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Impact of Rhamnolipid on Skin Wound Regeneration in Rats

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Abstract

Aims: This study aimed to investigate the skin wound healing potential of Rhamnolipid in vivo employing an experimental rat model. **Materials and Methods**: Rhamnolipid was purchased from a commercial source (Sigma) diluted in normal saline at 5 mg/mL and applied topically on excision back wounds in healthy Wister rats, Hyaluronic acid cream was used as the positive control. Wounds were monitored for closure rate and biopsies were obtained for histology assessment at the end of the experiment. **Results**: Wounds of the Rhamnolipid-treated group were completely closed within 14 days compared to 68.25 and 92.63 % closure rates for the untreated and Hyaluronic acid-treated groups, respectively. Histology assessment revealed a significant re-epithelization of Rhamnolipid-treated wounds when compared to the other groups. Inflammation was significantly (P = 0.049) diminished in response to Rhamnolipid, while granulation was significantly (p = 0.043) enhanced. No significant (p = 0.111) difference in angiogenesis was encountered among the groups. **Conclusion**: The findings of the study highlight the need to further seize the potential activity of Rhamnolipid as a wound healer, anti-scarring, and antimicrobial agent, especially in the maxillofacial field.

تأثير رامنوليبيد على تجديد جرح الجلد في الفئران

الملخص

الأهداف: تهدف هذه الدراسة إلى التحقق من قابلية التئام الجروح المحتملة من رامنوليبيد في الجسم الحي في نموذج الفئران التجريبية. **المواد وطرائق العمل**: تم الحصول على الرامنوليبيد من مصدر تجاري (سيغما) المخفف في المياه المالحة العادية في 5 ملغ/مل وتطبيقها موضعيا على استئصال الجروح الخلفية في الفئران نوع ويستر الصحية ، تم استخدام كريم حمض الهيالورونيك كعنصر تحكم إيجابي. تم رصد الجروح لمعدل الإغلاق وتم الحصول على الخزعات لتقييم الأنسجة في نهاية التجربة. **النتائج**: تم إغلاق جروح المجموعة المعالجة بالرامنوليبيد تماما في غضون 14 يوما مقارنة بمعدلات إغلاق 68.25 و 92.63 ٪ للمجموعات غير المعالجة والمجموعات المعالجة بحمض الهيالورونيك ، على التوالي. كشف تقييم الأنسجة عن إعادة تكوين ظهارة كبيرة للجروح المعالجة بالرامنوليبيد عند مقارنتها بالمجموعات الأخرى. تم تقليل الالتهاب بشكل ملحوظ (P = 0.049) استجابة للرامنوليبيد ، بينما تم تحسين التحبيب بشكل ملحوظ (P = 0.043). لم يكن هناك فرق كبير (P = 0.111) في تكوين الأوعية الدموية بين المجموعات. **الاستنتاجات**: تسلط نتائج الدراسة الضوء على الحاجة إلى مزيد من الاستيلاء على النشاط المحتمل للرامنوليبيد كمعالج للجروح ، ومضاد للتندب ، وعامل مضاد للميكروبات خاصة في مجال الوجه والفكين.

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INTRODUCTION

Having the face as the most showing part of the body, facial skin is most frequently subjected to damage either by trauma or by neoplastic events. Thermal, chemical, and infectious insults can also result in disruption of skin integrity causing skin wounds. Cosmetic and functional deficiency are the cardinal consequences of skin wounds to be concerned [(1)]. Wound healing is a complex process; however, it follows the main sequential steps of innate inflammation, blood vessel proliferation, and cellular migration and differentiation. Many elements are involved in these steps and sophisticated changes among blood cells, stem progenitor cells, soluble proteins, and tissue extracellular matrix are essentially developed. The release of inflammatory mediators causes chemotaxis of monocyte and neutrophil inflammatory cells to the wound site. These cells act by attacking bacterial invasion and production of angiogenic factors essential for fibroblast and keratinocyte activation [(2)]. Following the recruitment of inflammatory cells and fibroblasts and the formation of new blood vessels in the wound expanse, wound remodeling and regulation of the newly formed extracellular matrix take place to stabilize tissue tensile strength [(3,4)]. Overall, the process of wound healing should typically aim at preventing infectious deterioration of the open wound and accelerating wound closure via the regenerated tissue with minimal scar formation. A variety of natural and synthetic materials has been developed as wound healers such as natural polymers (e.g. chitosan) proteoglycan (e.g. collagen) and hyaluronic acid. They have been shown to work by modulating the process of wound healing, i.e. inflammation, angiogenesis, and cellular migration and differentiation [(5)]. Despite their advantageous effects, biomaterials, however, have some cons such as non-consistent degradation rates, and low modification ability in addition to their potential to induce immunological reactions [(6)].

Rhamnolipids is a glycolipid biosurfactant derived from *Pseudomonas aeruginosa*, composed of polar rhamnose moiety and nonpolar fatty acid moiety, this structure is responsible for the surface tension activity. Rhamnolipid has been reported to exert a significant antimicrobial effect against several Gram-positive and Gram-negative bacteria and fungi [(7,8)]. It has also been considered for wound healing potential and recommended for possible therapeutic application for diabetic, pressure, and venous stasis sores. The pointed-out effect was documented especially at low concentrations while higher concentrations were found toxic rather than curative. The latter effect was attributed to Rhamnolipid hemolytic characteristics while its healing-enhancing tendency was owed, though partially, to endorsing keratinocytes' proliferative

ability and wound closure rate [9]. By combining the antimicrobial and wound healing potentials previously monitored for Rhamnolipid, it sounds logical to further investigate its *in vivo* wound healing activity using a rodent model of excision wounds. The effect was judged visually and histopathologically in comparison to hyaluronic acid as the treatment control.

MATERIALS AND METHODS

Approval of the Study: The study was registered by the Oral and Maxillofacial Surgery Department/College of Dentistry Scientific Committee / Mosul University on 13 July 2023. Before starting the study, the Research Ethics Committee at the College of Dentistry / Mosul University (reference number UoM.Dent. 23/29 in 9 July 2023) granted ethical approval for its protocol.

Preparation of RH solution

Rhamnolipid was purchased from Sigma as a powder of 98 % purity. A solution in normal saline of 5 mg/mL RH [10] was prepared and kept at 4 ˚C for subsequent use.

Animal model design and preparation

Adult male healthy Wister rats (N=20) were included in the study. A pilot study was conducted on 2 animals to assess the toxicity of the applied RH. Two concentrations rh of 10 mg/ml and 5 mg/ml were tested. The high conc. appeared to induce skin ulcers and profound inflammation. Hence, 5 mg/ml was used subsequently. To induce skin wounds, the remaining eighteen animals were weighed and anesthetized by intraperitoneal injection of a combination of 5 mg/kg xylazine and 25 mg/kg ketamine [11,12]. Following dorsal hair removal and rubbing with ethyl alcohol 70%, wounds were created. Two full-thickness excisional wounds of 150 mm 2 areas were punched on the back of each animal using sterile sharp blades and scissors [13]. Animals were grouped into three groups, six in each one. Group 1 represented the untreated with test material negative control where skin wounds were left with no intervention until the end of the experiment. Animals of group 2 were treated with 1% Hyaluronic acid and 0.2% Chlorhexidine gel topically applied by cotton pellet applicator twice daily which represented the positive control group. Group 3 animals, the tester group, were treated with 5 mg/ml RH two times a day as a topical application by a cotton pellet. Treatment continued for 14 days and animals were caringly euthanized at the end of the experiment.

Histopathological assessment

Two full-thickness biopsies of the regenerated skin including the wound from the three groups were obtained at the end of the experiment. Tissue sections were processed and stained with hematoxylin and eosin. Descriptive expressions of the granulation tissue, angiogenesis, re-epithelialization, and inflammation of the skin of each group's animals were scored

and evaluated. Data were analyzed statistically by RM ANOVA for ranks test was used in the comparison of groups with utilized post hoc Duncan's multiple comparisons in the Sigma Plot software program for statistical analysis.

Rate of wound closure

The closure rate of the induced wounds of the three study groups was measured using a graduated ruler daily. The dimensions of the wounds were recorded and photographed. To calculate the rate of contraction, the following equation was applied [11]:

Rate of wound closure = Regenerated area*/Total wound area x 100 …….[Eq. 1]

[*; Regenerated area = Original wound area - Current wound area].

RESULTS

Assessment of wound closure rate

No death was reported in any of the study groups at any time during the experiment period. Wounds started to show signs of healing two days after surgical wound induction demonstrated by a blood clot and dark wound color. Visual monitoring of the wounds revealed the development of wound infection in the untreated control group shown as inflammation and pus formation at the wound spot. However, HA and RH groups' wounds were dry and clean (Figure 1). RH-treated wounds showed a significantly faster healing rate reaching a complete (100%) wound contraction at day 14 compared to 68.25 ± 3.3 and 92.63 ± 3.6 % closure rates of the untreated and HA-treated animals respectively (Table 1).

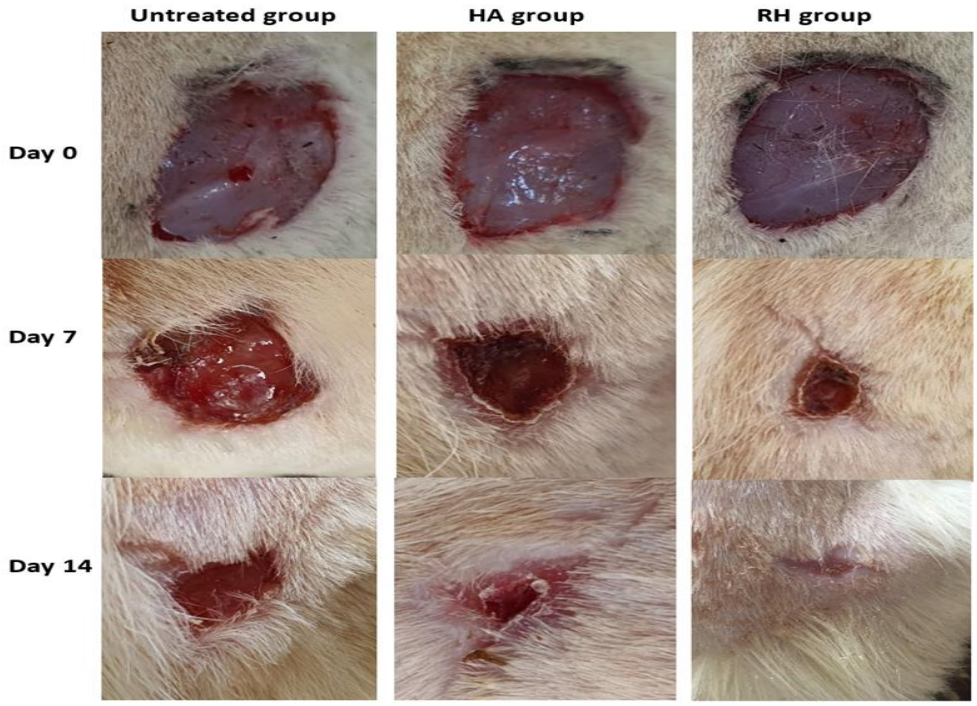

Figure 1. Rhamnolipid wound healing capacity. Untreated group; Untreated control group, HA group; hyaluronic acid treated group, RH group; Rhamnolipid treated group. Photos are representative of the experimental repeats of the study groups.

Table (1). Wound closure rate in response to treatment with rhamnolipid in comparison with hyaluronic acid as the positive control. Rate calculated according to equation 1. Data are expressed as the mean dimensions ± standard deviation.

	Wound closure rate (%)		
	Day 0	**Day 7**	**Day 14**
Untreated group	0.00 ± 0	25.74 ± 5.2	68.25 ± 3.3
HA group	0.00 ± 0	65.22 ± 4.5	92.63 ± 3.6
RH Group	0.00 ± 0	86.67 ± 2.9	100.00 ± 0.0

Histopathology of wounds

Examination of tissue sections of the treated wounds of the three study groups is depicted in Figure 2. Stained fields of the untreated control group and HA group revealed the absence of re-epithelization (score 0) but tissue granulation and angiogenesis were evident. The mucosal epithelium was sloughed indicating a slow rate of wound healing in the two control groups. Rareness of inflammatory cells (score 1) [14] was monitored in the untreated group with a scanty amount of granulation tissue and centrally present blood vessels. Hemorrhage was also observed in the untreated wound sections. Similarly, inflammatory crust with clot and mild infiltration of inflammatory cells (score 2) were reported in the section of HA treated group. A moderate amount of granulation tissue (score 3) with central blood vessels was the dominant histological feature in the HA group. On the other hand, the histology section of RH treated wound revealed an irregular full-thickness re-epithelization (score 3) of the wound area with a moderate amount of granulation tissue (score 3) and central and peripheral blood vessels (score 3), however, no infiltration of inflammatory cells was observed. When comparing these tissue healing criteria statistically using one way ANOVA test (Table 2), a significant difference (p = 0.043) was encountered in tissue granulation between RH and untreated groups. Re-epithelization in the RH group was dramatically significant (p = 0.005) in comparison to that of the HA and untreated group. Moreover, the RH group exhibited a significantly (p = 0.049) reduced inflammation when compared to the untreated group but not the HA-treated group. No significant difference (p = 0.111) was calculated among the three groups in terms of angiogenesis.

Table (2). Scores of granulation tissue, angiogenesis, re-epithelialization, and inflammation of rat skin wound healing process.

Groups / Criteria	Untreated Group	HA Group	RH Group	*P*-Value
Granulation tissue	2.66± 0.33 A	2.00±0.00 AB	3.66± 0.33 B	0.043*
Angiogenesis	2.33±0.33 A	1.66±0.33 A	3.00±0.00 A	0.111
Re-epithelialization	0.66±0.33 A	0.33±0.33 A	3.00±0.00 B	0.005**
Inflammation	1.33±0.33 A	2.66±0.33 AB	0.66±0.33 B	0.049*

Data expressed as Mean ± standard error SE
The different letters in rows mean there is a significant difference at $p \leq 0.05$.

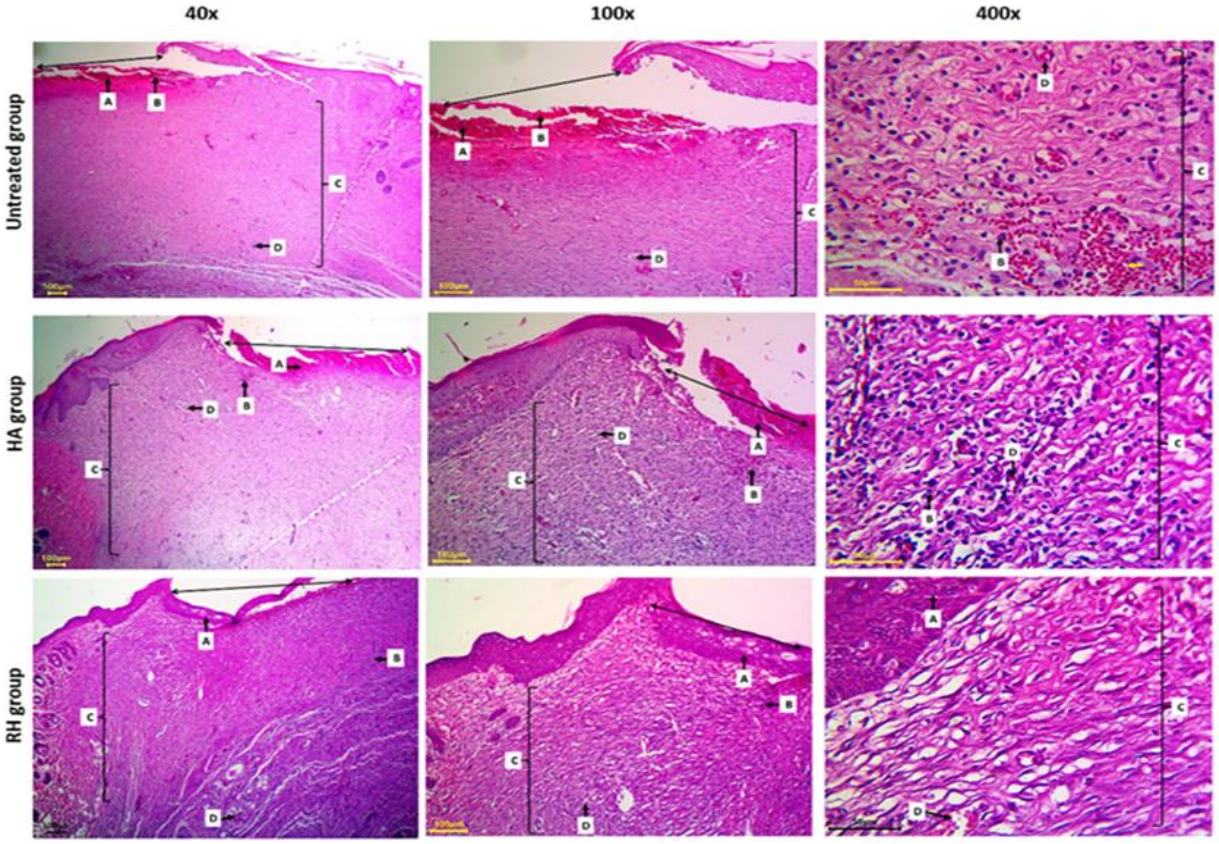

Figure 2. Representative photomicrograph of rat skin wound healing. Histopathology of a tissue section of the untreated control group showing the site of wound with sloughing the mucosal epithelium (↔), inflammatory crust with clot (A), infiltration of inflammatory cells (score 1) (B), granulation tissue (score 2) (C) with angiogenesis (score 2) (D) and without re-epithelialization (score 0). H&E stain, 40X. Magnifying the image at 400x shows the granulation tissue (C) with angiogenesis (D), infiltration of inflammatory cells (B) and hemorrhage (yellow arrow), HA group showing the site of wound with sloughing the mucosal epithelium (↔), inflammatory crust with clot (A), infiltration of inflammatory cells (score 2) (B), granulation tissue (score 3) (C) with angiogenesis (score 2) (D) and without re-epithelialization (score 0). H&E stain, 40X. Magnifying the image at 400x shows the granulation tissue (C) with angiogenesis (D), infiltration of inflammatory cells of polymorph-nuclear cells (B), and RH group showing the site of wound with well re-epithelialization (score 3) (↔,A), granulation tissue (score 3) (C) with angiogenesis (score 3) (D), infiltration of inflammatory cells (score 1) (B). H&E stain, 40X. Magnifying the image at 400x shows the site of wound with well re-epithelialization (score 3) (A), granulation tissue (C) with angiogenesis (D), without infiltration of inflammatory cells.

DISCUSSION

The incidence of facial injuries including soft tissues and skin loss fluctuates between 17% - 69%, due to different social and economic factors in addition to the need for skin reconstruction as an aid to regenerate skin in this sensitive area. Up to the writing of this article, there are no documented clinical applications of Rhamnolipid as a wound healer, therefore it was unable to compare the present study finding with the effectiveness of Rhamnolipid-containing wound healing products. Hyaluronic acid has been developed to provide a restorative bed for excision wounds with dermal loss [5,15]. This study originally compared the glycolipid biosurfactant Rhamnolipid with the commercial HA cream as the positive control in treating wounds induced in laboratory rodents. Wounds, especially full thickness, encompass the harm involving the dermal and epidermal layers and can outspread to the subcutaneous layer. Several processes are involved in skin wound repair which are complex but well-orchestrated [16]. Biosurfactants including Rhamnolipid have been studied widely for a variety of medical and industrial applications. They are considered to be non-toxic, less skin-irritant, or even anti-irritant with high skin compatibility [17]. Therefore, they have placed themselves among the skin cosmetic medications. In this experiment, complete closure of the induced excision wounds was obtained within two weeks of commencing RH treatment. A study conducted on experimental rats revealed a complete closure within 10 days of administrating an ointment containing Rhamnolipid at a concentration of 5 g/l [10]. The faster healing rate encountered in the latter study may be due to the better solubility and dispersion of the Rhamnolipid in the ointment formulation considering the amphiphilic property of Rhamnolipid [18]. It may also be due to the different congener activity between the extracted Rhamnolipid and the commercial one used in the current study. It might also be due to the diverse microbial flora [11]. Histology assessment, as reliable affirmative evidence of healing, revealed a significant granulation, re-epithelization and reduced inflammation of the RH-treated animals. Healing events include the formation of the epidermal layer, connective tissue biosynthesis, and deposition involving collagens which subsequently leads to the process of re-epithelization of the injured skin [19]. This faster healing rate of the RH-treated group in comparison to the HA positive control may be attributed to the ability of the Rhamnolipid to upregulate tissue level of the transforming growth factor-beta 1 (TGF-β1) [20]. Normally, inflammatory cells influx to the site of injury which is a characteristic feature of the early phase of cutaneous wound remodeling [21,22]. Histology assessment of the healed wounds demonstrated that RH-treated skin had minimal inflammatory cells than the untreated skin wounds. Since Rhamnolipid has been shown to stimulate chemotaxis

and chemokinesis of leukocytes [23], it might be that the administered Rhamnolipid stimulates the early enrollment of inflammatory cells to the site of the injury but with no tenacious abnormal gathering of neutrophils and monocytes within the wounded skin area. Re-epithelization, defined as the final step of proliferation, involves the relocation, multiplication, and differentiation of the epithelial cells from the wound boundaries to re-surface the defective tissue. This step requires the establishment of a granulation tissue bed. RH-treated wounds had a significantly higher level of granulation tissue than that of the negative control and thereby it seems reasonable to say that RH facilitates wound closure by enhancing granulation. Despite the high blood supply to the maxillofacial region, wounds in this area are highly susceptible to getting infected. Having Rhamnolipid potentially approved to exhibit antimicrobial activity [7,8] is an added advantage to using it as a wound healer. This may explain the advanced healing rate of the RH-treated group compared to the positive HA control where the latter has no evident antimicrobial effect [24]. Similarly, studies reported the antistaphylococcal activity of Rhamnolipid via its soap-like effect in penetrating and disintegrating bacterial cell walls [25-27] and suggested that the Rhamnolipid healing effect may be owed to protecting the wound from microbial contaminants. In addition, ideal wound healers should not only enhance the wound's healing rate but also protect against scar formation. Fibrosis, by definition, involves scar formation and excessive production of extracellular matrix by connective tissue. It has been found that overemphasis on the inflammatory response of wound healing events, upsurges growth factor levels which subsequently increases fibroblast counts, and overproduction of collagen and ECM establishing scar formation [28-30].

CONCLUSION

The findings of this study highlight the possibility of using Rhamnolipid to enhance the process of wound healing. It also raises the potential ability of Rhamnolipid to act as an anti-scaring agent minimizing the defects of chronic wounds. Studies can be directed towards formulating RH in easier applicable vehicles alone or in combination with other agents of wound healing potentials.

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Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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