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THE EFFECT OF TOPICAL THERAPIES ON
ULCER HEALING AND THE WOUND
MICRO-ENVIRONMENT IN
DIABETES MELLITUS

FRANCES RACHEL HENSHAW
A thesis submitted in fulfilment of the requirements for the degree of:
Doctor of Philosophy
Discipline of Medicine
University of Sydney
December 2014
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INTRODUCTORY STATEMENT

The studies presented in this thesis are the result of original research carried out while the author was enrolled for the degree of Doctor of Philosophy in the Faculty of Medicine, University of Sydney. These studies were conducted in the Department of Endocrinology, Sydney Medical School, the University of Sydney.

All experimental work carried out for this thesis is entirely my own original work except where stated otherwise in the text. The work presented in this thesis has not been submitted for a degree or a diploma in any other university.

Frances Henshaw

December 2014
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As a clinical podiatrist and scholar, it has been quite a challenge to juggle my clinical workload with the demands of a Ph.D. The laboratory work in particular was a very steep learning curve for me and logistically presented many challenges. I wish to convey my sincere thanks to all who have helped me throughout the course of this thesis.

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I also acknowledge Diabetes Australia for supporting the growth factor therapy animal studies in my work through a competitive grant and to the Endocrinology and Diabetes Research Foundation the University of Sydney and the Podiatry Board of NSW Research Fund for supporting my clinical propolis feasibility study, the latter through a competitive funding process.
I also like to acknowledge the use of Dr Michael Rolph as a freelance editor for this work.

Dr Rolph has a Ph.D in virology and immunology and assisted with the scientific editing.
NATIONAL AND INTERNATIONAL SCIENTIFIC MEETING ABSTRACTS AND MANUSCRIPTS ARISING FROM THIS THESIS

Henshaw F, McLennan S, Bonner J, Twigg S.
Topically applied connective tissue growth factor (CTGF) accelerates healing in a diabetic rodent model. *Annual Scientific Meeting of the Australian Diabetes Society, August 2009.*

Henshaw F, McLennan S, Bonner J, Twigg S.
Wound closure in a diabetic animal model is accelerated by topical connective tissue growth factor (CTGF). *National Conference of the Wound and Tissue Repair Society, March 2010, Perth, WA.*

Henshaw F, McLennan S, Bonner J, Twigg S.
Recombinant human connective tissue growth factor (rhCTGF) accelerates wound healing and increases collagen deposition when applied topically to diabetic rodent wounds. *The Global Conference on Amputation Prevention, Diabetes Management, and Diabetic Foot and Diabetic Wound Care, March 2011, California, USA*

Henshaw F.R., McLennan S.V, Twigg S.M.
Henshaw F.R., Bolton T., Nube V., Hood A., Veldhoen D., McKew G., McLeod C., McLennan S.V., Twigg SM.

Topical application of the bee hive protectant propolis is well tolerated and improves diabetic foot ulcer healing in a pilot study. American Diabetes Association 74th Annual Scientific Sessions, June 2014, San Francisco, USA.

Henshaw FR, Yue DK, Lo L, Boughton P, Bonner J, McLennan SV, Twigg SM.

Topically Applied Connective Tissue Growth Factor (CTGF/CCN-2) Accelerates Wound Healing In A Diabetic Rodent Model. (Manuscript in second stage of review, Wound Repair and Regeneration).

Henshaw FR, McKew G, Bolton T, Nube V, McLeod C, McLennan SV, Twigg SM.

Topical Application of the Bee Hive Protectant Propolis is Well Tolerated and Holds Promise as Additional Therapy In Human Diabetic Foot Ulcers: A Feasibility Study.


Henshaw FR, Twigg SM, McLennan SV.

What’s the buzz: Bee products and their potential value in diabetic wound healing.

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<td>Ankle-brachial pressure index</td>
</tr>
<tr>
<td>AGEs</td>
<td>Advanced glycation endproducts</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Activated protein C</td>
</tr>
<tr>
<td>αSMA</td>
<td>Alpha smooth muscle actin</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BIM</td>
<td>Bersoft image measurement</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>C5a</td>
<td>Complement component 5a</td>
</tr>
<tr>
<td>CMP</td>
<td>Collagen mimetic peptides</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>CYR61</td>
<td>Cysteine rich angiogenic inducer 61</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra cellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EEP</td>
<td>Ethanolic extract of propolis</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EPC</td>
<td>Endothelial progenitor cell</td>
</tr>
<tr>
<td>ePTFE</td>
<td>Expanded polytetrafluoroethylene</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and drug administration</td>
</tr>
<tr>
<td>HBOT</td>
<td>Hyperbaric oxygen therapy</td>
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<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>IGF</td>
<td>Insulin like growth factor</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>KGF</td>
<td>Keratinocyte growth factor</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility antigen</td>
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<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<tr>
<td>NDS</td>
<td>Neuropathy disability score</td>
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<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>NFκB</td>
<td>Nuclear factor kappa beta</td>
</tr>
<tr>
<td>NHMRC</td>
<td>National health and medical research council</td>
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<tr>
<td>NWPT</td>
<td>Negative wound pressure therapy</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating Cell Nuclear Antigen</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PDWHF</td>
<td>Platelet derived wound healing factor</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PGF</td>
<td>Placenta growth factor</td>
</tr>
<tr>
<td>PMSi’I</td>
<td>Phenylmethylsulphonyl</td>
</tr>
<tr>
<td>PPR</td>
<td>Pentatricopeptide repeat</td>
</tr>
<tr>
<td>PPF3</td>
<td>Phospholipid platelet factor 3</td>
</tr>
<tr>
<td>PRP</td>
<td>Platelet rich plasma</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation endproducts</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomised controlled trial</td>
</tr>
<tr>
<td>rhCTGF</td>
<td>Recombinant connective tissue growth factor</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>-----------</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
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<tr>
<td>RT-qPCR</td>
<td>Reverse transcription quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<td>SMA</td>
<td>Smooth muscle actin</td>
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<td>STZ</td>
<td>Streptozotocin</td>
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<td>TBST</td>
<td>Tris buffered saline, Tween</td>
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<td>TGFβ</td>
<td>Transforming growth factor beta</td>
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<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll like receptors</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
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<tr>
<td>TSP 1</td>
<td>Thrombospondin repeat type 1</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<td>VN</td>
<td>Vitronectin</td>
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<td>VPT</td>
<td>Vibration perception threshold</td>
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## LIST OF REAGENTS

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<tr>
<td>β-mercaptoethanol</td>
<td>Sigma, ST Louis, MO, USA</td>
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<tr>
<td>Benchmark™ Prestained Protein Ladder</td>
<td>Invitrogen, Carlsbad, CA, USA</td>
</tr>
<tr>
<td>Bovine serum albumin, Cohn analogue Minimum 98%</td>
<td>Sigma, ST Louis, MO, USA</td>
</tr>
<tr>
<td>Bromophenol blue</td>
<td>Sigma, ST Louis, MO, USA</td>
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<td>Dulbecco’s modified eagles medium (DMEM)</td>
<td>Gibco-BRL, Grand Island, NY, USA</td>
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<tr>
<td>ECL plus western blot detection system USA</td>
<td>Amersham Biosciences, Piscataway, NJ, USA</td>
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<td>Ethanol</td>
<td>Fronine Laboratory Supplies, NSW</td>
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<td>Foetal Calf Serum (FCS)</td>
<td>JRH Biosciences, VIC</td>
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<tr>
<td>Glycine</td>
<td>BDH, Poole, Dorset, UK</td>
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<tr>
<td>Hibiscrub</td>
<td>Molnlycke, Frenchs Forest, NSW</td>
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<tr>
<td>Insulin (human, Protophane®, 100IU/ml)</td>
<td>Novo-Nordisk, Malmo, Sweden</td>
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<tr>
<td>Ketamine</td>
<td>Troy Laboratories, NSW</td>
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<tr>
<td>Methanol</td>
<td>Fronine Laboratory supplies, NSW</td>
</tr>
<tr>
<td>Paraformaldehyde</td>
<td>Sigma, ST Louis, MO, USA</td>
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<tr>
<td>Phosphate buffered saline (PBS x 10)</td>
<td>Amresco, St Louis, MO, USA</td>
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<tr>
<td>Roswell Park Memorial Institute medium</td>
<td>Sigma, ST Louis, MO, USA</td>
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<td>Sodium Azide</td>
<td>Sigma, ST Louis, MO, USA</td>
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<td>Streptozotocin</td>
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<td>Superscript</td>
<td>Invitrogen, Mt. Waverley, VIC.</td>
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<td>Tri-reagent</td>
<td>Sigma, ST Louis, MO, USA</td>
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Tris
Triton X-100
Xylazine
3,3’- diaminobenzidine

Sigma, ST Louis, MO, USA
Sigma, ST Louis, MO, USA
Bayer, Pymble, NSW
Vector Labs, CA, USA
SUMMARY

Cutaneous wounds in patients with diabetes typically show abnormal healing. Such ulcers are characterised by chronicity, persistent inflammation, copious exudate, hypergranulation, increased bacterial load and reduced ability to heal. Diabetic foot ulceration is a cause of significant morbidity and financial burden. This thesis examines the effect of two topical therapies on healing in wounds in individuals with diabetes.

The protein connective tissue growth factor (CTGF) is induced during normal wound healing, whereas it is deficient in diabetic wounding models. Topical application of CTGF to rodent diabetic and control wounds in an established model was examined in this thesis. CTGF treated diabetic wounds showed an accelerated closure rate compared with vehicle treated diabetic wounds. Healed incision site skin was able to withstand more strain before breaking in CTGF treated rat wounds compared with untreated wounds. Granulation tissue examined after CTGF treatment in wounds in rats with diabetes showed collagen-IV accumulation compared with vehicle treated diabetic animals. The α-smooth muscle actin in fibroblasts and endothelial cells was increased in CTGF treated diabetic wounds compared with untreated diabetic wounds, as was macrophage infiltration. Wound breaking strength data showed that untreated diabetic wounds were able to tolerate significantly less final strain before breaking than either treated or untreated control wounds; the CTGF treated diabetic wounds had a slightly higher mean final strain (2.32 MPa) than the untreated diabetic wounds (2.20 MPa), however this did not reach statistical significance.

Propolis is a naturally occurring potent anti-inflammatory bee-derived protectant resin that is reported to aid wound healing in general but it has not been studied systematically in wounds.
in a cohort of humans with diabetes. To determine if propolis shows efficacy in a pilot study of foot ulcer healing in diabetic human subjects and if it is well tolerated, serial consenting subjects (n=24) with diabetic foot ulcers were studied. Propolis was applied topically at each clinic review for 6 weeks. The controls were a prespecified historic control group who were previously treated at the same clinic. Ulcer healing data compared with the control group of n=84 showed that wound closure rate was increased in propolis treated vs control ulcers. Post-debridement wound fluid analysed for viable bacterial count and the pro-inflammatory matrix metallo-proteinase, MMP-9 activity (a pro-inflammatory protease), showed that active MMP-9 and bacterial counts were significantly reduced from baseline in propolis treated ulcers vs. controls. Wound fluid CTGF was not clearly induced by propolis, although only early time points in wound healing were examined. No adverse effects of propolis treatment were reported.

In summary, this novel data shows that topical CTGF improves wound healing in diabetic rats. When combined with evidence that endogenous CTGF is induced as human diabetic foot ulcers heal, it provides rationale for considering studies of CTGF as therapy in human diabetic foot ulcers. The propolis pilot study indicates for the first time in a human diabetic foot ulcer series that topical propolis may enhance wound closure and it is well tolerated. A multi-site randomised controlled of topical propolis in diabetic foot ulcers now appears to be warranted.
Chapter 1

OVERVIEW OF DIABETES AND ITS COMPLICATIONS

Introduction

The purpose of this chapter is to provide a brief overview to the themes that will be examined more broadly in subsequent chapters this thesis.

Prevalence of diabetes

Diabetes mellitus is a serious metabolic condition and one of the world’s leading chronic diseases, affecting more than 381 million adults worldwide ~8% of the world’s adult population (Guariguata, Whiting et al. 2014). Moreover, 45.8%, or 174.8 million of all diabetes cases in adults are estimated to be undiagnosed (Beagley, Guariguata et al. 2014).

The burden of diabetes is becoming increasingly prevalent. Boyle, Honeycutt et al. (2001) estimated that in 2001 11 million US citizens suffered from diabetes, representing 4% of the total population. By 2050 this number is predicted to increase to 29 million or 7.2% of the population (Boyle, Honeycutt et al. 2001). Similarly, the International Diabetes Federation, (2014) estimates for current and prospective diabetes each continue to rise (Guariguata, Whiting et al. 2014). The Australian experience indicates that from 1989–90 to 2011–12, the prevalence of diabetes in the general population more than doubled, from 1.5% to 4.2%. The prevalence of diabetes rose in all population groups measured and rates of diabetes were similar across geographic regions, with some exceptions according to the Australian Institute of Health and Welfare (AIHW 2013). Between 2001 and 2004–05, the prevalence of
diabetes among Aboriginal and Torres Strait Islander Australians was more than three times that of non-Indigenous Australians (AIHW 2013).

There are two main types of diabetes, which vary considerably in their pathogenesis, clinical features and therapeutic approaches. The most common is type 2 diabetes which accounts for 85-90% of diabetes patients and type 1 diabetes accounting for 10%-15%. There are other, less common types of diabetes, including those occurring due to corticosteroid therapy, end stage liver disease, or from monogenic gene defects such as maturity onset diabetes of the young (MODY), (Fajans 1987; Wang, Tekeuchi et al. 1999).

Type 2 diabetes results from relative insulin deficiency typically with insulin resistance and some degree of impaired insulin secretion. In contrast, type 1 diabetes results from an absolute inability of the pancreas to produce insulin, attributable to a destructive autoimmune process. Most subjects with type 2 diabetes have reached middle age when they are diagnosed although it can also occur in youth (Diabetes UK, 2012). Type 2 diabetes is usually managed successfully through a controlled diet and exercise combined with oral hypoglycaemic medication and/or incretin mimetic treatment. In subjects for whom this is ineffective, parenteral insulin therapy is used. People with type 1 diabetes tend to become symptomatic earlier in life and typically are diagnosed before 30 years of age. Type 1 diabetes is managed through intensive diabetes management, including multiple times daily insulin therapy and control of oral carbohydrate intake. In each type of diabetes mellitus, assessment for diabetes complications is required on a regular basis, in order to detect and treat certain complications early and to prevent their progression.
Complications Associated with Diabetes

Despite improvement in effective management regimens, people with diabetes are prone to develop a variety of secondary complications that may result in permanent and irreversible damage to organs and tissues. Such complications include atherosclerotic cardiovascular disease, diabetic nephropathy, retinopathy and diabetic foot disease. The latter may give rise to wounds that fail to heal satisfactorily, which can then lead to lower limb amputation.

The aetiology of these complications is generally multifactorial and not attributable to a single root cause. The rate of onset and the severity of diabetic complications may be influenced directly by the severity of hyperglycaemia in diabetes, as well as by the dyslipidaemia and hypertension usually present in type 2 diabetes. In addition indirect factors such as genetic susceptibility to diabetes and its complications and environmental issues such as cigarette smoking and sedentary lifestyle can also effect the development of diabetic complications. DCCT (Diabetes Control and Complications Trial (DCCT 1996)) and UKPDS (1999) (U.K. Prospective Diabetes Study) (King, Peacock et al. 1999).

Diabetic Foot Ulcers

Foot ulceration is common in people with diabetes mellitus (Moulik, Mtonga et al. 2003) occurring in up to one quarter of people who have diabetes and commonly recurs in those who develop the complication (Singh, Armstrong et al. 2005). Foot ulceration as a consequence of diabetes is the most common reason for lower limb amputation, accounting for some 50-70% of non-traumatic lower limb amputations (Boulton and Vileikyte 2000; Boulton, Vileikyte et al. 2005). One-third of the US$116 billion directly apportioned to diabetes care in the US in 2007 was used to treat foot ulceration (Driver, Fabbi et al. 2010).
The pathogenesis of foot ulceration is complex. While most foot ulcers will heal in people with diabetes, some do not, and ulcer recurrence rates are high, around 50% (Dubsky, Jirokowska et al. 2013).

Major causes of diabetic foot ulceration include:

- neuropathy (sensory, motor, and autonomic deficits), especially reduced sensation in the feet due to a distal sensori-motor bilateral peripheral neuropathy;
- peripheral arterial disease causing ischaemia with delayed ulcer healing; often a combination of both ischaemia and neuropathy co-exist, further impairing ulcer healing compared with either condition alone;
- other important factors as referenced below include: mechanical foot deformity such as occurs secondary to peripheral neuropathy with calluses or after amputation; and ulcers complicated by secondary bacterial infection, a common and serious complication of diabetes mellitus. Increase of infections in patients with diabetes is known to depend upon an immunosuppressive state. The relationship between hyperglycemia and immune function is controversial and relevance of hyperglycemia and/or hyperinsulinemia to immunosuppressive mechanisms is currently unclear. (Tanaka, 2008)

An important deleterious effect associated with diabetes is the formation of sugar-derived substances called advanced glycation end products (AGEs). AGEs form at a constant but slow rate in the normal body, however, their formation is markedly accelerated in diabetes because of the increased availability of glucose.

The effects of glycosylation, that is accumulation of AGEs are observed in the lower extremities of people with diabetes. Skin thickness and bone density decreases; tendons thicken; muscles and fat pads atrophy; joints mobility is reduced; and fat pads migrate
distally. These local soft tissue changes also alter a patient’s gait, putting them at risk of foot ulceration’ (Wrobel 2010). In terms of ulcer precipitants, as opposed to the predisposing factors described above, a foot ulcer may result from an initiating injury such as acute trauma or from repetitively/continuously applied mechanical stress, usually attributable to neuropathy (Reiber, Vileikyte et al. 1999).

For example, new foot-wear combined with excessive walking is a common foot ulcer precipitant. In addition, tinea pedis can cause loss of skin integrity and risk of subsequent bacterial infection, which with continued trauma and suboptimal foot ulcer management, can further compromise healing (Xu, McLennan et al. 2007, Lipsky, Berendt et al. 2004 and Armstrong, Lipsky et al. 2004).

Diabetic foot ulcers are notably slow to heal and according to Jeffcoate et al. (2008) even with organised delivery of standardised care only 33% of the ulcers that are deep to tendon or bone will heal (Jeffcoate, Lipsky et al. 2008). In 2007-08 4.1 people per 100,000 were hospitalised for lower limb amputations in Australia as a result of diabetes (AIHW, 2013). A cost-analysis and cost of a single patient episode of an amputation in Australia attributable to a diabetic foot ulcer was estimated during that decade to be $26,700 (Ray, Valentine et al. 2005).

Historically diabetic foot ulcers have been neglected in healthcare research and planning, with clinical practice based more on opinion than as a result of methodologically robust evidence-based enquiry (Jeffcoate and Harding 2003). Furthermore, associated pathological processes are often not well understood by health care professionals and administrators and tend to be communicated inadequately (Jeffcoate and Harding, 2003).
Pathogenesis of Wound Healing in Diabetes

As described previously, the delayed wound healing in diabetic foot disease is attributable to a variety of factors including peripheral arterial disease, peripheral neuropathy, foot deformity and secondary bacterial infection (American Diabetes Association (ADA) 1999; Margolis, Kantor et al. 2000). Furthermore, the wound microenvironment in diabetes is reported to be abnormal and pathogenic factors lead to delayed ulcer closure and suboptimal granulation tissue formation with abnormal composition of extra-cellular matrix (ECM) (Lobmann, Ambrosch et al. 2002). These pathogenic factors appear to emanate from diabetes impairing key processes in wound healing including, as described below, the normal cellular functions involved in cutaneous wound healing. It has also been proposed that persistent inflammatory infiltrates associated with bacterial colonisation in the wound contribute to delayed healing in diabetes (Zhao, Hochwalt et al. 2010; Loots, Lamme et al. 1998).

In diabetic patients ulceration is characterised by chronicity, copious exudate, hypergranulation, increased bacterial load and reduced healing capacity (Berlanga-Acosta, Schultz et al. 2012). At a cellular level, in diabetic wounds, inflammatory cytokines, local proteases, reactive oxygen and nitrogen species produce a cytotoxic and pro-degradative wound environment and delayed healing arises from the resulting adverse local metabolic environment (Berlanga-Acosta, Schultz et al. 2012). In particular, pathological persistence of a pro-inflammatory high protease activity wound microenvironment and inflammatory cell infiltrate in foot ulcers in diabetes has been well recognised (Liu, Min et al. 2007).

A cytokine/chemokine mediated imbalance between synthetic and degradative matrix pathways is thought to be responsible for reduced formation of productive granulation tissue that is able to aggregate cells and proteins. Therefore the quantity and quality of extra-
cellular matrix (ECM) is reduced (Lobmann, Ambrosch et al. 2002). Human wounds in diabetes also exhibit an excess of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α), which leads to increased protease activity and consequently, persistent inflammation and delayed healing (Lobmann, Ambrosch et al. 2002).

ECM accumulation is controlled by matrix metalloproteinases (MMPs), which degrade ECM, and MMP tissue inhibitors or TIMPs (Visse and Nagase, 2003, Gill and Park, 2008). Growth factors are also involved in the regulation of ECM. Connective tissue growth factor (CTGF), is a profibrotic growth factor capable of inducing fibroblast proliferation, migration and adhesion - suggesting it may play a role in ECM accumulation (Moussad and Brigstock 2000). CTGF is also known as CCN2, and the term CCN is derived from the first three members of the family discovered, namely CYR61, CTGF, and NOVH (Shi-Wen, Leask et al. 2008). Throughout this thesis, the term CTGF will be used for this protein.

Altered levels of CTGF gene expression and protein levels have been reported in tissues and biological fluids of diabetic subjects, especially when associated with excess fibrosis. This includes diabetic nephropathy (Wang, Denichilo et al. 2001), diabetic cardiomyopathy, and retinopathy (Hinton, Spee et al. 2004), in which CTGF levels are elevated. This contrasts to findings in skin, with baboon skin studies showing a deficiency of CTGF in diabetic wound tissue compared with wound tissue in non-diabetic control animals (Thompson, McLennan et al. 2010). These results suggest that CTGF may have therapeutic benefit in wound healing although CTGF as therapy has not been examined in cutaneous wounds in diabetes.

In addition to CTGF, naturally occurring substances, or ‘neutraceuticals’ isolated or purified from foods have a potential physiological benefit in wound healing. Propolis is one such
neutraceutical: it is a bee-derived product with reported antibacterial and anti-inflammatory properties and noted effects on wound cytokines including suppression of inflammatory cytokines which are overexpressed in diabetic wounds (Reinhold, Ansorge et al. 2003). Propolis has yet to be trialled in human subjects with foot ulcers in diabetes.

The following literature review will discuss in more detail:

a) the pathogenesis of complications in diabetes, focusing on wound healing;
b) current and emerging wound healing therapies in diabetes; and
c) the therapeutic potential of growth factors, specifically CTGF, and the beehive protectant propolis, to aid the wound microenvironment and promote wound healing in diabetes.
LITERATURE REVIEW

2.1 Tissue damage in diabetes

The complications of diabetes can generally be categorised into macrovascular (coronary artery disease, peripheral arterial disease and stroke) and microvascular complications (diabetic nephropathy, neuropathy and retinopathy). The Diabetes Control and Complications Trial, (DCCT 1996) and U.K. Prospective Diabetes Study, 1999 (King, Peacock et al. 1999) established that hyperglycaemia is the initiating cause of diabetic tissue damage and that targeting this parameter across many years can help to reduce both microvascular and macrovascular complications of diabetes. Indeed, while abnormal blood lipids and systemic hypertension also increase the risk of organ complications from diabetes, it is hyperglycaemia presence that defines diabetes mellitus and also causes complications in both type 1 and type 2 diabetes.

Despite the enormity of the burden of diabetic complications, no single theory can explain hyperglycaemia-induced biochemical changes that contribute to pathology in diabetes (Rafehi, El-Osta et al. 2011); however, several mechanisms have been implicated in the pathological process, and these are shown in the schematic Fig. 2.1 overleaf:
Biochemical pathways implicated in mediating adverse effects of hyperglycaemia include: protein kinase C, advanced glycation, aldose reductase and hexosamine pathways, and excessive reactive oxygen species (Brownlee 2005). Currently poorly defined genetic susceptibility factors are thought to place some people more at risk of diabetes complications than others, and the adverse effects of these pathways (Rafehi, El-Osta et al. 2011). While these metabolic pathways are thought to mediate end-organ damage, notably targeting of each alone has not to date been shown to prevent complications in human diabetes (Brownlee 2005).
The delayed healing in diabetic ulcers is, according to the cited studies, attributable initially to hyperglycaemia, however control of hyperglycaemia has not been shown to aid wound healing in an experimental mouse model (Berdal and Jenssen, 2013) nor in any human intervention studies to date. Furthermore, factors common to other chronic wounds (e.g. decubitus and venous ulcers) also contribute to the failure of diabetic wounds to heal (for example, increased macrophage and B-cell infiltration) indicating that a number of factors are responsible for the recalcitrance of these wounds (Loots, Lamme et al. 1998). Whilst peripheral neuropathy and peripheral arterial disease can be prevented in people with diabetes by long term blood glucose control, across many years (DCCT, 1996; King, Peacock et al. 1999), relatively acute improvement of blood glucose has not been shown to aid foot ulcer healing in people with diabetes. Indeed, it may be that blood glucose control is most important to prevent and stabilise pre-existing diabetes complications as reported in the DCCT (DCCT 1996), but that such a strategy is much less able to reverse these complications, at least acutely.

2.1.1 Diabetic foot ulceration - epidemiology

In the developed world, 2-3% of patients with diabetes are likely to have a foot ulcer at any given time point (Abbott Carrinagton et al. 2002; Muller, de Grauw et al. 2002). The lifetime risk of a patient with diabetes developing a foot ulcer may be as high as 25% (Singh, Armstrong et al. 2005) and diabetes increases the risk of lower extremity amputation 10- to 20-fold (Wrobel, Mayfield et al. 2001). The incidence of lower-extremity amputation in people with diabetes ranges from 2.1 to 13.7 per 1000 patients (Bartus and Margolis 2004).

The cost to healthcare systems of providing care for diabetes-related foot ulcers is high. According to the Fremantle Diabetes Study (Davis, Norman et al. 2006) the average length of
hospital stay for a diabetes foot ulcer admission was 31 days and the cost was AUD$17,089 in 2000 (Davis, Norman et al. 2006). In the United States, 25-50% of the costs associated with diabetes-related care were attributed to the care of diabetes foot ulcers National Institute for Health and Clinical Excellence (NICE 2011). A Canadian study found that the cost of major events attributable to diabetes foot ulcers such as lower limb amputations generates a greater financial burden than the cost of treating the ulcer alone (Goeree, Lim et al. 2009). Following amputation due to foot ulceration in diabetes, mortality rates are higher than those rates for most malignancies (Armstrong, Wrobel et al. 2007): 13-40% mortality at 1 year, 35-65% at 3 years and 39-80% at 5 years, in each case mainly due to cardiovascular disease (Singh, Armstrong et al. 2005). The presence of a foot ulcer in a person with diabetes diminishes quality of life scores by 10-40% compared with the general population, indicating a significant social burden (Armstrong, Lavery et al. 2008).

2.1.2 Care of foot ulcers in diabetes

Standardised protocols of care using an interdisciplinary team approach have been shown to lower rates of complications and amputations in patients with foot ulcers in diabetes (O’Loughlin, McIntosh et al. 2010). Up to a 50% reduction in the risk of major lower-limb amputation is seen in patients with diabetes receiving evidence-based multidisciplinary treatment (Van Damme and Limet 2005). The American Diabetes Association (ADA, 1999) concluded that preventative care teams - that is, multidisciplinary teams that utilise patient education, risk assessment tools and therapeutic footwear - can reduce amputation rates by 50-85% (Mayfield, Reiber et al. 1998). The importance of early recognition of the high-risk foot is frequently underestimated in both inpatient and outpatient settings, and this is attributable to the asymptomatic nature of the condition and a reluctance to conduct routine foot screening (Boulton, Armstrong et al. 2008), with time constraints often cited as the
reason for the latter. In people with diabetes who develop a foot ulcer, the cornerstone of foot care is based on: careful assessment of the foot ulcer, including precipitating and predisposing factors, and the coordinated administration of four key elements of care:

- pressure offloading
- debridement and dressings and, where indicated,
- antibiotic therapy, and/or
- revascularisation (Kruse and Edelman 2006)

The individualisation of such treatments to a specific patient in a balanced manner across the multiple health care disciplines is key to optimising foot ulcer healing in diabetes (Boulton, Meneses et al. 1999, Wraight, Lawrence et al. 2005). Podiatrists, the treating physicians, surgeons and nursing staff all need to contribute to care in a coordinated manner in multidisciplinary clinics, along with broader disciplines such as microbiology and radiology. Wound biopsy studies are a safe, reliable and effective way of assessing tissue composition within a wound (Panuncialman, Hammerman et al. 2010) and radiological techniques allow the progression of pathological processes to be better quantified so treatment can be targeted accordingly. Despite these interventions and the introduction of standardised guidelines, many ulcers still do not heal and others are recalcitrant. It is essential that healing in wounds in diabetes is optimised in order to improve patient outcomes and to minimise use of limited health care resources. By doing so, gold standard outcomes of ulcer healing can be achieved and the need for hospital admission and extensive amputation can be minimised.
2.2 Pathological events in diabetic foot ulceration

A comprehensive understanding of wound healing in diabetes has yet to be realised (Blakyni and Jude 2006; Waugh and Sherratt 2006). However, many important factors that disrupt healing in people with diabetes have been identified, and are addressed in this section.

The pathology in diabetic foot ulcers is primarily attributable to co-existing ‘extrinsic’ factors—that is, neuropathy and vascular disease (Adler, Boyoko et al. 1999). Studies by the US Department of Health and Human Services, Centers for Disease Control and Prevention, observed that about 20% of foot ulcers in people with diabetes are caused primarily by peripheral arterial disease. In ~50% of cases neuropathy is the primary underlying cause and in ~35% both neuropathy and peripheral arterial disease are present (Control and Prevention 2011). Bacterial infection is considered a complication of diabetic foot ulceration as opposed to a cause but is included in this section as it delays ulcer healing. At a cellular and tissue level, intrinsic pathogenic factors are also implicated in the failure of diabetic wounds to heal. The ‘intrinsic’ pathophysiology of the diabetic wound is discussed alongside ‘normal’ wound healing in detail in section 2.3.

2.2.1 Peripheral arterial disease

Peripheral arterial disease is a manifestation of systemic atherosclerosis that is associated with an increased risk of death and ischemic events (Hirsch, Criqui et al. 2001). It is commonly diagnosed when pulse waveforms are abnormal, intermittent claudication is reported and/or ankle brachial pressure indices (ABPI) are less than 0.9 (Hirsch, Criqui et al. 2001). It must be noted that the ABPI is not a definitive measurement of peripheral arterial disease as calcified arteries may produce abnormally high readings (Palumbo, Melton et al. 2001).
More specific tests such as Doppler flow velocity can assist with diagnosis of peripheral arterial disease. People with diabetes are twice as likely to have peripheral arterial disease as those without (Gregg, Sorlie et al. 2004; Maly, Chovanec et al. 2010). Early research into wound healing in diabetes suggested that small vessel or microvascular disease is a major culprit in the failure of healing in diabetic wounds (Schramm, Dinh et al. 2006). It was assumed that microangiopathy, with endothelial proliferation in arterioles and basement membrane thickening in capillaries, delayed both the entry of essential cells into the wound and the clearance of metabolic by-products from the wound (Siperstein, Unger et al. 1968). Subsequent studies have shown that whilst microvascular disease contributes to abnormal wound healing and may persist in the diabetic ulcer (due to abnormal vessel permeability, functional ischaemia and an impaired neurogenic vasodilatory response), macrovascular disease is also a major co-contributor to morbidity (Donnelly, Emslie-Smith et al. 2000).

The prevalence of macrovascular disease is substantially elevated in people with diabetes and is characterised by the presence of atherosclerosis (Gaede, Jepsen et al. 2003). Factors that may contribute to the increased atherosclerosis in people with diabetes include increased expression of endothelial adhesion factors and platelet dysfunction (Boyle and Boyle 2007). People with diabetes are especially predisposed to atherosclerosis in distal vessels (vessels at and below the knee, for example, the tibial artery), as well as the usual larger arteries such as those in the pelvis and proximal leg (such as the femoral artery). Hypoperfusion resulting from macrovascular disease has a profoundly detrimental effect on wound healing in distal tissues. Table 2.1 outlines studies that have linked peripheral arterial disease to ulcer development and outcomes in diabetes.
Table 2.1: Studies identifying risk factors for foot ulceration, amputation and mortality due to peripheral arterial disease in diabetic populations.

<table>
<thead>
<tr>
<th>Study</th>
<th>Participants</th>
<th>Outcomes</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winkley, Stahl et al. 2007</td>
<td>253</td>
<td>Moderate ischaemia (an ABPI &lt;0.7) conferred a threefold risk of mortality to subjects with diabetic foot ulcers (HR 2.74, 95% CI 1.46-5.14). Microvascular complications predisposed individuals to recurrent ulcerations (HR 3.34, 95% CI 1.17-9.56).</td>
<td>The sample size was relatively small for this type of study</td>
</tr>
<tr>
<td>Eurodiale study (Prompers, Huijberts et al. 2007).</td>
<td>1,088 patients from 14 centres</td>
<td>The Eurodiale study linked a high prevalence of foot ulceration to baseline ischaemia with Europeans who present with a new diabetic foot ulcer and also linked ischaemia to increased risk of ulceration, amputation and mortality (p&lt;0.001)</td>
<td>There was a high dropout rate amongst older patients and those with deep ulcers, suggesting that these ‘worse’ ulcers were excluded and the actual prognosis is worse than the report suggests</td>
</tr>
<tr>
<td>North-West Diabetes Footcare Study Abbott (Abbott, Carrington et al. 2002)</td>
<td>1035</td>
<td>Reduced foot pulses (&lt;2/4) were shown to confer the risk of new foot ulcers.</td>
<td>Palpation of pedal pulses is associated with a high degree of inter-observer variability and high false positive/negative rates</td>
</tr>
<tr>
<td>The Fremantle Diabetes Study (Davis, Norman et al. 2006).</td>
<td>531</td>
<td>An ankle-brachial pressure index (ABPI) &lt;0.9 was an independent</td>
<td>If used alone, the ABPI can underestimate angiographic</td>
</tr>
</tbody>
</table>
A relatively small study of 253 subjects by Winkley et al (2007) showed that microvascular complications can explain recurrent ulceration (Winkley, Stahl et al. 2007). The Eurodiale study (2007) linked a high prevalence of ulceration to baseline ischaemia in Europeans presenting with a new foot ulcer in diabetes (Prompers, Huijberts et al. 2007). In contrast, some studies have failed to show a correlation between peripheral arterial disease and amputation (Faglia, Favales et al. 2001; Jude, Oyibo et al. 2001), but this could be attributed to the study pre-plan that people with severe peripheral arterial disease (ABPI <0.5) were excluded. Furthermore, some relatively minor amputations may be neglected in some databases, leading to an underestimate of the total number of amputations performed.

<table>
<thead>
<tr>
<th>Study</th>
<th>Methodology</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jude, Oyibo et al. 2001</td>
<td>136 arteriograms were analysed</td>
<td>People with diabetes had greater severity of arterial disease below the knee (p = 0.02); people with diabetes were five times more likely to require an amputation (41.4 vs. 11.5%, odds ratio [OR] 5.4, p &lt;0.0001). Mortality was higher in the group with diabetes (51.7 vs. 25.6%, OR 3.1, p = 0.002), and people with diabetes who died were younger at presentation than patients without diabetes (64.7 +/- 11.4 vs. 71.1 +/- 8.7 years, p = 0.04).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High proportion of hypertensive patients in diabetic group. This study showed that whilst diabetes conferred certain risks, it failed to show a correlation between peripheral arterial disease (PAD) and amputation.</td>
</tr>
</tbody>
</table>
The Fremantle Diabetes Study (2006) reported that an ABPI <0.9 is an independent predictor of both lower extremity amputation and mortality in people with diabetes who have a foot ulcer (Davis, Norman et al. 2006). Furthermore, this study reported that other microvascular manifestations of diabetes—nephropathy and retinopathy—were also associated with the risk of amputation.

Reduced foot pulse palpation (≤2/4 pulses palpable) was shown by the North-West Diabetes Footcare Study (Abbott, Carrington et al. 2002) to confer increased risk of new foot ulceration (Abbott, Carrington et al. 2002).

Peripheral oedema is not uncommon in people with diabetes, associated with heart failure, end-stage renal failure, and in some cases can be caused by medications (such as calcium channel blockers), or venous insufficiency (Apelqvist, Larsson et al. 1992). Peripheral oedema was found intercurrently in almost 50% of a cohort of people with diabetes and a foot ulcer with severe lower limb ischaemia. Peripheral oedema compromises vascular dynamics, and it precipitates tissue ischemia and impaired healing by increasing the distance required for diffusion of oxygen from capillaries to the ulcer. Oedema may reduce clearance of ulcer metabolites and degradation products from the ulcer site and its presence is closely associated with amputation and death yet it remains understudied in terms of optimal wound care, including in the setting of peripheral arterial disease (Apelqvist, Larsson et al. 1992). The evidence presented, whilst not conclusive, suggests that peripheral vascular disease is a contributing factor to foot ulceration in patients with diabetes.
2.2.2 Neuropathy

Diabetic neuropathy is defined as “the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes, after the exclusion of other causes” (Boulton, Gries et al. 1998). Neuropathic effects in diabetes mellitus can involve the sensory, motor and autonomic systems. Patients with loss of sensation commonly have decreased perception of pain. The loss of nociception that occurs in diabetic neuropathy is a major contributor to foot pathology by reducing the patient’s awareness of ulceration and infection. Some people with diabetes have reduced peripheral sensation; others have only painful neuropathic symptoms, with loss of sensation, while both conditions sometimes exist. There is no specific treatment for diabetic neuropathy where loss of sensation occurs, although there are many drugs to improve symptoms of painful neuropathy should they be present. Factors contributing to the development of diabetic neuropathy are not well understood, although some biochemical pathways, such as aldose reductase may mediate the effects of elevated blood glucose to cause nerve damage, whether this be directly on peripheral nerves or on the blood vessels supplying these nerves – the so-called vasa nervorum (Feldman, Stevens et al. 1997). Other than chronic hyperglycaemia clinical factors associated with diabetic neuropathy include hyperlipidaemia, hypertension, consumption of alcohol, and presence of obesity (Huizinga and Peltier 2007). The association between hyperglycaemia and diabetic peripheral neuropathy was best established with the DCCT trial (1996) where tight control of blood glucose levels led to a reduction in peripheral neuropathy onset of up to 60% in people with diabetes (DCCT, 1996). Various hypotheses to explain the aetiology of diabetic peripheral and autonomic neuropathy exist:
(i) Metabolic – Increased intracellular glucose as a result of hyperglycaemia is converted to sorbitol and fructose, which can cause nerve cell injury;
(ii) Vascular – Endoneural vascular resistance to hyperglycaemia results in secondary capillary damage and axonal degeneration;
(iii) Autonomic – Immunogenic alteration of capillary endothelial cells;
(iv) Neurotrophic – Deficiency of nerve support factors such as nerve growth factor (NGF) can result in reduced nerve proliferation and survival;

More recently through advanced neuroimaging studies the central nervous system has been implicated in diabetic peripheral neuropathy (Selvarajah, Wilkinson et al. 2008). Important work by Tesfayes’ group (2012) has shown that peripheral neuropathy concomitantly affects the central as well as peripheral nervous system (Tefsaye and Selvarajah 2012). This finding goes some way to explaining components of peripheral neuropathy such as allodynia, where pain is elicited with non-painful stimuli. Work by Zambreanu et al (2005) has shown this to be attributable to abnormalities in the spinal cord (Zambreanu, Wise et al. 2005)

It is probable that interplay between these factors forms the basis for the clinical features of diabetic peripheral neuropathy and that no single factor is responsible for diabetic peripheral neuropathy. Loss of protective sensation (sensory neuropathy) is detrimental to wound healing as sufferers are vulnerable to physical, chemical and thermal trauma. Ongoing, unnoticed trauma is a common consequence of neuropathy as is the development of increased areas of high pressure on weight-bearing surfaces. Indeed it is postulated that pressure per se
can accelerate cellular ageing (Wlaschek, Scharffetter-Kochanek et al. 2005). Motor neuropathy can lead to foot deformity (such as hammer toes and prominent metatarsal heads) and subsequently to areas of increased pressure from weight bearing or from poorly fitting shoes.

Loss of neuronal input to local vasculature (autonomic neuropathy) may result in abnormal vasodilation and oedema, or vasoconstriction and ischaemia (Aring, Jones et al. 2005), resulting in fissures, cracking and callouses, which can contribute to foot ulceration. Anidrotic skin changes may also reduce tensile strength of the skin making it more prone to ulceration. Table 2.2 summarises the outcomes of studies investigating neuropathy as a risk factor for diabetic foot ulceration.

Table 2.2: Diabetes studies identifying neuropathy as a risk factor in foot ulceration and ulcer outcomes including recurrence.

<table>
<thead>
<tr>
<th>Study</th>
<th>Participants</th>
<th>Outcomes</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winkley, Stahl et al, 2007</td>
<td>253</td>
<td>Vibration perception threshold (VPT) of ≥25 volts was a risk factor for ulceration and for mortality.</td>
<td>VPT is not itself independently associated with ulcer recurrence.</td>
</tr>
<tr>
<td>The Fremantle Diabetes Study (Davis, Norman et al. 2006).</td>
<td>531</td>
<td>Neuropathy was found to be an independent risk factor for foot ulceration.</td>
<td>The data were observational and not collected in a randomised fashion, which detracts from its validity.</td>
</tr>
<tr>
<td>Abbott, Vileikyte et al. 1998</td>
<td>1035</td>
<td>People with diabetes with peripheral neuropathy (DPN) had an annual first ulcer incidence risk which was higher than those with no DPN, rendering DPN a key ulcer risk factor.</td>
<td>The rate was much higher in the year subsequent to the study, indicating that patients were more compliant whilst being studied.</td>
</tr>
<tr>
<td>North-West Diabetes Footcare Study Abbott (Abbott, Carrington et al.)</td>
<td>9710</td>
<td>An abnormal Neuropathy Disability Score (NDS) of ≥6/10 positively correlated with risk of new</td>
<td>NDS is a composite score calculated by reporting ankle reflex, pinprick , and temperature sensation, vibration sense tested with</td>
</tr>
</tbody>
</table>
Abbott et al. (1998) demonstrated that people with diabetes who exhibit peripheral neuropathy (DPN) have an annual first ulcer incidence risk of 7.2% compared to 4.9% in a comparable population with no neuropathy rendering DPN a key risk factor (Abbott, Vileikyte et al. 1998). Reiber et al. (1999) found that neuropathy characterised by inability to feel the 10g monofilament and also Vibration Perception Threshold (VPT) of ≥ 25volts, to be risk factors for the development of foot ulcers (Reiber, Vileikyte et al. 1999). The Fremantle Diabetes Study (2006) also found neuropathy to be an independent risk factor for foot ulceration (Davis, Norman et al. 2006).

Winkley et al. (2007) studied a cohort of 253 people with diabetes with their first foot ulcer and found that a diminished VPT is also a risk factor for ulceration, and also for mortality but that VPT is not itself independently associated with ulcer recurrence (Winkley, Stahl et al. 2007). Similarly a study by Peters et al. (2007), examining risk factors for recurrence of foot disease in diabetes found that whilst neuropathy itself is not shown to be responsible for ulcer recurrence, having an index ulcer on the plantar hallux confers risk for ulceration (Peters, Armstrong et al. 2007). Liang (1998) identifies neuropathy as a known risk factor for toe amputation (Laing, 1998). It is likely that these findings are due to increased peak plantar pressures.

<table>
<thead>
<tr>
<th>Year</th>
<th>Authors</th>
<th>Study Details</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002</td>
<td>Peters, Armstrong et al. 2007</td>
<td>Examining risk factors for recurrence of diabetic foot disease, this study found that whilst neuropathy itself was not responsible for ulcer recurrence, an index ulcer on the plantar hallux conferred risk for subsequent ulceration (p&lt;0.05)</td>
<td>It is likely that this finding is due to increased peak plantar pressures, attributable to DPN. The study is also small, likely with type II error.</td>
</tr>
</tbody>
</table>

Abbott et al. (2007) demonstrated that people with diabetes who exhibit peripheral neuropathy have an annual first ulcer incidence risk of 7.2% compared to 4.9% in a comparable population with no neuropathy rendering DPN a key risk factor. Reiber et al. (2007) found that neuropathy characterised by inability to feel the 10g monofilament and also Vibration Perception Threshold (VPT) of ≥ 25volts, to be risk factors for the development of foot ulcers. The Fremantle Diabetes Study also found neuropathy to be an independent risk factor for foot ulceration.

Winkley et al. (2007) studied a cohort of 253 people with diabetes with their first foot ulcer and found that a diminished VPT is also a risk factor for ulceration, and also for mortality but that VPT is not itself independently associated with ulcer recurrence. Similarly a study by Peters et al. (2007), examining risk factors for recurrence of foot disease in diabetes found that whilst neuropathy itself is not shown to be responsible for ulcer recurrence, having an index ulcer on the plantar hallux confers risk for ulceration. Liang (1998) identifies neuropathy as a known risk factor for toe amputation. It is likely that these findings are due to increased peak plantar pressures.
pressures which are in turn attributable to peripheral neuropathy. Whilst there is controversy as to whether neuropathy is itself an independent risk factor for ulceration, the evidence presented suggests it is a major contributing factor to foot ulceration in patients with diabetes.

### 2.2.3 Other risk factors for diabetic foot ulcers, delayed healing and recurrence

Univariate predictors of healing failure, as described by the Ghanassia (2008) group include smoking, end-stage renal failure and popliteal stenosis. When pooled into a multivariate model, smoking and renal failure were both found to be independent predictors of poor healing (Ghanassia, Villon et al. 2008).

Research by Lin et al. (2009) established a link between micro/macrovascular complications and depression (Lin, Heckbert et al. 2009). Furthermore, Ismail et al. (2007) reported depression as a factor associated with worse healing outcomes for foot ulcers in diabetes (Ismail, Winkley et al. 2007). Depression is often associated with poor self-care (Gonzalez, Peyrot et al. 2008) which may explain the positive correlation between this type of ulceration and depression.

A cross-sectional study of 670 subjects used both odds ratio and logistic regression analysis to conclude that diabetes duration, cigarette smoking, aging and microalbuminuria showed a strong relationship with occurrence of foot ulcers in diabetes (Guerrero-Romero and Rodríguez-Morán 1998). These factors have all been shown to cause vascular damage, which is itself a predictor of diabetes foot ulceration. The findings from this study are supported by similar findings in several other studies (Delbridge, Appleberg et al. 1983; Apelqvist and Agardh 1992). In the large Eurodiale study (2007), clinical heart failure was
found to be risk factors that adversely affect foot ulcer healing rates. (Prompers, Huijberts et al. 2007).

2.2.4 Infection

Bacterial infection in a foot ulcer can be defined as a foot ulcer having observable clinical symptoms and/or signs of inflammation such as redness, swelling, heat, and when neuropathy is absent, pain. At least two of these features are thought to be required for a clinical diagnosis of foot ulcer infection (Boulton, Meneses et al. 1999). In addition, other features to indicate infection may be present such as cellulitis, purulent exudate, and/or sinus tracking and deep probing of an ulcer (Boulton, Meneses et al. 1999; Joseph, Lipsky et al. 2010). Underlying arterial insufficiency and neuropathy often diminish the classic features of infection of redness, heat and swelling. Therefore, vigilant screening for infection is necessary. ‘Secondary clues’ that may indicate infection including in a foot ulcer in a person with diabetes, are:

- those related to the ulcer: a positive probe-to-bone test; an ulcer present for >30 days; friable granulation tissue presence; wound edge undermining; copious and/or malodourous wound exudate; and a wound caused by trauma;
- patient related factors: history of recurrent ulcerations especially at a single site; and end-stage renal failure being present (Wounds International, 2013).

For many years, bacterial infection has been regarded as a complication of ulcers in people with diabetes and also as a factor that exacerbates ulcer healing as opposed to being a primary causal factor (Robson and Heggers 1970). It is estimated that 50-80% of foot ulcers in people with diabetes will become clinically infected. In addition, clinical infection portends a worse prognosis for healing - approximately 20% of patients with an infected foot ulcer will
require an amputation (Wu, Driver et al. 2007). Carlson and Reed (2003) found clinical ulcer infection even if not involving bone and especially abscess presence, or osteomyelitis to each be a risk factor for toe amputation (Carlson and Reed, 2003).

Bacterial presence, at least to a level of clinical infection, adversely affects ulcer healing (Jones, Edwards et al. 2004). Robson and Heggers (1970) reported that regardless of tissue type, any bacteria present at a level equal to or greater than $10^6$ organisms/g tissue will impair tissue healing (Robson and Heggers 1970), and this level of bacterial load is found in research studies when clinical infection is present (Edmonds and Foster 2004). According to data from the Australian Wound Management Association (AWMA 2011) the level of bacterial load in an ulcer necessary to cause clinical bacterial infection is also proportionate to virulence of the bacterial strain (AWMA 2011).

Data has linked bacterial colonising load in a wound and in its eschar with adverse outcomes. Chronic ulcers typically feature heavy bacterial colonisation (Schneidner, Vildozola et al. 1983; Dowd, Wolcott et al. 2008). Bacteria in these types of wound exist sub-clinically in adhesive biofilm communities. These biofilm communities are more resistant to anti-microbial therapy than acute wounds (Rhoads, Wolcott et al. 2008). James et al. (2008) found that 60% of chronic wounds were colonised by bacteria in a persistent biofilm, compared with only 6% of acute wounds (James, Swoggers et al, 2008). Foot ulcers are a common chronic wound which heal at a much slower rate than acute wounds, and limited data suggest that debriding a biofilm from a chronic wound, whether it be clinically infected or not, will aid ulcer healing possibly through reduction in the bacteria present in the biofilm (Rhoads, Wolcott et al. 2008). It is not known whether the bacterial burden found in most ulcers in diabetes is a consequence of the longevity of the wound, local wound hypoxic
conditions, neutrophil dysfunction as a deficit in bactericidal killing, and/or effects of the hyperglycaemia or its derivatives such as advanced glycation end-products (Falanga and Falanga 2005).

It remains unclear whether antibiotic therapy should be considered in clinically uninfected ulcers in people with diabetes. In one study a relationship between bacterial load and ulcer healing in diabetes was established from bacterial colony counts on aerobic blood agar plates derived from wound fluid obtained from post-debridement ulcer swabs. Results showed increased bacterial colony count positively correlated with reduced ulcer healing rate (Xu, McLennan et al. 2007). In that particular work most ulcers were infected clinically and the lack of high level data to support use of antibiotic treatment in clinically uninfected ulcers has led to recommendation in international guidelines of such antimicrobial use, either topically or systemically, for only clinically infected ulcers (IDF 2005). For the research presented in this thesis, an infected ulcer in diabetes is one that fulfils criteria for being clinically infected according to the Boulton group (1999) and stated at the beginning of this section (2.2.4) (Boulton, Meneses et al. 1999).

### 2.2.5 Classification of foot ulcers in diabetes

Systems exist to grade ulcers according to their characteristics and severity. These can assist with wound monitoring, treatment planning, comparing service delivery and in predicting patients ulcer healing outcomes (Oyibo, Jude et al. 2001). The table below (Table 2.3) shows a comparison of three commonly used wound classification systems.

---

- **Table 2.3**: Comparison of three wound classification systems.
  - **System A**: Characteristics and severity grading.
  - **System B**: Wound monitoring assistance.
  - **System C**: Treatment planning comparison.
  - **System D**: Service delivery prediction.
  - **System E**: Patients ulcer healing outcomes prediction.

---
Table 2.3: A comparison of wound classification systems.

<table>
<thead>
<tr>
<th>System: University of Texas</th>
<th>Description</th>
<th>Advantages/Disadvantages</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A 4-grade x 4-stage matrix to assess degree of infection and ischaemia.</td>
<td>Comprehensive description of ulcer types. Useful monitoring tool. Does not formally include ulcer depth.</td>
<td>Armstrong, Lavery et al. 1998</td>
</tr>
<tr>
<td>Wagner</td>
<td>A 6-grade system assessing ulcer depth, perfusion and infection.</td>
<td>Does not adequately address all aspects of infection and ischaemia. Some ulcers may fall between the 6 categories.</td>
<td>Wagner 1981</td>
</tr>
<tr>
<td>PEDIS</td>
<td>A descriptive table assessing perfusion, size, depth, infection and neuropathy using 4 grades.</td>
<td>User friendly, good for less experienced practitioners, but some ulcers may fall outside the stated parameters.</td>
<td>Schaper 2004</td>
</tr>
</tbody>
</table>

For the purposes of the research presented in this work, the validated University of Texas wound classification system has been used to categorise ulcers (Armstrong, Lavery et al. 1998). This system is viewed as comprehensive and was already adopted in the clinic where the study took place, meaning that both the staff and study personnel were familiar with its use and reliable data could be collected.

2.3 Wound Healing and impairments to the wound microenvironment commonly present in diabetes

Cutaneous wound healing is the complex multicellular process by which the skin repairs itself following injury. This process is highly organised and occurs as a series of events leading to barrier restoration and functional recovery of skin strength (Barrientos, Stojadinovic et al. 2008). In the case of wounded skin, healing of the dermis with wound closure occurs through re-epithelialisation and wound contraction (Inoue, Kratz et al. 1995; Sardari, Dehgan et al. 2006). Following haemostasis, normal wound healing can be depicted schematically with serial and overlapping phases, as shown in Figure 2.2.
At the cellular level, wound healing is commonly impaired in diabetes. Some of the events that are dysregulated in wound healing in diabetes are depicted schematically in Figure 2.3. The following text section (2.3) will address these phases in detail, focusing on healing in both ‘normal’ and ‘diabetes’ situations.

**Figure 2.2** Schematic diagram showing the three main stages of normal wound healing in humans following the ‘haemostasis’ stage. The time course reflects healing by secondary intention in a cutaneous wound, with re-epithelialisation occurring in the later proliferative phase, as described in the text section 2.3. Adapted from Falanga (Bentzen 2006).

<table>
<thead>
<tr>
<th>Time</th>
<th>Phases</th>
<th>Main cell types</th>
<th>Specific events</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hours</td>
<td>HAEMOSTASIS</td>
<td>Platelets</td>
<td>Platelet aggregation and release of fibrinogen fragments and other proinflammatory mediators</td>
</tr>
<tr>
<td></td>
<td>Fibrin plug formation,</td>
<td>Neutrophils, monocytes</td>
<td>Selectins slow down blood cells and binding to integrins → diapedesis</td>
</tr>
<tr>
<td></td>
<td>Release of growth factors,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytokines, hypoxia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days</td>
<td>INFLAMMATION</td>
<td>Macrophages</td>
<td>Hemidesmosome breakdown → keratinocyte migration</td>
</tr>
<tr>
<td></td>
<td>Cell recruitment and chemotaxis, wound</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>debridement</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weeks to</td>
<td>PROLIFERATION</td>
<td>Keratinocytes, fibroblasts,</td>
<td>Cross-talk between MMPs, integrins, cells cytokines → cell migration,</td>
</tr>
<tr>
<td>months</td>
<td></td>
<td>endothelial cells</td>
<td>ECM production</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Myofibroblasts</td>
<td>Phenotypic switch to myofibroblasts from fibroblasts</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>REMODELLING</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Scar formation and revision,</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ECM degradation, further contraction and</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>tensile strength</td>
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<td></td>
</tr>
</tbody>
</table>

Table: Phases, Main cell types, Specific events.
2.3.1 Haemostasis

The most immediate event following wounding is haemostasis. Platelets flow from injured blood vessels and adhere to surfaces of the wound, especially to collagen (via carbohydrate molecules on the collagen surface) (Gentry 1992). Adenosine diphosphate and 5-HT (serotonin) are then released from granules within platelets, allowing them to aggregate via expression of ‘sticky’ glycoproteins on their cell membranes (Gentry 1992). The aggregated platelets are not strong enough to sustain haemostasis but this early clumping causes phospholipid platelet factor 3 to be revealed on the platelet surface. Phospholipid platelet factor 3 is central to the conversion of prothrombin to thrombin and subsequent haemostatic fibrin plug formation. The resulting plug maintains structural support prior to the deposition of collagen (Midwood, Williams et al. 2004).
2.3.2 Inflammatory phase

Wound inflammation is characterised by the sequential recruitment of immune cells, release of inflammatory chemokines and cytokines and local infiltration of leukocytes (Stadelmann, Digenis et al. 1998). Inflammatory mediators such as prostaglandins and thromboxanes are released from ruptured cell membranes. This causes spasm of local blood vessels, which stems loss of blood and prevents removal of essential inflammatory cells from the area (Stadelmann, Digenis et al. 1998). After a short time lapse, the vasoconstriction is reversed, largely by the action of histamine, leading to vasodilation (Witte and Barbul 1997). Histamine also increases vascular permeability, allowing larger proteins and inflammatory leukocytes to cross the vessel wall. The subsequent movement of proteins into the extravascular area increases the osmolarity of the wounded tissue. Thus, water is drawn into the wound area along with the larger proteins, causing a localised oedema.

The plasminogen activation system potentiates the early inflammatory response via intra-cellular signalling and the induction of cytokines (Ploplis, French et al. 1998; Lighvani, Baik et al. 2011). Damaged cells release intracellular components such as heat shock proteins and transcription factors, which act to limit blood loss and attract anti-inflammatory cells to the wounded area (Nathan 2002). Under the influence of chemotactic molecules, leukotrienes and prostaglandins are synthesised and work synergistically to attract leukocytes, particularly monocytes to the site of injury (Ploplis, French et al. 1998; Lighvani, Baik et al. 2011).

Various studies have reported abnormalities in chronic wounds in diabetes compared with acute wounds. Reduced leukocyte numbers, especially monocytes, are noted in wounds in diabetes; this is attributable to defective chemotaxis and inhibition of proliferation (Pierce 2001). Phenotypic changes in the leukocytes for example their increased the generation of
cytokines such as TNF-α, IL-1, and IL-6 and enhanced the production of O₂⁻ (Ding, Kantarci et al. 2007; Brownlee, Cerami et al. 1988) also contribute to impaired wound healing, although the underlying mechanisms are largely unknown (Loots, Lamme et al. 1998). Elevated levels of chemokines and proinflammatory cytokines observed in wounds in diabetes may result in changes in the ECM composition that impede the healing process (Agren, Werthen et al. 2007). Rather than progressing through the usual phases of wound healing, wounds in diabetes become ‘fixed’ predominantly in the inflammatory phase (Agren, Werthen et al. 2007). This occurs because the normal feedback mechanisms that down-regulate the inflammatory stage are impaired, leading to an increased and persistent inflammatory response. These abnormalities are explored below.

Fibronectin, a major component of ECM, plays a role in the regulation of MMPs. The relative deficiency of fibronectin in chronic wounds is thought to be related to increased degradation rather than decreased synthesis. This is illustrated by the finding that the level of fibronectin mRNA is stable while fibronectin degradation products are widespread in wound fluid from subjects with diabetes, (Ongenae, Phillips et al. 2000). The deficiency in fibronectin results in an imbalance in levels of MMPs and their tissue inhibitors, the TIMPs, which contributes to impaired wound healing (Lobmann, Ambrosch et al. 2002). Browse et al. (1982, 1998) postulated that impaired healing in chronic wounds is partially attributable to the development of a fibrin peri-wound cuff (Browse and Burnand 1982; Browse 1988). The cuff, a result of abnormal fibrinolytic activity, prevents gaseous exchange and traps some leukocytes, which in turn release cytokines, growth factors and platelet activating factor at the wound periphery. These factors further impair wound healing through their local effects on vascular permeability and by causing endothelial damage (Browse and Burnand 1982; Browse 1988)
2.3.2.1 Neutrophil infiltration

Neutrophils are the earliest leukocytes to infiltrate the wound site from the blood (Singer and Clark 1999). They migrate rapidly, within hours, to the wounded area, attracted by fibronectin and growth factors including platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) released from platelets and kinins such as complement component 5a and platelet activation factor. Neutrophils release pro-inflammatory factors such as interleukins -6 and -8 and macrophage chemoattractant protein-1, 4 to 12 hours post wounding (Kondo, Ohshima et al. 2002). Chemoattractant release by neutrophils leads to an influx of monocytes, which explains the sequential appearance of these two types of leukocytes in acute inflammation. The inflammatory actions of neutrophils are regulated by mast cells.

In normal wounds, neutrophils are scarce after 72 hours; however, neutrophils persist in wounds in diabetes, prolonging the inflammatory state (Wetzler, Kampfer et al. 2000). The excess of neutrophils, combined with decreased TGF-β and insulin-like growth factor IGF-I, leads to a persistent inflammatory wound environment with the accumulation of excessive amounts of MMPs, especially MMP-1 and MMP-9 (Lobmann, Ambrosch et al. 2002). These MMPs are not matched in quantity by their regulators, the TIMPs. This inflamed, protease-enhanced environment leads to uninhibited tissue degradation.

The capability of macrophages to phagocytose neutrophils, which is typically a key landmark in the conclusion of the inflammatory stage, is impaired in wounds in diabetes (Lecube, Pachon et al. 2011). In diabetes this deficit in wound macrophage phagocytosis of neutrophils is at least partly attributable to the increased presence of pro-inflammatory
mediators, which as described below, maintains an inflammatory rather than a reparative macrophage phenotype (Lecube, Pachon et al. 2011).

2.3.2.2 Macrophage infiltration

In response to chemoattractants such as TGF-β and fragments of ECM as well as neutrophils in wounds, monocytes migrate into wounds where, under the influence of pro-inflammatory mediators, they become activated macrophages (DiPetro 1995). In diabetes wounds exhibit a persistence of inflammation by neutrophils persisting in the wound and a relative lack in macrophages (Ochoa, Torres et al. 2007). The macrophage lack may in part be due to diminished plasminogen in the early inflammatory response: Shen et al. (2012), showed that administering plasminogen to diabetic mice normalised the relative inflammatory cell numbers (Shen, Guo et al. 2012).

Macrophages in normal healing appear to be derived mainly from circulating monocytes, which are themselves derived from bone marrow. Recent studies in mice by Brancato and Albina (2011) characterised distinct populations of circulating monocyte phenotypes that have the capacity to migrate to wounds. These monocytes displayed specific macrophage phenotypes upon activation, according to their monocyte precursor type (Brancato and Albina, 2011). Mesenchymal stem cells migrate from bone marrow to the wound area under the influence of various chemokines and differentiate into a variety of cells including macrophages (Sasaki, Abe et al. 2008).

Once a monocyte has entered a normal wound it differentiates into a macrophage and becomes activated. The activated macrophage has an increased oxygen requirement, it can bind to ECM via integrin receptors, it expresses higher levels of major histocompatibility
antigens, and has increased capacity for antigen presentation (Janeway, Travers et al. 2001). As a consequence of differentiation and activation the ability of macrophages to proliferate is diminished. Upon exposure to cytokines, danger-associated molecular patterns and interferons, macrophages acquire either M1 (inflammatory) or M2 (reparative) characteristics. Local helper T cells are instrumental in determining the proportions of inflammatory/reparative macrophages in a normal wound (Stout and Suttles 1997). The inflammatory type macrophage produces interleukins -1, -6, and -12 and TNFα and other chemoattractants (Koh and DiPietro 2011). As the normal wound inflammatory stage nears conclusion, the macrophage alters to a reparative cell state; this change is a result of ingestion of apoptotic neutrophils (Fadok, Bratton et al. 1998).

In wounds in diabetes this act of neutrophil phagocytosis by macrophages, which would normally cue macrophages to reprogramme themselves from inflammatory to reparative cells, is diminished and therefore macrophages continue in their inflammatory state in diabetes and inflammation persists in these wounds (Mosser and Edwards 2010).

2.3.2.3 Advanced glycation end products

Advanced glycation end products (AGEs) cause cellular ageing and have a range of detrimental effects on wound healing (Schmidt, Yan et al. 1999). AGEs are formed through a series of non-enzymatic reactions mainly between glucose and proteins. Accumulation of AGE proteins in wounds in diabetes is directly proportional to glycaemia and high glucose levels. The mechanisms by which AGEs cause cellular damage may include structural modification of proteins and stimulation of cellular responses via AGE receptors such as the receptor for AGEs, known as RAGE (Schmidt, Yan et al. 1999). AGEs may both bind to ECM and modify its function. Commonly this induces cellular oxidant stress pathways
(Schmidt, Yan et al. 1999). Preclinical and clinical data indicate that AGE activity in the wound microenvironment in diabetes can lead to dysregulation of: cytokines, MMPs, growth factor expression, and inflammatory cell infiltration and function (Schmidt, Yan et al. 1999; Goova, Kislinger et al. 2001).

2.3.2.4 Other Molecules in Wound Healing

Various growth factors, cytokines and proteases have been shown to regulate aspects of wound inflammatory processes in vitro, giving rise to the concept that these different classes of molecules also regulate important phases of wound healing in vivo. These molecules are discussed in Table 2.4.

<table>
<thead>
<tr>
<th>Molecule &amp; Origin</th>
<th>Function</th>
<th>Dysfunction in Diabetes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pro-inflammatory Cytokines and Neuropeptides</strong>&lt;br&gt;E.g. Substance P&lt;br&gt;Secreted by small nerve fibres</td>
<td>Sensitise local mast cells, and activate neutrophils and other leukocytes via the local release of histamine and arachidonic acid to produce chemokines and proinflammatory cytokines such as TNFα; increase the permeability of blood vessels to allow wound infiltration of macrophages. Substance P increases fibroblast proliferation via natural killer (NK) receptors, tachykinins promote angiogenesis in vivo and in vitro.</td>
<td>High levels of pro-inflammatory TNFα and also the neuropeptide substance P, persist in diabetic wounds. Landis et al. hypothesised that impaired neutrophil apoptosis could be attributable to elevated and prolonged TNFα expression.</td>
<td>Pradhan, Cai et al. 2011; Kurkowski, Kummer et al. 1990; Brain 1997; Landis, Evans et al. 2010; Nieto-Vazquez, Fernández-Veledo et al. 2008.</td>
</tr>
<tr>
<td><strong>Toll like receptors</strong> (TLRs)&lt;br&gt;E.g. TLR-3</td>
<td>TLRs recognise invading microbes and promote the production of inflammatory</td>
<td>Dasu et al. reported that increased TLR -2 activity prolonged inflammation in wounds in diabetes,</td>
<td>Kawai and Akira 2006; Macedo, Pinhal-Enfield et al.</td>
</tr>
<tr>
<td>Part of the innate immune system</td>
<td>cytokines, chemokines and interferons which are essential in the development of the adaptive immune response by upregulated antigen presenting cells. suggesting that in some cases TLRs in these wounds may be detrimental to wound healing.</td>
<td>2007; Dasu, Thangappan et al. 2010.</td>
<td></td>
</tr>
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</tbody>
</table>
| **Proteases**  
E.g. MMP-2 and MMP-9  
Secreted (e.g. collagenases, gelatinases, and stromelysins) or membrane-bound; require activation via catalytic cleavage to become fully active. | MMPs are synthesised as proenzymes. There are four tissue inhibitors of MMPs (TIMP-1 to TIMP-4), Leukocytes are both sources of, and targets for, pro-inflammatory cytokines such as IL-1β and TNFα, which are themselves mediators of Nuclear factor kappa beta (NFκB) and ROS.  
MMP levels are increased in the inflammatory phase of normal wound healing (Madlener, Parks et al. 1998). During the inflammatory phase in, for example, post-surgical wounds, expression of MMP-9 pro- and active forms is increased and its actions are largely pro-inflammatory.  
Levels of the activated form of MMP-9 correlate with neutrophil attraction to wounds through its ability to cleave IL-8.  
The excess of neutrophils, combined with decreased TGFβ and IGF-I, leads to the accumulation of excessive amounts of MMPs, especially MMP-9. These MMPs are not matched in quantity by their regulators, the tissue inhibitors of metalloproteinases (TIMPs), leading to uninhibited tissue degradation. High glucose concentrations also increase the levels of MMPs.  
A high ratio MMP-9 to TIMP-1 protein in post-debridement wound fluid predicts poor wound healing outcomes. Bennett and Schultz postulate that increased MMP levels in chronic wounds are responsible for the decrease in growth factors due to proteolytic activity of MMPs. | Madlener, Parks et al. 1998; Watelet, Claey s et al. 2004; McLennan, Bonner et al, 2008; Schleicher and Friess 2007; Liu, Min et al. 2007; Bennett and Schultz 1993; Ladwig, Robson et al. 2002. |
| **Reactive oxygen species (ROS)**  
E.g. 4-hydroxy-2-nonenal (a marker of ROS).  
Released by cells in response to degraded tissue fragments. | ROS have cytotoxic and signalling roles. They are crucial in wound healing. When present in high levels they cause oxidative stress, which subsequently exacerbates inflammation.  
In wounds in diabetes, ROS activate a number of pro-inflammatory pathways and cause defective angiogenesis in response to ischaemia. | Dissemond, Goos et al. 2002; Giacco, Brownlee et al. 2010. |
2.3.3 Proliferative phase

Inflammatory macrophages produce several growth factors such as PDGF, basic fibroblast growth factor (bFGF) and TGFβ (Rappolee, Mark et al. 1988). These attract fibroblasts and endothelial cells to wound sites and encourage proliferation of these cells (Katz, Alvarea et al. 1992).
al. 1991). Reparative macrophages help to remodel wound ECM; they produce IGF-I and PDGF. Macrophages and macrophage-derived growth factors are essential in initiating the formation of new tissue in wounds tissues and they assist in the transition between inflammation and repair (Leibovich and Ross 1975). It is this transition that is often delayed resulting in impaired healing in wounds in diabetes (Tellechea, Leal et al. 2010).

Macrophage signalling, especially in reparative macrophages, induces fibroplasia (Hunt, Knighton et al. 1984). The majority of fibroblasts found in wounds are recruited from local tissues although a small proportion are bone marrow-derived. Fibroblasts are responsible for the synthesis and deposition of the ECM and the remodelling of ECM into a collagenous matrix (Clark, Nielsen et al. 1995). Their presence marks the onset of the proliferative stage of healing.

A fibroblast is able to synthesise many ECM components including collagen, glycosaminoglycans, glycoproteins and elastic fibres. Fibroblast migration is governed by growth factors, primarily PDGF, which upregulates syndecan-4 (a proteoglycan) and other integrins in order to attract fibroblasts to the wounded area. In diabetes, fibroblast proliferation is reduced, causing a delay in healing (Loots, Lamme et al. 1999). This extent of this proliferative defect is proportional to hyperglycaemia (Hehenburger, Heilbom et al. 1998).

Growth factor degradation through proteolysis is quite common in subjects with diabetes (Roth, Piekarek et al. 2006) reflecting the increased inflammatory and protease active environment in these wounds (Wetzler, Kampfer et al. 2000). This notion is supported by Wlaschek et al (1997), who showed that the most prominent isoform of PDGF is degraded by
wound fluid from chronic venous leg ulcers and that the loss of PDGF stability could be prevented by addition of specific protease inhibitors (Wlaschek, Peus et al. 1997).

Fibroblast-derived enzymes such as collagenases and stromelysin may partially proteolyse ECM in order to cleave pathways for cell migration. In the latter stages of healing, fibroblasts differentiate into more specialised, activated cells, termed myofibroblasts, which have contractile and remodelling functions. The phenotypic transition of fibroblast to myofibroblast is precipitated by changes in the local mechanical microenvironment (Midgley, Rodgers et al. 2013). Fibroblasts are usually protected from cellular invasion by foreign bodies and by proteolytic degradation by cross-linked fibres within the ECM. However, when tissue is injured, this protection is lost as the ECM is continuously remodelled and fibroblasts acquire contractile stress fibres. The acquisition of these stress fibres is the intermediate step between fibroblast and myofibroblast, and at this stage the cell is commonly referred to as the ‘protomyofibroblast’ (Tomasek, Gabbiani et al. 2002). When granulation is complete fibroblasts die by apoptosis (Akasaka, Ono et al. 2010) and the cell-rich granulation tissue transforms into a relatively acellular collagenous tissue.

Once granulation tissue has developed, keratinocytes lose their anchorage to basement membranes and under the influence of multiple growth factors such as epithelial growth factor (EGF) and IGF-I migrate into wounds for the purpose of re-epithelialisation (Nickoloff, Mitra et al. 1988). VEGF is a key growth factor in the proliferative phase. It induces endothelial cell proliferation and migration and vasopermeability which is required for wound healing (Aiello and Wong 2000). VEGF protein levels are reduced in chronic non-healing wounds due to protease activity (Lauer, Solberg et al. 2000). Motility and proliferation of keratinocytes is controlled by chemicals such as nitric oxide and by growth
factors (Witte, Barbul et al. 2002). More recent studies have shown that contact with fibroblasts stimulates migration and proliferation of keratinocytes, and that without such contact keratinocyte proliferation slows significantly (Wang, Wang et al. 2012).

Angiogenesis must occur concurrently with fibroblast, keratinocyte and endothelial cell proliferation as this proliferation is dependent on the local availability of oxygen. Angiogenesis involves the activation of local endothelial cells and circulating endothelial progenitor cells by growth factors such as VEGF. Under the influence of these angiogenic factors, endothelial cells develop pseudopodia and migrate through the ECM to form new blood vessels. Fibrin is a potent promoter of endothelial cell migration (Potter, Linge et al. 2006) and has also been implicated in angiogenesis. MMPs contribute to angiogenesis by digesting basement membrane, which permits cell proliferation and angiogenesis (Lansdown, Sampson et al. 2001). In a hypoxic environment macrophages and platelets produce angiogenic factors to promote angiogenesis. These factors are then switched off when sufficient blood flow is restored to reverse the hypoxia (Greenhalgh 1998). Simultaneously endothelial cell migration and proliferation is reduced when perfusion is adequate.

2.3.4 Wound contraction and remodelling

Following the proliferative stage, a wound must undergo a period of remodelling in order to acquire tensile strength. Collagen molecules replace the ECM proteins; the predominant type of collagen switches from type III in early wounding to type I later (Robins, Milne et al. 2003). Collagen fibrils become stabilised via inter and intra-molecular cross linking and proteoglycans organise the collagen and contribute to tensile strength. This process may take many months and, in comparison to normal skin, the wounded area will always have a tensile strength deficit (Robins, Milne et al. 2003).
As normal wounding is resolved, cells associated with later wound healing such as myofibroblasts, undergo apoptosis and are phagocytosed. Redundant blood vessels regress and wound contracture is completed (Robins, Milne et al. 2003). Wound contraction and remodelling is mediated by myofibroblasts, which give wound tissue muscle-like characteristics of contraction and resistance to deformation, as first described by Abercrombie (1956) (Abercrombie, James et al. 1956). Another phenotypic hallmark of the myofibroblast is its expression of high levels of α-smooth muscle actin (αSMA) (Darby, Skalli et al. 1990). Due to its abundance in myofibroblasts, αSMA is the most commonly used molecular marker for myofibroblasts. For this reason αSMA was used as a parameter to quantitate wound healing in the studies reported in this thesis.

In diabetes, collagen levels are markedly reduced due to both decreased collagen synthesis and increased collagen degradation (Spanheimer, Umpierrez et al. 1988). This contributes to the delay in overall healing, and a loss of tensile strength in foot ulcers in diabetes (Spanheimer, Umpierrez et al. 1988). The presence of advanced glycation of ECM in diabetes is thought to contribute to the increased ‘stiffness’ in intact skin and in healed wounds in diabetes with an associated loss of wound tensile strength (Spanheimer, Umpierrez et al. 1988).

An overview of normal wound healing and the factors that contribute to impaired healing in the wound in diabetes has been presented. The aim of this work was to explore the possibility of improving wound healing using topical therapies. Currently many therapies exist whose purpose is to enhance wound healing in diabetes. The following section discusses these therapies.
2.4 Current therapies in foot ulceration in diabetes

Foot ulcers in diabetes are chronic and complex wounds that often have a profound and sometimes devastating impact on the quality of life of the sufferer and cause a significant financial burden to healthcare providers. For this reason there is an urgent need to improve wound healing in people with diabetes. Current treatment regimens, as discussed in section 2.1.2 include offloading, debridement, dressing, infection control and revascularisation (Kruse and Edelman 2006). Other therapies including negative wound pressure (NWPT), vacuum assisted closure (VAC), skin substitutes, hyperbaric oxygen and various growth factor therapies (Hopf, Humphrey et al. 2001). Autologous platelet plasma gel (Driver, Hanft et al. 2006), ultrasound (Ennis, Foremann et al. 2005) and various autologous engineered scaffoldings (Uccioli, Giurato et al. 2011) have also been investigated as potential wound healing therapies and are discussed in this section.

In Australia the National Health and Medical Research Council (NHMRC) National Evidence-Based Guideline for the Prevention, Identification and Management of Foot Complications in Diabetes’ (NHMRC 2011) have graded the evidence of current wound healing interventions based on NHMRC guidelines, (NHMRC 2009). In order for continuity when assigning grades of recommendation, the NHMRC has formulated descriptors of the type of evidence that can be attributed to each grade of recommendation. These guidelines are aimed at facilitating clinical decision making for health professionals in order to optimise patient outcomes, and are shown in Table 2.5.
Table 2.5 Definition of NHMRC grades of recommendations.

<table>
<thead>
<tr>
<th>Grade of recommendation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Body of evidence can be trusted to guide practice.</td>
</tr>
<tr>
<td>B</td>
<td>Body of evidence can be trusted to guide practice in most situations.</td>
</tr>
<tr>
<td>C</td>
<td>Body of evidence provides some support for recommendation(s) but care should be taken in application.</td>
</tr>
<tr>
<td>D</td>
<td>Body of evidence is weak and recommendation must be applied with caution.</td>
</tr>
</tbody>
</table>

Adapted from National Health and Medical Research Council (NHMRC 2009).

National Evidence-Based Guideline for the Prevention, Identification and Management of Foot Complications in Diabetes wound care guideline is shown in Table 2.6 (NHMRC 2011).

In general, Grade A or B Recommendations are high levels of Recommendation, whilst Grade C or D are much weaker Grades.

NHMRC guidelines state that:

‘Grades of recommendation are intended to indicate the strength of the body of evidence underpinning the recommendation. This should assist users of the clinical practice guidelines to make appropriate and informed clinical judgments. Grade A or B recommendations are generally based on a body of evidence that can be trusted to guide clinical practice, whereas Grades C or D recommendations must be applied carefully to individual clinical and organisational circumstances and should be interpreted with care’ (NHMRC 2009).
Table 2.6: Synopsis of NHMRC guidelines regarding established wound care practices.

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Description</th>
<th>Level of Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Topical hydrogel dressings</strong></td>
<td>May be considered for autolytic debridement to assist the management of non-ischaemic, non-healing ulcers with dry, non-viable tissue.</td>
<td>Grade B</td>
</tr>
<tr>
<td><strong>Pressure reduction</strong></td>
<td>Otherwise referred to as redistribution of pressure or offloading, is required to optimise the healing of plantar foot ulcers.</td>
<td>Grade B</td>
</tr>
<tr>
<td><strong>Total contact cast or other device rendered irremovable</strong></td>
<td>Offloading of the wound can be achieved with the use of a total contact cast or other device rendered irremovable.</td>
<td>Grade B</td>
</tr>
<tr>
<td><strong>Topical negative pressure therapy</strong></td>
<td>May be considered for foot ulcers in specialist centres, as part of a comprehensive wound management program.</td>
<td>Grade B</td>
</tr>
<tr>
<td><strong>Hyperbaric oxygen therapy</strong></td>
<td>May be considered for foot ulcers in specialist centres, as part of a comprehensive wound management program.</td>
<td>Grade B</td>
</tr>
<tr>
<td><strong>Larval therapy</strong></td>
<td>May be considered for foot ulcers in specialist centres, as part of a comprehensive wound management program.</td>
<td>Grade C</td>
</tr>
<tr>
<td><strong>Skin replacement therapies</strong></td>
<td>May be considered for foot ulcers in specialist centres, as part of a comprehensive wound management program.</td>
<td>Grade B</td>
</tr>
<tr>
<td>• <strong>Cultured skin equivalents</strong></td>
<td></td>
<td>Grade B</td>
</tr>
<tr>
<td>• <strong>Skin grafting</strong></td>
<td></td>
<td>Grade D</td>
</tr>
</tbody>
</table>

Adapted from The National Evidence-Based Guidelines: Prevention, Identification and Management of Foot Complications in Diabetes, (NHMRC 2011).
As yet no therapy has attained an ‘A’ grading, indicating that the pinnacle of wound healing therapies has yet to be reached. These therapies along with other emerging wound treatments are discussed in detail in the remainder of this chapter.

2.4.1 Dressings

Most dressings are designed to create and maintain a moist wound healing environment, which is optimal for wound healing (Bishop, Walker et al. 2003). Factors that must be considered when choosing a wound dressing include:

- Location and size of wound
- Amount and type of exudate
- Type of wound tissue and periwound tissue
- Compatibility with other therapies (e.g. casts)
- Wound bioburden/infection risk
- Patient pain, quality of life and wellbeing
- Cost and ease of application

The table below is a synopsis of the types of dressings available. Briefly, alginates, foams, polyhexamethylene biguanide (PHMB) and silicones are useful for heavily exudating wounds due to their absorbent capacity. Honey, silver, PHMB and iodine are useful for critically colonised wounds; however, systemic antibiotic therapy is necessary when a wound is clinically infected or increasing in size (Brown 2006).

<table>
<thead>
<tr>
<th>Type</th>
<th>Actions</th>
<th>Indications/use</th>
<th>Precautions/contraindication</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Alginites</strong></td>
<td>Absorb fluid</td>
<td>For use in moderate to highly exuding wounds</td>
<td>Do not use on dry/necrotic wounds Use with caution on friable tissue (may cause bleeding)</td>
</tr>
<tr>
<td></td>
<td>Promote autolytic debridement</td>
<td>Designed to be used to fill cavities, these dressings often exist in the form of rope or ribbon and may be combined with silver for antimicrobial activity</td>
<td>Do not pack cavity wounds tightly</td>
</tr>
<tr>
<td></td>
<td>Control moisture</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Conform to the wound bed</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Foams</strong></td>
<td>Absorb fluid</td>
<td>Moderate to high exuding wounds</td>
<td>Do not use on dry/necrotic wounds or those with minimal exudate</td>
</tr>
<tr>
<td></td>
<td>Deliver moisture control</td>
<td>Special cavity presentations in the form of strips or ribbon</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Conform to the wound bed</td>
<td>Low adherent versions available for patients with fragile skin</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Combined presentation with silver or PHMB for antimicrobial activity</td>
<td></td>
</tr>
<tr>
<td><strong>Honey</strong></td>
<td>Rehydrates the wound bed</td>
<td>Sloughy, low to moderate exuding wounds Critically colonised wounds or with clinical signs of wound infection</td>
<td>May cause 'drawing' pain (osmotic effect) Known sensitivity</td>
</tr>
<tr>
<td></td>
<td>Promotes autolytic debridement</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antimicrobial action</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hydrocolloids</strong></td>
<td>Absorb fluid</td>
<td>Clean, low to moderate exuding wounds Combined presentation with silver for antimicrobial activity</td>
<td>Do not use on dry/necrotic wounds or high exuding wounds May encourage over-granulation</td>
</tr>
<tr>
<td></td>
<td>Promote autolytic debridement</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hydrogels</strong></td>
<td>Rehydrate wound bed</td>
<td>Dry/low to moderate exuding wounds Combined presentation with silver for antimicrobial activity</td>
<td>Do not use on highly exuding wounds or where anaerobic infection is suspected May cause maceration</td>
</tr>
<tr>
<td></td>
<td>Control moisture</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Promote autolytic debridement</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Iodine</strong></td>
<td>Antimicrobial action</td>
<td>Critically colonised wounds or clinical signs of infection Low to high exuding wounds</td>
<td>Do not use on dry necrotic tissue Known sensitivity to iodine Short-term use recommended (risk of systemic absorption)</td>
</tr>
<tr>
<td><strong>Low-adherent wound contact layer (silicone)</strong></td>
<td>Protect new tissue growth Atraumatic to periwound skin Conformable to body</td>
<td>Low to high exuding wounds Use as contact layer on superficial low exuding wounds</td>
<td>May dry out if left in place for too long Known sensitivity to silicone</td>
</tr>
<tr>
<td><strong>PHMB</strong> (polyhexamethylene biguanide)</td>
<td>Antimicrobial action</td>
<td>Low to high exuding wounds Critically colonised wounds or clinical signs of infection May require secondary dressing</td>
<td>Do not use on dry/necrotic wounds Known sensitivity</td>
</tr>
<tr>
<td>Odour control (e.g. activated charcoal)</td>
<td>Odour absorption</td>
<td>Malodorous wounds (due to excess exudate) May require antimicrobial if odour due to increased bioburden</td>
<td>Do not use on dry wounds</td>
</tr>
<tr>
<td>Protease modulating</td>
<td>Active or passive control of wound protease levels is achieved</td>
<td>Clean wounds that are not progressing despite correction of underlying causes, exclusion of infection and optimal wound care</td>
<td>Do not use on dry wounds or those with leathery eschar</td>
</tr>
<tr>
<td>Silver</td>
<td>Antimicrobial action</td>
<td>Critically colonised wounds or clinical signs of infection Low to high exuding wounds Combined presentation with foam and alginates for increased absorbency.</td>
<td>Some may cause discolouration Known sensitivity Discontinue after 2 weeks if no improvement and re-evaluate</td>
</tr>
<tr>
<td>Polyurethane film</td>
<td>Moisture control Breathable bacterial barrier Transparent (allows visualisation of wound)</td>
<td>Primary dressing over superficial low exuding wounds Secondary dressing over alginate or hydrogel for rehydration of wound bed</td>
<td>Do not use on patients with fragile/compromised periwound skin Do not use on moderate to high exuding wounds</td>
</tr>
</tbody>
</table>

Other more advanced dressings (e.g. collagen and bioengineered tissue products) may be considered for wounds that are not healing adequately; these are discussed in section 2.4.6.

Dressings may be useful delivery vehicles for the wound healing therapies such as those tested in the studies within this thesis. Whilst wound dressings are useful in helping to optimise the wound environment and/or reducing bacterial burden, no single dressing fulfils all the requirements to heal a foot ulcer in diabetes (Hilton, Williams et al. 2004) and further research is warranted in this area.

### 2.4.2 Pressure reduction and total contact casts

As previously discussed, the feet of patients with peripheral neuropathy are subject to areas of high pressure forces that frequently result in ulceration. It is important to offload at-risk areas of the foot and redistribute these pressures more evenly. Total non-weight-bearing using crutches or a wheelchair provides effective offloading, but levels of patient compliance are poor. Shoes and in-shoe devices such as orthoses and insoles are well tolerated in the
ambulant patient but their offloading capacity is limited (16-52% offloading improvement compared to flat rubber-soled shoes) (Cavannah and Bus 2011).

It is generally accepted that the best ‘compromises’ between patient ambulation and offloading, are the total contact cast (TCC) and the removable cast walker (RCW). In clinical trials the RCW improved offloading by 64-92% compared to control rubber-soled shoes (Lavery, Vela et al. 1996). Subsequent studies by Armstrong et al. (2001) showed the TCC to be more effective in healing diabetic foot ulcers than the RCW or modified half shoe (Armstrong, Nguyen et al. 2001). Patients with removable devices wear them less and ambulate more than those issued with non-removable TCCs (Armstrong, Nguyen et al. 2001). The irremovable TCC is therefore considered the ‘gold standard’ of offloading, but it can complicate dressing changes. This problem is frequently circumvented by bi-valving the cast and fixing the two halves in place with bandaging, which facilitates removal of the cast for wound visualisation and dressing changes.

2.4.3 Negative wound pressure therapy (NWPT)

NWPT accelerates wound healing by increasing local blood flow, aiding the formation of granulation tissue and decreasing bacterial load (Argenta and Morykwas 1997). Multicentre studies showed NWPT to be more effective than advanced moist wound therapy (hydrogels) in healing diabetic wounds (Blume, Walters et al. 2008). NWPT over lower extremity wounds with exposed bone greatly reduced the amount of tissue oedema, thus decreasing the surface area of the wound as shown in in studies by DeFranzo et al. (2001) where profuse granulation tissue was observed to form rapidly in the wounds treated with NWPT and these complex wounds showed improved healing (DeFranzo, Argenta et al. 2001).
Due to the expensive and cumbersome nature of NWPT systems and issues with maintaining a good vacuum seal around the intricate architecture of the foot, NWPT is more useful in large, complex wounds with an ischaemic component rather than as an everyday therapeutic option for less complex ulcers.

2.4.4 Hyperbaric oxygen therapy (HBOT)

The therapeutic use of oxygen under pressure is known as hyperbaric oxygen therapy (HBOT) and has been used to assist wound healing. HBOT has been shown experimentally to improve wound tissue hypoxia, enhance perfusion, reduce oedema and down regulate inflammatory cytokines, whilst promoting fibroblast proliferation, collagen production and angiogenesis (Gill and Bell 2004). HBOT has also been proposed as a bactericidal/bacteriostatic treatment through its enhancement of the effects of anti-microbial drugs and its ability to restore leukocyte function by increasing tissue oxygen tension (Çimşit, Uzun et al. 2009).

There have been few trials of HBOT. The reported trials have been largely in favour of HBOT (Baroni, Porro et al. 1987; Abidia, Laden et al. 2003) but they have been consistently flawed in design and have not provided compelling evidence, being based mostly on case studies. Other limitations of these studies include small cohorts, poor therapy compliance, a lack of investigator or patient blinding, inadequate descriptions of the types of wounds enrolled, and disparities in allocation of treatment (Lipsky and Berendt 2010). Recently the Cochrane review (Kranke, Bennett et al. 2012) of nine HBOT clinical trials (of which 8 enrolled diabetic foot ulcers) concluded that:

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“In people with foot ulcers due to diabetes, HBOT significantly improved the ulcers healed in the short term but not the long term and the trials had various flaws in design and/or reporting that means we are not confident in the results. More trials are needed to properly evaluate HBOT in people with chronic wounds; these trials must be adequately powered and designed to minimise all kinds of bias.” (Kranke, Bennett et al. 2012).

This conclusion highlights the need for further research into this controversial and expensive therapy. It is likely that other topical therapies will in the long term be more cost effective and easier to administer than HBOT and unless HBOT shows remarkable results in high quality clinical trials it is unlikely to become a mainstream therapy.

2.4.5 Larval therapy

Also known as ‘maggot therapy’ or ‘biosurgery’, larval therapy is a popular tool for the debridement of sloughy and necrotic material, especially in painful ischaemic wounds. Larvae have two mechanisms of action. Firstly their mandibular hooks mechanically break down tissue. Secondly the larvae produce secretions containing proteolytic enzymes that further break down devitalised tissue into an ingestible form that the larvae then feed on (Chambers, Woodrow et al. 2003). Larval therapy is not a cosmetically or psychologically acceptable treatment for some individuals; however, the ability of larvae to debride most wounds in 3-5 days renders them both clinically and financially attractive as a wound therapy.

2.4.6 Skin substitutes

Bioengineered skin substitutes have been fabricated according to specific functional objectives (Boyce and Warden 2002), using both artificial and natural ECM proteins (Raeber,
Examples of artificial skin substitute materials include polyethylene glycol macromers, polylactide and poly-ethylene terephthalate. Natural materials used as artificial ECM include hyaluron, fibronectin, collagen and alginates.

Balasubramani et al (2001) classified skin substitutes into three classes according to their preparation and composition (Balasubramani, Kumar et al. 2001):

**Class I skin substitutes** consist of epidermal-equivalent-only, naturally occurring or biological dressing substitute, e.g. amniotic membrane or synthetic dressing substitute, e.g. synthetic polymer sheet (Tegaderm®, Opsite®), (Halim, Khoo et al. 2010).

**Class II skin substitutes** encompass dermal components from processed skin or fabricated with collagen and other matrix proteins e.g. Apligraft® (Organogenesis, Inc., Canton, MA, USA, and Novartis Pharmaceuticals Corp., East Hanover, NJ, USA) composed of type I bovine collagen and allogeneic keratinocytes and neonatal fibroblasts.

**Class III skin substitutes** possess distinct dermal and epidermal components and are popularly referred to as composite skin e.g. Dermagraft® (Advanced BioHealing, La Jolla, CA, USA) is a bioabsorbable polyglactin mesh seeded with allogeneic neonatal fibroblasts (Kumar 2008).

Problems and limitations with existing skin substitutes include impaired vascularisation due to poor anastamoses between graft and bed areas (O'Ceallaigh, Herrick et al. 2006); absence of differentiated structures such as sweat glands, hair follicles and pigment; and subsequent scarring. Skin substitutes also suffer from the high financial costs associated with research, development, cell sourcing and biocompatibility (Metcalf and Ferguson 2007). Therefore, this type of therapy is often overlooked in favour of less expensive topical wound treatments. Such scaffolds do, however, have the potential to be used as delivery vehicles for topical...
treatments such as growth factor therapies (Geer, Swartz et al. 2005; Wang, Leong et al. 2008).

Aside from existing wound treatments for diabetic foot ulcers, advanced wound healing therapies are constantly being developed as understanding of regenerative processes and potential interventions in wound healing advance. The following section explores the evidence and strength of emerging therapies that are still in various stages of research and development.

2.5 Emerging Wound Healing Therapies in Diabetes

2.5.1 Topical growth factors as therapy

Growth factors are polypeptide proteins that regulate cell proliferation, differentiation, organ growth and metabolism of target cells (Hopf, Humphrey et al. 2001). Studies conducted to test the efficacy of growth factors in treating foot ulcers in diabetes are summarised in the table below (Table 2.8) and the biologic actions of growth factors are discussed in detail in Sections 2.6 and 2.7.

Table 2.8: Studies of growth factors in human foot ulcer healing in diabetes.

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Study Title/Author</th>
<th>Experimental design/cohort</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic Fibroblast Growth Factor (bFGF)</td>
<td>Application of basic fibroblast growth factor may reverse wound healing impairment in diabetes. Robson, Phillips et al. 1992</td>
<td>Randomised, blinded, placebo-controlled human trial. n=50</td>
<td>Histologically, fibroblast and capillary counts were elevated in treated subjects, all of whom had longstanding pressure sores. More patients treated with bFGF achieved &gt; 70% wound closure (p &lt; 0.05).</td>
</tr>
<tr>
<td><strong>Epidermal Growth Factor (EGF)</strong></td>
<td>Intra-lesional injections of recombinant human epidermal growth factor promote granulation and healing in advanced foot ulcers in diabetes: multicentre, randomised, placebo-controlled, double-blind study.</td>
<td>Multicentre, double-blind, placebo-controlled trial. n=149</td>
<td>Granulation tissue covering ≥ 50% of the ulcer at 2 weeks was achieved by 19/48 controls versus 44/53 in the 75 µg EGF group [odds ratio (OR): 7.5; 95% confidence interval (CI): 2.9-18.9] and 34/48 in the 25 µg EGF group (OR: 3.7; 1.6-8.7). No drug-related severe adverse reactions were reported;</td>
</tr>
<tr>
<td>---</td>
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<td>---</td>
</tr>
<tr>
<td><strong>Granulocyte Colony Stimulating Factor (G-CSF)</strong></td>
<td>Effects of granulocyte-colony stimulating factor in the treatment of foot infection in diabetes.</td>
<td>Double-blind, placebo-controlled trial. n=30</td>
<td>Duration of hospitalization (26.9 +/- 2.0 vs. 28.3 days; P&gt;0.05, NS), duration of parenteral antibiotic administration (22.9 +/- 2.0 vs. 23.3 +/- 1.9 days, p &lt;0.05), time to resolution of infection (23.6 +/- 1.8 vs. 22.3 +/- 1.7 days, p &lt; 0.05), and need for amputation (13.3% vs. 20%), P&gt;0.05, NS) were similar between the G-CSF and the standard groups</td>
</tr>
<tr>
<td><strong>Trial of recombinant granulocyte colony-stimulating factor as adjunctive therapy for limb-threatening foot infection in diabetes.</strong></td>
<td>Randomised prospective controlled trial. n=40</td>
<td>The administration of G-CSF for 3 weeks as an adjunctive therapy for limb-threatening diabetic foot infection was associated with a lower rate of amputation within 9 weeks after the commencement of standard treatment (p≤0.038). No significant differences in wound closure were observed between the groups.</td>
<td></td>
</tr>
<tr>
<td>Platelet Derived Growth Factor (PDGF)</td>
<td>Efficacy of topical recombinant human platelet derived growth factor on wound healing in patients with chronic lower limb ulcers in diabetes. Jaiswal, Gambhir et al. 2010.</td>
<td>Randomised, blinded, placebo-controlled human trial. n=50</td>
<td>At the end of 10 weeks, 18 (72%) ulcers had healed in control group and 15 (60%) in test group (p &gt;0.05). Three ulcers in the control group showed &gt;75% reduction in size compared to 2 in the test group (p &gt;0.05). There was no statistically significant improvement in ulcer healing rates after the use of topically applied rhPDGF.</td>
</tr>
<tr>
<td>Clinical evaluation of recombinant human platelet – derived growth factor for the treatment of lower extremity ulcers in diabetes. Steed et al. 1995.</td>
<td>Double-blind, placebo-controlled, multicentre study. n=118</td>
<td>Twenty-nine (48%) of 61 patients randomized to the rhPDGF-BB homodimer group achieved complete wound healing during the study compared with only 14 (25%) of 57 patients randomized to the placebo group (p=0.01). The median reduction in wound area in the group given rhPDGF-BB was 98.8% compared with 82.1% in the group given placebo (p =0.09). There were no significant differences in the incidence or severity of adverse events between the rhPDGF-BB and placebo groups.</td>
<td></td>
</tr>
<tr>
<td>Randomised clinical trial comparing OASIS wound matrix to Regranex (PDGF) gel for ulcers in diabetes. Niezgoda, Van Gils et al. 2005.</td>
<td>Randomised, prospective, controlled multicentre trial at 9 outpatient wound care clinics. n=73</td>
<td>After 12 weeks of treatment, 18 (49%) OASIS-treated patients had complete wound closure compared with 10 (28%) Regranex-treated patients. The sample size was not large enough to demonstrate that the incidence of healing in the OASIS group was statistically superior (p =0.055). The study showed that treatment with OASIS is as effective as Regranex in healing full-thickness diabetic foot ulcers by 12 weeks.</td>
<td></td>
</tr>
<tr>
<td>Effectiveness of recombinant human platelet-derived growth factor for the treatment of diabetic neuropathic</td>
<td>Retrospective cohort study. n=24,898 of whom 9.6%</td>
<td>RhPDGF was more effective than standard therapy in both helping a wound to heal (relative risk (RR) was 1.32 [1.22, 1.38] and preventing amputation, RR</td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Methodology</td>
<td>Results</td>
<td></td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Efficacy and safety of a topical gel formulation of Recombinant Human Platelet-Derived Growth Factor-BB (Becaplermin) in patients with chronic neuropathic diabetic ulcers.</td>
<td>A phase III randomised placebo-controlled double-blind study. n=382</td>
<td>Compared with placebo gel, becaplermin gel 100 µg/g significantly increased the incidence of complete wound closure by 43% (50 vs. 35%, p = 0.007) and decreased the time to achieve complete wound closure by 32% (86 vs. 127 days; estimated 35th percentile, p = 0.013).</td>
<td></td>
</tr>
<tr>
<td>Efficacy of recombinant human platelet-derived growth factor (rhPDGF) based gel in diabetic foot ulcers: A randomised, multicentre, double-blind, placebo-controlled study in India.</td>
<td>A randomised, multicentre, double-blind, placebo-controlled study. n=111</td>
<td>Efficacy evaluations carried out at 10 and 20 weeks showed that a significantly higher (p &lt; 0.01) percentage of patients (40% higher at 10 weeks and 32% higher at 20 weeks) in the rhPDGF-based gel-treated group achieved complete healing compared to the placebo-treated group. The average time to healing was significantly shorter in the treatment group (46 days) compared to the placebo group (61 days) at 10 weeks (p&lt;0.001) and also significantly shorter in the treatment group (57 days) versus the placebo group (96 days) at 20 weeks (p &lt; 0.01). The incidence of adverse events was low in both groups.</td>
<td></td>
</tr>
<tr>
<td>Treatment of nonhealing foot ulcers in diabetes with a platelet-derived growth factor gene-activated matrix (GAM501): Results of a Phase 1/2 trial.</td>
<td>Pilot study to evaluate the safety, maximum tolerated dose, and preliminary biological activity of GAM501. n=15</td>
<td>Following treatment with a replication-defective adenovirus encoding (PDGF)-B, there were no apparent differences in wound closure between the doses. However, the study was underpowered.</td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Design</td>
<td>Results</td>
<td></td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>-------------------------------</td>
<td>-------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Recombinant human platelet-derived growth factor-BB (becaplermin) for healing chronic lower extremity ulcers in diabetes. Embil, Papp et al 2000.</td>
<td>Open-label clinical evaluation. n=134</td>
<td>Complete healing of ulcers was achieved in 57.5% of patients, with a mean time to closure of 63 days and a recurrence rate of 21% at 6 months. Of the potential factors affecting ulcer healing, only drug compliance (p &lt; 0.001), dressing compliance (p &lt; 0.01), the presence of infection (p &lt; 0.01), baseline ulcer area (p &lt; 0.05), and baseline total wound evaluation score (p &lt; 0.05) were significantly associated with healing. Results of this study further confirmed the efficacy and safety of becaplermin gel for the treatment of lower extremity diabetic ulcers.</td>
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<tr>
<td>Efficacy and safety of becaplermin (recombinant human platelet-derived growth factor-BB) in patients with nonhealing, lower extremity diabetic ulcers: a combined analysis of four randomised studies. Smeill, Wieman et al. 1999</td>
<td>Combined and separate analyses of four multicentre, randomised, parallel group studies n=922</td>
<td>Becaplermin gel-100 µg/g significantly increased (p=0.007) the probability of complete healing compared with placebo gel. Becaplermin gel-100 µg/g significantly decreased (p=0.01) the time to complete healing compared with placebo gel. Therefore, it is effective and well tolerated in patients with full thickness lower extremity ulcers in diabetes.</td>
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In general, it has been difficult to maintain full bioactivity of proteins applied to wounds due to protein instability in the protease-rich environment of the wound (Choate and Khavari 1997). This field of therapy has realised some clinical outcomes and holds promise (Buchberger, Follmann et al. 2010), but so far only one growth factor derived treatment, PDGF, has Therapeutic Goods Administration (TGA) approval.

PDGF-BB (marketed as Regranex) has FDA (Food and Drug Administration) approval in the US for the treatment of ‘lower extremity diabetic neuropathic ulcers that extend into the
subcutaneous tissue or beyond, and have adequate blood supply’ (Dailymed, 2011). A multicentre trial concluded that treatment with PDGF-BB-containing becaplermin gel at a dose of 100 µg/g once daily, in conjunction with good ulcer care, is an effective and well tolerated wound therapy in patients with diabetic foot ulcers. The treatment improved both the total number of ulcers healed and the overall wound healing rate (Smiell, Wieman et al. 1999; Embil, Papp et al. 2000; Senet 2004). Some studies have questioned the efficacy of PDGF-BB (Mandracchia 2001), including in a non-clinical trial, post-marketing setting (Papanas and Maltezos 2010). PDGF-BB has not been compared with other additional treatment modalities, especially bioengineered skin substitutes and extracellular matrix proteins (Papanas and Maltezos 2010). Growth factor in a liquid or gel form might not be the best approach to target the growth factor to the cells directly involved in wound healing (Margolis, Crombleholme et al. 2000). Studies incorporating PDGF with an adeno-associated viral vector have shown that this method of delivery can result in a prolonged and more localised delivery. Improved distribution of growth factors to wound sites may enhance their efficacy in the future (Chen and Giannobile 2002, Koria2012)

A post-marketing retrospective case-control study involving 1622 becaplermin initiators and 2809 matched control subjects did not indicate concern in long term (more than 6 years) follow-up (RR, 1.0; 95% CI, 0.5-2.3) the adjusted rate ratio for cancer mortality among those who received three or more tubes relative to those who received none was 5.2 (95% confidence interval 1.6-17.6) (Ziyadeh, Fife et al. 2011). The level of evidence in such studies is not high, due particularly to lack of randomisation and inadequate power. As an increased mortality rate secondary to cancer was reported in patients treated with more than three tubes of becaplermin, a boxed Product Information Warning for Regranex (becaplermin gel) has been required by the FDA (Papanas and Maltezos 2010). Current recommendations
indicate this agent should be used only when the anticipated benefits outweigh the potential harm, and it should be used, if at all, with caution in those with diagnosed systemic malignancy (Papanas and Maltezos 2010). These findings should be taken into account when developing other growth factors as wound therapies.

2.5.2 Autologous Platelet Rich Plasma (PRP)

Platelet-rich plasma (PRP) is defined as the portion of the plasma fraction of autologous blood having a platelet concentration above baseline (Mehta and Watson 2008). PRP has also been referred to as platelet-rich concentrate, autologous platelet gel, platelet-enriched plasma, and platelet releasate. It is a rich source of chemotactic and mitogenic growth factors (Steed, Goslen et al. 1992). Being autologous, PRP is safe to use as it is produced from the patients own blood as required (Lacci and Dardik 2010).

Few PRP studies exist that are scientifically rigorous; however, small-scale trials showed that PRP-treated wounds healed more quickly than controls (Knighton, Ciresi et al. 1986; Driver, Hanft et al. 2006; Saad Setta, Elshahat et al. 2011). Limitations to these studies included small sample size, manufacturer sponsorship and compliance issues. More research is warranted in this field.

2.5.3 Stem cells

The ability of stem cells to self-renew and give rise to subsequent generations of cells has led to them being targeted as therapies in many situations where regeneration is required, with varying degrees of success (Weissman 2000).
Stem cells have yet to be studied in human wound healing in diabetes. Studies in diabetic rats have shown that topical transplantation of a clonal population of embryonic stem cells \((5 \times 10^6)\) by injection enhanced diabetic wound healing during the early stage of healing. The rats also showed markedly elevated VEGF, EGF and fibronectin, suggesting that some of the effect of the embryonic stem cells was through induction of growth factors (Lee, Choi et al. 2011). Kim et al. (2012) found mesenchymal stem cells significantly enhanced diabetic wound healing and that topical proinflammatory reaction and suppression of CD45 expression was reduced in the mesenchymal stem cell group (Kim, Zhang et al. 2012). VEGF and EGF were also increased, in line with the findings of Lee et al. (2011) (Lee, Choi et al. 2011).

This promising data could, if confirmed, lead to wound regeneration through stem cell therapy in humans in the future, including autologous therapy. However, a greater understanding of the mechanisms of human stem cell differentiation is needed before such an approach using stem cells will be widely accepted as a wound healing therapy.

### 2.5.4 Other Peptides/Proteins

Some bioactive peptides have been shown to successfully stimulate wound healing in diabetes: a selection of those that have been deemed to show potential in early stage development are discussed below.

#### 2.5.4.1 Activated protein C

Activated protein C (APC) is a plasma protease derived from its precursor, protein C, which circulates in plasma. APC has anti-inflammatory and pro-apoptotic properties and contributes to wound healing, particularly in the granulation and remodelling phases (Jackson, Xue et al.
2005; Whitmont, Fulcher et al. 2013). A pilot study showed APC to be an effective treatment for longstanding venous ulcers that were resistant to conventional treatment (Whitmont, Reid et al. 2008). Recent studies have shown diabetic wounds to have low levels of protein C compared with controls (Whitmont, Fulcher et al. 2013). This finding combined with the known pro-angiogenic properties of APC (Xue, Thompson et al. 2006) makes it a particularly attractive treatment for complex diabetic and ischaemic ulcers.

2.5.4.2 Vitronectin complexes

The ECM protein vitronectin is an adhesive glycoprotein, abundant in blood plasma and different ECM sites. Particularly upon tissue injury/repair and remodelling, vitronectin acts as a potent matricellular factor, coordinating cell migration with pericellular proteolysis and growth factor signalling at sites of tissue remodelling (Preissner and Reuning 2011; Upton, Cuttle et al. 2008).

Upton et al. (2011), hypothesised that because wound healing is driven by interactions between ECM proteins and growth factors, not just by growth factors alone, a vitronectin/growth factor complex might enhance wound healing (Upton, Wallace et al. 2011). A single arm pilot study using chronic wounds supported this hypothesis (Upton, Wallace et al. 2011).

2.5.4.3 Nor Leu

NorLeu\(^3\)-A(1-7) is an analogue of the naturally occurring peptide, angiotensin 1-7. Its mechanisms of action when administered topically include induction of progenitor proliferation (such as epidermal stem cells and hematopoetic progenitors which then
differentiate into different, more specialised cell types) and accelerated vascularisation, collagen deposition and re-epithelialisation (Rodgers, Verco et al. 2011).

NorLeu$^3$-A(1-7) increased flap survival in a nicotine-induced mouse skin wound model by promoting angiogenesis (Baykan, Gunay et al. 2012). Administration of NorLeu$^3$-A(1-7) peptides in diabetic mice accelerated collagen deposition by 2- to 6-fold, increased rates of re-epithelialisation and induced angiogenesis. Increased infiltration of the wound by macrophages and neutrophils was also observed at day four (Rodgers, Roda et al. 2003).

These positive animal studies led to a human pilot study, which concluded that topical NorLeu$^3$-A(1-7) is safe and effective in the healing of foot ulcers in subjects with diabetes and that a larger scale, higher level of evidence study of this therapy is justified (Balingit, Armstrong et al. 2012).

2.5.5 Anti-inflammatory therapies in foot ulceration in diabetes

Given that inflammation typically persists in diabetic foot ulcers, there is much interest in anti-inflammatory therapies. However there is no history of large-scale human trials of primary anti-inflammatory therapy for diabetic foot ulcers (Falanga and Falanga 2005; Eldor, Raz et al. 2004). Topical agents that have mainly anti-inflammatory effects and that have been examined in limited pilot studies in human diabetic foot ulcers include curcumin (Appendino, Belcaro et al. 2011), bark extract Bensal HP (Jacobs and Tomczak 2008) and natural honey (Makhdoom, Khan et al. 2009). In each case, the agent was quite well tolerated, but due to study design and numbers enrolled, no firm conclusions about efficacy could be made.
Many therapies directed at healing diabetic wounds are currently in development, and other existing therapies such as NWPT and HBOT show potential but lack robust clinical evidence of a therapeutic effect in wound healing. Such therapies are frequently prohibitively expensive and logistically difficult to administer. It is therefore logical to investigate therapies that are less costly and can be easily administered as a topical agent. This thesis seeks to test two such therapies, the growth factor CTGF and the bee-hive product propolis, which are discussed in sections 2.7 and 2.8.

The following section discusses the role of growth factors in wound healing in order to provide context to the rationale for testing CTGF as a wound therapy.

2.6 Roles of growth factors in wound healing in diabetes

As an immediate response to injury, platelets release a variety of growth factors. These growth factors then attract essential cells and proteins to the wound area and thus stimulate tissue regeneration. During wound healing, growth factors are secreted by various cells such as macrophages, fibroblasts and endothelial cells and, as well as being important chemotactic agents, they regulate mitogenesis, angiogenesis and matrix production. Studies using a combination of PDGF and IGF-I topically applied to partial thickness porcine wounds, provide evidence that growth factors work synergistically in wound healing (Lynch, Colvin et al. 1989; Greenlagh, Albertson et al. 1993).

Growth factor levels are decreased in chronic wounds and wounds in diabetes. This is primarily due to protein degradation or sequestration, rather than reduced gene expression or a translational defect in protein production (Dvonch, Murphey et al. 1992; Brown, Breeden et
al. 1994; Bitar and Labbad 1996). Bennet and Schultz (1993) postulated that increased MMP levels in chronic wounds are responsible for the decrease in growth factors through proteolysis (Bennet and Schultz 1993). However, other studies have suggested that growth factors are not so much deficient as ‘trapped’ within the peripheral fibrin cuff that surrounds wound capillaries (Falanga, Moosa et al. 1987). Regardless of whether growth factors are absolutely deficient or merely trapped, there is much interest in the potential of exogenous growth factors to accelerate wound healing.

PDGF is released from the α-granules of platelets. It is a major mitogen, induces fibroblast and smooth muscle cell proliferation, is chemotactic to leukocytes, and has the ability to upregulate ECM deposition through stimulation of fibronectin and collagen synthesis (Heldin and Westermark 1999). As discussed in section 2.5.1, PDGF was the first growth factor to be approved for use in a wound, (Knighton, Ciresi et al. 1986), but it has attracted some controversy due to its carcinogenic potential and poor outcomes in some wound healing studies.

2.6.1 Basic fibroblast growth factor (bFGF)

bFGF has been approved in China for topical therapeutic wound use. It is chemotactic for inflammatory cells such as macrophages and neutrophils, and mitogenic to fibroblasts and keratinocytes (Fu, Sun et al. 2000). Importantly, it also has a pro-angiogenesis role via its proliferative effect on vascular smooth muscle and endothelial cells (Robson, Phillips et al. 1992).

Animal models of excisional wound healing by Hebda’s (1990) and Stenberg’s (1991) groups indicated the potential of bFGF to enhance wound healing and to overcome the inhibition to
wound contraction caused by bacteria in a healthy porcine model (Hebda, Klingbeil et al. 1990; Stenberg, Phillips et al. 1991). bFGF promotes the proliferation of almost all cells associated with wound healing (Richardson, Trinkaus-Randall et al. 1999). A gelatine sheet containing bFGF promoted neo-epithelialisation, granulation, angiogenesis and wound closure in mice (Miyoshi, Kawazoe et al. 2005). Reductions in angiogenesis and granulation tissue formation were observed in burn wound and infection wound models of diabetic mice compared to normal mice, and bFGF treatment restored these functions significantly (Okumura, Okuda et al. 1996).

2.6.2 Vascular Endothelial Growth Factor (VEGF)

In wounds, VEGF is produced by neutrophils, platelets and macrophages. Acting primarily through tyrosine kinase receptors expressed on endothelial cells, its primary function in wounds is the induction and maintenance of wound vasculature (Carmeliet 2000). Actions of VEGF include increases to the influx of inflammatory cells, migration and proliferation of endothelial cells, and vascular permeability. There are five isoforms of VEGF: VEGF, VEGF-b, VEGF-c, VEGF-d and Placenta Growth Factor (Saaristo, Tammela et al. 2006). The bioactivity of VEGF is decreased in diabetic wound, which is thought to be a consequence of the highly proteolytic wound environment in such wounds (Roth, Piekarek et al. 2006).

Diminished production of VEGF and decreased angiogenesis are thought to contribute to impaired tissue repair in diabetic patients (Galiano, Tepper et al. 2004). In one study, VEGF-treated wounds in diabetic mice demonstrated increased epithelialisation, increased matrix deposition and enhanced cellular proliferation, which was associated with double the rate of healing compared to untreated mice (Galiano, Tepper et al. 2004). These findings were
supported by Saaristo et al. (2006) who showed that VEGF-c enhanced angiogenesis and lymphangiogenesis in wounds in diabetic mice and significantly accelerated wound healing (Saaristo, Tammela et al. 2006).

It is likely that as well as acting directly on the local vasculature, VEGF mobilises bone marrow-derived endothelial progenitor cells, which also contribute to angiogenesis (Asahara, Takahashi et al. 1999). The function of endothelial progenitor cells is compromised in people with diabetes, which contributes to the higher rate of vascular defects in such patients (Tepper, Galiano et al. 2002). Therefore, it seems plausible that VEGF therapy in diabetics might be able to reverse deficits in the function of endothelial progenitor cells function.

2.6.3 Epidermal growth factor (EGF)

EGF is an essential growth factor for epithelial cell proliferation and wound healing (Huo, Qiu et al. 2009). Released initially from α-granules of platelets and subsequently by macrophages and keratinocytes (Loot, Kenter et al. 2002) EGF promotes mitogenesis of fibroblasts, endothelial cells and keratinocytes. EGF in gelatine-microsphere dressings improved healing in both diabetic and non-diabetic rats (Dogan, Demirer et al. 2009). A US-based study showed topical EGF to have a moderately positive effect on wound healing, enhancing burn wound healing by 1-4 days (Brown, Breeden et al. 1994). However, a similar study by Cohen et al. (1995) found that EGF had no demonstrable accelerative effect on healing (Cohen, Crossland et al. 1995).

2.6.4 Transforming growth factor –beta (TGF-β)

TGF-β has been implicated widely in wound healing. It has a broad spectrum of actions, including cell migration and proliferation, synthesis of ECM, angiogenesis and remodelling
TGFβ affects all cell types that are involved in wound healing and is present in high concentrations in platelet α-granules and it is also present in T-lymphocytes and monocytes (Wang, Han et al. 2006). Of its three isoforms, TGFβ type 1 is most common in human wounds (representing 85% of TGFβ found in wound fluid). It is notable that the type 2 isotype predominates in fetal tissues which do not scar (Longaker, Bouhana et al. 1994). Cowin et al. (2001) found low levels of TGF-beta1 and TGF-beta2 in fetal murine wounds compared with adults and corresponding low rates of scarring in the fetal tissue (Cowin, Holmes et al. 2001). This indicates that the expression of TGF-beta and its receptors in adult and fetal wounds could be important in the absence of scar formation that is observed in the fetus. TGFβ has perhaps the most broad spectrum of action of all the known growth factors. It plays a central role in angiogenesis by recruiting and activating inflammatory cells that secrete endothelial mitogens. TGFβ-mediated chemotaxis leads to the influx and activation of inflammatory cells. TGFβ also has a key role in the elaboration of matrix by contributing to the synthesis of matrix proteins and by controlling the secretion of proteases so as to favour the accumulation of ECM proteins (Ksander and Olsen 1993). Expression of TGFβ by fibroblasts is impaired in diabetic foot ulcers (Jude, Blakytny et al. 2002). Studies using aminoguanidine to block AGE formation have been shown to restore TGFβ levels; it is therefore likely that AGE accumulation in the diabetic ulcer contributes to the impaired TGFβ levels (Yavuz, Tugtepe et al. 2005).

TGFβ is a powerful inducer of CTGF in connective tissue cells but not in some other cell types such as epithelial cells (Bradham, Igarashi et al. 1991; Frazier, Williams et al. 1996). Following brief exposure to TGFβ, fibroblasts are able to express CTGF for prolonged periods (Grotendorst, Okochi et al. 1996), studies by Igarashi et al. (1996) showed that
CTGF mRNA is not observed in the absence of exogenous TGFβ (Igarashi, Nashiro et al. 1996) indicating a link between these two factors.

TGFβ has multiple direct effects mediated by Smad and mitogen-activated protein kinase pathways. Smad3-null mice show impaired chemotaxis of inflammatory cells in response to TGF-β1 therapy and decreased ability of inflammatory cells to auto-induce TGFβ (Lakos, Shu-Jen et al. 2004; Yang, Letterio et al. 1999). It is probable that the effects of TGFβ are also partly due to the downstream effects of CTGF.

Animal models have been used to evaluate the efficacy of exogenous TGFβ in wound repair. Topical application of TGFβ has consistently been shown to increase the tensile strength of incisional wounds in rats and to accelerate healing (Ammann, Beck et al. 1990; Jones, Curtsinger et al. 1991; Beck, DeGuzman et al. 1993). Dosing profiles indicate that the efficacy of TGFβ follows a bell curve profile. The tensile strength of TGFβ-treated punch biopsy wounds in pigs and guinea pigs was also increased following healing by secondary intention (Ksander, Chu et al. 1990). Intravenous injection of TGFβ up to 24 hours prior to wounding has been shown to enhance wound healing. TGFβ-treated wounds also show increased collagen content, as measured by Picrosirius red staining (Pierce, Tarpley et al. 1992). This increased wound tensile strength and collagen indicate that TGFβ is a key growth factor in wound healing.

TGFβ-1 knockout mice showed accelerated re-epithelialisation during incisional wound repair, in comparison with wild-type mice (O’Kane and Ferguson, 1997; Koch, Roche et al. 2000). Conversely, overexpression of TGFβ in an animal model accelerated the rate of wound closure in partial thickness wounds by promoting keratinocyte migration (Tredget,
Demare et al. 2005). In full-thickness wounds transgenic mice overexpressing the TGFβ-1 transgene in keratinocytes exhibited delayed healing after burn injury and TGFβ-1 slowed the rate of wound re-epithelialisation (Yang, Letterio et al. 2001). This conflicting data suggests that improved wound-healing outcomes may be achieved by selectively blocking the negative effects of TGFβ, although it does potentially limit the role of TGFβ-1 as a therapy to aid overall cutaneous wound healing.

2.6.5 Other growth factors

Other growth factors that may ameliorate wound healing and that are deficient in chronic wound milieu include placenta growth factor (PGF), insulin-like growth factors (IGFs) and hepatocyte growth factor (HGF).

An adenoviral vector containing placenta growth factor (PGF) showed it to significantly accelerate healing in diabetic wounds, improving granulation tissue formation, maturation, vascularisation and monocyte recruitment (Cianfarani, Zambruno et al. 2006). PDGF, FGF2, and VEGF mRNA levels were also increased, suggesting a synergistic effect of PGF with induced endogenous growth factors to aid wound healing.

Circulating IGF-I and IGF-II are transferred from blood to local sites of production via a sequence of binding proteins. It has been postulated that alterations in levels of these binding proteins and increased levels of IGF antagonists are associated with defective wound repair in diabetes (Loot, Kenter et al. 2002). Topical IGF-I therapy has been shown to aid wound healing when used alone (Beckert, Haack et al. 2007) and also when used in combination with other growth factors (Lynch, Colvin et al. 1989).
HGF stimulates proliferation and migration of epidermal keratinocytes and melanocytes. It also increases MMP-2 and -9 levels and their related activities (cell migration, angiogenesis and ECM remodelling) (Yoshida, Matsumoto et al. 2004). Transgenic overexpression of HGF in mice resulted in increased vascularisation and granulation tissue formation, (Toyoda, Takayama et al. 2001) while incisional wounds in Wistar rats showed enhanced healing following intradermal administration of HGF (Ono, Yamashita et al. 2004).

Growth factors have the potential to heal human diabetic wounds, as discussed in section 2.5.1. PDGF –BB is the only growth factor treatment currently approved by the FDA, and it is expensive, controversial and not available in Australia. The current literature lacks robust research to determine whether the growth factor CTGF is a safe and effective agent in healing diabetic wounds. CTGF has, however, shown potential as a wound healing therapy in a Baboon burn model (Liu, Shi et al, 2007). Its structure, function and wound healing potential is described below.

2.7 Connective Tissue Growth Factor

2.7.1 Structure and regulation in normal skin

First identified as a secreted protein in 1991, (Bradham, Igarashi et al. 1991), CTGF, also known as CCN2, is a 32-38 kDa member of the CCN family, a group of proteins that share a common modular structure (Bork 1993). CTGF comprises 349 residues and is organised into a signal peptide and four domains (Figure 2.4) designated as:

- Insulin-like growth factor binding protein domain at the amino-terminus;
- Von Willebrand factor type C module domain;
- Thrombospondin repeat type 1 domain;
- C-terminal module domain (CT) (Yoshida and Munakata 2007).

This structural organisation suggests that different domains of CTGF might be responsible for different signals in the modulation of biological activities.

**Figure 2.4: CTGF structure**
The five exons of CTGF, which encode the single peptide (SP) and four domains (Adapted from Gupta, Clarkson et al. 2000).

CCN2 is induced during normal wound healing in the absence of diabetes. This includes in preclinical models, in a transient manner in keratinocytes some 7 or more days after skin full thickness porcine wound generation (Wang, Olsen et al. 2001) and in dermal fibroblasts in murine models of wounding including stented excisional wounds and PVA subcutaneously implanted sponge models of wounding (Alfaro, Deskins et al. 2013). The CTGF promoter appears to be activated in such wounding in normal rodent skin (Kapoor, Liu et al. 2008). In contrast to normal wound healing, excessive skin CTGF is implicated as a causal factor in skin scarring including hypertrophic scar and keloid formation (Igarashi, Nashiro et al. 1996) and in skin diseases dominated by sclerosis such as systemic sclerosis (Fonseca, Lindahl et al., 2008).
where in each case CTGF over-expressed compared with levels in normal skin and in normal, non-scarring wound healing.

2.7.2 CTGF bioactivity

Whilst the mechanism of action of CTGF has yet to be fully understood, a picture of its functions does exist: a series of studies have shown that CTGF stimulates fibroblast proliferation and differentiation, thus enhancing ECM production (Daniels, Van Bilsen et al. 2009). CTGF induces ECM proteins such as collagen and fibronectin (Frazier, Williams et al. 1996). CTGF promotes cell adhesion through an integrin-heparin sulphate proteoglycan dependent pathway. The specific integrin used in this pathway by CTGF varies between different cell types (Babic, Chen et al. 1999). In addition, CTGF has chemotactic and mitogenic properties and can promote cell differentiation (Bradham, Igarashi et al. 1991; Yosimichi, Nakanishi et al. 2001). CTGF can induce cell migration and proliferation by upregulating integrin expression on target. Other integrins, induced by reactive oxygen species, have been shown to inhibit CTGF-mediated migration (Chang, Shih et al. 2004). Therefore local factors (such as oxygen levels) are likely to influence the actions of CTGF (Chang, Shih et al. 2004).

CTGF may also influence angiogenesis. Shimo et al. (1999), demonstrated that CTGF promotes angiogenesis in vivo (Shimo, Nakanishi et al. 1999). However transgenic mice that constitutively overexpress CTGF show decreased expression of VEGF and decreased angiogenesis at growth plates (Ivkovic, Yoon et al. 2003). As discussed above, it is probable that local conditions in target tissues determine the effects of CTGF.
Despite considerable research, a specific signalling receptor for CTGF has not been identified (Leask, Abraham et al. 2003). Chen et al. (2001) demonstrated that CTGF is secreted through the Golgi and degraded in the endosome via a low density lipoprotein receptor (Chen, Segarini et al. 2001). However, it is not clear whether this is a true signalling reaction or merely a mechanism by which excess CTGF degraded. Wang et al. (2010) hypothesised that CTGF could signal in some cells through the nerve growth factor receptor, tyrosine kinase A (Wang, McLennan et al. 2010).

CTGF acts as a regulator of other growth factors but may in turn be regulated by such factors (Arnott, Nuglozeh et al. 2007). CTGF regulates the expression of VEGF under certain conditions (Nishida, Kondo et al. 2009). Abreu et al. (2002) showed that binding of CTGF to TGF-β led to enhanced TGF-β mediated gene transcription in Xenopus cells (a genus of frogs widely used as a model for molecular and cell biology studies), (Abreu, Ketpura et al. 2002), while Igarashi et al. (1993) demonstrated that TGF-β was able to induce CTGF in human skin fibroblasts, thus lending weight to the co-regulatory concept (Igarashi, Okochi et al. 1993).

### 2.7.3 CTGF regulation by exogenous agents

Exogenous substances have the ability to affect the expression of CTGF. This was explored by Gressners group, (2008), who established that caffeine was able to inhibit TGF-β-stimulated CTGF expression in hepatocytes through effects on the PPAR-gamma and SMAD 2/3 pathways (Gressner, Lahme et al. 2008). Other exogenous regulators such as the saturated fatty acid palmitate and cigarette smoke have been shown to induce CTGF (Churg, Tai et al. 2006; Yu, Birke et al. 2012).
2.7.4 CTGF as a biomarker, predictor and mediator of disease in diabetes

Elevated CTGF gene expression and protein levels have been reported in the tissues of people with diabetes (Riser, Denichilo et al. 2000). Jaffa et al. (2008) demonstrated that elevated plasma CTGF-N fragment was a predictor of macrovascular disease in type 1 diabetes, indicating a strong link between CTGF and some diabetic complications (Jaffa, Usinger et al. 2008). Similarly, CTGF has been implicated in the development of diabetic nephropathy, hepatopathy, cardiomyopathy and retinopathy (Wang, Denichilo et al. 2001; Twigg, Cao et al. 2002; Paradis, Perlemuter et al. 2001; Rachfal and Brigstock 2003; Candido, Forbes et al. 2003; Way, Isshiki et al. 2002; Tikellis, Cooper et al. 2004) and expansion of the ECM occurs within tissues in diabetes. It has been postulated that this ECM aberration is largely responsible for the diabetes-related complications mentioned above. The mechanisms by which CTGF is able to influence ECM accumulation are not well understood and multiple mechanisms may be involved.

Specific diabetes-related stimuli have been found to induce CTGF expression \textit{in vitro}. These include metabolic factors such as AGEs (Twigg, Chen et al. 2001), ROS (Park, Kim et al. 2001) and high blood glucose levels (Murphy, Godson et al. 1999). Candido et al. (2003) showed that by treating diabetic animals with a breaker of AGEs, CTGF levels were reduced to normal (Candido, Forbes et al. 2003). The increased low-density lipoproteins commonly associated with hyperlipidaemia have also been shown to induce CTGF expression \textit{in vitro} (Sohn, Tan et al. 2006). CTGF induction has therefore been implicated not only as a marker but as a potential mediator of pathology in tissues in diabetes where excessive fibrosis may occur, such as the kidney, heart, liver, complicated atheromatous plaques and the diabetic eye (Twigg and Cooper 2004).
In contrast to the many tissues affected by fibrosis in diabetes, in which CTGF is induced, CTGF appears to be deficient in wounds in diabetes. In baboon studies, CTGF levels were reduced in sterile subcutaneous wound tissue of diabetic animals compared with non-diabetic controls (Thompson and McLennan, 2010). Interestingly, in this work, CTGF mRNA levels in the wound tissue were not different between diabetic and control animals. Also in this work wound fluid from diabetic animals was found to proteolyse CTGF (Thompson and McLennan, 2010), suggesting that CTGF in diabetic wounds may undergo proteolysis. In wounds in diabetic animals the theme of growth factor degradation through proteolysis is quite common (Roth, Piekarek et al. 2006) reflecting the increased inflammatory and protease active environment in diabetic wounds (Wetzler, Kampfer et al. 2000).

The MMPs, their TIMPs and CTGF appear to be linked in a serially regulated manner involving feedback mechanisms. For example, MMPs are secreted by endothelial cells in response to CTGF (Kondo, Ohshima et al. 2002) and, in other systems, CTGF induces MMPs, such as MMP-1 (Ishibuchi, Abe et al. 2010) and MMP-2 (Chintala, Liu et al. 2012). In turn, CTGF degradation is induced by a number of MMPs, including MMP-9 (Hashimoto, Inoki et al. 2002). CTGF induces the MMP natural inhibitor TIMP-1 in renal mesenchymal cells (McLennan, Wang et al. 2004) and, of more relevance to skin, recombinant human CTGF (rhCTGF) increases each of TIMP-1 to TIMP-4 in porcine skin fibroblasts (Hashimoto, Inoki et al. 2002). If this same mechanism exists in wounded skin then it could contribute to the benefits of CTGF wound healing therapy by limiting CTGF degradation.

As previously discussed, the ability of CTGF to regulate the bioactivity of other growth factors (such as TGF-β) may contribute to its role wound healing. CTGF expression in dermal fibroblasts is induced potently by TGF-β through an elaborate interplay between
various transcriptional factors (Shi-Wen, Leask et al. 2008). It has also been postulated for many years that CTGF is required for, or possibly synergistically augments TGF\(\beta\) actions in fibroblasts (Grotendorst 1997). Indeed, in a mouse model, subcutaneous injection of either CTGF or TGF-\(\beta\) resulted in only transient granulation but a more persistent response was observed when these two factors were injected in combination (Mori, Kawara et al. 1999). This supports the concept that fibroblast proliferation, activation and migration, which characterise granulation and, therefore, ECM accumulation, are influenced by CTGF.

CTGF induces \(\alpha\)SMA in fibroblasts, converting them to a myofibroblast phenotype. Activated fibroblasts - or ‘myofibroblasts’ - express \(\alpha\)SMA, which has been shown to ameliorate wound contraction and remodelling (Chen, Shi-Wen et al. 2005). As discussed in section 2.3.3, \(\alpha\)SMA induction in diabetic wounds is reduced and this contractility and remodelling is delayed. Conversely, contractility is protracted in fibrotic conditions such as scleroderma in which the \(\alpha\)SMA-producing myofibroblast phenotype persists (Whitfield, Finlay et al. 2003).

### 2.7.5 CTGF levels in wound fluid of human diabetic foot ulcers

A series of studies examined CTGF levels in post-debridement human wound fluid from 32 human subjects with chronic diabetic foot ulcers by Western immunoblot over a prolonged period of wound healing (>3 months). Demographic information describing the cohort is shown in Table 2.9. High CTGF protein levels in wound fluid correlated with improved wound healing rates in diabetic foot ulcers Dr L. Lo (personal communication, July 2008) as indicated in Figure 2.5. Data were calculated with \(n=38\), for all ulcer aetiology (with \(p<0.001\)), and with \(n=32\) after excluding post-surgical ulcers (also \(p<0.001\)). Together with the known bioactivities of CTGF—chemotactic for macrophages, pro-angiogenic, induction
of SMA, and ECM accumulation—the finding that high levels of CTGF correlates with increased ulcer healing in human diabetic foot ulcers further supports the rationale that applying rhCTGF to wounds may improve healing in diabetic foot ulcers.

**Table 2.9: Demographics of subjects and ulcer type in study analysing CTGF in wound fluid of diabetic foot ulcers by Western immunoblot, using in-house anti-CTGF antibody.**

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<thead>
<tr>
<th>Patient demographics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Study subject number (n)</td>
<td>32</td>
</tr>
<tr>
<td>Age (years)*</td>
<td>64.1±1.8</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>17.6±1.9</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>75</td>
</tr>
</tbody>
</table>

**Ulcer type**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuropathic only</td>
<td>29</td>
</tr>
<tr>
<td>Neuro-ischemic</td>
<td>3</td>
</tr>
</tbody>
</table>

* all data are mean ±SD

**Figure 2.5: Wound healing rate and CTGF in wound fluid.**
Plot showing % change per 10 days in CTGF in wound fluid post-debridement and wound healing in the human diabetic foot ulcers described in Table 2.9. Each data point represents a patient in the study and typically three analyses were performed in each patient across a 12 week or longer period. Linear regression shows a highly significant trend towards wound healing rate correlating with rate of increase in CTGF in human foot ulcer fluid (p<0.001). The data for n=32 (without the post-surgical ulcers) is shown.
Topical application of CTGF has not yet been studied in any diabetic wound model. It is notable, however, that topical application of rhCTGF to acute burns in monkeys resulted in accelerated cutaneous wound closure and granulation tissue induction (Liu, Shi et al. 2007). Thus considering the known bioactivities of CTGF, its relative deficiency in wounds in diabetes, that it is induced as wounds heal in human diabetes, and that CTGF therapy accelerated wound healing in a monkey burns model (Liu, Shi et al. 2007), there is a rational basis to consider CTGF as topical therapy in cutaneous wounds in diabetes.

Whilst CTGF is an endogenously produced factor which could, which has properties that may be favourable to wound healing in diabetes, the following section introduces the concept of substances that occur within nature, but not within the human body which may hold therapeutic value. In particular the bee-derived product propolis is in order to provide context for its testing as a wound healing therapy in diabetic foot ulcers within this thesis.

### 2.8 Neutraceuticals

Many innovative therapies are being developed to treat diabetic wounds. As previously discussed, many are prohibitively expensive and/or logistically difficult to deliver. NWPT and HBOT are two prime examples of therapies that are both costly and logistically challenging to deliver.

To improve healing rates in recalcitrant diabetic wounds in a cost effective manner, it is rational to investigate natural products, which are inexpensive and readily available. The relatively low cost of developing existing natural products or ‘neutraceuticals’ into wound
healing therapies makes them particularly attractive. Such therapies generally have low side
effect profiles as they are often already used for human consumption—for example, as foods.

2.8.1 Bee-derived wound therapies

Since ancient times bee-derived products have been used as medicines and as wound healing
therapies (Najafi, Vahedy et al. 2007). Recently there has been a revival of interest in bee-
derived products, but to date the role of these products in diabetic wound healing has not
been studied. Bee or ‘Apitherapy’ products are of particular interest as they have established
anti-bacterial and anti-inflammatory properties, are inexpensive and generally well tolerated.

Bees nest in colonies that are headed by a single fertile female, the queen, who is usually the
only egg layer in the colony. Tasks such as foraging for nectar to make honey, producing
royal jelly to feed the queen and larvae, cleaning, removing debris from the hive, and
producing the resinous substance called propolis to protect the hive from pathogens are
carried out by the worker bees, which are all female. These clearly defined functions are
thought to have enabled the bee to survive a variety of evolutionary challenges. Apitherapy
products are produced by bees and can be harvested from their nests. Of these products, only
four—honey, propolis, royal jelly and bee venom—have been researched for their potential
as wound healing therapies. Their composition is shown overleaf in Table 2.10
Table 2.10: Composition of apitherapy products used in wound healing.

<table>
<thead>
<tr>
<th>Bee Product</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Honey</td>
<td>20% water, 70–75% reducing sugars, 5-10% sucrose (Grembecka and Szefer 2013).</td>
</tr>
<tr>
<td>Royal Jelly</td>
<td>60% water, 5% lipids, 15% protein, 20% sugars (Xu and Gao 2013).</td>
</tr>
<tr>
<td>Propolis</td>
<td>50% resin and vegetable balsam, 30% wax, 10% essential oils, 5% pollen and 5% organic debris (Bankova 2005; Menniti-Ippolito, Mazzanti et al. 2008).</td>
</tr>
<tr>
<td>Bee Venom</td>
<td>88% water, 6% melittin, 6% combination of other enzymes and amino acids, carbohydrates, phospholipids and physiologically active amines (Alia, Laila et al. 2013).</td>
</tr>
</tbody>
</table>

The composition of bee products shows a large amount of regional variation, depending on the local species of bee, the plants that they feed on, and climatic and environmental conditions (Gheldof and Engeseth 2002; Righi, Negri et al. 2013). The most well studied and commonly used bee products are derived from the honey bee (*Apis mellifera*), which is native to Europe, Africa and western Asia. This species has also been introduced to other continents, starting in the 17th century, and is now found all around the world, including east Asia, Australia and North and South America (Winston, Dropkin et al. 1981).

*Apis mellifera* has several sub-species or regional varieties, including the Italian bee (*Apis mellifera ligustica*), European dark bee (*Apis mellifera mellifera*) and the Carniolan honey bee (*Apis mellifera carnica*). Each species differs in its ability to produce the various types of bee products, with some subspecies being better suited to producing certain bee products than others. For example, the Russian honey bee yields more propolis than the Irish honeybee. However, most colonies are capable of producing at least some of each of these products. The principal components of the various bee products are shown in Table 2.10. They vary quite markedly in composition, particularly water and carbohydrate content.
The occurrence of chronic foot ulcers in persons with diabetes is, as previously discussed, due to several inter-related factors that cause local wound related abnormalities (Sibbald and Woo 2008). Some of the factors that prevent diabetic foot wounds from healing, such as persistent inflammation and increased bacterial burden could potentially be ameliorated with apitherapy/bee products. Preclinical research into the use of apitherapy products in wound healing suggests that honey, propolis, royal jelly and bee venom have the potential not only to promote healing in ‘normal wounds’ but also to attenuate the chronic inflammation, oxidative stress, bacterial burden and immunodeficiency that thwarts healing in diabetic wounds.

Within the hive ecosystem, honey and royal jelly are foodstuffs, bee venom is involved in defensive roles (killing intruders) and signalling (releasing pheromones that warn other bees of attack) and the prime function of propolis is to protect bees against disease. Bees coat the internal walls of their hives with a thin layer of propolis to sterilise the comb and keep their hives free of bacteria. Given this specific function and its proven efficacy in preclinical wound healing studies, it is likely that propolis, out of all the bee products, holds the greatest potential as a wound healing product.

2.8.2 Propolis

Propolis is a resinous hive product produced by bees (Figure 2.6). The name derives from the Greek words Pro (defence of) and Polis (city), and the name reflects the importance of this substance as a hive protectant. Propolis consists of plant buds that are collected on the hind legs of worker bees and then masticated. Bee salivary enzymes are added through this process and the resulting product is then mixed with wax (Ghisalberti 1979; Bankova, de Castro et al. 2000). Within the hive, propolis is used to fill cracks and crevices to prevent
insect invasion (Daugsch, Moraes et al. 2008). It is also used to ‘embalm’ hive invaders that the bees are able to kill but cannot transport out of the hive, thus preventing problems associated with decomposition (Sforcin 2007).

Figure 2.6: Propolis in situ in a commercial beehive. Photograph courtesy of Yves Ginat, Apiarist, Woodbridge, Tasmania

Propolis has been used in ‘medicine’ since the time of the Pharaohs (Lamprecht 1994). It is collected from the hive superstructure by scraping, usually performed in autumn following honey extraction. Propolis is sticky when freshly collected and at a hive temperature of 35°C. It hardens as temperatures drop and is quite brittle at 5°C (Ghisalberti 1979).

Following collection, propolis must be evaluated and processed before therapeutic use. Excess wax is removed by cold water washing. The propolis is then dissolved in a solvent (usually 95% ethyl alcohol) and filtered to remove large particles and foreign bodies (Burdock 1998). Propolis can be administered orally or topically. Photoacoustic spectroscopy has shown penetration of propolis into wounded skin to be excellent (Sehn, Hernandes et al. 2009). In contrast to honey, which also has antibacterial properties (Al-Waili, Salom et al. 2011), propolis is not prone to cause maceration around the wound edge and it also has potent antibacterial and anti-inflammatory properties (de Moura, Negri et al. 2011, Ramos, Miranda et al. 2007).
2.8.3 Composition and toxicity

The composition and colour of propolis vary according to the region from which it originates. (Bankova 2005; Menniti-Ippolito, Mazzanti et al. 2008). However, whilst the composition of propolis may show regional variation, its biological properties appear to remain similar regardless of origin.

The composition of propolis is complex. Over 200 constituents of propolis have been identified (Marcucci 1995). The most biologically-active fractions of propolis are flavanoids and esters of caffeic acid (Russo, Longo et al. 2002). These substances also occur naturally in foodstuffs, and studies show that the amount introduced into the body by ingestion or topical application of propolis is negligible by comparison. Flavanoids are easily metabolised, and no flavanoid residues accumulate in the body after oral or topical administration of propolis (Havsteen 1983; Havsteen 2002).

No adverse effects were observed when propolis was administered to rodents in relatively large doses (700 mg/kg) (Dobrowolski, Vohora et al. 1991). Avouret-Grand et al. (1993) reported the oral lethal dose of propolis in mice to be >7340 mg/kg, which indicates a low toxicity (Arvouet-Grand, Lejeune et al. 1993). Irritancy testing on guinea pig skin showed that even at its highest dilution of 20%, propolis was not an irritant (Hausen and Wollenweber 1988). Human data on contact allergens in persons with leg ulcers showed propolis to have a relatively low sensitisation rate (4%) compared to 21% for wool tar and 10% for Balsam Peru (both used in fragrance). The preponderance of occupational allergic contact dermatitis seen in apiarists (Hausen and Wollenweber 1988; Gulbahar, Ozturk et al. 2005) is thought to be due to caffeic acid derivatives found in propolis and for this reason propolis should be used with caution in people with an allergic disposition.
2.8.4 Actions of propolis

As a topical agent propolis is reported to be an effective treatment to aid resolution of cuts and abrasions (Okonenko 1985) and inflamed throats (Pang and Chen 1985). Its mechanisms of action, which may have relevance to wound healing in diabetes are discussed in the following section.

2.8.4.1 Anti-inflammatory activity of propolis

The anti-inflammatory effects of propolis are well-established (Ledon, Casaco et al 1997; Mirzoeva and Calder 1996; Sforcin 2007) and are largely attributable to caffeic acid (Grunberger, Banerjee et al. 1988; Chan, Wen et al. 1995). Studies by Jin et al. (2005) showed that caffeic acid phenyl ester (CAPE) in propolis is a potent inhibitor of MMP-9 (Jin, Chung et al. 2005). Temiz et al. (2008) hypothesised that propolis-treated rat colon anastomoses would heal more quickly and showed increased bursting strength due to decreased collagenolysis attributable to CAPE action on MMP-9 (Temiz, Aslan et al. 2008). Propolis treatment has been shown to reduce the persistent inflammation that characterises diabetic wounds by normalising neutrophil and neutrophil elastase levels (McLennan, Sakar et al. 2009). It has been proposed that propolis reduces inflammatory exudates in diabetic rodents and improves the wound closure rate in diabetic wounds through its widely reported anti-oxidant effects (Vieira, Laranjinha et al. 1998; Nagaoka, Banskota et al. 2002; Mercan, Kivrak et al. 2006).

Reduced inflammatory cell infiltration was observed in propolis-treated rabbit corneal injuries compared to controls; this anti-inflammatory effect was comparable to that achieved with dexamethasone induction of cytokine production by spleen cells, thus demonstrating
the potential of propolis to reduce chronic inflammation such as that observed in diabetic foot ulcers (Missima and Sforcin 2008).

### 2.8.4.2 Antimicrobial activity of propolis

The exposed subcutaneous tissues of wounded skin are prone to contamination and colonisation by a variety of microorganisms. Robust evidence exists to support the antimicrobial properties of propolis (Bonvehi, Coll et al. 1994; Kosalec, Pepeljnjak et al. 2005, Marghitas, Mihai et al. 2010; Ozklap, Ozcan et al, 2010). The inhibitory concentration of propolis is 400 times greater than that of tetracycline against *E. coli* and more than 50 times higher against *S. aureus* and *B. subtilis*. The composition of propolis determines its efficacy against *P. aeruginosa* in vitro (Pepeljnjak and Kosalec 2004), with the extracts of propolis being particularly effective (Aliyazıcıoglu, Sahin et al. 2011).

To test the anti-microbial activity of propolis, its activity against *Micrococcus luteus* was assessed using microcalorimetric analysis of bacterial cultures in different growth phases. The addition of propolis extracts to these cultures resulted in a strong decline in heat production, a prolongation of the lag phase and the introduction of a second lag phase, indicating that propolis is both bacteriostatic and bactericidal (Lamprecht 1994). The antibacterial activity of propolis especially against *P. aeruginosa* and *S. aureus*, is thought to be largely attributable to the phenolic acid fraction (Lamberte, Cabrera et al. 2011; Bankova, Christov et al. 1995). For example, no antibacterial activity against *S. aureus* was observed in a batch of propolis with a low phenol count, unlike batches with a higher phenol count, which had substantial antibacterial activity (Bankova, Christov et al. 1995).
Propolis has also been shown to inhibit the proliferation of fungal elements such as Candida (at concentrations of 3-10 mg/mL) (Metzner, Schneidewind et al. 1977) and viruses. Propolis mediated inhibition of the HIV (human immunodeficiency virus) virus has also been observed. The mechanism of propolis’ HIV antiviral property in CD4+ lymphocytes occurs via, inhibition of viral entry (Gekker, Hu et al. 2005).

2.8.5 Propolis in wound healing

Several studies have examined the potential of propolis to promote wound healing. Propolis has been deemed to have a low-allergen profile which makes it particularly suitable to study as a wound healing therapy (Menniti-Ippolito, Mazzanti et al. 2008). In excisional rat wounds, propolis improved wound contraction rates compared to petroleum jelly treated controls (Pillai, Palsamy et al. 2010). The wounds treated with propolis had higher levels of hydroxyproline (indicating collagen formation), hexosamines and uronic acid (responsible for ECM accumulation and chemoattraction). There was also a significant increase in DNA, RNA and protein in the wound at day 7 in the propolis treated animals, indicating accelerated hyperplasia associated with wound healing. Similar findings of improved wound healing and increased hydroxyproline levels were observed in propolis-treated rat colon anastamoses (Temiz, Aslan et al. 2008).

The altered cell composition in the wounds of propolis treated animals was shown to favour healing. As described above, topical propolis treatment prevented the persistent elevation in neutrophil levels otherwise seen in a diabetic rat model of impaired wound healing (McLennan, Sakar et al. 2009) and increased fibroblast proliferation in rat colon wounds (Temiz, Aslan et al. 2008). Sehn et al.(2009) observed that propolis treatment promoted wound healing by stimulating keratinocyte proliferation (Sehn, Hernandez et al. 2009).
A small-scale observational human study comparing thrice-daily application of propolis skin cream to silver sulfadiazine in the treatment of burns showed some beneficial effects (Gregory, Piccolo et al. 2002). Time to wound closure was reduced in the propolis group (9.09 days versus 10.96 days) and inflammation was reduced. Whilst bacterial colony counts in wound samples were not different between the two groups, those treated with propolis were anecdotally found to have decreased discomfort, possibly due to analgesic properties that have been previously associated with propolis (Ledon, Casaco et al. 1997; Paulino, Dantas et al. 2003). The study authors also noted that propolis is less expensive than silver sulfadiazine cream and does not confer such a risk of allergy that is associated with silver sulfadiazine therapy.

2.8.6 Propolis as an investigational therapy

Investigation into the properties of propolis has been largely confined to animal models. There have been few clinical studies on the therapeutic efficacy of propolis. Furthermore, even though the main actions of propolis appear consistent across sources from various geographic regions and countries, propolis is not a standardised product and its composition is largely determined by its geographical area of origin.

The mode of action of propolis is controversial and that whilst its wound healing properties are largely associated with its anti-microbial and anti-inflammatory actions, rather than acting as a pure tissue regenerator, propolis may have other healing properties that might be derived from fatty acids, vitamins and minerals that have yet to be fully investigated (Burdock 1998). A lack of large-scale, well-conceived, robust clinical trials precludes bee products from becoming more accepted as wound therapies. Given the magnitude of the problem of diabetic foot ulceration, there is an urgent need to systematically study bee products in human
diabetic foot ulcers to determine if they may improve healing outcomes. Propolis in particular could be of value in wound healing in diabetes because it is known to have potent anti-inflammatory and anti-bacterial properties and it is known that diabetic foot ulcers fail to heal largely because of excessive inflammation and excessive bacteria which propolis might ameliorate.

The burden conferred by a diabetic foot ulcer in terms of both financial and social costs has been outlined as have the abnormalities in the diabetic wound microenvironment and the probable causative factors of these abnormalities. Using this understanding, many therapies are emerging that aim to accelerate healing in diabetic wounds. This work aims to explore the potential of two therapies, CTGF and propolis, to improve healing in diabetic wounds and to normalise the wound environment.

2.9 Hypotheses and aims of this thesis

Diabetic foot ulcers are a significant cause of morbidity and mortality and are characterised by persistent inflammation and a dysregulated wound microenvironment. The growth factor CTGF has a multitude of biological actions. Its expression is induced in wound tissue and increases in human wound fluid as the wounds heal. Prior to this work the effect of topical rhCTGF on wound healing had not been tested in a wound model in diabetes.

In an experimental rodent model of diabetes, the beehive protectant propolis has already shown by our research group to improve wound healing. A logical extension of this existing work was to test the safety and efficacy of topical application of propolis in a cohort of people with diabetes who have foot ulcers.
Study hypotheses:
1. CTGF improves cutaneous wound healing in a diabetic rodent model.
2. Propolis is a well-tolerated treatment for human foot ulcers in diabetes.
3. Propolis will improve healing in human diabetic wounds.

Study Aims
The overall, integrated aims of this series of studies were to investigate the capacity of topical therapies (CTGF and propolis) to improve healing in wounds in diabetes and to gain an understanding of their effect on the wound microenvironment. The objectives related to the respective hypotheses were:

1. To determine the effect of topical administration of rhCTGF on wound closure, strength and wound histology in non-diabetic and diabetic cutaneous wounds in an experimental rodent model.
2. To trial topical propolis in a small-scale human feasibility study of foot ulceration in diabetes, observing safety, acceptability and patient tolerability and side effect profile, and conducting a preliminary analysis of wound closure rates compared with historic controls, including to calculate sample size requirements for a full scale randomised control trial of this therapy.
3. To undertake analysis of wound fluid CTGF, MMP’s -2 and -9, and viable bacterial load in propolis-treated wounds compared with historic controls.

Collectively, the outcomes from this novel work could advance understanding in the wound microenvironment and in the future development of topically applied therapies to improve healing in foot ulcers in diabetes.
Chapter 3

METHODOLOGY

3.1 Introduction

This series of studies in my PhD explores two wound therapies in diabetes - the growth factor CTGF and the bee-hive protectant propolis. CTGF has not previously been trialled in diabetic wounds, so this study will be conducted in a rodent model of wound healing. Propolis has shown promise as a therapy to heal rodent diabetic wounds so will be tested in a small-scale human study of foot ulcers in diabetes. This section explores the methodologies used to test the study hypotheses.

3.2 Experimental models of wound healing

An experimental animal model can be defined as “a living organism with an inherited, naturally acquired or induced pathological process that, in one or more respects closely resembles the same phenomenon in humans” (Isselhard 1986). To substantiate the ability of any factor to ameliorate wound healing, in vivo experimentation is necessary. Ethical constraints largely prohibit studies of wound therapies in humans that have not been examined in non-human settings, especially when such therapies may well have adverse effects, such as in the case of protein growth factors. Thus, the use of animal models of wound healing in the diligent search for improved wound care is inevitable.
Ideally, a perfect analogue of the human clinical situation would be used in a preclinical wounding study, including in diabetes. Unfortunately such an analogue has not yet been established (Dorsett-Martin and Dorsett-Martin 2004). The choice of model in wound healing depends upon several factors such as type of investigation, stage of investigation (acceptable degree of simplification), outcome measurement and recruitment criteria. Logistical constraints such as species availability, housing facilities, husbandry requirements, time and budget must also be considered (Gottrup, Agren et al. 2000).

3.2.1 Species and breeding

Animal models of wound healing have been developed in many species including the rat, mouse, hamster, guinea pig, pig and dog (Greenhalgh and Greenhalgh 2005). Small animals such as rodents are more easy to procure and less difficult to maintain than a larger animal such as a fully grown pig. For this reason the rat was chosen for this work. However, this animal has its limitations, in imitating the true clinical picture, which are addressed in the following section.

3.2.2 Selection of wounding models

The choice of wounding model is dependent on the type of healing and/or the stage of the healing process that is to be investigated (Gottrup, Agren et al. 2000). A factor that often confounds attempts to mimic human wound healing in animal models is wound contraction. Many of the animals used in wound healing studies are ‘loose skinned’—that is, they have a subcutaneous panniculous carnosus muscle which contributes to repair via contraction and collagen formation (Cross, Naylor et al. 1995). Indeed, it has been reported that 90% of rodent model wound closure can be attributed to contraction (Levenson, Geever et al. 1965). The human is not loose skinned and caution must be exercised when drawing parallels with
other animal models. The pig has skin similar to human skin and is, therefore, often the animal of choice in cutaneous wound studies. However, its major drawback is that pig skin often heals too rapidly to study the effect of wound healing interventions. In addition, financial and ethical considerations may prohibit use of porcine models (Winter 1962). For the purposes of this work a rat model was used and wounds were splinted with a transparent film to minimise wound contraction so that healing more closely approximated the human state.

### 3.2.3 Wound models

Several wound models enable measurement of granulation tissue and wound contraction and are therefore more akin to the clinical situation. These can be grouped as ‘tissue models’, which mimic clinical wounds (incisional, excisional, burn), or ‘artificial models’, which create an artificial defect using a foreign implanted material (chambers, sponges etc.).

Cutting of the skin with a sharp blade will inflict an accurate, reproducible wound. In the landmark 1929 paper “The healing of wounds as determined by their tensile strength”, Howes and Harvey (1929) described the incisional wound model as four phases through which the histology and biochemistry of the wound alters over time and wound tensile strength increases progressively, reaching a maximum at around the 60-80th day postoperatively (Howes and Harvey 1929). Subsequently, the approach has become a benchmark in the evaluation of breaking and bursting strength in skin and other tissues. However, this model has limitations in wound healing studies as the skin defect is relatively small and quick to heal.
Split thickness models are less commonly used. These scrape-type lesions are not readily reproducible in hairy animals and healing is often too rapid to determine clear differences between treatment groups in the porcine model; nor do they imitate the deeper wounds seen in human diabetic foot ulcers. Another partial thickness wound, the blister, has been used extensively to evaluate epidermal regeneration (Kiistala and Mustakallio 1964). Blisters are easily reproducible, but have little pertinence to the deeper dermal wounds that are clinically problematic.

An excisional wound is created when a more significant volume of target tissue is removed (Falanga, Schrayer et al. 2004; Reid, Said et al. 2004). The filling of the resulting void allows for more detailed histological and biochemical analysis. In addition, this type of wound lends itself to the topical application of wound agents and subsequent study of their value and was therefore chosen for the animal studies in this work. This model more closely approximates the human foot ulcer than an incisional model. Following wound closure, scarring and strength can also be quantitated. The major disadvantage of this model is that the wound heals by contraction as well as by re-epithelialisation from the wound edges, but this can be minimised with splinting techniques.

The chronic wound is uncommon in laboratory animals. Therefore, various animal models have been adapted to reflect the local and systemic conditions that might prevent a wound from healing in humans.
3.3 Diabetes

Diabetes contributes significantly to the morbidity and mortality associated with chronic wounds (Boulton and Vilekyte 2005, 2001; Goodson and Hung 1977). It is therefore unsurprising that many animal models of diabetes have been developed for the purpose of studying wound healing. These models fall into two categories of induced diabetes: genetic or chemically-induced diabetes.

3.3.1 Genetic models of diabetes

Several research groups have used the db/db mutant mouse to study the effects of diabetes in wound healing. Clinical signs of diabetes exhibited by this animal include obesity, hyperphagia, hyperinsulinaemia and hyperglycaemia (Tsuboi, Shi et al. 1992). Diabetes in the genetically diabetic mouse known as the db/db mouse is due to a single gene mutation on the leptin receptor on chromosome 4. Db/db mice are infertile, which necessitates that heterozygous carriers be used to propagate the mutation. This model also exhibits an abnormal immune system, which is perhaps its major shortcoming (Bell and Hye 1983).

The ob/ob mouse is another genetic model of diabetes in which the animal is genetically predisposed to overeating and becomes obese. In contrast to the db/db mouse, no other abnormalities have been observed in this model. Whilst initially obese, hyperinsulinaemic and hyperglycaemic, this state does not persist and the animal eventually achieves normoglycaemia (Genuth 1969). The transient nature of diabetes in this model renders it less useful in long-term wound studies.
Genetically diabetic strains have also been characterised in other species including guinea pigs, Celebes apes (Macaca Nigra), Yucatan swine, Keeshond dogs, outbred baboons, and South African hamsters.

3.3.2 Chemically-induced models of diabetes

Animals can be rendered diabetic by chemical treatment. Two agents—alloxan and streptozotocin (STZ)—are commonly used for this purpose, both of which selectively destroy pancreatic beta cells. Both substances cause toxic glucose analogues to preferentially accumulate in pancreatic beta cells causing their death (Lenzen 2008). The action of alloxan is more rapid than that of STZ. For this reason, STZ was the preferred agent in the studies within this thesis as ketosis is less likely to occur with its use. Our laboratory has used a variety of models across the years in cutaneous and subcutaneous wound healing in diabetes; in particular, despite its short duration of wound healing, we have found that full thickness dorsal skin wounds in STZ-induced diabetic wild-type rats (after 6 weeks of diabetes) provide a good model to study topical application of therapies (McLennan, Bonner et al. 2008). For this reason, the STZ-induced diabetic rat was used in the studies within this thesis.

3.3.3 Rationale for the preclinical wounding model chosen in this thesis

Following due consideration, the studies of rhCTGF in diabetic wound healing were undertaken in a rat model of STZ-induced diabetes, studying full thickness skin wounds. This decision was made because: the model is widely recognised as a reliable model of diabetes mellitus; the cutaneous model of excisional biopsy is also widely recognised and has been established and published in our research laboratory, including in studies of ulcer closure and dermal changes; and the cutaneous model enables direct application of rhCTGF
to the wound. Some potential disadvantages of the model are that it is of type 1 diabetes rather than the more common type 2 diabetes; that rat skin differs significantly from human skin, with wounds healing to some degree by contraction, which does not occur in healing human skin wounds; and that the wound healing defects in diabetic rats in this model are relatively mild, with only small differences compared with ulcers in non-diabetic control rats. By splinting the ulcers using a reliable, established method using the dressing Tegaderm (Chung, Peplow et al. 2010) and by following an established, published protocol for 6 weeks of diabetes induction, then wounding and monitoring healing in our research laboratory (McLennan, Bonner et al. 2008), many of the negatives of this model can be overcome, thus enabling a reliable study of rhCTGF in diabetic skin wounds.

3.4 Types of clinical investigation to address the utility of an investigational agent

Evidence-based medicine aims to close the gap between research and everyday clinical practice (Sackett, Rosenberg et al. 2007). The risks and benefits of scientifically developed treatments need to be assessed in a clinical arena to predict outcomes and merit in medical practice.

Clinical evidence is rated or ranked according to a number of parameters, including freedom from biases, which reduce quality and impact of research. Several rating systems exist but, in general, evidence falls into one of the five categories described below.

3.4.1 Randomised controlled trials

Randomised controlled trials (RCTs) are considered the gold standard of evidence-based medicine (Britton, McKee et al. 1998). Blinding, randomised allocation of treatment/placebo
and strict inclusion/exclusion criteria are used to minimise bias. However strict inclusion/exclusion criteria may limit generalisation in a study i.e. the study group may be a homogenous cohort but not representative of a real, more generalised population (Lovato, Hill et al. 1997; McKee, Britton et al. 1999).

### 3.4.2 Observational studies

Observational type studies provide weaker empirical evidence, greater potential for confounding bias and are therefore most valuable in generating preliminary evidence to be used in future studies rather than as stand alone evidence (Concato, Shah et al. 2000).

### 3.4.3 Case control studies

Case control studies are limited as stand alone evidence as they are often retrospective, which in itself can confer bias, and methodology may be flawed. These studies are, however, valuable in establishing research direction in poorly researched areas or areas where large studies are not feasible, such as rare complications (Towler 2001; Vandenburgoucke and Pearce 2012).

### 3.4.4 Expert opinion

Expert opinion based on clinical experience, descriptive studies, or reports of expert committees have been shown to be a more powerful tool for changing clinical practice than national evidence-based guidelines (Lomas 1991); however, the evidence they provide is not robust and must be treated with caution (McManus, Wilson et al. 1998).
3.4.5 Pilot or feasibility studies

A pilot or feasibility study is a small scale version or ‘trial run’ of a full scale study (Anderson and Prentice 1999; Bowen, Kreuter et al. 2009). Such studies may be indicated when scant studies/data exists within the study field, partnerships need to be forged and tested, no in depth information into the intervention or target population is available, or previous similar studies have not worked (troubleshooting). Pilot or feasibility studies are usually used as justification for a subsequent RCT (internal study) but can also be a stand alone piece of work (external study) (Lancaster, Dodd et al. 2004). In the latter case, external controls can be used, such as appropriate historic controls, albeit with recognition of the potential biases introduced by such an approach as articulated by the Therapeutic Goods Administration (TGA 2001).

The major reason to conduct a pilot study is to determine sample size necessary to power a larger trial (Browne 1995; Feldman and Fleischer 1999). The pilot study can also be viewed as a feasibility study if aspects of the study design need to be examined prior to undertaking a larger randomised controlled trial. A general rule is that approximately 30 participants in a study will allow estimation of a parameter (Browne 1995). A pilot/feasibility study may also be used to test the integrity of a study methodology (Shih, Ohman-Strickland et al. 2004) and to test questions that pertain to recruitment, sampling, instrumentation, analysis and interpretation.

The analysis of a pilot study should be mostly descriptive (Loscalzo 2009). It is important not to place too much emphasis on statistical analysis (Arain, Campbell et al. 2010), and the results from such a small scale study should be interpreted with caution. In particular, a decision to proceed or terminate evaluation of an intervention based on the results of a pilot
study is hazardous because it is possible that the decision will be derived from false positive or false negative results (Type 1 or 2 error) (Fain 2010). After propolis was found to have wound healing properties in a diabetic rodent model, (McLennan, Bonner et al. 2008) the next logical step was to run a pilot study to test it in human studies, following the recommendations of Browne, 1995, including to permit further estimations for larger scale RCT parameters (Browne, 1995).

3.5 Logistics of clinical trials

The most important principle of clinical trial design is to answer one question well. Secondary questions and analyses should be reserved for generating subsequent hypotheses (Kestle 1999). The study question should be a single sentence and is key to the study (Beitz 2006). The sample of subjects to be studied should be clearly defined using inclusion/exclusion criteria. Underlying pathophysiology must also be clearly defined. In the case of wounds, this would include wound assessment criteria, which in these studies was defined as percentage change in wound area from original size, determined from wound tracings.

Appropriate ‘standard care’ must also be determined. Several wound studies have been criticised for using inappropriate standard care—for example, Steed compared becaplermin, a topical preparation containing PDGF, with saline-soaked gauze dressings, although the latter was widely considered an outdated therapy and therefore not an appropriate control (Steed, 1995). For this reason, it was decided that propolis-treated patients would receive usual care (debridement, topical and systemic anti-microbial therapies and pressure offloading as necessary). This approach minimised bias when compared to control subjects who received
similar therapy, but without propolis, and ensured that subjects were not deprived of what is currently regarded as the gold standard of care.

3.6 Outcomes

Endpoints and surrogate endpoints in any clinical study must be clearly defined, and methods of analysis should be established (Stanley 2007). The primary outcome should be specifically defined (Kestle 1999); in these studies, complete wound healing was the primary outcome. For wound care therapeutics, FDA guidelines require all wound care products to heal a wound (Armstrong, Boulton et al. 2009), which was the basis for choosing complete wound healing as the primary endpoint.

Successful wound healing is often a series of smaller triumphs, such as a reduction of local pressure or wound healing across a fixed time. Wound therapies should perhaps be considered in terms of intermediate/surrogate endpoints (Pocock, Geller et al. 1987; Gandhi, Murad et al. 2008). Surrogate outcomes such as wound healing rate after the first four weeks of care have been shown to be excellent predictors of subsequent wound healing (Margolis, Kantor et al. 2000; Sheehan, Jones et al. 2003). Such endpoints are therefore useful in some studies, especially smaller scale pilot studies, which tend to be of shorter duration. Therefore, rate of wound healing at all time points was used as a surrogate endpoint, as was analysis of wound cellular content (such as collagen and macrophages in animal studies) and markers of healing (such as MMPs) in human wound fluid.
3.7 Rationale for the method chosen for the clinical intervention

After careful consideration, the decision was made to use a feasibility/pilot study to investigate the effects of propolis, and to use an external historical control group. As described in the literature review in this thesis, topical application of propolis has not been studied systematically in human diabetic foot ulcers. In fact, only one case study could be found in which it was applied in a diabetic foot ulcer, and this was in combination with other agents (Lofty, Badra et al. 2006).

It was decided that as a single centre study, the historical controls could be identified pre-hoc from the same Diabetes High Risk Foot Service as for the propolis study, based on the study selection criteria. This approach was justified because the methods of treatment and the senior medical staff in the clinic had not changed in recent years, thus allowing a valid control group to be identified. This method enabled series of study subjects for the propolis topical application to be examined in an appropriate time interval from the single site, High Risk Foot Service.

Retrospective or historical data has been used successfully in many studies (Lawrence, Wraight et al. 2004). Indeed, analysis of cancer studies by Vickers et al. (2007) showed that over half of all phase II trials required historical data to determine a null response rate (Vickers, Ballen et al. 2007).

“In the absence of important biases in the study setting, the retrospective method could be regarded, according to sound statistical method, as the study method of choice” (Mantel and Haenszel 1959)
In order to support the quantitative endpoints described above in the propolis human foot ulcer study in diabetes, qualitative data was collected and ranked so the clinicians’ experiences of the propolis study could be quantitatively described. I determined the Likert scale to be the most appropriate technique to collect, analyse and summarise individual clinicians’ opinions as to whether propolis is a safe, tolerated and practical therapy and whether the study design was suitable to be used for a randomised controlled trial. The Likert scale is regarded as the most appropriate, accurate method of enquiry and analysis of such data. The strength of the Likert scale lies in its capacity to provide a quantitative means of understanding what essentially is qualitative data (i.e. individual rankings) as articulated by (Uebersax, 2012).

The major disadvantage of a feasibility/pilot study is that in the absence of randomisation and large study subject numbers, efficacy can only be inferred. Therefore there will almost certainly be a lack of homogeneity between the two patient cohorts in a pilot study. Controlled trials tend to overestimate efficacy of test therapies and tests of statistical significance carried out in such studies are less reliable than in randomised trials (Jadad, Moore et al.1996; TGA 2001). Specifically, as described earlier in this chapter, biases, both conscious and subconscious, can be introduced into a study when external controls are used.

These biases were limited in degree (TGA 2001) by the following approaches that were adapted in this current feasibility/pilot study: the historic control group entry criteria were defined at the study design phase; the control group, as historic controls external to the prospective propolis study, were derived from the same clinic and similar patient phenotypes and under the same treatment conditions as per the propolis treated group; application of the propolis was not undertaken by the regular clinic staff, and any loss of continuity in their
regular care of patients was minimised; and the main study efficacy end-point of ulcer healing with reduction in ulcer area was objective and documented real-time in the controls and the propolis study subjects by the regular treating staff using the same method of acetate tracings.

While the work hours and intensity required to undertake the pilot/feasibility study was expected to be highly demanding for a single Ph.D. student, especially in the absence of a research nurse or assistant dedicated to the clinical study, it was concluded that a pilot/feasibility study could be addressed through one site. In addition, exploratory studies of the effect of propolis on the wound microenvironment by examining the stored wound fluid post-debridement was undertaken through use of the Endocrinology Research Laboratories, University of Sydney facilities by me as the Ph.D. student (FH). In addition, microbiological analysis of the wound fluid was undertaken as a collaboration with Dr Geraldine McKew at the Microbiology facilities and Royal Prince Alfred Hospital, Camperdown.

3.8 Statistics

Results are expressed as mean ± standard error of the mean (SEM) or mean ± standard deviation (SD), each where indicated. Statistical advice for analyses undertaken was provided by a statistician employed by the Central Clinical School in Sydney Medical School, Dr Anne-Sophie Veillard. Statistical significance was determined using Number Crunching Statistical Software package (NCSS, Kaysville, Utah, USA). Methods used where appropriate, were: Student’s t–test for single comparisons, analysis of variance (ANOVA) for multiple comparisons, and the Chi squared test for small groups with discrete data, or the Mann Whitney U-test for non-normally distributed data. Post-hoc correction for multiple
comparisons was undertaken by Bonferroni multiple comparison test. Statistical significance was accepted at $p<0.05$. 
Chapter 4

RECOMBINANT HUMAN CONNECTIVE TISSUE GROWTH FACTOR THERAPY IN A DIABETIC RODENT MODEL OF WOUND HEALING

4.1 Abstract

Some topical growth factor therapies, such as PDGF, have shown utility in diabetic wounds. CTGF is a 32-38 kDa member of the CCN family, a group of proteins sharing a common modular structure. Also known as CCN-2, CTGF is capable of promoting cell adhesion; is chemotactic for inflammatory cells, especially macrophages; is mitogenic; and promotes cell differentiation enhancing ECM production including in skin (Daniels, Van Bilsen et al. 2009). CTGF levels are increased in human wound fluid as wounds heal including in diabetes Dr L. Lo (personal communication, July 2008 and section 2.7.5) whereas it is decreased in baboon wound tissue in diabetes compared with controls without diabetes (Thomson, McLennan et al. 2010). CTGF applied topically has shown efficacy in preclinical models of cutaneous burns (Liu, Shi et al. 2007) but to date its role has not been studied in diabetic wound healing, including in preclinical settings.

This chapter addresses the laboratory production of rhCTGF, the rodent model of delayed wound healing and the effects of rhCTGF as topical therapy in the model.
4.2 Introduction

CTGF is a 32-38 kDa member of the CCN family, a group of proteins that sharing a common modular structure. In non-human primates (baboons), recent studies have recently shown that intact CTGF protein is deficient in diabetic wound tissue compared with wound tissue in non-diabetic animals (Thomson, McLennan et al. 2010). In this study, the levels of pro-inflammatory mediators and proteases were increased in the wounds of diabetic animals and CTGF protein accumulation in wounds was found to be delayed. To date, the effect of topical application of CTGF to diabetic wounds has not been described in any animal model.

The aims of this study in a preclinical model of wound healing in diabetes were to:

(i) examine whether topically applied rhCTGF improves wound healing rate; and

(ii) determine whether CTGF treatment improves wound cellular content and breaking strength in a well-defined model of diabetic rodent skin wounding.

Ethics approval for induction of diabetes in animals and creation of wounds was obtained from the Animal Research Ethics Committee, Sydney South Western Area Health Service (SSWAHS), # 2D10024.

4.3 Methods and materials

4.3.1 Cell culture and rhCTGF protein production

The rhCTGF for treatment of wounds was produced using an adenoviral vector (Agilent technologies, La Jolla, CA, USA). The process being described in detail in a previous publication (Tan, McLennan et al. 2008). The CTGF cDNA open reading frame was cloned,
sequenced and inserted into the AdEasy vector (Agilent technologies, La Jolla, CA, USA) using the BamHI and Kpn1 sites.

After insertion of the vector, the recombinant adenovirus was then replicated in mammalian cells. The adenoviral vector was transfected into cells from the 911 cell line that had previously been cultured in RPMI and at 37°C and 5% CO₂. At confluence, the cells were subdivided by trypsinisation and grown to 75% confluence. At this point the cells were transfected by addition of the adenoviral vector containing CTGF to cells in Dulbecco’s Modified Eagles Medium (DMEM; Sigma-Aldrich Co, Castle Hill NSW) and 0.05% BSA. The cells were maintained at 37°C in 5% CO₂. The medium, which contained rhCTGF, was collected at 24 and 48 hour intervals and filtered using a 0.2 µm Ministart filter (Sartorius AG, Goettingen, Germany).

4.3.2 Purification of rhCTGF

A heparin-sepharose affinity column (Hi Trap Heparin HP, Amersham Biosciences, Uppsala, Sweden) was used to purify CTGF from the conditioned medium. The column firstly was equilibrated with phosphate-buffered saline (PBS) and then the filtered medium was allowed to pass through the column at a rate of 0.2 ml/min. The unbound protein was washed from the column using PBS. The rhCTGF that had bound to the column was then eluted using a high salt solution (0.1M NaCl in PBS) and fractions of 1 ml were collected. Excess salt was removed from the fractions by dialysis overnight in 4l PBS using molecular porous membrane tubing (molecular weight cut-off 12 000 – 14 000; Spectra/por, Spectrum Medical Industries, Los Angeles, CA). The dialysed fractions were stored at -80°C prior to quantification of rhCTGF protein concentration and verification of CTGF biological activity.
4.3.3 Quantitation of rhCTGF

To determine the concentration of the purified rhCTGF protein, 20 µl of each fraction was loaded into individual lanes of a 12.5% solution of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and run under standard conditions (120V for 1 hour or until the dye front reached the bottom of the gel). The resulting protein bands were visualised with Coomassie blue dye. A Phoretix image analysis system (Nonlinear Dynamics, Newcastle-Upon-Tyne, UK) was used to determine the concentration of CTGF at the 38 kDa band. This was compared with a protein of known concentration (BSA standard).

4.3.4 Assessment of rhCTGF bioactivity

To quantify the bioactivity of the rhCTGF, its capacity to stimulate fibronectin production in cultured fibroblasts was investigated. Normal human fibroblasts (ATCC, Manassas, VA, USA, cat. CRL1474) were grown to confluence in RPMI containing 10% FCS. The cells were then trypsinised and plated into 6-well plates. Forty-eight hours later the media was removed and replaced with RPMI containing 0.1% albumin and rhCTGF in prescribed concentrations (100, 250, 500, 1000 ng/ml). After a further 48 hours the media was removed and the RNA extracted from the cell layer using Tri Reagent (Sigma-Aldrich). The RNA was reverse transcribed with Superscript III (Invitrogen, Mt Waverley, VIC) and fibronectin gene expression measured by quantitative real-time PCR using primers previously validated (Tan, Mc Lennan et al. 2008). The CTGF used in this study was shown to induce fibronectin expression by four fold at 48 hours which was comparable to the CTGF synthesised by Tan et al. 2008 in which bioactivity had been proven (Tan, McLennan et al, 2008).
4.4 Rodent wounding studies

4.4.1 Induction and maintenance of diabetes in Sprague-Dawley rats

Male Sprague-Dawley rats (n=52) aged between 6-7 weeks were purchased from Australian Laboratory Supply (Perth, Australia), this number had previously proved adequate to yield meaningful data in similar treatment vs. control rodent wound healing experiments (McLennan, Bonner et al. 2008). Type 1 diabetes was induced in 26 animals using STZ (60 mg/kg i.p., Calbiochem, Sydney, Australia) over 3 consecutive days. Animals whose blood glucose measurements were greater than 15 mmol/L after 3 consecutive days of STZ administration were considered diabetic, and those that did not achieve this (n=3) were excluded from the study. Subsequently, animals were maintained on 2-4 IU of insulin (Mixtard, Novo-Nordisk, Malmo, Sweden) every second day to prevent weight loss and ketoacidosis. The insulin was administered every second day, according to blood glucose levels determined with a blood glucose meter, to maintain a target blood glucose level of ~15 mmol/L. All animals had access to standard chow and water ad libitum.

4.4.2 Skin wounding

Seven weeks after the induction of diabetes, diabetic (n=23) and control (n=29) animals were anesthetised using ketamine (85 mg/kg, Pfizer, Sydney, Australia) and xylazine (5 mg/kg, Bayer, Leverkusen, Germany). Dorsal skin was shaved and depilated (Veet depilatory cream, Reckitt Benckiser, Hull, UK). In order to prevent bacterial infection the dorsum of each animal was swabbed with solution of 10% povidone-iodine prior to wounding. In each animal a total of four full-thickness skin wounds were created using an 8mm biopsy punch (Stieffel Laboratories, NSW, Australia) with two wounds on each side of the midline, as shown in Fig. 4.1. The wounds included the panniculus carnosus exposing the underlying
dorsolateral skeletal muscle fascia. At the time of wounding all animals were treated with a single dose of parenteral antibiotic (ampicillin: 50 mg/kg).

Figure 4.1: Wounding locations on rat dorsum.
Full thickness wounds were created using an 8mm punch biopsy.

4.4.3 CTGF treatment

The rhCTGF used for this study was prepared as described in detail in section 4.3 (Tan, McLennan et al. 2008). To examine the effect of CTGF on wound healing, 1µg of the purified rhCTGF in PBS was applied to each of two wounds: one each in an antero-right location and one postero-left. The remaining two wounds were treated with PBS only. The wounds were occluded with a transparent film dressing (Tegaderm, 3M, NSW) which was secured on all sides using Hypafix tape (Smith and Nephew, Victoria).

After an interval of 24 hours the animals were anaesthetised (section 4.4.2 of this chapter) and treated with a second dose of CTGF or PBS vehicle to the same wounds. The wounds were again occluded using Tegaderm and Hypafix tape. This dosing schedule was chosen because the CTGF had to be made in house in a long, low yielding process and was therefore scarce but this dose had previously be shown as efficacious in previous in vitro studies.
CTGF is important in initiating healing, firstly by ‘priming’ the wound and secondly to potentiate healing. On this basis administration at early time points was deemed appropriate.

4.4.4 Wound visualisation and retrieval

In one series of experiments, the effect of CTGF on wound closure was determined by tracing the circumference of the wounds onto transparencies on the day of wounding and at twice-weekly intervals thereafter (days 1, 3, 7, 10, 14 and 21), or until terminated. This time frame was used as it has been shown to produce data reporting wound closure accurately up to day 14, at which time most wounds have healed. In a parallel series of experiments, rats from each group were terminated at day 21 specifically tissue for breaking strength analysis. The tissue containing the wound was excised and either

(i) fixed in paraformaldehyde (4% in PBS) for immunohistochemical analysis; or

(ii) snap frozen in liquid nitrogen for determination of wound breaking strength, thus yielding one treated and one untreated wound for each assay.

4.4.5 Wound area measurement

Wounds were observed during closure and serial wound area measurements were taken. Various methods of wound area measurement were trialled. Visitrak, (a portable digital device marketed by Smith and Nephew for wound measurement (Smith & Nephew Surgical Pty Limited, North Ryde, NSW)), was found in my preliminary work to be insufficiently sensitive to measure wounds less than 10 mm diameter (data not shown). The ARANZ Silhouette system (Merivale, Christchurch, New Zealand), which is designed to capture digitally wound photographs, measurements and clinical notes, was ineffective in this preclinical study due to difficulty in keeping the animals adequately still for routine photography purposes. However, acetate tracings quantitated using Image J software were found to be an efficient method of wound area analysis. It is noted that this method has also
been used for monitoring diabetic wound healing in foot ulcers in diabetes high risk foot clinics such as at Royal Prince Alfred Hospital and for animal wounding studies and found to be a robust method attributable to its reproducibility (Thomson, McLennan et al. 2010).

Wound circumference tracings were translated into computer images using pen-tablet software (Bamboo, Wacom, USA) for quantification of wound area using ImageJ software (Research Services Branch, NIMH, USA). Wound closure was then determined and expressed as a percentage of the original wound size.

4.4.6 Breaking strength

For the measurement of wound breaking strength, healed excised wounds from day 21 were cut into rectangular strips with the width being the diameter of the healed wound area and the length being the diameter of the wound area plus one centimetre on each side to allow attachment to the tension meter. The width, length and thickness of wound pieces were measured using calipers.

To measure tensile strength, the tissue was first glued to balsa wood pieces and then the tissue and the balsa wood pieces were clamped into the jaws of an Elf 3400 tension meter (BOSE EnduraTec, MN, USA). A constant load (45N) at a crosshead speed of 10 mm/min was then placed on the skin until it failed causing the skin to rupture. The cross-sectional area of the skin was measured before the load was applied. The stress and strain required to break the wound and Young’s modulus (tensile strength) was calculated using these values.

4.4.7 Analysis of wound cellular content
The excised paraformaldehyde-fixed, paraffin-embedded wounds were sectioned (5 μm sections) perpendicular to the wound surface. Sections obtained at day 7 and 14 were stained with specific antibodies for CTGF, CD68, collagen IV, αSMA or PCNA (proliferating cell nuclear antigen) (Abcam, Cambridge, MA). Following antigen retrieval, the slides were incubated in a solution of 3% hydrogen peroxide for 10 minutes and washed in Tris-buffered saline (TBS). Non-specific binding was blocked by incubation in 10% v/v goat serum and, after washing with TBS, the sections were incubated for 60 minutes at room temperature (RT) with primary antibodies diluted in TBS. A further TBS wash was carried out before incubation with a secondary antibody for 30 minutes. Cells were visualised using peroxidase-conjugated secondary antibodies followed by 3,3’-diaminobenzidine chromogen (Vector Laboratories, Burlingame, CA). The antibodies used, supplied by Vector Laboratories, Ca, USA are shown in Table 4.1.

**Table 4.1: Primary and secondary antibodies used in immunohistochemical staining**

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Concentration</th>
<th>Supplier</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CTGF</td>
<td>1: 400</td>
<td>In house (ab196) (Tikellis, Cooper et al.2004)</td>
<td>Goat anti-rabbit</td>
</tr>
<tr>
<td>Anti-Collagen IV</td>
<td>1: 200</td>
<td>Abcam (ab86042)</td>
<td>Goat anti-mouse</td>
</tr>
<tr>
<td>Anti-CD68</td>
<td>1: 800</td>
<td>Abcam (ab955)</td>
<td>Goat anti-mouse</td>
</tr>
<tr>
<td>Anti-αSMA</td>
<td>1: 400</td>
<td>Abcam (ab5694)</td>
<td>Goat anti-rabbit</td>
</tr>
<tr>
<td>Anti-PCNA</td>
<td>1: 500</td>
<td>Abcam (ab15497)</td>
<td>Goat anti-rabbit</td>
</tr>
</tbody>
</table>
4.4.8 Analysis of cell density and staining intensity

The mean staining intensity of CTGF in epithelial and granulation tissue was assessed semi-quantitatively by two independent observers blinded to animal condition and wound treatment status using the following scoring system:

0 = no staining;
1 = weak staining;
2 = moderate staining; and
3 = strong staining.

The same scoring system was used to analyse the intensity of collagen IV staining and $\alpha$SMA staining in fibroblasts and epithelial cells. The number of macrophages (CD68 stained) in 20 sequential fields of each section was determined (at 100x magnification under oil) by a single observer blinded to condition and treatment status. The number of PCNA-positive cells was counted in three sequential fields of each section within each wound and the number of positive keratinocytes on the epithelial edge was recorded as a percentage of total keratinocytes in a section from each wound, again by a blinded observer.

4.5 Results

4.5.1 The effect of CTGF on wound closure in diabetic and control rats

To test the therapeutic effects of CTGF on wound healing, full-thickness wounds were established in diabetic and non-diabetic rats and then treated with topical CTGF. Wound healing was assessed over a 14-day period with wound breaking strength examined on day 21. Data for the measurement of macroscopic wound closure expressed as percentage change in the initial wound area at selected time points is shown at Table 4.3.
Table 4.2 Abbreviations and n values for the wound closure rate studies.

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Day 7 n=</th>
<th>Day 14 n=</th>
<th>Treatment Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>C + PBS</td>
<td>58</td>
<td>44</td>
<td>Control + Phosphate Buffered Saline</td>
</tr>
<tr>
<td>C + CTGF</td>
<td>58</td>
<td>44</td>
<td>Control + Connective Tissue Growth Factor</td>
</tr>
<tr>
<td>DM + PBS</td>
<td>46</td>
<td>26</td>
<td>Diabetes + Phosphate Buffered Saline</td>
</tr>
<tr>
<td>DM + CTGF</td>
<td>46</td>
<td>26</td>
<td>Diabetes + Connective Tissue Growth Factor</td>
</tr>
</tbody>
</table>

Table 4.3: Epithelial closure rates.

<table>
<thead>
<tr>
<th>Group</th>
<th>DAY 1</th>
<th>DAY 3</th>
<th>DAY 7</th>
<th>DAY 10</th>
<th>DAY 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>C+PBS</td>
<td>85.7±1.5</td>
<td>57.7±2.1</td>
<td>25.2±0.9*</td>
<td>18.0±0.6</td>
<td>13.0±0.4*</td>
</tr>
<tr>
<td>C+CTGF</td>
<td>81.7±1.4*</td>
<td>52.2±1.8*</td>
<td>25.6±0.9*</td>
<td>16.5±0.4</td>
<td>13.2±0.3*</td>
</tr>
<tr>
<td>DM+PBS</td>
<td>92.8±1.6</td>
<td>61.7±1.7</td>
<td>31.0±1.1</td>
<td>20.7±0.8</td>
<td>15.9±0.5</td>
</tr>
<tr>
<td>DM+CTGF</td>
<td>85.4±1.4</td>
<td>57.5±2.2</td>
<td>28.4±1.2*</td>
<td>16.1±0.7*</td>
<td>13.6±0.4*</td>
</tr>
</tbody>
</table>

Results are expressed as percentage of initial wound size (mean ± SEM), *p<0.05 compared with untreated diabetic wounds (DM + PBS alone), each at the same time point, by ANOVA.

Wound closure was slower in diabetic animals (DM) than in control (non-diabetic) animals (C); (at day 7, C = 25.2 ± 0.9 % original size, DM = 31.0 ± 1.1 % original size; mean ± SEM *p<0.05. In CTGF-treated diabetic rats, macroscopic wound closure was significantly enhanced on days 7, 10 and 14 compared to the non-treated diabetic group (Table 4.3 and Fig. 4.2). In contrast to the effect of CTGF in diabetic wounds, non-diabetic wounds treated with CTGF did not show improved closure rates relative to controls (Fig.4.2).
Figure 4.2 Wound area reduction after cutaneous wounding and CTGF effects. Wound area reduction was significantly enhanced on days 7, 10 and 14 in the CTGF-treated diabetic group (DM+CTGF) compared with the vehicle treated diabetic group (DM+PBS), *p<0.05 by ANOVA. For clarity, only these significant differences are shown in the figure. All statistically significant differences and SEM data are shown in Table 4.3.

4.5.2 The effect of CTGF on wound breaking strength in diabetic and control rats

The effect of CTGF on the wound breaking strength was measured at day 21 when the wounds were healed (control n=30 wounds, diabetes n=30 wounds). As shown in Table 4.4, analysis by ANOVA showed that untreated diabetic wounds tolerated lower levels of final strain before breaking than either CTGF-treated or untreated control wounds (*p<0.005) (average 2.20 MPa compared to 3.08 and 3.45 MPa respectively), indicating that diabetic wounds are less robust. These results are shown in Table 4.4 (A).
Differences between the amounts of strain levels required for the wound to fail was assessed by comparing grouped data from diabetic animals (whether treated with CTGF or PBS) and controls (whether treated with CTGF or PBS). Control wounds were able to tolerate a greater strain before breaking than diabetic wounds (\(^*p<0.05\), Student’s t-test). In tear strain and final strain measurements, diabetic wounds required less strain to fail than control wounds (tear strain 0.79 MPa compared to 1.11 MPa \(^*p<0.01\); \(^\sim p<0.05\) for final strain 2.25 MPa compared to 3.27 MPa; using Student’s t-test) (Table 4.4 (B)). The diabetic wounds tended to have a lower mean Young’s modulus than the control wounds. Thus whilst they may be less stiff, the data is not statistically significant.

Data was collated into groups of PBS- or CTGF-treated (regardless of diabetic status) (Table 4.4(C)). Young’s modulus (or stress in proportion to strain) was found to be greater in CTGF-treated wounds than untreated wounds (\(*p<0.05\) by unpaired t-test) indicating that healed CTGF-treated wound sites were more stiff and less extendable than the untreated wounds (Table 4.4 (C)).

**Table 4.4: Breaking strength of wound sites.**

<table>
<thead>
<tr>
<th></th>
<th>Modulus</th>
<th>Tear Strength</th>
<th>Tear Strain</th>
<th>Ult. Strength</th>
<th>Final Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>C + PBS</td>
<td>0.93 ± 0.09</td>
<td>0.56 ± 0.06</td>
<td>1.17 ± 0.10</td>
<td>1.06 ± 0.01</td>
<td>3.45 ± 0.22</td>
</tr>
<tr>
<td>DM + PBS</td>
<td>0.87 ± 0.13</td>
<td>0.46 ± 0.05</td>
<td>0.71 ± 0.06</td>
<td>0.81 ± 0.13</td>
<td>2.20 ± 0.16*</td>
</tr>
<tr>
<td>C + CTGF</td>
<td>1.21 ± 0.11</td>
<td>0.53 ± 0.05</td>
<td>1.04 ± 0.10</td>
<td>0.89 ± 0.06</td>
<td>3.08 ± 0.27</td>
</tr>
<tr>
<td>DM + CTGF</td>
<td>1.01 ± 0.17</td>
<td>0.53 ± 0.05</td>
<td>0.87 ± 0.18</td>
<td>0.86 ± 0.10</td>
<td>2.32 ± 0.22</td>
</tr>
</tbody>
</table>
### Breaking strength

#### Student’s t-test

<table>
<thead>
<tr>
<th></th>
<th>Modulus</th>
<th>Tear Strength</th>
<th>Tear Strain</th>
<th>Ult Strength</th>
<th>Final Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL CONTROLS</td>
<td>1.06 ± 0.07</td>
<td>0.49 ± 0.21</td>
<td>1.11 ± 0.07</td>
<td>0.98 ± 0.06</td>
<td>3.27 ± 0.17</td>
</tr>
<tr>
<td>ALL DIABETES</td>
<td>0.94 ± 0.10</td>
<td>0.49 ± 0.16</td>
<td>0.79 ± 0.09*</td>
<td>0.84 ± 0.08</td>
<td>2.25 ± 0.13*</td>
</tr>
</tbody>
</table>

#### Student’s t-test

<table>
<thead>
<tr>
<th></th>
<th>Modulus</th>
<th>Tear Strength</th>
<th>Tear Strain</th>
<th>Ult Strength</th>
<th>Final Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL PBS ONLY</td>
<td>0.90 ± 0.07</td>
<td>0.52 ± 0.04</td>
<td>0.99 ± 0.07</td>
<td>0.97 ± 0.05</td>
<td>2.96 ± 0.19</td>
</tr>
<tr>
<td>ALL CTGF TREATED</td>
<td>1.13 ± 0.09*</td>
<td>0.53 ± 0.03</td>
<td>0.97 ± 0.09</td>
<td>0.88 ± 0.05</td>
<td>2.78 ± 0.20</td>
</tr>
</tbody>
</table>

(A) Data from the four groups are as shown; by ANOVA, *p<0.01 compared with untreated control and also with CTGF-treated control; (B) Collated data are shown for all non-diabetic (control) rats and all diabetic rats; by Student’s unpaired t-test, *p<0.01 vs non-diabetic controls (for tear strain); ˜p<0.05 vs non-diabetic controls (for final strain). (C) Collated data are shown for all PBS-treated rats and all CTGF-treated rats; by Student’s unpaired t-test, *p<0.05 vs all PBS treated animals (for Modulus). Results are expressed as mean ± SEM.

### 4.5.3 The effect of CTGF on wound tissue composition in diabetic and control rats

The effect of CTGF on cells involved in wound healing including macrophages, activated fibroblasts and endothelial cells was investigated. The intensity of staining of collagen-IV as a marker of wound healing was examined along with, PCNA as a marker of cell proliferation and CTGF protein in the wound tissue.

#### 4.5.3.1 Macrophage counts in wound tissue

By ANOVA, no differences in macrophage number were observed across the four groups (Fig. 4.3). The number of macrophages, identified by CD68 staining, was ded to be greater in CTGF-treated animals (control and diabetes combined) than in non-CTGF treated animals (control and diabetes combined) at day 7 (p<0.05 by ANOVA). The macrophage count
declined in both treated and untreated groups by day 14, and no statistically significant differences between the groups were observed at this timepoint (Fig.4.3B).

(A) (B)

Figure 4.3: Macrophage count averaged per 20 fields in rat wounds. Tissue macrophages were counted in 20 fields per wound in each of the experimental groups. No statistical difference was observed across each of the groups at day 7 or 14 (panel (B)). Results are expressed as mean ± SEM.

4.5.3.2 The affect of CTGF treatment on activated fibroblasts and mature vascular endothelial cells in wound tissue in diabetic and control rats.

Intensity of staining by α-SMA, a marker of activated fibroblasts and endothelial cells, was measured at days 7 and 14. There were very few activated fibroblasts in diabetic wounds at day 7, either treated with CTGF or untreated. Compared with untreated diabetic animals at day 7, CTGF-treated control animals showed significantly more intense fibroblast staining (Fig 4.4) compared with both treated and untreated non-diabetic animals (*p<0.05 by ANOVA) indicating that healing was more advanced in the CTGF treated control group. Other comparisons were not statistically significant for α-SMA scores.
Figure 4.4: Alpha smooth muscle actin staining scores in fibroblasts in respective wounds. The α-SMA staining scores in wound fibroblasts in control and diabetic rats, with and without CTGF treatment is shown at (A) day 7 and (B) day 14. *p<0.05 vs DM + PBS, and DM + rhCTGF by ANOVA. Results are expressed as mean ± SEM.

There were no differences in α-SMA staining in blood vessels in the four experimental groups (Fig. 4.5). However, when the groups were combined, reduced staining was observed at day 7 in diabetic (treated and untreated combined) compared to control animals (treated and untreated combined) (*p<0.05 by unpaired t-test).

Figure 4.5: Alpha smooth muscle actin staining scores in blood vessels of wounds. Wounds from diabetic and non-diabetic rats treated with CTGF or PBS were stained with α-SMA and staining scores in the endothelial cells was assessed. (A) Day 7 and (B) Day 14 Results are expressed as mean ± SEM.
4.5.3.3 The effect of CTGF treatment on collagen-IV content in wound tissue in diabetic and control rats.

Collagen-IV staining was decreased in untreated diabetic wounds at day 7 (*p<0.05 vs. control+CTGF treated rats). At day 14, CTGF-treated diabetic wounds showed more collagen-IV staining compared with all other groups (untreated diabetic wounds and CTGF-treated and untreated controls) (**p<0.005 by ANOVA), (Fig. 4.6). Representative images are shown (Fig. 4.7). Increased levels of collagen are typical of wound healing.

![Figure 4.6](image)

**Figure 4.6 Collagen-IV staining in wound tissue.**
Intensity of collagen-IV in wounds in control and diabetic rats treated with PBS or CTGF. (A) At day 7 and (B) at day 14. *p<0.05 compared to control + CTGF treated animals; **p<0.005 compared to the other groups. Data were analysed by ANOVA and results are expressed as mean ± SEM.
4.5.3.4 The effect of CTGF treatment on expression in wound tissue in diabetic and control rats.

PCNA is a marker of proliferating cells and has been measured in preclinical models elsewhere to examine wound healing (Liu, Shi et al. 2007). The number of PCNA-positive and PCNA-negative cells in the total wound (both epidermis and dermis) was measured by immunohistochemical staining. The staining data were expressed as the total number of PCNA-positive cells and as a percentage. Cell counts were made by a single observer who was blinded to the tissue source of the wounds.

The data indicated that diabetic wounds showed no clear differences in PCNA nominally (Fig 4.8) or as total number of positive cells (Fig. 4.9), across any of the groups.
Figure 4.8: Percentage of PCNA-positive cells in wound tissue.
PCNA-positive cells expressed as a percentage of the total number of cells in wounds from diabetic and non-diabetic rats treated with PBS or CTGF. (A) Day 7 and (B) day 14. Results are expressed as mean ± SEM.

Figure 4.9: Mean number of PCNA-positive cells.
Total number of PCNA-positive cells in wounds from diabetic and non-diabetic rats treated with PBS or CTGF. (A) Day 7 and (B) day 14. Results are expressed as mean ± SEM.
4.5.3.5 CTGF protein in wound tissue of diabetic and control rats.

CTGF protein was examined in wound tissue macrophages, fibroblasts and endothelial cells by immunohistochemistry. Intensity of CTGF staining was measured (as described in section 4.3.7) and CTGF tended to be marginally deficient in diabetic animals compared with control animals but this was not statistically significant. Treatment with rhCTGF tended to increase CTGF protein in both treated groups at day 7 and 14 compared to controls, although the relative increase of CTGF protein in the treated groups was not statistically significant at day 7 or 14, after the CTGF was applied as per protocol on days 1 and 2 only. This result suggests that the application of topical rhCTGF to a wound may cause only a small non-sustained, transient increase in CTGF levels and that the CTGF measured at days 7 and 14 was produced endogenously.

(A)         (B)

Figure 4.10: rhCTGF in wound tissue samples.

CTGF staining intensity score in wounds of diabetic and non-diabetic rats treated with PBS or CTGF. Results are expressed as mean ± SEM. There were no significant differences between the groups, as determined by ANOVA.
4.6 Discussion

In diabetic wounds, several abnormalities can contribute to impaired healing including; prolonged inflammation, impaired angiogenesis, decreased synthesis of collagen and defective macrophage function (Wang, Li et al. 2009). CTGF already has been shown to hold potential value in contributing to wound healing (Chang, Shih et al. 2004) and can accelerate the healing rate of burns in rhesus monkeys (Liu, Shi et al. 2007).

The data from this chapter demonstrate that rhCTGF applied topically accelerates parameters of wound healing including wound closure in vivo and collagen-IV induction in particular. CTGF is known to act through signalling pathways to induce fibroplasia (Sonnylal, Shi-Wen et al. 2010). Previous studies have shown CTGF can significantly induce collagen protein expression (Gore-Hyer, Shegogue et al, 2002). TGFβ which is mediated by CTGF is also able to induce collagen. Topical application of CTGF improves wound healing towards normal in a diabetic rodent model of full thickness cutaneous wound healing. The main endpoint, rate of epithelial closure as a percentage of original wound size, was accelerated in the diabetic rats treated with CTGF. Some other wound healing end-points were not affected significantly by CTGF treatment, including CTGF staining, α-SMA and PCNA, and the breaking strength of wounds.

Occlusion of wounds using a semi-permeable film dressing ‘Tegaderm’ further validated the model used, as occlusion enabled more accurate visualisation of the wound since no scab or foreign matter distorted or contaminated the wound. Furthermore, in diabetic rodent models the film dressing exerts a ‘splinting’ effect on wound margins and also on contraction. This promotes healing through re-epithelialisation (Chung, Peplow et al. 2010) which
approximates human wound healing, rather than through contraction which predominates in non-splinted rodent wounds.

CTGF did not affect wound closure rates in control rats suggesting that CTGF is able to normalise certain deficits found within diabetic wounds without affecting healing in normal wounds. It is thought that CTGF may normalise wound healing through the augmentation of chemotaxis and mitosis and/or through up-regulation of downstream mediators such as TGF-β. These actions might attenuate the persistent inflammation that is detrimental within the diabetic wound.

The beneficial effect of rhCTGF therapy on epithelial closure was found to occur quite early on in wound healing - a significant effect on diabetic wounds was observed by day 7 after wounding. In this current work, macrophage numbers were increased in wounds treated with CTGF, regardless of diabetes status and may have contributed to the enhanced healing observed in the diabetic wounds.

The CTGF effect may have contributed to macrophage induction in diabetic wounds: increased macrophage infiltration observed within CTGF treated diabetic wounds at day 7 co-segregated with accelerated healing in these wounds. Indeed, the influx of macrophages may contribute to healing, for example the application of macrophages to diabetic rodent wounds has been shown to accelerate wound healing and epithelial closure (Waugh, Sherratt et al. 2006). CTGF is expressed transiently in wounds and this is probably why CTGF protein was not increased at days 7 and 14. The relatively mild differences in macrophages between treated and untreated groups was relatively mild probably due to the modest dosing of CTGF and limited study power. Macrophages upregulate healing through their inflammatory and
reparative phenotypes and the balance between inflammatory and repair macrophages is crucial for successful healing (DiPietro, 1995). Inflammatory macrophages synthesise a range of growth factors which in turn attract fibroblasts and endothelial cells and promote their proliferation (Stout, Jiang et al. 2005). Reparative macrophages also support ECM remodelling (DiPietro, 1995).

The finding that Young’s modulus (a numerical description of the ability of a material to withstand changes in length under tension or compression in one direction, in this case tension), was found to be lower in the diabetic animals than in the controls is consistent with recent literature (Greenwald, Shumway et al. 1993). Young’s modulus was found to increase (t-test p<0.05) in the group of animals treated with CTGF (regardless of diabetes status) with attendant increase in tensile strength observed. The observed increase in collagen-IV after CTGF treatment may contribute to the greater tensile strength of the wound sites. Collagen contributes to wound strength and its increased accumulation in the wound would reasonably explain the increased strength and strain observed in CTGF-treated wounds. These observations are similar to studies with other growth factors. For example, PDGF treatment was shown to induce collagen and cause increased strength in corneal tissue wounds (Murali, Hardten et al. 1994).

Collagen-IV was increased in CTGF-treated diabetic ulcers when compared with untreated diabetic ulcers and controls. Collagen-IV is found primarily in basement membranes, its formation shows progression of the wound to remodelling and therefore end stage repair. Collagen-IV peptides have been shown to promote cell adhesion and migration in corneal epithelial cells suggesting that it can contribute directly to the wound healing process (Cameron, Skubitz et al. 1991). The collagen content of a wound is determined by the
balance between collagen production and degradation (Stadelmann, Digenis et al. 1998) with MMP-9 being a key regulator of collagen-IV degradation (Falanga and Falanga 2005). The direct effects of CTGF on MMP activity have not been studied extensively, although it has been reported that CTGF upregulates the expression of TIMP-1, an MMP inhibitor, in mesangial cells (McLennan, Wang et al. 2004). Therefore, one possible mechanism for the increased collagen content in the diabetic wound is CTGF-mediated upregulation of TIMPs leading to inhibition of MMP-9 and reduced collagen degradation.

Fibroblasts derived from foot ulcers in type 2 diabetes patients show diminished proliferative capacity and hypertrophy (Loots, Lamme et al. 1999). This cellular hypertrophy is characterised by a halt in the cell cycle at the G1 phase, with continued production of cellular proteins, leading to an increase in the overall size of the cell (Wolf and Ziyadeh 1999). Previous cell cycle analysis has revealed that prolonged exposure to high concentrations of glucose arrests mesangial cells in the G1 phase with the concomitant induction of hypertrophy (Wahab, Weston et al. 2002). The process is initiated in response to exposure to high concentrations of glucose. While it might be expected then that PCNA measures in the current work may have exposed differences in cell proliferation however none were observed across wounds in rats with or without diabetes and no rhCTGF effect was observed on this end-point. In addition cell-cycling and phase of tissue cells were not examined.

Relatively small changes in epithelial closure between control and diabetic untreated animals were observed in these studies. Nonetheless, CTGF was effective at accelerating wound closure in diabetes. Future studies may benefit from adopting alternative animal models with greater differences in epithelial closure between diabetic and non-diabetic wounds e.g., a pig
model, which has tighter skin than a rat; or an ischemic model. Ethical and logistical issues prevent the use of these models at this University’s facilities at this stage.

The novel rodent data described in this chapter which indicates the efficacy of topical rhCTGF to induce wound healing in a diabetic rodent model of cutaneous wound healing, complements other data related to CTGF in wounds in diabetes. As described in the literature review section 2.7.5, research colleagues in the same laboratory have found across a series of samples in 32 study subjects with diabetes and foot ulcers, a positive association between immunoreactive CTGF in post-debridement ulcer fluid and ulcer healing. Published data in a primate (baboon) model of wounding in diabetes indicates that CTGF is deficient in wound tissue in diabetes (Thompson, McLennan et al. 2010). On the basis of the current results using CTGF as therapy in a rat cutaneous model in diabetes, combined with the work of others addressing endogenous CTGF regulation in human and non-human primates with diabetes, it is suggested that early-phase studies of topical CTGF in human foot ulcers in diabetes, should now be considered.
Chapter 5

A PILOT STUDY OF PROPOLIS AS A WOUND HEALING THERAPY IN A COHORT OF HUMAN DIABETIC FOOT ULCERS

5.1 Abstract

Propolis has potent anti-inflammatory and anti-bacterial properties and previous small animal studies have indicated its potential to heal cutaneous diabetic wounds. Propolis has a low side-effect profile and is TGA approved for therapy of abrasions, however, no systematic study of the use of propolis in humans with diabetic foot ulcers has been published. In this chapter, the results of a pilot study are presented for 24 subjects with chronic diabetic foot ulcers who received six consecutive weeks of topical propolis treatment applied post-debridement, (in addition to standard care), followed by six weeks of follow-up in monitoring of wounds and patient progress.

5.2 Introduction

The beehive protectant propolis has been shown to have anti-inflammatory and antibacterial actions (Aliyazıcıoglu, Sahin et al. 2011). The complex composition (comprising over 200 compounds) and regional variation in propolis (Marcucci 1995) means it is not possible to determine its active ingredients exactly. However, an extensive literature implicates caffeic acid poly-ester compounds (CAPE) and phenols as probable mediators of at least some of its anti-inflammatory and anti-bacterial properties (Bankova, Christov et al. 2005).
Propolis-treated diabetic rats showed improved wound healing and normalised neutrophil count, and the treatment was safe and well-tolerated treatment (McLennan, Sakar et al. 2009). This novel data indicated propolis to accelerate wound healing in a rodent model of diabetes (McLennan, Sakar et al. 2009). As far as it has been possible to ascertain with any degree of certainty that is the only published study to show that propolis has efficacy in diabetic wounds, albeit in a preclinical environment.

Considering the efficacy of propolis in rodent diabetic wounds and that topical administration has been well-tolerated (section 2.8.5) (Menniti-Ippolito, Mazzanti et al. 2008), it was determined that propolis should be trialed in human diabetic foot ulcers. As propolis has not been systematically examined in human diabetic wounds, it was decided that the correct approach was an initial pilot study to test the study methodology as well as the acceptability, tolerability and safety of topical propolis.

5.3 Methods and materials

5.3.1 Study subjects

Patients with type 1 or type 2 diabetes who attended the High Risk Foot Clinic at Royal Prince Alfred Hospital, Sydney, and who fitted the study inclusion criteria (outlined below) were approached to take part in this study between January and June 2011.

The protocol was approved by the ethics committee of Sydney South West Area Health Service (SSWAHS) (study ID: X10-0266, and HREC/10/RPAH/473) NSW, Australia, and informed consent was obtained from each enrolling patient.
Thirty patients were recruited of whom six patients were subsequently removed from the study, four of whom failing to attend appointments and did not reschedule. A further patient was removed from the study when their wound deteriorated requiring a VAC (Vacuum Assisted Closure) NWPT system, which clinic staff considered sufficiently severe to justify that patient’s exclusion from the study. Three other patients declined to take part in the study and several others were ineligible because they did not fulfill the study criteria. The final number of serial consenting patients successfully taking part in and completing the study was twenty four.

In general, the control subjects and the propolis-treated subjects were similar in demographic and ulcer characteristics (Table 5.1). Notably patient age, diabetes duration and gender distribution were not different nor were the HbA1c levels documented at the time of clinic therapy or the history of past foot ulceration (by student’s t-test). The ulcer area (mainly forefoot site), University of Texas staging and grading of ulcers were also similar in the propolis and control groups (Table 5.1). However, the propolis group had a ~two-fold higher rate of historic amputation (usually a digit), (p<0.01 by student’s t-test) and a greater than two-fold greater prevalence of neuro-ischaemic ulceration compared with the control group (p<0.05 by student’s t-test). The control group had a higher rate of recurrent ulceration but this was not statistically significant (Table 5.1). In both groups, the predominant ulcer location was on the plantar surface of the forefoot or a digit.
Table 5.1: Demographic details of propolis-treated and historic control populations.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Propolis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient number (n)</td>
<td>84</td>
<td>24</td>
</tr>
<tr>
<td>Males (%)</td>
<td>76.2</td>
<td>84.0</td>
</tr>
<tr>
<td>*Age (years)</td>
<td>63.1±13.7</td>
<td>58.1±11.2</td>
</tr>
<tr>
<td>*Type 2 diabetes (%)</td>
<td>86.9</td>
<td>77.3</td>
</tr>
<tr>
<td>*Diabetes duration (years)</td>
<td>17.7±16.5</td>
<td>18.3±9.2</td>
</tr>
<tr>
<td>Smoker (%)</td>
<td>62.3</td>
<td>63.6</td>
</tr>
<tr>
<td>*HbA1c level (%)</td>
<td>8.8±2.0</td>
<td>8.2±1.6</td>
</tr>
<tr>
<td>Previous amputation (%)</td>
<td>15.7</td>
<td>32.0</td>
</tr>
<tr>
<td>Previous foot ulcer (%)</td>
<td>59.6</td>
<td>56.0</td>
</tr>
<tr>
<td>*Ulcer area (mm²)</td>
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<td>240±561</td>
</tr>
<tr>
<td>Texan grade (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>71.3</td>
<td>77.3</td>
</tr>
<tr>
<td>II or III</td>
<td>28.7</td>
<td>22.7</td>
</tr>
<tr>
<td>Texan stage (%)</td>
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<td>31.8</td>
</tr>
<tr>
<td>A</td>
<td>65.0</td>
<td>54.5</td>
</tr>
<tr>
<td>B</td>
<td>13.7</td>
<td>13.7</td>
</tr>
<tr>
<td>C or D</td>
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<td>68.3</td>
</tr>
<tr>
<td>Ulcer type (%)</td>
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<td>22.7</td>
</tr>
<tr>
<td>Neuropathic</td>
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<td></td>
</tr>
<tr>
<td>Neuro-ischaemic</td>
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<td>9.0</td>
</tr>
<tr>
<td>Post-surgical or pressure/trauma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A recurrent ulcer site (%)</td>
<td>21.5</td>
<td>13.6</td>
</tr>
<tr>
<td>Antibiotic therapy - oral (%)</td>
<td>80</td>
<td>75</td>
</tr>
</tbody>
</table>

*Data expressed as mean ± SD.

A foot ulcer of four weeks duration or more was classified as a chronic ulcer, as per the American Diabetes Association (ADA 1999). The exclusion criteria included severe ischaemia as these wounds are unlikely to heal in the absence of revascularisation (Stadelmann, Digenis et al. 1998; Prompers, Huijberts et al. 2007) and, on reviewing the
literature, propolis is not shown to have anti-ischaemic or angiogenic properties. Ischaemia was determined as an ABPI of <0.8 or where vessel calcification was suspected, determined on the result of diagnostic Doppler testing. Since propolis’ anti-microbial properties have not previously been tested on wounds in human subjects with diabetes, individuals with severe infection (defined as requiring IV antibiotics/hospital admission) were excluded from the study. This was based on the advice of senior clinical staff who deemed it inappropriate to enrol patients requiring intensive antibiotic interventions at the early pilot stage of a clinical trial. Participants with wounds that were deemed clinically infected by clinic staff but did not require IV antibiotics and did not have evidence of osteomyelitis were permitted to enrol in the study and to continue with oral antibiotic therapy as prescribed by the attending physician. This was consistent with the control group (described below).

As an external control, ulcer healing results were compared with a cohort of recently-treated historical controls (n=84) from Royal Prince Alfred Hospital High Risk Foot Clinic patients with ulcers who were subject to the same inclusion/exclusion criteria and were receiving ongoing care in the same foot clinic. Notably, the standard care provided in the foot clinic was the same for the historic controls as it was for the propolis study. All study subjects who would have qualified for the propolis study and who were treated in the same foot clinic in the recent years (2008-2010) prior to study recruitment for the were included as controls. The standardised approach to treating diabetic foot ulcers in the clinic was the same in 2008-2010 as it was during the period of the propolis study (2011-2012) and so permitting a valid comparison between these two cohorts. Similarly, the attendant senior medical, nursing and allied health staff were in continuity across the period 2008-2012 and had previously been shown to have a high inter-tester reliability when measuring wound areas for research purposes (Xu, McLennan et al. 2007).
Experienced clinical staff comprising (title; initial):

Endocrinologist ST
Nurse Manager TB
Podiatrist VN
Podiatrist DV
Podiatrist AH

collaborated with the researcher (FH) to establish study inclusion/exclusion criteria that were consistent with ethical requirements—that is to enrol patients who were able to provide informed consent and were deemed low risk for side effects (such as allergy) and adverse events. Standard care (as described in section 5.3.5) was maintained so as not to disadvantage participating patients by withdrawing what is currently considered to be the gold standard of care and also to maintain continuity with the control group. The study design was structured so that the trial could be undertaken with minimal disruption to the daily activities within the clinic.

5.3.2 Study entry inclusion criteria
1. Aged 18-80 years inclusive.
2. Type 1 or type 2 diabetes mellitus.
3. A diabetic foot ulcer of four weeks or greater duration.
4. Must provide written informed consent.

5.3.3 Study entry exclusion criteria
1. Severe pedal ischaemia indicated by an ankle brachial pressure index (ABPI) <0.7.
2. Severe lower limb sepsis requiring IV antibiotics and/or hospital admission.
3. Current enrolment in any other investigational clinical drug trial.
4. Known allergy to propolis, honey, royal jelly, Elastoplast, fragrance mix, or balsam of Peru.
5. Female subjects who were pregnant or lactating.
6. History of any clinically significant disease as determined by the chief investigator.

5.3.4 Investigational therapy

Propolis (Honey Spring Variety, batch number 7232, Vastrade, Lidcombe NSW), which has TGA approval in Australia for use on wounds and abrasions, was administered topically each time the patient attended the clinic for 6 weeks or until the ulcer healed, whichever occurred first. For any non-healing ulcers where surgery was indicated, propolis therapy was ceased prior to surgery. Each subject was followed up for a further 6 weeks after propolis treatment ceased or until their wound healed, whichever occurred first. The study was conducted over a 12 week period as this is a well-recognised and documented time frame in wound healing studies (Marrgolis, Kantor et al. 1999). At each visit, wound area was measured post-debridement using acetate tracing (described in section 5.3.6). Results were compared to historical controls previously described.

Propolis is a brown, odoriferous agent and it was not possible to blind the study subjects or investigators to its treatment. A thin and even coating was painted onto the entire wound surface with a sterile cotton bud, ensuring that the entire wound bed was covered. Due to the fluid consistency of propolis, it was deemed inadvisable to apply a large amount of the agent to the wound as it would run off and soil the dressings, which could increase the frequency of dressing change and therefore cause a difference between the treated subjects and their comparator controls. The study investigator (FH) who applied the propolis was not involved in ongoing patient care (described in section 5.3.5) or in determining ulcer area. The propolis
was applied at the conclusion of each scheduled treatment, just prior to application of
dressings, to minimise any potential bias that could arise from a change in routine care. On
average the wounds were seen in the clinic every 10.5 days, which was in keeping with the
average time between Foot Clinic attendances for the control group (10.4 days).

Figure 5.1: Propolis bottle – the variety used in the study

5.3.5 Standard care

The average time between visits was 10.5 days, with most individuals being seen weekly or
fortnightly for standard care, as is usual practice in the Foot Clinic. This timeframe of
application was maintained for the study in order not to interfere with standard care and to
achieve consistency with the historic controls. Patients in both groups received routine
wound debridement and appropriate optimised offloading for ulcers (offloading types
included CAM walkers, post-op shoes, felt padding, orthopaedic grade footwear and total
contact orthoses) (Liu, Min et al. 2009 ). The types of dressings used in the two groups were
also similar with almost all patients in each group receiving foam dressings (mainly Biatain)
and, occasionally, anti-microbial dressings (mainly Iodosorb) (Liu, Min et al. 2009 ).
Antibiotics were prescribed as deemed appropriate by the clinical staff (including an
endocrinologist, podiatrist and nurse) who were highly experienced in judging signs of local
wound infection such as redness, swelling, discharge and heat, in addition to systemic signs
such as fever, increased body temperature and malaise. Wound swabs were used to culture bacteria on these occasions where there was uncertainty as to whether a wound was infected.

There was little variation in oral antibiotic therapy between the control and propolis treated groups (Table 5.2), with 80% and 75% of patients (n= 66 and 18), respectively, being on oral antibiotic treatment at study recruitment. Anti-\textit{Staphylococcus aureus} antibiotic therapy predominated in each group with 66 of 84, and 18 of 24, (75.5 and 75% respectively), in each group receiving this treatment.

\textbf{Table 5.2: Antibiotic therapy used predominantly during the course of study period.}

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Control</th>
<th>Propolis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes %</td>
<td>80</td>
<td>75</td>
</tr>
<tr>
<td>No %</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>Antistaph (A/S) only %</td>
<td>34.5</td>
<td>50</td>
</tr>
<tr>
<td>A/S + Anaerobic (An) %</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>A/S + Gram –ve %</td>
<td>18</td>
<td>15</td>
</tr>
<tr>
<td>A/S + An + Gram -ve %</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Gram -ve only %</td>
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<td>0</td>
</tr>
<tr>
<td>An only %</td>
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<td>0</td>
</tr>
<tr>
<td>A/S + Anti-MRSA (MR) %</td>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>A/S + Anti- Pseudomonas %</td>
<td>1.5</td>
<td>0</td>
</tr>
<tr>
<td>A/S + MR + An %</td>
<td>1.5</td>
<td>0</td>
</tr>
</tbody>
</table>
5.3.6 Wound area measurement

Wound area was measured using acetate tracings. Two pieces of acetate paper were cut to a size slightly larger than the wound. One piece of paper was swabbed with Hibiscrub (Molnlycke, Frenchs Forest, NSW) to prevent wound contamination and placed over the wounded area whilst the second sheet of acetate was placed on top of the first and an outline of the wound area traced using a permanent marker pen with a 2mm tip. The acetate tracings were scanned onto a PC and wound area was quantitated using Bersoft image software (Lunenburg, Nova Scotia, Canada). This system superseded the ImageJ system used in Chapter 4 as it was able to measure multiple scanned acetate images simultaneously. These measurements were used to calculate percentage change in wound area per wound per visit during the trial period.

Wound healing, for the purposes of this study, was defined as epithelialisation of the entire wound area, as defined by the clinic staff. This definition was based on the work of Lazarus et al. (1994) who described a minimally healed wound as ‘characterised by the restoration of anatomic continuity, but without a sustained functional result’ (Lazarus, Cooper et al. 1994). Within the time frame of the study it would be unlikely that wounds would heal to the point of full functionality and therefore minimal healing, defined as epithelialisation, was accepted as the healing parameter.

5.3.7 Clinical staff questionnaire

Staff (n=4; nursing and main podiatry staff) who were involved in the patient care of the propolis treated cohort, but not propolis administration, were asked to complete a questionnaire using a 7-point Likert scale. The questionnaire reproduced at Table 5.3 was designed to provide feedback regarding the study design and execution.
Table 5.3: Likert scale questionnaire administered to foot clinic staff (n=4).

<table>
<thead>
<tr>
<th>Question</th>
<th>Strongly agree</th>
<th>Neither</th>
<th>Strongly disagree</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trial design</strong></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>The study inclusion/exclusion criteria were appropriate</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Ultra-vulnerable populations may have been under-represented in the study</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trial participants received usual wound care as well as Propolis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>The comparator group (patients seen in the same clinic within the previous 5 years) would have received similar standard care</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>The wound assessment tools (Texas grade/stage/tracings) are accurate/validated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinic staff are adequately experienced and skilled to carry out wound assessment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Propolis therapy</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adverse side effects were reported</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propolis had a positive effect on wound healing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propolis treated wounds showed deterioration in wound area/border</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propolis appears to be easy to apply</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propolis had an unpleasant odour</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propolis treated wounds became malodourious</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propolis was a safe treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propolis was well tolerated by patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propolis is suitable for use over exposed tendons</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propolis is suitable for use over exposed bones</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propolis is suitable for use in patients with osteomyelitis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propolis is suitable for use in patients with severe PVD (ABPI &lt;0.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cost is important when choosing a wound therapy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>At $8 for 50ml, propolis represents good value as a wound therapy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I would consider using propolis as a diabetic foot ulcer treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I would consider using propolis as a diabetic foot ulcer treatment following the results of larger scale trials</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Further research may enhance the scope for using Propolis (e.g. On more critical wounds)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Further exploratory endpoints such as wound closure rate, bacterial count and wound protein composition in terms of key MMPs and CTGF were also examined in order to determine whether or not it was feasible to subsequently consider a larger, more comprehensive study of propolis as a topical wound healing therapy, and the results from these studies are outlined in Chapter 6.

5.4 Results

5.4.1 The effect of Propolis treatment on wound healing.

Weeks 1 and 3 (the second and fourth weeks after treatment with propolis was commenced, the first week being week 0) propolis treated ulcers showed a significantly improved healing rate compared to historical controls, (*p<0.005 and **<0.05 respectively by Mann-Whitney U-test) (Fig.5.1 (A)). The percentage of patients whose ulcers were fully healed at weeks 4, 5 and 7 was higher in the propolis treated group than the controls (*p<0.05) by Chi-squared test (Fig.5.1 (B)).

Table 5.4: Number of active participants at each time-point during the propolis trial

<table>
<thead>
<tr>
<th>Week</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>84</td>
<td>84</td>
<td>84</td>
<td>83</td>
<td>81</td>
<td>81</td>
<td>79</td>
<td>73</td>
<td>67</td>
<td>62</td>
<td>54</td>
<td>53</td>
<td></td>
</tr>
<tr>
<td>Propolis</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>23</td>
<td>22</td>
<td>22</td>
<td>21</td>
<td>19</td>
<td>18</td>
<td>17</td>
<td>15</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

Numbers reduce as wounds heal
Figure 5.2: Percentage wound area compared to original size and percentage wounds healed in propolis vs historic control populations. (A) Percentage wound area. *p<0.005 and **0.05 by Mann-Whitney U-test. (B): Percentage of patients whose ulcers had healed per week. *p<0.05 by Chi-squared test. Results in A are expressed as mean ± SEM.
Figure 5.3: Representative image of a neuropathic ulcer
Panel (A) shows initial size at week 0 and panel (B) shows a reduction in size by 64% at week 4 following topical treatment with propolis.

5.4.2 The effect of Propolis on wound healing in patients treated with antibiotics

Patients were divided into two groups: 1) those who were being treated with antibiotic therapy and 2) those who were not and were therefore not considered to be clinically infected.
Figure 5.4: Wound healing rate of propolis and control wounds with and without concurrent antibiotic therapy. (A) Percentage wound area compared to original size in propolis vs. historic control populations who were receiving concurrent antibiotic therapy. *p<0.001 and **p<0.05 by Mann-Whitney U-test. (B) Percentage wound area compared to original size in historic control populations who were receiving concurrent oral antibiotic therapy compared with those who were not. **p<0.05 by Mann-Whitney U-test. (C) Percentage wound area compared to original size in propolis-treated populations who were receiving concurrent oral antibiotic therapy compared to those who were not. No differences were observed between the groups. All results are expressed as mean ± SEM.
Wounds of patients who were receiving systemic antibiotic therapy and topical propolis showed improved healing at weeks 1, 3 and 4 compared to wounds that received systemic antibiotics only (*p<0.001 at week 1 and **p<0.05 at weeks 3 and 4 by Mann Whitney U-test) (Fig. 5.4 (A)). No such differences were seen when the non-antibiotic treated control and propolis treated groups were compared. Control wounds receiving no antibiotics showed accelerated healing at weeks 2 and 3 compared to antibiotic treated wounds ( **p<0.05 by Students t-test, Fig. 5.4 (B)). No differences were observed between healing rates of antibiotic and non-antibiotic treated, propolis-treated groups as shown in Fig. 5.4 (C).

No adverse effects of propolis therapy were reported by patients enrolled in the propolis study, thus none were reported in progress ethics reports.

5.4.3 Clinical staff questionnaire

The four clinical staff addressing a Likert scale questionnaire, gave quantitative responses to questions posed about:

i) the trial design; and

ii) the propolis therapy.

These responses were graded 1-7, with 1 indicating strong agreement with the statement and 7 indicating strong disagreement, and are summarised in Fig. 5.5 (A and B). The total of the scores for each respondent is given for each answer. Overall a score of less than 16 indicates a tendency to agreement, a score of 16 is neutral across the group, and a score of greater than 16 indicates a tendency to dis-agree with the respective statement made. The staff responses were, in general similar between respondents for each question with the exception of respondent 2 whose responses were quite different to the other 3 respondents for the
questions: ‘Ultra vulnerable populations may have been underrepresented’ and ‘Adverse side
effects were reported’. Whilst respondent 2 did not disagree with these statements as
strongly as their colleagues, their responses were more neutral than their colleagues. Given
that no adverse effects were reported or communicated to research staff it is most it is likely
that this staff member was indicating that their opinion regarding the study population and
adverse effects was neutral, and not negative.

Overall, the staff were satisfied with all aspects of the trial design, suggesting that the
selection criteria, methods of wound assessment, patient care and experience of clinical staff
in assessing the wounds were adequate (Fig. 5.5(A)). They also opined that, in their
observation, the propolis trial group was comparable to the historical control group (Fig.
5.5(A)).

In terms of the therapy itself, staff indicated through their responses that they believed
propolis to generally be a safe, well-tolerated treatment that was easy to apply and they would
consider using again (Fig. 5(B)). It did not cause significant harm to the wound border or bed
nor did it have an unpleasant odour profile (Fig. 5(B)).

Clinical staff responses indicated that they would be reluctant to apply propolis onto exposed
tendon or bone or in areas with an osteomyelitic and/or severe ischaemic component due to
the paucity of evidence regarding the safety and efficacy of propolis in more ‘high risk’
pateints at this time. It was considered that more research is needed into propolis as a therapy
and if this research was undertaken, then the clinical staff would be more inclined to use it
more broadly as a wound therapy. The staff did not consider propolis to be expensive, but
nor was cost a significant detriment when choosing a wound therapy (Fig. 5.5).
Figure 5.5: Graphical representations of the total Likert scale responses from 4 clinical staff members.
5.5 Discussion

This chapter has examined the feasibility of topical propolis as a therapy in diabetic foot ulceration. Propolis was found by patients and Foot Clinic allied health staff to be a well-tolerated and very acceptable therapy. The lack of adverse effects in patients receiving propolis therapy is not surprising considering that the treatment is generally thought to have a low side effect profile in the general population and it is available over the counter both as a topical preparation and as an oral preparation.

Propolis has been used since ancient Greek and Roman times for its medicinal properties. As a topical agent it is reported to be an effective treatment to aid resolution of cuts and abrasions (Okonenko 1985) and also to treat inflamed throats (Pang and Chen 1985). It has not been systematically studied in diabetic foot ulcers. Some years ago, our laboratory reported that propolis topical therapy as a single application improved ulcer healing rate in full-thickness skin wounds in diabetic rats (McLennan, Bonner et al. 2008). In that study, the healing rate was improved to levels similar to those seen in non-diabetic animals. This current research took this therapy one step further in conducting a pilot study into propolis therapy for human diabetic foot ulcers.

To gauge the potential efficacy of propolis, ulcer healing rate was measured and compared with a cohort of historic controls. The data suggest that propolis may have a beneficial effect on ulcer closure rate. The change in ulcer area favouring propolis was consistent with the known bioactivity of propolis and its time course of administration in that greater differences in healing rate were observed in the first six weeks of the study when Propolis was administered (Fig.5.2(A)). The rate of healing slowed when propolis therapy ceased after 6 weeks treatment, further supporting the role of propolis in wound healing. This finding
suggests that the action of propolis is relatively transient and that more frequent or persistent dosing may increase its effects. Considering the potent anti-inflammatory action of propolis it is notable that the improved ulcer healing was seen most clearly after an initial dose of propolis therapy (Fig.5.2(A)). The finding of increased epithelial closure in propolis-treated wounds compared to controls is consistent with the animal work previously undertaken in a rat model, where one application of topical propolis normalised ulcer closure in the diabetic rats with full thickness ulcers (McLennan, Bonner et al. 2008).

In the control group, healing rates were improved at weeks 2 and 3 in the wounds that did not receive antibiotic therapy. It is logical that these wounds heal more rapidly, as they lack the complicating factor of infection. In contrast, the healing rate between the antibiotic treated and non-antibiotic treated propolis-treated wounds was similar. This finding suggests that the anti-bacterial properties of propolis prevented a delay in healing that otherwise would occur in infected wounds. This is particularly salient in light of the recent finding that propolis potentiates the potency of most antibiotics, especially those active against *S. aureus* (Wojtyczka, Dziedzic et al. 2013). Future studies should examine these preliminary data in a larger RCT design.

This study was not intended to definitively determine if propolis was effective in foot ulcer healing, however, it did show that propolis was safe, well-tolerated and easy to use. Sheehan et al (2003) reported that achieving 50% wound healing by week 4 is a robust predictor of long-term healing prognosis: these authors found that if a wound was not 50% healed at week 4 then there was only a 9% chance of it healing within 3 months (Sheehan, Jones et al. 2003). Power calculations based on this pilot study show that in order for propolis-treated wounds to show a 25% improvement in healing compared to wounds receiving standard care at week 4 with a 90% confidence interval (CI) (that is, for the propolis-treated group to be 42% of
original size and control wounds to be 56% of original size), 68 subjects would need to be recruited in each arm of the study (propolis/control). For a higher confidence interval of 95% (that is, for the propolis-treated group to be 30% of original size and control wounds to be 50% of original size), 103 subjects would need to be recruited in each arm of a randomised controlled study. Both of these calculations assume variance as in this study, with 80% power needed to indicate a statistically significant difference in treated vs untreated groups.

Clinical staff involved in the trial reported that propolis had a positive effect on wound healing. This assumption would have been made based on a series of casual observations as clinical staff were not directly involved in analysing the data. It is nevertheless encouraging that feedback was positive. The main contention discernable from the Likert scale responses was that the clinical staff indicated they would be reluctant to use propolis as a therapy on wounds that are considered more ‘critical’—that is, wounds that probe to bone/tendon or that have an element of osteomyelitis or ischaemia. It is not surprising that clinical staff adopted this conservative stance given the preliminary nature of the evidence to support propolis at this early stage, and that such wounds were excluded from the study design. It is encouraging that questionnaire responses indicated that clinical staff were engaged with the concept of propolis as a wound therapy and would welcome more research in this area.

Clinical staff did not have great concern regarding the financial cost of wound therapies. It is likely that this is because their clinic is established and relatively well-resourced as its primary concern is to effect appropriate clinical outcomes. It is widely known that health complications such as foot ulcers in diabetes are more prevalent in lower socio-economic groups (Dalstra, Kunst et al. 2005) and therefore the low cost of propolis as a wound therapy may increase its appeal to clinics that are less well-resourced; individuals who finance their
wound therapies themselves; and in developing countries where many modern wound therapies are unavailable or prohibitively expensive. This research does not in any way detract from the established therapies and multi-disciplinary approach undertaken by the diabetes High Risk Foot Service, which were described in detail in Section 2.4 of this thesis. However it suggests that topical propolis therapy applied in the clinic in addition to established therapies may be effective especially in helping some ulcers to heal more rapidly and that it is well-tolerated.

In summary, the results indicate that topical propolis is well-tolerated, highly acceptable to patients and staff, and may have efficacy in promoting healing rate in human diabetic foot ulcers above that seen by current optimised multi-disciplinary foot ulcer care.

The following chapter extends the study of propolis by undertaking exploratory analysis of proteins and viable bacteria in diabetic foot ulcer wound fluid, in propolis-treated diabetic ulcers.
Chapter 6

THE EFFECT OF PROPOLIS ON HUMAN WOUND CHEMOKINES

6.1 Abstract

Propolis is a naturally-occurring substance that contains more than 200 chemicals. It is derived from bees, through plant buds that are collected on the hind legs of worker bees. It is applied by bees to their hives as a protectant resin. The most biologically-active fractions of propolis are reported to be flavanoids and esters of caffeic acid. In this chapter, post-debridement human wound fluid extracted from propolis-treated wounds was examined and MMP, CTGF and viable bacterial number and type reported. These results were compared to previous analyses of wound fluid from foot ulcers in historic controls that did not receive propolis treatment but had similar ulcers and demographic characteristics.

6.2 Introduction

Wound healing is impaired in diabetes, and this has been shown to contribute a significant burden to society. One-third of US$116 billion directly apportioned to diabetes care in the US in 2007 was used to treat foot ulceration (Driver, Fabbi et al. 2010). Growth factors such as intact CTGF are deficient in diabetic wounds (Thomson, McLennan et al. 2010) and the balance between proteases and their inhibitors is also disturbed (Muller, Trocmé et al. 2008).
Recent work has shown in animal models that both CTGF (chapter 4 of this thesis) and propolis (McLennan, Bonner et al. 2008) are able to improve wound healing and that increased MMP-9 predicts poor wound healing (Liu, Min et al. 2009).

To explore the potential modes of action of propolis, wound fluid collected from propolis-treated human wounds (as described in section 5.3.5) was tested for MMP-2 and -9 by zymography and CTGF by western immunoblot and the results were compared to control wounds. Viable bacterial counts after propolis therapy were also determined. Some of the wounds were small and/or dry and it was not possible to extract sufficient wound fluid to test for all biomarkers. However, given that this was a preliminary study, the results are useful to test research methodologies and laboratory techniques and to target more specific outcome measures for future studies.

6.3 Methods and Materials

6.3.1 Wound fluid collection

Wound fluid was collected from patients enrolled in the propolis trial. On each occasion, following ulcer debridement, but prior to the application of propolis, 2 x 25µl samples of wound fluid were obtained from study subjects using a calibrated sterile paperpoint tip (Meta Biomed Co., Elmhurst, NY). This was mixed with 100 µL sterile PBS and frozen at -80°C. The wound fluid solution was transferred to the University of Sydney Central Clinical School Endocrine Research Laboratory and analysed in the laboratory for wound fluid CTGF by Western immunoblot, MMPs by zymography and bacterial colony count by serial dilution and culture on agar plates, using established techniques in each case (Liu, Min et al. 2009; Thompson, McLennan et al. 2010; Xu, McLennan et al. 2007. Some wounds were dry and it was not possible to collect enough fluid from these wounds for analyses. For this reason,
fluid was collected at visits 1, 2 and 3 because after this time the majority of wounds were unable to yield sufficient fluid as they were dry or had healed.

On average, the time between visits was 10.5 days, with most individuals being seen weekly or fortnightly for standard care (as described section 5.3.5), as is usual practice in the High Risk Foot Clinic. Therefore it was decided that changes in CTGF, MMP and bacterial load levels be calculated as a percentage change per 10 days. Rate of change in wound area (%) was also calculated over the same period. The data for the propolis-treated wounds were compared to a cohort of patients derived from the historical control cohort described in section 5.3.1 that had wound fluid analysed in the same manner in previous studies. This included CTGF measured in 10 controls (from the cohort described in section 2.7.5) (Dr L. Lo, personal communication July 2008), MMP-2 and -9 in 39 controls (Liu, Min et al. 2009) and bacterial load in 32 controls (Xu, McLennan et al. 2007). The demographic details of these three cohorts of patients are described below (Tables 6.1, 6.2 and 6.3).

6.3.2 Study subjects

Comparison of the control subjects with propolis-treated subjects whose wound fluid was analysed for CTGF indicated that in general, the two groups were of similar age and ulcer type. The patients in the propolis groups had larger wounds (average of 139.7 mm² compared to 83.1 mm², not statistically significant) and that these patients had on average a longer duration of diabetes (17.5 years vs. 11 years, p<0.05 by student’s t-test).
Table 6.1: Demographic details of historic control patients used in the CTGF wound fluid analysis.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Propolis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient number (n)</td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>Males (%)</td>
<td>90</td>
<td>82</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>58.0±9.7</td>
<td>57.4±11.5</td>
</tr>
<tr>
<td>Diabetes duration (yrs)</td>
<td>11.0±5.9</td>
<td>17.5±5.1</td>
</tr>
<tr>
<td>Ulcer area (mm²)</td>
<td>83.1±79.2</td>
<td>139.7±170.6</td>
</tr>
<tr>
<td>Tex grade (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 or I</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>II or III</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Tex stage (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A or B</td>
<td>100</td>
<td>93.4</td>
</tr>
<tr>
<td>C or D</td>
<td>0</td>
<td>6.6</td>
</tr>
<tr>
<td>Ulcer type (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neuropathic/neuro-ischaemic</td>
<td>90</td>
<td>87</td>
</tr>
<tr>
<td>Post-surgical</td>
<td>10</td>
<td>6.6</td>
</tr>
<tr>
<td>Trauma</td>
<td>0</td>
<td>6.6</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD.

The demographics of patients whose wound fluid was analysed for MMPs are displayed in Table 6.2. The control and propolis cohorts showed similar HbA1c levels, although the propolis treated group had a generally higher proportion of subjects with type 1 diabetes (33.3% compared to 17.7% in the control group, p<0.05 by student’s t-test) and the control group had larger (average area of 168.3 mm² compared to 139.7 mm², not statistically significant) and more severe ulcers in terms of Texas grade and stage (p<0.001 by student’s t-test)
Table 6.2: Demographic details of historic control patients used in the analysis of MMP - 2 and -9

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Propolis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient number (n)</td>
<td>39</td>
<td>15</td>
</tr>
<tr>
<td>Males (%)</td>
<td>79</td>
<td>80</td>
</tr>
<tr>
<td>*Age (yrs)</td>
<td>62.0±11</td>
<td>57.4±11.5</td>
</tr>
<tr>
<td>*HbA1c</td>
<td>8.4±2.1</td>
<td>8.3±1.9</td>
</tr>
<tr>
<td>*Ulcer area (mm²)</td>
<td>168.3±79.2</td>
<td>139.7±170.6</td>
</tr>
<tr>
<td>Tex grade (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 or I</td>
<td>70</td>
<td>100</td>
</tr>
<tr>
<td>II or III</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>Tex stage (%)</td>
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<td></td>
</tr>
<tr>
<td>A or B</td>
<td>71</td>
<td>93.4</td>
</tr>
<tr>
<td>C or D</td>
<td>29</td>
<td>6.6</td>
</tr>
<tr>
<td>Ulcer type (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neuropathic/neuro-ischaemic</td>
<td>77.5</td>
<td>86.8</td>
</tr>
<tr>
<td>Post-surgical</td>
<td>17.7</td>
<td>6.6</td>
</tr>
<tr>
<td>Trauma</td>
<td>4.8</td>
<td>6.6</td>
</tr>
</tbody>
</table>

*Data are expressed as mean ± SD.

Comparison of patients whose wound fluid was analysed for bacterial count showed the two cohorts were similar in terms of age, ulcer type and ulcer Texas stage and grade. The propolis-treated cohort had a generally longer duration of diabetes (mean 19.9 years compared to 14.6 years in the control group) and smaller wounds (90.8 mm² compared to 170 mm²), although these parameters were not statistically significantly different across the groups.
Table 6.3: Demographic details of historic control and propolis groups used in the analysis of bacterial load.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Propolis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient number (n)</td>
<td>32</td>
<td>11</td>
</tr>
<tr>
<td>Males (%)</td>
<td>69</td>
<td>82</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>60.0±9</td>
<td>58.5±9.9</td>
</tr>
<tr>
<td>Diabetes duration (yrs)</td>
<td>14.6±10.1</td>
<td>19.9±10.6</td>
</tr>
<tr>
<td>HbA1c level (%)</td>
<td>7.9±1.4</td>
<td>8.8±1.9</td>
</tr>
<tr>
<td>Ulcer area (mm$^2$)</td>
<td>170±312.6</td>
<td>90.8±81.5</td>
</tr>
<tr>
<td>Tex. Grade (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 or I</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>II or III</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tex. Stage (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A or B</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>C or D</td>
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<td>0</td>
</tr>
<tr>
<td>Ulcer type (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neuropathic/neuro-ischaemic</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Other</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Concurrent antibiotic therapy (%)</td>
<td>83</td>
<td>73</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD.

6.3.3 Measurement of biomarkers in wound fluid

6.3.3.1 Western immunoblot to measure wound fluid CTGF

For the analysis of diabetic wound fluid, CTGF was measured by Western immunoblot. For each lane of the Western blot, 6.6 µL of wound fluid solution (collected was mixed with 17 µL of Laemmli sample loading buffer (5 ml 1M Tris pH 8.8, 2 g SDS, 10 ml glycerol, 100 mg bromophenol blue in 20ml distilled water and 2% v/v β-mercaptoethanol/L) and the solution made up to 45 µL with 21 µL PBS. Separation of the proteins was achieved on
SDS-PAGE using 4-15% graded mini TGX gels (BioRad) in running buffer (3 g Tris, 14.4 g glycine, 10 g SDS) at 150V for 45 min. Proteins on the TGX gel were then transferred onto a nitrocellulose membrane (BioRad Transfer Mini) using a BioRad Turbo Transblot SD Semi-Dry Transfer Cell. The nitrocellulose membranes were blocked using 5% (w/v) skim milk powder in Tris-Buffered Saline and 0.05%Tween (TBST) for 30 minutes at RT and then subjected to 3 x 5 minute washes with TBST.

Membranes were probed for CTGF using an in-house manufactured CTGF antibody (CTGF 196 end terminal (Tikellis, Cooper et al. 2004) antibody diluted 1:1000 in TBST for two hours at RT with gentle agitation, as previously described (Thomson, McLennan et al. 2010). Following the incubation with primary antibody, the membrane was washed with 3 x 5 minute washes in TBST and incubated with horseradish peroxidase-conjugated anti-rabbit IgG at a 1:20000 dilution (Sigma, Vector laboratories, CA, USA) at RT with gentle agitation for one hour. Membranes were washed for 3 x 5 minutes in TBST and immunoreactive proteins were detected by chemiluminescence using the ECL plus Western Blot Detection System (ECL) (Amersham Biosciences, Piscataway, NJ, USA). The light emitted by the labelled protein was detected by ChemiDoc fluorescence and chemiluminescence gel documentation system (Bio-Rad, Gladesville, NSW). Protein was quantitated using semi-quantitative methods by Phoretix 1D Advanced Version 3 (NonLinear Dynamics Ltd., USA) all as previously described (Thomson, McLennan et al. 2010).

Some samples had been freeze-thawed more than once which could potentially lead to some CTGF degradation, affecting the validity of the results. To test this, one wound fluid sample from a study subject was aliquoted into three eppendorf tubes in triplicate. One of the three samples was frozen at -80°C; then the second was frozen and thawed twice in the same
manner; the third was frozen and thawed four times. All samples were thawed and assessed by CTGF Western immunoblot the average results are shown below in Table 6.4. These were not significantly different.

<table>
<thead>
<tr>
<th></th>
<th>1 Freeze</th>
<th>2 Freezes</th>
<th>4 Freezes</th>
</tr>
</thead>
<tbody>
<tr>
<td>65kDa</td>
<td>5.97 x 10^6</td>
<td>5.85 x 10^6</td>
<td>6.79 x 10^6</td>
</tr>
<tr>
<td>26kDa</td>
<td>2.18 x 10^6</td>
<td>3.07 x 10^6</td>
<td>2.23 x 10^6</td>
</tr>
<tr>
<td>Total</td>
<td>8.14 x 10^6</td>
<td>8.92 x 10^6</td>
<td>9.02 x 10^6</td>
</tr>
</tbody>
</table>

### 6.3.3.2 Measurement of wound fluid MMP-2 and -9 by gelatine zymography

MMP-9 and MMP-2 in human wound fluid were measured by zymography. For this analysis, 1µl of wound fluid (in 4µl of PBS, as diluted on collection) was run on each gel. The fluid was mixed with 2µl sample loading buffer (5ml 1M Tris pH 6.8, 2g SDS, 10ml glycerol, 100mg bromophenol blue in 5ml distilled water). The proteins were separated on SDS-PAGE using an 8% polyacrylamide gel with 3% stacking gel in running buffer (60.6g Tris, 288g glycine, 20g SDS/l) at 130V for 55 min. The gels contained 1mg/ml gelatine, which had been fluorescently labelled with 2-methoxy-2, 4-diphenyl-3-dihydrofuranone. For each assay, standards of known concentrations of MMP-9 and MMP-2, as well as a pre-stained molecular weight marker (Invitrogen, CA, USA), were run on the gels along with the samples.

The gel was incubated for 30 min with gentle agitation in 50ml Buffer #1 at RT (25 ml 2M Tris, 2ml 10% NaN₃, 1µL1M ZnCl₂, 25ml 100% Triton X-100, pH7.5, in distilled water). Buffer #1 was drained and the gel was incubated for 30 min with gentle agitation in 50ml Buffer #2 at RT (25ml 2M Tris, 2ml 10% NaN₃, 1µl 1M ZnCl₂, 25ml 100% Triton X-100, 10ml 1M CaCl₂, pH7.5, in distilled water). Buffer #2 was drained, and the gel was covered
with plastic and incubated at 37°C overnight with gentle agitation in Buffer #3 (25ml 2M Tris pH 7.5, 2ml 10% NaN₃, 1µl 1M ZnCl₂, 10ml 1M CaCl₂, pH 7.5 in distilled water). The gel was photographed under long-wave ultraviolet light illumination and MMP activity was quantitated using semi-quantitative methods by Phoretix 1D Advanced Version 3 (NonLinear Dynamics Ltd., USA).

In this technique, gelatinases (including MMP-9 and MMP-2) hydrolyse the copolymerised protein substrate and are seen as dark bands on a grey background. MMPs were identified by the position of the bands at their specific molecular weight (57 and 83 kDa for active MMP-2 and -9 respectively). Activity of the MMPs was calculated from the digital image, and percentage change in intensity was calculated by comparison with the loaded standard.

6.3.3.3 Measurement of wound fluid bacterial load

Wound fluid was mixed with PBS as described in section 6.3. Ten microlitres of the fluid-PBS mixture was then serially diluted (10⁻² to 10⁻⁷), streaked onto blood agar plates, and aerobically incubated for 24 h at 37°C. Bacterial load was quantified by counting the CFUs on each plate. The bacterial species were identified using standard microbiological techniques, including gram stain and microscopic examination. Previous studies have verified the reproducibility of sampling in a post-debridement wound fluid sample by this method (Xu, McLennan et al. 2007).
6.4 Results

6.4.1 MMP-2 and -9 levels in wound fluid from propolis-treated and control wounds

Wound fluid samples from the propolis-treated study participants were analysed by zymography to measure levels of active (as opposed to latent ‘pro’) MMP-2 and -9 at visits 1, 2 and 3, as presented in the representative image (Fig.6.1).

![Zymograph Image]

**Figure 6.1: Representative zymograph showing MMP-2 and MMP-9**
The standard (STD) is a known quantity of MMP-2 and MMP-9. WF, wound fluid from propolis treated diabetic ulcers in duplicate at visit 1 in lanes one and two, and visit 2 in lanes three and four.

The results for active MMP-2 and MMP-9 (n=15) (i.e. the small molecular weight form of each of the MMPs) were then compared with data from a previous study in which wound fluids were gathered from subjects (n=39, not treated with propolis) that had received standard wound care over a similar time period (described in section 5.3.5). This control cohort had attended the same foot clinic and received similar care to the propolis group in this study (Liu, Min et al. 2009). Wound fluid samples were analysed by zymography to measure levels of active (as opposed to latent ‘pro’) MMP-2 and -9, expressed as log_{10} values at visits 1, 2 and 3. Analysis of fluid obtained at visits 1 and 2 are presented in duplicate shown in the representative image (Fig. 6.1). The rate of change in MMP levels was calculated as a percentage change over 10 days. Rate of change in wound area was also calculated over the
same period. The changes in MMP levels and wound healing rate were transformed logarithmically in order to account for outliers, making graphical representation of these data more clear.

Percentage change in active MMP-9 levels was lower between log-transformed data from propolis-treated subjects and the historical control group in the first time period (between visits 1 and 2) (*p < 0.05 by Mann-Whitney U-test; % change /10 days -0.68 (propolis) vs. 2.64 (controls) mean ± SEM; Fig. 6.2 (A)). During the second time period (between visits 2 and 3), active MMP-9 was significantly lower in propolis-treated wounds (% change /10 days, 0.05 (propolis) vs. 0.64 (controls), mean ± SEM). This difference in active MMP-9 between propolis and control treated wounds was statistically significant (**p < 0.005 by Mann-Whitney U-test, as shown in Fig. 6.2 (B). Wound healing rate over the same time points was increased in the propolis-treated groups compared to controls at visits 1-2 (p < 0.005) and visits 2-3 (p < 0.001), as shown in Fig. 6.2 (C).

Across visits 1 to 2, and 2 to 3, there were no differences in the change in MMP-2 levels between propolis-treated and the historic controls (Fig. 6.2 (A and B)).
Figure 6.2. MMP-2 and MMP-9 changes in post-debridement wound fluid. Active MMP-9 and active MMP-2 changes from (A) visit 1 to visit 2 and (B) visit 2 to 3, each per 10 days, in control and propolis-treated wounds. (C) Change in wound healing rate over these time points. Data are mean ± SEM. The y-axis data is log transformed in each case. At visits 1-2 and 2-3, the difference in the change in active MMP-9 between the propolis and control groups were significant; *p<0.05 and **p<0.005 by Mann Whitney U-test. No such differences were seen in MMP-2 levels. Propolis-treated wounds healed at a significantly greater rate at both time points (*p<0.005 and **p<0.001 by Mann Whitney U-test). Results are expressed as mean ± SEM.
6.4.2 CTGF levels in wound fluid from propolis-treated and control wounds

CTGF was measured by Western immunoblot in the wound fluid collected from propolis-treated diabetic foot ulcers, as described in section 6.3.1.1. CTGF exists in differing molecular forms, as shown (Fig. 6.3) and was found to be consistent with previously published data (Tan, McLennan et al. 2008), (Thomson, McLennan et al. 2010), (Tikellis, Cooper et al. 2004). The main immunoreactive band was observed at 26 KDa in this wound fluid series. As CTGF is predicted to occur at 32-38 KDa on full length, this band likely represents a partially proteolysed, C-terminal form of CTGF whilst the less intense higher molecular mass material may be a homodimer of this protein (Thomson, McLennan et al. 2010). For these studies, CTGF immunoreactivity detected in each entire lane of the gel was determined by densitometry as previously described (Tan, McLennan et al. 2008), with the background non-specific signal in the gel subtracted.

![Figure 6.3: Representative Western immunoblot showing bands of CTGF immunoreactivity.](image)

CTGF was measured in the propolis-treated subjects from whom adequate wound fluid was obtained (n=15). These results were compared with a group of historic controls (n=10) whose wound fluid had been analysed for CTGF described from a cohort of patients described in the literature review (section 2.7.5) set out at Table 6.1. This cohort of patients was also derived from those used as controls in the propolis wound closure study. Wound
healing rate as a percentage of original wound area was also calculated for both groups. The changes in immunoreactive CTGF and wound healing rate were transformed logarithmically in order to account for outliers, making graphical representation of these data more clear.

The change in CTGF levels per 10 days was not different between the two groups (Fig 6.4 (A) and (B)). Furthermore, within the propolis group when CTGF changes across the visits indicated were related to ulcer healing rate, there was no significant correlation found (not shown).

Figure 6.4: Change in total immunoreactive CTGF in post-debridement wound fluids (A) and change in wound healing rate (B). (A) Change in the level of CTGF over a 10-day period in propolis-treated and control patients. (B) Change in wound healing rate over a 10-day period. Data are mean ± SEM.
6.4.3 Bacterial load in wound fluid from propolis-treated and control wounds.

Wound fluid obtained at visits 1 and 2 from each study subject was analysed for bacterial load from propolis-treated wounds and control wound, as previously summarised in table 6.3. The results are reported as change in CFU per 10 day period. The bacterial species identified in the wound fluid are presented in Fig. 6.5 as a pie chart with each slice reflecting specific bacterial CFUs expressed as a percentage of the total number of bacteria detected by species (per ml wound fluid). The size of the pie chart is larger for visit 1 than visit 2, reflecting differences in total colony count (26% less at the second visit for the propolis-treated wounds).

![Pie charts showing relative amounts and types of bacteria at visit 1 and visit 2.](image)

**Figure 6.5: Pie charts showing relative amounts and types of bacteria at visit 1 and visit 2.** The different bacteria cultured on blood agar after 24 hours are shown. The average time difference between visit 1 and visit 2 was 10 days and was within the range of 1-2 weeks.

Following the first application of propolis, bacterial count was reduced in the whole cohort, from a total of 118.4 CFU to 87.6 mean per sample, ± SEM. Whilst this was not statistically
significant, possibly due to variation between study subjects, it indicates a 26% reduction in bacterial burden in the wound. The most noticeable decrease in bacterial species between visits 1 and 2 was for *P. aeruginosa*. This bacterial species was cultured from the wound fluids of three subjects and it was not possible to perform further statistical analysis for these samples.

The data from the propolis treatment group was compared with a cohort of 48 patients in a comparable published cohort (Xu, McLennan et al. 2007) whose wound bacterial count had previously been determined and calculated as a percentage change per 10 days (Fig. 6.6). The bacterial count showed a decrease of less than 1% in the control group over 10-day period as opposed to 26% in the propolis-treated group.

**Figure 6.6: Graph of % change in bacterial colony forming unit counts in the first 10 days.** The colony count had decreased by 26% in the Propolis treated group compared with no demonstrable decrease in controls * p<0.0001. Results are expressed as mean ± SEM.

The bacterial count decreased markedly in the propolis-treated group compared with historic controls, p<0.001 ± SEM (Fig.6.6).
6.5 Discussion

The previous chapter focused on the end-points of safety and tolerability in addition to the acceptance of propolis as weekly topical therapy delivered in a diabetes multi-disciplinary foot clinic. The feasibility data was encouraging; pilot data examining the potential wound healing efficacy of propolis was also examined and found to hold some promise. This chapter further explored the wound fluid microenvironment post-debridement in propolis-treated ulcers.

CTGF is known to be induced during normal wound healing (Frazier, Williams et al. 1996) but little is known regarding its levels in diabetic wounds. Studies by Thomson et al. (2010) using a baboon diabetic model showed that this wound tissue contained less CTGF at 4 weeks post-wounding compared with controls (Thomson, McLennan et al. 2010). Furthermore unpublished data Dr L. Lo, (personal communication, July 2008) has shown in wound fluids from diabetic patients the wound fluid CTGF correlates positively with wound healing. Propolis is known to possess anti-inflammatory properties so observing the levels of CTGF in propolis treated tissues assisted in determining whether CTGF is upregulated by propolis. However the extent to which wound fluid CTGF is affected remains unknown despite being investigated as part of this study, as endogenous CTGF could be examined over a relatively short time period only.

The changes in CTGF levels in post-debridement wound fluid in propolis-treated wounds were not statistically different to untreated controls over a 10-day time period. The similarity between the two groups may be attributable to the narrow (10 day) time period of measurement. CTGF induction may not have been stimulated during this relatively early
phase of wound healing following propolis topical application: as described in the literature review (section 2.7.5) changes in CTGF immunoreactivity may take series of weeks to be observed in wound fluid in human diabetes. The data based on Western immunoblot analysis appear reliable and it is unlikely that technical issues affected the experimental outcomes as the techniques used have been trialled extensively in previous studies. In cultured dermal fibroblasts propolis has no direct effect within hours or days on CTGF expression J. Jia, (personal communication, 8 October 2009) and it is possible that any link between propolis treatment and wound fluid CTGF is indirect, possibly secondary to general effects on wound healing per se.

Previous studies over a more protracted period (3 months+) showed that increases in CTGF in the wound correlate positively with wound healing rate, section 2.7.5 (Fig. 2.4). Given the data presented at section 2.7.5, it would be interesting to investigate these changes over a longer timeframe of 12 or 16 weeks in order to gain a more comprehensive, longitudinal understanding of the effect of propolis on CTGF.

MMPs are involved in wound healing and continue to persist in inflamed wounds; causing excessive degradation of the ECM; and contributing to delayed healing that is observed in persistently inflamed wounds such as diabetic foot ulcers. The protease MMP-9 in wound fluid is important as a marker and potential mediator of foot ulcer healing in diabetes. In contrast, high levels of persistent active MMP-9 correlate with poor wound healing and predict future non-healing (Ladwig, Robson et al. 2002). Reductions in active MMP-9 correlate with increased wound healing rates and collagen content in a diabetic rat model (Aparecida Da Silva, Leal-Junior et al. 2013 ). For this reason, these were examined in the post-debridement wound fluid of propolis-treated wounds. In the current study, propolis-
treated wounds showed significantly greater reductions in active MMP-9 than the untreated controls. One potential mechanism to explain this activity is that caffeic acid, a component of propolis, can inhibit expression of MMP-9 (Jin, Chung et al. 2005). In addition to being a marker of wound healing, MMP-9 can potentiate inflammatory responses (Gill and Parks 2008) and therefore, a propolis-mediated decrease in MMP-9 could attenuate and resolve persistent inflammation in diabetic wounds and leading to accelerated healing.

Wound fluid could not be obtained in all cases for serial analysis due to dry, very small or healed wounds, which limited the analysis. Owing to limited amounts of wound fluid being available, other pro-inflammatory proteins and markers were not measured. Given larger fluid samples, factors such as TNF-α, IL-1, prostaglandin metabolites and neutrophil elastase could be examined since these are key mediators in the healing process.

Bacterial load was decreased in propolis-treated wounds by 26% over 10 days whilst untreated control wounds showed no significant change in CFU count. Therapy comprising topical propolis and where wounds were deemed to be clinically infected (73%), systemic antibiotics, resulted in this reduction of viable bacterial count. By contrast control wounds which received systemic antibiotic therapy alone if deemed clinically infected (83%) did not show such a reduction. This is in keeping with other studies that have recognised propolis’ antibacterial properties (Bonvehi, Coll et al. 1994; Lamprecht, 1994; Bankova, Christov et al. 1995).

As wounds heal, bacterial colony counts generally fall even where wounds that are not overtly clinically infected (Xu, McLennan et al. 2007). Clinical data from our group has shown that there is a cross-sectional association between lower bacterial CFU count and
human foot ulcer healing rate (Xu, McLennan et al. 2007). Moreover, Edmonds et al. (2004) found in a study of diabetic foot ulcers, that even when an ulcer was not clinically infected, antibiotic therapy can reduce the incidences of hospitalisation and amputation (Edmonds and Foster 2004). Bacteria are destructive in the wound environments in a variety of ways. For example, their ability to secrete proteolytic enzymes can lead to an increase in MMP secretion that may cause an alteration in the delicate balance of MMP/TIMP activity in wounds. Further studies with a larger patient cohort will be needed to rigorously test the potential bactericidal activity of propolis and to explore more precisely which bacteria are sensitive to propolis.

In summary, the exploratory laboratory examinations of post-debridement wound fluid in propolis-treated diabetic foot ulcers indicated a trend towards the effectiveness of propolis as a therapy. Despite the small sample size and limited size of the comparator historic controls, the data were found to be consistent with the published ability of propolis to reduce wound inflammation and exert bactericidal activity (Kosalec, Peleljinjak et al. 2005; Ledon, Casaco et al. 1997; Lamberte, Cabrera et al. 2011). Collectively, the wound fluid data parallel the improved ulcer healing rate seen by propolis therapy and help to form the basis for larger, multicentre randomised controlled trials of propolis as an intervention in addition to standard optimised care in foot ulcers in diabetes.
Chapter 7

DISCUSSION AND CONCLUDING REMARKS

7.1 Introduction

Foot ulceration commonly is seen in patients with diabetes causing significant mortality and morbidity (Boulton and Vileikyte 2005; Moulik, Mtonga et al. 2003). The incidence of foot ulcers in people with diabetes ranges from 1.0% to 4.1%, and the incidence of lower-extremity amputations ranges from 2.1 to 13.7 per 1000 in such patients (Bartus and Margolis 2004). Current treatment regimens include coordinated multi-disciplinary approaches to offloading, debridement, dressing, infection control and revascularisation (Kruse and Edelman 2006). Evidence-based guidelines aims to standardise clinical decision making, therefore benefitting patient outcomes and streamlining health care systems (Wraight, Lawrence et al. 2005). The benefits of this approach have been shown in the context of diabetes: in applying evidence-based multidisciplinary treatment a 50% reduction in major lower-limb amputation risk has been achieved in people with diabetes (Van Damme and Limet 2005).

In addition to the traditional interventions mentioned above, other therapies have been developed to assist healing in wounds in diabetes. These include negative wound pressure (NWPT), skin substitutes, hyperbaric oxygen and various growth factor therapies (Hopf, Humphrey et al. 2001). Autologous platelet plasma gel (Driver, Hanft et al. 2006), ultrasound (Ennis, Foremann et al. 2005) and various autologous engineered scaffoldings
(Uccioli, Giurato et al. 2011) have also been investigated as potential wound healing therapies. Despite these interventions and the introduction of standardised guidelines, many ulcers still do not heal and others are recalcitrant. It is essential that healing in wounds in diabetes is optimised in order to improve patient outcomes and to minimise use of limited health care resources.

7.2 CTGF as a wound therapy

CTGF promotes the production of ECM and inhibits its degradation (Wang, McLennan et al. 2010). CTGF also functions by affecting cellular responsiveness to other cytokines such as TGF-β (Arnott, Nuglozeh et al. 2007). Studies have shown that rhCTGF in human wounds correlates with wound healing rate (section 2.7.5). To explore this finding, rhCTGF was added to diabetic and control rodent wounds using an established model in order to test whether CTGF could improve wound healing. The main conclusion arising from this work was that rhCTGF improved epithelial closure and improved collagen-IV induction in rats with diabetes, in a statistically significant manner.

As described in section 2.7.5, while some recent reports have implicated CTGF implicated CTGF in post-wound scar formation and keloid, macroscopic excessive scarring in the treated CTGF wounds was not observed in this study, although the planned limited study duration may have precluded the possibility of determining such a change. It is possible that CTGF as topical therapy could induce scarring including in foot ulcer healing in diabetes. Furthermore, systemic adverse effects of CTGF topical therapy to induce ECM and scarring in non-cutaneous tissues is a realistic concern as described in the literature review in section 2.7.4. In particular, increased CTGF in an environment of diabetes could contribute to
systemic effects such as scar formation in kidney, heart and liver and it could also potentially act as a cancer progression factor if cancer is present (Twigg and Cooper 2004). However, as discussed in section 2.5.1, the growth factor PDGF has give rise to such concerns for foot ulcers in human diabetes yet it has been registered as a therapy for this indication. It is envisaged that future preclinical studies will need to confirm that CTGF has bioactivity to induce healing in diabetes models in skin wounding. Toxicology studies will also be necessary to examine for degree of systemic absorption when CTGF is delivered to cutaneous ulcers. Methods to minimise systemic CTGF levels from such administration should be investigated.

7.3 Propolis as a wound therapy

Based on the known anti-inflammatory and anti-microbial activity of propolis, we conducted a pilot study to determine its potential for treatment of human diabetic ulcers. It has not been possible to study CTGF in this manner as CTGF is not currently approved for use in human subjects. However, propolis is TGA approved for human use and was chosen to be trialled in a small-scale human study. Propolis is not a novel wound therapy per se, it has been used for thousands of years as a traditional wound therapy (Najafí, Vahedy et al. 2007) and has been shown to reduce MMP-9 and neutrophil counts in a rodent model of diabetic wound healing (McLennan, Sakar et al. 2009). It has not previously been systematically studied in foot ulcers in human subjects with diabetes.

Wounds in individuals with diabetes suffer from persistent inflammation and tend to be heavily and poly-microbially colonised (Dowd, Wolcott et al. 2008). In such wounds, bacteria persist in adhesive biofilm communities, which further promotes chronic
inflammation. These bacteria are more resistant to anti-microbial therapy and healing is delayed as a result (Rhoads, Wolcott et al. 2008). The known anti-inflammatory and antibacterial properties of propolis therefore, combined with positive preclinical data (McLennan, Bonner et al. 2008), make it a logical target for a human wound healing study. Previous studies of propolis have indicated that it is well tolerated, minimally allergenic and low cost therapy. The human study results shown in Chapter 5 were consistent with the animal studies, showing improved wound healing with propolis treatment. The rate of healing slowed when propolis therapy was ceased after 6 weeks, further supporting the therapeutic role of propolis in wound healing. The trial design was positively rated by the clinical staff who were involved in the study and Likert scale responses indicated that the clinical staff viewed propolis to be a safe and well tolerated therapy warranting further investigation.

In this pilot study, historical controls were used. Retrospective data has been used successfully in many studies elsewhere. In work by Lawrence et al. (2004), a retrospective analysis of patient records concluded that variability in care ought to be addressed in order to optimise outcomes when using historical controls (Lawrence, Wraight et al. 2004). The controls had received standard care in the same multidisciplinary setting as the propolis-treated subjects and were demographically similar, justifying their pre-specified plan as a valid comparator to the pilot study. In the absence of any systematic study of propolis in people with foot ulcers in diabetes, the study team was keen to undertake an examination of tolerability and safety, as well as practicality of propolis as therapy added to routine clinical care. After considering this study’s outcomes it can be concluded that the study was successful: tolerability and safety as well as practicality were addressed within the confines of the small sample size. Furthermore efficacy data enabled calculations of best-estimate numbers required to treat in a multicentre, prospective randomised controlled trial format.
Mechanisms by which propolis may lead to improved wound healing were also examined. Propolis-treated wounds had less active MMP-9 than untreated controls. High levels of MMP-9 have been shown to correlate with poor wound healing and predict future healing (Ladwig, Robson et al. 2002; Liu, Min et al. 2009). Sufficient wound fluid could not be collected from the propolis-treated wounds to permit effective measurement of other proteins or for cytokines involved in wound healing. Many wounds were small or dry and did not yield adequate post-debridement fluid. Ideally, fluid would have been collected from all wounds over a longer period allowing a more comprehensive analysis of wound proteases, their inhibitors and chemokines. This would have enabled a connection between propolis treatment and CTGF induction to be established. As discussed previously this may have occurred at a later time point than was considered in this study. These may be significant considerations for future studies.

Propolis treatment decreased bacterial load (CFU) significantly in diabetic wounds. Studies by my colleagues in our laboratory found that increased bacterial count predicts poor wound healing in neuropathic ulceration, the predominant ulcer type in this study. Therefore, the capacity of propolis to reduce bacteria is an important finding and it may go some way to explaining some of propolis’ efficacy in ulcer healing (Xu, McLennan et al. 2007).

Multiple studies previously have found that propolis has a capacity to reduce bacterial load in wounds (Kosalec, Pepeljnjak et al. 2005; Marghitas, Mihai et al. 2010; Özkalp and Özcan 2010). This finding suggests the need for further investigation of the capacity of propolis to reduce bacterial burden and the maturation and differentiation of biofilms in clinical infection (Lamberte, Cabrera et al. 2011).
It has been established that the composition of propolis determines its efficacy at least against bacteria (Pepeljnjak and Kosalec 2004). Yet studies have not yet been undertaken in skin ulceration in preclinical models in diabetes. Differences in the antibacterial activities of propolis are observed, depending on region of origin and procedure used for the preparation of propolis.

Propolis comprises over 200 individual constituents. At this stage it is not productive to isolate each of these as it is more practicable to direct research into propolis as a composite instrument. It may be for subsequent research to isolate the individual compounds for analysis. Potency could also be studied in subsequent preclinical series in vivo, as published work indicates the efficacy of propolis may be dose dependent (Pepeljnjak and Kosalec 2004).

The mechanism of propolis delivery could be improved through its incorporation into a film or dressing through impregnation. Gelatine-based films plasticised with sorbitol for ease of application, have the capacity to deliver antimicrobial activity and their polyphenol concentration remains stable for 177 days of storage (Bodini, Sobral et al. 2013). Combining propolis which is known to have anti-inflammatory and anti-bacterial properties with such a scaffold could potentially deliver sustained anti-inflammatory and anti-bacterial elements to diabetic wounds and promote their healing over a prolonged time frame in line with work published by Bodini et al. (2013) in which this method was used to deliver extracts of propolis to wounds, (Bodini, Sobral et al. 2013). In the human study in this thesis, propolis was easily applied as a thin film across the entire ulcer bed post-debridement. However, it is anticipated that to further aid ease of use, full strength (that is, neat) propolis impregnated
sorbitol dressings could be used in subsequent RCTs. By this means propolis could be applied freshly with each change of dressing which in usual foot ulcers in diabetes occurs roughly every 3 days. This approach would enable an ulcer to receive more constant exposure to propolis than the current study.

7.4 Future studies and directions

The positive outcome of the animal trial of rhCTGF suggests that CTGF could be examined in a pilot study in human foot ulceration for people with diabetes. However growth factors in protein form are prone to proteolysis. Moreover, it has been proposed that application of a growth factor in a liquid or gel form might not be the best approach to target the growth factor to the cells directly involved in wound healing (Margolis, Crombleholme et al. 2000) and alternative methods of delivery may be more effective. For PDGF delivery, adeno-associated viral vectors were found to deliver a more localised and prolonged effect (Chen and Giannobile 2002). This type of vector to CTGF therapy is a promising approach. In particular, localisation of the effects of CTGF to the wound, as able may be crucial, because whilst its deficiency in diabetic wounds may be detrimental to wound healing, CTGF has been shown to be over-expressed in diabetic heart (Way, Isshiki et al. 2002), liver (Paradis, Perlemuter et al. 2001; Rachfal and Brigstock 2003) and kidney tissues (Twigg, Cao et al. 2002), potentially leading to fibrotic damage.

A larger scale, multicentre blinded randomised controlled trial (RCT) to determine if propolis, in combination with standard wound care, significantly improves the healing of diabetic foot ulcers compared with standard wound care alone should be performed, with assessment of wound size and closure. Based on power calculations made from the data
collected in this study, at least 68 subjects would need to be recruited into each arm of an RCT. A multicentre study is indicated, including at least four diabetes multidisciplinary foot clinics.

For a study such as this, the following protocol would need to be established: The study patients should be treated with topical propolis to complete wound closure, as our preliminary studies observed wound healing rate decline in propolis-treated wounds when propolis treatment ceased at 6 weeks. Other acceptable and commonly used time points to predict longer term wound healing rate include 4 weeks and 12 weeks (Sheehan, Jones et al. 2003). These intervals could also be analysed as surrogate endpoints and, importantly, if the term of the trial precluded the parameter of complete wound healing from being achieved, these surrogate endpoints would allow an analysis of unhealed subjects in the trial.

To date angiogenic properties have not been described for propolis. At this stage propolis would not be expected to share the same efficacy in critically ischaemic that pro-angiogenic treatments such as activated protein C (Whitmont, Reid et al. 2008). For this reason, patients with an ischaemic limb must be excluded from the proposed trial. The threshold for ischaemia would be set at an ABPI of 0.7 or below, a commonly used cut-off point for ischemia. Patients with an abnormally high ABPI (>1.2) would also be excluded due to the likelihood of them having arterial calcification that precludes an accurate APBI reading from being obtained. The proposed trial would be blinded, and an acceptable placebo for propolis would need to be developed. The propolis would be applied post debridement as a thin topical layer by a healthcare professional in a clinical setting blinded to treatment group, at a standardised dose calculated per mm² wound area. Standard wound care would also be administered to all participants.
Statistical analysis of results would examine *incidence of closure* endpoints, using categorical techniques (e.g., Chi-square,) and for *time-to-closure* endpoints, outcome survival analyses would be performed (e.g. Kaplan-Meier).

Secondary endpoints that may provide some mechanistic insight include wound fluid analysis and wound tissue histology. The calibrated paperpoint (described in section 6.3.1) enables a uniform amount of fluid to be drawn from a wound (providing the wound is large enough). Wounds of less than 5 mm diameter usually do not yield enough fluid for laboratory testing, so wounds would need to be excluded from the wound fluid analysis of the study when they reached this size. A wound biopsy would enable histological changes to be monitored, such as macrophage, neutrophil, fibroblast and endothelial cell infiltration. Wound biopsies are a safe procedure that do not delay overall healing of the chronic wound, so there should be no major ethical issues pertaining to the modification to include ulcer biopsy within this arm of the study (Panuncialman, Hammerman et al. 2010).

### 7.4.1 CTGF and propolis as potential combination therapy.

The concept of combined anti-inflammatory and a growth factor therapy is attractive as synergism could potentially occur in wound healing, including in diabetes where inflammation persists and growth factor proteolysis is recognised. The data from our pilot study indicated that wounds heal more quickly and express higher levels of CTGF when treated with topical propolis. Whether CTGF is directly induced by propolis is uncertain and such could not be detected in the short term examination in the current pilot. Studies of human and preclinical wounding for example by mechanical measures or burns, show that wound tissue CTGF is induced more than one week after wounding, and the studies described
in the literature review in this thesis (2.7.5) found that some months were required in human ulcers in diabetes for CTGF to increase as human ulcers heal in diabetes. It may be that the link between propolis and CTGF is less direct and that if propolis is used as therapy, then CTGF should be analysed over subsequent months, as well as in the initial weeks after propolis application.

If propolis is shown to induce CTGF across some days or weeks in wounds in people with diabetes, then this could occur through induction of CTGF gene expression and new protein synthesis and/or through inhibition of CTGF degradation. As described in the literature review in section 2.7.1 depending on the model used, it takes many days to some weeks for CTGF be induced during wound healing including normal healing. Furthermore, in baboon studies CTGF levels were reduced at 2 and 4 weeks in sterile subcutaneous wound tissue of animals with diabetes compared with non-diabetic controls (Thompson and McLennan, 2010). In that study, CTGF mRNA levels in the wound tissue were not different between diabetic and control animals. Also in that study, wound fluid from diabetic animals was found to proteolyse CTGF through MMP activity including MMP-9 (Thompson and McLennan, 2010) suggesting that CTGF in diabetic wounds may experience proteolysis. In wounds in animals with diabetes the theme of growth factor degradation through proteolysis is quite common (Roth, Piekarek et al. 2006), reflecting the increased inflammatory and protease active environments in wounds in diabetes (Wetzler, Kampfer et al. 2000). Hence any increased CTGF after propolis therapy in preclinical or clinical studies in ulcers in diabetes may be attributable to decreased CTGF proteolysis since MMP-9, a powerful proteinase, was significantly decreased in propolis-treated wounds in the current pilot. This decrease in MMP-9 could prevent degradation of CTGF, leading to enhanced CTGF-mediated wound healing through its chemotactic, proliferative and angiogenic functions.
7.4.2 Therapeutic delivery systems

The study in Chapter 4 shows that CTGF can augment the self-healing capacity of wounds by enhancing one or more processes important for healing. However, the mechanisms to apply topical growth factors in clinical settings remains limited attributable to lack of robust delivery systems and biomaterial carriers (Koria 2012). In general, it has been difficult to maintain full bioactivity of proteins applied to wounds due to protein instability in the protease-rich environment of the wound (Choate and Khavari 1997). This suggests that protease inhibitors added with CTGF could potentially augment the enhanced wound healing due to topical CTGF application. This notion is supported by Wlaschek et al (1997), who identified that the most prominent isoform of PDGF, PDGF-PDGF is degraded by wound fluid from chronic venous leg ulcers, and that the loss of PDGF stability could be prevented by addition of specific protease inhibitors. For example, the serine protease inhibitor phenylmethylsulphonyl fluoride could prevent the degradation of PDGF by chronic wound fluids (Wlaschek, Peus et al. 1997).

Anionic collagen mimetic peptides (CMPs) were shown in studies by Wang et al (2008) to exhibit specific binding affinity to type I collagen substrates and to attract vascular endothelial growth factors (VEGFs). Subsequent enhanced morphological features of endothelial cells showed that these newly developed CMPs could be used to direct proliferation and differentiation of cells in collagen scaffolds by promoting localisation and sustained delivery of growth factors (Wang, Leong et al. 2008). Collagen gelatinase sponge scaffolds have been developed that release positively charged growth factors such as bFGF in a sustained manner. Such scaffolds and also CMPs hold promise as vehicles for growth factors, including CTGF for skin regeneration (Kanda, Morimoto et al. 2012). In another analysis, Geer et al (2005) conjugated keratinoocyte growth factor (KGF) into a fibrin matrix.
to achieve ongoing, localised KGF infiltration into wounds, in concert with wound cellular 
demand (Geer, Swartz et al. 2005). Strategies such as this to optimise delivery of topical 
therapies ‘on demand’ would potentially accelerate wound healing.

Upton et al. (2008) reported that complexes comprising IGF and IGF-binding proteins that 
are bound to the ECM protein vitronectin (VN) significantly enhanced cellular functions 
related to wound repair (Upton, Cuttle et al. 2008). Moreover, the delivery of growth factors 
in this manner resulted in therapeutic benefit at doses 1/10th–1/2,000th of those previously 
trialed with growth factor alone. Given the high costs associated with growth factor therapy, 
these results indicate that relatively low dose CTGF (eg less than 0.1 mg of CTGF, rather 
than the 0.4 mg or more used in PDGF studies) in combination with vitronectin may be both 
effective and cost saving. Recent studies have shown that propolis treatment stimulated 
significant increases in vitronectin complexes (Olczyk, Komosinska-Vassev et al. 2012), 
providing a further dimension to this field.

7.5 Study limitations

In the first study the growth factor CTGF was assessed for its ability to heal wounds. A more 
highly powered study would enable more robust conclusions to be derived from the studies. 
For example macrophage numbers appeared to be normalised by CTGF in diabetic wounds 
but this was not statistically significant, possibly due to type II error due to small numbers. 
Dosing of CTGF was also relatively limited, as only 2 doses were administered. A more 
sustained delivery of CTGF to the wound might result in greater observable effects of this 
treatment.
The propolis study was carefully described as a pilot study and therefore one must be mindful of this in drawing conclusions from the results. Obviously a larger scale, randomised controlled study would allow more reliable results to be shown but this was not the purpose of this work. As previously discussed it was difficult to obtain adequate amounts of wound fluid for analysis of propolis treated wounds. Ideally larger wounds would be included as they usually yield more fluid and this would be taken into account in future study designs. Also in future studies a qualitative element could be added to ascertain the patients opinions regarding the propolis treatment. This would add another dimension to the study.

7.6 Concluding remarks

In conclusion, it is important to recognise that with good care and standardised evidence-based protocols, most wounds in people with diabetes will heal. Not all people with diabetes have access to this optimal care and amputation rates amongst patients with diabetes remain unacceptably high. It is important to improve healing rates in order to improve patient quality of life and to decrease demands on funding and resources. This work has shown in preliminary studies that wound healing rates can be improved by using topical agents to manipulate the wound microenvironment. These agents are potentially easy to administer and cost-effective when compared to other wound healing therapies. Laboratory studies also indicated that these agents have the capacity to improve the quality of the healing tissue within a wound in diabetes so that they are able to more closely approximate a normally healing, granulating non-diabetic wound.

The novel studies of topical rhCTGF in ulcers in rats with diabetes indicate that like some other growth factors, such as PDGF and bFGF, CTGF improve ulcer healing including
wound closure and quality of dermal tissue in subjects with diabetes. Future studies will need to examine defining optimal doses and methods of CTGF administration to ulcers to enhance its local effect and minimise its potential systemic adverse effects. It is envisaged that CTGF delivery as protein or in a viral vector or protective scaffold, possibly even with propolis, may ultimately, after series of supportive studies, be demonstrated to aid ulcer healing in humans with diabetes.

The study of propolis efficacy and tolerability in human foot ulcers in diabetes and the exploratory, laboratory-based examinations of post-debridement wound fluid in propolis-treated diabetic foot ulcers demonstrate the potential of propolis as an effective therapy. Its anti-inflammatory and anti-bacterial properties were demonstrated. Although this was a pilot study, the data obtained were promising and consistent with these published positive activities of propolis. Collectively, these promising data promoting propolis as therapy forms the rational basis to consider a larger, multi-centre randomised controlled trial with propolis as a topical therapy to optimise foot ulcer care in people with diabetes.


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