Modulation of inflammation by Cicaderma® ointment accelerates skin wound healing.

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Cicaderma’s effect on inflammation and skin wound healing

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PMNs: polymorphonuclears
MMP: matrix metalloproteinases
Abstract

Skin wound healing is a natural and intricate process taking place after injury, involving different sequential phases such as hemostasis, inflammatory, proliferative and remodeling that are associated with complex biochemical events. The interruption or failure of wound healing leads to chronic non-healing wounds or fibrosis-associated diseases constituting a major health problem where unfortunately medicines are not very effective. The objective of this study was to evaluate the capacity of Cicaderma® ointment to accelerate ulcer closure without fibrosis and to investigate wound healing dynamic processes. We used a necrotic ulcer model in mice induced by intradermal adriamycin injection and after eleven days, when the ulcer area is maximal, we applied vaseline or Cicaderma® every 2 days. Topical application of Cicaderma® allowed a rapid recovery of mature epidermal structure, a more compact and organized dermis and collagen bundles compared to vaseline group. Furthermore, the expression of numerous cytokines/molecules in the ulcer was increased 11 days after adriamycin injection compared with healthy skin. Cicaderma® rapidly reduced the level of pro-inflammatory cytokines, mainly TNF-α and others of the TNF pathway which can be correlated to a decrease of polymorphonuclears recruitment. Interestingly, the modulation of inflammation through TNF-α, MIP-1α, IL-12, IL-4 and M-CSF was maintained 9 days after the first ointment application, facilitating the wound closure without affecting angiogenesis. These cytokines appear to be potential targets for therapeutic approaches in chronic wounds. Our results confirm the use of Cicaderma® to accelerate skin wound healing and open new avenues for sequential treatments to improve healing.
INTRODUCTION

Skin wound healing involves a series of complex processes that need the interaction of cytokines and growth factors produced by many different specialized cells. During normal wound healing, these orderly events can be classified in four overlapping phases including inflammation, formation of granulation tissue, re-epithelialization and matrix formation/remodeling. When these stages are delayed for more than a few weeks, wound consequently heals unusually slowly such as in diabetic foot ulcer (Jeffcoate and Harding, 2003) or more generally necrotic ulcer (Disa et al., 1998). This defines clinically the chronic wound, one of the most common disorders, which severely impairs the quality of life of the patient and creates a huge financial burden on the healthcare system. Classically caused by a variety of events such as trauma, exposure to heat, cold, corrosive material or radiations, problems with blood circulation, skin ulcers are open wounds often accompanied by the sloughing of inflamed tissue. In order to improve this situation, many different therapeutic approaches have been tested to accelerate healing processes without fibrosis in the scar. For example, in several animal models for wound repair, a significant increase of healing was obtained by topical application of growth factors such as keratinocyte growth factor (Shannon et al., 2006; Henemyre-Harris et al., 2008), basic fibroblast growth factor (Akita et al., 2008), transforming growth factor (Cho et al., 2010) and platelet-derived growth factor (Yan et al., 2011). However, costs and side effects restricted the use of these compounds and opened the way for new approaches like the use of protectors or co-receptors of these growth factors such as mimetics of endogenous sulfated glycosaminoglycans (Garcia-Filipe et al., 2007; Barbier-Chassefiere et al., 2009).

Since antiquity, plant extracts such as St. John’s Wort (Hypericum perforatum L.), have been used to treat wounds within folk medicine in various countries. However, in the
majority of cases, the composition and the scientific evidence of their efficiency remain to be established. Interestingly, Cicaderma® ointment is mainly marketed in Europe and has been used since the middle of the 20th century in the treatment of wounds, superficial burns of limited extent and insect bites. This ointment is prepared by the extraction of fresh *Calendula officinalis* L., *Hypericum perforatum* L., *Achillea millefolium* L. aerial parts in vaseline, mixed with hydroalcoholic extract of *Ledum palustre* L.. Separately, some of these extracts, known to inhibit inflammation processes and matrix metalloproteinases (MMP) activity (Dell’Aica et al., 2007a; Dell’Aica et al., 2007b; Ozturk et al., 2007; Suntar et al., 2011), are traditionally used to improve wound healing.

In order to better understand the claimed use of Cicaderma® in wound healing and to identify new therapeutic targets, we tested its effects in a model of necrotic skin ulcer healing that reproduces human chronic wound (Barbier-Chassefiere et al., 2009) and investigated the dynamic processes implicated in wound healing. For the first time, the expression levels of forty cytokines/molecules involved in the inflammation processes were analyzed when the ulcer reached its maximal area and during wound healing.
MATERIALS AND METHODS

Animal model of skin ulceration

Housing of animals and anesthesia were performed following the guidelines established by the Institutional Animal Welfare with the European guide for care and use of laboratory animals. Standardized skin ulceration was performed by intra-dermal adriamycin (Doxorubicin Teva® 0.2%) injection on the shaved dorsum of male Swiss mice (Janvier, Le Genest-St-Isle, France) as described previously (Barbier-Chassefiere et al., 2009). Briefly, animals were anesthetized intraperitonally with sodium pentobarbital (Céva Santé Animale, Libourne, France). The back of the mice was shaved with a hair clipper and depilated with Veet® depilatory cream. Two days after depilation, mice received 150 µL of a 2 mg/mL adriamycin solution by intra-dermal injection on the depilated area. The maximum of skin ulcer area was reached eleven days after adriamycin injection. This day (day 1) was the first day of treatment with Cicaderma® (Laboratoires Boiron, France) or vaseline (the main excipient of Cicaderma®) which were then applied topically every two days on the ulcer. Ulcers were photographed every two days (i.e. 1, 3, 5, 8, 10, 12, 14, 17, 19 and 21) and cleaned until their complete closure. The lesion size was measured three times using the ImageJ software (Rasband, 1997-2012) for each ulcer and the mean was calculated. Biological samples for histological analysis were taken from sacrificed animals on days 1, 3, 5, 10, 17, 19 or 30 after the first application of the ointment whereas biochemical analysis were done on days 1, 5 and 10.

Histological studies

Skin samples were fixed in formaldehyde-buffered solution and embedded in paraffin wax. Serial 8 µm sections were prepared. Staining with Masson trichrome was used to study
skin regeneration of five mice in each experimental group (Junqueira et al., 1979). For collagen deposition study, tissue samples were stained with Sirius Red and pictures were taken using Leitz laborlux 12 PolS microscope under polarized light. For inflammatory cell studies, sections were prepared as above and stained with May-Grunwald-Giemsa. Polymorphonuclear (PMNs) blue spots (x250) were counted in three sections per mouse and for three mice in each group (Barbier-Chassefiere et al., 2009). For blood-vessel density evaluation, paraffin-embedded sections of skin samples on days 3, 5 and 10 after adriamycin injection were rehydrated using a decreasing percentage alcohol series as classically described. Anti-CD31 primary antibody (1:25 dilution; BD Biosciences) was incubated at room temperature for 2 h, and washed in 3% BSA/PBS. Then sections were incubated with a second antibody-FITC (anti-rat IgG, 1:100 dilution; Jackson ImmunoR) for 2 h at room temperature and after 3 washes, mounted with Vectashield® mounting medium (Erba et al., 2011). Specific fluorescence intensity was evaluated using TECAN Infinite M1000. The blood vessel density corresponded to the fluorescence of a section labeled by CD-31 antibody minus the auto-fluorescence of this section without CD-31 labeling of three sections per mouse and for three mice.
Biochemical studies

Proteins were extracted from skin sample following manufacturer instructions with slight modifications. Skin biopsies were minced and incubated in the sample diluent buffer (from Quantibody® Mouse Inflammation Array 1, Raybiotech) for 1 h at 37 °C before homogenization using a Potter-Elvehjem glass-Teflon homogenizer. The homogenate was centrifuged for 5 minutes at 13 000g to remove debris and insoluble material. Aliquots of the supernatant were assayed for total protein content by BCA method or stored at -80 °C until analysis.

These protein extracts (200 µg/mL) were used with Quantibody® Mouse Inflammation Array 1 (Raybiotech) to quantify forty cytokines in the kinetics of wound healing skin subjected to vaseline or Cicaderma® treatment. The binding of each cytokine on the membrane was revealed by autoradiography and quantified by the Protein Array Analyzer for ImageJ program developed for the ImageJ software (Carpentier, 2010). Each assay was performed in duplicate from three mice per experimental group for each day tested.

Statistical analysis

All results reported in text and figures are the mean (±SEM) of independent determinations. Differences between the means in two groups were evaluated using Student’s paired $t$ test; $p$ values < 0.05 were considered significant.
RESULTS

Ulcer area studies

Intradermal injection of adriamycin was previously reported to induce a necrotic skin ulcer, which regenerates spontaneously in mice and constitutes a good model to study the first phases of skin wound healing (Barbier-Chassefiere et al., 2009). As shown in Fig. 1, the measurement of the ulcer area indicates that topical applications of vaseline, from day 1 until day 21 did not modify the kinetics of ulcer closure compared with untreated skin. However, topical treatment with Cicaderma® ointment induced an acceleration of healing compared with the vaseline-treated group by reducing the surface area of ulcer by 20-25% in the first week of treatment. Interestingly, Cicaderma® application significantly induced the complete closure of ulcer 2 days earlier than what is observed with vaseline. Macroscopic observations indicated that ulcers treated with Cicaderma® appeared less inflamed, less red and thick than those treated with vaseline (Fig. 2).

Histological studies

a. Dermal reconstruction

The effects of Cicaderma® application on dermal reconstruction have been assessed by histological studies using Masson staining throughout ulcer closure. On day 1, corresponding to the maximum of the ulcer area, the total destruction of the epidermis and dermis was clearly observed as compared with the mature healthy skin (Fig. 3a-b). Then the different steps of the healing processes were followed daily. Major modifications including epidermal-cell proliferation, illustrated by thickening edges throughout the ulcer were noticed in both Cicaderma® and vaseline treated-mice. Interestingly, these effects occurred from day 2, just after one topical application of the ointment and were still persistent on day 5 (Fig. 3c-f). On day 10, the reconstitution of organized skin layers seemed to proceed for the animals.
treated with Cicaderma® and the thick skin still reflected an important activation (Fig. 3h). In contrast, vaseline induced the production of new tissue in the dermis and hypodermis, but its organization seemed more anarchic (Fig. 3g) suggesting the development of fibrosis in these animals. These differences between the two treatments were still observed on day 17, mainly in layers below the epidermis that seemed better organized in Cicaderma® treated animals (Fig. 3i-j). This was confirmed by the organization of collagen (stained in green) which was less pronounced in sections treated with vaseline than in those treated with Cicaderma®. Finally, at day 29 this results in a greater thickness of the epidermis in vaseline-treated animals than in Cicaderma®’s (Fig. 3k-l). Regarding the number of epithelial-cell layers, a faster maturation of epidermal structures is induced by Cicaderma®. Moreover the compact and organized dermis in the healing zone exhibited more common features with normal skin than the one observed with vaseline treatment.

b. Collagen organization

The histological studies also allowed us to compare collagen organization in the dermis treated with vaseline or Cicaderma® by Sirius red staining, which was visualized by polarized light. The collagen network was studied in the edges of the ulcer at early stages of wound healing (day 1, 3 and 5) or at later stages, inside the healing ulcer area. When the ulcer reached its maximal area (Fig. 4a), collagen fibers displayed heterogeneity, appearing fragmented and with longer and disorganized fibers with horizontal and vertical crossing alongside. This was probably due to the normal degradation of these fibers by proteases in the ulceration processes. On day 32, collagen was more abundant, without any organization between fibers in vaseline-treated ulcers (Fig. 4b). In contrast, treatment with Cicaderma® induced the presence of better defined intertwining fibers and reflecting the initiation of structural organization of collagen bundles (Fig. 4c). This was confirmed distinctly on day 5 and 10 (Fig. 4d-f vs e-g). At this time, collagen fibers of Cicaderma®-treated ulcers were slim
and well-defined with clear interlacements that reflected organization of these fibers, whereas
the crosswise organization of collagen bundles was not so apparent for vaseline treatment.
When ulcers were finally closed, the collagen organization started to appear in vaseline-
treated animals, while in those treated by Cicaderma® an increased amount of fibers and a
better organization in collagen network were observed (Fig. 4h-j vs i-k).

c. Polymorphonuclears infiltration

Inflammation was evaluated through the measurement of PMNs recruitment at the
dges of the ulcer (Fig. 5a). The number of PMNs was high when the ulcer reaches its
maximal area (day 1) and decreased by two fold on day 3 following the first treatment with
vaseline. In contrast, the topical application of Cicaderma® maintained the high number of
PMNs in the wound. On day 5, PMNs increased in the vaseline-treated group at the level of
day 1, and diminished by 30% on day 10. Cicaderma® treatment allowed the progressive
inhibition of PMNs recruitment until day 10, at levels significantly lower than with vaseline
treatment, suggesting a reduction of inflammatory processes in the latter steps of wound
healing.

d. Angiogenesis

CD31 staining was performed to analyze microvessel formation during the
angiogenesis phase of wound healing (Vecchi et al., 1994). Blood vessel density was not
modified by either treatment, but significantly decreased at day 10, compared to day 3 and 5
(Fig. 5b).

Biochemical studies

a. Inflammatory status at the maximal ulcer area

Before evaluating whether Cicaderma® modulates the inflammatory response during
the wound healing process, we first measured the level of forty inflammatory
cytokines/molecules in skin ulcer samples when the ulcer reached its maximal area (day 1) and compared it to normal skin (Fig. 6). Eleven days after adriamycin intradermal injection (day 1), the majority of molecules (34/40) were significantly increased in the non-treated ulcer, except GM-CSF, IFN-γ, IL-12p70, SDF-1, TCA-3 and TIMP-2 whose levels remained unchanged. Just before the first application of the ointment, TIMP-1 level was remarkably increased by ten times, whereas BLC, CD30-L, Eotaxin, Eotaxin-2, Fas-L, Fractalkine, G-CSF, IL-3, IL-6, IL-12p40p70, KC, MIP-1α, MIP-1γ, sTNF RI and sTNF RII were augmented by more than two fold. Other important molecules involved in wound healing, such as TNF-α, IL-4 and IL-10, rose from 100 to 200%.

b. Effect of Cicaderma® and vaseline treatments

Based on these observations obtained on day 1, we compared the levels of all cytokines in ulcers treated with vaseline or Cicaderma® on days 5 and 0 after induction by adriamycin. Overall, in comparison to vaseline treatment, the levels of the majority of these cytokines/molecules synthesized in the ulcer were reduced by Cicaderma® at day 5 and/or day 10.

At day 5 (Fig. 7a), Cicaderma® reduced significantly the amount of molecules involved in the TNF pathway such as TNF-α, sTNF RI, sTNF RII by 24%, 43% and 35%, respectively. Moreover, this treatment decreased classical pro-inflammatory cytokines/molecules such as IFN-γ, IL-2, IL-12p40p70, IL-12p70, MIP-1α and MIP-1γ. Surprisingly, others known molecules involved in wound-healing such as IL-1α, IL-1β or GM-CSF were not significantly modified by the two treatments (data not show). Interestingly, the amounts of some anti-inflammatory cytokines were also reduced by topical application of the ointment. Cicaderma® reduced IL-4 level by 23% and maintained a stable level of IL-10 four days after the first application while vaseline increased IL-10 by about 40% (Fig. 7a).
G-CSF and M-CSF, described as hematopoietic molecules, were significantly reduced by Cicaderma®, whereas their amount was not influenced by vaseline compared to day 1 (Fig. 7a). Moreover, the level of the metalloproteinase inhibitors TIMP-1 and TIMP-2 involved in collagens and extracellular matrix degradation were reduced after Cicaderma® treatment by 68 and 33% respectively. Finally, the detection of Fas-Ligand, involved in the regulation of apoptotic cell death, was also diminished by 50% in Cicaderma®-treated ulcer compared to vaseline group (Fig. 7a).

On day 10, the healing processes continued and we noticed that there were no statistical differences between the two treatments for the majority of proteins (27/40) as shown on Fig. 7b. However, thirteen cytokines/molecules expression were significantly decreased by 15%-35% by Cicaderma® compared with vaseline treatment (Fig. 7b). Cicaderma® was able to reduce significantly the level of the pro-inflammatory cytokines Fractalkine, IL-12p70, IL-17, MIP-1α, and TNF-α, but also of the anti-inflammatory cytokines (IL-4, IL-13) or other molecules MCP-1, M-CSF, Lymphotactin, Rantes, SDF-1, TIMP-2. The amount of pro-inflammatory cytokines IL-17 and Rantes were reduced by respectively 33 and 35% (Fig. 7b). The hematopoietic molecules MCP-1 and SDF-1 were decreased by 29 and 26%, while the anti-inflammatory cytokine IL-13 was maintained stable close to the level observed on day 1 in Cicaderma®-treated ulcer, preventing the increase induced by treatment with vaseline (Fig. 7b). Lastly, vaseline application increased TIMP-2 by 25% whereas Cicaderma® reduced significantly the level by 18% (Fig. 7b).

Interestingly, five molecules involved in inflammation IL-12p70, IL-4, M-CSF, MIP-1α, and TNF-α were significantly diminished both on days 5 and 10 (Fig. 8). Therefore, Cicaderma® seemed to modulate the inflammation during the wound healing by maintaining a reduced level of these specific factors compared with vaseline. As presented in the histological studies on day 5 (Fig. 3e-f and 4d-e) and day 10 (Fig. 3g-h and 4f-g), the
regulation of these five molecules was probably implicated in epidermal-cell proliferation, in better collagen organization and quicker re-epithelialization of the skin. As for day 5, TNF-α, the major cytokine involved in inflammation, was maintained stable by Cicaderma® treatment, while vaseline-treated ulcer induced a higher level than at day 1 (Fig. 8a). However, the level of TNF-α with Cicaderma® remained higher than in healthy skin. Whereas the level of M-CSF and IL12p70 in vaseline-treated ulcer remained unchanged from day 1 and close to those observed in normal skin (Fig. 8b-c), Cicaderma® reduced significantly the concentration of these cytokines at a lower level than on day 1 and healthy skin. Interestingly, Cicaderma® reduced the level of IL-4 from the first application at a level close to the one of healthy skin, while IL-4 level remained equivalent to day 1 and higher than in normal skin after vaseline application (Fig. 8d). The concentration of MIP-1α (Macrophage Inflammatory Protein) in Cicaderma®-treated ulcer was significantly lower to the one observed with vaseline treatment but remained higher than in normal skin (Fig. 8e).
DISCUSSION

Non-healing wounds remain a major health problem and new treatments are required to accelerate the ulcer closure. Using a mouse model of skin ulcer induced by intradermal injection of adriamycin (Barbier-Chassefiere et al., 2009), the kinetics of the ulcer closure was studied in the presence of the Cicaderma® ointment. To focus on skin wound healing processes, topic treatment with Cicaderma® only started at day 1 when the ulcer reached its maximal area. This treatment is compared to the classical clinical treatment using vaseline, which provides a protective moist environment that facilitates re-epithelialization, wound healing and is classified by the Food and Drug Administration as a skin protectant (Food and Drug Administration, 2003).

The hemostasis/inflammation phases

Eleven days after adriamycin injection, the area of the ulcer is maximal. Cell damage, blood vessels injury, degradation of collagen network induced by many toxic effects of adriamycin, including the production of an important oxidative stress, lead to clot formation. This clot is considered as an important reservoir of molecules such as cytokines, growth factors which are involved in the chemoattraction of various cells during the early steps of healing (Frank et al., 2000; Marin et al., 2001). In our ulcer model, eleven days after adriamycin injection, the expression of cytokines/molecules was completely modified compared to healthy skin (Fig. 5). Among the numerous molecules classically involved in the early steps of hemostasis/ inflammation phases of wound healing, Rantes, MCP-1, MIP-1α, MIP-1γ, Eotaxin, Fractalkine were significantly increased, which correlates with the recruitment of inflammatory cells in the wound bed, as demonstrated by PMNs counting (Fig 5a). PMNs are described as a major source of pro-inflammatory cytokines IL-1α, IL-1β, IL-6, and TNF-α, which are over-expressed in our ulcer model, to stimulate newly attracted
monocytes to differentiate into M1 macrophages. These pro-inflammatory cytokines can also be released by endothelial cells and peripheral blood monocytes in response to thrombin stimulation (Mahdavian Delavary et al., 2011). In addition to these inflammatory cytokines, we noticed a significant four fold increase in M-CSF, a hematopoietic cytokine described as an important chemoattractant for PMNs.

**Effects of Cicaderma on the granulation phase**

Interestingly, from its first topical applications (days 3 and 5), Cicaderma® induced a significant acceleration of ulcer closure as shown by the macroscopic measurement of the ulcer area. The effect of Cicaderma® allowed not only the recovery of a mature epidermal structure, a more compact and organized dermis, but also a rapid improvement of the collagen bundles organization close to mature healthy skin. Differences observed in collagen fibers network can be notably related to *Hypericum perforatum* L., which has been described as an inhibitor of MMP-2 and MMP-9 activities (Dell'Aica et al., 2007a). *Hypericum perforatum* L. down-regulates the expression of both MMPs through the inhibition of ERK1/2 signaling pathway (Dona et al., 2004). Besides, the activation of the fibroblasts and their increased collagen production by *Hypericum perforatum* L. (Ozturk et al., 2007), strengthen the beneficial effects of Cicaderma® in the granulation phase of wound healing. *Hypericum perforatum* L. was also described to reduce *in vivo* the recruitment of PMNs attaching to the vascular endothelium at the site of injury leading to a diminution of inflammation and angiogenesis (Dell'Aica et al., 2007b). This can be linked to the decrease of PMNs, considered as inflammatory markers, in the wound bed of Cicaderma® treated-ulcer. This reduction in the PMNs number is not only mirrored by the decrease of hematopoietic cytokines levels such as G-CSF and M-CSF, but also by the reduction of MIP-1α and TNF-α.
levels, in accordance with the improvement of the granulation phase seen in our histological studies.

According to the central role of TNF-α proposed by Weinstein and Kirsner in the pathogenesis of wound healing (Weinstein and Kirsner, 2010), the reduction in TNF-α level associated to the decrease of the TNF receptors sTNF RI and sTNF RII induced by Cicaderma® at day 5 is of importance to explain the acceleration of ulcer closure. The decrease of TNF-α could participate in the down-regulation of numerous inflammatory cytokines, i.e. IL-1, IL-6, IL-12, IL-17 but also the synthesis of cell surface adhesion molecules involved in keratinocytes proliferation, in PMNs migration and adhesion to endothelium (Mahdavian Delavary et al., 2011). In the granulation phase, pro-inflammatory cytokines such as TNF-α and other molecules of the TNF pathway are produced by macrophages/monocytes predominantly recruited by MIP-1α (Kondo and Ishida, 2010). However, the reduction of anti-inflammatory cytokines IL-4 and IL-10 by Cicaderma® suggests that this ointment did not simply act as an anti-inflammatory system enhancer but as a general modulator regulating both pro and anti-inflammatory processes such as TNF-α (Liechty et al., 2000; Werner and Grose, 2003). Interestingly, Calendula Officinalis L. was able to reduce levels of TNF-α, IFN-γ and IL-6 in an in vivo model of inflammation (Preethi et al., 2009) and to promote re-epithelialization in a skin excision model (Preethi and Kuttan, 2009). Other studies suggested that the specific lack of endogenous IFN-γ can reduce IL-12 levels and this significantly enhances granulation tissue formation and wound closure (Ishida et al., 2004). Thus, according to these findings, the beneficial and similar effects of Cicaderma® from day 5 on the granulation tissue could be attributed to the presence of Calendula Officinalis L. in the ointment.
Effects of Cicaderma on the angiogenesis and remodeling phases

In our ulcer model, the angiogenesis phase appeared to begin before day 5 as shown by the labeling of endothelial cells (Fig 6b). On day 3, 5 and 10, angiogenesis was not modified by Cicaderma® treatment, but further studies should be used to confirm this finding. However, Cicaderma® modified the pattern of Fractalkine, IL-13, IL-17, Lymphotactine, Rantes and SDF-1 only at day 10, whereas only IL-4, IL-12, MCP-1, M-CSF, TIMP-2 and TNF-α were decreased both on day 5 and 10. While highlighting the importance of these cytokines in the regulation of skin wound healing, these findings suggest that 1) the remodeling phase of the wound healing process starts between days 5 and 10, and 2), Cicaderma® treatment acts possibly on both re-epithelialization and remodeling phases. Furthermore, our results show for the first time, the involvement in skin wound healing processes of IL-17, an inducer of the production of many other cytokines (IL-6, G-CSF, GM-CSF, IL-1β, TGF-β, TNF-α) and chemokines including IL-8 and MCP-1 (Akdis, 2010), by fibroblasts, endothelial or epithelial cells, keratinocytes, or macrophages. Fractalkine, secreted by monocytes/macrophages and endothelial cells in response to inflammatory mediators and oxidative stress, was decreased by Cicaderma® treatment. This could reduce macrophage and fibroblast accumulation (Ishida et al., 2008), endothelial cells production of VEGF (Ryu et al., 2008) and finally angiogenesis (Clover et al., 2011). On day 10, because SDF-1 is proposed to simultaneously promote re-epithelialization and delay contraction, the reduction of SDF-1 level by Cicaderma® implies the slow-down of the re-epithelialization phase and the acceleration of wound contraction which is classically described to improve skin wound healing in rodents (Sarkar et al., 2011). On the contrary, the important decrease of G-CSF level for both treatments suggests the reduction of wound contraction. Thus, Cicaderma® could play an important role in the improvement of wound healing kinetics by participating in the fine tuning of the contraction regulation. Furthermore, Cicaderma® did not modify the
level of GM-CSF described to be essential for scarless wound repair. The low level of GM-CSF is classically associated with a stronger macrophages infiltration in the wound, with an increase of angiogenesis and a better healing (Fang et al., 2009). Thus, the inability of Cicaderma® to modify GM-CSF level in this model while downregulating a few others (Fig. 7b) may be hypothesized to contribute and promote faster wound healing, without keloid scar formation (Yeh et al., 2009). Indeed, lymphotactin level can be linked to the presence of PMNs which are the main producers this cytokine. The reduction of lymphotactin level induced by Cicaderma®, could be therefore correlated to the diminution of PMNs in the wound bed, and would be of particular interest for improving healing and for reducing the risk of keloid scar formation. In addition, the slim, defined and well organized collagen fibers in Cicaderma®-treated group, reinforce the improved quality of the final remodeling of the wound, with less inflammation and no keloid scar formation.

In conclusion, this study demonstrated that Cicaderma® ointment, a mix of well known natural extracts, modulates inflammation, promotes re-epithelialization and accelerates skin wound healing in a skin ulcer model in mice (Fig. 9). This ointment can be proposed to accelerate the healing of small wounds, and its potential use in major diseases such as burns and radiodermatitis should be considered. Moreover, our data suggest the possibility it specifically regulates the first steps of wound healing through the modulation of a few cytokines, whose regulation clearly makes a potential target for new pharmacological therapies in chronic wounds pathologies. Finally, our data strengthen the emerging concept of a sequential treatment to improve wound healing and involving different pharmacological tools.
Authorship contribution

Participated in research design: Morin, Caredda and Courty.
Conducted experiments: Morin, Roumegous and Barbier-Chassefière.
Performed data analysis: Morin, Roumegous and Carpentier.
Wrote or contributed to the writing of the manuscript: Morin, Garrigue-Antar, Caredda and Courty
References


Footnotes:

Unnumbered footnote:

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Legend of Figures

**Figure 1.** Effect of Cicaderma® on the ulcer size. Eleven days after Adriamycin injection (day 0), vaseline (dotted line) or Cicaderma® (solid line) were applied topically to the ulcers every 2 days. The surface area of each ulcer was measured as described in Methods and reported at each time point as the percentage of the surface area at baseline (day 1). Ulcer areas of animals without any treatment were represented by small dotted line. Each result is the mean (±SEM) of three independent determinations in each of 10 mice. Statistical analysis was performed using Student’s paired t test (*p < 0.05, **p < 0.01, ***p < 0.001).

**Figure 2.** Macroscopic aspect of ulcer wound healing. Eleven days after Adriamycin injection (day 1), vaseline or Cicaderma® were applied topically to the ulcers every 2 days.

**Figure 3.** Histological effects of vaseline and Cicaderma® treatment on skin ulcers. Tissue samples were stained with Masson trichrome as described in Methods. (a) Mature healthy skin; (b) Ulcer 11 days after Adriamycin intradermal injection; (c, e, g, i, k) Vaseline-treated ulcer after 2, 4, 9, 16 and 29 days of treatment (D3, D5, D10, D17 and D30 respectively); (d, f, h, j, l) Cicaderma®-treated ulcer after 2, 4, 9, 16 and 29 days of treatment (D3, D5, D10, D17 and D30 respectively). Bar= 2.0 mm

**Figure 4.** Histological effects of vaseline and Cicaderma® treatment on skin ulcers. Tissue samples were stained with Sirius red as described in Methods. Representative pictures from the area corresponding to the ulcer edges (day 1, 3, 5) or inside the healing ulcer area at later stages (day 10, 17, 21) are shown. (a) Ulcer 11 days after Adriamycin intradermal injection; (b, d, f, h) Vaseline-treated ulcer after 2, 4, 9 and 16 days of treatment (D3, D5, D10
and D17 respectively); (c, e, g, i) Cicaderma®-treated ulcer after 2, 4, 9 and 16 days of treatment (D3, D5, D10 and D17 respectively). Bar= 100 µm

**Figure 5.** a) Leucocytes recruitment at the ulcer edges. Vaseline and Cicaderma® treatment at day 3, 5 and 10 were established after May-Grunwald coloration of paraffin sections. Inflammatory cells were counted in ten independent fields of three sections of three mice of each experimental group. Results are expressed as the mean ± SEM. b) For blood-vessel density evaluation, paraffin-embedded sections of skin samples at day 3, 5 and 10 after adriamycin were used. Specific fluorescence intensity linked to anti-CD31 primary antibody was evaluated using TECAN Infinite M1000. The blood vessel density corresponded to the fluorescence of a section labeled by CD-31 antibody minus the auto-fluorescence of this section without CD-31 labeling of 3 sections from 3 mice. Results are expressed as the mean ± SEM.

**Figure 6.** Cytokine modifications at the maximum of the ulcer area (day 1). Cytokines were extracted as described in Materials and Methods and their quantification was done using Quantibody® Mouse Inflammation Array 1 from Raybiotech. Results are expressed as % of cytokine level in normal skin and represent the mean ± SEM of three animals in duplicate. Statistical analysis was performed using Student’s paired t test (*p < 0.05, **p < 0.01, ***p < 0.001).

**Figure 7.** Cytokine levels after treatment by vaseline or Cicaderma® (day 5 and day 10). a) Cytokines statistically modified after 4 days (day 5) of Cicaderma® treatment; b) GM-CSF level and cytokines statistically modified after 9 days (day 10) of Cicaderma® treatment.
Cytokines were extracted as described in Materials and Methods and their quantification was done using Quantibody® Mouse Inflammation Array 1 from Raybiotech. Results are expressed as % of cytokine level measured at the maximum of the ulcer area (day 1) and represent the mean ± SEM of three animals in duplicate. Statistical analysis was performed using Student’s paired t test (*p < 0.05, **p < 0.01, ***p < 0.001).

**Figure 8.** Level of TNF-α (a), M-CSF (b), IL-12p70 (b), IL-4 (c) and MIP-1α (c) in normal skin, at the maximum of ulcer area (D1), 4 days (D5) and 9 days (D10) after the first application of vaseline or Cicaderma®. Cytokines were extracted as described in Materials and Methods and their quantification was done using Quantibody® Mouse Inflammation Array 1 from Raybiotech. Results are expressed in % of cytokine level measured at the maximum of ulcer area (day 1) and represent the mean ± SEM of three animals in duplicate. Statistical analysis was performed using Student’s paired t test (*p < 0.05, **p < 0.01, ***p < 0.001 vaseline vs cicaderma; #p < 0.05, ##p < 0.01, ###p < 0.001 compared to normal skin).

**Figure 9.** Sequence of events associated with normal and Cicaderma®-treated wound healing.
vaseline

Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Normal Wound Healing

Adriamycin injection

-11 hemostasis/inflammation

Cicaderma treatment

Reduction of ulcer area
Organized collagens
Maintain of PMNs

Reduction of ulcer area
Organized collagens
Decrease of PMNs
Decrease of cytokines

Maximum of ulcer area
Epidermis and dermis destruction
Increase of inflammatory cytokines

Un-organized collagens
Decrease of PMNs

Un-organized collagens
Increase of PMNs

Un-organized collagens
Increase of PMNs

Uni-organized collagens

Angiogenesis

Re-epithelialization

Remodeling

Wound closure

Collagen fibers organization

Figure 9