CHAPTER 4.0

SKIN IRRITATION AND IN-VIVO STUDY
4. Skin Irritation and *In-vivo* Study

4.1 Introduction

Skin irritation safety testing and risk assessment for new products, and the ingredients they contain, is a critical requirement before market introduction. In the past, much of this skin testing required the use of experimental animals. However, new current best approaches for skin corrosion and skin irritation testing and risk assessment are being defined, obviating the need for animal test methods. Several in vitro skin corrosion test methods have been endorsed after successful validation and are gaining acceptance by regulatory authorities.

4.2 Skin Irritation Study

In skin irritation study 20 Albino Wistar rats of either sex, weighing between (200-250 g) was used. Animals were separated into 4 groups (n=5). Hairs were removed from the back of rats by the use of depilatories and area 4 cm$^2$ was marked on both the sides. One side considered as control whereas the other as experimental. After hair depletion (wait for 24 hrs) formulation was applied (250 mg/rats) daily for 7 days and observation was made for any sensitivity and the reaction if any was graded as. A - No reaction, B - Slight, patchy erythema, C - Slight but confluent or moderate but patchy erythema, D - Moderate erythema, E - Severe erythema with or without edema (Kulkarni and Jain, 2001).

4.3 *In vivo* Animal Study in Rats

The in vivo study was carried out in rats to evaluate the therapeutic potential of 0.25% SSD nanosuspension and 0.25% SSD nanogel containing AV-gel in the treatment of second degree burns. Albino Wistar rats (n = 6 per group) were used in the study. The Animal Ethics Committee, Faculty of Pharmacy, Integral University, Lucknow, U.P., India, approved the experimental protocol for conducting *in vivo* study. All experimental
procedures on animals were carried out as per ethical guidelines of the committee. Thirty-six male Albino Wistar rats (200–250 g) were kept in separate cages with food (*ad libitum*) and water. Ketamine hydrochloride (50 mg/kg) in addition to 5 mg/kg diazepam intramuscular injection was used as anesthetic agents to anesthetize each rat on day 0. Dorsum hairs were removed from the rats prior to injury followed by cleaning with povidone iodine. A deep second-degree dermal burn wound (burn area, 4.9 cm²) was developed by exposing the shaved dorsum part of each rat for 5 s to stainless steel stamp (2.5 cm in diameter) heated over a 600-W heater for 5 min (Morsi et al., 2014). The rats were arbitrarily separated into six groups. First, a negative control group animals without any burn injuries. The rats of the second group were burned but left untreated (control group). Rats in group III were treated by 0.25% nanosuspension (quantity applied—250 mg). Rats in group IV were treated by 0.25% SSD nanogel containing AV-gel (quantity applied—250 mg). Rats in group V were treated by AV-gel post burning (quantity applied—250 mg). Rats in the last group were treated using 1% marketed cream (quantity applied—250 mg). The developed preparation was used once-a-day for 14 days to the wound region at a thickness of about 3–5 mm and the experimental animals were examined each day and the time (in days) for acquiring absolute epithelialization of wound was considered as the time of wound healing progression (healing time). After burn wound grafting, the wound boundaries were drawn on clear paper showing a scale (millimeter) at 48 h intervals during the experimental phase for 14 days. The complete wound healing was considered as a percentage of wound contraction and epithelialization time (Shegokar and Müller, 2010).

To determine the wound contraction (%), the wound region on day 0 was taken as 100% by using the following Eq. (4.2):

\[
\text{Wound contraction (\%) } = \frac{\text{Wound area on day zero} - \text{Wound area on specific day}}{\text{Wound area on day zero}} \times 100 \\
\text{.............4.2}
\]
Images of the wounds region were used for visual comparative examination. A One-way ANOVA followed by Newman–Keuls multiple comparison post test was employed to analyze the data received.

4.3.1 Histopathological Study

Autopsy samples were obtained from the treated skin region of rats in different groups and were kept for 24 h in the formalin solution (10%). Tap water was used for washing the samples followed by serial dilutions with alcohol. Xylene was used to clear the specimens and fixation was done in paraffin at 56°C for 24 h. Paraffin tissue blocks were used for cutting a section of 4 μm areas by means of a sledge microtome. The tissue sections fixed on paraffin tissue blocks were put on glass slides and deparaffinized, followed by staining with hematoxylin and eosin dyes for histopathological evaluation of skin sections using electric light microscope (Morsi et al., 2014).

4.4 Results

4.4.1 Skin Irritation Study

The primary irritation index of the nanogel, nanosuspension and AV-gel was noted to be A; no reaction/no irritation was observed on the skin of the rats, while in case of marketed formulation the primary irritation index was noted to be B. This may indicate that the SSD nanogel formulation of silver sulfadiazine is safe when applied to human skin.

4.4.2 In Vivo Animal Study in Rats

The marketed formulation causes slight irritation to burn wound area upon application of nanogel formulation but no irritation was observed in the case of 0.25% SSD nanogel containing AV-gel, 0.25% SSD nanosuspension, and AV-gel. The 0.25% SSD nanogel containing AV-gel and AV-gel cause the experience of cold feeling due to
moisture (high water content of AV-gel), which may be helpful to give relief to the patient from burn in wound. SSD nanogel containing AV-gel treated burn wound appeared to heal better than SSD nanosuspension, AV-gel alone, marketed formulation, and control group. The results were concluded upon gross examination through close pictures of the burn area and percentage of wound contraction. The assessments made among groups were completely based on wound size on a specific day. The assessment of burn wound contraction was done in every 2-day time period and ended on the 14th day.

Figure 4.1 shows the wound healing time succession in burn wound management with the prepared nanogel formulation and control group. The maximum percentage wound healing reduction was observed in 14 days in the animals treated with 0.25% SSD nanosuspension, 0.25% SSD nanogel containing AV-gel, AV-gel alone, and 1% SSD marketed cream with the values as 85.27, 93.63, 61.59, and 79.05%, respectively, whereas control group animals exhibited 49.65% wounds healing at the end of study (Table 4.1). Besides, in the starting week of the experiment, control group animals exhibited the appearance of inflammation and pus formation, while in treated animals no measurable inflammation and pus were observed. The images of developed wounds on 1st and 14th day of group 2–6 are presented in figure 4.2.
Figure 4.1: Percentage wound contraction progresses in wound treated with different formulations and control group.

Figure 4.2: Representative photographs of burn wounds in rats.
Table 4.1: Burn wound healing in rats in terms of percentage reduction in wound treated with different formulations and control group.

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th>0.25% nanosuspension</th>
<th>0.25% nanogel</th>
<th>Aloe vera gel</th>
<th>1% Marketed formulation</th>
<th>Control</th>
<th>0.25% nanosuspension</th>
<th>0.25% nanogel</th>
<th>Aloe vera gel</th>
<th>1% Marketed formulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>335.06 ± 1.584</td>
<td>340.88 ± 1.275</td>
<td>343.23 ± 0.7101</td>
<td>339.97 ± 0.3905</td>
<td>340.47 ± 0.8027</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>279.11 ± 1.218</td>
<td>271.63 ± 1.084</td>
<td>273.85 ± 0.9283</td>
<td>291.27 ± 0.928</td>
<td>274.73 ± 1.275</td>
<td>16.69</td>
<td>20.32</td>
<td>20.22</td>
<td>14.33</td>
<td>19.31</td>
</tr>
<tr>
<td>4</td>
<td>260.61 ± 1.005</td>
<td>245.91 ± 0.9417</td>
<td>243.77 ± 0.9671</td>
<td>256.48 ± 0.5207</td>
<td>250.83 ± 0.3632</td>
<td>22.22</td>
<td>27.87</td>
<td>28.98</td>
<td>24.56</td>
<td>26.33</td>
</tr>
<tr>
<td>6</td>
<td>247.73 ± 0.9755</td>
<td>188.49 ± 1.706</td>
<td>190.83 ± 1.03</td>
<td>239.91 ± 0.42</td>
<td>198.56 ± 0.6432</td>
<td>26.07</td>
<td>44.71</td>
<td>44.41</td>
<td>29.44</td>
<td>41.69</td>
</tr>
<tr>
<td>8</td>
<td>211.24 ± 1.113</td>
<td>152.89 ± 1.519</td>
<td>140.54 ± 0.7797</td>
<td>200.75 ± 0.6524</td>
<td>174.31 ± 0.9047</td>
<td>36.96</td>
<td>55.15</td>
<td>59.06</td>
<td>40.96</td>
<td>48.81</td>
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<tr>
<td>10</td>
<td>195.42 ± 1.113</td>
<td>124.24 ± 0.8206</td>
<td>109.71 ± 0.5789</td>
<td>174.78 ± 0.5842</td>
<td>145.41 ± 0.6812</td>
<td>41.68</td>
<td>63.56</td>
<td>68.04</td>
<td>48.59</td>
<td>57.29</td>
</tr>
<tr>
<td>12</td>
<td>179.45 ± 0.5144</td>
<td>83.06 ± 1.751</td>
<td>43.85 ± 0.7283</td>
<td>154.52 ± 0.5669</td>
<td>99.49 ± 0.5795</td>
<td>46.45</td>
<td>75.64</td>
<td>87.23</td>
<td>54.55</td>
<td>70.78</td>
</tr>
<tr>
<td>14</td>
<td>168.72 ± 0.962</td>
<td>50.24 ± 0.8474</td>
<td>21.89 ± 1.157</td>
<td>130.61 ± 1.092</td>
<td>71.36 ± 0.5197</td>
<td>49.65</td>
<td>85.27</td>
<td>93.63</td>
<td>61.59</td>
<td>79.05</td>
</tr>
</tbody>
</table>
4.4.3 Histopathological Study

The images of histopathological sections of different groups of rats skin is illustrated in figure 4.3(A-E). Section of rat’s skin from the wound area of groups II, III, IV, V and VI were examined. After 14 days, good healing with epithelization, fibroblast proliferation, and collagen deposition was seen in groups III and IV, while lesser degree of healing was seen in group V and VI. Group II showed the least degree of healing with angiogenesis and granulation tissue still persisting in the wound area. After 14 days, complete regeneration of epidermis and granulation tissue formation were seen in group IV. These are the excellent results in contrast with other test groups and marketed formulation treated group. At the end of 14th day, in group VI regeneration of epidermis was not accomplished due to cytotoxicity of SSD to fibroblasts, in this dosage form.
Figure 4.3: Photomicrographs of histopathological sections representing burned skin of rat groups after treatment with different formulation for 14 days. A = Group II, B = Group III, C = Group IV, D = Group V, E = Group VI.
4.5 Discussion

Fox and Modak described that the effectiveness of SSD results from its quiet and steady reactions with body fluids having sodium chloride and serum, which allows a slow and sustained release of silver ions into the wound surroundings. The nanosized SSD having large surface area helps in faster interaction with bacteria (Venkataraman and Nagarsenker, 2013). Hence, SSD (0.25%) nanosuspension and nanogel show higher efficacy in contrast to marketed preparation with micron-sized drug particles. The outcomes obtained from the in vivo studies were agreeable with literature. The images of developed wounds on the 1st and 14th day of groups II–VI are presented in figure 4.2. In control group animals, reduction in the burn wound area was seen from 14th day onward, might be due to the immune response of animals. By virtue of description, the gel has a tridimensional arrangement with polymeric networks, which are able to hold a huge sum of water inside their structures and swell up without dissolving. This indicates that they have the capability to soak up exudates that maintain wetness on the wound surface.

Furthermore, gels exhibited excellent permeability to water droplet and oxygen, as well as mechanical features that are concerned to physiological soft tissues of burn injuries (Nascimento et al., 2009). In the present work, Carbopol 940 was employed as a gel former due to its tremendous gel-forming quality at low concentration, biodegradable and biocompatible nature as well as nontoxic to the human body. In addition to Carbopol 940, the inclusion of AV-gel in this formulation improve the gel consistency, bioadhesive property of gel and reducing the concentration of the gelling agent, as well as also imparting therapeutic potential in the management of burn wounds as a wound healing promoter. Shear rate is inversely proportional to the viscosity of the gel, which revealed the pseudoplastic or shear thinning properties of the gel formulation. This could be
beneficial because the gel becomes more fluid when it is being applied to the burn wound surface, which leads to an easier and less painful application.

Furthermore, the viscosity would most likely increases while the stress is discontinued (a shear rate equal to zero) and resistance will create in the gel to flow from the burn wound surface (Nascimento et al., 2009). In our result, the nanosuspension-based AV-gel was able to develop a thin bioadhesive film over the wound surface (second day of treatment). Because of this bioadhesivion, a bioadhesive film adheres to the wound surface as a protective layer against the bacterial attack to avoid further infection. In addition to the physical defense, the developed nanogel should freely release the SSD in order to efficiently reduce the bacterial loads. The protracted contact of the bioadhesive film to the wound superficies besides the proficient delivery of SSD for 24 h possibly would be able to get rid of the frequent application of SSD. The second-degree burn wounds healing process is an optimistic response to burn injury which includes wound contraction, wound execution, and repairing of the effective obstruction (Govindarajan et al., 2004). Burn wound healing is achieved with the involvement of three overlapping phases that includes inflammation, granulation tissue formation, as well as remodeling. Burn wound healing involves, primarily the conversion of granulation tissue into scar tissue, secondly remodeling of collagen that includes, collagen degradation followed by formation of a large number of collagen bundles, and an increment in the number of intermolecular cross-linkage. Fibroblasts, macrophages, epidermal cells, and endothelial cells release the matrix metalloproteinase’s enzyme that controls wound healing process. The degree of formation of collagen, in this context, is directly related with formation of tissue mass with high tensile strength for protection against the wound. Because of the collagen remodeling and the established intra and intermolecular cross-linkages, the strength of the wounded tissue is restored (Singer and Clark, 1999). In this experiment,
the medicinal effects of AV-gel on the healing of burn wounds (second degree) were assessed in rats. AV-gel possibly showed a direct action on the wound healing processes altogether, which revealed to facilitate the rate of contraction of the wound area. Subramanian et al. also reported the therapeutic performance of AV-gel in the course of wound healing is due to the availability of mannose-6-phosphate (Subramanian et al., 2006). The presence of prostaglandin and bradykinin hydrolyzing enzymes (carboxypeptidase and bradykinase) in A. vera is the key components, which seems to be effective in the reduction of pain and inflammation which occurs in burn wound (Tizard et al., 1989; Davis et al., 1994). The polysaccharide mannose-6-phosphate isolated from A. vera has been hypothesized to be active growth substances, involved in epithelialization during wound healing process (Govindarajan et al., 2004; Davis et al., 1994). Davis et al. reported that the interaction of mannose-6-phosphate with fibroblast receptors facilitates the fibroblastic proliferation, which in due course assist to endorse collagen deposition and tissue reorganization (Davis et al., 1994). The another polysaccharide acemannan has been isolated from A. vera leaf gel which takes part in the regulation of white blood cell activity in the course of wound healing (Boudreau and Beland, 2006; Tamura et al., 2009). Kuzuya 2001 reported the presence of an organic compound anthraquinones in A. vera that minimizes the chances of infection in burn wound due to its antibacterial property (Tamura et al., 2009; Kuzuya et al., 2001). AV-gel may possibly have growth factors like mitogenic polypeptides, which are considered as wound healing promoters. All of these therapeutic outcomes have an optimistic effect during the course of burn wound healing. Wound healing process is an equitable phenomenon by means of intrinsic and extrinsic effects. Bacterial colonization (bacterial count >10^5 organisms/g of tissue) is a foremost extrinsic factor that delays and interferes with the wound healing, though topical antibacterial drugs applied to wound area tend to
restore the bacterial load within a wound, but they themselves are reported to retard wound healing (Muller et al., 2003). As a result, the SSD nanogel comprising of AV-gel and Carbopol 940 seems to be effectively useful for burn wounds healing and infection. The silver-containing compounds like SSD have been reported to be an effective antimicrobial agent that acts against almost all microbes (e.g., bacteria, fungi, and some viruses). But on the other hand, SSD has been known to have a cytotoxic effect toward fibroblasts and keratinocytes in vitro and therefore tends to retard wound healing in vivo (Sandri et al., 2014). The addition of AV-gel in SSD nanosuspension would possibly prevent the delay in wound healing, by minimizing the cytotoxicity via reducing the dose of SSD and maintaining its antimicrobial properties. Finally, histopathological examination carried out on burn wound skin isolated from SSD nanosuspension, SSD nanogel, AV-gel, and marketed formulation-treated rats confirms the excellent wound healing activity of the developed SSD nanogel formulation containing AV-gel.
4.6 Conclusion

The developed SSD (0.25%) nanogel showed better wound healing response in comparison to 1% SSD marketed gel formulation. In the course of wound healing process, re-epithelialization was more efficient in nanogel treated animals due to availability of AV-gel in the formulation. In burn wound healing, the cell proliferation and anti-inflammatory effects may possibly be due to AV-gel. 1% SSD formulation is one of the most commonly used formulation for bacterial wound infection, which are reported to have verifiable deleterious effects on wound healing. Addition of AV-gel to SSD nanosuspension reversed the wound healing retarding effects of SSD in the animal model. The incorporation of AV-gel in SSD nanogel demonstrated the additive therapeutic potential for faster burn wound healing, which will lessen the trauma of the patients suffering from second-degree burn wounds.