Sensory Peptides as Neuromodulators of Wound Healing in Aged Rats

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An intact nociceptor system of primary afferent sensory nerves is important for the initiation of the inflammatory process and successful tissue repair. Dysfunction of this system with age could be a contributing factor for delayed wound healing in the elderly. This study was designed to examine the role of sensory nerves (using capsaicin-pretreated rats) and the effect of aging on the healing of a thermal wound induced on the interscapular region (using a CO₂ laser). The healing endpoint was the time when full wound contraction had occurred. The ability of the sensory peptides, substance P (SP) and calcitonin gene-related peptide (CGRP), in modulating wound healing in aged rats was examined by taking into account the modulatory interaction effects between these peptides. A blister model in the rat hind footpad combined with a laser Doppler flowmeter was used initially to establish these vascular interaction effects.

The results showed a significant delay in thermal wound healing in both the capsaicin-treated and aged rats. Using the blister model, we demonstrated the ability of SP and CGRP to induce a limited and a prolonged vasodilator response, respectively. When tested together, SP attenuated the vascular effect of CGRP, an effect that was reversed using the tachykinin antagonist spantide II. When injected at the wound site, SP and CGRP were equally potent in promoting wound healing in aged rats. The beneficial effect of taking the interaction effects into account was most evident in a group of rats that received the tachykinin antagonist as part of their initial treatment with CGRP and 6 h later, an injection of SP; the time to complete wound closure was 11.3 ± 0.6 days compared to 21.0 ± 0.9 days in aged controls. We contend that wound healing in aged rats can be accelerated by exogenous administration of sensory peptides. Furthermore, modulatory interaction effects between sensory peptides should be taken into consideration when designing any treatment protocol that purports to accelerate wound healing.

With aging there is also a decrease in the capacity of individuals to maintain appropriate inflammatory and repair processes. For example, both animal (Khalil, 1993) and human (Eaglstein, 1989) studies have suggested that wound healing, as measured by wound closure time, is impaired with age. We have recently provided evidence in aged rats for changes in sensory nerve function at both pre- and postterminal levels (Khalil et al., 1994). We have also documented a decrease in peptide synthesis, content, and release in old rats and an overall decrease in neurogenic inflammation with antidromic nerve stimulation, and raised the possibility that these changes may have important implications for wound healing and repair mechanisms.

The overall aim of this study was to investigate the concept that the decline in sensory nerve function with age contributes significantly to poor healing qualities of tissues in aged animals. We examined the role of sensory nerves and the effect of aging on the healing of a thermal burn induced on the interscapular region of the rat. In the second part of our study, we made use of our previous findings, that tachykinin receptors in old rats are upregulated (as a consequence of a preterminal defect in peptide release mechanisms of sensory nerves) to investigate the role of substance P (SP) and calcitonin gene-related peptide (CGRP) in modulating wound healing in old rats, with specific emphasis on the importance of the modulatory interactions of these peptides.

METHODS

Animals. — Young control and capsaicin-pretreated rats (3 months old) and aged (24 months old) Sprague-Dawley male rats were used. For capsaicin pretreatment, neonatal
rats were given a single subcutaneous dose of 50 mg/kg of capsaicin on 2-4 days of life. The purpose of this treatment was to permanently destroy small diameter sensory nerves. Capsaicin was prepared in normal saline containing 10% Tween-80 and 10% alcohol. The effectiveness of neonatal capsaicin pretreatment was tested before each experiment using the eye-wiping response to topical application of capsaicin (0.1% in alcohol) to the cornea. Denervation with capsaicin was later confirmed by assaying SP levels in the skin. Data are only included if capsaicin pretreatment results in >80% depletion in SP levels.

**Thermal burn.** — The fur on the interscapular region was removed with animal clippers and a cosmetic depilatory agent. At least 24 h after this treatment, rats were anesthetized with sodium pentobarbital (65 mg/kg i.p.) and a thermal burn was induced in the treated region by using a CO2 laser (four consecutive stimulation periods each at 25 W power, 0.5-sec duration, and spot diameter of 10 mm). This technique delivers standard energy levels over a given area of skin and therefore gives a similar and reproducible thermal injury. This injury results in a large circular wound area (2 cm²). The wound area increases to 2.5-3 cm² by day 2 due to progressive loss of the microcirculation because thermal injury damages capillary endothelial cells. This shape and size of wound allow for healing primarily by contraction and re-epithelialization over approximately 3 weeks in old rats. It is also known that round wounds do tend to contract more uniformly, rarely deform into two or more wounds, and are associated with less measurement error (Richey et al., 1989).

**Measurement of healing.** — The area of burn was traced daily for the first 6 days and every 48 h thereafter and measured with a digital planimeter. Measurements were made by an observer who was unaware of the treatment status of the animals. Wounds were left uncovered and observed daily. Any lightly adherent eschar was removed with forceps. Although scab formation was minimal, when it occurred, the scab was gently removed. This was done to keep all wounds comparable as previous studies by Snowden et al. (1982) showed that scab formation induces a transient decrease in the rate of wound contraction. It was also easier to accurately measure open wound area without the overlying scab. The healing endpoint was determined as the time when full wound contraction had occurred. It should be noted that although wound contraction is only one parameter of wound healing, it accounts for large portions of wound closure in full-thickness wounds.

The modulatory interaction effect between sensory peptides by using the blister model. — Young and old rats (3 and 24 months old, respectively) were anesthetized with pentobarbital sodium (60 mg/kg i.p.). Further doses of 15 mg/kg anesthetic were administered to ensure that rats stayed under anesthesia. Body temperature of the rats was maintained at 37°C.

A blister was induced in the right hind footpad of the rat by applying a suction pressure of -40 kPa for approximately 30 min by using a metal suction cap heated to 40 °C (Khalil and Helme, 1989, 1990; Khalil et al., 1994). The surface epithelium was removed and the rat foot secured in a perspex chamber with inlet and outlet ports. Ringer’s solution was perfused over the blister base at 4 ml/h by means of a peristaltic pump (Microperpex S; LKB, Sweden). Relative blood flow was monitored over time by a laser Doppler flowmeter (Periflux, PF2B; Perimed, Sweden) via a probe placed in a central port immediately above the blister base; relative blood flux (volts) was continuously monitored on a chart recorder. At the end of the experiment, animals were killed by barbiturate overdose. An initial equilibration period of 30 min was allowed, during which time a stable baseline was achieved. In control experiments with young and old rats, either SP or CGRP (both at 1 μM) were perfused for 30 min or 10 min, respectively, over the blister base. To examine possible interaction modulatory effects between CGRP and SP, in a separate set of experiments, 1 μM of CGRP was perfused for 10 min followed by a 10-min poststimulation period with Ringer’s solution; then SP (at 0.1, 1 or 10 μM) was perfused for 30 min. Furthermore, the involvement of SP receptor in the modulatory effects exerted by SP on the vasodilator response to CGRP was examined in another group of rats. Subsequent to perfusion of CGRP and Ringer’s solution, an effective tachykinin antagonist (Spantide II) (Folkes et al., 1990; Hakanson et al., 1991; Xu et al., 1991) was perfused together with SP (all at 1 μM).

**Drug injection.** — Peptide (200 μl) was injected intradermally at four sites 1 cm distant to the burn edge twice daily (6—8 h apart) for 5 days after thermal injury. Six groups of 2-year-old rats were used: group 1, saline; group 2, SP; group 3, CGRP; group 4, SP and CGRP; group 5, CGRP after SP antagonist, and group 6, CGRP (after SP antagonist) and SP. In all groups except group 6, the same treatment was used twice daily. However, group 6 received Spantide II as part of the initial treatment with CGRP and 6 h later an injection of SP alone. The rationale behind using the SP antagonist before CGRP injection was to allow the latter to maintain its vasodilator response. All peptides were used at 10 μM. Five days of treatment were chosen because our previous studies showed that old rats and capsaicin-pretreated rats healed significantly more slowly than adult rats, with the most significant difference in wound size occurring in the first 6 days. Both groups showed a lag period of around 4 days before wound contraction began.

**Drugs.** — Nembutal (pentobarbital sodium) was from Boehringer Ingelheim Ltd., Australia. SP and CGRP were from Auspep, Australia, while the SP antagonist (Spantide II) was from Peninsula Laboratories.

**Statistics and expression of data.** — All results are expressed as mean ± SEM (n = 7-8 in each group). Statistical analyses were performed using a two-way (group × days) repeated measures analysis of variance (ANOVA) followed by post hoc Duncan’s pairwise comparisons. A p-value < .05 was considered significant.
RESULTS

Wound healing in young, old, and capsaicin-pretreated rats. — The time required to complete wound closure was monitored in three groups of rats, young (3 months; control), capsaicin-pretreated rats at 3 months of age, and 2-year-old rats. Young control rats showed an early increase in wound size that reached a maximum by day 2 followed by healing, manifested as a gradual reduction in wound size. Complete wound closure was achieved at 14.9 ± 0.2 days (see Figure 1). Old rats and young capsaicin-pretreated rats showed a different profile of healing compared with young controls. In both groups, the wound reached maximum size by day 2 (similar to young control rats); however, there was a lag phase before an actual reduction in wound size observed by day 5. Complete wound closure occurred at 21.1 ± 0.3 and 21.3 ± 0.6 days for old and capsaicin-pretreated rats, respectively (Figure 1).

Effect of SP or CGRP on skin microvasculature in young and old rats. — Perfusion of 1 μM SP over the blister base induced a vasodilatation (VD) response that is characterized by tachyphylaxis within 15 min in young rats. This vasodilator response was significantly enhanced in old rats, while the subsequent tachyphylaxis was less prominent (Figure 2A). The area under the response curve for 30-min perfusion in young rats was 29.3 ± 5.3 cm² as opposed to 55.9 ± 6.8 cm² in old rats.

Perfusion of 1 μM CGRP for 10 min over the blister base induced an immediate VD response in young rats that reached maximum within 1–2 min and was maintained for more than an hour after cessation of stimulation. A similar response was obtained in old rats; however, the poststimulation period showed a greater amplitude of response compared to young rats (Figure 2B). The area under the response curve for 10-min perfusion followed by 10-min poststimulation with Ringer’s solution in young rats was 45.7 ± 4.9 cm² compared with 64.8 ± 7.2 cm² in old rats.

Modulation of the vascular response to CGRP by SP. — In these experiments, 1 μM CGRP induced a vasodilator response that was maintained during the poststimulation period with Ringer’s solution. Subsequent SP perfusion at 1 μM terminated the existing vasodilator response to CGRP (Figure 3A). The modulatory effects exerted by increasing concentrations of SP (0.1, 1, and 10 μM) on the vasodilator response to 1 μM CGRP were dose dependent (Figure 3B). The higher the concentration of SP, the quicker it terminates the vasodilator response to CGRP. The possibility that this modulatory effect of SP on the vasodilator response to CGRP is mediated via an action on NK-1 receptor was examined by using an NK-1 receptor antagonist (Spantide II). When this antagonist was concomitantly perfused with SP (all at 1 μM), it was able to prevent SP from terminating the vasodilator effect of CGRP (Figure 3A).

Effect of SP and/or CGRP treatment on wound healing in old rats. — The effect of two daily i.d. injections of 200 μl SP or CGRP (both at 10 μM) on wound healing in old rats is shown in Figure 4. Old rats treated with SP showed less enlargement of the wound compared to saline-treated con-
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Figure 3. (A) Tracing of a typical record of relative blood flow monitored by using a laser Doppler flowmeter, showing that subsequent perfusion of SP can terminate an existing vasodilator response to CGRP (both peptides used at 1 μM). The figure also shows that the modulatory effect of SP can be prevented by concomitant perfusion of the tachykinin antagonist (Spantide II) at 1 μM. (B) The modulatory effect exerted by SP on the vasodilator response to CGRP is dose dependent. The higher the concentration of SP, the quicker it terminates the vasodilator response to CGRP.

Figure 4. Effect of two daily i.d. injections of 200 μl of CGRP or SP (10 μM each) or combined treatment with the two peptides on the wound size and healing in old rats. Old rats treated with SP showed an accelerated wound closure that was significantly different from control by day 4 onward, with complete wound closure occurring at 16.3 ± 0.4 days. Treatment with CGRP increased wound size compared with controls during the first 5 days, with this difference reaching statistical significance on day 4. A significant reduction in wound size in response to CGRP was determined by day 8 onward, with complete wound closure achieved at 17.1 ± 0.4 days. The combined treatment resulted in complete wound closure at 17.3 ± 0.6 days compared with 16.3 ± 0.4 and 17.1 ± 0.4 days for SP or CGRP, respectively.

Effect of a combined treatment of a SP antagonist with CGRP on wound healing. — In an attempt to prolong the vasodilator effect of CGRP, we preceded the CGRP treatment (10 μM) with an injection of the tachykinin antagonist (Spantide II) (10 μM). This treatment significantly reduced the time to complete wound closure, with healing achieved at 14.9 ± 0.3 days compared with 17.1 ± 0.4 days using CGRP alone (Figure 5). This combined treatment, however, still had the disadvantage of an increased wound size during the first 4 days, which was significantly greater than control. A significant healing effect was observed by day 6.

Effect of a combined treatment of a SP antagonist and CGRP followed by a SP treatment on wound healing. — In an attempt to overcome the early phase of increased wound size and at the same time accelerate wound healing, we combined the prolonged CGRP treatment [achieved by preceding the injection of CGRP with an injection of the SP antagonist (see above)] with a second injection of SP 6 h later. This protocol resulted in the most rapid healing, with complete wound closure at 11.3 ± 0.3 days compared to 21.1 ± 0.9 days in saline-treated old control rats (Figure 6).
Our results demonstrate that both old and capsaicin-pretreated groups showed delayed wound contraction and predispose to trophic disorders of the skin. Support for this conjecture comes from clinical observations that sensory neuropathies may be associated with persistent skin ulcers (Bockers et al., 1989) and connective tissue diseases (Hagen et al., 1990). Experimentally, ablation of capsaicin-sensitive afferent neurones reduces the survival of a musculocutaneous flap (Kjatansson et al., 1987) and can lead to appearance of persistent skin wounds (Maggi et al., 1987a), aggravation of acid-induced skin lesions (Maggi et al., 1987b), and formation of keratotic-like lesions in the cornea of small rodents.

A role of sensory afferent neurones in wound healing can also be envisaged from the ability of SP, neurokinin A (NKA), and CGRP to stimulate the proliferation of endothelial cells (Haegerstrand et al., 1990), arterial smooth muscle cells (Nilsson et al., 1985; Payan et al., 1986), and skin fibroblasts (Nilsson et al., 1985). These actions are likely to play a role in the angiogenesis of healing tissue, and SP is in fact able to induce neovascularization of the vascular rabbit cornea (Ziche et al., 1990b). SP is also known to cause neurite outgrowth in embryonic chick dorsal root ganglia (Narumi and Fujiita, 1978) and cultured neuroblastoma cells (Narumi and Maki, 1978). Furthermore, SP stimulates DNA synthesis in cultured arterial smooth muscle cells and human skin fibroblasts, and this effect is inhibited by a tachykinin antagonist (Nilsson et al., 1985). All these effects may be involved in wound healing and trophic functions of C-afferent fibers.

Dysfunction of sensory afferent neurones with age is likely to have a bearing on the pathophysiology of the skin. Both animal (Khalil, 1993) and human studies (Eaglstein, 1989) show that wound healing is impaired with age. Wound contraction begins later and proceeds more slowly. The major reason for delayed healing appears to be a prolonged lag phase between injury and the start of wound contraction.

Wound healing is a complex process, and several models have been used to study distinct aspects of the overall event. Full-thickness wounds, including our thermal burn wound, are known to heal by contraction as well as re-epithelialization. It should be noted that full-thickness burn wounds in humans could be complicated by excessive connective tissue remodeling and permanent scar formation. In this study, wound size and time to complete wound closure were measured following a standard thermal injury. We used the change in wound size, as monitored by the open wound area, as the outcome measure of wound healing. We believe that our measurements documenting the change in surface wound area over time could be representative of two aspects of wound healing — namely, contraction and re-epithelialization. However, because quantitative histological and immunohistochemical techniques were not applied in this study, the authors concede that assessment of wound size as reported here might not exactly reflect the true extent of wound contraction or re-epithelialization.

In the first part of the study, the importance of sensory nerves and the effect of aging on wound healing using our thermal wound model were examined in capsaicin-pretreated rats and in 2-year-old rats, respectively.

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significantly prolonged time for wound closure. The greatest differences in wound area occurred in the first 6 days following injury, with both old and capsaicin groups showing a delay of around 5 days before wound contraction was initiated (Figure 1). The similarity in the profile of delayed healing in the old group and capsaicin-pretreated group supports our proposition that a decrease in sensory nerve function with age (Khalil et al., 1994) could play an important role in delayed wound healing in old rats.

Previous studies by Cohen et al. (1987) examining the effect of age on wound repair documented a slower rate of repair of full-thickness cutaneous wounds in aged mice which was attributed to a decline in macrophage function. Our data are in agreement with this study, taking into account the evidence that sensory peptides are important modulators of macrophage function (Lotz et al., 1988; Brunelleschi et al., 1990).

After we established the importance of sensory nerves and the effect of aging on wound healing by using our thermal burn model, we then used another injury model (the blister model) to demonstrate the importance of the interaction between sensory peptides in modulating microvascular blood flow.

Using the blister model in the rat hind footpad, we demonstrated the ability of SP to terminate an existing vasodilator response to CGRP (Figure 3A). Previous studies (Brain and Williams, 1988) reported that combined administration of SP and CGRP in human skin can limit the vasodilator activity of CGRP. Our results not only confirm but also extend these previous reports by providing evidence that this modulatory inhibitory effect exerted by SP on the vasodilator activity of CGRP is dose dependent. The higher the concentration of SP, the quicker its ability to terminate response to CGRP (Figure 3B). In addition, by using the blister model we also provided evidence that the presence of the tachykinin antagonist (Spantide II) can prevent this SP-induced modulation, thus allowing CGRP to maintain a prolonged vasodilator response (Figure 3A).

We then proceeded to investigate the ability of these peptides to modulate wound healing in old rats with a thermal burn injury. In this part of the study, we administered sensory peptides by intradermal injection around the injury in an attempt to improve wound healing in old rats. We designed treatment protocols that took into consideration our previous finding that (a) postinjury sensory receptors are upregulated with age (Khalil et al., 1994), and the evidence from this study that (b) SP can terminate an existing vasodilator response to CGRP, and (c) that exogenous application of a tachykinin antagonist before CGRP can prolong the vasodilator response to exogenously administered CGRP. The rationale for using a SP antagonist was to counteract the influence of any endogenously released SP from sensory nerves around the wound that might act to terminate the action of similarly released or exogenously administered CGRP.

The results showed that exogenous administration of SP or CGRP independently decreased healing time from 21 ± 0.5 days to 16.3 ± 0.4 and 17.1 ± 0.4 days, respectively. The profile of the healing was, however, different between the two treatment groups. The group that received SP showed a significant healing effect manifested by a decrease in wound size that was observed by day 4, thus overcoming the initial wound deterioration effect. On the other hand, the group that received CGRP showed an initial increase in wound size during this early phase, and a significant healing effect was only observed by day 8 onward. The reason for the initial increase in wound size in response to CGRP treatment could be attributed to its potent vasodilator activity. Previous studies by Sasaki et al. (1991) showed that topical application of papaverine, a smooth muscle relaxant, on dorsal skin wounds in the rat resulted in inhibition of wound contraction due to its effects on the organization of actin filaments of fibroblasts. We also observed that prolongation of the vasodilator activity of CGRP by prior administration of a SP antagonist resulted in a further increase in the wound size during the early phase, which argues in favor of the proposition that the increase in wound size by CGRP could be related to its potent vasodilator activity. Despite a similar increase in wound size in response to CGRP in the presence of a SP antagonist, it should be mentioned that this treatment protocol resulted in a faster time to complete wound closure (14.9 ± 0.3 days) compared with 17.1 ± 0.4 days in response to treatment with CGRP alone.

The ability of CGRP to accelerate wound healing despite the early increase in wound size could be attributed to the known trophic effects exerted by this peptide, including its ability to influence innervation either directly as suggested by Karanath et al. (1990) or indirectly through its angiogenic properties (Haefnerstrand et al., 1990). In support of this argument, previous studies by Kjatansson and Dalsgaard (1987) showed that the ability of CGRP to increase survival of a musculocutaneous flap was an effect independent of its vasodilator activity. It should be noted that part of the initial increase in wound size in the presence of CGRP and the SP antagonist could be related to the ability of the antagonist to block the healing effect of the endogenously released SP.

SP is also capable of exerting similar trophic functions and may stimulate DNA synthesis in arterial smooth muscle cells and skin fibroblasts (Nilsson et al., 1985) and is able to promote the growth of capillary vessels (Ziche et al., 1990b). These effects, combined with a limited vasodilator activity compared to CGRP, might have allowed SP to exert a potent significant healing effect earlier than CGRP by overcoming the early delayed phase in wound contraction.

In an attempt to optimize the effect of sensory peptides on wound healing, we then combined the two treatments that induced the most significant healing effect — namely the prolonged CGRP effect (achieved by preceding the injection of CGRP with an injection of the SP antagonist) followed by a second injection of SP 6 h later in an attempt to overcome the early delay phase. This treatment protocol resulted in an early healing effect, characterized by a significant reduction in wound size from day 4 onward together with a significant reduction in time to complete wound closure, an improvement of 46% over saline-treated controls.

Therefore, our data provide direct evidence for the ability of sensory peptides to accelerate wound healing and indirect evidence to suggest a possible role for sensory peptides in modulating wound contraction and re-epithelialization. This latter proposition is supported by previous studies by...
Watcher and Wheeland (1989) who provided evidence that topical application of capsaicin accelerated wound contraction and re-epithelialization of full-thickness wounds in swine. The authors suggested that the effects of capsaicin might be mediated via the release of SP from sensory nerves.

Our current findings demonstrating the ability of sensory peptides to accelerate tissue healing should not undermine the important role of locally acting growth factors in the regulation of wound repair. It is well known that endogenous growth factors are released at the wound site and play a necessary part of the natural wound healing process (for a review see Martin et al., 1992). Studies by Nanney (1990) and Stoscheck et al. (1992) provided evidence that epidermal growth factor (EGF) stimulates the rapid epithelialization of skin wounds with an increase in EGF-receptor expression in the epidermis during the proliferation and migration of epidermal keratinocytes. Furthermore, Seyfer et al. (1990) demonstrated that cell-bound EGF is processed into multiple forms and that the mechanisms of processing EGF and the time course of these events may explain the lag phase of wound healing. Recent studies by Tsuboi et al. (1995) demonstrated that coadministration of insulin-like growth factor (IGF) and IGF-binding protein 1 stimulates wound healing of full-thickness wounds of diabetic mice and normal rabbits. In another study by Robson et al. (1994), the authors provided evidence that topical application of granulocyte-macrophage colony stimulating factor inhibits the retardation of wound closure produced by bacterial contamination.

It is certainly true that sensory peptides and growth factors could have an overlapping range of actions that are essential for tissue repair, including a role in angiogenesis, cell proliferation and migration, synthesis of extracellular matrix, wound contraction, and re-epithelialization. In support of this notion, a recent immunohistochemical study by Manek et al. (1994) using ischaemic skin flaps demonstrated an increase in CGRP-immunoreactive nerve fibers and raised the possibility that CGRP may be able to influence innervation either directly, as suggested by Karamat et al. (1990), or indirectly through its angiogenic properties (Haeagerstrand et al., 1990). SP is also known to exert a powerful mitogenic action on different cell types which, like fibroblasts (Nilsson et al., 1985; Ziche et al., 1990a), endothelial cells (Ziche et al., 1990b), and macrophages (Brunelleschi et al., 1990), are involved in tissue repair. In addition to the ability of SP to stimulate the proliferative responses of smooth muscle cells and skin fibroblasts (Nilsson et al., 1985), it also stimulates cytokine production by mononuclear cells and is capable of stimulating the production of interleukin 1, transforming growth factor α, and EGF by eosinophils and macrophages (Lotz et al., 1988). Therefore it is reasonable to suggest that the ability of sensory peptides to modulate wound healing could be attributed partly to their ability to induce the production of certain growth factors by inflammatory cells.

In summary, we have examined the role of sensory nerves and the effect of aging on wound healing of a thermal burn induced on the interscapular region of the rat. We have provided evidence to support the notion that sensory nerves are important for the initiation of healing and tissue repair and showed that wound healing is significantly delayed with age. The results also provide evidence that sensory peptides accelerate wound healing in aged rats and that the interaction between these peptides can influence their ability to modulate the healing process. This study will bring valuable advances in the clinical management of impaired wound healing with age and raises the possibility of the future use of similar treatment protocols to accelerate wound healing in aged humans.

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