammation.[23] Barker et al.[6] and Scott et al.[24] reviewed the effect of sensory nerve elements on wound healing and hypertrophic scarring respectively.

Denervated animal models

Animal models with surgical sensory skin denervation have been used in wound healing studies. In rats, the hindlimb,[25] back region[26,27] and groin region (denervated groin flap)[4,15] were mainly denervated. The back region and ears of mice[28] and the hindlimb of pigs[29] were also denervated. Wounds on these regions have been studied for alterations in the duration/extent of wound contraction, tensile strength, epithelisation and healing phases. For rats, systemic capsaicin application is an alternative method of skin denervation.[25] Several experimental studies have demonstrated that denervation of a skin region impairs wound healing characteristics.[3,5,26–29] Conversely, Ranne et al.[4] found no histologic difference in denervated rat groin skin wound healing. Wallengren et al.,[25] also following the depletion of at most 70% of C-fibres via both capsaicin and peripheral neurotomy, demonstrated normal wound healing in rat skin.

In this study, we used an excessive dermal scarring model in rabbit ear.[8] Our main reasons for choosing this model were: the consistent formation of excessive dermal scarring found in many previous studies,[10,30–33] the presence of surgically manipulable nerves in the related skin region and the ability to measure the extent of scarring by quantitative means using a predetermined method (SEI). The scarcity of hair follicles in this skin region (ventral ear) was also an advantage.

In addition to the GAN and LON, the auriculotemporal branch of the trigeminal nerve, the auricular branch of the vagus nerve and the cervical sympathetic chain also contribute to the sensory innervation of rabbit ears.[7] Having already considered this knowledge, we avoided performing any dissection or excision in these minor nerves for two reasons. First, 'removing' all sensory nerves of the ear would need broader exposure and extensive dissection, which may cause vascular compromise. Any vascular disturbance may directly affect the wound healing dynamics and dermal scarring attitudes distal to the area. This might cause a discrepancy between the denervated and non-denervated wound groups. Second, the above-mentioned nerves mostly innervate the proximal regions of the ear skin. Excisional wounds were applied to more distal ventral regions of the ear; this particular point did not cause any problem. In view of these circumstances, we simply harvested a horizontal proximally based skin flap on the ventral 1/3 middle-proximal border of the right ears and cauterised fine neurovascular bundles. Accompanied with excisions from the GAN and LON, this procedure increased the possibility of sensory denervation of the middle 1/3 right ventral ear skin, on which the scar model was applied subsequently.

Besides being nociceptors, sensory nerve endings also have secretory functions. The retrograde invasion of primary afferent neuron arborisations forms antidromic (anterograde) action potentials in neighbouring branches. This phenomenon is known as 'axon reflex'.[34] Anterograde action potentials may also be formed in the level of dorsal root ganglia. By this type of mechanism of stimulation, the free nerve endings (nociceptors) of the skin may secrete neuropeptides. The neuropeptides in skin mediate communication among free nerve endings, immune cells and skin cells.[35,36] Figure 8 demonstrates a summary of events related to this concept.

After being synthesised in the soma, neuropeptides are transmitted to nerve endings through accelerated axonal transport.[37] Therefore, a decreased level of neuropeptides in distal parts may be expected after surgical discontinuation of sensory nerve trunks. It has also been demonstrated that skin sensory denervation (via capsaicin) decreases tissue neuropeptide levels.[3,38,39]

The mean SEIs of the right side (denervated) scars are significantly lower than those of the left side (non-denervated)

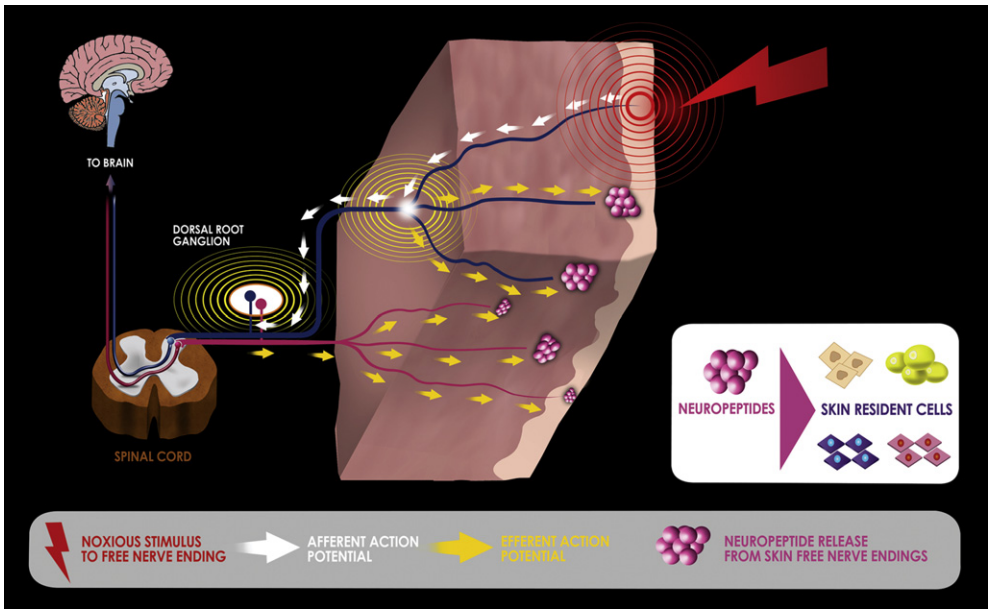

Figure 8 The noxious stimuli to free nerve endings normally induce retrograde (afferent) action potentials. However, on the arborization points of sensory neurons retrograde (afferent) action potentials may invade the neighbouring branches to form antidromic (anterograde, efferent) action potentials, which may cause neuropeptide release from their free endings. Released neuropeptides have proliferative effect on many types of skin resident cells. The dorsal root ganglia may also be involved in the formation of antidromic (anterograde, efferent) action potentials in sensory nerves.

scars. Additionally, no correlation between inflammatory cell count and SEI was found in our study. One may find this 'peculiar', considering the close relationship between excessive dermal scarring and robust inflammation. However, assessing the 'pathologic inflammation' related with 'excessive scarring' may require more sophisticated methods. Methods to evaluate the amount and activity of inflammatory and fibrogenic mediators in scar specimens before and after intervention would also have to be performed. This point indicates a drawback of our study.

As it seemed to be the best time for comparing scar formation between the test and control groups in our preliminary study, after photographing the scars, all of the animals were euthanised and the scars were harvested on day 28 after wounding. Although this model can yield scars that remain prominent beyond 48 days, the prominence of the scars tends to decrease with time.[8] As any inevitable loss of prominence of the scars with time may interfere with our findings, we decided to harvest the scars from both groups at a specific time, when excessive scarring was still prominent. Definitely, this short period of time and the healing physiology of the model may not fully coincide with real human hypertrophic scars and keloids. On the other hand, the eventual decrease in excessive dermal scarring in denervated skin may encourage us to conduct further studies and find new treatment applications. Surgical denervation, nerve block, direct local anaesthetic applications or implantation of slow-releasing local anaesthetic preparations to prevent or treat problematic scars may be considered in the future.

Human skin is innervated by sensory and autonomic (postganglionic parasympathetic and sympathetic) nerves. The sensory nerves of the skin are able to function in both an afferent and an efferent manner.[40] Therefore, the potential role of this bidirectional connection between the skin and the central nervous system (CNS) in many pathological skin conditions must be considered. 'The mind-and-skin connection' may manifest itself as in the case of eczema or psoriasis, both of which may be worsened by emotional stress.[41,42] A similar condition, however, may also be valid for human excessive dermal scarring.

Considering the complex pathophysiology of excessive dermal scarring, many intervening factors may be addressed. To rely on a single factor (a cell type, a mediator, a physical condition, etc.) in our attempt to find biological truths related to excessive dermal scarring may be unsatisfactory. Having regard to this point, in this study we demonstrated that the 'surgical sensory denervation of the related skin region in a rabbit ear excessive dermal scarring model resulted in less prominent scars'. Understanding the exact role of sensory nerves and neural mediators in excessive dermal scarring requires more sophisticated and more comprehensive studies to be carried out.

Conflict of interest statement

The authors have no financial interest associated with the publication of this article. None of the authors has commercial associations, stock ownership, equity interests or patent licensing arrangements related to information presented in this article.

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