CHAPTER -5

Discussion
Wound healing is an interaction of various complex cascades of cellular and biochemical actions leading to the restoration of structural and functional integrity with regain of strength of injured tissues. It involves continuous cell-cell interaction and cell matrix interactions that allow the process to proceed in different overlapping phases such as inflammation, wound contraction, reepithelialization, remodeling & formation of granulation tissue with angiogenesis (270).

Chronic wounds are characterised by impaired blood flow and oxygen supply, decreased collagen and fibronectin synthesis (due to protein malnutrition), impaired local immune cell defenses and decreased anabolic activity with decreased levels of growth factors (271). In normal process of wound healing in response to tissue loss, fibroblasts proliferate and migrate into the extracellular matrix. Enhanced healing activity has been attributed to increased collagen deposition by fibroblasts and increased angiogenesis in granulation tissue (272).

NPWT is used in the treatment of acute and chronic wounds and reported to be associated with improved rates of healing (273, 151) Various studies have shown that continuous NPT improves wound healing by inducing fibroblast, endothelial cell proliferation, and by increasing the amount of granulation tissue formation (274) and appears to be particularly useful in wounds having copious production of exudate (275). The evidence supporting the use of negative pressure therapy in the treatment of chronic wounds are very few, as per available data, a total of 418 peer-reviewed articles, 484 abstracts, 61 book references, 18 randomized clinical trials (RCTs) and 43 reviews on TNP therapy were published (276).

However there are only few studies proved the efficacy of negative pressure therapy on chronic wound healing using histological and biochemical parameters. Limited access dressing (with definite intermittent negative pressure regimen) have shown to be effective in treatment of acute and chronic wounds which was proved clinically (151) but there was a lack of histological and biochemical evidences.

5.1 Histological Study

Numerous studies have shown that wound healing can determined by both semi-quantitative (wound re-epithelisation; presence of: inflammatory cells, fibroblasts, new vessels, and collagen) and quantitative methods (polymorphonuclear leucocytes/tissue macrophages ratio, percentage of re-epithelisation, area of the granulation tissue) (277).

Franco Bassetto et al (2012) found from histological study, that wounds treated with NPT developed healthy granulation tissue, with high proliferation index, increased in blood vessels density and decreased chronic inflammation, oedema and underwent progression eventually towards stable tissue (278).
In our study the histological score of wounds were almost similar on day 0 both in LAD [Figures- 4.4.1, 4.4.3, 4.4.5 (H&E), and 4.6.1, 4.6.3, 4.6.5 (VG)] and conventional dressing group [Figures- 4.5.1, 4.5.3 (H&E) and 4.7.1, 4.7.3 (VG)], median (Q₁, Q₃) = 5 (3, 7) vs 4 (3, 6). Wound healing was markedly improved in LAD group [Figures- 4.4.2, 4.4.4, 4.4.6, 4.4.7 (H&E) and 4.6.2, 4.6.4, 4.6.6 (VG)], median (Q₁, Q₃) = 9 (7, 9) after 10 days of treatment than compare to conventional dressing group 6 (5, 7.25) [Figures- 4.5.2, 4.5.4 (H&E) and 4.7.2, 4.7.4 (VG)]. The median (Q₁, Q₃) on day 10 - day 0 (LAD versus conventional dressing group = 3 (2, 4.25) versus 2 (1.75, 4); P =0.008) [Table-4.3]. Consistent with these findings the histologic score of wounds from the LAD group was significantly higher with decreased cellular filtration and increased fibroblasts, collagen deposition, increased the number of capillary vessels than compare to conventional dressing group.

5.2 Biochemical Study

5.2.1 Hydroxyproline

Collagen is a fibrous protein component of the connective tissue and provides a structural framework to the tissue. Hydroxyproline content has been used as an indicator to determine collagen biosynthesis. It is one of the biomarkers indicating wound healing process. (279). Hydroxyproline can be measured in wound granulation tissue at day 10 after wounding as it represent the points of peak collagen deposition and remodeling (181).

It has been shown from numerous studies that application of NPWT improves wound healing by inducing fibroblast thereby collagen formation and endothelial cell proliferation, with increase in the rate and degree of granulation tissue formation that stimulate wound repair (280).

In our study the mean (±SD) of hydroxyproline content (µg/mg dry weight of tissue) in LAD group was (78.2±22.21) significantly higher than conventional dressing group (29±14.85) (P= 0.001) [Table-4.4].

The present study confirms that LAD exerts beneficial effect on chronic wounds by increasing collagen synthesis.

5.2.2 Hexosamine

Hexosamine is an important part of the extracellular matrix and a main glycosaminoglycan secreted during tissue repair. Increased hexosamine content reflects the stabilisation of collagen molecules by enhancing electrostatic and ionic interactions with it, which in turn
reflects remodeling of the new extracellular matrix produced hence, enhanced hydroxyproline and hexosamine synthesis provides strength to repaired tissue and stimulates healing (281). In the present study, the mean (±SD) of hexosamine content (dry weight of tissue) in LAD (9.6±2.71) group is higher than conventional dressing group (8.0±1.43); P= 0.047 [Table-4.5]. The present study confirmed that LAD exerts beneficial effect on chronic wounds by increasing hexosamine synthesis which reflects formation of extracellular matrix.

### 5.2.3 Total Protein

Protein is another important constituent of extracellular matrix. High protein content confirms positive effects towards cellular growth, proliferation, granulation tissue formation and epithelisation (281). In the present study, mean (±SD) total protein (mg/g wet tissue weight) in LAD versus conventional dressing (13.8±7.04 versus 9.61±4.24; P= 0.013) [Table-4.6], which is significantly higher in LAD group than the conventional dressing group. The present study confirms that LAD prevents breakdown of protein in ground substance of the ECM from various proteases.

### 5.2.4 Antioxidants

Antioxidants have been clearly known to accelerate wound healing. Several studies have shown that antioxidants level increases as wound proceeds to healing state. Study by Martin. A (1996) proved that antioxidants enhance the healing of infected wounds by reducing the damage caused by oxygen radicals (282). Glutathione (GSH) is a major non protein thiol antioxidant compound present in living organisms, which serves a significant role in antioxidant defense mechanism. GSH and thiol (SH) acts as an effective antioxidant, protecting the cellular components from oxidative damage caused by ROS. Depletion of glutathione may play an important role in delayed healing (283). Prolonged overproduction of reactive oxygen species cause severe tissue damage which leads to impaired wound healing and may even lead to damage to DNA within cells involved in the normal healing process. Enzymatic antioxidants play key role in healing of delayed wounds. Catalase converts excessive H₂O₂ in wound tissues in to H₂O and O₂. GPx and GST are ROS-inducible enzyme essential factor in defense against oxidative tissue damage and extremely important in the cytoprotection during cutaneous wound healing (284). However there are no NPWT studies available in the literature which studied on antioxidants. In the present study, LAD versus conventional dressing group mean (±SD) GSH (7.9±2.0 versus 6.7±2.5 µMole/mg tissue protein; P= 0.035), thiol (27.2±7.75 versus 22.74±7.16
µMole/mg tissue protein; \( P = 0.285 \), GPx (145.3 ± 74.7 versus 115.9±37.4 µMoles NADPH oxidised/min/mg of tissue protein; \( p=0.003 \), GST (26.5±11.9 versus 17.4±7.5 µMoles CDNB conjugate formed/ min/mg of tissue protein; \( P= 0.023 \), CAT (1.7±1.0 versus 1.16±0.80 IU/sec/mg of tissue protein; \( P= 0.068 \) [Table-4.7].

The present study confirms study that LAD prevents breakdown of antioxidants by free radicals and from various proteases.

5.2.5 Malondialdehyde [MDA]

ROS is the natural byproduct of the normal oxygen metabolism and plays a role in cell signaling and homeostasis. The mechanism of free radical generation and their disposal procedures are changed in delayed wound healing, increased oxidative stress, together with elevated levels of ROS, could result in damage and strand breakage of cellular DNA. In the study by James (2003), states that reactive oxygen species have been implicated in the impaired healing of human chronic wounds and observed a significant (\( P < 0.01 \)) elevation of the allantoin: uric acid percentage ratio, a marker of oxidative stress, in wound fluid from chronic ulcers compared acute surgical wound fluid (285). There is a lack of evidence related to the effect of NPWT on oxidative stress.

The present study reveals that oxidative stress biomarker MDA significantly decreased after 10 days of treatment in LAD group (mean MDA (±SD) =13.6±6.62 nMole/mg of protein) than that of conventional dressing group (9.80±3.35 nmole/mg of protein); \( P= 0.006 \) [Table-4.8]. The present study confirmed that positive effects of LAD on to wound bed by reducing damage by reactive oxygen species.

5.2.6 MMP-2 [Gelatinase A]

Yager (1996), in his study, examined the wound fluid from acute surgical wounds and nonhealing pressure ulcers for the presence of several matrix metalloproteinases. He confirmed the presence of two major gelatinases with molecular masses of 72 kDa (matrix metalloproteinase-2) and 92 kDa (matrix metalloproteinase-9) by the method of gelatin zymography. Levels of matrix metalloproteinase-2 and matrix metalloproteinase-9 were elevated more than 10-fold and 25-fold, respectively, in fluids from pressure ulcers compared with fluids from healing wounds. Author observed that, presence of excessive levels of activated forms of matrix degrading enzymes at the wound surface of pressure ulcers may impede the healing of these wounds (286). Another study conducted by Wysocki (1993) to determine if metalloproteinase levels were elevated in human chronic wound fluid and observed that markedly increased levels of MMP-2 and MMP-9. Results suggest that non-
healing ulcers develop an environment containing high levels of activated metalloproteinases, which may result in chronic tissue turnover and failed wound closure. Very few studies have shown that reductions in MMP-9/ MMP-2 expression in the wound bed under NPWT (287). Consistency with the above studies and results, in the present study after 10 days of treatment, chronic wounds treated with LAD group (0.59±0.25 ng/mg of protein) shown significant reduction in MMP-2 activity than compare to conventional group (0.51±0.39 ng/mg of protein); \( P= 0.046 \) [Table-4.9].

The result of the present study confirm that LAD prevents the detrimental effect of MMP’s to the ECM of the wound bed and facilitates the formation of healthy granulation tissue.

5.2.7 Nitric Oxide

In immunocompromised states wound healing can be interrupted as a result of impairing the production of effector molecules such as nitric oxide (NO). Several studies have revealed that nitric oxide influence wound healing by reducing inflammation, increase collagen deposition, blood vessel formation and keratinocyte proliferation (288). Chronic wounds treated with NPWT have shown to increase NO thereby blood flow to wounds. Study by Sano (2013) investigated the role of nitric oxide (NO) in the mechanism of blood flow increase in the wound bed during negative pressure wound therapy (NPWT) and found that wounds treated with NPWT has significant increase of blood flow was observed. These findings suggest that NO synthesis is involved in the wound bed microcirculatory change induced by NPWT (289).

Consistency with the above results, in the present study NO is increased significantly in LAD group (1.13±0.52) than conventional dressing group (0.60±0.36) after 10 days of treatment \( P= 0.019 \) [Table-4.10] thereby confirming the clinician’s observation by increasing the vascularity in the wound bed.

5.2.8 Wound Surface pH

The pH measurement is defined as the negative logarithm of the activity of hydrogen ions in an aqueous solution, used to express the acidity and alkalinity on a scale of 0 to 14. Wound surface pH is neglected parameter still now. But very few studies have investigated the relationships between wound pH and healing of chronic wounds. Study by Sayegh [1988] (253) confirmed that skin graft were successful on chronic wound if the pH value is above 7.4. Recently, Shukla et.al [2007], have conducted study on pH in acute wounds and diabetic foot ulcers. They measured wound fluid pH using litmus paper and observed improvement in wound status with associated reductions in wound pH and
concludes that with decrease in wound pH, wounds progress from ‘unhealthy’ status towards a ‘granulating’ or ‘healing’ status (269).

Greener et al (250) in an in-vitro study suggested that MMPs at the wound bed may be sensitive to changes in pH and concludes that manipulation of wound fluid to lower pH values might improve healing rates in non-healing wounds.

The present study reveals that wound surface pH of LAD versus conventional dressing group on day 0 = 8.4±0.35 versus 8.30±0.38 and on day 10 =7.5±0.43 versus 7.9 ± 0.47. After 10 days of treatment wound surface pH significantly reduced to (0.9± 0.52) in LAD group compared with conventional dressing group (0.40±0.26); P = 0.048 [Table-4.11].

It is evident from the present study that LAD plays important role in modulating the wound pH and provide optimal pH to facilitate healing.

5.3 Correlation

5.3.1 Hydroxyproline and MMP-2

Correlation study on day 10 in LAD group between hydroxyproline and MMP-2 shown significant negative correlation (Pearson correlation coefficient r= -0.329; P= 0.033). [Figure-4.15B]. Conventional dressing group on day 10 revealed no significant correlation (Pearson correlation coefficient r = 0.88; P= 0.579) [Figure-4.16B]. From correlation study it is evident that in chronic wounds, LAD reduces the catabolic activity of MMP-2 leading to increased deposition of hydroxyproline.

5.3.2 Reduced glutathione (GSH) and Malondialdehyde (MDA)

Correlation study on day 10 in LAD group between GSH and MDA revealed a negative correlation r = -0.279; P= 0.074) but not statistically significant [Figure-4.17B]. In conventional dressing group on day 10 revealed no significant correlation (Pearson correlation coefficient r =0.125; P= 0.429) [Figure-4.18B]. From correlation it is evident that in LAD group after 10 days of treatment oxidative stress is markedly reduced.

5.3.3 Wound surface pH and MMP-2

Correlation study on day 10 in LAD group between wound surface pH and MMP-2 revealed a significant positive correlation r = 0.344; P= 0.026) [Figure 4.19B]. In conventional dressing group on day 10 revealed no significant correlation (Pearson correlation coefficient r= -0.110; P= 0.486) [Figure-4.20B]. From correlation it is evident that proteases (MMP-2) level in chronic wounds may be regulated by wound surface pH.