Chapter 7

*In Vivo* Wound Healing Activity of E1

7.1 INTRODUCTION

Of the four bioactive cryptic peptides identified in the previous chapters, the peptide E1 demands explicit mention because of its multiple activities. It has already been assayed in Chapters 3, 5 and 6 to be an effective antioxidant and cell adhesion peptide along with *in vitro* wound healing capabilities. In this chapter, the bioactivity of E1 was tested via an *in vivo* model; by applying E1 as a healing agent.

The wound healing process is a complex and dynamic series of events restoring cellular structures that begins at the moment of injury and can continue for months to years. It involves 4 overlapping steps; hemostasis, inflammation, proliferation and remodeling, each with multiple carefully regulated sub-steps with various dependence relations among themselves (Ramos et al 2011; Tabatabhai et al 2011). Each step influenced through a myriad of activities including radical scavenging, cellular recruitment and stress relief. Determining the biochemical and biophysical effects of E1 on the several phases of wound healing could possibly substantiate the *in vivo* bioactive nature of E1.

The first phase hemostasis begins as an immediate response to an injury; the aggregation of platelets leads to the formation of a blood clot, resulting in a temporary seal of the wound. The vasculature constricts to decrease the blood flow, reducing the extent of haemorrhage. The platelets release vasodilators and platelet-derived growth factor (PDGF) which makes the blood vessels ‘leaky’ and recruits the next set of cells to the wound site for the next phase, the inflammation phase (Mutsaers et al 1997, Sharrott and Dallon, 2002).

During the second phase, neutrophils and macrophages are attracted to the wound site where, with the help of resident mast cells, they initiate destruction of invading microorganisms and clearing the cellular debris of the wounds (Traci, 2008). They too, act as a source of chemoattractants and growth factors including epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor beta (TGF-β) and tumour necrosis factor alpha (TNF-α) which appear to direct the next phase.

In the third stage (the proliferative stage), the hormones released in the previous phase induce endothelial cell and fibroblast migration towards the centre, thus forming
new blood vessels and a new granulation tissue (Mutsaers et al 1997). Fibroblasts further stimulate migration and proliferation of keratinocytes at the wound edge, resulting in complete coverage of the wound by a neoepidermis, propagating epithelialization (Schäfer and Werner, 2008; Ramos et al 2011).

The fourth or the remodeling phase consists of rapid synthesis and cross-linking of newly formed collagen matrix and their organization. Fibroblasts produce and deposit large quantities of matrix proteins with a major share being collagen types I and III, which increases the tensile strength of the wound. The balance between types I and III collagen is maintained by TGF β. The final process in normal wound healing is resolution of the scar. In skin, the high rate of collagen synthesis within a wound recedes to normal levels by 6-12 months.

Rapid wound healing is dependent on a number of factors including proper synchronization of the phases (Schultz and Wysocki, 2009). A wound healing agent with the ability to influence multiple phases of the healing engages in phase-synchronization resulting in lower healing time periods. This study was therefore undertaken to evaluate the activity of this multifunctional peptide E1 as an in vivo bioactive agent.

7.2 MATERIALS AND METHODS

7.2.1 ANIMALS

Male albino Wistar strain rats in the weight range of 500-600g and 10 months of age were used for this study. The animals were maintained on clean, sterile, polyvinyl cages and fed with commercial rat food from M/s Hindustan Lever Ltd, India {mixed with wheat flour in the ratio 1:1 (w/w)}. Food and water were provided ad libitum to the animals. All processes were carried out according to the stipulations of the IACUC.

7.2.2 CHEMICALS

Pepsin, L-hydroxyproline, glucuronic acid, HEPES buffer {2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid}, chloramine-T and MTT {(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} were obtained from Sigma, St. Louis, USA. p-dimethyl aminobenzaldehyde and Folin’s phenol reagent were obtained from SD Fine Chemicals Limited, Mumbai, India. Plates and reagents for chemotactic assay were procured from Cell Biolab, San Diego, USA. Methyl cellosolve was obtained from E. Merck, Darmstadt, Germany. All other reagents were of analytical grade.
7.2.3 CHEMOTACTIC ASSAY OF E1

The chemotactic properties of E1 were measured through a trans-well cell migration assay (Augustin, 2004). Blood was collected from a healthy donor and the leukocytes isolated by the use of Hi-Sep LSM (from HiMedia, India). Different amounts of E1 was dissolved in 150µl Hank’s balanced salt solution (HBSS) along with 150mM HEPES buffer and added to the feeder tray wells at final concentrations ranging from 1-1000nM. A fixed number of cells suspended in 100µl of HBSS were added to the membrane chamber and the cover replaced. The plates were incubated for 3h in a 5% CO₂ chamber at 37°C. The number of cells migrated was measured by dislodging cells from the bottom of the permeable support and the feeder chambers followed by MTT assay in a new 96-well plate. Absorbance was measured at 550nm with reference at 630nm in a Bio-Rad microplate reader. The chemotactic peptide N-formylmethionyl-leucyl-phenylalanine (FMLP) and BSA were used as the positive and negative control respectively. In a separate set-up, E1 was degraded with trypsin in the ratio 1:50, extracted with 3.4mM acetic acid and re-subjected to chemotactic assay along with suitably adjusted control. The amino acid sequence of E1 was uploaded to PeptideCutter in ExPASy to check for trypsin cleavage sites. The assay was repeated with a fixed concentration of E1 and degraded E1 in the bottom chamber and increasing number of cells in the upper chambers. A checkerboard assay was conducted with differing amounts of peptide E1 in the upper and lower chambers in the presence of a fixed number of cells to ascertain its chemotactic properties.

7.2.4 WOUND CREATION AND SAMPLE APPLICATION

A 2cm² full thickness open excision wound was made in the back of 36 rats as reported in earlier studies (Panchatcharam et al 2006). The animals were divided into two groups; control and test. 200µl of the peptide solution was applied topically to the test rats at a fixed concentration of 60µM, once daily for a period of 12 days. 200µl of physiological saline was applied to the control rats for a similar duration of time. Six rats were sacrificed at different time intervals. The wound tissues were removed on the 4th and 8th days’ post-wound infliction and used for biochemical analyses.

For incisional wound creation, 12 rats were divided into two groups of six; they were anaesthetized and two paravertebral long incisions of 5cm were made through the skin and cutaneous muscles at a distance of 1.5cm from the vertebra. After wiping the wound dry, intermittent sutures were applied via a surgical nylon thread and a curved
needle (no. 11), 1cm apart. For the test rats, 60mM of the peptide solution was added to the wound on a daily basis whereas the control group received same volume of saline. The sutures were removed on day 7 and tensile strength was measured on the 8th day by the method of Vogel (1970) as described in Section 7.2.6.

7.2.5 BIOCHEMICAL PARAMETERS

Grannulation tissues from the test and control groups were collected and washed well in cold 0.9% saline. Protein from wet granulation tissues was extracted with 5% trichloroacetic acid as per Porat et al (1980) and was estimated by the method of Lowry et al (1951). The protein was redissolved in 0.1M Na$_2$HPO$_4$-NaH$_2$PO$_4$ buffer, pH 7, followed by addition of 5ml of CuSO$_4$-tartarate reagent. The solution was incubated at 28°C for 30min, following which 0.5ml of 1:1 diluted Folin phenol agent was added and the developed colour was read at 660nm. BSA was used as standard.

For collagen and hexosamine estimation, the tissue samples were defatted in chloroform: methanol (2:1) and dried in acetone before use. Collagen was quantified using the method of Woessner (Chapter 2, section 2.2.2). Hexosamine was quantified by the method of Elson and Morgan (1933). 5mg of lyophilized wound tissue sample was hydrolysed with 5ml of 2N HCl at 110°C for 7h in a sealed glass tube. The residue was evaporated to dryness, dissolved in a known volume of water and a series of diluted solutions prepared. The samples were treated with 1ml of freshly prepared 2% acetyl acetone dissolved in 0.5M sodium carbonate and boiled for 15min. The solutions were cooled in tap water, followed by additions of 5ml 95% ethanol and 1ml of Ehlrich’s reagent. The red colour developed was read after 30min at 530nm. N-acetyl amino sugar was used as standard.

Lipid peroxide levels were calculated via the thiobarbituric acid method (Marreilha dos Santos et al 2008). To 0.1ml of the acid-hydrolyzed and water-reconstituted tissue, 0.9ml of 10% TCA and 2ml of 0.67% thiobarbituric acid reagent were added and the resultant solution was boiled in a water bath for 20min. After cooling at room temperature, the samples were diluted with 2ml of distilled water, vortexed and centrifuged to obtain a clear solution. The pink colour developed was read at 532nm. A standard curve was made with MDA in isotonic saline.

For ascorbate estimation by Omaye et al 1979, a known amount of the tissue was added to water to create a suspension followed by addition of 10% ice-cold TCA. The mixture was centrifuged at 3500g for 20min. 0.5ml of the supernatant was mixed with
0.1ml of dithiocarbamate, mixed well and incubated at 37°C for 3h. This was followed by addition of 0.75ml 65% H₂SO₄ and incubation at 28°C for 30min. The yellow colour developed was read at 520nm. Ascorbic acid was used as the standard.

7.2.6 BIOPHYSICAL PARAMETERS

The period of epithelialization was taken as the number of days for shedding of eschar without any raw wound left behind. The rate of wound contraction was determined by tracing the wound surface on to a transparent graph sheet and measuring the surface area by planimetric method. Reduction in wound size was determined as percentage area of the original wound dimensions, measured using the formula:

\[ \% \text{ contraction} = (\frac{W_o - W_t}{W_o}) \times 100 \]

Where Wo and Wt represents the initial and the day-specific wound size respectively.

The shrinkage temperature of the wound tissue was measured through the method of Borasky and Nutting (1949). A small piece of the granulation tissue was moistioned with a drop of water and fitted onto a heating stage fitted with an optical microscope. The tissue was heated at a constant rate with the help of a tungsten lamp and continually observed. The temperature at which the tissue started to shrink was noted as the shrinkage temperature.

The tensile strength was determined as follows. After sacrifice, the back of the animals was shaved and a flap of skin containing the wound gap removed. The skin flap was placed between 2 pieces of polypropylene material of known thickness and the total thickness measured by callipers. The actual thickness of the skin is calculated by the difference of the total thickness and the thickness of the materials. Two dumb bell shaped specimens are cut with a special punch in a direction perpendicular to the vertebra and the skin pelts were fixed between the clamps of an Intron instrument. The specimens were preloaded at 0.2N and were made to undergo a 10mm min⁻¹ displacement rate at room temperature till fracture. The force-elongation curve was recorded at a sampling rate of 5Hz. The data included the values of the load as a function of time and the value of the displacement of the moving plate, also as a function of time. Elimination of the time factor from both data sets allowed the applied force to be calculated as a function of the displacement induced. The maximum force recorded was defined as the breaking strength and the tensile strength was calculated by dividing the breaking strength by the cross sectional area of the specimen.
7.2.7 STATISTICAL ANALYSIS

Values given for the chemotactic assay and the biochemical and biophysical parameters are reported as mean ± standard deviation. The data were analyzed for statistical significance using ANOVA for larger groups and student’s t test for comparing two groups. P values less than 0.05 were considered significant.

7.3 RESULTS AND DISCUSSION

7.3.1 CHEMOTACTIC ACTIVITY OF E1

As depicted in Table 7.1, the chemotactic activity displayed by E1 was observed to be dependent on peptide concentrations. Activity decreased at higher concentrations, possibly due to the recoiling nature of collagen peptides and/or the desensitization of chemotactic receptors on the leukocytes. Maximum activity displayed by E1, although lower than FMLP (p<0.01), was at 100nM with 30% migrated cells.

Interestingly, the activity increased 1.8 times with similar amounts of the degraded peptide. The activity was also found to be dependent on the number of cells seeded in the upper chamber, as is evident from Table 7.2. For lower cell counts, 42-45% cells were found to migrate but the % of migratory cells decreased somewhat with increasing cell concentration. However, with a 500 fold increase in cell count, from left to right, a 255 fold and a 367 fold increase in cell migration was observed with E1 and degraded E1 respectively.

E1 in the degraded form allowed a significantly increasing number of cells (p<0.02) to migrate although the pattern of migration was similar to that of the intact peptide. Checkerboard analysis results displayed in Table 7.3 exhibited a number of interesting results. First, the migration of cells increased 36 times in the presence of a 100× higher concentration of E1 in the bottom chamber and decreased to 25 and 7.5 times with the increase of E1 in the top chamber. In fact, there was a 12 time decrease in migration with a 100× increase of E1 in the top chamber. Second, moving diagonally downwards the table, similar migratory cell counts are noticable for equal concentrations of E1 in the top and bottom chambers.
Table 7.1 Results of cell migration assay with different concentrations of E1, protease degraded-E1 and FMLP.

| Concentration (nM) | Cell count ($\times 10^4$) ± SD in the presence of |  |
|--------------------|---------------------------------|-----|-----|-----|
|                    | E1                              | E1 degraded | FMLP |
| 1                  | 0.5±0.2                         | 0.8±0.1    | 2.5±0.6 |
| 10                 | 1.1±0.1                         | 2.8±0.2    | 3.9±1.0 |
| 100                | 3.8±0.5                         | 5.9±0.7    | 7.4±2.3 |
| 1000               | 1.0±0.4                         | 1.8±0.5    | 4.8±1.0 |

Table 7.2 Results of cell migration assays conducted with 100nM E1 and degraded E1 with different cell counts in the top chamber.

<table>
<thead>
<tr>
<th>Chemotactic agent</th>
<th>Cell count ($\times 10^4$) ± SD in top chamber</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>E1</td>
<td>0.045±0.003</td>
</tr>
<tr>
<td>E1 degraded</td>
<td>0.06±0.007</td>
</tr>
</tbody>
</table>

Table 7.3 Migratory cell count with varying concentrations of E1 in the top and bottom chambers.

<table>
<thead>
<tr>
<th>Concentration of E1 in bottom chamber (nM)</th>
<th>Concentration of E1 in top chamber (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0.4±0.2</td>
</tr>
<tr>
<td>10</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>100</td>
<td>3.6±0.5</td>
</tr>
</tbody>
</table>
7.3.2 BIOCHEMICAL PARAMETERS

Table 7.4 depicts the collagen, total protein, hexosamine, ascorbate and linoleic acid levels on the 4th and 8th day. A preliminary glance at the data indicates a consistent difference between the test and control groups in most of the cases.

LPO was detected through the detection of malondialdehyde (MDA), a degradation product of LPO which reacts with TBA to form a coloured product. The 1.3 and 5.7 fold reductions in the LPO levels on the 4th and 8th day, respectively ensured E1’s potential antioxidant activity which neutralized the ROS load at the wound site. The difference of LPO levels on the 4th and 8th day were found to be significantly different (p<0.001 and p<0.00001 at 95% confidence level, respectively) than those of the control. Ascorbate was converted to dehydroxyascorbate, which upon reaction with phenyl hydrazine results in coloured crystals soluble in sulphuric acid. Ascorbate amounts on the 8th day were found to increase 3 folds for the control and 2.7 folds for E1-treated groups from their respective values on the 4th day. This difference was significant (p=0.03) for the 4th day but the 8th day ascorbate levels were almost similar; in fact, the difference between the two levels were statistically insignificant (p>0.05, at 95% confidence level).

Both O and hexosamine react with Ehrlich reagent to produce a colored condensation product and therefore, is used as the basis for their detection. On the 4th and the 8th day, the amount of collagen produced in the test group was 2.36 and 2.85 times that of collagen present in the control group. In both cases, the values for E1 were significantly higher than the control values with p=0.002 for the 4th day and p=0.0005 for the 8th day at a confidence level of 95%. As noted from the table, hexosamine content was moderately increased on the 4th day but the difference was significant from that of the control-treated group (p=0.005). On the 8th day, there was almost two fold increase in the treated group when compared to the control group (p=0.001 at a confidence level of 95%). Protein estimation was carried out through Cu (II) coordination by the peptide bonds followed by the reduction of phosphotungstic acid through aromatic residues. Protein production followed a similar pattern as with other parameters. On both the 4th and 8th day, protein production was more than 3 times that of the control group suggesting of the active remodeling process. The difference between the two groups was significant on both occasions; p=0.0006 and p=0.003 for the 4th and 8th day respectively.
Table 7.4 Effect of E1 on five biochemical parameters.

<table>
<thead>
<tr>
<th></th>
<th>Day 4</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LPO (mg/100g body weight)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.15 ± 0.20</td>
<td>4.09 ± 0.16</td>
</tr>
<tr>
<td>Test</td>
<td>1.57 ± 0.23c</td>
<td>0.71 ± 0.14a</td>
</tr>
<tr>
<td><strong>Ascorbate (mg/100g body weight)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.44 ± 0.1</td>
<td>7.72 ± 0.75</td>
</tr>
<tr>
<td>Test</td>
<td>3.63 ± 0.54c</td>
<td>9.09 ± 0.69d</td>
</tr>
<tr>
<td><strong>Collagen (mg/100g body weight)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.1 ± 0.11</td>
<td>2.07 ± 0.44</td>
</tr>
<tr>
<td>Test</td>
<td>2.6 ± 0.23b</td>
<td>5.96 ± 0.54a</td>
</tr>
<tr>
<td><strong>Hexosamine (mg/100g body weight)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.44 ± 0.09</td>
<td>0.6 ± 0.08</td>
</tr>
<tr>
<td>Test</td>
<td>1.4 ± 0.21b</td>
<td>2.62 ± 0.16a</td>
</tr>
<tr>
<td><strong>Protein (mg/100g body weight)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.78 ± 0.1</td>
<td>2.32 ± 0.43</td>
</tr>
<tr>
<td>Test</td>
<td>5.41 ± 0.31a</td>
<td>8.5 ± 0.56b</td>
</tr>
</tbody>
</table>

Values are expressed in mean ± SD for six animals. Level of significance expressed as a \( p<0.001 \), b \( p<0.01 \), c \( p<0.05 \) and d \( p\geq0.05 \) as compared using t-test at 95% confidence level.

7.3.3 BIOPHYSICAL PARAMETERS

The %wound contracted had a markedly different value for the control and the test samples. As displayed in Fig. 7.1a, the difference was highly significant in the 4\(^{th}\) (\( p<0.0002 \)) and 12\(^{th}\) (\( p<0.0002 \)) days with the highest difference being on the 8\(^{th}\) day (\( p<0.00006 \)). Fig. 7.1b displays the period of epithelialization, which was found to be reduced in the test rats from the control rats by a factor of 1.75. Fig. 7.1c displays the tensile strength of tissues from the control and test groups; a significant increase by a factor of 2.2 was observed for the test groups on the 8\(^{th}\) day. Fig. 7.1d registers the difference in shrinkage temperature of the collagen extracted from the control and the test tissues. Fig. 7.3 displays the photographs showing the rate of wound contraction.
a. The % of wound contraction in control group (white) and test group (grey) at different days. Level of significance, as calculated by t-test; 4\textsuperscript{th} and 12\textsuperscript{th} day: $p<0.001$, for 8\textsuperscript{th} day: $p<0.0001$ at a confidence level of 95%.

b. Period of epithelialization in control and test groups; $p<0.0001$ at a confidence level of 95%.

c. Tensile strength of 8\textsuperscript{th} day wound tissue for control and test groups; $p<0.0001$ at a confidence level of 95%.

d. Shrinkage temperature of 8\textsuperscript{th} day wound tissue for control and test groups; $p<0.0001$ at a confidence level of 95%.

Results are given as mean of triplicate samples ± SD.
7.3.4 ACTIVITY OF PEPTIDE E1

Externally administered bioactive agents can influence one or more steps of wound healing (Balekar et al 2012). Antimicrobial, haemostatic and antioxidative compounds, the latter mostly flavonoids-like or terpene-like in nature, have been isolated from phytochemicals and used to improve the healing phases (Okoli et al 2007). Bioactive peptides have also been known to play a key role in wound healing; one of the well-studied examples being the human tripeptide GHK, which accelerates tissue remodeling (Pickart, 2008). Based on the sequence and its previously assayed in vitro antioxidant activity, E1 was expected to act in a multi-functional manner, influencing more than one phase in the wound healing activity cascade.

7.3.4.1 EFFECT ON LEUKOCYTE RECRUITMENT

Chemotactic activity of most peptides is dependent on both sequence and structure. Earlier studies report that the presence of certain residues like G, P, R and F promote chemotaxis. Presence of an imino group makes the sequence rigid and reduces torsional movements, possibly leading to better interaction with chemotactic receptors. It is also known that presence of certain residues like R as the N- or C-terminus acts as a chemotaxis promoter, as in bradykinin (Pfister et al 1995; Kohidai et al 2002; Afonso et al 2013).

RGD peptides have previously been known to act as chemotactic agents (Senior et al 1992) and the results of this study further confirm such reports. E1, along with containing the RGD sub-sequence also displayed 33% G and 19% P. The activity of E1 was somewhat less when compared to FMLP, but this was probably because of the larger size of E1. It has been reported that smaller peptides are more efficient at chemotactic effect, possibly because they can interact with the chemotactic receptor of the concerned cell in a far more effective manner (Laskin et al 1986). The fact that degraded E1 could support more than twice the chemotactic activity adds some credit to the claim.

The virtual peptide cutter at ExPASy identified three cleavage sites for trypsin, and four for elastase, as depicted in Fig. 7.2. The absence of large hydrophobic residues restricts the action of chymotrypsin reducing the cleavage chances to be in the range of 2-6%. Cleavage with trypsin occurs after positively charged residues and results in peptides with a C-terminal R or K residue, most notably a 1.5kDa N-terminal fragment with four GP sequences and a C-terminal R. The smaller size, increased amount of GP and a terminus R residue possibly enhanced the chemotactic properties of degraded E1.
The checkerboard analysis helps to differentiate between chemotactic and chemokinetic agents. A truly chemotactic agent induces quicker and a directional cell migration than in control upon exposure to a gradient. As seen in Table 7.3, equal concentrations of peptide E1 in the upper and lower compartment does not lead to a stronger migration to either chamber when compared to the controls. However, a glance down each column would reveal increasing cell migration with increased E1 concentration in the bottom chamber. Based on these two facts, E1 was deduced to be a true chemotactic agent. So possibly, E1 could recruit a higher number of leukocytes to the affected area ensuring rapid healing.

7.3.4.2 LOWERING OXIDATIVE STRESS

A second level in which E1 might have acted could be in reducing the stress generated from ROS. ROS, produced in the second stage, acts as one of the crucial regulators of the wound healing process (Wlaschek and Scharffetter-Kochanek, 2005; Sen and Roy, 2008). The wound site has two distinct sources of ROS; from the respiratory burst by phagocytic cells and a continuous production of low amounts by enzymes of the NOX family present in fibroblasts, keratinocytes and endothelial cells. While the former is involved in removing invading pathogens; the latter, drives a wide array of biological processes, including gene expression and angiogenesis in the granulation phase (Roy et al 2006). In the absence of substantial wound contamination, neutrophil infiltration ceases within 1-2 days after injury and most of these cells become
entrapped within the wound clot. However, if excess contamination of the wound occurs, neutrophils persist and the ROS generated by them can lead to significant tissue damage (Steiling et al 1999). ROS scavenging is achieved by a group of native antioxidants including redox enzymes, organic molecules along with compounds derived from food; vitamin E and C, carotinoids and phenolic compounds (Sen and Roy, 2008, Bedard and Krause, 2007).

The amount of lipid peroxidation is known to be a good indicator of the severity of the wound. The level of lipid peroxides (LPO) is found to be drastically reduced in the E1 treated rats in the 8th day tissues when compared to the controls. This is in accordance with the results of section 3.3.3.1, Chapter 3, where E1 exhibited comparatively better lipid peroxidation inhibition, displaying better LA auto-oxidation inhibition on the 5th and 6th days of incubation. The data presented here also supports a previous study which states that lipid peroxidation inhibition by administered agents can lead to faster healing in case of diabetes-impaired wounds (Altavilla et al 2001). Inhibition of LPO requires the peptide to be lipid-accessible. E1 displays an ample number of hydrophobic residues with % occurrence of 5.6% T, 11.1% A along with 2.8% of two strictly hydrophobic residues, I and V, each. These residues increase the lipid-solubility of E1, ensuring a higher accessibility to the LPO radicals.

Vitamin C is a cofactor for the enzymes prolyl and lysyl hydroxylase, two key enzymes important for collagen synthesis. It is also a powerful natural antioxidant all by itself, used in quenching free radicals, chelating Fe (II) and often helping in the regeneration of other natural antioxidants including vitamin E (Guo and DiPietro, 2010). Shah et al (1971) has reported the increase in ascorbate almost to four fold during the differentiation phase of wound healing. The increase in ascorbate levels on the 4th day were more for E1-treated group but by the 8th day, both groups exhibited almost similar levels. Ascorbate, along with being a cofactor in collagen synthesis is a general antioxidant. E1 being a moderately powerful antioxidant could share some of the ROS load along with ascorbate and other ROS scavengers in the test group. The control group, due to lack of an externally added antioxidative agent, would be dependent solely on naturally available antioxidative molecules including ascorbate and would probably use them exhaustively. This would ultimately increase the amount of ascorbate accumulated in the control-treated wound when compared to the E1-treated group, as was found in the study.
7.3.4.3 EFFECT ON FIBROBLAST RECRUITMENT AND WOUND CONTRACTION

Integrins have a large tissue distribution and almost all the cells involved in wound healing, including neutrophils, keratinocytes, endothelial cells and fibroblasts, display one or the other sub-type of integrin. The RGD sequence found in E1 is considered to be a well-documented integrin-recognition motif used by several ECM components, especially, fibronectin (Ruoslahti, 1996). However, a fraction of the total number of motifs remains inaccessible to the receptors in the native triple helical tightly packed collagen (Ruggiero et al, 1996). In degraded collagen, the motifs are ‘exposed’ or it undergoes a conformational change, which allows it to interact with integrin receptors, activating cell adhesion (Eliceiri and Cheresh, 1999). The RGD motif in E1 may have a role in fibroblast recruitment to the wound site, which would have helped to accelerate collagen deposition.

During the proliferative phase, there is an abundance of fibroblasts and endothelial cells in the reparative dermis. Capillary growth, formation of granular tissue and synthesis of collagen are the primary activities characteristic of this stage (Katayama et al, 1991). Adequate synthesis of collagen is required for the final phase of healing and indicates a quicker healing. Collagen mass in normal tissues is dependent on the balanced relation between rate of synthesis and degradation. A rapid increase of collagen production in the test rats is indicative of quicker formation of granulation tissue resulting in faster re-epithelialization.

Although collagen exhibits a triple helical spiral conformation, heat sensitive cross links prevent it from recoiling to a random form. However, upon heating, the intramolecular cross links are rendered ineffective and the collagen chains recoils. The temperature at which this occurs is termed as shrinkage temperature (ST) and the magnitude of this reaction is such that collagen will shrink to one-third of its physiological length. The temperature thus becomes an excellent indicator of the strength of inter and intramolecular bonding. A relatively younger granulation tissue will have less cross linked collagen and would thus display a lower ST, wheras a granulation tissue formed through faster wound healing would display a larger ST owing to the mature collagen formed in the wound bed. Since 70% of skin is collagen and since the granulation tissue also consists of newly deposited collagen, shrinkage temperature of the wound tissue would be representative of the maturity of collagen fibers and the rate of
wound repair. A higher shrinkage temperature, supported by increased collagen deposition in test tissues, indicated mature collagen and faster wound healing.

Fibroblasts produce large quantities of collagen which are ultimately responsible for imparting tensile strength to the scar. Collagen is first detected in the wound around the third day post-injury after which the levels increase rapidly for approximately 3 weeks. The collagen is initially deposited in a seemingly haphazard fashion and these individual collagen fibrils are subsequently reorganized, by cross-linking, into regularly aligned bundles oriented along the lines of stress in the healing wound. Since the results obtained in Chapter 5 reveals that peptides can increase adhesion and proliferation of a fibroblastic cell line, even under substantial stress, it was concluded that the strength of the tissue was due to the larger deposition of collagen, arising from the presence of higher fibroblast count.

Shrinkage temperature and tensile strength of collagen is dependent on the degree of cross linking. A higher value for both parameters demands the presence of cross linked, well arranged collagen fibers, as would be expected if the collagen production was more. This increase can possibly be attributed to the application of E1 as earlier reports suggest that products of collagen degradation may influence collagen synthesis (Gardi et al 1994). Collagen peptides have also been reported to stimulate collagen biosynthesis in both in vitro and in vivo model although the exact mechanism is yet unclear (Katayama et al 1991). Overall, the data confirms that the application of the peptide had a consistent effect on the collagen production which could have ultimately affected the remodeling phase.

Within the wound bed, fibroblasts produce collagen as well as glycosaminoglycans and proteoglycans, which are major components of the ECM. Hexosamine is a primary component essential for building GAGs and proteoglycans and its quantification would determine the amount of ECM being produced during the proliferative and the remodelling stages. GAGs form a hydrated gel-like ground substance in which the collagen molecules are embedded. GAGs are charged and can possibly influence the deposition and orientation of newly synthesized ECM components through ionic interaction (Munakata et al 1999, Bastiaansen-Jenniskens et al 2009). Increased fibroblast recruitment possibly enhanced the rate of GAG and other ECM component synthesis signifying an active remodeling. The increase in production of ECM was also supported by the increased protein content of the wound tissue in test group.
Fig. 7.3 Photographical representation of wound closure in control and test rats for twelve days.
The level of protein synthesis in the wound site is known to be an efficient marker of the rate of healing (Costarelli and Emery, 2009). The increased fibroblast recruitment into the site due to the RGD sequence in E1 could also be one of the reasons for the overall increase in protein and in particular, collagen levels of the wound.

Tracking and documenting the changes in the wound area can act as an accurate marker of the rate of healing and wound contraction. Cutaneous wound healing primarily occurs through two independent processes, contraction and epithelialization.

Wound contraction is the centripetal displacement of the wound edges facilitating gap closure at 5–15 days after the injury. Wound contraction is hallmark of the proliferative phase and is carried out by activated fibroblasts or myofibroblasts that migrate into the damaged tissue in response to cytokines locally released from inflammatory and resident cells. In response to mechanical challenge, activated fibroblasts, along with depositing massive amounts of ECM, acquire contractile stress fibers composed of cytoplasmic actins. This leads to establishment of a large number of cell-cell and cell-ECM networks, generating the force necessary for wound contraction (Hinz et al 2007). A faster wound contraction demands a large population of activated myofibroblasts and consequently, large amount of ECM synthesis and deposition. The significant increase in wound contraction for test rats probably stems from the holistic influences of E1 in recruiting cells, reducing ROS damage and increasing collagen production.

Epithelialization is accompanied by the migration of keratinocytes into the injured tissue. This step is defined as the reconstitution of an organized, stratified epithelial layer that permanently covers the wound, leading to restoration of functionality. The time period for epithelialization for the test groups were found to be almost half of that required by the control groups, indicating a faster wound closure. Upon completion of epithelialization, the reconstructed ECM again takes over the mechanical load and myofibroblasts perish by massive apoptosis, indicating the transition from granulation tissue to a scar tissue (Laplante et al 2001).

To sum up, the peptide E1 could significantly affect the various biochemical and biophysical parameters on multiple levels of the wound healing process. The summary of the action of E1 is shown in Fig. 7.4.
Fig. 7.4 Schematic representation of the action of E1 at different stages of the wound healing process. E1 initiates chemotaxis, lowers ROS stress and recruits fibroblasts leading to faster contraction and healing.
7.3.5 COMPARISON WITH OTHER WOUND HEALING PEPTIDES

A therapeutic agent selected for the treatment of wounds should ideally improve one or more phases of healing without producing deleterious side effects. In this respect, the application of E1 as a wound healing agent displayed significant effects. Collagen peptides have previously been known for chemotactic activity. It has been demonstrated that the peptides PGP, POG and PO can demonstrate chemotactic effect on peripheral blood neutrophils and thus enhance the second phase of wound healing (Shigemura et al 2009). The peptide GHK in complex with Cu$^{2+}$ and biotin has displayed potential to increase all phases of wound healing viz., inflammation, fibroblast proliferation, connective tissue formation and remodeling of wound tissue (Arul et al 2007; Pickart, 2008). The pentapeptide KTTKS, a subfragment of type I collagen has been found to be the minimum sequence necessary to stimulate collagen synthesis from fibroblasts (Katayama et al 1993). Even chemically modified peptide Ac-PHSRN-NH$_2$ form plasma fibronectin, has been known to stimulate reepithelialization and wound closure in obese diabetic mice (Livant et al 2000). Although with diverse sequence of residues, most of the peptides, including E1, displayed a higher % occurrence of hydrophilic residues when compared to collagen.

The efficient wound repairing peptide E1 may just be one among many cryptic peptides that are ‘released’ during ECM degradation caused by migrating cells leading to modulation of physiological processes, including endothelial morphogenesis and angiogenesis (Rhodes and Simons, 2007). Even applying collagenases derived from Clostridium histolyticum have proven beneficial for wound healing because they degrade the collagen matrix giving rise to bioactive fragments that stimulate wound closure (Demidova - Rice et al 2011).

To summarize, a significant decrease in lipid peroxides, especially on the 8$^{th}$ day hinted at the in vivo antioxidant capacity of E1. A significant increase in general protein synthesis along with amino sugars and collagen synthesis was observed in the E1-treated group. Wound contraction and reepithelialization was found to be faster for test group and the collagen produced displayed higher tensile strength, indicating faster wound healing. The results substantiated the ability of E1 to act as an in vitro and in vivo multifunctional bioactive peptide. It also opens up the possibility that cryptic peptides from collagen plays significant roles in regulating physiological processes and could possibly be used safely as a pharmaceutical agent.