CHAPTER-3

Materials and Methods
3.1 Materials

3.1.1 Chemicals and Reagents

3.1.1.1 General Chemicals

**Merck, India**
- Ammonium Chloride
- Calcium Chloride
- Hydrogen chloride
- Hydrogen peroxide
- Potassium Chloride
- pH paper strips
- Sodium Acetate
- Sulphuric acid
- Sodium Carbonate
- Sodium Chloride
- Sodium Hydroxide
- Sodium Nitrite

**SD Fine Chemicals Ltd, Boisar**
- Napthyethenediamine dichloride (NEED)
- Ortho-Phosphoric acid
- Sulphanilamide
- Thiobarbituric acid,
- Tri-chloroaceticacid

**Sigma Aldrich (St Louis, USA)**
- 1,1,3,3-Tetramethoxypropane
- Bovine serum Albumin
- Ethyl alcohol
- Glutathione reductase (from Baker’s yeast)
- Glycerol
- Haematoxylin and eosin stain
- Mounting media - DPX
- Para-dimethylaminobenzaldehyde
- Standard Gluthathione
Materials and methods

Standard L-Hydroxyproline
Standard D (+) – Glucosamine hydrochloride
Standard Catalase
Van Gieson stain

Blue Star – Mumbai, India
Glass slides, (size 75mm × 25mm)
Glass coverslip, (size 22 × 60mm)

3.1.1.2 Commercial Kits

SEA100Hu ELISA Kit for Matrix Metalloproteinase 2 (MMP-2) (Usen Life Science Inc. USA) [Appendix –A].

3.1.1.3 Instruments

Cold centrifuge- REMI CM 12 plus (REMI Pvt. Ltd, India)
Elisa reader - LISA plus (Rapid Diagnostic Pvt. Ltd, India)
Embedding machine (Leica EG1150 H) (Leica Microsystems, India)
GENESYS 10S UV-Vis spectrophotometer (Thermo Scientific, USA)
Hot Air Oven (Bottom Heater)-Bio Technics, (India)
Incubator- (Asian exporters-India)
Microtome (Leica RM2255) (Leica Microsystems, India)
Microscope - Olympus (CX21i Microscope), (India)
Microphotography-Olympus (PM20 photomicroscope), (India)
pH meter- EUTECH pH 700 (EUTECH instruments, India)
Remi tissue homogeniser-RQ 127A/D (REMI Pvt. Ltd, India)
Weighing machine (Sartorius, Germany)

3.2. Methods

3.2.1 Patient Selection and Recruitment Criteria

The study is prospective randomised clinical trial which registered to Clinical Trials Registry-India, NATIONAL INSTITUTE OF MEDICAL STATISTICS (ICMR, Government of India) - CTRI number: CTRI/2015/01/005419 [Appendix-B].
The main body of this thesis consists of a prospective, randomised study on chronic wounds of duration more than 4 weeks, conducted in the Department of Plastic Surgery and Burns,
Kasturba Hospital and also patients were recruited from Department of General Surgery, District Hospital, Udupi (Government of Karnataka), duration of the study was from January 2012 to December 2014. The primary objective of the study was to evaluate the effect of LAD on chronic wound by assessing selective histological and biochemical parameters, and compare with that of conventional dressing. Consenting patients who met the inclusion/exclusion criteria outlined below participated in the study. Following recruitment and collection of baseline data, participants were randomly allocated to either LAD or conventional dressing group.

3.2.1.1 Patient Selection Criteria

**Inclusion criteria**
- Patients with non-healing chronic wounds of more than 4 weeks
- Age of 12-65 years

**Exclusion criteria**
- Age less than 12 years and more than 65 years
- Diabetic patients
- Patients with collagen disorders
- Leprosy patients
- Pregnant women
- Patients with liver cirrhosis
- HIV +ve patients

3.2.1.2 Ethics

The study protocol, patient information sheet and patient consent form were approved by an Institutional Ethics Committee (IEC) Kasturba Hospital [Appendix C] and a Government District Hospital, Udupi [Appendix D].

3.2.1.3 Randomisation

Simple randomization, patients were allocated by generating tables of random numbers through www.random.org, intervention model was parallel assignment and masking was open label.
3.2.1.4 Patient Population

Two hundred and fifteen patients ailing from chronic wounds were enrolled in to the study. After examined inclusion and exclusion criteria and give written informed consent [Appendix-E], 140 patients were randomized and allocated to the LAD group (n=70) and conventional dressing group (n=70) as shown in Consolidated Standards of Reporting Trials (Consort) flow chart [Figure-3.1]. Out of 140 patients, 56 participants (30 in the LAD group and 26 in the conventional dressing group), were lost to follow up from the study. In remaining 84 patients, LAD group has 40 patients where 20 (50%) were women and 20 (50%) men and in conventional dressing group, out of 44 patients, 20 (45.4%) were women and 24 (54.6%) were men.

Figure 3.1: Consolidated Standards of Reporting Trials (Consort) flow chart
In LAD group mean patient age was 38.3 (±10.56) years, range (12 to 60 years) and mean wound size at the time of admittance was 19 cm² (range 9-40 cm²) and in conventional dressing group mean age is 35.3 (±14.0) years, range (17 to 65 years) and mean wound size at the time of admittance was 18 cm² (range 10-39 cm²).

3.2.1.5 Screening Protocol

All eligible patients were screened for inclusion and exclusion criteria, and signed the informed consent form approved by IEC at the start of the study. The patients wound history was recorded; area of the wound was measured using a transparent ulcer grid measurement sheet, so that its area could be determined.

3.2.2 Treatment of Patients

Patients were randomly allocated to one of the two treatment regimens

3.2.2.1 LAD group

Patients were treated with Limited access dressing (intermittent negative pressure and moist environment) [Figure-3.2].

Figure 3.2 Application of LAD to the patient
3.2.2 Conventional Dressing group

Conventional closed dressing group -Patients were dressed daily with 5% povidone iodine solution soaked gauze.
Wounds were washed daily both LAD and conventional group prior to dressing by povidone iodine.

3.2.3 Biopsy Procedure

Biopsies were obtained at day 0 (before dressing) and day 10 from the wound using a sterile surgical blade No.22 (Lister). The wound was infiltrated with 1% plain Lignocaine before taking biopsy. The tissue was then scooped out of the wound bed by shave biopsy. Haemostasis was achieved by gentle pressure with an absorbent dressing. The first biopsy was taken on day 0 and second biopsy on day 10, as it is point for the second biopsy because wound demonstrated that the maximal cellular activity, in response to wound manipulation, occurs at this time.

3.2.3.1 Storage of the Granulation Tissue

Biopsies were rinsed in ice-old PBS (0.1 mol/L, pH 7.0 -7.2) to remove excess blood and bisected longitudinally by placing a scalpel over the mid-point of the tissue and pressed firmly with one smooth movement. One half of the biopsy was placed in 10% formalin for histological study and other half was snap frozen in liquid nitrogen at –80°C in labeled cryovials, for estimation biochemical parameters.

3.2.4 Tissue Preparation for Histological Study

Wound biopsies on days 0 and 10 were collected, and preserved in labeled plastic container in 10% formalin for at least 24 h at room temperature for fixation.

3.2.4.1 Preparation for Histological study

After fixation, perpendicular sections to the anterior-posterior axis of the wound were dehydrated through graded alcohol series [50% (24 h), 70% (24 h), 90% (12 h) and 100% (12 h)], and cleared in xylene. The specimens were then carefully embedded (Leica EG1150 H) in paraffin wax (m.p. 56 °C) and orientated so that sections could be cut vertically through the thickness of the wound biopsy. After solidification, 5-µm sections were cut using microtome (Leica RM2255) and mounted onto poly-L-lysine coated slides.
3.2.4.2 Staining [255]

The effects of treatment were monitored histologically by H&E and Vangieson staining.

a. Hematoxylin and eosin staining (H&E staining)

Sections were deparaffinized in xylene and rehydrated in decreasing concentrations of alcohol (100%, 90%, 70% and 50%) to water and placed in haematoxylin solution for 5 minutes. The Sections were then washed under running water till all the excess stains were washed away clearly (Blue). Immers the sections in the eosin solution for 1-2 minutes and dipped in 1% acid alcohol. Dehydrated the sections in increasing concentrations of alcohol (50%, 70%, 90%, 100%) and cleared with xylene. After drying the slides were mounted and coverslipped in DPX. All the slides were viewed in Olympus microscope at 20X and 40X magnifications.

b. Vangieson staining (VG staining)

Sections were deparaffinized in xylene and rehydrated in decreasing concentrations of alcohol (100%, 90%, 70% and 50%) to water. Placed the sections in the working Iron Hematoxylin, (By mixing equal parts of solution A & Solution B) for 10 to 20 minutes. Sections should be over stained, as they will slightly decolorise by the picric acid, wash slides in running tap water for 2 minutes. This step will be staining the nuclear details (Nuclei) in black and dip slides in 1% acid alcohol in order to remove any excess Iron Hematoxylin. Wash slides in running water for 10 minutes for bluing. Placed sections in Van Gieson solution for 10 minutes to stain collagen and immersed in 95% alcohol to remove the excess nonspecific staining. Dehydrated with 95%, 100% alcohol, and cleared in xylene and coverslip with a DPX mounting media. All the slides were viewed by means of an eyepiece grid under the Olympus microscope from 20X to 40X magnifications.

3.2.4.4 Histological Scoring of Slides

Each slide was given a histologic score ranging from 1 to 12, with 1 corresponding to no healing and 12 corresponding to a completely reepithelialized wound (Table 3.1), according to the method described by Greenhalgh DG 1990 [256]. The scoring was based on the degree of cellular invasion, granulation tissue formation, vascularity, and reepithelialization. The histologic score was assigned separately by two pathologists. The code describing treatment to the patient’s was broken after the scoring was completed by pathologists.
Table 3.1 Scale for histologic study [adopted from Greenhalgh et al. (256)]

<table>
<thead>
<tr>
<th>Score</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-3</td>
<td>None to minimal cell accumulation, no granulation tissue or epithelial travel.</td>
</tr>
<tr>
<td>4-6</td>
<td>Thin, immature granulation that is dominated by inflammatory cells but has few fibroblasts, capillaries, or collagen deposition, minimal epithelial migration.</td>
</tr>
<tr>
<td>7-9</td>
<td>Moderately thick granulation tissue, can range from being dominated by inflammatory cells to more fibroblasts and collagen deposition, extensive neovascularization, epithelium can range from minimal to moderate migration.</td>
</tr>
<tr>
<td>10-12</td>
<td>Thick, vascular granulation tissue dominated by fibroblasts and extensive collagen deposition, epithelium partially to completely covering the wound.</td>
</tr>
</tbody>
</table>

3.2.5 Tissue Preparation for Biochemical Study

3.2.5.1 Tissue Preparation for Hydroxyproline and hexosamine estimation

The granulation tissues were dried at 60°C for 24 h. It was weighed and kept in glass stoppered test tubes. 6N HCL was added in each tube so that it contained 40 mg of the dried granulation tissue per ml of acid. The tubes were kept in 100°C 24 h for hydrolysis in incubator. The hydrolysate was then cooled and excess of acid was neutralized by 10N NaOH using phenolphthalein/ Methyl red as an indicator. The volume of neutral hydrolysate was diluted to a concentration of 20 mg/ mL of dried granulation tissue in the final hydrolysate with distilled water. The hydrolysate was used for the estimation of hydroxyproline and hexosamine.

3.2.5.2 Tissue Preparation for estimation of Antioxidants (GSH, Thiol, GPx, GST, CAT), Lipid peroxidation biomarker (MDA), MMP-2, Nitric oxide.

Tissue samples were rinsed in ice-old PBS (0.1mol/L, pH 7.0-7.2) to remove excess blood thoroughly, weighed and minced in to small pieces. The tissues were homogenized by Remi tissue homogenizer (RQ 127A/D) in ice-cold 0.2 M phosphate buffer (pH 7.4). Homogenates were centrifuged at 12,400g for 30 min in cooling centrifuge (REMI CM 12 plus) and
supernatant was then used for determine total protein, antioxidants (GSH, GPx, GST, CAT, Thiol), lipid peroxidation biomarker (MDA).

For MMP-2 assay, samples were homogenized in PBS. The resulting suspension was sonicated with an ultrasonic cell disrupter or subjected to two freeze-thaw cycles to further break the cell membranes. After that, the homogenates were centrifuged for 5 minutes at 3000g, supernatant were removed and aliquot store at -80°C.

For Nitric oxide (NO) assay tissue was homogenized in isotonic solution of PBS containing 10mM N-ethylmaleimide (NEM) and 2.5 mM EDTA. The addition of NEM/EDTA serves the purpose of blocking SH-groups and inhibiting transition metal-catalyzed transnitrosation reactions, preventing artificial nitrosation, as well as thiolate- and ascorbate-mediated degradation of endogenous RSNOs and nitrite [257].

3.2.6 Estimation of Biochemical Parameters

3.2.6.1 Estimation of Hydroxyproline (OHP) [258]

1. 0.1 mL of hydrolysate samples were pipetted into clean tubes, volume made up to 0.5 mL with distilled water.

2. From the stock solution of standard hydroxyproline 1.6 mL was taken and diluted up to 100 mL with distilled water. From this 0.5 ml (8 µg) was pipetted into a clean test tube.

3. To this mL each of 2.5N NaOH, 0.01M CuSO₄ and 6% H₂O₂ were added. Immediately test tubes were placed in a water bath at 80°C for 16 minutes and then cooled for 5 minutes.

4. To this 2 mL of freshly prepared 5% solution of para-dimethyl amino benzaldehyde in n-propanol and 4 mL of 3N H₂SO₄ were added. Test tubes were once again placed in hot water at 80 °C for 15 minutes and then cooled for 5 minutes.

5. The optical density (O.D.) of the pink colour of these samples was compared to that of standard hydroxyproline of known concentration samples at 540 nm using GENESYS 10S UV-Vis spectrophotometer for the estimation of hydroxyproline and concentration was expressed in µg/mg of dry weight of the tissue.
3.2.6.2 Estimation of Hexosamine [259]

0.5 mL of the hydrolysate fraction was taken. To this 0.5 mL of acetyl-acetone reagent was added. The mixture was heated in boiling water bath at 60°C for 20 minutes, and then cooled under running tap water. 1.5 mL of 90% alcohol was added and allowed for 30 minutes. The colour intensity was measured in GENESYS 10S UV-Vis spectrophotometer at 540 nm against blank prepared by using distilled water instead of hydrolysate. Hexosamine content was determined from the standard curve prepared by using D (+) – glucosamine hydrochloride and concentration was expressed in µg/mg of dry weight of the tissue.

3.2.6.3 Estimation of Total protein [260]

Total protein content in the tissue homogenate was estimated by the method of Lowry et al. 0.5 mL of hydrolysate was pipette out in to the test tube, make up to 2 mL with distilled water. This is followed by addition of freshly prepared alkaline copper tartarate solution 4 ml. Keep it for 10 minutes at room temperature. Add 0.5 mL of Folin’s reagent to each test tube, mixing vigorously while adding. Blank is prepared simultaneously and allowed to stand for 10 minutes to develop color. Readings are taken only after 30 minutes [because color would not be developed fully before 30 minutes]. The absorbance was measured at 540 nm in GENESYS 10S UV-Vis spectrophotometer and expressed in mg/g of tissue. Standards were treated similarly using Bovine serum albumin [BSA] at concentrations of 0, 20, 40, 60, 80, and 100 µg/ mL in 0.1M phosphate buffer at pH 7.4.

3.2.6.4 Estimation of Reduced Glutathione (GSH) [261]

50 µL tissue supernatant was added to the 1.8 mL of the Ellman's reagent (0.1 mM of 5, 5'-dithiobis -2-nitrobenzoic acid) prepared in 0.3 M phosphate buffer with 1 % of sodium citrate solution) was added. After completion of the total reaction absorbance of the solution was measured at 412 nm against blank in the spectrophotometer. Absorbance values were compared with a standard curve generated from known concentration of GSH and expressed as µMole/mg of tissue protein.

3.2.6.5 Estimation of Thiol [262]

Aliquots of 0.5 mL of the homogenate were mixed with 1.5 mL of 0.2M PBS buffer (pH 8.2) and 0.1 mL of 0.1 mM of DTNB. The mixture was brought to 0.5 ml with absolute methanol. The color was allowed to develop for 15 min. Absorbance of the clear supernatant was read at 412 nm and expressed as µMole/mg of tissue protein.
3.2.6.6 Estimation of Glutathione peroxidase (GPx) [263]

GPx activity was determined by using cumene hydrogen peroxide as substrate. The standard assay mixture contained in 1.0 mL final volume: 50 mmol/L tris buffer, pH 7.4; 0.14 mmol/L β nictoinamide adenine dinulciated phosphate (reduced form, NADPH); 1.0 mmol/L glutathione; 1.0 kU/L glutathione reductase; 0.5 mmol/L cumene hydrogen peroxide; and a rate limiting amount of glutathione peroxide (tissue homogenate supernatant fraction). The reaction mixture read at 340 nm for 5 minutes at an interval of 60 seconds. Glutathione peroxidase activity is expressed as the amount of enzyme required to oxidize 1.0 µmole/min of NADPH oxidised/mg of tissue protein.

3.2.6.7 Estimation of Glutathione S-transferase (GST) [264]

Glutathione S-transferase activity was determined according to the procedure described by Habig et al. The reaction mixture consists of 0.1 M potassium phosphate (pH 6.5), 1.6 mM GSH and 1 mM CDNB. The reaction was monitored spectrophotometrically at 340 nm for 5 minutes at an interval of 60 seconds. The specific activity of GST was expressed as µmol GSH-CDNB conjugate formed/min/mg of tissue protein.

3.2.6.8 Estimation of Catalase (CAT) [265]

Catalase activity was measured by the method of Aebi. The reaction mixture contained 1ml of tissue sample homogenate and reaction was started by the addition of 1.0 mL of freshly prepared 30 mM H₂O₂. The rate of decomposition of H₂O₂ was measured spectrophotometrically from changes in absorbance at 240 nm. Activity of catalase was expressed as IU/sec/mg of tissue protein.

3.2.6.9 Estimation of Malondialdehyde [266]

Malondialdehyde (MDA) a measure of lipid peroxidation was measured spectrophotometrically in homogenate, according to the method of Ohkawa. Where MDA determined by using 1 mL of Trichloroacetic acid [TCA] 10% and 1 mL of thiobarbituric acid [TBA] 0.67% and were then heated in a boiling water bath at 100°C for 30 min. Mixture was cooled under tap water and centrifugation at 12.200g for 10 min, Thiobarbituric acid reactive substances [TBARS] were determined by the absorbance at 535 nm and expressed as nMole/mg of protein.
3.2.6.10 Estimation of Matrix metalloproteinase-2 (MMP-2) [267]

The assay was carried out on the human granulation tissue samples. A total of 100 mg of tissue was homogenized in 1mL ice-cold lysis buffer. Subsequently, homogenates were centrifuged at 3000g for 5 minutes at 4°C, and supernatants were stored at −80°C until use. Matrix metalloproteinase 2 (MMP-2) were measured using prefabricated ELISA kits, according to manufacturer protocol (R&D Systems, Uscn Life Science Inc. USA). Plates were read at 450 and 540 nm in ELISA reader and concentrations were calculated using a 4-point standard curve and expressed as ng/mg of protein.

3.2.6.11 Estimation of Nitric oxide [268]

Nitric oxide (NO) biosynthesis was measured by determining nitrite levels in the wound tissues. Wound tissues were homogenized in hypotonic saline and centrifuged. Nitrite concentrations were determined with Griess reagent by the method of Green et al. Briefly, the supernatant was mixed with freshly prepared Griess reagent (0.1% NEED, 1% sulphanilamide and 5% phosphoric acid in a 1:1:1 ratio), incubated at 37°C for 30 mins and absorbance was measured at 543 nm using UV-visible spectrophotometer. Sodium nitrite was used as standard. Nitrite levels are expressed in terms of µMole/ mg tissue.

3.2.6.12 Measurement of Wound surface pH [269]

The surface pH was measured by means of a pH paper (strip) placed on the surface of the wound.

3.3 Statistical Analysis

Statistical analysis for histological study between the groups was performed Mann-Whitney U test and data were expressed as median (Q1, Q3) [median and interquartile range (IQR)] and biochemical parameters were compared by applying Student's t-test, data were expressed as Mean ± Standard Deviation (SD) using the SPSS software 15th version package. P value < 0.05 was considered as significant. When appropriate, statistical uncertainty was expressed by the 95% confidence level.