EFFECT OF DENERVATION ON BURN WOUND HEALING

SUMMARY. The skin is a natural barrier between the interior milieu of the organism and its environment. This barrier has multiple physiological functions and may be affected by an array of pathologies including wounds and burns. The present study aims to determine the effect of the nervous system on wound healing. Specifically, this study tested the effect of denervation by chemical ablation on the burn wound healing process using guanethidine for denervation of the sympathetic postganglionic neurons and resiniferatoxin for denervation of the sensory capsaicin-sensitive fibres. Animals were divided into 8 different groups: (1) control group, (2) sensory denervated and burned, (3) sensory denervated non-burned, (4) sympathetic denervated and burned, (5) sympathetic denervated non-burned, (6) vehicle sensory burned, (7) vehicle sympathetic burned, (8) non-denervated burned. We measured different morphologic and biochemical parameters such as wound surface area, histological alterations and mast cells. In addition, NGF, IL-1β, IL-6 and IL-8 levels were determined using the ELISA technique. The gross observations, the histological data including mast cell modulation, as well as the molecular data, speak in favour of a significant delay in burn wound healing caused by sensory denervation. On the other hand, results support the positive role of sympathetic denervation in speeding up the healing process. The dual effect of the nervous system on burn wound healing is being documented in an animal model for the first time.

Keywords: burn wound healing, sympathetic denervation, capsaicin fibres denervation, inflammation, cytokines, NGF

RÉSUMÉ. La peau est une barrière naturelle entre le milieu intérieur et son environnement. Elle a des fonctions physiologiques multiples et peut être atteinte par de nombreuses pathologies parmi lesquelles plaies et brûlures. Cette étude a pour but d'évaluer les effets du système nerveux sur la cicatrisation et plus particulièrement ceux de la dénervation chimique par guanéthidine des neurones sympathiques postganglionnaires ainsi que celle des fibres sensitives à capsaïcine par résiniferatoxine. Des animaux ont été répartis en 8 groupes : (1) contrôle, (2) dénervation sensitive + brûlure, (3) dénervation sensitive sans brûlure, (4) dénervation sympathique + brûlure, (5) dénervation sympathique sans brûlure, (6) solvant de résiniferatoxine + brûlure, (7) solvant de guanéthidine + brûlure, (8) pas de dénervation + brûlure. Nous avons mesuré plusieurs paramètres morphologiques et biochimiques parmi lesquels la surface brûlées, les anomalies histologiques et la fonction mastocytaire. NGF, IL1β, IL6 et IL8 ont été mesurés par méthode ELISA. L’observation clinique, les données histologiques dont la modulation mastocytaire ainsi que les données moléculaires orientent vers un ralentissement de la cicatrisation après dénervation sensitive alors que la dénervation sympathique l’accélère. C’est la première fois que ces effets opposés des dénervations sélective est observée chez l’animal.

Mots-clés: brûlure, cicatrisation, dénervation sympathique, dénervation sensitive algésique, inflammation, cytokines, NGF

278
Introduction

A burn is defined as damage inflicted to a body’s tissue caused by heat, electricity, sunlight or radiation.¹

In superficial wounds, damage is limited to the epidermis, leaving the dermis intact. Superficial partial-thickness wounds involve superficial layers of the dermis.²

Deep partial-thickness wounds extend to deep dermis layers.² In full-thickness wounds the subcutaneous tissues are also damaged.³

Wound healing is a complex process formed of overlapping stages: immediate homeostasis followed by inflammation, proliferation, and finally remodelling or maturation. The process is initiated directly after wounding and might last for several months. It is a dynamic process that is highly regulated by cellular, humoral and molecular mechanisms.⁴

Cytokines regulate inflammatory and immune responses during wound healing by activating various cells.⁵ Exogenously administered IL-1 has been shown to promote healing of partial-thickness wounds in swine.⁶ TNF-α level is elevated in chronic wounds, and its expression diminishes as healing progresses.⁵ GM-CSF (Granulocyte-macrophage colony-stimulating factor) influences the activity of keratinocytes and fibroblasts and increases the production of vascular endothelial growth factor (VEGF).⁷

Growth factors are synthesized and secreted by many of the cell types involved in wound healing.⁸ The most relevant growth factor families for wound healing are the epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor β (TGF-β), platelet-derived growth factor (PDGF) and VEGF.⁵ Each of these cells plays a role in each phase of wound healing.⁹,¹⁰

The nervous system is involved in wound healing, and it has been reported that wound healing is delayed in denervated cutaneous wounds.¹¹-¹³

In neurogenic inflammation, dermal and epidermal nerve endings secrete proinflammatory cutaneous neuropeptides, such as substance P (SP) and calcitonin gene-related peptide (CGRP), in the proximal direction.¹⁴

Resiniferatoxin (RTX) is an excitotoxic agonist for transient receptor potential vanilloid receptor 1 (TRPV1).¹⁵ When activated, TRPV1 results in action potentials in nociceptive sensory nerves like capsaicin-sensitive fibres and some Aδ fibres. Stimulation by agonists such as RTX at high concentrations leads to impaired local nociceptor function for extended periods.¹⁶ On the other hand, guanethidine has been shown to diminish noradrenaline accumulation and dense-cored vesicles of sympathetic nerves in vitro in a dose-dependent manner,¹⁷ resulting in a chemical sympathectomy.¹⁸

NGF has a critical role in survival, differentiation and function of peripheral sensory and sympathetic nerves and brain neurons of mammals.¹⁹ The removal of the tissues that store large amounts of NGF affects recovery in wounded mice and delays skin healing considerably, while exogenous NGF added at the site of injury markedly accelerated wound contraction rate.²⁰

This study aimed to determine the effect of various components of the nervous system on wound healing via their effect on the inflammatory response triggered by burn wound infliction in a rat model; specifically, by assessing the effect of denervation by chemical ablation on the burn wound healing process using guanethidine (G) for the sympathetic postganglionic neurons and resiniferatoxin (RTX) for the capsaicin-sensitive fibres.

Materials and methods

This study is a prospective study approved by the Institutional Animal Care and Use Committee at the American University of Beirut. We used clinical observation and gross inspection of wound healing, comparison of histopathological changes over time and measurement of the changing rates of NGF, IL-1β, IL-6, and IL-8 at various time points to assess the effect of 2 types of denervation on the healing process.

Study population

A total of 143 adult female Sprague-Dawley rats (250-300g) were used in this experiment. The animals were randomly assigned to 2 major groups:

Group one: 54 rats to assess the effect of resinifer-
atoxin (RTX). They were divided into 3 subgroups:
1. Subgroup A (RTXB) consisted of 30 rats that were subjected to burning and treated with RTX;
2. Subgroup B (RTXnB) consisted of 15 rats that were treated with RTX but were not subjected to burn;
3. Subgroup C (VRTX) consisted of 9 animals that were subjected to burning and received only the vehicle for RTX (absolute alcohol).

Group two: 54 rats, subdivided into similar subgroups, with the only difference being treatment with guanethidine (G) instead of RTX. Similarly, there were three other subgroups:
1. Subgroup D treated with guanethidine and subjected to burn (GB);
2. Subgroup E treated with guanethidine only (GnB);
3. Subgroup F burned and treated with the vehicle (VG) (physiological saline).

Group three included 30 rats that were all subjected to burning without any treatment, and group four consisted of 5 rats that were considered as normal healthy controls.

Rats that underwent a standardized burning procedure were left to heal without any dressing or topical treatment. They were housed throughout the experiment on 12 h light/dark cycles with temperature of about 22 to 24°C. They had access to standard rodent chow and water. Animals from each group were sacrificed at multiple time points on D0, 3, 8, 14, 21 and 28.

Pre-operative preparation

Burning was performed under deep anaesthesia with a mixture of atropine (atropine sulfate, Laboratoire Aguettant, 0.05 mg/kg) and chlorpromazine (Largactil®, 8 mg/kg), injected intra-peritoneally as pre-anaesthetics and followed 10 min later by an intraperitoneal injection of ketamine (Ketalar®, 50 mg/kg).

The backs of all animals were shaved one day before the burning procedure. Following the Ossipov (1999) protocol, sensory ablation was achieved by intra-peritoneal injection of resiniferatoxin, 0.1 mg/kg dissolved in 100% pure ethanol. Desensitization was verified after 3 days by indifference to a corneal application of capsaicin in the RTX group. Peripheral block of sympathetic efferents was performed according to Coderre et al.’s protocol, whereby guanethidine (1-[2-guanidinoethyl] octahydroazocine) monosulfate (1:1) (from Sigma) was used to block the sympathetic efferents. It was dissolved in sterile saline, 30 mg/ml, and injected subcutaneously in the area of the burn one hour before burn.

Burning

A modified version of the aluminium stamp described by Knabl et al. was used. The desired temperature was maintained and controlled via an electronic temperature controller with a thermo-coupling feedback sensor. A burn area of 4.9 cm² was produced by applying an ordinary soldering iron (20 W) retrofitted with a 2.5 cm diameter aluminium stamp. The desired temperature of 80°C was reached after preheating the device for 15 mins. The stamp was applied for 55 sec to produce a consistent deep partial thickness burn. The iron was held vertically with no additional pressure to ensure a reproducible experimental burn.

Biopsy and observational phase

Wounds were inspected on a daily basis and findings were documented for edema, debridement, exudation, quality of the healing wound and re-epithelialization. Photos of the wounds were also taken with an mm-graded scale in frame and wound surface was measured in square cm (area = πr²) of the burn as it progressed.

On D0, 3, 8, 14, 21 and 28, punch biopsies of 3.5mm diameter were taken, under deep anaesthesia, from the rostral (for light microscopy) and caudal parts (for ELISA) of the burn areas. Animals were then sacrificed.

Fixed biopsies were embedded in paraffin and 5 µm thick sections were stained with Toluidine Blue and Hematoxylin-Eosin for routine microscopy. H&E slides were photographed by Olympus E330 camera connected to a CX41RF Olympus light microscope. The TB slides were photographed at 2048x1536 pixel resolution, using a VanGuard microscope fitted with MU300 camera with 3.1MP Aptina color CMOS and an AmScope capturing software version 3.7.3036. Mast cell count was reported as high (7-10 or more mcpf), moderate (4-6 mcpf), low/normal (1-3 mcpf) and very low (0-1 mcpf).
Caudal biopsy samples were used for the quantitative assessment of IL-1β, IL-6, IL-8 and NGF using ELISA. Biopsies were snapped directly into liquid nitrogen then stored in deep freeze at -80°C until processing. Collected tissues were homogenized for 45 sec on ice at 21000 rpm using a homogenization probe (Tissue Tearor, Polytron, Biospec Products, Inc.) along with freshly prepared ice-cold extraction buffer (Tris 100mM, NaCl 150mM, EGTA 1mM, EDTA 1mM, Triton X-100 1%, Sodium deoxycholate 0.5%; pH=7.4; 1000µl/tissue) and protease inhibitor cocktail tablets (Roche Diagnostics, Mannheim, Germany; 2 tablets/100 ml). The homogenates were then centrifuged at 4°C for 1 hour at a speed of 11,000 rpm (15,000xg) and the supernatants were collected, in sterilized test tubes, and stored at -80°C.

ELISA was used to evaluate IL-1β, IL-6, IL-8 and NGF in the supernatants of the homogenized tissues. The protein concentration of each sample was first determined using the BCA protein assay according to the manufacturer’s guidelines (Bio-Rad Laboratories, Hercules, CA). The concentrations of NGF were detected using the four-day ELISA kit (R&D Systems, Minneapolis, MN) and following the protocol provided by the manufacturer. The concentrations of each cytokine were determined using a modified two-site sandwich ELISA as previously described. Data were then analyzed using a four-parameter logistics curve-fit by Ascent Software for iEMS Reader. Cytokine levels were expressed as picograms per milligram protein.

The protein concentration in the supernatant was quantified using the DC Protein Assay following the manufacturer’s instructions (DC Protein Assay Reagent Kit, Bio-Rad) with minor modifications. Samples were pipetted as duplicates (5 µl/well) in a 96 well microtiter plate (Nunc). Each plate was inserted into a plate reader (iEMS Reader MF, Labsystems, Finland) to read the optical density of each well at an absorbance of 750 nm. Data were analyzed using Ascent Software for iEMS Reader.

**Statistical analysis**

Microsoft Excel 2013 software was used to determine statistical significance using student T-test double sided with unequal variance when comparing one group to another. Significance was considered for values less than 0.05 (P<0.05).

**Results**

No mortality was recorded in any group during the course of the study, and likewise no burn wound infection was noted clinically in any of the animals.

As presented in Fig. 1, daily observation of the animals showed that at D0, in the non-denervated burned group (nDB), the wounds were well delineated with an elevated rim and a high degree of erythema (+3). On the other hand, the guanethidine treated animals (GB) presented with a well-delineated softer burn and...
little erythema (+1), while the resiniferatoxin treated animals (RTXB) reacted similarly with little erythema (+1). The burned areas in the RTXB and GB groups were relatively softer than controls.

On D3, (+3) erythema persisted in the nDB group with a relatively harder crest, than in GB (+2) and RTXB (+1); no other major changes were noted.

After the first week, the wounds in the nDB animals showed erythema of rims (+2) with dryness and hardening of the crusts as well as partial elevation of the rims. On the other hand, the GB animals exhibited less hardening with elevation of the rims and less necrosis and partial erythema (+2). The RTXB depicted a clinical picture similar to GB with less erythema (+1).

After two weeks, the crust was partially detached in the nDB group, uncovering a well-vascularized bed (+2). In the GB group, the crust was completely detached with excellent wound bed vascularization (+3). On the other hand, the RTXB wounds maintained hard elevated crusts with a rim of erythema (+2).

After 3 weeks (D21), the crust was completely sloughed in the GB group but partially maintained in the nDB group. In the RTXB group, sloughing was partial. Vascularization in all groups was good, however it was best so in GB- and least so in RTXB-treated rats compared to the nDB group.

The burn wounds were checked for the last time on D28. They had all healed with hairs starting to grow, however, the RTXB still had a very loose crust attached and healing was less advanced.

**Burn wound area**

Measurements of the wound surface area showed that during the first week (D0 to D8), there were no changes in burn wound size. However, they significantly differed by the end of the fourth week, with the GB group having a smaller surface area (1.08 ± 0.06) compared to the RTXB (2.27 ± 0.06) and nDB group (1.72 ± 0.06) (Table I).

<table>
<thead>
<tr>
<th>Time Points</th>
<th>nDB</th>
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<th>RTXB</th>
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<tr>
<td>D28</td>
<td>1.72 ± 0.06</td>
<td>1.08 ± 0.06</td>
<td>2.27 ± 0.06</td>
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Table I - Average wound area (in cm2) ± SEM of different experimental groups at all time points

Data in Table II, comparing the surface area of the wounds inflicted in the GB group against the nDB group, show no significant difference except at D28 (p-value = 0.002). Similarly, comparing RTXB against nDB groups on D28 we observed a significant difference (p-value = 0.003) with a smaller surface area in the nDB group. When comparing GB against RTXB, there were marked differences at all time points, with better healing in the GB group.

**Histological inspection**

Microscopic changes in the non-burned skin in the CTRL, GnB and RTXnB categories showed normal skin histology with distinct well-layered epidermis, continuous ducts and glands well delineated along with a well-organized dermis.

The non-denervated burn wounds (nDB) showed...
disrupted and sloughed epidermis with edema and disorganized dermis with destroyed glands and ducts. Intact remnants of such glands were maintained in the wound bed. The collagen bundles in the upper dermis were also disorganized. The picture remained as such until D8. On D14, there was clear evidence of re-epithelialization; however, the glands and ducts did not fully recover and collagen bundles were becoming reorganized to a good extent. By D21 the wounds were fully healed; however, the glands and ducts were not fully organized, and by D28 the skin over the wound area was back to normal.

The GB group showed a similar pattern to nDB, except that the edema was less on D3; by D8 the epidermal and dermal layers showed less disorganization than the nDB group, with the growing of distinct epithelial layers (one or two). On D14, more organized and thicker epidermis and well-organized dermis were observed but the adnexa were not fully recovered until D21.

On the other hand, in the RTXB group the picture on D0 and 3 was similar to nDB. On D8, however, disorganization remained very distinct and epidermal growth was minimal; otherwise, the picture was pretty similar to that of D8 for nDB and GB. Moreover, the picture on D21 was pretty similar to D14 of GB and nDB, and the picture on D28 was similar to D21 in other groups, with a reduction in healing in the RTXB group (Fig. 2).

**Mast cell alterations**

There were alterations in the relative number of mast cells in the various groups compared to the CTRL controls (2-3 mcpf) (Fig. 3).

In the nDB group, mast cells on D0 were about 6 mcpf, and kept increasing on D3 to 7-8 mcpf on average; they then decreased on D8 with 4-6 mcpf. On D14 they went back to D0 levels (4-6 mcpf), then increased again on D21 to 7 or more, to settle on D28 at 4-6 mcpf.

In the GB group, the picture on D0 was similar to nDB (4-6 mcpf), and they maintained a similar presence on D3. They then decreased on D8 to undetectable levels and later increased on D14 to 7 or more mcpf, remaining numerous (4-6 mcpf) on D21. They increased again to 7 or more mcpf on D28.

In the RTXB group, on D0 the picture was similar to nDB or GB, however undetectable levels of mast cells were noted on D3, 8 and 14. In the last week of the experiment the number went up to 7 or more mcpf.

In the VG group, the count started similar to nDB at D0 and began to rise on D3 (4-6 mcpf), continuing to increase until D14 (7 or more mcpf). However, the number became moderate for the last 2 weeks of the experiment, with a cell count of 2-4 mcpf.
VRTX presented a higher count than CTRL on D0: 4-6 mcpf. Later, on D3 and 8 the count decreased to the level of the controls (2-3 mcpf) and then rose back to a moderate count of 4-6 mcpf for the rest of the experiment.

**Molecular parameters**

Multiple molecular parameters were assessed in the various groups at all time points considered, including NGF, IL-1β, IL-6 and IL-8. Grossly, NGF levels were different in different groups and at different time points, oscillating around control levels (Fig. 4). IL-1β exhibited undetectable levels in non-burned normal healthy skin and in the RTX-treated non-burned skin (RTXnB) (Fig. 5b) as well as extremely low levels in the GnB group, ranging between 2.81 ± 0.2, 3.68 ± 0.4, and 6.82 ± 0.4 (Fig. 6b). Nevertheless, GB against RTXB also showed significant differences at two time points, D8 and D14, where p-values were 0.03 and 0.029, respectively (Figs. 7b, 8). IL-6 concentrations started high in all burned skin groups, however in the non-denervated group (nDB) levels of IL-6 were significantly higher, 1523 ± 58 pg/mg, compared to 1062 ± 101 pg/mg and 1068 ± 78 pg/mg in the RTXB and GB groups, respectively (P < 0.05) (Figs. 7c, 9c, 10c and 11). RTXB, when compared against GB groups, presented statistical significance at three time points, D14, 21 and 28, with respective p-values of 0.03, 0.0004 and 0.01 (Figs. 7c, 9c and 10c). The data collected on IL-8 showed that levels of denervation of burned skin decreased significantly during the first week with RTXB and the first 3 days in the GB
groups compared to the non-denervated burned group (nDB). The concentration levels in the 3 burned groups later became closer. However, concentrations of IL-8 in the non-burned and control groups were undetectable (Figs. 7d, 9d and 10d). When comparing RTXB against GB, there was a difference only on D8, with p-value = 0.000001 (Figs. 7d and 12).

In brief, the results, when grouped by treatment type, exhibited multiple alterations in the concentrations of NGF, IL-1β, IL-6 and IL-8 (Tables IV-VIII and Figs. 4-12).

**Non-denervated burns (nDB)**

**NGF:** NGF decreased from D0 to D3 (14.5 pg/mg to 7.9 pg/mg), then at D8 there was an increase to 11 pg/mg. This rise continued to D14, which showed a value of 16 pg/mg, with a significant statistical difference to D0 (p < 0.05), then fell again at D21 to 11 pg/mg, thus presenting a statistically significant difference to D14 of 0.005. On D28, there was a rise back to the same value as D0 (14 pg/mg), showing a statistical significance against D21 with a p-value = 0.04, slightly higher than normal healthy controls (12.32 pg/mg) (Fig. 10a).

**IL-1β:** IL-1β increased slowly but steadily during the experiment from 134 ± 15 on D0 to 353 ± 36 on D28. Comparing the nDB concentrations against those of the CTRL presented a significant difference at all time points, with p-values of 0.01, 0.02, 0.02, 0.02, 0.008 and 0.002 for D0, 3, 8, 14, 21 and 28, respectively (Fig. 10b). Fig. 6b also shows a trend of steady increase. When comparing one time point to its previous one, no statistical significance was present. However, comparing concentrations at D0 against D14, D21 and D28, the difference was statistically significant with p-values < 0.05.

**IL-6:** IL-6 had an initial concentration of 1523 pg/mg at D0, falling to 311 pg/mg on D3, (p-value = 0.018). The fall continued to 81 pg/mg on D8 (p-value = 0.018) against D3. On D14 the concentration decreased even more to 34 pg/mg with a statistical significance against D8 (p-value = 0.023). On D21 the concentration rose again significantly to 155 pg/mg (p-value = 0.0001) against D14. The concentration then rose again on D28 to 406 pg/mg (p-value = 0.031) when compared to D21. All time points
showed statistical significance against D0 with p-values of 0.018, 0.007, 0.006, 0.008 and 0.014 for D3, 8, 14, 21 and 28, respectively. Furthermore, comparing nDB against CTRL data, they showed statistically significant differences at all time points, with p-values of 0.01, 0.017, 0.013, 0.0001 and 0.02 for D0, 3, 8, 21 and 25, respectively (Fig. 10c).

**IL-8**: Fig. 10d shows the average concentrations of IL-8 at various time points. At D0 the concentration scored 7 pg/mg, to rise significantly on D3 to 125 pg/mg (p-value = 0.002). The concentration continued to rise on D8 to 241 pg/mg, with a statistical significance against D3 (p-value = 0.004). This concentration decreased on D14 to 201 pg/mg, then to 178 pg/mg on D21. This decrease continued to D28 to reach 107 pg/mg, again showing statistical significance (p-value = 0.020) compared to D21. Similarly, D3, D8, D14, D21 and D28 showed statistical significance against D0, with p-values of 0.002, 0.0005, 0.0027, 0.0012 and 0.0007, respectively (Fig. 10d). Furthermore, the comparison of nDB vs. CTRL presented significance at all time points D3, 8, 14, 21 and 28 with respective p-values of 0.006, 0.002, 0.008, 0.004 and 0.004 (Fig. 10d).

**RTXB treatment**

Denervation by RTX of burned wounds altered the concentrations of various parameters at the various time points (Fig. 9).

**NGF**: Concentrations of NGF in the RTXB group oscillated. Fig. 9a shows the NGF level in the RTXB group at D0 to be 12 pg/mg. This level rose to 16 pg/mg at D3, presenting a statistical significance with p-value = 0.04. It then fell to 11 pg/mg at D8, with p-value = 0.01, when compared with controls, followed by another decrease to 10 pg/mg at D14. On the other hand, at D21, NGF concentration increased to 13 pg/mg, to then fall again to a value of 8 pg/mg at D28 after healing (p-value = 0.03) (Fig. 9a).

**IL-1β**: As shown in Fig. 9b, IL-1β in the RTXB group picked up after two weeks (185 ± 25) to reach its maximal level in the third week (534 ± 43) and remain high (471 ± 39) until the end of the experiment. Concentrations in the RTXB group against those of CTRL also showed significant differences at 4 time points, with p-values of 0.01, 0.05, 0.006, and 0.01 at D3, D14, D21, and D28.
D21 and D28, respectively (Fig. 9b). Fig. 9b shows the trend of increasing concentrations of IL-1β in the RTXB group. The slight increase at the first four time points showed no significance when compared to D0 and D3. However, significance was present on D14, 21 and 28 compared to D0 and D3, with a rise in IL-1β concentration from 185 pg/mg to 534 pg/mg, with a p-value = 0.02 (Fig. 9b).

**IL-6**: Fig. 9c represents the concentrations of IL-6 within the RTXB group. At D0 the concentration scored 1062 pg/mg, to fall gradually on D3 to 928 pg/mg without showing a statistical significance against D0. The fall continued to D8, presenting a concentration of 285 pg/mg with a statistical significance against D3 (p-value = 0.05). On D14 the concentration was significantly decreased to 40 pg/mg (p-value< 0.05), to then rise on D21 to 165 pg/mg, with a statistical significance compared to D14 (p-value = 0.0001), and to 194 pg/mg on D28. IL-6 values on D8, D14, D21 and D28 showed a statistical significance against D0 with p-values of 0.019, 0.011, 0.016 and 0.017, respectively. However, there was a significant drop in IL-6 with time. Such a drop was slower in the RTXB compared to the nDB or GB groups. RTXB against CTRL showed significant differences at five time points, D0, D3, D8, D21 and D28, with p-values of 0.02, 0.04, 0.047, 0.0002 and 0.0005, respectively (Fig. 9c).

**IL-8**: IL-8 was suppressed significantly from D0 (0 pg/mg) to D3 (37 pg/mg) and D8 (16 pg/mg), then increased steadily until D14 (184), to reach a maximum on D21 (247 pg/mg), remaining high on D28 (191 pg/mg) (Fig. 9d). However, when looking into the expressions of IL-8 in the RTXB group, the concentration at D0 was undetectable, then rose to 37 pg/mg at D3 to then fall again on D8 to 16 pg/mg. On D14 there was an abrupt significant increase to 181 pg/mg (p-value = 0.02). The increase continued until D21, up to 247 pg/mg, then fell to 191 pg/mg on D28. Values at D8, D14, D21 and D28 time points showed statistical significance against D0, with p-values of 0.001, 0.014, 0.0008 and 0.005, respectively (Fig. 9d). However, when comparing the RTXB against the CTRL group, they also showed significance at four time points, D8, D14, D21 and D28, with p-values of 0.002, 0.028, 0.002 and 0.010 respectively (Fig. 9d).

**GB treatment**

The changes in the various parameters in the guanethidine treatment group were close to those of RTX (Fig. 7).

**NGF**: NGF oscillated around the CTRL level, with the lowest concentrations on D8 (10.74 pg/mg) and the highest on D21 (12.87 pg/mg) (Fig. 7a). The changes were less pronounced than RTXB. Fig. 7a shows the statistical difference when compared to RTXB changes. The NGF levels in the GB group ranged from 10.74 pg/mg to 12.87 mg/pg. Such values were close to the normal control of 12.32 mg/pg. At D0 the level of NGF was 12.06 pg/mg. It then rose a little at D3 to 12.14 pg/mg and at D8, then the concentration fell to 10.74 pg/mg before rising again on D14 to 11.42 pg/mg. The rise continued until D21, with a concentration of 12.87 pg/mg, and then at D28 a fall to 11.74 pg/mg was noted. In this group, no statistical significance existed at any time point compared to controls (Fig. 7a).

**IL-1β**: Fig. 7b shows a marked decrease in concentration of IL-1β from 48 pg/mg to 38 pg/mg on D0 to D3. However, on D8 the concentration increased significantly to 324, showing a p-value = 0.0003. The rise continued on D14 to 585 pg/mg (p-value = 0.04). On D21, the concentration dropped to 378 pg/mg with no significance against D14 but still significant compared to D0 (p-value = 0.04). On the other hand, on D28 the concentration increased to 388 pg/mg with no significance compared to D21 but was still significant when compared to D0 (p-value = 0.03) (Figs. 8, 7b). Looking at the comparison among the experimental groups, GB data against CTRL data showed significant differences on D3, 8, 14 and 28, with p-values of 0.05, 0.001, 0.009 and 0.04, respectively. It is noteworthy that IL-1β had a different expression pattern in the GB group (Fig. 7b).

**IL-6**: The changes in IL-6 concentrations were sim-
ilar to nDB and slightly different from RTXB. IL-6 concentrations decreased sharply from D0 (1068 pg/mg) to D3 (38 pg/mg) and remained very low throughout the duration of the experiment (Fig. 7c). Comparing IL-6 levels in the GB group against those of the CTRL groups, they exhibited significant differences at four time points, where p-values are 0.005, 0.008, 0.001 and 0.002 for D0, 3, 8 and 14, respectively. Furthermore, in the GB group, IL-6 concentration reached 1068 pg/mg on D0, very close to RTXB, then fell significantly to 300 pg/mg on D3 (p-value = 0.004). On D8 the concentration rose again to 433 pg/mg, then fell again on D14 to 261 pg/mg, also showing a statistical significance against D8 (p-value = 0.04). On D21, however, a very low concentration of 38 pg/mg was detected with a statistical significance when compared to D21. In brief, concentrations at all time points showed statistical significance against D0 with p-values, of 0.004, 0.008, 0.003, 0.003 and 0.003, respectively (Fig. 7c).

IL-8: Fig. 7d shows the average IL-8 concentrations at the different time points. At D0, the concentration was 9 pg/mg, then it fell at D3 to 3 pg/mg. On D8, the concentrations rose sharply to 196 pg/mg to show a statistical significance against D3 (p-value = 0.0000001). On D14, the concentration decreased significantly to 132 pg/mg, thus presenting a statistical significance against D8 (p-value = 0.017). It then rose on D21 to 163 pg/mg, and on D28 the concentration reached 288 pg/mg with a statistical significance against D21 (p-value = 0.050). The time points D8, D14, D21 and D28 showed statistical significance against D0 (p-value <0.05) (Fig. 7d). A comparison of the GB group IL-8 values against those of the CTRL group values showed significant differences at four time points, D8, 14, 21 and 28, with respective p-values of 0.0000001, 0.002, 0.049 and 0.004.

RTXnB and GnB

In the absence of burn, RTX by itself lead to a decrease in NGF and IL-6 for 2 weeks. For the rest of the duration, NGF increased while IL-6 remained low (Figs. 5a and 5c). On the other hand, RTX suppressed IL-1β and IL-8 throughout the experiment (Figs. 5b and 5d). As for guanethidine, in the absence of burn, it also suppressed NGF for two weeks and IL-8 throughout the experiment (Figs. 6a and 6d). Moreover, IL-1β decreased slowly from D8 to D21, while IL-6 increased sharply during the second week (D8-D14) before decreasing sharply later on (Figs. 6b and 6c).

Discussion

The results of this study demonstrated the important role of sensory and postganglionic sympathetic fibres in the healing process of skin burn injury. The denervation of the sympathetic postganglionic neurons seemed to hasten the process, with an earlier shedding of the crust and better vascularization compared to the non-denervated group. On the other hand, the denervation of the capsaicin-sensitive fibres by resiniferatoxin delayed the inflammation process, vascularization and sloughing of the crust for almost a week compared to the nDB group. Towards the end of the experiment (D28), the burn surface was reduced significantly in the GB and nDB groups, while the crust was widely maintained in the RTXB group. Such results are in line with reported data in the literature. Our data showed that re-epithelialization was relatively slow in the first two weeks, then it picked up faster in the GB group than in the RTXB group, which was well delayed. Consequently, by D28, the guanethidine-treated rats had the best results of least surface area with better vascularization. Similar findings have been reported in the literature, documenting that sympathetic innervation is very important in regulating vascularity.

In this study, guanethidine helped to maintain a more favourable microenvironment for the healing process, while resiniferatoxin did not. However, it is important to note that the dermis has a very important effect on the epidermal layer and both layers “cross talk”. In our study, the dermis was not fully destroyed, suggesting that in a partial-thickness burn, sympathetic inhibition may not be complete and might recover in 2-3 weeks, thus leading to a rebound and to a modulation of alterations in the levels of cytokines and growth factors involved or even affecting cell populations involved in healing.
Mast cells

Many studies have indicated that mast cells degranulate when subjected to stress or to the antidromic effect of sensory fibres. It has been reported that sensory denervation impairs skin inflammation induced by mast cells. Our data are in line with the reported literature, showing that mast cells increased during the inflammatory phase in the regular nDB to go back to normal control levels later. However, in the RTXB group there was a drop and even absence of mast cells during the first two weeks of the healing process, to reappear later during the last healing phase. On the other hand, in the GB group the effect on mast cells was similar to RTXB, but less drastic; mast cells were absent only for a short time during the first week, to later regain their presence during the remodelling phases.

Changes in molecular parameters per treatment

Our data showed that burning the skin stimulated significant alterations in tested parameters; it increased the pro-inflammatory cytokine (IL-1β) and down-regulated to variable extents the anti-inflammatory cytokine (IL-8). Denervation delayed for one week in GB or two weeks in RTXB the up regulation of pro-inflammatory cytokines and inhibited the production of IL-8 in the early phases. On the other hand, NGF had non-significant alterations.

RTX blocked the sensory neurons from secreting NGF and other peptides hence the low levels of NFG in the RTXnB groups. After burning, NGF was back to around CTRL levels. This may be due to the fact that NGF is usually secreted by different cell types present at the wound site, including fibroblasts, mast cells, T-cells and keratinocytes. Keratinocytes produce and secrete active NGF, stimulating neuritic outgrowth. NGF also acts on keratinocytes, inducing them to migrate and proliferate, promoting re-epithelialization.

In our study, NGF levels decreased more in denervated non-burned rats (RTXnB) than GnB, nDB or in controls, indicating a successful denervation by RTX with maximal effect compared to guanethidine or controls.

In the RTXB group, after an initial non-significant increase, NGF decreased throughout the experiment and so did IL-6, which also decreased slowly in the first three days, then sharply to zero levels by D8, to remain low later on. In addition, IL-1β presence was delayed and started to increase significantly after the first week, therefore delaying the inflammatory homeostasis phase. Such a response corresponded to an increase in IL-8.

During the experiment, NGF did not seem to vary significantly among the various groups, as was the case for IL-6, which dropped significantly to zero levels in the nDB and later in the RTXB group but remained present for more time in the GB group, until the end of the second week. This leads us to conclude that sensory neurons are needed for the upregulation of NGF production by keratinocytes and mast cells. The sensory nerve fibres entering wounds may contribute to keratinocyte stimulation and epithelial proliferation at the wound edges and hence more NGF and IL-1β secretion.

Our results are mostly in agreement with the reported literature concerning skin wounds and the nervous system. The inflammatory cytokines that are secreted with barrier disruption by wounds or burns can recruit and trap inflammatory cells in the dermis and epidermis and store an array of cytokines and growth factors. These preformed cytokines: interleukin-1α and β, NGF and tumour necrosis factor-α (TNF-α), among others, are released from the keratinocytes, mast cells and granular cells in response to minimal external perturbations. Levels of IL-1β, IL-6 and IL-8 released at the site of injury are indicative of progress in burn wound healing. In our case, the presence of burn and sensory denervation decreased or inhibited IL-6, a possible indication of delayed inflammatory response and re-epithelialization. Simultaneously, changes leading to repair are aided by cytokines like IL-6, triggering proliferation and rebuilding of the epidermis. However, it is proven that the mitogenic effect of IL-6 can inflict a dramatic delay in re-epithelialization for keratinocytes in IL-6 knockout mice. Thus, IL-6 appears to be crucial for the initiation of the healing process via its mitogenic effect on keratinocyte and neutrophil recruitment. It decreased dramatically in the denervated rats, in particular, in the RTXB group, which may be due to the delayed inflammatory process. On the other hand, IL-8, being an anti-inflammatory cytokine, is also expressed in healing skin wounds. In vivo application of IL-8 stimulated re-epithelialization on human skin grafts in a chimeric
mouse model. Data in our study showed that the increase in IL-8 coincided with the progress of the healing process.

The inflammatory process can sustain the abnormal skin condition initiated by the primary barrier disruption.46 Acute and chronic disruption of the cutaneous permeability barrier increases messenger ribonucleic acid (mRNA) levels for TNF, granulocyte-macrophage colony-stimulating factor (GMCSF), IL-1α, IL-1β and IL-6 in the epidermis.47 It could also be the opposite case; decreasing inflammation severity by guanethidine could enhance the healing process for a shorter time, similar to what we have observed.

As reported in this study, in controls, IL-1β concentration at D0 after injury inflection was 134 pg/mg, more than double the concentration for the RTXB (52 pg/mg) and the GB group (48 pg/mg). Moreover, IL-6 concentration on D0 was 1523 pg/mg, which was also higher than the RTXB (1062 pg/mg) and GB group (1068 pg/mg). However, it took more time for IL-8 to pick up.

This difference might be explained by the suppression of the inflammatory process in the first week (GB group) and second week (RTXB group). Richards et al. found a significantly reduced cell count of monocytes and macrophages in the granulation tissue of the sensory denervated flap compared with his controls.48 As the inflammatory phase picked up pace, IL-1β concentrations started to rise and IL-6 concentrations continued to decrease in the homeostasis and proliferation phases. The pace was, however, slower for RTXB compared to the GB or nDB group. IL-1β levels were almost significant at all time points in the RTXB, GB and nDB groups compared with the control group. However, NGF did not seem to have much influence in this case or was not much affected, since the low concentration levels were close and not significantly different in all groups. On the other hand, IL-8 increase corresponded very well to progress in the healing process.

It is important to note that only female animals were used in this experiment. It has been reported that estrogen has an inhibitory role on the inflammatory process after injury in female rodents.49,50 Moreover, the percentage of re-epithelialized biopsy surface was demonstrated to be significantly larger in male patients.51 Based on this information, cytokine levels in this study may have been affected by the estrogen cycle. Hence, in burn studies conducted on females, time points should take into consideration the estrogen cycle, which is about 5 days in female rats.

Conclusion

In conclusion, denervation by RTX of sensory fibres delayed the healing time of burn wounds. On the other hand, denervation by guanethidine of the sympathetic nerves created a better environment for healing. The clinical significance of this finding has yet to be determined.

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