A Comparative Topical Wound Healing Study Of 5-Amino Salicylic Acid As Ointment And Gel By In-Vitro And In Vivo Models

Moola Joghee Nanjan Chandrasekar, R. Mouleeswaran, Jayakumar Chenniah, Shivaramakrishnan Balasubramanian

Abstract— Wound healing is a restoration process of tissue repair in response to an injury. Treating wounds is a clinical challenge for physicians. Few limitations being donor site morbidity, infection and pain. Our therapeutic direction is to initiate the re-epithelization and enhanced curative activity in a shorter duration of time without side effects. 5-Amino salicylic acid (5-ASA) is amino salicylate drug used to treat ulcerative colitis. It exerts the mucosal healing at inflated surface of colon. This surface action of 5-ASA has not been explored for external purpose to cure wounds. In this study, the wound healing potency of 5-ASA by was studied by in vitro and by in vivo methods. By in vitro scratch wound assay using HaCaT cell lines and by excision wound model in Wistar rat, the healing potential of 5-ASA was studied in gel and ointment formulations. The scratch assay showed promising cell migration in 5-ASA at 125.629μg/ml. Excellent wound healing was seen at maximum degree of wound contraction at 99.5% in 5-ASA 2.5% Gel in 12h day and at 99.5% in 5-ASA 2% Ointent in 15th day in both treated groups compared to control. 5-ASA showed promising wound healing ability topically. Our future direction is to explore the molecular insight of 5-ASA in initiating this tissue restitution and its enhanced curative activity of an injury in a shorter duration of time. By this future direction, it might be possible to reposition 5-ASA for topical uses in different wounds.

Key-words: 5-Aminosalicylic acid; scratch assay; excision wound; re-epithelization

1 Introduction

Wound healing is a restoration process of tissue repair in response to an injury. This process is a sequential event of three phases: inflammation, fibroplasia, and maturation. Biological events in wound healing begin with haemostasis leading to an inflammatory response, the formation of granulation tissue, and the remodeling. Each of these phases is controlled and regulated by biologically active polypeptides called growth factors. Growth factors interact with specific cell-surface receptors to control the process of tissue repair through differentiation and metabolism of cells. In the first phase, thromboxane, serotonin and histamine initiate the hemostatic response to control hemorrhage within the wound space. The activated platelets aggregate and initiate the blood clotting cascade through both the intrinsic and extrinsic systems. In the phase of fibroplasia, there is an increased cellular infiltration of keratinocytes, platelets, fibroblasts, macrophages, endothelial cells. The macrophage controls the degradation of extracellular connective tissue by enzyme secretion and phagocytosis. It also regulates remodeling of the wound matrix. The released growth factors initiate the process of collagen matrix formation by fibroblasts. The process of angiogenesis begins with the formation of capillaries from existing capillary networks, stimulated by factors. Epithelialization begins through the proliferation of cells at the edge of the wound. In the maturation phase, wound remodeling follows. Hyaluronidase, plasminogen activators, collagenase, and elastase also are involved in wound remodeling. This process reduces any further cell migration and allows cell differentiation to occur to the original tissue integrity [1] [2] [3]. New wound healing strategies are emerging including tissue growth factors in the wound microenvironment [4] [5]. 5- Amino salicylic acid (5-ASA) is amino salicylate anti-inflammatory drug used to treat mild to moderate ulcerative colitis (UC) and Crohn’s disease. It acts by decreasing the synthesis of prostaglandins through cyclooxygenase (COX) pathway and also decreases the mobilisation of leucocyte at the inflamed site. Further, they also scavenge hydroxyl radical, reduce peroxide and nitric oxide formation. It is also poorly absorbed in the intestine [6] [7]. By virtue of this, it produces pharmacological effect of mucosal healing on the lumen rather than underlying tissues in UC.

By its pharmacokinetics, 5-ASA exerts the mucosal healing at the surface of inflamed site and prevents the reemission of UC [8]. 5-ASA caused a significant enhancement of epithelial cell migration and proliferation in vitro in non-transformed small-intestinal epithelial cell line IEC-6. This effect produced was dose dependent action through tissue growth factor (TGF-β) independent mechanism [9]. However, till date no study has confirmed the effectiveness of 5-ASA binding to tissue growth factors for wound healing. In the current hypothesis, it is proposed to evaluate this effect of tissue regeneration ability of 5-ASA for an accelerated wound healing process upon its topical administration.

2 Methods

2.1 Cell Migration Assay

HaCaT cells were used for this assay. The medium containing 5-ASA was then added after cell adhesion. Cells were incubated in DMEM medium for the indicated time. The cell migration of HaCaT cells was subsequently determined by the method described by [10].

2.2 Experimental Animals

Adult albino Wistar rat of either sex of (180 - 220 g) were obtained from the Central Animal House, JSS College of Pharmacy, Ootacamund. The animals were housed under laboratory conditions (relative humidity 65 ± 2%, temperature 22 ± 1°C and 12 h light and dark cycle). They were fed with standard rodent pellet diet (Gold Mohar, Lipton –India, Ltd.) and purified water ad libitum. The study was approved by the institutional animal ethics committee for animal care and use (JSSCP/IAEC/M.Pharm/PH.COLOGY/06/2017-18).

2.3 Formulation of 5-ASA as Gel and Ointment.

The dose was selected based on the reported data in the paper titled topical 5-amino salicylic acid: a treatment for aphthous ulcer [11]. The selected dose at 2.5 % w/w was then formulated into simple gel (using HPMC as base of gel) and 5% w/w was formulated into simple ointment; then tested for acute dermal irritation studies. Following which, it was then studied for excision wound model.

2.4 Acute Dermal Irritation Test

Acute dermal irritation test was performed by using albino rabbits as per OECD guidelines 404, where the drug-5ASA was applied in a single dose to the skin of the rabbit; untreated skin areas of the test animal served as the control. The degree of irritation was observed for signs of erythema and oedema. The scoring of erythema and oedema formation was scored on a scale of 0 - 4. The results obtained were tabulated.

2.5 Evaluation of Wound healing activity by excision wound model

Excision wound model was used to evaluate the wound-healing activity of 5-ASA by studying the rate of contraction of wound and re-epithelization. The study was approved by the Institutional Animal Ethical Committee of JSS College of Pharmacology, Ooty who carried this research work in collaboration with the other authors. Mob.+91-9862001429, E-mail: shivaram.krisna@jssuni.edu.in

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Pharmacy, Ooty (T.N) registered under CPCSEA, India. Animals were anesthetized with ketamine (5 mg/kg i.p.) and xyazine (2 mg/kg i.p.). The skin was shaved using an electrical trimmer and disinfected with 70% alcohol. A uniform wound of 2.00 cm in diameter was excised from the nape of the dorsal neck of each rat with the aid of a round seal as described [12]. Precautionary measures and the surgery were done precisely to avoid injuring the muscle layer. The wound area was measured immediately by placing a sheet of transparent tracing paper over the wound and tracing. The tracing paper was placed on sheet of 1 mm2 graph paper, and the wound tracing was traced. The squares were counted, and the area was recorded using a digital Vernier caliper as described [13]. Group 1 animals served as the Control group and they were dressed with 0.2 mL of sterile distilled water twice daily; served as the control group [14]. Group 2 animals were dressed topically twice daily with 5-ASA 2.5% gel. Group 3 animals were dressed topically twice daily with 5-ASA 2.5% ointment. The treatment was given for 14 days. All animals were sacrificed on day 15. The wound closure area of each animal was assessed by tracing the wound on days 0, 3, 6, 9, 12 and 15 after wounding surgery using transparent paper and a permanent marker under light keratine and xyazine anesthesia as described [15]. The wound areas recorded were measured using graph paper. The percent wound healing values on these days were determined as reduction in wound size area expressed in mm2 and percentage contraction of wound.

2.6 Antioxidant Measurement from Granulation Tissue
Wound tissue was collected on day 15 for measurement of the tissue anti-oxidant levels. The level of glutathione was determined in the granulation tissue from wound tissue area by the method described by [15].

2.7 Estimation of Hydroxyproline Content
Wound tissues were analyzed for hydroxyproline content, which is basic constituent of collagen. The collagen composed of amino acid (hydroxyproline) is the major component of extra-cellular tissue, which gives strength and support. Breakdown of collagen liberates free hydroxyproline and its peptides. Measurement of hydroxyproline hence can be used as a biochemical marker for tissue collagen and an index for collagen turnover as described in [16]. The hydroxy proline were analysed by Tecan Multi-Detection Microplate Reader at 540nm.

2.8 Histological Evaluation of Wound Tissues
On day 15, skin specimens from the wound areas were fixed in 10% buffered formalin and were processed by a tissue processing machine. The wound area was assessed by staining a 5 μm section with H/E staining procedure.

2.9 Statistical Analysis
All values are reported as the mean ± S.E.M., and the statistical significance of differences among groups was assessed using one-way Anova. A value of P < 0.05 was considered significant.

3 Results
3.1 Cell Migration Assay
In our study, cell migration was evaluated for Control (Normal), 5-ASA as depicted in the Figure 1. This assay was performed in HaCaT cells and observed at the time interval of 0hr, 8hrs, and 24hrs. Cell migration showed very promising results for the drug 5-ASA. Migrating cells like keratinocytes play a critical role in wound healing that enable them to secrete proteins and proliferate. This helps in acceleration of wound healing.

![Figure 1 showing the Scratch assay (Cell Migration) in Control [A] and 5-ASA induced migration [B]](image)

3.2 Acute Dermal Irritation Test
Acute dermal irritation test was performed in albino rabbit. Upon application of 5-ASA in a simple ointment and simple gel formulation on the skin patches of albino rats showed no erythema or oedema over the duration of the study. The signs and the scoring was performed under two tests namely- initial test and confirmatory test. By the observations and scoring it is clear indicative that 5-ASA is classified a non-irritant drug and was confirmed as per the OECD guidelines 404.

3.3 Evaluation of Wound healing activity by excision wound model
Reduction in mean wound area and Percentage reduction in mean wound area of all study groups were recorded on day 0, 3, 6, 9, 12, 15. The rats treated with 5-ASA 2.5% ointment and 5-ASA 2.5% gel showed varying degree of wound contraction when compared to untreated group. 5-ASA 2.5% gel showed the unsurpassed wound healing and contraction when compared to other treated groups.

<p>| Table 1 showing the effect of Test Formulations on reduction in mean wound area mm2 |
|---------------------------------|----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Day</th>
<th>Control</th>
<th>5-ASA 2.5% Gel</th>
<th>5-ASA 2.5% Ointment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Day</td>
<td>152.5±0.00</td>
<td>133.1±0.00</td>
<td>150.1±0.00</td>
</tr>
<tr>
<td>3 Day</td>
<td>127.1±4.50</td>
<td>80.1±9.41</td>
<td>117.2±24.38</td>
</tr>
<tr>
<td>6 Day</td>
<td>98.1±5.72</td>
<td>70.9±11.57</td>
<td>79.0±7.15</td>
</tr>
<tr>
<td>9 Day</td>
<td>55.0±4.71</td>
<td>4.0±23.01</td>
<td>42.7±64.93</td>
</tr>
<tr>
<td>12 Day</td>
<td>16.9±1.57</td>
<td>5.9±9.00</td>
<td>13.9±1.73</td>
</tr>
<tr>
<td>15 Day</td>
<td>3.9±1.57</td>
<td>0.0±0.00</td>
<td>0.9±0.00</td>
</tr>
</tbody>
</table>

Data expressed as Mean ± SEM (n=6) by One way ANOVA; statistical significance: *P<0.05 vs. Control; values expressed in mm2.
**Figure 2** showing the effect of test formulations on reduction in mean wound area (mm²)

**Table 2** showing the effect of Test Formulations

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>5-ASA 2.5% Gel</th>
<th>5-ASA 2.5% Ointment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Day</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3rd Day</td>
<td>16.13</td>
<td>60.68*</td>
<td>21.91</td>
</tr>
<tr>
<td>6th Day</td>
<td>35.67</td>
<td>88.96*</td>
<td>47.32*</td>
</tr>
<tr>
<td>9th Day</td>
<td>63.88</td>
<td>97.30*</td>
<td>70.84*</td>
</tr>
<tr>
<td>12th Day</td>
<td>87.90</td>
<td>99.61**</td>
<td>90.71**</td>
</tr>
<tr>
<td>15th Day</td>
<td>96.49</td>
<td>100.0**</td>
<td>99.40**</td>
</tr>
</tbody>
</table>

Data expressed as Mean ± SEM (n=6) by One way ANOVA; statistical significance: *P<0.05 vs. Control; values expressed in %.

**Figure 3** showing the effect of test formulations on Percentage reduction in Mean wound area

**Table 3** showing the tissue GSH levels at Day 15

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GSH</th>
<th>% increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.95±0.03</td>
<td>----</td>
</tr>
<tr>
<td>5-ASA 2.5% Gel</td>
<td>1.65±0.45</td>
<td>*** 173.68%</td>
</tr>
<tr>
<td>5-ASA 2.5% Ointment</td>
<td>1.05±0.02***</td>
<td>110.92%</td>
</tr>
</tbody>
</table>

Data expressed as Mean ± SEM (n=6) by One way ANOVA; statistical significance: *P<0.05 vs. Control.

**Figure 4** showing the images of the different healing stages from Day 0, Day 3, Day 12

**Table 4** showing the tissue Hydroxyproline levels at Day 15

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hydroxyproline (mg/100 mg of Skin tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.36±1.03</td>
</tr>
<tr>
<td>5-ASA 2.5% Gel</td>
<td>6.16±0.28***</td>
</tr>
<tr>
<td>5-ASA 2.5% Ointment</td>
<td>6.18±0.30***</td>
</tr>
</tbody>
</table>

Data expressed as Mean ± SEM (n=6) by One way ANOVA; statistical significance: *P<0.05 vs. Control.

**3.4 Antioxidant Measurement from Granulation Tissue**

The control group showed tissue GSH levels from 0.95±0.03. Treatment with 5-ASA 2.5% as gel and ointment prevented the depletion of GSH levels as indicated by the % increase of GSH values in treatment group.

**3.5 Estimation of Hydroxyproline Content**

**3.6 Histological Evaluation:**

**Figure 5** showing the histopathological examinations of skin samples at Day 15.
Control [A], 5-ASA 2.5% Ointment [B] & 5-ASA 2.5% Gel

4 Discussion
The initial inflammatory responses to injury provide the necessary framework to the subsequent production of a new functional barrier and next stage of wound healing is proliferative phase, it lasts for 3-14 days. In this phase of healing, cellular activity predominates. The major events during this phase are the creation of permeability barrier (i.e. re-epithelialization/wound contraction), the establishment of appropriate blood supply (i.e., angiogenesis), and reinforcement of the injured dermal tissue [18]. Contraction of the wound begins soon after wounding and peaks at 2 weeks. The degree of wound contraction varies with the depth of wound. The depth of full-thickness wounds, contraction is an important part of healing and accounts for up to 40% decrease in the size of the wound. Fibroblasts are the predominant mediator of this contractile process because of their ability to extend and retract. During wound healing, fibroblasts are gradually modulated into myofibroblasts, which are characterized with actin microfilament bundles. The cells within the wound align along the lines contraction, and contraction of the wound occurs in directions of skin tension lines. The role of contraction is proportional to the cell number and inversely proportional to the collagen concentration. Revascularization or angiogenesis of the wound proceeds in analogous with fibroplasia. Capillary buds sprout form blood vessels adjacent to the wound and extending the wound space [18]. This is the continuous supply of materials for the formation of new tissue. The transition from granulation tissue to scar involves reorganization and maturation of collagen fibers. In a normal wound, homeostasis between collagen synthesis and breakdown is achieved within 3 weeks of injury [20]. Treatment of wound is a simple process yet is intricate in choosing the correct strategy. Our therapeutic direction for treating a wound is to use a drug which can effectively repair the traumatized tissue. This can be achieved by healing the epithelial or connective cells, inflammatory cells, platelets and fibroblasts to hasten the process of tissue regeneration and restoration at a faster rate [22]-[23]. 5-ASA was examined for the potential for wound healing by tissue regeneration. It was assessed by both in-vitro and in-vivo models for establishing the ability of 5-ASA to act on the fibroplasia and maturation phase. For suitable topical administration, conventional drug formulations of gel and ointment were made. In-vitro scratch assay is particularly suitable for studying the effects of cell-matrix interaction and cell-cell interaction on mimetic cell migration during wound healing. This procedure is well-suited with imaging of living cells during migration to monitor intracellular events. In vivo model was performed to study the wound healing activity of 5-ASA in gel and ointment formulations at 2.5% concentration in Wistar Albino Rats. This is conclusive by the reduction of wound by mean surface area and percentage reduction in 14 days treatment. In the present study, the pharmacological assessment is limited to wound healing potential of 5-ASA on topical administration by in vitro and in vivo models. However, it may be planned and designed to assess the tissue regeneration by other pharmacological models such as endothelial cell tube formation for assessing in-vitro angiogenesis, CAM assay for angiogenesis, Masson's trichrome (Masson's trichrome staining by Claude L. Pierre Masson method) and analysis of cell migration in vitro. Nature protocols. 2007;2(2):329-33.

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