Determination of potential diagnostic markers of wound healing

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Background

Ulceration of the lower limb represents the most advanced stage of chronic venous insufficiency [1, 2]. The point prevalence of chronic ulceration of the leg in an urban Australian community was 1.1 per 1000 population in 1993 [3]. Ulcers were a chronic problem with 24% having been present for more than 1 year. There was also an increasing prevalence with age; 90% of patients with venous ulcers were 60 years or older [3, 4]. Venous disease is the commonest cause of leg ulcers and is present in 50% to 90% of cases [5, 6]. The costs associated with the long-term care of these chronic wounds are substantial [6, 7]. Overall chronic venous disease has been estimated to account for 1 to 3% of the total health care budgets in countries with developed health care systems [3, 6, 8].

Venous disease has a significant impact on quality of life and work productivity [9-11]. Venous ulcers usually remain unhealed for extended periods [12]. In one Scottish study, 45% of patients reported episodes of ulceration lasting for more than 10 years [13]. Venous ulcers are variable in size, ranging from small to the full circumference of the leg [8, 12]. Leg ulcer recurrence is recognized as a significant issue, with up to one third of patients develop four or more episodes [14] and the overall recurrence rates have been reported to be around 45-70% [13, 15]. Pain and poor quality of life associated with VLU remain a current problem [10, 11].

Clinical management of chronic wounds can be complex. There are a vast array of treatments that can influence wound healing, including an extensive range of wound dressings and devices, topical and systemic medications, various surgical procedures and adjunctive therapies such as hyperbaric therapy and electrical stimulation [16, 17]. Currently the gold standard for identifying if a wound is in a healing phase or non-healing phase, is to measure the ulcer size and/or surface area and to compare that with the measurement in a subsequent week. Repeated measurements over time are therefore needed before the healing status of the wound can be determined.

There is a need for a reliable biomarker which can determine the phase of wound healing at the time at which the wound is assessed. Potential biomarkers for wound healing include assessments on wound fluid, blood, urine and physiological assessments of the wound and surrounding tissues[18]. In this study six biomarkers will be evaluated (1) pH of wound fluid, (2) oxidative status of the wound fluid, (3) temperature of the wound bed, (4) calcium levels in the wound fluid, (5) lysolecithin levels in the wound fluid, and (6) dermal oedema.

The wound bed pH of chronic venous leg ulcers and pressure ulcers was found to be alkaline or neutral when compared to intact surrounding skin; it was also found to be an acidic state during epithelialisation [19]. Chemical acidification of the wound bed has been shown to increase the healing rate in chronic venous leg ulcers [19].

Reactive oxygen species (ROS) are derived from the 250 gm of oxygen (approx) that humans use each day; of this 2–5% is converted to reactive oxygen species (ROS)[20]. ROS assist in cellular signalling, angiogenesis and efficient defence against invading pathogens [21]; However, excessive production of ROS or impaired detoxification of these aggressive molecules can cause oxidative stress [20]. Direct measurement of reactive oxygen molecules is not possible in a wound due to their short half-life, and as

a consequence, it is necessary to rely on the measurement of oxidative products as well as the measurement of the endogenous antioxidant capacity in order to assess the oxidative status of the wound bed [22]. Uric acid: allantoin ratio, malondialdehyde and 8-isoprostane are some of the indicators which have been used to quantify the oxidative stress assessed in research literature [22, 23]. 8-isoprostane is produced by the random oxidation of tissue phospholipids by oxygen radicals [24]. Thus, 8-isoprostane has been proposed as a reliable biomarker of oxidative stress and it has been found to be associated with non-healing venous ulcers [25].

Temperature in the skin surrounding wounds has been observed to rise during the process healing of a wound [26]. Infrared noncontact thermometry is a fast and stable method of measuring temperature that is relatively independent of the user [27, 28] .

Calcium is known to affect cellular functions that include cellular mitosis, neutrophil exocytosis, superoxide production, remodelling of embryonic epithelium, and growth factor regulation [29]. It is possible that specific cells involved in wound healing respond differently to elevated levels of calcium. Research shows the addition of 5 mmol/day calcium inhibits wound closure significantly compared with the normal wound. Addition of verapamil (Ca channel blocker) to the calcium-treated wounds partially reverses the defective wound closure [29].

Lysolecithin and phospholipase-A2 are derivatives of lecithin and result from lipid peroxidation of cell membranes. These are toxic to cells and have been associated with conditions such as necrotising pancreatitis and myocardial infarction. They have been associated with non-healing venous leg ulcers in preliminary studies [30].

Oedema is considered a key pathogenic factor in the development of venous leg ulcers [7]. Recent quantitative research conducted by this department have assessed the reliability of the measurement of dermal thickness using high frequency ultrasound (HFU) as an indicator to quantify the dermal oedema, in the patients with venous leg ulcers [31]. This work has also demonstrated that oedema reduces when patients have compression bandaging applied to their legs, which has been shown to be associated with better ulcer healing.

In addition other standard blood chemistry parameters will be assessed as well as an array of growth factor, cytokines, and proteases and their inhibitors.

In this study a number of factors that have previously been shown to be associated with the healing of venous leg ulcers will be evaluated to determine whether there is sufficient separation in the levels between healing and non-healing phases of wounds for these factors to be of value as diagnostic tests to assess the phase of healing at a single time of assessment.

Hypothesis

The healing phase of chronic wounds can be determined at any time point by measuring one or more wound related factors (biomarkers)

Overall aim

To determine whether specific factors that can be measured in wounds have suitable cut-off levels that can be used to determine the healing phase of a wound.

Specific aims

1. To measure at weekly intervals in venous leg ulcers the following factors

- pH of wound fluid
- oxidative status of the wound (measured by 8-isoprostane, total antioxidative status and allantoin/uric acid ratio)
- temperature of the wound measured by non-contact infra-red thermometer with dual laser targeting (Shenshen Huazhui Tech Lab)
- calcium levels of the wound
- lysolecithin levels in the wound
- dermal oedema in the leg (DermaScan Protocol)
- standard biochemical electrolytes
- a panel of growth factors, cytokines, proteases and their inhibitors

2. To measure at weekly intervals the ulcer sizes and to determine the healing phase of ulcers at each time point.

3. To determine individual variability of each factor

4. To determine whether each factor has a suitable cut-off level that can be used to determine ulcer healing phase.

Methods

Design:

A prospective observational study.

Participants:

Participants will be recruited from the Leg Ulcer Clinic at Fremantle Hospital and will have chronic ulcers on their lower limbs that have a venous aetiology either alone or in association with other causes of impaired healing in order to reflect the spectrum of participants who are seen in most clinical settings. It is planned to recruit a total of 40 subjects to the study.

Entry criteria:

• Male or female subjects over the age of 18 years

• Proven evidence of venous disease on photoplethysmography or Duplex scan

• Ulcer greater than 2 cm^2 in area

• Ankle brachial indices greater than 0.5 (to exclude participants with severe arterial disease)

• Able to give informed consent

Study duration:

Participants will continue in the study for 12 weeks or until the ulcer has reduced in size to a stage that it is no longer producing sufficient exudate that can be collected for the analyses in the study

Study evaluations:

It the initial study visit, demographic parameters will be documented, and data from the standardised assessment of ulcer aetiology that is performed at the Fremantle Hospital Leg Ulcer Clinic will be recorded. Participants will attend for study assessments at weekly intervals, and at the initial visit and each of these subsequent visits the following will be performed –

• Collection of wound fluid from the ulcer using a standardised methodology that is also used by other groups in the CRC

• Evaluation of dermal thickness of the skin adjacent to the ulcer using high frequency ultrasound

• Measurement of temperature from the surface of the wound – this will be performed at each visit using an infrared thermometer and may also be recorded from temperature sensors incorporated into the wound dressings if these are available to the study. Temperature will be recorded from the wound surface and from several control sites on the body – contra-lateral leg, upper limb, and chest.

Assessment of healing status:

Prior to commencing the data collection for the study, an evaluation will be performed of the variability of ulcer area measurement using the tracing and Visitrak method. These data will be used to determine the percentage change in ulcer size that can be regarded as real change. At each visit the size of the ulcer will be measured by tracing the ulcer area onto a polyurethane sheet and the ulcer area will be calculated using the Visitrak planimetry device. These measurements will be performed in duplicate by two assessors who have no knowledge of the other assessor's measurement. Classification of ulcer healing status will be determined for each visit except for the first and final visits, and will be performed at the completion of data collection for each participant. Healing status will be based on the following

> • Healing phase – a reduction in ulcer size for the week before and the week after the visit.

> • Non-healing phase – no change or an increase in ulcer size for the week before and the week after the visit

• Indeterminate healing phase – a different change in ulcer size for the week before and the week after the visit.

All assessments and measurements that are performed on wounds will be performed without reference to previous measurements.

Wound fluid evaluations:

The wound fluid will be placed on ice and will be taken to the laboratory. The pH will be measured with a pH meter and the fluid will then be spun to remove particulate matter and will be stored at -80°C until ready for measurement of different parameters. The parameters will be measured at the completion of the study, and will include the following

- oxidative status of the wound fluid
    - 8 – isoprostane levels
    - Allantoin and uric acid (to determine allantoin / uric acid ratio)
    - Total oxidative status
- calcium level of the wound fluid
- lysolecithin levels in the wound fluid
- dermal oedema in the wound
- standard biochemical electrolytes
- a panel of growth factors, cytokines, proteases and their inhibitors, and related molecules that may have an impact on wound healing, using Multiplex ELISA Assays

The study analysis will be performed by comparing the levels of each factor in the non-healing and healing phases of wound healing, and by determining cut-off scores that give the best separation between the two phases. Only factors that have a high level of discrimination between the two healing phases will be considered as potential diagnostic tools.

Treatment of ulcer:

Participants will undergo the standard treatment of their ulcer that is appropriate for the cause of their ulcer and that is recommended by the Leg Ulcer Clinic at Fremantle hospital. The treatment will be documented at each visit.

This study will be conducted in Vascular Research Laboratory at Fremantle Hospital. Forty participants will be recruited who meet the inclusion and exclusion criteria and who give informed consent.

Data analysis:

1. Age, gender and ulcer aetiology will be summarised using descriptive statistics

2. For each factor, Receiver Operating Characteristic (ROC) curves will be created using the sensitivity and specificity of a potential cut-off point for dermal oedema that equals to a healing phase. For a factor

to be useful as a diagnostic marker, the area under the curve would be expected to be greater than 0.8 and preferably greater than 0.9.

All data will be analysed using IBM SPSS version 19.

Demographic data will be summarised using mean and standard deviation for continuous variables and percentages for categorical variables.

Sample size:

It is anticipated that there will be a maximum of 10 data points for each of the 40 participants, and each data point will be categorised as healing, non-healing or indeterminate as above. Allowing for indeterminate healing phases and reduction in ulcer sizes prohibiting wound fluid collection, it is anticipated that there will be 150 - 200 data points for analysis. In order to demonstrate an area under the curve of 0.70 a sample of 44 per group (non-healing and healing data points) is required. To determine greater areas under the curve, smaller sample sizes per group are needed. Allowing for an uneven distribution of healing and non-healing data points, the sample size of 40 participants will be more than adequate.

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