Chapter VIII
10.1. INTRODUCTION

Wounds are inescapable events of life. Wound may arise due to physical, chemical or microbial agents and wound healing has been one of the earliest medical problems. Healing is essentially a recovery mechanism and represents an attempt to maintain normal anatomical structure and function. Healing of wound takes place in a direction away from its normal course and it is common to have none, under or over healing. Treatment is therefore aimed at either shortening the time required for healing or minimizing the undesired consequences. Fish is used by the patients in the post-operative period with the belief that it promotes wound healing and reduces post-operative pain and discomfort. It is known to contain polyunsaturated fatty acids that can regulate prostaglandin synthesis and hence induce wound healing (Bowman and Rand, 1980; Gibson, 1983). Certain amino acids like glycine, aspartic and glutamic acid are also known to play an important role in the process of wound healing (Chyun and Griminger, 1984). These amino acids were largely expressed in the innate defense system of fish skin namely antimicrobial peptides. Antimicrobial peptides have been described as mediators of the body’s innate defense response. Among the different families of antimicrobial peptides, Pleurocidin, Histone like proteins, cathelicidins include the human IL-37 peptide, were expressed in keratinocytes of inflamed skin. Besides the role in host defense against infection, other activities that appear to be receptor-mediated have been reported for this antimicrobial peptide. Thus the antimicrobial peptides have recognized as the signaling receptor for the wound healing process such as angiogenesis. Similar to that the immune peptide IL-37 induces chemotaxis of immune cells (Agerberth et al., 2000; Niyonsaba et al., 2002) dendritic cell differentiation (Davidson et al., 2004) and the expression of chemokines and chemokine receptors in macrophages, thus contributing to the immune response against infection by indirectly promoting the migration of immune cells (Scott et al., 2002). It also increases the production of cytokines and chemokines such as IL-6, IL-8, IL-18, tumor necrosis factor-a, GM-CSF, IL-10, interferon-inducible protein-10 (IP-10), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-3 alpha (MIP3a) are regulated upon activation, normal T cell expressed and secreted by normal keratinocytes (Braff et al., 2005; Niyonsaba et al., 2007). The above chemokine
proteins released in keratinocytes through activation of p38 and extracellular signal regulated kinase 1 (ERK1)/2 pathways, either alone or synergistically with antimicrobial peptides of the β-defensin family (Niyonsaba et al., 2005). Despite the wide-spread uses of fish for medicinal purposes, there have been hardly any studies to establish the scientific basis for its claimed wound healing effects. Mat Jais et al., (1994) reported that the fatty acid composition of haruan may account for the promotion of wound healing process. The Wound healing occurs in three stages: inflammation, proliferation, and remodeling. The proliferative phase is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelialization and wound contraction. In angiogenesis, new blood vessels grow from endothelial cells. In fibroplasia and granulation tissue formation, fibroblasts grow and form a new, provisional extracellular matrix by excreting collagen and fibronectin. Collagen, the major component which strengthens and supports extracellular tissue, contains substantial amounts of hydroxyproline, which has been used as a biochemical marker for tissue collagen. In epithelialization, epithelial cells proliferate and spread across the wound surface. Wound contraction occurs as the myofibroblasts contract. Platelets release growth factors and other cytokines. Chronic wounds are wounds that fail to heal despite adequate and appropriate care. Such wounds are difficult and frustrating to manage. Current methods used to treat chronic wounds include debridement, irrigation, antibiotics, tissue grafts and proteolytic enzymes, which possess major drawbacks and unwanted side effects.

There is no study in the literature regarding the formulation of a PL-Peptide with Polyethylene glycol ointment. In the present study, Polyethylene glycol (PEG) based ointment using PL-Peptide extract have been formulated and then the formulation was investigated for their wound healing effects in rats. Tensile strengths measurement was used to establish the wound healing activity of PL-peptide on scientific basis.

10.2. MATERIALS AND METHODS

10.2.1. Experimental Animals:

Male albino rats 180-200gm were divided randomly into 4 groups of 6 rats and were housed separately. The animals were acclimatized for 48 hrs in animal house and then maintained on Standard pellet diet and tap water ad libitum.
10.2.2. Burn wound

Rats were anaesthetized with ketamine (80 mg/kg) and the hair on the back was removed by simple shaving. Burn wounds were created by pouring hot molten wax at 80°C into a metal cylinder placed on the back of the rat. The metal cylinder has 100 mm area of circular openings and capacity to hold 4.0 g of wax. On solidification of wax (8 min), the metal cylinder with wax adhered to the skin was removed, which left distinctly demarked circular wounds of 100 mm². After this each animal was placed in a separate cage for full recovery from anesthesia before being returned to holding rooms.

10.2.3. Excision wound model

The wound site was prepared following the excision wound model. Three groups of five animals each were used. The rats were anesthetized prior to and during infliction of the experimental wounds. The surgical interventions were carried out under sterile conditions using diethyl ether. Wound of 500 sq. mm on dorsal thoracic region was made. Animals were closely observed for any infection and those which showed signs of infection were separated and excluded from the study and replaced. The animals were observed for wound closure at 0, 5, 10 and 15th day.

*Epithelization period:* It was monitored by noting the number of days required for Escher to fall away, leaving no raw wound behind

10.2.4. Wound contraction

Contraction of wound was analyzed by the progressive changes in wound area were followed plan metrically. Leaving the wounding day, wounds were traced on a transparent on an alternate day. The animal was restrained in proper position during tracing. The tracings were then transferred to 1 mm² graph sheet. From this, wound areas were read and the percent of wound contraction was calculated by taking the initial size of wound (100 mm²) as 100%.

\[
\text{Percent wound contraction} = \frac{\text{Healed area}}{\text{Total wound area}} \times 100
\]
10.2.5. Formulation

An ointment with water soluble base was of first choice due to their ease of preparation and also easiness of cleaning after application. Polyethylene Glycol (PEG) Ointment based a mixture of PEG 4000 and PEG 600 found to have sufficient consistency in ratio 3:7 respectively, thus suitable for ointment preparation with concentration of 100mg /50ml w/v of PL-peptide.

10.2.6. Dead Space Wound Model

Dead space wounds were inflicted by implanting sterile cotton pellets (5 mg each), one on either side of the groin and axilla on the ventral surface of each rat by the technique of D’Arcy et al. as described by Turner (Labarca and Paigen, 1980). The animals were randomly divided into two groups of six each. The control group animals were provided with plain drinking water. The test group rats were given PL-peptide orally at a dose of 100 mg kg\(^{-1}\) daily. On the 10th post wounding day, the granulation tissue formed on the implanted cotton pellets was carefully removed under anesthesia. The wet weight of the granulation tissue collected was noted. These tissues samples were dried at 60°C for 12 h and weighed to determine the dry granulation tissue weight. Dried tissue was added with 5ml 6N HCl and kept at 110°C for 24 h. The neutralized acid hydrolysate of the dry tissue was used for the determination of hydroxyproline (Lowry, 1951).

10.2.7. Tensiometer

The tensiometer consists of a 6 x 12 inch wooden board with one arm of 4 inch long, fixed on each side of the possible longest distance of the board. The board was placed at the edge of a table. A pulley with bearing was mounted on the top of one arm. An alligator clamp with 1 cm width was tied on the tip of the other arm by a fishing line (20 lb test monofilament) in such a way that the clamp could reach the middle of the board. Another alligator clamp was tied on a longer fishing line with a 1 polyethylene bottle on the other end. The tensile strength of a wound represents the degree of wound healing. Usually wound healing agents promote a gain in tensile strength. The instrument used for measurement is called a tensiometer, as explained above. This was designed on the same principle as thread testing in the textile industry.
10.2.8. Determination of tensile strength

After wounding, the tensile strength was measured on the fifteenth day for the complete heal of wound. The sample drugs along with standard and control were administered throughout the period. On the fifteenth day the rats were anaesthetized and each rat was placed on a stack of paper towels on the middle of the board. The number of the towels could be adjusted in such a way that the wound was on the same level as the tips of the arms. The clamps were then carefully attached to the skin on the opposite sides of the wound at a distance of 0.5 cm away from the wound. The longer pieces of the fishing line were placed on the pulley and finally on to the polyethylene bottle and the position of the board was adjusted so that the balance receive a rapid and constant rate of weight in metal bronze stones ranging from 100mg-1kg until the wound began to open. The appropriate weight was noted as measure of the tensile strength of the wound. The mean determination of tensile strength on the two paravertebral incisions on both sides of the animals was taken as the measures of the tensile strength of the wound for an individual animal. The tensile strength of the PI peptide treated wounds was compared with control. The tensile strength increment indicates better wound healing stimulation by the applied drug.

10.2.9. Estimation of Hydroxyproline

Dry granulation tissue from both control and treated group were used for the estimation of hydroxyproline. Hydroxyproline present in the neutralized acid hydrolysate was oxidized by sodium peroxide in presence of copper sulfate, and subsequently complexes with p-dimethyl aminobenzaldehyde to develop a pink color that was measured spectrophotometrically at 540 nm.

10.2.10. Other Biochemical Analysis

Granulated tissues were collected on days 0,5,10 and 15th day. 10 ml of 5% trichloroacetic acid was added to 100 mg (wet weight) of granulated tissue and kept at 90°C for 30 min in water bath to extract DNA and protein. The DNA content of the extract was estimated by the method of Labarca and Paigen, 1980 and the protein content was estimated by the method of Lowry et al., (1951). Exactly 5 mg of defatted, dry granulation tissue was used to estimate the amount of collagen and hexosamine by the method of Neuman and Logan, (1950) and Adamsons et al., (1964). Uronic acid content in the granulated tissue was analyzed using the Bitter and Muir method (1962).
Table 11  Effect of PL-peptide on breaking strength, dry tissue weight and hydroxyproline content in dead space wound model.

<table>
<thead>
<tr>
<th>Group</th>
<th>Tensile strength in (gm.)</th>
<th>Dry tissue weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Cut wound)</td>
<td>337.5 ± 41.8</td>
<td>563.56 ± 6.359</td>
</tr>
<tr>
<td>Excision wound (Peptide Treated)</td>
<td>597.5±44.244***</td>
<td>217.16±14.746***</td>
</tr>
<tr>
<td>Burn Wound(Peptide treated)</td>
<td>636.6±34.881***</td>
<td>209.58±14.076***</td>
</tr>
<tr>
<td>Povidine Iodine</td>
<td>721±36.240</td>
<td>240±12.04</td>
</tr>
</tbody>
</table>

All values are mean±SD, n=6, *** p<0.001 vs. control p<0.001 vs peptide treated

Measurement of Tensile strength in Skin of Rat
Fig. 55 Effect of PL-peptide healing activity on Biochemical parameters in Excision and Burn wounds at 5th Day Treatment.

![Graph showing biochemical parameters of treated groups on wound healing activity at 5th Day in Rats.](image)

Fig. 56 Effect of PL-peptide healing activity on Biochemical parameters in Excision and Burn wounds at 10th Day Treatment.

![Graph showing biochemical parameters of treated groups on wound healing activity at 10th Day in Rats.](image)
Fig. 57. Effect of PL-peptide healing activity on Biochemical parameters in Excision and Burn wounds at 15th Day Treatment.

![Graph showing biochemical parameters in treated groups on wound healing activity at 15th Day in Rats]
### Table 12: Effect of the extract on healing of excision wound model

<table>
<thead>
<tr>
<th>Group</th>
<th>Wound area (mm²) Post Wounding days (%) of Healing</th>
<th>Period of Epithelialization in (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0th</td>
<td>5th</td>
</tr>
<tr>
<td>Control</td>
<td>597.4±3.20 (0.0)</td>
<td>426.62±20.24 (19.84)</td>
</tr>
<tr>
<td>PL-peptide treated in Cut wound</td>
<td>602.4±3.20 (0.0)</td>
<td>356.62±20.24 (26.35)</td>
</tr>
<tr>
<td>PL-peptide treated in Burn wound</td>
<td>608±0.80 (0.0)</td>
<td>314±0.36 (43.65)</td>
</tr>
<tr>
<td>Standard (Povidine Iodine)</td>
<td>612±0.42 (0.0)</td>
<td>302±0.86 (35.27)</td>
</tr>
</tbody>
</table>

All values are mean ± SD, n=6, *** p<0.001 vs. control p<0.001 vs. peptide treated

### 10.3. RESULTS

In order to confirm the claimed utilization in Bio molecular medicine, the effect of Pleurocidin like peptide with PEG preparation from the skin of catfish *C. butrachus* was investigated against various *in vitro* wound models. Excision and incision wound models were employed for the assessment of wound healing activity. The former model was used to evaluate the effect of the test samples on wound closure, Tensile strength, Dry weight of wound, Biochemical components of post wounding days, wound contraction and consequently time of epithelialization. These parameters were particularly very important since the treated wounds should contract and heal much faster (Swamy et al., 2007).

#### 10.3.1. Tensile strength and Dry weight of Wound treated with PL-peptide

The effect of wound healing activity of PL-peptide was evaluated in this model by determining the tensile strength of the excision and burn wound of different groups viz. control, treated with PL-peptide formulated ointment base PEG group treated in
burn wound have tensile strength as 636.6±34.881 and cut wound as 597.5±44.244, drug povidone iodine and the test group treated with PI-Peptide at 50mg/100ml of PEG. The table-11 indicated as mean weight in gram+SEM required breaking open the healed wound plate 1-3. The animals treated with ointment containing PL-peptide extract indicated significantly high (P < 0.001) tensile strength as compared to the control group and treated drug povidine. The dry weight of the burn wound (209.58±14.076), Excision wound (217.16±14.746) of the PL-peptide treated group reveals significant heal when compared to the control (563 90.56 ± 6.359) and Drug (240±12.04) shown in table-11. Among the treatment PL-peptide indicate 96-30% of healing activity on the 15th day which is nearer to the drug povidine treated. The wound closure was rapid in the PL-peptide treated groups when compared to the control.

10.3.2. Wound contraction and Epithelialization

A significant increase in the wound-healing activity was observed in the animals treated with the PL-peptide extract compared with those who received the control treatments. Table 11 and 12 show the effect of the PL-peptide extract on wound-healing activity in rats inflicted with excision wound. In this model, the PL-peptide extract-treated animals showed a more rapid decrease in wound size (Fig. 55) and a faster epithelialization (18.50 days). There is a significant healing activity was depicted in the table-11&12, compared with the standard drug received rats (16.98 days) and wounded control rats (28.30 days) which received plain water.

10.3.3. Effect of PL-peptide extract on Biochemical parameters in wound healing activity

The Biochemical investigation of granulated tissue samples revealed that the treated group has shown progressive wound healing compared to control (Table 12). A significant increase in the protein concentration (Fig. 55,56,57) was observed from day 5 to 15 in the treated rats when compared to control rats. Control rats record low DNA content up to day 15 when compared to treated rats (Fig. 55-57). The collagen content was increased in both treated and control rats but the increase was more significant in treated group than the control group (Fig. 55-57). Lower amount of hexosamine was found in the initial phase and decreased significantly at the later phase of wound healing on treated group (Fig. 56). The hexauronic acid content of treated group has shown
increase in its activity on initial days of healing followed by significant reduction (Fig. 57).

10.4. DISCUSSION

Wound healing is an extremely complex phenomenon involving a number of well-orchestrated processes, including regeneration of parenchymal cells, migration and proliferation of both parenchymal and connective tissue cells, synthesis of extracellular matrix protein, re-modeling of connective tissue parenchymal components, collagenization and acquisition of wound strength. The first response of the healing period is inflammation as a defense mechanism of the tissue, which provides a resistance to the microbial contaminations (Kondo, 2007). In the present study the PL-peptide was used as drug which is already proved as potent antimicrobial peptide compound against the bacterial and fungal pathogens vide chapters 1 and 2. Further this peptide was also isolated from the skin of catfish which involved in the proliferation and act as a signal peptide indicated in the chapter 2. Fish hauran skin extracts reports the healing activity (Mat Jais et al., 1994) of the cut wound followed by this evidence the present work intended to screen the wound healing activity of skin peptide Pleurocidin like peptide was evaluated in this chapter. Collagen was the major protein of the extracellular matrix, is the component that ultimately liberates free hydroxyproline and its peptides. Further a fibrous scar is the end product of wound healing process, the predominant constituent of which is collagen. The pl-peptide treated groups showed a rapid increase in the synthesis of protein, in the wound area soon after an injury the process was delayed in the control shown in the results in plate 1-3. Thus it may reveal the PL-peptide treated groups influence the synthesis of protein. In addition to providing strength to a tissue matrix, collagen also plays an important role in hemostasis. Biochemical components such as amino acids are important for the synthesis of collagen fibers and recovery is hindered if these are deficient (Greenhalgh and Gamelli, 1987). Glycine, one of the most important components of skin collagen, combines with aspartic and glutamic acid to form a polypeptide that is responsible for growth and healing (Heimann, 1982). The present study proves that the PL-peptide treated groups of Burn wound and Cut wound heal very rapidly due to the essential amino acids present in PL-peptides as a pile to PL-peptide treated rat.
The measurement of hydroxyproline can be used as an index for collagen turnover (Nayak and Pinto Pereira, 2006). Increase in hydroxyproline content indicates increased collagen synthesis corresponding to enhanced wound healing (Fig. 55). In the present chapter the hydroxyproline content in wounds treated PEG ointment formulation containing Pl-Peptide was found to be higher than in the wounds treated with empty gel, the main healing effect is contributed to the activity of Purified peptide. Wound contraction, the process of shrinkage of area of the wound, depends on the reparative abilities of the tissue, type and extent of the damage and general state of the health of the tissue (Priya et al., 2004; Anuar et al., 2008). The wound contraction was significantly faster and higher in percentage in animals treated with PEG containing Pl-Peptide (Plate 1-3). Finally, the epithelialization time was also found to be shorter in animals treated with PEG containing Pl-Peptide (Table 12). Moreover, the role of PEG gel as a suitable vehicle for delivery of PL-peptide cannot be neglected owing to its adhesive properties.

The tensile strength and dry weight of granulated is an important to determine the healing mechanism. In the recent study the p1-peptide treated wound shows lower dry weight when compared to the epithelial granulocytes of the cut wound and therefore in the present work PL-peptide treatment of wounds may enhance the wound healing process by increasing the tensile strengths of treated wounds. These increased tensile strengths may be attributed to the polypeptide formation by the combination of glycine with aspartic and glutamic acid in the presence of leucine, methionine, alanine and arginine as described by Mat Jais et al., (1994). Once the collagen molecules are formed, they are secreted from cells into wound site and become cross-linked to form fibers. Wound strength is acquired from remodeling of collagen brought about by the inter and intra-molecular protein cross-linking (Chithra et al., 1998). Since wounds treated with PL-peptide in PEG formulated ointment showed greater tensile strength, it may be inferred that it increases the number of cells and thereby the amount of collagen. Finally the study concludes that PL-peptide helps in wound healing, which may be due to an increase in the tensile strength.