The Safety and Efficacy of Mupirocin Topical Spray for Burn Wound Healing in A Rat Model

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ABSTRACT
This study evaluated the effect of mupirocin topical spray on burn wound healing in a rat model. Fifteen male Sprague Dawley rats were used to create full-thickness burns on the rat dorsum using a cylindrical stainless steel rod. The rats were topically treated with normal saline solution (NSS), mupirocin spray, ointment, and solution. The wound size and morphological evaluation were investigated by photographs and clinical criteria for wound healing. The histology was observed by hematoxylin and eosin (H&E) staining assay. The immunohistochemical study was evaluated by detection of transforming growth factor-beta 1 (TGF-β1), and the ratio of matrix metalloproteinase-9 to the tissue inhibitor of matrix metalloproteinase-1 (MMP-9/TIMP-1) was quantified using the enzyme-linked immunosorbent assay (ELISA) assay. A complete healing was observed at 28 days in all treatments. Mupirocin formulations accelerated the wound healing faster than NSS in size. However, the clinical criteria indicated a desirable skin appearance in the mupirocin spray and ointment treated groups. The histological evaluations showed no differences between the treatments while the immunohistochemical study revealed that all treatments reduced the level of TGF-β1 over time, particularly on day 28 in the mupirocin spray and ointment treated groups. The MMP-9/TIMP-1 ratio was significantly lower in the mupirocin spray and ointment treated groups than in the NSS and mupirocin solution groups. This study shows the safety and efficacy in the use of mupirocin topical spray. The topical mupirocin spray is an alternative suitable for development as a human topical anti-infective and wound protection spray.

Keywords: mupirocin, topical spray, burns, wound healing, animal model.

INTRODUCTION
An intact skin is vital to preserve body fluid homeostasis, thermoregulation and protect against systemic infection by acting as a physical barrier. However, burn injury, which is one of the most widespread accidents and the major cause of mortality, cause an inflammatory reaction due to the direct heat effect on the blood vessel and cytokine mediators of inflammation, leading to rapid edema formation. In addition, a breach of skin integrity provides an area for microorganism infection at the skin surface.

Although many advances have been made in burn injury managements, undesirable outcomes in wound healing process are still observed. Therefore, several drugs or substances have been used or developed for treatment and enhancement of burn wound healing. To successfully treat the burns, it is necessary to improve the use of modern medical technology. Our previous study successfully developed a topical spray formulation of mupirocin, which is an alternative antibacterial agent for the treatment of skin infection in burn wounds, particularly methicillin-resistant Staphylococcus aureus (MRSA). The composition included Eudragit E100 as a film-forming agent, polyethylene glycol 400 (PEG400) as a plasticizer, and glycerol as a humectant, with the promising wound healing applications of the sprayed film as a wound dressing. The optimized formulation generated a rapid formation of thin film with a burst release of the drug displaying an antibacterial activity against superficial bacterial infection such as S. aureus and S. epidermidis. The film provides a physical protection that can prevent an entry of microorganisms from external environment, and also permits adaptation to body contours by which the mechanical properties of the film may be increased by repeated spraying. Significantly, air and moisture can be permeated through the film to maintain a favorable environment for wound healing and avoid a maceration due to excess moisture.
MATERIALS AND METHODS

Animal preparation

All procedures were followed with the guideline approved by The Animal Ethics Committee, Prince of Songkla University (Ref.40/2014). Fifteen male Sprague Dawley rats (290-330 g, 6-8 weeks old) were obtained from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. The rats were caged at 25±2 °C, subjected to a 12- to 12-hour light-dark cycle, and allowed ad libitum access to standard rodent diets and water. The animals were acclimatized for one week before use. The rats were anesthetized by intraperitoneal injection with 50 mg/kg of pentobarbital sodium (Nembutal®, Oak Pharmaceuticals, Inc., Lake Forest, IL, USA). The dorsal hair of the rats was shaved with an electric shaver together with a depilatory cream (Veet, Reckitt Benckiser, Larkana, Pakistan). The area for the burn infliction was 10±2 cm x 8±2 cm. The rat dorsum was marked by lines that divided the dorsum into four equal areas. Each quadrant accommodated a single burn wound (Figure 1). The skin was disinfected with 70% ethanol (Siribuncha Co., Ltd., Nonthaburi, Thailand) and then left to dry and equilibrated to ambient temperature for 3 min before the burn infliction.

Burn wound creation

The rats were categorized equally into five groups. A number of rat in each group was three. Four groups were used as an experimental group to create a full-thickness burn at day 0. Another untreated group was used as a negative control. To make the oval-shaped burn wounds, a cylindrical stainless steel rod of 1 cm diameter was used for the infliction of the burn. The temperature of the rod was monitored with a thermocouple (model 51 II, Fluke Corporation, Everett, WA, USA). The rod was heated up to 100±5 °C using a gas cartridge burner before immediately placing the rod vertically onto the skin for 10 s without additional pressure.

Sample treatments

The rats were topically treated with NSS (Klean & kare, A.N.B. Laboratories Co., Ltd., Bangkok, Thailand) on the wound at the left upper quadrant as a control wound. Mupirocin topical spray in a concentration of 2% w/v was sprayed once in a volume of 50 μL metered by an actuator on the wound at the right upper quadrant, other dosage forms of mupirocin in the same amount were compared including 2% w/v mupirocin solution (50 μL) that was applied to the wound at the left lower quadrant, and 50 mg of 2% w/w mupirocin ointment (Bactroban, Lot: 1740053C, SmithKline Beecham, Rizal, Philippines) was applied to the wound at the right lower quadrant. All treatments were equivalent to 1 mg of mupirocin. Each sample was applied with a blinding cover sheet to ensure that there was no cross-contamination among the treatment groups upon application. The samples were reapplied once daily for 28 days after cleaning with NSS.

Wound size measurement and morphological evaluation

The wound size was measured by taking daily photographs for a total of 28 days. A circular reference with a diameter of 1 cm was placed parallel to permit correction for the distance between the camera and the rats. The wound area was calculated by ImageJ software (1.42q/Java 1.6.0.10, Bethesda, MD, USA) and expressed as a percentage of wound contraction compared to the original area using the following equation:

Percentage wound contraction = \((A^0 \times A^1 / A^0) \times 100\)

where \(A^0\) is the wound area on the first day of burn infliction and \(A^1\) is the wound area on the \(x^{th}\) day of burn infliction.
The clinical criteria for the morphological evaluation of the wound healing were scored according to secretion type (purulent-1, sanguineous-2, serous-3, none-4), wound secretion (heavy-1, moderate-2, low-3, none-4), wound color (dark grey-1, creamy-2, reddish-3, bright red-4), and scar formation (stiff-1, moderate-2, soft-3, none-4). Histologic and immunohistochemical analysis
The rats from a specific group on days 0, 7, 14, and 28 were euthanized using a high dose of pentobarbital sodium. Their skin tissue was then collected from the wounds. The tissue was excised and fixed in 10% neutral buffered formalin and then processed as paraffin-embedded tissue and cut into 5-µm-thick sections. The sections were stained using H&E staining kit (Sigma-Aldrich, St. Louis, MO, USA) according to the standard H&E staining protocol. The sections were observed under a light microscope equipped with a digital camera (BX61, Olympus, Tokyo, Japan) at a magnification of X40 and X100. The histological parameters for assessment of the healing were the amount of granulation tissue, inflammatory infiltrate, collagen fiber orientation, and pattern of collagen.
For the immunohistochemical study, TGF-β1 was detected in the immersion fixed paraffin-embedded sections using mouse anti-TGF-β1 monoclonal antibody.

Figure 2: (A) Skin photographs of wound contraction in rat dorsal full-thickness burn on day 0, 3, 5, 7, 14, 21 and 28 after application with NSS and different formulations of mupirocin including spray, ointment and solution. Photographs were taken from a representative animal of each group. (B) The percentage of wound contraction in the different treatments on day 3, 7, 14, 21, and 28. Data represented in mean ± SD of 3 burns. * p < 0.05, ** p < 0.01.
Figure 4: Histological images of post-burn full-thickness wound on day 7, 14 and 28 in the different treatments of NSS, mupirocin spray, mupirocin ointment, and mupirocin solution. The representative images were obtained from at least 3 specimens. Arrows indicate inflammatory cell infiltration. (Hematoxylin and eosin staining; scale bars = 1,000 and 200 μm in a magnification of X40 and X100, respectively).

ELISA, the skin was homogenized in 3 mL of extraction buffer containing 10 mM Tris pH 7.4, 150 mM NaCl, and 1% Triton X-100 per gram of tissue using a homogenizer. The homogenates were transferred to 1.5 mL Eppendorf tubes, centrifuged at 13,000×g for 10 min at 4 °C, and the supernatant was stored at −80 °C until analyzed.

Statistical analysis
Statistical evaluation was tested using SPSS software (Version 20.0, IBM Corp., Armonk, NY, USA). All data are presented as the mean values ± standard deviation (SD) in at least three replicates (unless indicated). Statistical significance was determined by paired or unpaired Student's t-tests. The statistical differences between the means of multiple groups were identified using one-way analysis of variance (one-way ANOVA) and a p-value<0.05 was considered statistically significant.

RESULTS
Healing effect of mupirocin topical spray on the morphology of a burn wound
The photographs and percentages of wound contraction after burn infliction and following the treatment of different formulations for 28 days are shown in Figure 2. Skin morphology observation and wound size measurements showed that full-thickness burn wounds consumed a total time of 28 days for complete healing in all treatments. However, mupirocin formulations accelerated wound healing observed by the exfoliation of the eschar faster than the NSS treated group. The mupirocin formulations reduced the wound size in half compared with the original, particularly on day 14.
(p<0.01) and day 28 (p<0.05). Although there was no difference in wound healing efficiency among the mupirocin formulations, a contrast in the morphology of skin was observed by assessment of the wound healing criteria (Table 1). The results showed that the secretion type and amount of wound secretion were similar...
between the treatment groups indicating a low to moderate amount of all secretion types were found within the first 7 days. A small amount of serosanguineous secretion was observed on day 14. On day 21 onward, the secretion was completely absent. For wound color, the results revealed that a dark grey wound was observed in the NSS and mupirocin solution groups in the first 7 days, while the wounds with mupirocin spray and ointment were creamy in color. On day 14, the wounds were bright red in the mupirocin spray treated group that was affected by the reflection of a transparent film covering the wound area, whereas a reddish color was observed in the ointment group, and a mixture of dark grey with reddish color was observed in the NSS group and particularly in the mupirocin solution group. On day 21 to 28, the results showed that the wound color in the mupirocin spray and ointment groups turned into a creamy color, but a reddish color remained in the NSS and mupirocin solution groups. Scar formation between the treatments showed that the mupirocin spray and ointment produced a softer scar than the NSS or mupirocin solution during the first 7 days of post-burn. However, there were no differences from day 14 onward. The stiff scar gradually became softer and completely formed a soft scar without any contracture at the ends.

**Healing effect of mupirocin topical spray on the histology and immunohistochemistry of the burn wounds**

The histological evaluation of normal skin and full-thickness wound on the rat dorsum on day zero is shown in Figure 3. The burn wounds showed attenuated epidermis layer with thermal coagulative damage to the dermis and involvement in subcutaneous fat and subjacent skeletal muscle. The depth of tissue damage was around 2.3 mm. High magnification (X100) showed obvious thermal damage of the connective tissue at the original burn site. Figure 4 shows the representative histological images obtained from at least 3 specimens of the post-burn full-thickness wounds on days 7, 14, and 28 after treatment with the different samples. The qualitative evaluation showed that on day 7, separation of the necrotic and non-necrotic layers was observed with serum oozing and numerous neutrophils in the areas of separation, mainly in the mupirocin solution group. Also, early re-epithelialization had occurred. On day 14, serum oozing and numerous neutrophils at the upper part remained with granulation tissue formation at the base of the ulcer. The pattern and orientation of the collagen fibers were mixed, and the re-epithelialization process showed an incomplete basement membrane. However, complete wound healing was observed in all treatments on day 28 which indicated the completion of re-epithelialization with fibrous scar without any inflammatory infiltrates. The immunohistochemical staining evaluation is shown in Figure 5. The results show that brown staining of TGF-β1 progressively increased after burning, particularly on day 7, and was predominantly localized in the epidermis layer, hair follicles, and sebaceous glands. All treatments gradually reduced the level of TGF-β1 along the timeline of the study period especially on day 28, which found a significant decrease of TGF-β1. The stained layer of TGF-β1 in NSS and mupirocin solution groups was higher than those in treated groups with the mupirocin spray and ointment.

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**Table 1: Morphological parameter assessments for wound healing property of a normal saline solution, mupirocin spray, mupirocin ointment, and mupirocin solution on full-thickness burns in a rat dorsum.**

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Day</th>
<th>NSS</th>
<th>Mupirocin spray</th>
<th>Mupirocin ointment</th>
<th>Mupirocin solution</th>
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<tbody>
<tr>
<td>Secretion type</td>
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<tr>
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<td>1, 2, 3</td>
<td>1, 2, 3</td>
<td>1, 2, 3</td>
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<tr>
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<td>2, 3</td>
<td>2, 3</td>
<td>2, 3</td>
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<tr>
<td></td>
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<td>4</td>
<td>4</td>
<td>4</td>
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<td></td>
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<td>Wound color</td>
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</table>

The results were averaged from 3 rats from each group. Secretion type (purulent-1, sanguineous-2, serous-3, none-4), wound secretion (heavy-1, moderate-2, low-3, none-4), wound color (dark grey-1, creamy-2, reddish-3, bright red-4), and scar formation (stiff-1, moderate-2, soft-3, none-4).
**Effect of mupirocin topical spray on the biomarkers for wound healing**

The quantification of the MMP-9/TIMP-1 ratio in post-burn of full-thickness tissue on days 7, 14, and 28 after treatment with the different formulations is shown in Figure 6. The results show the lowest of MMP-9/TIMP-1 ratios was observed in mupirocin spray and ointment treated groups that were significantly lower than the other groups on days 7 and 14, respectively (p<0.05). However, all treatments indicated low levels of the biomarkers on day 28 in which the MMP-9/TIMP-1 ratio in the mupirocin spray and ointment treated groups were lower than the NSS and mupirocin solution groups.

**DISCUSSION**

Using an animal as the wound healing model can replicate human physiology and predict therapeutic outcomes. Rats are commonly used for wound healing studies since they are easily available, easy to handle, have an accelerated rate of healing, and the experiments can be conducted on a large number at any point in time. However, there are differences in the skin anatomy between rats and humans. For example, the major mechanism of wound healing in rats is contraction while the major mechanisms involved in human skin healing occur through the processes of re-epithelialization and granulation tissue formation.

Heated instruments are one of the most commonly used methods to create a burn confliction. The reproducible method used in the current study followed the method described by Cai et al. The burn wounds were consistent in uniformity, size, and depth and the burn duration of 10 s resulted in a full-thickness burn injury.

In this study, mupirocin topical spray demonstrated possible activity in the burn wound healing particularly in a high risk of wound infection to avoid the development and spread of drug-resistant Gram-positive bacteria. However, the properties of components in the formulation directly affected accelerated wound healing because membrane fluidity plays an essential role in the wound healing process. Therefore, a humectant such as glycerol in the mupirocin spray is necessary for the preservation of wound moisture and prevention of drying of the film formulation. In this study, a transparent film that covered the wound area was observed. This moisture retention property of the film is useful in facilitating the process of wound healing, accelerating angiogenesis, and increasing the breakdown of dead tissue and fibrin. Even if the samples in themselves did not promote wound healing, there were no growth factors or wound healing associated cytokine production in the formulations. However, NSS and the mupirocin solution, that consisted of the drug dissolved in absolute ethanol, obviously showed an undesirable inflamed skin in terms of darkness and redness in wound color and stiffness in scar formation in comparison with mupirocin spray and ointment caused by the non-moisture preservation of NSS and the dehydration effect of ethanol.

TGF-β is a family of growth factors comprised of three isoforms involved in a number of essential cellular functions. TGF-β has been known as a potent stimulus of connective tissue accumulation such as the synthesis of extracellular matrix proteins including collagens, fibronectin, TIMP-1, and plasminogen activator inhibitor-1 or even TGF-β itself. Furthermore, it is associated with the wound healing process such as inflammatory response, angiogenesis, cell proliferation and migration, cell apoptosis, and matrix remodeling. There is a study found that hypertrophic derived fibroblast and scar tissue produced more mRNA and protein for TGF-β1 than normal skin, suggesting a possible role of TGF-β1 in scar formation. Interestingly, chronic and non-healing wounds often show a loss of TGF-β1 signaling. This study showed a significant difference of TGF-β1 in terms of intensity and the area of TGF-β1 localization, which was obviously found on day 28 between the NSS and mupirocin solution groups, and the mupirocin spray and ointment treated groups. It is possible to explain that the wound healing process occurs faster in mupirocin spray and ointment treated groups even if there was a greater intensity of TGF-β1 but found only in the upper part of the epidermal layer indicating that the healing process was nearly complete, while TGF-β1 in the NSS and mupirocin solution treated groups remained in the entire layer of epidermis meaning a slower process of wound healing. Moreover, this explanation correlates with the quantification of the MMP-9/TIMP-1 ratio that the higher the intensity of TGF-β1 in the mupirocin spray and ointment treated groups the lower non-healing wound biomarkers in comparison with the NSS and mupirocin solution groups.

Wound healing can be assessed by various biomarkers and defined as an indicator to evaluate the progress of a disease or the effects of treatment. MMPs constitute a family of zinc and calcium-dependent endopeptidases that function in the breakdown of the extracellular matrix. They play an important role in many normal physiological processes such as tissue remodeling. MMPs also mediate the critical steps in every phase of wound healing. MMP-9 is produced by a variety of normal and transformed cells including neutrophils, monocytes, macrophages, keratinocytes, fibroblasts, endothelial, and epithelial cells. It exerts physiological and pathological angiogenic and remodeling effects on the vasculature to heal properly at an appropriate level in the correct location and precise duration. TIMPs are specific inhibitors that bind with MMPs. The mammalian TIMP family includes four isoforms that are regulated during development and tissue remodeling. TIMP-1 stimulates erythropoiesis, inhibits angiogenesis, and is an anti-apoptotic agent for B cells. The MMP-9/TIMP-1 ratio is one of the biomarkers associated with a non-healing wound. Chronically elevated levels of MMPs and reduced levels of TIMPs have been correlated with healing-impaired wounds. The results indicated not only mupirocin spray was nontoxic to skin tissue, it also facilitated the process of wound healing by reducing the ratio of non-healing wound. On the contrary, the ratio was dominant in mupirocin solution treated group, particularly in the first inflammatory phase on day 7.
resulted from that mupirocin solution was prepared by an absolute ethanol that can cause tissue dehydration, inflammation and necrosis. In addition, the results also conformed to the histological evaluation showing numerous inflammatory cells detected in mupirocin solution treated group.

CONCLUSIONS
The present study shows the efficacy and safety use of mupirocin topical spray on rat skin as a wound dressing. Wound healing properties were demonstrated by accelerated wound contraction with desirable physical appearance outcomes, completion of the re-epithelialization process, and a lower level of non-healing and inflammatory wound biomarkers within 28 days. Mupirocin in a topical spray formulation is an alternative substitution suitable for development as a human topical anti-infective and wound protection spray.

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CONFLICT OF INTEREST
The authors report no declarations of interest.

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