Pharmacological modulation of wound healing in experimental burns

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1. Introduction

Tissue damage triggers a cascade of events aimed at rapid repair [1]. Healing of wounds, however, is one of the most complex biological events after birth as a result of the interplay of different tissue structures and a large number of resident and infiltrating cell types constituted mainly by leukocyte subsets (neutrophils, macrophages, mast cells, and lymphocytes), which serve as immunological effector cells and as a source of inflammatory and growth-promoting cytokines [2]. In both primary and secondary healing, the process consists of a series of events characterized by distinct, but overlapping phases: inflammation, epithelialization, connective tissue deposition and contraction involving the overlapping action in time and space of specific cells and a multitude of cytokines [3,4]. Though burns have traditionally been considered as special types of wounds requiring specialized management protocols, healing of burn wounds does not differ in any way from healing of any other type of wound [5,6] and can be compromised by endogenous as well as exogenous factors.

Factors involved in wound healing and their interdependence are not yet fully understood; nevertheless, new prospects for therapy to favor speedy and optimal healing are emerging. Reports about wound healing modulation by local application of simple and natural agents abound even in the recent literature, however, most are anecdotal and lack solid scientific evidence. We describe the effect of silver sulfadiazine and moist exposed burn ointment (MEBO), a recently described burn ointment of herbal origin, on mast cells and several wound healing cytokines (bFGF, IL-1, TGF-β, and NGF) in the rabbit experimental burn model. The results demonstrate that various inflammatory cells, growth factors and cytokines present in the wound bed may be modulated by application of local agents with drastic effects on their expression dynamics with characteristic temporal and spatial regulation and changes in the expression pattern. Such data are likely to be important for the development of novel strategies for wound healing since they shed some light on the potential formulations of temporally and combinatorially optimized therapeutic regimens.

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The normal healing response begins the moment the tissue is injured [4]. If the tissue is skin, then repair involves re-epithelialization, formation of granulation tissue and contraction of underlying wound connective tissues. This concerted effort by the wounded cell layers is accompanied and might also be partially regulated by a robust inflammatory response, in which first neutrophils and then macrophages and mast cells migrate from nearby tissues and the circulation [1]. Acute inflammation takes place and vascular leakage occurs via several mechanisms like endothelial contraction and cytoskeletal reorganization or by formation of endothelial gaps in venules. Various cells release pro-inflammatory and anti-inflammatory cytokines as well as various growth factors. In addition, low molecular-weight compounds from the serum of injured blood vessels and from degranulating platelets and mast cells fill the injured area. The functions of these different compounds are interdependent [7–19]. Though this inflammatory response is crucial for fighting infection, recent studies suggest that depletion of one or more of the inflammatory cell lineages can even enhance healing, giving some insight that various inflammatory cells that are recruited with overlapping time courses to the wound site might do more harm than good [1]. Regulation of inflammatory cells migration to sites of tissue damage might therefore guide therapeutic strategies for modulating the inflammatory response [1] and ultimately influencing healing. Moreover, by understanding the functional relationships of the biological processes of normal compared to abnormal wound healing, hopefully new strategies can be designed to treat the pathological conditions [4].

Cell–cell and cell–matrix interactions are extremely important in all stages of the repair process. These interactions are controlled by multiple cytokines and growth factors, including transforming growth factor beta (TGF-β), tumor necrosis factor alpha (TNF-α), interleukin 1 alpha (IL-1α), interleukin 6 (IL-6), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), epidermal growth factor (EGF), keratinocyte growth factor (KGF), granulocyte-monocyte colony stimulating factor (GMCSF), and nerve growth factor (NGF) among others. It is worth noting that any given cytokine has multiple actions on the various phases of the healing process. Its effect is almost never restricted to one single phase, and not infrequently, actions of any specific cytokine at various stages may look contradictory [11,20–23]. It was demonstrated also that some cytokine levels could be used as outcome predictors of sepsis in burn, and they could correlate with prognosis such as: IL-6, TNF α, IL-8, IL-10, TGF-β [24–26]. Thermal tissue injury also induces activation of γδ T cells present in the various layers of the skin. The latter stimulates the macrophages that have been already ‘primed’ via interferon gamma (IFN-γ) to produce interleukin 12 (IL-12) which is known to stimulate pro-inflammatory cytokine production. The primed macrophages, however, will eventually respond to a secondary stimulus (primarily sepsis) by a hyperactive response (macrophage hyperactivity = increased productive capacity for pro-inflammatory mediators) leading to further release of inflammatory mediators, both pro and anti, erratically leading to further tissue injury with increased production of immunosuppressive mediators [9,27–29].

As our knowledge of the basic mechanisms of wound healing and body’s response to injury is expanding to the biomolecular level, new prospects for therapy are emerging. It is not science fiction any more to imagine that the effect of “positive” growth hormones and cytokines may be enhanced and that of “negative” factors suppressed through molecular or genetic manipulation [8]. Timely intervention with exogenous mediators may also be possible to favor a better wound healing. Topical application of cytokines like certain anti-inflammatory cytokines such as interleukin 8 (IL-8) or PDGF, though not very effective, or other agents such as emu oil [30–32] that inhibit pro-inflammatory cytokine production, were shown experimentally to promote wound healing. Some molecules like penta decapptide BPC 157 as a topical cream preparation, among many others, were also reported to improve burn wound healing through interaction with cytokines. It was demonstrated that wounds treated with positively charged cross-linked diethylaminoethyl dextran (CLLD) beads exhibit increased healing potentials probably because of 2.1 times increase in mast cells than control wounds [33]. All these observations demonstrate the fact that wound healing mechanisms may not need sophisticated gene therapy modalities to be drastically altered.

MEBO (Moist Exposed Burn Ointment) (Julphar, Gulf Pharmaceutical Industries, UAE) was patented in the USA in 1995, and is composed of six herbal extracts having as active ingredient β-sitosterol in a base of beeswax and sesame oil. It is the basis of MEFT (Moist Exposed Burn Therapy) popularized two decades ago by Xu Rongxiang [34] from the Beijing Chinese Burn Center and offers the advantages of a moist environment for wound healing by simple ointment application without the need for an overlying secondary cumbersome bulky and expensive dressing. The importance of hydration as the single most important external factor responsible for optimal wound healing has been extensively studied [3,5,6,8,21,22,35–37]. Moisture retaining dressings provide a protective barrier, prevent eschar formation, reduce dermal necrosis seen in wounds that have been allowed to dry, and significantly accelerate wound re-epithelialization [38]. Healing under both wet and moist environments is significantly faster than under dry conditions. Speed of healing, however, in moist or wet wounds may not be identical though not significantly different [39]. It seems that the optimum environment would be an intermediate gelatinous environment between moist and dry such as seen under highly vapor permeable dressings [39,40]. However, despite mounting evidence and appreciation of the biologic factors of moist environments and their ability to promote rapid infection-free re-epithelialization [29,41–45], the advantages of moisture retentive dressings on wound healing are often offset by their impracticality, particularly when they need to be applied over extensive wounds [7]. Though MEBO was introduced as a topical agent that favors wound healing by moisture retention and offsets the many drawbacks of the classical moisture retentive preparations, the clinically observed effects of this agent on wound healing cannot be solely explained by moisture retention alone. We postulate that its effect is mediated by an array of cytokine modulations. This experimental study is designed to investigate the effect of MEBO as compared to Silver sulfadiazine on mast cells and bFGF, IL-1, TGF-β, and NGF in the rabbit experimental burn model.

2. Materials and methods

2.1. Animals

With the approval and following the guidelines and recommendations of the Animal Research Board at the American University of Beirut, 21 New Zealand rabbits were included in the study. The animals' weight ranged from 1.8 to 2.0 kg. Three days prior to the experimental burn, the backs of the animals were shaved with a standard electric shaving machine. Just before inflicting the burn, the area was depilated with a commercial depilatory cream (Veet®) to obtain a smooth and hairless skin. The animals were kept under standard laboratory conditions and veterinary supervision with no restrictions on water and food.

2.2. Anesthesia

The animals were anesthetized by intramuscular injection of 3 mg/kg Ketamine and 5 mg/kg xylazine 2% (Chanazine®) an alpha adrenergic agonist. Booster injections of up to one-half of the initial dose were administered as needed in order to ensure that the rabbits were painfree during the infliction of the burn and subsequent dressing application.

2.3. Burn injury

The aluminum stamp described by Knabl et al. [123] was modified. An electronic temperature controller with a thermocouple type feedback sensor was added in order to allow precise optimal temperature monitoring at the burning surface of the stamp. A dimmer was also integrated to provide further temperature control. Continuous confirmation and monitoring of the aluminum stamp temperature was hence attained. The round stamp of 2.5 cm diameter was fitted to the ordinary soldering iron (20 W) producing a burn area of approximately 5 cm². The desired stamp temperature of 80 °C was reached 15 min after switching on the electric current. It was then applied for 25 s to produce a deep partial thickness burn. In each animal, four deep partial thickness (DPT) burns were produced on the right and left paravertebral areas. About 2–3 cm intact skin was maintained between burn areas. Total burn area was less than 10% of the animal’s total body surface area (TBSA) (Fig. 1).

2.4. Treatment

The animals were divided into three groups. Group one (G1) was the control group in which the burn wounds were covered by a simple saline dressing without any topical ointment treatment. GII had daily application of MEBO® and in GIII Silver sulfadiazine (Flammazine™—Smith & Nephew), the gold standard in burn treatment, was applied.

2.5. Dressing

The dressings were changed daily after sedating the rabbits with intramuscular injection of 0.4 ml/kg of 2% xylazine. Previously applied silver sulfadiazine was cleansed with saline solution. MEBO® was only wiped with dry gauze. Treatment ointment was reapplied and new gauze dressings were held in position with a self adhering wrap-around bandage (Mollelas® haft, Lohmann Rauscher International, Rengsdorf) (Fig. 1).


Fig. 1 – (A) Modified Aluminum stamp with an electronic temperature controller and a thermocouple type feedback sensor. (B) Burn injury. (C) Fully anesthetized animal with self adhering dressing.
2.6  **Burn tissue assessment**

Punch biopsies were taken from the cephalad and caudal burn areas on days 0, 1, 2, 3, 4, 5, 6, 9, 12, and 15. The tissues biopsied from the cephalad area were fixed in 4% paraformaldehyde for routine microscopy staining and those biopsied from the caudal area were frozen in liquid nitrogen for ELISA immunoassays of cytokines. Specimens for light microscopy were processed according to the standard methods and stained with Hematoxylin and Eosin, Periodic Acid-Schiff, or Toluidine Blue.

2.7  **Transepidermal water loss measurement (TEWL)**

The TEWL of the non-biopsied burn area and of the surrounding skin was recorded on days 0, 1, 2, 3, 4, 5, 6, 9, 12, and 15 with Dermalab 900 (Denmark) [46]. The measurement of transepidermal water loss (TEWL) expressed in grams per hour per square meter is an important non-invasive method for assessing the efficacy of skin integrity as a protective barrier. The stratum corneum forms a barrier against diffusion of water through the epidermis. As a consequence, the measurement of TEWL provides information concerning the integrity of the epidermis. For this purpose the DermaLab system equipped with a TEWL probe was used. This is based on the vapor pressure gradient estimation method of Nilsson.

2.8  **Measurement tools and immunoassay procedures**

1. The expression of basic fibroblast growth factor (bFGF) protein was detected immunohistochemically in paraffin-embedded sections (Fig. 5). Rabbit anti-fibroblast growth factor (bFGF) IgG, primary antibody (Lot # Q19, AB-33-NA) (R&D Systems Inc., Minneapolis) at a dilution 1/400 was used and FITC-Goat Anti-rabbit IgG was used as a secondary antibody at a 1/1000 dilution according to a standard method [47]. To report the degree of immunofluorescence, a subjective semi-quantitative scale of 0–3 was followed. Zero stands for no fluorescence (negative control without the primary antibody), 1 for low grade fluorescence, 2 medium grade fluorescence and 3 high grade fluorescence.

2. Mast cells density and distribution: Mast cells in burned skin were studied by two different methods using formalin fixed, paraffin-embedded 5 μm thick sections. The sections for the routine light microscopy were stained with Toluidine Blue, a basic dye that stains metachromatically mast cells. Direct immunofluorescence staining using FITC-labeled anti-mast cell antibody on paraffin fixed tissue section was also performed. This antibody targets all mast cells.

3. Enzyme-linked immunoabsorbant assay (ELISA): Frozen skin biopsies from the three groups at all time points were used for the determination of the levels of IL-1β, TGF-β1 and NGF by the two-site enzyme-linked immunoabsorbant assay (ELISA). Skin biopsies were removed under deep anesthesia, snap frozen on solid CO2 and stored at −70 °C before processing. The tissues were homogenized in phosphate-buffered saline (PBS, containing 0.4 M NaCl, 0.05% Tween 20, 0.5% bovine serum albumin, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA and 20 KI/ml aprotinin). The homogenates were centrifuged at 12,000 × g for 60 min at 4 °C and stored at −70 °C until further use. The IL-1β, TGF-β1 and NGF levels were measured by a two-site enzyme-linked immunoabsorbant assay (ELISA) as described previously by Safieh-Garabedian et al. [48]. The IL-1, TGF-β1 and NGF levels were also measured by using a Promega immunoassay kit (Promega Corporation, USA) as instructed by the manufacturer. For the IL-1β assay, we used purified polyclonal sheep anti mouse IL-1β antibody to coat high binding microtiter plates. Recombinant mouse IL-1β (National Institute for Biological Standard and Control, England) was used as the standard and a biotinylated, immunoaffinity purified polyclonal sheep anti mouse IL-1β was used as a recognition antibody. For the immunoassay, the experiments were repeated at least three times. The measured levels for each cytokine at each time interval were averaged for each experimental group. Comparisons were made between the three groups of rabbits for each time interval. The degree of significance of differences between these pairs was calculated by one-way ANOVA followed by the Bonferroni post-hoc test.

3.  **Results**

Morphological, immunological and molecular parameters were used to evaluate the efficacy of Moist Exposed Burn Ointment (MEBO) and to assess its effects on deep partial thickness burns compared to silver sulfadiazine, the gold standard local agent commonly used for burn management.

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Fig. 2 – Transepidermal water loss (TEWL) measurements. Increased water loss in the silver sulfadiazine group observed beyond day 10.
3.1. Transepidermal water loss (TEWL)

Immediately after the burn, TEWL was at its highest value in all three groups. Water loss diminished progressively as healing progressed and the epithelium covered the wound surface. As shown in Fig. 2, delta TEWL was plotted against time, i.e., the difference between the burned and non-burned skin of the same animal at the same time point. The lowest water loss values all through the experiment were recorded for the MEBO group seconded by the saline group and most water loss was registered in the silver sulfadiazine group (Fig. 2).

3.2. Macroscopic and microscopic findings

Close inspection of the wound was done every day and clinically evaluated in order to document re-epithelialization, edema, debridement, exudation and quality of the healed wound. The rabbits treated with MEBO showed accelerated epithelial repair in the burns and none of the burns became infected (Fig. 3). In general, the skin around the burns was soft and looked normal except in the silver sulfadiazine group in which it was very dry. The quality of the scars appeared to vary between the three groups. Immediately following healing, the scar formed by MEBO was relatively softer while that of the

Fig. 3 – Photomicrographs of the burned skin. G1 (control), G2 (MEBO), G3 (silver sulfadiazine). Better epithelialization is observed in G2 as compared to G3 as early as D3 progressing to epithelial layering close to normal by D12 and 15. More edema and inflammatory cells infiltration are observed in G 1 from D3 decreasing by D12 and 15.
silver sulfadiazine was drier than both saline and MEBO. We observed better scar quality with MEBO treatment paralleling faster regeneration of the new epithelium in this group and better debriding effect by day 7. Clinical healing in the other groups was relatively delayed with sloughing observed only on days 9–10.

The microscopic findings on day 0 in all three groups were similar. The upper third to half of the dermis was affected by the burn injury. Most epidermal layers and basement membrane were disrupted. Some hair follicles were externalized and partially destroyed with strong edema (+2). Edema was assessed by light microscopy through the presence of spaces filled with structureless lightly stained PAS positive material. On day 3, the MEBO group showed less edema (+1) than the other two groups (+2) with moderate invasion of inflammatory cells (less than the saline or silver sulfadiazine) including mast cells. In addition, collagen bundles remained disorganized in all three groups to the same extent, but the basal layer showed partial bridging with the epithelial cells surrounding the hair follicles in the MEBO group only. By day 6, the MEBO group showed an advanced degree of epithelialization and basement membrane continuity being established between hair follicle covering epithelium and the basal layer of the epidermis. A distinct space between old and new regenerating layers of epithelium was noted. In addition, there were persistent inflammatory cells, less edema (+1) and more mast cells compared to the other two groups in which early re-epithelialization could be observed but without establishment of continuity of the basement membrane. On day 9, the MEBO group showed more advanced re-epithelialization and layering with continuous basement membrane in addition to a better organization of the collagen bundles including the fibroblasts alignment. There was no obvious edema and normalized inflammatory cells presence including mast cells. The other two groups showed a picture similar to day 6 of MEBO. On day 12, the MEBO treated group had normal skin

Fig. 4 – Mast cell immunofluorescence. Peak fluorescence observed on D3 in G2, while levels remained high after the initial increase in G1 decreasing on D9. Fluorescence in G3 increased gradually.
architecture while the other two groups showed only continuity of the basal layer of epidermis with the hair follicles and a continuous basement membrane. In brief, the other 2 groups showed a picture similar to day 9 of MEBO. The experiment was discontinued on day 15 after complete epithelialization in the silver sulfadiazine and saline groups. The histological studies showed an overall early recovery and regeneration in the MEBO group (±3 days less) with less edema. Less deepening of the burn pathology due to progressive secondary injury with clinically better quality.
burn surface was also observed in this group as well same as healthier and smoother para-burn area that appeared very dry in the silver sulfadiazine group with delayed hair growth.

3.3. Mast cells

The immunodetection of mast cells depicted changes similar to what has been observed on routine light microscopy by Toluidine Blue staining (Fig. 4). Day 0 revealed very low activity around blood vessels in the dermis in all groups. On day 3, the saline group showed high activity (+3) which was sustained especially in between the upper and deep dermis until days 12 and 15 whereby a low to moderate activity was detected especially next to the scar (Fig. 4). The MEO group showed high mast cell activity (+3) at day 3 in the dermis and particularly in the sub-epidermis. This activity decreased dramatically by day 6 (+1.5) especially around hair follicles and blood vessels and continued so by day 9. Days 12 and 15 revealed low to moderate activity (+1.5). The sulfadiazine group showed little mast cell activity all through the experiment compared to the other two groups except at day 12. At days 3, 6, and 9 the activity was low (+1) and characterized by the presence of isolated mast cells in the dermis and close to the basal epidermal surface. Day 12, however, showed high activity everywhere in the tissue (+3) and decreased to moderate by day 15 (+2).

3.4. Cytokine activities

Modulation of the bFGF as assessed by immunofluorescence and modulation of IL-1, TGF-β and NGF assessed by ELISA assays depicted various activities in the different groups and at different time points of the 15 days of observation.

3.5. bFGF

The indirect immunofluorescence data using the anti-basic fibroblast growth factor antibody showed that by day 0, all groups had a low detectable level of florescence. In the saline control group, the detected activity of bFGF on day 1 was (+1) similar to the other two groups. However, this activity increased to (+2) by day 6. During the rest of the experiment, days 9, 12, and 15, the activity of bFGF in the saline control group went back to (+1). The bFGF activity was mostly observed within the hair follicles, in large cells of the dermis probably in mast cells, or around some glandular acini. In the MEO group, the starting level of D1 (+1) was similar to saline group. However, by day 3 the activity started to rise to (+3). This high activity was maintained all through the duration of the experiment on days 6, 9, 12, and 15. In the silver sulfadiazine group, however, the starting activity of bFGF detected by D1 was (+0.5) and this level was maintained as such all through the 15 days of the experiment (Fig. 5).

3.6. IL-1

In the saline group, the control group, IL-1 concentration in the burn tissue remained stable at a level between 50 and 75 pg/mg from day 0 to day 9 then increased significantly to almost three times to 153 and 160 pg/mg on days 12 and 15, respectively, coinciding with burn wound healing. In the silver sulfadiazine group, however, IL-1 fluctuated between trace amounts to a maximum of 50 pg all through the experiment period. In the MEO group, IL-1 concentration followed a bell shape curve of activity starting from trace amounts directly after the burn on day 0 like the other two groups then increased to 125 pg by day 3, peaked to 220 pg on day 6, then decreased to 100 by day 9 and stayed at about 50 pg on days 12 and 15 (Fig. 6).

3.7. TGF-β1

In the saline group, TGF-β1 fluctuated between days 1 and 6 and then peaked on days 9, 12, 15, paralleling the trend observed with IL-1. In the silver sulfadiazine group, the values remained fluctuating similar to IL-1 levels all through the experiment (17,000 pg on days 2, 9, and 12 to 25,000 pg on days 0, 6, and 15). The MEO group, however, expressed relatively the highest levels on day 2 (50,000 pg) and 9 (54,000 pg) and decreased back to 35,000 pg on day 15. MEO seemed to
enhance TGF-β expression same as IL-1 correlating with earlier healing of the burn wound (Fig. 6).

3.8. NGF

In the saline group, NGF maintained relatively low levels ranging between hardly detectable amounts through out the whole experiment. Similarly, in the silver sulfadiazine group, the NGF levels were even relatively lower than in the saline group except that some activity was clearly expressed on day 3 (13 pg/mg) and on day 6 (10 pg/mg). On the other hand, in the MEBO group some activity was expressed on day 2. This activity almost doubled on day 3, and peaked to 22 pg/mg on day 9. This correlated very well with the growth of neurites and observed early healing in the MEBO treated group (Fig. 6).

4. Discussion

Major burn is a particularly severe form of trauma characterized by a hypermetabolic state. This vulnerable state compromises the immune system and attenuates wound healing. Moreover, it causes tissue damage by membrane destabilization and energy depletion at the cellular level, resulting in tissue necrosis [15,49]. A logical therapeutic approach to promote recovery after burn would therefore, be to block the immediate triggering of the inflammatory cascades that result in prolonged metabolic imbalances. A second component of the therapy would be to enhance wound healing, several molecular elements of which are regulated in part by components of the inflammatory cascade [15]. The prospects that the effect of “positive” growth hormones and cytokines may be enhanced and that of “negative” factors suppressed through molecular or genetic manipulation open new therapeutic venues [21]. Findings suggest that an endogenous growth factor mediated pathway during wound repair may be amenable to exogenous manipulation [50]. As promising as it may be, research into this domain has yet to overcome numerous obstacles the least of which is still our incomplete understanding of the intricate mechanisms involved.

Local application of cytokines as proteins has been shown to be ineffective and of little clinical value due to enzymes and proteases locally present in the wound and because of lack of adequate receptors [15,51,52]. Large amounts of systemic cytokines needed for the desired therapeutic effects may result, however, in serious side-effects limiting their potential therapeutic utility in burn treatment [15]. Although gene therapy is emerging as an effective therapeutic approach to improve clinical outcomes after thermal injury [53], numerous hurdles still need to be overcome before these new technologically advanced modalities become practical for routine clinical usage [21]. Cytokine modulation by local application of therapeutic agents is an extremely appealing modality. This study has clearly demonstrated that various local burn wound care regimens have drastically different effects on the various cellular elements and cytokines involved in the healing process. Interpretation of these effects and determination of the exact significance of specific cellular and cytokine modulation on wound healing, in isolation or in combination with other cells and cytokines, remain to be clearly made.

As expected, TEWL in the MEBO treatment group was the lowest confirming previous findings that the ointment is an effective moisture retentive agent [6,37,38]. In contradistinction, silver sulfadiazine does not provide the optimal moist conditions for wound healing and therefore should not be used beyond its stated goal of prevention and treatment of burn wound sepsis. As for the effect of the two agents on inflammation, in particular on mast cells (MC’s), it was clearly divergent and different from the control group which showed sustained MC activity from days 3 to 12. MEBO exhibited an early peak activity and silver sulfadiazine a delayed peak activity. The MC has an important role in trauma and in the process of wound healing [54]. When activated, it was proven to control via a wide array of newly formed or preformed mediators released by degranulation the key events of the healing phases: triggering and modulation of the inflammatory stage, proliferation of connective cellular elements and final remodeling of the newly formed connective tissue matrix [55]. In view of this profound and complex effect of mast cells on wound healing, we can safely postulate that the two agents have different effects underlying some serious implications regarding wound healing mechanisms.

The past decade has witnessed a renaissance of mast cell research ‘beyond allergy’, along with a more systematic exploration of the surprisingly wide range of physiological functions that mast cells may be involved in [56]. Under physiological conditions, skin mast cells preferentially localize and are in close anatomic contact with the basement membrane of the nerves’ and vessels’ endothelial cells and with hair follicles. This observation, which dates back to Paul Ehrlich, intuitively suggests that these enigmatic, multifaceted protagonists of natural immunity are functionally relevant to many more aspects of tissue physiology than just to the generation of inflammatory and vasodilatory responses to IgE-dependent environmental antigens [56]. Mast cells accumulate at sites of injury [57]. Their role in wound healing, inflammation, fibrosis and epidermal hyperproliferation has been well documented and extensively studied [53,58]. On the other hand, mast cell hyperplasia has been documented in different pathologies [59]. Though the functional significance of the accumulation of mast cells in these processes is largely unknown [59], it can be safely said that their increased numbers are affiliated with a broad spectrum of pathologic skin conditions, including ulcers, atopic dermatitis, neurofibromatosis, hemangiomas, keloids, and hypertrophic scars, a significant sequela of wound healing in both traumatic and surgical injuries. It has also been proposed that mast cells play a primary pathophysiological role in these disorders and that their presence represents more than merely a secondary event [33].

Mast cells accumulate in general in tissues undergoing angiogenesis such as during tumor growth, wound healing, and tissue repair [60]. In addition, the number of MC’s surrounding a wound site was shown to increase rapidly and peak on days 2–3. MC’s numbers then steadily return to normal levels as healing progresses. It seems that increased recruitment and activation of mast cells alters the ischemic microenvironment and promotes vascular regeneration [60]. Cytokines are of particular importance for mast cell recruitment, development, and function [59]. Upon stimulation, mast
cells release a heterogeneous group of factors that promote inflammation and influence cell proliferation [57] including a potent neutrophil chemoattractant. As described in the literature, experimental data suggests that the multifactor increase in trypsin positive MC’s in the burn wound is the result of increased recruitment of MC’s or MC’s precursors. This recruitment is paralleled by the expression of monocytic chemoattractant protein-1, selective IL-4 immunoreactivity and a significant contribution to the cytokine network of wound repair via a plethora of biological mediators. These secreted mediators: IL-1β, NGF, TGF-β, bFGF as well as other pro-inflammatory and anti-inflammatory cytokines differentially modulate cutaneous wound repair depending on the time of degranulation and the quantity of mediators released. They stimulate fibroblast proliferation, and enhance the recruitment of inflammatory cells to the burn area.

Mast cells definitely participate in the regenerative processes, but their influence on epithelialization has not been well studied. In one study, mast cells are assumed to control epidermal regeneration and to impair epithelialization of chronic ulcers [61]. It is reported that accumulated mast cells in the close proximity of the epithelium edge and their chymase may impair keratinocyte adherence and migration [62]. It is clear, however, that MC’s stimulate keratinocytes and coordinate keratinocyte proliferation stimulated by NGF. In turn, stimulated keratinocytes produce also a vast repertoire of cytokines. These cytokines include interleukins, growth factors, colony stimulating factors and chemokines. These keratinocytes derived cytokines regulate the immune and inflammatory responses through their receptors on Langerhans cells, dermal fibroblasts, endothelial cells and infiltrating T cells. In addition, keratinocytes secretory products attract leukocytes from the circulation to inflammatory foci. This recruitment of immunocytes to the site of inflammation is also a major role ascribed to mast cells.

Most significant is the finding that the release of mast cell granules is commonly associated with inflammation and fibrosis [63], and that fibrotic lesions are associated with increased densities of mast cells [4]. Moreover, it has been experimentally demonstrated that fibroblast populated collagen lattice (FPCL) cast with mast cells show enhanced lattice contraction suggesting that that mast cell–fibroblast gap junction intercellular communication (GJIC) may play a role in fibrosis [63]. In a recent study, however, no relationship was found between mast cell, T-lymphocyte and macrophage numbers and the formation of normal or hypertrophic scars. In this study, hypertrophic scar formation was significantly associated with an increased number of epidermal Langerhans cells [64]. These results indicate that the epidermal immune barrier plays an important role in the development of hypertrophic scars [64]. Other studies suggest that mast cells modulate the recruitment of neutrophils into sites of injury, yet indicate that mast cells are unlikely to exert a major influence on the proliferative response within healing wounds [57]. Even with these findings, the correlation between mast cells and hypertrophic scarring previously documented cannot be dismissed due to the unequivocal mast cell functions in type I immune responses and in the pathogenesis and management of allergic diseases [56]. The importance of the mast cell in regulating healing processes has been clearly demonstrated. As a matter of fact a surplus or deficit of degranulated biological mediators causes impaired repair, with the formation of exuberant granulation tissue (e.g. keloids and hypertrophic scars), delayed closure (dehiscence) and chronicity of the inflammatory stage [55]. Experimental studies demonstrated that the collagen aggregation at the wound edges in mast cell deficient (W/Wv) mice was tighter and less interwoven compared with normal mice [65]. Moreover, several factors released by mast cells are fibrogenic.

Irrespective of this apparent controversy, and even though MC’s may participate in tissue remodeling in the late phase of wound healing [65], it is clear that only temporary mast cell activation is needed in healing wounds [58]. The effect of MEBO on MC recruitment, in contradistinction to silver sulfadiazine and control groups, must be viewed from that perspective. Though it may be beyond the scope of the present experimental study, it may be justified to postulate that the improved scar quality observed following MEBO treatment of healing wounds described in previous studies [50–52] may be due, at least in part, to limited recruitment of MC’s by MEBO ointment to the first 3 days of the healing process. The significance of this differential MC’s activity among the three study groups and its exact impact on the quality of healing is difficult to ascertain from this present study. More quantitative studies taking into consideration MC groups and specific secretory products are needed.

Numerous studies implicated fibroblast growth factors (FGFs) as key molecules during the initiation of the cellular proliferation, differentiation, migration and matrix deposition that characterize wound healing [66–70]. Basic fibroblast growth factor (FGF-2) is a critical mediator of angiogenesis that is essential for normal reproduction and wound healing [66,67]. Higher levels of bFGF were clearly shown to promote healing [71,72]. Moreover, basic fibroblast growth factor (bFGF) enhances early apoptosis contributing to decreased cellularity in granulation tissue resulting in reduction of scar formation [73]. In an interesting experimental study in rats, combined local administration of bFGF and hepatocyte growth factor HGF gene immediately after skin incision made the healing process proceed in a way closer to tissue regeneration through the induction of apoptosis and increasing the apparent regeneration of the dermis allowing for “scarless wound healing” [74]. A gelatin sheet incorporating basic fibroblast growth factor was demonstrated also to enhance sternal perfusion and accelerate sternal bone healing following dehiscence in large animals [75]. bFGF has also a broad mitogenic spectrum. It stimulates proliferation of cells from mesodermal, ectodermal, and also endodermal origin. It also regulates migration and differentiation of target cells or works as cytoprotective and supports cell survival under stress conditions. Studies also demonstrated a correlation between reduced bFGF expression/responsiveness and wound healing disorders. Data from experimental animals showed that the lack of bFGF leads, among other things, to delayed healing, a retardation in the rate of re-epithelialization, and a reduced collagen deposition at the wound site. It is obvious now that wound healing and its quality could be improved by bFGF. Healing time is significantly shortened [76]; granulation tissue formation, and collagen maturation are accelerated [77]. bFGF stimulates
also cell proliferation of the basal cell layer in the regenerating epithelium speed up epithelialization [77].

In our experimental study, most strikingly, sustained high levels of bFGF activity were detected only in the MEBO treated group and were least detected in the silver sulfadiazine group. As expected, immunofluorescence activity was detected around the hair follicles, and in the cells of the dermis and the basal keratinocyte layer. The moisture retentive ointment could have had either a direct effect on bFGF producing cells like fibroblasts, mast cells, macrophages, endothelial cells and other dermal cells, or an indirect effect through the early recruitment of mast cells to the wound site with subsequent stimulation of the bFGF producing cells by cytokines or mediators released by mast cells. The production and maintenance of the bFGF activity demonstrated experimentally in the MEBO group, correlated well with the observed hyperproliferation of the epidermal layers and early re-epithelialization. Relatively more organized collagen bundles formation was also observed.

IL-1 (IL-1α and IL-1β) is a pleiotropic pro-inflammatory cytokine responsible for fundamental functions in wound healing, inflammation, and host anti-tumor responses [78–81]. IL-1 can induce apoptosis [82] or antagonize apoptosis depending on the cell type [83]. IL-1β increases cell recruitment, tissue growth, and production of key wound healing factors and accelerates the deposition of collagen [84]. A small amount of IL-1 is necessary for host defense and wound healing [85], particularly in a challenging environment [86], whereas overproduction of IL-1 can hinder the early phase of wound healing [87,88]. IL-1 differentially regulates the expression of other cytokine and related genes, thus adding to the body of evidence that IL-1 is a major mediator of inflammatory reactions [89]. Moreover, IL-1, synergistically with other cytokines like tumor necrosis factor (TNF)-alpha or interferon (IFN)-gamma, and induces production of hepatocyte growth factor HGF (one of the vital factors for wound healing), in human dermal fibroblasts [90]. Pro-matrix metalloproteinase MMP-1 protein levels, which play a pivotal role in wound healing, are significantly increased by IL-1β [91].

In our experimental model, IL-1 levels remained low all through the duration of the experiment in the silver sulfadiazine group, increased towards the end of the experiment on day 12 to 150 pg/mg in the control group, while in the MEBO group showed an early rise on day 3 to 120 pg/mg, peaked on day 6 to 200 pg/mg, then decreased thereafter to baseline levels. Interpreting these results may be difficult in view of the complex and often contradictory effects of IL-1. However, the early and limited rise in IL-1 may be beneficial to wound healing, this probably explains somewhat why observed healing in the MEBO group was better than in the control group in which IL-1 levels increased towards the end of the observation period and probably were sustained further thereafter, and why healing was also better than in the silver sulfadiazine group in which IL-1 activity remained low. It is worth mentioning here a murine study in which restraint stress (RST) was observed to have a negative impact on wound healing. This was correlated to reduced frequencies of IL-1β mRNA-expressing leukocytes for the first 5 days [92]. The reverse trend was observed in the MEBO group. Another study worth mentioning also is about the effects observed in an in vitro scar compression model. Compression of hypertrophic scars induced secretion of IL-1β two-fold higher compared to basal condition strongly increasing apoptosis in the hypertrophic scar dermis [93] further stressing the role of IL-1 in optimal healing and better scarring.

Neurotrophins and their receptors are trophic factors that are known to play important roles in cutaneous tissues, nerve development and wound healing. Among the neurotrophins, the nerve growth factor (NGF), a pleiotropic modulator of wound-healing and inflammatory responses [94], is the prototype for the neurotrophin family of polypeptides and is one of the earliest used for clinical studies [95] and it is a neurotrophic and immunomodulatory factor contributing of the control of cutaneous morphogenesis, wound healing and inflammatory responses [96]. NGF was originally recognized for its properties in stimulating growth and differentiation of neurons. It is now also considered responsible for several activities in the immune system and beyond [97]. Even though it is clearly a pro-inflammatory factor [180] the neurotrophin nerve growth factor (NGF) is pro-angiogenic [98]. Vasoactive neuropeptides dilate blood vessels and deliver serum proteins to the wound. They induce also pain which indirectly protects from further injury [99]. This may partly explain why sensory innervation is so critical for wound healing. It may explain also the good healing effect of MEBO on neurotrophic ulcers already observed in a previous clinical study [38].

NGF was demonstrated to be an important component of wound healing and tissue repair process in vivo and in vitro [98,100–102]. NGF stimulates proliferation and inhibits apoptosis of keratinocytes and hence improves wound healing kinetics by promoting epithelialization [100]. It was also reported that NGF has a potent effect on fibroblast migration, a favorable factor for early re-epithelialization. Clinical experience has clearly shown that the topical administration of NGF is effective in accelerating the healing of surgical corneal wounds [103]. NGF promotes also healing of diabetic foot ulcers unresponsive to conventional therapies after 5–14 weeks of treatment [104]. It may also be an effective therapy for patients with severe pressure ulcers [105]. Experimental studies demonstrated that local NGF administration prevented diabetes-induced expressional alterations, enhanced reparative capillarisation, and accelerated wound healing. This was associated with a three-fold increase in endothelial cell proliferation, while apoptosis was reduced by 50%. NGF increased also the release of immunoreactive vascular endothelial growth factor-A (VEGF-A) [98]. In one study, the NGF-incorporated collagen-treated rats required shorter duration for the healing with an increased rate of wound contraction. Histological and electron microscopical evaluations revealed the activation of fibroblasts and endoplasmic reticulum and therefore increased level of collagen synthesis and wound healing due to NGF application [106].

NGF levels with both silver sulfadiazine and MEBO treatment were more than in the control group. Levels in the MEBO group were, however, higher with a peak at day 6, while levels in the silver sulfadiazine group were higher in the first day but did not reach the peak levels of the MEBO group thereafter. NGF was up-regulated to 22.5 pg/mg in the MEBO group by day 9 in contradistinction to trace levels detected in the silver sulfadiazine and saline groups with high not
exceeding 5–10 pg/mg. This coincided with complete histological healing of the burn site by day 9 observed in the MEBO group. It is worth mentioning here that the MEBO treatment group had also simultaneously higher levels of TGF-β. Transforming growth factor-β1 is considered the main profibrogenic modulator. Recently, findings indicate that NGF, via TGF-beta induction, is likely to be involved in the healing or fibrotic processes during some pathological conditions [107], hence the simultaneous increase in the levels of both factors may be clinically significant in inducing early collagen deposition and, hence, better healing.

Central to wound repair is transforming growth factor (TGF), a cytokine secreted by several different cell types involved in healing [108]. Even though various TGF isoforms may have opposing and contradictory actions [109], transforming growth factor-beta (TGF-β), in general, has surfaced from abundant research as a key signal in orchestrating wound repair [110]. It induces genes involved in fibrosis, inflammation, cell proliferation, cytoskeletal organization, and apoptosis [111] and stimulates fibroblasts to differentiate into myofibroblasts, whereas fibroblast growth factor and heparin (FGF/β) induce myofibroblasts to “de-differentiate” into fibroblasts. TGF-β induces expression of smooth muscle alpha actin (SMalphaA) and its incorporation into stress fibers, a phenotype of differentiated myofibroblasts. Additionally, TGF-β induces the expression of fibronectin and fibronectin integrins [112]. Its action individually on keratinocytes, fibroblasts, endothelial cells, and monocytes, which are the major cell types involved in wound repair, has been recently reviewed stressing its multipotent role in the process of wound healing [110,113]. More specifically, the cytokine transforming growth factor-β1 (TGF-β1) substantially influences synthesis of extracellular matrix, fibrosis, and neoangiogenesis during wound healing in a dose-dependent manner [114].

The role of TGF-β in fibrosis is well documented [115]. TGF-β is not only a major mediator of normal wound healing, its role is critical as well in various pathological conditions involving fibrosis [111]. It induces genes involved in fibrosis [111] and its fibrogenic actions are mediated through connective tissue growth factor (CTGF) [115]. TGF-β stimulates the differentiation of myofibroblasts, a hallmark of fibrotic diseases [111]. There is evidence that it is also involved in keloid formation [116]. It has been recently determined that if TGF-β signaling is not terminated at the proper time, there will be overexpression of extracellular matrix such as seen in keloids [116]. In an experimental study, less fibrosis was observed and prominent collagen types I-IV fibres production was minimized by neutralisation of the TGF-β1 activity by specific polyclonal antibodies (poAB) resulting in inhibition of the cytoplasmatic expression TGF-β1-mRNA [114]. Moreover, inhibition of TGF-beta during early corneal wound healing reduces myofibroblast differentiation and fibrosis [109]. On the other hand, up-regulation of TGF-β in the early stages of wound healing has been demonstrated to promote cutaneous wound repair [117,118]. It is obvious that modulating the effects of TGF-beta on fibrosis and adhesion formation will have tremendous significance in various clinical situations [119].

Experimentally, the MEBO group showed persistent elevated levels of TGF-β1 peaking on day 2 to 50,000 pg/mg and to 54,000 pg/mg on day 9. Levels then dropped back to 28,000 and 35,000 pg/mg on days 12 and 15, respectively. The observed early peaks of TGF-β1 on may have a great significance in the early phase of healing. In comparison, the silver sulfadiazine group TGF-β1 values oscillated between 16,000 and 25,000 pg/mg all through the experiment and those of the saline group started as 15,000 on day 1, and increased by day 9 to 30,000 pg, day 12 to 40,000 pg and reached 50,000 pg on day 15. This ascending trend may have continued thereafter. Different TGF-β1 kinetics among the three groups may not only due to the different treatment modalities. Cytokine interdependence and mutual up-regulation or down-regulation may play an important role. IL-1 that has up-regulated in the MEBO group on day 5, and not in the other two groups, probably inhibited the increase of TGF-β1 expression observed on day 6 through its IL-1 ra receptor.

5. Conclusion

Knowledge about wound healing kinetics is still limited. Factors involved and their interdependence are not yet fully understood. Nevertheless, new prospects for therapy are emerging for inflammation and cytokine modulation among which is gene therapy. Wound healing mechanisms, however, may not need sophisticated therapeutic modalities to be drastically altered. Reports about wound healing modulation by local application of simple and natural agents abound even in the recent literature [120,113]. The results described in this study demonstrated that the multitude of inflammatory cells, growth factors and cytokines present in the wound bed may be modulated by application of local agents with drastic effects on their expression dynamics with characteristic temporal and spatial regulation and changes in the expression pattern. Such data are likely to be important for the development of novel strategies for wound healing since they shed some light on the potential formulations of temporally and combinatorially optimized therapeutic regimens. One observation though that warrants special attention and further investigation is the lesser deepening and progression of the burn pathology observed in the MEBO group. This finding is extremely important and joins the efforts exerted at present to find ways to minimize secondary tissue injury following burns. One proposed therapeutic modality is topical heparin [121,122]. MEBO might prove to be a much convenient and safer alternative.

This experimental study confirmed our previous impression that the observed beneficial effect of the moisture retentive ointment MEBO on wound healing and scar quality cannot be explained only by moisture retention. It has demonstrated also an extremely important fact, namely, that topical agents may have profound effects on wound healing kinetics and should not be used indiscriminately without understanding the basic mechanisms involved. Silver sulfadiazine, undoubtedly is a very efficient antibacterial agent, however, its effect on wound healing is rather negative. Its use, therefore, should be tailored to each particular situation. It must be stressed also that the various available silver sulfadiazine preparations may not have the same effects on the wound bed and on the healing mechanisms. Other agents
or base vehicles present in the preparation being used may have an effect of their own that should not be attributed to silver sulfadiazine as such. The ultimate effective topical burn therapy remains in the choice of a product with a superior profile of antimicrobial activity over cellular toxicity. Topical intervention should be modified as well as the wound status is changing opting for the treatment modality with optimal modulation potential of wound healing kinetics.

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