A PILOT RANDOMIZED CONTROLLED TRIAL OF NANOCRYSTALLINE SILVER DRESSING AGAINST MANUKA HONEY DRESSING AND CONVENTIONAL DRESSING IN HEALING DIABETIC FOOT ULCER

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A Pilot Randomized Controlled Trial of Nanocrystalline Silver Dressing against Manuka Honey Dressing and Conventional Dressing in Healing Diabetic Foot Ulcer

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CERTIFICATE OF ORIGINALITY

I hereby declare that this thesis entitled “A pilot randomized controlled trial of nanocrystalline silver dressing against manuka honey dressing and conventional dressing in healing diabetic foot ulcer” is my own work. To the best of my knowledge and belief, it reproduces no material published or written, nor material that has been accepted for the award of any other degree or diploma, except where due acknowledgement has been made in the text.

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ABSTRACT

INTRODUCTION

One of the common complications of diabetes is diabetic foot ulcer (DFU). Some individuals receive multiple surgeries and even amputation. Up to now, there is no gold standard for the topical treatment of DFU. Among all topical interventions, the use of nanocrystalline silver (nAg) and manuka honey (MH), which have similar effects in terms of antibacterial and anti-inflammatory actions, is becoming more widespread. Based on the literature, there is no evidence on using nAg so the effect of nAg dressing on DFU healing is unknown.

STUDY AIMS

There were two study aims in this pilot study. The first one was to test the feasibility of the study design and the acceptability of the interventions. The secondary one was to test the preliminary effect of nAg dressing against MH and conventional dressings on DFU healing.

METHODS

This study was an open-label randomized controlled trial using a three-group design: i) nAg with alginate (the experimental group); ii) MH with alginate (the comparison group); iii) conventional dressing (paraffin tulle) (the control group). A total of 31 eligible participants (11 in the nAg group, 10 in the MH group and 10 in the conventional group) were recruited after they had been discharged from two hospitals and from one GOPD which are all under the operation of Hospital Authority. The inclusion criteria included living in community settings,

being type 2 DM patients, being aged 40 or over and having a foot ulcer larger than 1 cm in diameter and located at or below the malleolar region of the foot. The exclusion criteria included HbA1c level ≥ 10%, an ankle brachial index ≤ 0.4, an ulcer exposing bone or joint, osteomyelitis, severe wound infection, known allergy to the tested materials, known case of venous ulcer, tumor or autoimmune disease.

All the participants received the interventions in an orthopedic nurse clinic of a regional hospital (hospital A). Each participant visited the clinic once each week in the first 4 weeks and then once every two weeks from the 5^{th} to 12^{th} week of this study. In each clinic visit, the student investigator carried out the specialized nursing care, which included patient education on self-care management in diabetic control and foot care, providing off-loading method, cleaning the wound and sharp debridement as well as applying the topical dressings to each participant according to the group allocation. The outcomes for preliminary effect of nAg included the proportion of complete healing; ulcer size reduction; the concentration of biochemical markers [total protein, matrix metalloproteinase-9 (MMP-9), tumor necrosis factor-alpha (TNF-α) and interleukin-1 alpha (IL-1α)] in wound fluid, bacteriology; and severity of wound infection. All study outcomes were assessed in each clinic visit, except the concentration of biochemical markers that were assessed within the first 4 weeks.

In the feasibility and acceptability tests, the comments and feedback from the participants, research assistants and intervention providers were collected. In

addition, the student investigator also self-reflected and self-reviewed on the whole study process.

RESULTS

Some issues were identified from the feasibility and acceptability tests and they were required to improve in the main study. These feasibility issues included inappropriate and inadequate numbers of study centers, non-blinding of group allocation on collection and analysis of wound fluid, insufficient frequency and long duration of clinic visit, insufficient length of trial period, lengthy education session, inadequate pain control on sharp debridement, inadequate skills of research assistants and inappropriate instrument for outcome measures. In the acceptability issues, the unsatisfactory adherence of on foot care and off-loading in middle-aged participants and the possible barriers to these issues were also identified.

The result on preliminary effect of nAg showed that the percentages of participants who experienced complete ulcer healing and ulcer size reduction were the highest in nAg group (nAg>MH>conventional groups) but there was no significant difference among the three groups. Besides, there was no significant difference in concentration of total protein, MMP-9, TNF-α and IL-1α than MH and conventional dressing groups in MMP-9 concentration among groups. The concentration of all biomarkers did not align with the ulcer healing in terms of ulcer size reduction. There was no obvious trend of concentration changes of all biomarkers. In bacteriology and severity of wound infection, there were also no differences among groups. The major reasons for the insignificant results

included small sample size as well as specialized nursing care on foot care and serial sharp debridement that further reduced the differences among groups. In addition, the short duration of observation period in wound fluid analysis may result in the non-alignment of clinical and laboratory outcomes.

CONCLUSION

To conclude, it was the first pilot trial to explore the feasibility, acceptability and preliminary effect of nAg on DFU. The preliminary test this study included clinical and laboratory data, both results triangulated together to address the study objectives. This study approach extended the scope of nursing research in wound care. This pilot study contributed to the preparation of future main study and as a reference of other similar studies and also the potential development of evidence-based nursing clinical practice. The recommendations to address the issues identified from the feasibility and acceptability tests would improve the internal and external validity of the main study. Overall, the present study result served as a foundation of the study design of future main study and other similar studies.

Publication and Presentations

Publications

1. Tsang, K.K., Kwong, E.W.Y., Woo, K.Y., To, T.S.S., Chung, J.W.Y., Wong, T.K.S. (2015). The Anti-Inflammatory and Antibacterial Action of Nanocrystalline Silver and Manuka Honey on the Molecular Alternation of Diabetic Foot Ulcer: A Comprehensive Literature Review. *Evidence-Based Complementary and Alternative Medicine*. doi: 10.1155/2015/218283.
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Conference Presentations

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List of abbreviations

ABI	Ankle brachial index
AE	Adverse event
Ag	Silver
AGEs	Advanced glycation end products
CI	Confidence interval
CMI	Custom-made insoles
COX-2	Cyclooxygenase-2
DFI	Diabetic foot infection
DFU	Diabetic foot ulcer
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
DPN	Diabetic peripheral neuropathy
DUSS	Diabetic ulcer severity score
E. coli	Escherichia coli
ECM	Extracellular matrix
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
GCP	Good clinical practice
GEE	General estimating equation
GOPD	General outpatient department
HbA1c	Hemoglobin A1c
HK	Hong Kong
HR	Hazard ratio
HRQOL	Health-related quality of life

IL-1-α	Interleukin 1-alpha
iNOs	Nitric oxide synthase
ISDA	Infectious Disease Society of America
IWGDF	International Working Group on Diabetic Foot
KGF	Keratinocyte growth factor
KM	Kaplan-Meier
MAID	Multiple ulcer, area, pedal pulses, ulcer duration
MGO	Methylogloxal
MH	Manuka honey
MIC	Minimum inhibitory concentration
MMP	Matrix metalloprotease
MW	Meggit-Wagner
nAg	Nanocrystalline silver
NCT	Nerve conduction test
O&T	Orthopedics and Traumatology
OM	Osteomyelitis
P. aeruginosa	Pseudomonas aeruginosa
PAD	Peripheral artery disease
PDGF	Platelet-derived growth factor
PMNLs	Polymorphonuclear leucocytes
RCT	Randomized controlled trials
RCW	Removable cast walker
ROS	Reactive oxygen species
RR	Relative risk
S. aureus	Staphylococcus aureus

SAE	Severe adverse event
SD	Standard deviation
SEWSS	Saint Elian Wound Score System
SOPD	Specialty outpatient department
SSD	Silver sulphadiazine
SWME	Semmes-Weinstein monofilament examination
TBI	Toe brachial index
TGF-β	Transforming growth factor-beta
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
TIMP	Tissue inhibitor of metalloprotease
TCC	Total contact cast
UMF	Unique manuka factor
UT	University of Texas
VEGF	Vascular endothelial growth factor
WHO	World Health Organization
ZOI	Zone of inhibition

Chapter 1 – Introduction

1.1 Introduction

This chapter presents the relevant background of the present research study. Sections 1.2 to 1.4 include an overview of diabetes mellitus and its prevalence and impact both overseas and in Hong Kong. Sections 1.5 to 1.8 discuss the impact of diabetic foot ulcer and its care strategies. Finally, section 1.9 and 1.10 reveal the problem statement and research aim.

1.2. Diabetes mellitus

Diabetes mellitus (DM) is a chronic disease caused by inherited and/or acquired deficiency in insulin production by the pancreas, or by the ineffectiveness of the insulin produced. Such a deficiency results in increased concentrations of glucose in the blood, which in turn damage many of the body's systems, in particular the blood vessels and nerves (World Health Oganization, 2015). Type 1 diabetes is due to b-cell destruction, usually leading to absolute insulin deficiency. Type 2 diabetes is due to a progressive insulin secretory defect on the background of insulin resistance (American Diabetes Association, 2015). This form of diabetes accounts for ~90–95% of those with diabetes (American Diabetes Association, 2004). The current World Health Organization (WHO) diagnostic criteria for diabetes are fasting plasma glucose ≥ 7.0 mmol/l- or 2-hour plasma glucose ≥ 11.1 mmol/l (WHO, 2006), or hemoglobin A1c (HbA1c) ≥ 6.5% (WHO, 2011).

1.3. Prevalence of DM overseas and in Hong Kong

It is estimated that DM affects 8.3% of the global population, or 382 million people (International Diabetes Federation, 2013). This number continues to grow, making DFU a major public health problem (Tsourdi et al., 2013). In Hong Kong (HK), DM is a major cause of morbidity and mortality. It claimed about 12900 in-patient discharges and in-patient deaths in all hospitals, and 360 registered deaths in 2013. It was the tenth most common cause of death in Hong Kong, accounting for 0.86% of all deaths in 2014 (Department of Health, 2014). The true number of deaths from diabetes may be higher, since many deaths can be attributed to its late complications (Department of Health, 2015).

1.4. Impact of DM

Since DM has severe complications that can cause physical and psychological stress, and have a direct impact on caregivers, family and the healthcare system. People with DM have an increased risk of developing a number of serious health problems. Diabetes complications are divided into macrovascular and microvascular damage (World Health Organization, 2015). Macrovascular damage consists of cardiovascular diseases such as heart attacks and strokes. Microvascular damage includes damage to eyes, leading to blindness, to kidneys, leading to renal failure, and to nerves, leading to impotence, and diabetic foot disorders that include severe diabetic foot infections, leading to amputation. (World Health Organization, 2015; International Diabetes Federation, 2014). People with DM are also vulnerable to losses in specific domains of function,

including concentration, physical functioning, recovery to work, and social participation. Depression is not uncommon among working class people with DM (Stynen et al., 2015).

In addition, the quality of life of caregivers is affected, especially for those who are unemployed, less educated, or suffering from a medical problem (Awadalla et al., 2006). Besides patients, caregivers and families, the impact of DM affects the healthcare system in its entirety. The total estimated cost of diagnosed diabetes in 2012 was $245 billion, including $176 billion in direct medical costs and $69 billion in reduced productivity. People diagnosed with diabetes incur average medical expenditures of about $13,700 per year, of which about $7,900 is attributed to diabetes (American Diabetes Association, 2013).

1.5. Diabetic foot ulcer as one of the complications

Diabetic foot ulcer (DFU) is a common complication of DM. It is a "full-thickness" lesion of the skin, that is, a wound penetrating the dermis; lesions such as blisters or skin mycosis are not included in this system (International Working Group on Diabetic Foot, 2007). Indeed, the pathology is complicated. At the microscopic level, cellular impairment (Xu et al., 2013) and molecular dysfunction (Muller et al., 2008) make it difficult for the ulcer to heal. At the macroscopic level, diabetes compromises the circulation in the small blood vessels. The distal nerves are also affected because of the lack of a nutrient supply. This leads to circulation and sensation impairment, as well as foot deformity (Clayton et al. 2009). All of these contributing factors increase the

probability of minor injury. The minor trauma may not heal by itself, and may lead to foot ulcer.

1.6. Impact of diabetic foot ulcer

In each year, many people with DM are afflicted with the complication of DFU. Some of them receive multiple surgeries and eventually even undergo amputation. DFU was found to affect 4%-10% of people with diabetes mellitus (Singh et al., 2005) and to precede over 85% of amputations in this population of patients (Oyibo et al., 2001). The risk of complication increases with time. The cumulative incidence of DFU increases from 27.3% during the first year of diagnosis to 76.4% five years after the initial diagnosis. The rate of amputation increases from 12.5% to 47.1% over this time (Chu et al., 2014).

The cost of caring for DFU is exorbitantly high. The total cost for diabetes was $174 billion in the United States in 2007, with foot ulceration accounting for 24% to 31% (American Diabetes Association, 2008). Stockl et al. (2004) revealed that the average cost per DFU episode was $13,179, and greater in the case of deep ulcers with co-existing infection and circulation problems (as evaluated using the Wagner classification system). In addition, diabetes patients with DFU experienced both longer hospitalization stays and higher mortality compared with those without DFU (Nirantharakumar et al., 2013). Apart from the financial impact of DFU, patients with DFU suffer many limitations in their physical, social, and vocational activities (especially those who are required to undergo an

amputation), leading to poor health-related quality of life (HRQOL) (Valensi et al., 2005; Goodridge et al., 2006).

1.7. Care strategies for DFU

There are two main categories of care for DFU management: glycemic control and local ulcer care. Since microvascular complications including neuropathy are strongly related to HbA1c (Stolar, 2010), the control of blood sugar level can be achieved by lifestyle modification, diet and medication (Freeman, 2010). Sanghani et al. (2013) found that structural training on lifestyle modification was associated with significant reductions in HbA1c. Stulnig (2015) discovered that a protein-enriched and low glycemic index diet could improve glycemic control and body weight in type 2 diabetes patients.

Local ulcer care involves decreasing pressure on the local ulcer, especially in the plantar region, and applying topical and systematic anti-bacterial agents to combat wound infection (International Best Practice Guidelines, 2013). Special care is also a crucial part of treating DFU. Special care includes thorough cleansing of the affected limb before dressing, debridement of non-viable tissue, stimulating the vascularity of the avascular structure over the wound bed, and educating the patient on diabetic foot care. Finally, as a part of standard care, different kinds of topical dressing materials are used on the ulcer (International Best Practice Guidelines, 2013).

1.8. Local topical dressing materials for DFU

DFU is hard to heal. This is partly due to the biochemical alternation of the local micro-wound environment, which results in persistent inflammation (Blakytny et al., 2009). Another reason is the high local bioburden (Lobmann et al., 2005). After off-loading the local ulcer pressure and controlling the active infection and debridement of non-viable tissue, one of the standard treatment methods is the application of local topical dressing materials (Woo et al., 2013). There are many choices of topical dressing for DFU clinically, which can be roughly divided into three groups. The first is "conventional wound care products", such as paraffin tulle. The second is "advanced wound care products" like nanocrystalline silver (nAg) and manuka honey (MH). The third group is the expensive "next-generation DFU therapy", including living cell, scaffold and growth factor therapy. Since DFU arises as a result of multiple biochemical deficiencies, singular use of the new therapy is unlikely to be effective. Most often, this therapy only yields mild improvement in DFU repairs. At present, this "next generation therapy" is not widely used clinically (Futrega et al., 2014). The most commonly used materials are "conventional wound care products" and "advanced wound care products" because of their more reasonable price and the availability. Among "advanced wound care products", silver and honey are becoming increasingly popular for wound care management in DFU.

To summaries, because of the high impact of DFU on patients, family, caregivers and the health care cost as discussed above, the effective care strategies on DFU are required to minimize the impact. The care strategies include patient

education on glycemic control and self-care management as well as local wound care including off-loading, debridement, combating infection and the topical dressing application. Selection of evidence-based topical dressing materials is one of the important aspects to promote wound healing in wound management.

1.9. Research problem

Based on the in vitro evidence, nAg and MH appear to be helpful in DFU healing since a number of studies have shown consistent effects on antibacterial and anti-inflammatory action (Gordon et al., 2010; Wong et al., 2009; Adams et al., 2008; Chan et al., 2013), suggesting that they target the biochemical deficiencies of DFU. From the in vitro evidence, we know that nAg and MH target the cellular and biochemical alternation of wounds in the local environment. However, there is no clinical evidence to show the effect of nAg on DFU healing. So, the evidence on nAg on DFU healing is unknown.

1.10. Aim

As there is no previous study to test the effect on nAg on DFU healing, we conducted a pilot study instead of a main study to address the following aims,

- Investigate the feasibility of the study design
- Test the acceptability of the interventions
- Test the preliminary effect of nAg against MH and conventional dressings on healing DM foot ulcer

1.11. Summary

Chapter one discussed the background of this research area, and the importance and impact of DM and DFU on patients, caregivers, family and the healthcare system. The research problem and aim of the study were also identified.

Chapter 2 – Literature review

2.1. Introduction

This chapter reviews and reports the literatures directly relevant to this study area, and they are organized as follows. Sections 2.1 to 2.9 describe the background, context and rationale for developing this pilot study. Sections 2.10 to 2.13 provide the latest updates on what is already known about my research topic and identify the research gap in this field. The present research should have great relevance to clinical practice. Section 2.14 summarizes the background and importance of my research topic.

2.2. Normal wound healing

An acute wound normally heals in a strict order comprising four distinct but overlapping phases (Enoch & Price, 2004; Goldberg & Diegelmann, 2010): hemostasis, inflammation, proliferation and remodeling (Pradhan et al., 2007; Diegelmann & Evans, 2004). A complex network of biochemical pathways and sequential cellular interactions ensures an integrated progression of different distinct wound healing phases (Sibbald & Woo, 2008; Blakytny & Jude, 2006).

2.2.1. Hemostasis

Soon after injury, hemostasis occurs through vasoconstriction and platelet aggregation at the wound site (Goldberg & Diegelmann, 2010). Platelets are

exposed to and activated by the extracellular matrix in the vascular wall, such as fibrin, fibronectin, vitronectin, which provide the provisional matrix for cellular migration (Li et al., 2007; Enoch & Price, 2004; Clark et al., 1982). Once activated, the platelets undergo adhesion to form plugs and release many mediators and adhesive proteins. The alpha granules of the platelets contain cytokines and various growth factors, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and transforming growth factor-beta (TGF-β). These proteins initiate the wound healing cascade by attracting and activating fibroblasts, endothelial cells and macrophages. At the same time, the vasoactive amines (e.g. serotonin) increase the permeability of nearby blood vessels and allow for the migration of inflammatory cells (Blakytny & Jude, 2006; Enoch & Price, 2004).

2.2.2. Inflammatory phase

In the early inflammatory phase, polymorphonuclear leucocytes (PMNLs) are entrapped in the blood clot and release a wide variety of factors that act as chemoattractants for cells and peak in the first 24 to 48 hours (Enoch & Price, 2004; Szpaderska et al., 2003). The PMNLs then begin to adhere to the endothelial cells in the adjacent blood vessel wall. They kill bacteria by releasing degrading enzymes and oxygen-derived free radical species. Another leukocyte subset (mast cells) degranulates within a few hours. They release chemotactic factors such as vasoactive amines and histamine-rich granules that cause surrounding vessels to become leaky and are used to recruit neutrophils and

monocytes to the site of the injury (Goldberg & Diegelmann, 2010; Eming et al., 2007; Enoch & Price, 2004).

In the early inflammatory phase, neutrophils and monocytes are the dominant cell types. The activated neutrophils produce proteolytic enzymes such as matrix metalloproteases (MMPs) to remove necrotic debris and bacterial contaminates. In the late inflammatory phase (48 to 72 hours), monocytes transform into larger phagocytic macrophages and become the dominant cell type (Fonder et al., 2008; Blakytny & Jude, 2006; McLennan et al., 2006). Macrophages are used for releasing proteolytic enzymes (e.g. collagenase and elastin) to debride devitalized tissue and foreign debris, destroy remaining neutrophils, and kill and digest pathogenic bacteria (Li et al., 2007; Diegelmann & Evans, 2004). Macrophages and PMNLs secrete additional growth factors such as vascular endothelial growth factor (VEGF) and PDGF, which further stimulate the inflammatory response, initiate granulation formation and accelerate wound healing (Sibbald & Woo, 2008; Enoch & Price, 2004).

2.2.3. Proliferative phase

In the proliferative phase, the fibroblast is the predominant cell type. It is predominately regulated by PDGF and TGF-β (Goldberg & Diegelmann, 2010). This cell is responsible for producing a new matrix by attaching to the provisional fibrin matrix; it then begins to produce collagen as well as a stable extracellular matrix at the wound site (Goldberg & Diegelmann, 2010; Clark, 2001). In this stage, TGF-β is considered to be a master control signal that

regulates fibroblast functions (Robert & Sporn, 1993). TGF-β has several functions. Firstly, it can increase the overall production of matrix proteins. Secondly, it can decrease the secretion of proteases. Thirdly, it can also stimulate the tissue inhibitor of metalloprotease (TIMP) (Hall et al., 2003) to regulate the MMP level. Fourthly, it can attract macrophages, platelets and keratinocytes, which produce growth factors such as EGF, TGF-α to facilitate the process of epithelization (Diegelmann & Evans, 2004; Schultz et al., 1991). To sum up, the activities involved in these phases are fibroblast migration, collagen synthesis, angiogenesis, granulation tissue formation and epithelialization (Enoch & Price, 2004).

2.2.4. Remodeling phase

This is the final phase of wound healing, in which granulation tissue matures into a scar. Reorganization and the remodeling of tissue continue in the remodeling phase. The maturation of granulation tissue involves a reduction in the number of capillaries and a decrease in the amount of glycosaminoglycans. Cell density and metabolic activity decrease. Type III collagen is replaced by type I collagen, which is the dominant fibrillar collagen in the skin (Schultz et al., 2005; Falanga, 2005). Fibroblasts reorganize the collagen matrix, ultimately resulting in connective tissue compaction and wound contraction (Fonder et al., 2008).

2.3. Pathophysiology: pathway to ulcer

Diabetes causes several pathological changes to the lower limbs, including diabetic neuropathy, peripheral artery disease (PAD) and foot deformity. None of these in themselves will cause ulceration. Indeed, they are all contributing factors that affect ulcer development after a repetitive trauma (Clayton & Elasy, 2009; Rathur & Boulton, 2005; Reiber, 1999). When a patient has peripheral artery disease and the wound is characterized by trauma, recurrent in nature, deep to the bone, and has a duration longer than 30 days, the wound is even more prone to infection (Lavery, 2006).

2.3.1. Diabetic peripheral neuropathy

Diabetes affects all types of nerve fibers, both myelinated and unmyelinated (Sytze Van Dam et al., 2013). Diabetic peripheral neuropathy (DPN) causes impairment in sensation, movement and other aspects of health, depending upon the nerve affected (Noor et al., 2015). There are three components of neuropathy, comprising sensory, motor and autonomic aspects (Brownlee et al., 2008). Peripheral neuropathy plus foot deformity may account for 60% of ulcers (Plummer & Albert, 2008). The etiologies of diabetic neuropathy are multi-factorial. Indeed, glycemic control and the duration of diabetes are strongly correlated with DPN (Papanas & Ziegler, 2012). The major mechanisms causing hyperglycemic nerve damage are elevated levels of intracellular advanced glycation end products, protein kinase-C activation, increased flux through the polyol pathway and increased hexosamine pathway activity (Brownlee, 2005).

There are two issues that need to be considered with regard to the risk of injury. These are the risk of insensate foot subject to acute trauma (Cavanagh et al., 2001) and the chronic low-grade pressure associated with repetitive trauma (Plummer & Albert, 2008). When a patient loses the protective sensation due to repeated trauma and wearing overly tight shoes, it can cause tissue ischemia. Autonomic neuropathy also causes loss of function in the sweat and sebaceous glands, leading to dry skin on the foot. This in turn increases the chance of skin breakdown and infection. (Clayton & Elasy, 2009). All these contributing factors predispose the DM patient to diabetic foot ulcer (DFU).

2.3.2. Peripheral artery disease

PAD is a diffuse artherosclerotic vascular disease frequently present in diabetic patients (Potier et al., 2011). It is an important risk factor, causing foot ulcer, gangrene and amputation with odds ratios of 8.33, 62.07 and 20.14 respectively (Al-Rubeaan et al., 2015). It commonly affects the tibial and peroneal arteries of the calf. Endothelial cell dysfunction and smooth cell abnormalities result in decreasing endothelium-derived vasodilators, leading to vasoconstriction (Clayton & Elasy, 2009). Indeed, it is rare for PAD to cause ulceration directly. When there is minor trauma in a patient with PAD, this renders the patient prone to wound infection (odd ratio 1.9) (Lavery, 2006). Wound infection increases the demand of blood supply that is beyond the circulatory capacity, and ischemic ulceration develops (Brownlee et al., 2008). The ischemic status is further worsened by atherosclerosis in the lower limb and decreased angiogenesis in the diabetic wound (Brem et al., 2004).

2.3.3. Foot deformities

In a recent retrospective study of patients with diabetes, foot deformity was found in 27% of the study cohort from a Chinese tertiary hospital (Wu et al., 2015). Because of the imbalance of the flexor and extensor muscles in the foot, diabetic patients commonly experience foot deformity, with prominent metatarsal heads and clawing of toes (Brownlee et al., 2008). The cells of the skin react to persistent abnormal high pressure by increasing keratinization and turning into calluses, which are predisposed to foot ulceration (Arosi et al., 2015).

2.4. Alternation of healing in DFU

Most chronic non-healing wounds, including diabetic ulcer "stuck" in the inflammatory phase (Blakytny et al., 2009; Pradhan et al., 2007; Leung, 2007; Falanga, 2005; Lobmann et al., 2005). In addition, chronic wounds exhibit an imbalance between tissue deposition stimulated by growth factors and tissue destruction mediated by proteases in which the balance favors the destructive process (Cullen et al., 2002). DFU is also associated with the disruption of the above wound healing mechanism. A persistent inflammatory phase is commonly witnessed in histopathology and associated with a delay in the formation of mature granulation tissue. However, the reason for the extension of the inflammatory phase is still unclear (Blakytny et al., 2009; McLennan et al., 2006). This prolonged inflammatory reaction may be the result of bacterial contamination, bacterial infections (Lipsky et al., 2012b; Ogunlesi, 2008), and recurrent painless tissue trauma (Lobmann et al., 2005). The wound healing

deficiencies occur at both cellular and molecular levels (Bream & Tomic-Canic, 2007).

2.4.1. Cellular abnormalities

The exact mechanisms behind poor wound healing remain elusive (Blakytny & Jude, 2006). Loots et al. (1999) showed a diminished proliferative capacity and an abnormal morphology of fibroblasts in wounds related to diabetes. Galkowska et al. (2005) found in an in vitro study that the healing process of diabetic foot ulcers may be hampered by mechanisms that reduce the accumulation of leukocytes. Waltenberger et al. (2000) performed a chemotaxis assay using isolated monocytes from diabetic patients and found that monocytes are less responsible for the VEGF when compared with normal person. Using immunohistochemistry techniques, Usui et al. (2008) discovered that keratinocyte migration and differentiation were impaired along the margin of chronic ulcers in patients with diabetes mellitus. Albiero et al. (2011) discovered that delayed wound healing as a result of diabetes was associated with the defective recruitment, survival, and proliferation of BM-derived endothelial progenitor cells in mice. Macrophages isolated from diabetic mice also exhibit greater infiltration by inflammatory M1 macrophages and may contribute to impaired diabetic wound healing (Kanter et al., 2012).

2.4.2. Poor extracellular matrix formation and high levels of matrix metalloproteinases

The extracellular matrix (ECM) formation is defective in DFU. ECM creates a scaffold for cellular attachment, which is crucial for wound healing. Blakytny and Jude (2006) stated that the disruption in the formation of new ECM, as well as the diminished stimulation of cell proliferation, results in the lack of a proper scaffold for cellular attachment.

MMPs are zinc-dependent endopeptidases, and their inhibitors are called tissue inhibitors of TIMPs. They are excreted by a variety of connected tissue, fibroblasts, keratinocytes, pro-inflammatory cells such as neutrophil, and macrophage. These MMPs are regulated by hormones, growth factors, and cytokines in response to signals (Verma & Hansch, 2007; Smith, 2003). The functions of MMPs include influencing cell migration, promoting cellular proliferation apoptosis, modulating growth factors and their receptors, and degrading the structural components of ECM during the remodeling of tissue (Smith, 2003; Johnson et al., 1998).

In diabetic patients, hyperglycemia activates the pathways of the mitogen-activated protein kinase to stimulate the production of cytokine and promote inflammation (McLennan et al., 2008). The high level of MMPs is also the pathological alternation in DFU in biochemical terms. The over-expression of MMPs and elastase breaks down the components of ECM and inhibits growth factors (Sibbald & Woo, 2008). Lobmann et al. (2002) compared the MMP levels

of 20 patients with diabetic foot ulcers with those of 12 patients with traumatic ulcers. The results showed that the concentrations of MMP-1 and MMP-9 increased 65-fold and 14-fold respectively in the diabetic ulcer biopsies. Muller et al. (2008) conducted another cohort study on 16 patients with neuropathic diabetic ulcers. Their results echoed those of Lobmann's study. The levels of MMP-8 and MMP-9 decreased in the good healer group (wound size reduction more than 50% over 4 weeks) and remained stable in the poor healer group during the 12-week follow-up period.

Indeed, MMP-9 is the well-recognized MMP that plays an important role in normal healing (Widgerow, 2011b) and appears to be the major protease responsible for matrix degradation in chronic wound fluid (Widgerow, 2011a). MMP-9 is produced by a number of inflammatory cells, including neutrophils, macrophages and monocytes. It decreases in the normal wound healing process, and the expression declines in the proliferative phase (Rayment et al., 2008b; Moore et al., 2007).

2.4.3. High pro-inflammatory cytokines

To heal ulcers, pro-inflammatory cytokines can chemotactically draw inflammatory cells into the injured area (Schultz & Mast, 1999). From the basic science, interleukin 1 (IL-1) is produced by macrophages and neutrophils. It is a pro-inflammatory cytokine whose function is to recruit fibroblasts and keratinocytes, and perform collagen synthesis (Mohd Yussof et al., 2012; Lobmann et al., 2005). Tumor necrosis factor-alpha (TNF-α) is also mainly

secreted by neutrophils and macrophage. This pro-inflammatory cytokine TNF-α helps in collagen synthesis (Mohd Yussof et al., 2012). Macrophages and neutrophils are the dominant cells in the inflammatory stage of wound healing, and their activity is minimized in the proliferative stage (Digelmann and Evans, 2004). As a consequence, the levels of IL-1 and TNF-α decrease when the wound starts to heal. The findings from published studies support the above basic science. Lobmann et al. (2005) stated that the up-regulation of TNF-α and IL-1 stimulates the synthesis of MMP-1 and inhibits the synthesis of collagen. Trengove et al. (2000) studied the mitogenic activity of healing and non-healing ulcers. They found that the expression of pro-inflammatory cytokines TNF-α and IL-1 was down-regulated during the healing process. Shah et al. (2012) suggested that the cytokines, including TNF-α and IL-1, had the best potential to predict wound healing.

2.4.4. High oxidative stress

People with diabetes usually have hyperglycemia. There is increasing evidence to suggest a causal link between hyperglycemia and oxidative stress leading to cellular damage (Sibbald & Woo, 2008). Excessively high levels of free radicals cause damage to cellular proteins, membrane lipids, and cell nucleic acids, eventually leading to cell death (Maritim et al., 2003). In people with diabetes, free radicals (superoxide anion and hydroxyl radial) are formed in disproportionately high levels by glucose oxidation, the non-enzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins. The glycated proteins develop further reactions to form advanced glycation end

products (AGEs) (Van den Berg et al., 2008). The accumulation of AGEs causes the up-regulation of proinflammatory cytokines and MMPs that will degrade ECM through the production of reactive oxygen species (ROS) (Blakytny & Jude, 2006). The production of peroxynitrite anion and peroxynitrous acid (Soneja et al., 2005) can also lead to biological damage (Enoch & Price, 2004).

2.4.5. Angiogenesis

Angiogenesis is the formation of new blood vessels, which is the most critical component of wound healing. Macrophage and endothelial cells have an important role in angiogenesis development (Lingen, 2001). In diabetic patients, there are abnormalities of angiogenesis in many organs, including ulcers (Martin et al., 2003). Limited penetration of new blood vessels into the wound restricts the entry of inflammatory cells. In turn, the total number of factors released by these cells will decrease. The oxygen infiltration will be poor (Blakyntny & Jude, 2006). This phenomenon has a great impact in terms of impairing wound healing.

In summary, because of the factors regarding cellular and biochemical disturbances in diabetes, DFU is relatively difficult to heal when compared with non-diabetic foot ulcer. The molecular alternations of DFU are summarized in table 2.1.

Table 2.1 The cellular and biochemical alternations of DFU

Author	Nature of study	Cellular abnormalities
Loots et al., 1999	In vitro	Fibroblasts decrease proliferative capacity and abnormal morphology
Galkowska et al., 2005	In vivo	Leukocytes decrease accumulation
Waltenberger et al., 2000	In vitro	Monocytes in diabetic patients are less reactive to VEGF
Usui et al., 2008	In vivo	Impaired migration and differentiation of keratinocytes
Albiero et al., 2011	Animal study	Reduction in the recruitment, survival, and proliferation of endothelial progenitors at the site of the injury
Kanter et al., 2013	Animal study	Decrease in the polarization and activation of macrophages
		Poor ECM formation
Blakytny & Jude, 2006	Review	AGEs cause the up-regulation of MMPs and cytokines that degrade ECM through the production of ROS
Sibbald & Woo, 2008	Review	The over-expression of MMPs and elastase breaks down the components of ECM and inhibits growth factors
		High levels of MMPs
Lobmann et al., 2002	In vivo	MMP-1 and MMP-9 increased 65-fold and 14-fold respectively in diabetic ulcer biopsies
Muller et al., 2008	In vivo	MMP-8 and MMP-9 remained stable in the poor healer group but decreased in the good healer group

Table 2.1 The cellular and biochemical alternations of DFU (cont'd)

Author	Nature of study	High pro-inflammatory cytokines
Lobmann et al., 2005	Review	The up-regulation of TNF-α and IL-1 stimulated the synthesis of MMP-1 and inhibited the synthesis of collagen
McLennan et al., 2006	Review	Hyperglycaemia activates the pathways of mitogen-activated protein kinase to stimulate cytokine production and promote inflammation
Trengove et al., 2000	In vivo	Il-1, IL-6, and TNF-α are up-regulated in chronic non-healing ulcers
Chan et al., 2012	In vitro	Neutralization of TNF improves angiogenesis
		High oxidative stress
Van den Berg et al., 2008	In vitro	Free radicals (superoxide anion and hydroxyl radicals) are formed by the oxidative degradation of glycated proteins, which subsequently form AGEs
Soneja et al., 2005	Review	The production of peroxynitrite anion and peroxynitrous acid can lead to biological damage
		Angiogenesis
Kalluri & Zeisberg, 2006	Review	Fibroblast diminishes proliferative capacity which plays essential angiogenic roles by producing several pro-angiogenic cytokines such as fibroblast growth factors
Xu et al., 2012	Review	The number and function of endothelial progenitors are reduced in the circulation and within wounds for diabetic patients, which in turn decreases re-endothelialization and angiogenesis

2.5. Wound fluid analysis

As mentioned in the previous section, DFU has a number of biochemical alternations. According to the discussion in section 2.2.2, the biomarkers (e.g. MMPs) are secreted by inflammatory cells (Fonder et al., 2008). During the healing process, the inflammatory cells (neutrophils and monocytes) are replaced by fibroblasts (Goldberg & Diegelmann, 2010). In addition, the fibroblasts will secrete TIMP, which is an MMP inhibitor (Hall et al., 2003). Theoretically, the concentration of biomarkers is down-regulated when wound healing begins. Therefore, the concentration of biomarkers is an indicator of wound healing. Indeed, wound fluid is a new window for assessing the local microenvironment of wounds that cannot be evaluated by the analysis of serum biomarkers (Löffler et al., 2011). The concentration of wound fluid is influenced by the state of the wound and the phases of wound healing (Trengove et al., 1996). Thus, analysis of the wound fluid is a way to monitor the progress of wound healing. With reference to the discussion in the previous section, MMP-9 and cytokines IL-1 and TNF-α are the major biomarkers that are associated with wound healing.

There are three traditional approaches to collecting wound fluid. The first involved collecting wound fluid from the negative pressure drain (Nissen et al., 1998). However, this method could only be used for negative pressure wound therapy. The second method involved the use of absorbent materials of varying types, including Dextranomer beads in place for 24 hours (Cooper et al., 1994), foam dressing (Mendez et al., 1999), absorptive filter paper (Moseley et al.,

2004), and polyester-tipped applicators (Wyffels et al., 2010). When extracting wound fluids from the collection material, there was greater disparity in the methods used, with varying types of extraction buffers and times ranging from one hour to overnight (Ramsay et al., 2015). Some studies even found that collection of wound fluids from absorbent dressing may influence the biochemical properties of the wound fluids (Yager et al., 2007; Schmidtchen, 2000; Hoffman et al., 1999).

The third method was the placement of a semi-occlusive or occlusive dressing over the wound for varying amounts of time, followed by the removal of fluid from beneath through aspiration (Eming et al., 2010; Rayment et al., 2008b; Fernandez et al., 2008; Lauer et al., 2002). This method was simple and eliminated the need for extraction from the absorbent dressing. The disadvantage was that it required patients to remain at the collection site for extended time periods (Ramsay et al., 2015). Therefore, for the sake of simplicity and to prevent any variation from the restoration of wound fluid, this method was the most appropriate method for this study using topical dressing materials.

2.6. DFU classification

A validated classification system for DFU may help both clinicians and researchers in the everyday assessment and management of patients, or with the development and assessment of new therapies. There are numerous classification systems but some have not been validated (Karthikesalingam et al.,

2010) and there are no prognostic accuracy measures (Monteiro-Soares et al., 2014). The classic system was the Meggit-Wagner (MW) ulcer classification system (Wagner, 1987). This grading system classified ulcers into 6 grades: grade 0 (no open lesions), grade 1 (superficial diabetic ulcer), grade 2 (DFU with ligament, tendon, joint capsule or fascia involvement), grade 3 (deep ulcer with abscess or osteomyelitis or joint sepsis), grade 4 (gangrene localized to portion of forefoot or heel), and grade 5 (extensive gangrene of the foot). Obviously, this system did not consider all the characteristics of DFU, including PAD and DPN. The majority of patients were classified as grades 2 and 3. This system was considered to be very simplistic, linear and lacking the specificity of DFU description, failing to address the important parameters of ischemia and infection (Monteiro-Soares et al., 2014; Frykberg, 2002). Sun et al. (2012) found that MW may not be a sensitive tool for predicting DFU healing or amputation after comparing MW with clinical parameters such as white blood cell counts, albumin, and estimated glomerular filtration.

The University of Texas (UT) system (Lavery et al., 1996) was a little more complicated. It divided a wound into stages and grading. It included not only the Wagner system for grading on depth, but also the stage: A (no infection or ischemia), B (infection), C (ischemia) and D (infection and ischemia). Oyibo et al. (2001) and Gul et al. (2006) compared the Wagner and UT systems. Both concluded that the UT system made a better prediction of outcome. However, Santema et al. (2015) point out that neither MW nor UT are useful as a single instrument: they should always be used in combination with additional clinical information. They found that intra-observer agreement in the two systems was

low. The Cohen κ-value for MW was 0.415 [95% confidence interval (CI) 0.413-0.418] and for UT was 0.462 (95% CI 0.445-0.479). This implied that variance occurred when using MW or UT alone to describe the DFU condition. In addition, neither system assessed some of the specific parameters in DFU, such as size and neuropathy. Thus, the UT system may have clinical and research value when combined with other assessment parameters.

Another well-known classification is size (area and depth), sepsis, arteriopathy and denervation [S(AD)SAD], as developed by Macfarlane & Jeffcoate (1999). This system was composed of 5 parameters: size, depth, infection, arteriopathy and neuropathy, each scoring from 0 to 5. The parameters included in this system seemed to be more comprehensive and related to the risk factors in DFU healing. It was also validated by several studies (Treece et al., 2004; Chipchase et al., 2005; Ince et al., 2007; Abbas et al., 2008; Parisi et al., 2008). Unfortunately, the system has not undergone any assessment on inter-observer agreement.

The perfusion, extent (size), depth (tissue loss), infection, sensation (PEDIS) score was developed by IWGDF, consisting of the same 5 parameters as the S(AD)SAD (Schaper, 2004). This seemed to represent an evolution. The system was simple, with a clear definition and relatively small number of categories making it user-friendly (Schaper, 2004; Lipsky et al., 2012a). Abbas et al. (2008) compared the above classification systems, including WT, UT, S(AD)SAD and PEDIS, in Tanzania (Africa). The baseline demographic and disease severity in this cohort was different from those in the previous studies. The results showed that the correlation between clinical outcome and neuropathy/infection

contrasted with the previous study findings. This highlighted that the four systems may have different accuracy and precision in different countries and populations. Importantly, PEDIS proposed no final risk classification and reported no prognostic accuracy. Therefore, it cannot compare between groups in different risk categories and may not be suitable for research purposes when group comparison is required. Chuan et al. (2015) attempted to create a scoring system for PEDIS. However, they failed to discuss how to create this scoring system and did not report the missing data in this retrospective study, since the data included were quite complex. Their results may therefore be subject to bias.

The diabetic ulcer severity score (DUSS) consisted of four clinical parameters: palpable pedal pulses, probe to bone, and site and number of ulcerations. The aim of this score was to predict the probability of wound healing. Beckert et al. (2006) revealed its prognostic accuracy in 1000 patients, demonstrating that a one-point increase in the DUSS reduced the chance of healing by 35%. This was a good system for busy clinical purposes, since it had only four parameters and was easy to remember. In addition, no special equipment was needed in order to investigate these parameters. However, it did not account for perfusion and could not differentiate between neuropathic and neuro-ischemic ulcers, and therefore may not be suitable for research purposes. The multiple ulcer, area, pedal pulses, ulcer duration (MAID) scoring system was more-or-less the same as DUSS, except that the DUSS incorporated any bone exposed and the site of the ulcer (Beckert et al. 2009). Thus, MAID and DUSS shared the same advantages and disadvantages. They were not suited to research purposes.

The Saint Elian Wound Score System (SEWSS) contains 10 parameters (Martínez-De Jesús, 2010). This is five more than PEDIS, adding ulcer location, topographic aspects, number of affected zones (ulcers), odema and wound healing phase. The scoring of each parameter is from 0-3 or 1-3 and yields a total score of 6 to 30 points. It classifies wounds into grades 1, 2 and 3 with scores of ≤ 10, 11-20 and 21-29 respectively. The inter-observer agreement was Cohen κ 0.8. In a recent study, Huang et al. (2015) validated the score in a hospital in Shanghai, China. The prognostic accuracy showed that a one-point increase in the SWESS score reduced the probability of healing by 24%. The receiver operating characteristic analysis found a cut-off point of 17 and considered scores above this to relate to DFU with low healing potential. In a recent systematic review, Karthikesalingam et al. (2010) concluded that the choice of scoring system was influenced by the context in which it was needed and the population under study. Monterire-Soares et al. (2014) further revealed that the available systems had a poor evidence level due to the lack of validation studies. Therefore, there is no single validated DFU classification system suitable for all contexts. Although there has been only one validation study for the SWESS, both Shanghai and Hong Kong are well-developed cities. Their populations and patient characteristics are comparable. Based on the above review, the SWESS seems to be the most suitable scoring system for research on DFU in HK.

2.7. Diabetic foot assessment

2.7.1. Screening of DPN

The "gold standard" for identifying peripheral neuropathy is considered to be nerve conduction testing (NCT) (Jayaprakash et al., 2011). However, the use of NCT remains limited due to the limited availability of special laboratories, high costs and long waiting lists (Shehab et al., 2012). Apart from the gold standard, there is no solid conclusion on which clinical screening test is best at differentiating patients with diabetic neuropathy. Among all the screening tests for DPN, the Semmes-Weinstein monofilament examination (SWME) is a non-invasive, low-cost, rapid, and easy-to-apply test often used in clinical testing (Feng et al., 2009). The International Working Group on Diabetic Foot (IWGDF) recommended that SWME be used for sensory perception screening at least once a year (Bus et al., 2015). Feng et al. (2011) identified 6 studies, and the pooled relative risk (RR) of a positive SWME result versus that of a negative result ranged from 2.5 (95% CI 2.0-3.2) to 7.9 (95% CI 4.4-14.3) at a 1- to 4-year follow up. This means that diabetic patients with positive SWME results have 2.5–7.9 times a greater chance of developing ulceration than those with negative results.

Regarding the weight of the SWME, the 5.07/ 10 g monofilament is commonly used in clinical settings for the purposes of screening for DPN. McGill et al. (1999) found that the most suitable method of using the 5.07/ 10 g monofilament in the SWME was to categorize patients who could not feel either the plantar aspect of the first metatarsal or the fifth metatarsal as having neuropathy. The

combination of the two sites gave a reasonable 80% sensitivity and 86% specificity. For the number of sites tested by SWME, recent evidence shows that the accuracy of 3 (plantar aspect of big toe, and third and fifth metatarsal heads) or 4 testing sites was similar to that of 8 or 10 testing sites, with sensitivity 35.9-53.8% and specificity 73.9-84.7% (Feng et al., 2009; Baraz et al., 2014). The IWGDF (2015) also recommends the use of 3 testing points instead of 10.

2.7.2. Screening for PAD

Hinchliffe et al. (2015) recommends the use of non-invasive bedside tests, including the ankle brachial index (ABI), the toe brachial index (TBI) and the presence of triphasic pedal Doppler arterial waveforms to exclude PAD. Measuring ABI by Doppler ultrasound is a simple method to screen for PAD and to evaluate cardiovascular prognosis in both the general population and in patients with type 2 diabetes with a high degree of accuracy (Li et al., 2012; Gornik, 2009). ABI is defined as the ratio between the highest systolic blood pressure of the ankle (either posterior tibial or dorsalis pedis arteries) and the highest systolic pressure of the arm (brachial or radial arteries) (Faglia, 2011). It can provide objective data that serves as a standard for the diagnosis of lower extremity PAD.

It is well known that ABI will be falsely elevated in patients with artery calcification, such as patients with diabetes. Williams et al. (2005) evaluated 130 limbs in 68 individuals and found a reduction in the sensitivity of ABI from 71 to 38% for patients with detectable neuropathy. However, it is still a useful tool to

differentiate patients with high risk of PAD, for measurements of less than 0.9 and more than 1.3. (Potier et al., 2011).

A number of studies have been carried out to assess the diagnostic performance of ABI ≤ 0.90 to detect > 50% stenosis identified by imaging methods (Schroder et al., 2006; Williams et al., 2005; Premalatha et al., 2002; Allen et al., 1996), magnetic resonance angiography (Wilkinson et al., 2000) or angioplasty (Lijmer et al., 1996; Niazi et al., 2006; Guo et al., 2008). All of these published studies found the ABI to have reasonable specificity (83-99%) but relatively low sensitivity (69-79%). Lijmer et al. (1996) validated ABI in the lower extremities against angiography. The results showed a diagnosis threshold of 0.91, with sensitivity at 79% and specificity at 96%. Similar findings emerged from a more recent study with sensitivity at 63% and specificity 97% (Parameswaran et al., 2005). All the studies concurred with the high specificity and relatively low sensitivity nature of ABI measurement, especially for patients with DPN. This means that it can confidently be concluded that a patient with a low ABI value has poor arterial perfusion, but that high ABI may be falsely elevated due to medial arterial calcification and the non-compressibility of affected arteries. Therefore, the use of ABI can fulfill the research purpose of excluding patients with arterial insufficiency.

2.8. Risk factors affecting DFU healing

There are a number of risk factors that contribute to DFU healing. They can be further classified into personal, disease and local ulcer factors.

2.8.1. Personal factors

The first personal factor that affects DFU healing is **age.** Both a large multi-center and multi-national prospective cohort (n=1088) (Prompers et al., 2008) and a small, local retrospective cohort (n=340) (Leung et al., 2001) found that older age was the contributing factor for delayed wound healing. **Male gender** is the second personal factor hindering wound healing (Prompers et al., 2008). Marston et al. (2006) conducted a prospective randomized trial (n=245) and also found that being male involved an increased risk of non-closure of DFU. By contrast, Ince et al.'s cohort study (2007) reported that being male was not associated with DFU healing. The possible reason for this inconsistent finding is that the latter study was conducted in a single country, unlike Promper et al.'s cohort study involving multiple centers and countries. Therefore, the risk of selection bias is greater in Ince et al.'s study compared with Promper et al.'s cohort study.

Nutritional status also contributes to DFU healing. Both a prospective cohort study conducted in China (n=192) (Zhang et al., 2013) and a retrospective cohort study in HK (Leung et al., 2001) revealed that low serum albumin level was one of the obstacles hindering the healing of DFU. The **lower ambulatory status** of patients is also a personal risk factor affecting wound healing (Prompers et al., 2008; Pickwell et al., 2013).

2.8.2. Disease factors

There are several disease-related factors associated with patients' DM status. The control of diabetes and other diseases that affect the local circulation are obstacles to DFU healing. Both a large cohort (n=2480) (Gershater et al., 2009) and a small cohort (Ince et al., 2007) found that the long **duration of diabetes** was a factor in DFU healing impairment. In addition, the level of **HbA1c** reflects the control of diabetes. According to the American Diabetes Association, patients with well-controlled diabetes had HbA1c levels below 7% (American Diabetes Association, 2014). Marston (2006) conducted a secondary analysis and concluded that low HbA1c level had a significant effect on wound healing.

Co-morbidities including **PAD**, **heart disease** and **renal failure** also affect DFU healing (Gershater et al., 2009). PAD is supported by other studies as a risk factor in DFU healing (Pickwell et al., 2013; Prompers et al., 2008; Ince et al., 2007). Prompers et al. (2008) identified the presence of renal failure as a risk factor that affected ulcer healing. **Ischemia** also has co-morbidity with non-healing DFU (Leung, 2001), probably due to the fact that renal failure resulting from small vessel disease is highly correlated with PAD (Lepäntalo et al., 2012). In addition, heart disease, PAD and ischemia all affect the local tissue perfusion and hinder the healing of DFU (Leung, 2001).

2.8.3. Local ulcer factors

The parameters of the ulcers are additional risk factors that affect DFU healing. Margolis et al. (2002) analyzed a large cohort study with 31000 individuals, and

found that the longer **duration of ulcer** was associated with the delayed wound healing of neuropathic DFU. Margolis' study also discovered that larger **wound size** and increased **depth** of wound were significantly associated with the low probability of wound healing. A number of other studies supported these findings that wound size and wound depth were factors associated with DFU healing (Pickwell et al., 2013; Gershater et al., 2009; Prompers et al., 2008; Marston, 2006; Leung et al., 2001).

Ulcer location is another factor found to affect healing. Pickwell et al. (2013) analyzed the effect of ulcer location on time to heal in DFU, revealing that mid-foot [hazard ratio (HR) 0.77; 95% CI 0.64-0.92] and heel ulcers (HR 0.62; 95% CI 0.47-0.83) were more likely to heal than toe ulcers, probably because toe ulcers are more distal than mid-foot and heal ulcers, so that the circulation is compromised and it is more difficult for them to heal. **Ulcer infection** was also identified as a risk factor for healing by a number of studies (Pickwell et al., 2013; Prompers et al., 2008; Marston, 2006). Pickwell et al. (2013) found ulcer infection to have a negative influence on healing time. Marston (2006) revealed that episodes of infection during the study (RR 2.9; 95% CI 1.45-4.22) were associated with an increased risk of non-closure of DFU.

To conclude, there are some common risk factors contributing to DFU healing that were reported in previous studies. A summary of these risk factors is shown in Table 2.2.

Table 2.2. Risk factors affecting DFU healing

Personal factors	Disease factors	Local ulcer factors

• Age
• Gender
• Nutritional status
• Ambulatory status
	• Diabetic control
	  • Duration of diabetes
	  • HbA1c level
	• Co-morbidities
	  • Heart disease
	  • PAD
	  • Ischemia
	  • Renal failure
		• Ulcer duration
		• Ulcer size and depth
		• Ulcer location
		• Ulcer infection

2.9. Overview of treatment modalities

The treatment of diabetic ulcer is multi-directional and targeted at the above pathophysiology. The primary objective can be healing for healable DFU, and palliation for non-healable DFU (Woo et al., 2013). The holistic care of diabetic foot ulcer patients requires a multidisciplinary team approach (Braun et al., 2014). A cohort study showed that this approach could improve the process of care and clinical outcome for diabetic patients (DiPero et al., 2008). The standard treatment cannot be confined to local wound management alone. It should also focus on all of the factors mentioned in the previous section that affect DFU healing. The standard treatments are as follows.

2.9.1. Patient education for self-care management

Patient education should be an integral part of management and prevention (International Best Practice, 2013). In a Cochrane review, it pointed out that

there was insufficient evidence that limited patient education alone was effective in achieving clinically relevant reductions in ulcer improvement and amputation (Dorresteijn et al. 2014). However, this should be interpreted as insufficient robust evidence rather than evidence of no effect (Dorresteijn & Valk, 2012). In fact, education on DFU for patients' self-care management is considered as the cornerstone in managing DFU. Among healthcare providers, nurses have the most active and effective role in preventing amputation by educational intervention (Aalaa et al., 2012).

Several recent studies pointed out that diabetic foot care education resulted in a rise of knowledge, attitude and motivation so that patient could change their foot care practice (Beiranvand et al. 2015; Nemcová and Hlinková 2013). A recent single-blinded RCT also concluded that self-efficacy in foot care improved significantly after patient education by nurses (Seyyedrasooli et al., 2015). Ren et al. (2014) further showed that intensive nursing education of diabetic patients was highly significant in decreasing the rate of amputation in their two-year follow-up study. Abid-Hajbaghery and Alinaqipoor (2012) discovered that by using lecture and self-care practice demonstration by instructor had a higher ulcer size reduction significantly compared with lecture only. Nemcová and Hlinková (2013) found that the contents of the diabetic foot care education should include diet control, self-assessment on foot, choice of footwear, solution to problem foot and foot exercise. Chin et al. (2014) further discovered from the cohort that the lotion applying behavior was important in the prevention of DFU.

Therefore, from the best available evidence, intensive nursing education with self-care practice demonstration appears to effectively improve the clinical outcome of patients in ulcer management. The contents of the patient education must emphasize patients' responsibility for their own health and well-being (Yazdanpanah et al., 2015). Throughout education on the primary principles of diabetes and foot care, nurses can facilitate the active participation of patients and their family members in care (Aalaa et al., 2014).

2.9.2. Debridement

Debridement is one of the foundations of comprehensive care for DFU. It is widely accepted as a beneficial treatment for diabetic ulcers (Brem et al., 2006). However, not all wounds need and benefit from debridement. The decision to perform debridement should take into consideration whether complete wound closure is realistic and achievable (Woo et al., 2013). It should be performed after adequate perfusion has been established (Crawford & Fields-Varnado, 2013). There are several debridement methods, including autolytic, enzymatic, mechanical, surgical and biological (Enoch & Harding, 2003). The selection of the method of debridement should be determined by the wound condition, the presence or absence of infection, the vascularity of the wound and any anticoagulation medications (Crawford & Fields-Varnado, 2013).

Indeed, it is difficult to measure the effect of debridement alone. The administration of antibiotics, resting and off-loading before and after debridement are crucial in the clinical management of DFU. All these factors

contribute to the clinical outcome and were not well controlled in the available clinical studies. A recent Cochrane systematic review was conducted on the effect of various types of debridement on DFU. However, only six randomized trials were included in this meta-analysis. Five of them compared hydrogel plus good care with good care alone. Only one compared surgical debridement with conservative, non-surgical debridement. Thus, the pooled result could only compare the effect of autolytic debridement by hydrogel. The result suggested that hydrogel was significantly more effective in healing DFU compared with gauze dressings or standard care (RR 1.84; 95% CI 1.3-2.61) (Edwards & Stapley, 2010). However, the low quality of the trials on sharp and biological debridement hindered the pooling of results into this meta-analysis.

In practice, the gold standard technique for tissue management in DFU is regular, local, sharp debridement using a scalpel, scissors and/or forceps (International Best Practice Guidelines, 2013). Sharp debridement has been identified as an essential component of biofilm-based wound care (White, 2011). It should only be performed by health professionals with the relevant training and competencies (Turns, 2015). It involves the removal of all non-viable, infective tissue and surrounding callus (Brem et al., 2004), which can decrease bacterial counts, stimulate the production of local growth factors, reduce local pressure, facilitate wound drainage and evaluate the wound bed (Yazdanpanah et al., 2015). In a recent comprehensive review, the current best available evidence indicated that sharp debridement might have an important role to play when undertaking sharp debridement as a part of management of biofilm with the combination of standard care, although a high level of evidence was lacking

(White, 2011). In addition, a recent large retrospective review of 312744 wounds from 525 centers supported the correlation between routine frequent debridement and better clinical outcomes (Wilcox et al., 2013).

2.9.3. Off-loading

Unrelieved pressure not only impairs DFU healing but also increases the risk of complications (Brem et al., 2004). In order to allow a neuropathic ulcer to heal, repetitive pressure must be reduced or eliminated by external mechanisms or devices (Armstrong et al., 1998). This can be done by proper off-loading and pressure redistribution (Frykberg et al., 2006). The redistribution of plantar pressure should be considered for all individuals with DFU, especially those with neuropathic foot ulcer (Woo et al., 2013).

In general, off-loading devices can be classified into removal (e.g. custom-made footwear, removable walking brace) and non-removal types (e.g. total contact cast, non-removable walking brace). The total contact cast (TCC) is the gold standard of pressure redistribution over the plantar aspect of the diabetic foot (Armstrong et al., 2005). However, because the TCC is relatively difficult to remove, it is not suitable for patients who require frequent wound inspection (Yazdanpanah et al., 2015). Therefore, it is not suitable for DFU, which needs regular dressing. Another design of off-loading device is the removable cast walker (RCW). Because it can easily be removed, it is especially suitable for patients who need frequent wound inspection and dressing. However, patients themselves can also remove the RCW easily, thus their adherence is always in

question (Armstrong et al., 2003). In the last 10 years, RCT and case series studies have found that removable off-loading devices are as effective as TCC, provided there is good patient compliance (Van De Weg et al., 2008; Verity et al., 2008). However, another RCT compared the effect between the same design of RCW and TCC. The result showed that the healing of patients using the TCC was better (Armstrong et al., 2005), implying that patients' compliance with RCWs was difficult to monitor.

The final off-loading method discussed here is custom-made insoles (CMI). Evidence on plantar pressure distribution suggests that different designs of CMI could effectively decrease the shearing force (Lavery et al., 2005), peak pressure, and pressure over the metatarsal head and heel (Arts et al., 2015; Bus et al., 2004; Tsung et al., 2004). Evidence has revealed that CMI significantly reduced peak pressure and pressure over the metatarsal heads in patients with normal feet as well as those with foot deformity (Bus et al., 2004; Arts et al., 2015). In particular, contoured insoles were significantly better than flat insoles in reducing local peak pressure (Tsung et al., 2004). Four recent systematic reviews concluded that non-removable off-loading devices, regardless of type, are more likely to result in ulcer healing (RR 1.06, 95% CI 0.88-1.27; RR 1.17, 95% CI 1.01-1.36) than removable devices (Morona et al., 2013; Lewis & Lipp, 2013; Bus et al., 2008). Snyder et al. (2014) further revealed that there was a need for clinicians to improve patient compliance with off-loading devices when using non-removable off-loading devices. This means that effective off-loading can be achieved when patients' compliance is improved through education and monitoring. Patients with DFU usually need frequent dressings, especially for

high bacterial loading or infected ulcers. The TCC is not suitable for this type of patient. To conclude, CMI with regular monitoring is more suitable for patients with DFU.

2.9.4. Combating infection

Diabetic foot infection (DFI) is defined as a clinical syndrome characterized by local inflammation or purulence occurring in a site below the malleoli in a person with diabetes (Uçkay et al., 2014). Since bacteria are present on all open wounds, the DFU is the portal of entry of infection (Fonder et al., 2008). This is especially so for diabetic patients as a result of immunodeficiency, neuropathy and arteriopathy (Woo et al., 2013). The significant risk factors for DFI were wounds penetrating to the bone, wounds with a duration > 30 days, recurrent wounds, wounds with traumatic etiology, barefoot walking, history of previous amputation, PAD and neuropathy (Lavery et al., 2006; Peters et al., 2005).

The initial assessment of DFU should evaluate the patient's infectious status through local or systemic signs (Plummer & Albert, 2008). However, a patient might have an impaired neuroinflammatory response without manifesting typical signs of infection (Hobizal & Kukich, 2012). Therefore, secondary signs of infection should be assessed, such as wound friability, poor granulation, foul odor or delayed wound healing (Uçkay et al., 2014). A systematic infectious marker such as C-reactive protein is suggested to rule out any systematic signs of infection (Hobizal & Kukich, 2012; Uçkay et al., 2010).

Antibiotics are not always recommended. There is no evidence that treating a clinically uninfected wound with antimicrobials has any value in either preventing infection or improving ulcer healing (Uçkay et al., 2014). If the DFU is not infected, the use of antibiotics is not indicated (Brownlee et al., 2008) and topically applied antimicrobial agents are more effective (Steed et al., 2006). When the antibiotic is administered, the selected regimen should be as narrow spectrum as possible (Lipsky et al., 2012a). Severe infections require parenteral therapy, but mild to moderate infection can be treated with oral antibiotics that are highly bioavailable (Lipsky et al., 2012b). Osteomyelitis (OM) is not uncommon in diabetic ulcer. The probe to bone test is a common clinical test to evaluate OM on DFU [sensitivity 90%, specificity 79%) (Dinh et al., 2008). Plain film radiography was is important for the initial assessment of any OM (sensitivity 60%, specificity 67%) (Termaat et al., 2005). Treating DFU with osteomyelitis by debridement of the infected bone is more effective than the application of topical antibacterial dressings such as MH or nAg, since the penetration of the active ingredients of the topical dressing is only on the surface layers (Rigo et al. 2013).

2.9.4.1. *Classification of DFU infection*

The Infectious Disease Society of America (ISDA) and the International Working Group on the Diabetic Foot (IWGDF) have a clear classification of DFU. It is categorized as uninfected, mild, moderate or severe according to the clinical signs and laboratory findings (Lipsky et al., 2012a). The details are shown in table 2.3.

Table 2.3. ISDA and IWGDF classification of diabetic foot infection (DFI) (Lipsky et al., 2012a)

IDSA infection severity	Clinical manifestation of infection
Uninfected	▪ No symptoms or sign of infection ▪ Infection present, as defined by the presence of at least 2 of the following items: • Local swelling or induration • Erythema • Local tenderness or pain • Local warmth • Purulent discharge (thick, opaque to white or sanguineous secretion)
Mild	▪ Local infection involving only the skin and the subcutaneous tissue (without involvement of deeper tissues and without systematic signs as described below) ▪ If erythematous, must be > 0.5cm to ≤ 2 cm around the ulcer ▪ Exclude other causes of an inflammatory response of the skin (e.g. trauma, gout, acute Charcot, neuro-osteoarthropathy, fracture, thrombosis, venous stasis)
Moderate	▪ Local infection (as described above) with erythema > 2 cm, or involving structures deeper than the skin and subcutaneous tissues (e.g. abscess, osteomyelitis, septic arthritis, or fascitis) and ▪ No systematic inflammatory response signs (as described below)
Severe	▪ Local infection (as described above) with the signs of systematic inflammatory response syndrome, as manifested by ≥ 2 of the following • Temperature > 38°C or < 36°C • Heart rate > 90 beats/ min • Respiratory rate > 20 breaths/min or partial pressure of arterial carbon dioxide ($PaCO_2$) < 32 mmHg • White blood cell count > 12 000 or < 400 cells/ µL or ≥ 10% immature (band) forms

2.9.4.2. *Counting the bacteriology of ulcer*

Historically, the gold standard for determining wound bacterial bio-burden and obtaining wound specimen has been the quantitative tissue biopsy or wound

tissue culture (Bill et al. 2001; Gardner et al. 2007). Since tissue biopsy affected the healing of the small ulcer wound (Fleck, 2006), it was not suitable to use for small ulcer. Quantitative tissue swab culture has been suggested as a means to determine the wound bioburden. Bill et al. (2001) prospectively studied 38 patients with chronic wounds of various etiologies to evaluate the correlation between quantitative wound biopsy and swab culture. The result showed that there was a 79% in correlation with the 2 methods. The authors concluded that quantitative swab culture provides a valuable adjunctive method in the management of chronic wounds.

The most common wound swab technique is using wound exudate, Z-technique and the Levine technique. After removing the dressing, wound exudate apparent on the wound surface. By taking a wound swab, it was simply sampling the wound exudate. For the Z-technique, swab was taken by a broad Z-stroke over the entire wound bed (Gardner & Frantz, 2004). When using the Levine technique, select an area near the center of the wound free of necrotic tissue and debris. Cleanse the wound with normal saline; swab was then rotated over one cm^2 area for five seconds with sufficient pressure to extract fluid within the wound tissue (Gardner et al. 2006). The authors studied the diagnostic validity of the above three different swab techniques in chronic wound. The findings indicated that swab specimens collected from wound using Levine's technique performed better than swab specimens collected using either the wound exudate of Z-technique. Accuracy was the highest for swab specimens using the Levine's technique, which provided Sn 90%, Sp of 57%, PPV 0.77 and NPV 0.91. Angel et al. (2011) got a similar result and revealed that Levine's technique detected

more microorganisms in both acute and chronic wounds then Z-technique. They postulated that with the Levine technique, the pressure exerted was released those microorganisms within the soft tissue than the Z-technique, though the method of collecting the sample did not.

Gardner et al. (2007) further examined the diagnostic validity between semi-quantitative and quantitative swab culture by using the Levine's technique with reference to the gold standard of tissue culture. The main difference between 2 methods was that semi-quantitative method did not count the absolute amount of organisms on the agar plate. Quantification was expressed as 1+, 2+, 3+, or 4+ based on the number of quadrants that demonstrated bacterial growth. For the quantitative swab method, the quantification of each organism was accomplished by counting the number of colony forming unit (CFU) on each plate. The result found that swab specimens processed using quantitative processes provided a more accurate and valid findings that correlate well with culture findings from tissue specimens. In a narrative review, Rondos et al. (2013) revealed that only few studies in the literature compare wound swabs with biopsies for the diagnosis of chronic infected wound. The best sampling technique for taking a swab has not been identified. Until now, the Levine technique has been considered as the most reliable and valid method.

Slater et al. (2004) collected a wound swab before debridement and obtaining biopsy sample in post-debridement in 60 patients with DFU. The result showed that the swab identified all organisms isolated the tissue sample in those ulcers not extending to bone. Therefore, for those ulcers did not have bone exposed,

Levine's technique seems to be the most suitable method used for the detection of bacteriology.

2.9.5. Local wound care: cleaning and wound dressing materials

The objective of management should be decided before the commencement of local wound care. It is recommended that the ulcer be cleansed at each dressing change by boiled and cooled tap water, distilled water or saline solution (Crawford & Fields-Varnado, 2013). For palliative wound care on a non-healable wound, the objective of management is to keep the wound dry and avoid moisture to decrease the risk of infection (Woo et al., 2013). A topical antiseptic such as povidone iodine is a good choice if it is not contra-indicated. For a healable ulcer, maintaining a moist wound environment through exudate control is the golden principle (Hinchiffe et al., 2008). Importantly, exudate management and controlling the bio-burden is always a concern (Lipsky et al., 2012a).

In the past few decades, many dressing materials have been developed for the treatment of diabetic ulcers. The selection of dressings should allow for moist wound healing and controlling excess exudate, regardless of the type of topical dressing material used. The choice of dressing should be based on the size, depth and nature of the ulcer, such as dry, exudate or purulent (Lipsky et al., 2012a). Indeed, there are no studies that show the effectiveness of the conventional dressing. There are a number of anti-bacterial dressings used for decreasing and controlling bacterial loading on the wound bed, such as iodine-impregnated dressing (Vermeulen et al., 2010), polyhexamethylene biguanide (PHMB)-impregnated dressing (Napavichayanun et al., 2015), silver dressing (Jude et al.,

2007) and honey dressing (Lusby et al., 2005). The moist wound healing principle could facilitate epidermal cell migration, angiogenesis and collagen synthesis (Fonder et al., 2008). The next section presents an in-depth discussion of the effects of silver and honey dressings on DFU.

2.10. Dressing materials: silver and honey

2.10.1. History of silver and honey dressings

Among all types of topical anti-bacterial dressing, honey and silver are being used more frequently in wound care, including care of DFU. The use of silver as a prophylactic and treatment for infections and other diseases dates back to about 100 BC, when it was used for this purpose by the ancient Greeks and Romans (White, 2001). In the late nineteenth century, there was a resurgence of interest in using silver compounds to treat venereal diseases and eye infections (Lansdown, 2002a). The topical application of honey was also a common practice for centuries (Dunford et al., 2000) by the Egyptians, Greeks, Romans, and Chinese (Jull et al., 2008a). The early Egyptians of around 1650 BC were the first to use honey as a component in the topical treatment of wounds, as evidenced from the text of the Smith papyrus (Mwipatayi et al., 2004). However, the use of honey came to be considered outmoded around the 1940s, following the advent of antibiotics. With the recent increase in multi-resistant bacteria due to the overuse of antibiotics in the past few decades, the potential of honey and silver in the management of various chronic wounds has attracted new interest in the wound care community (Sare, 2008).

2.10.2 Types of silver and honey dressings

2.10.2.1. *Types of silver dressings*

There are two main types of silver dressing in the form of silver molecules, namely Ag and nAg. Ag dressing was developed earlier and nAg dressing is a relatively new type of silver dressing. Indeed, both Ag and nAg have a similar effect on antibacterial and anti-inflammatory actions. However, the nAg had a higher bioactivity (Fong & Wood, 2006) that could be exerted on the cell membrane (Sondi & Salopek-Sondi, 2004) and result in a more potent antibacterial action (Lok et al., 2006) when compared with Ag.

Takchenko and Karas (2012) revealed in vitro that nAg-coated dressing exhibited the most potent antibacterial activity when compared with ionic silver-containing dressing in terms of the time-kill assay and the zone of inhibition test. Edwards-Jones (2006) compared nAg dressing with two ionic silver dressings, and the result suggested that the nAg dressing was more effective than other silver dressings in providing a barrier function and antimicrobial activity against epidemic strains of methicillin-resistant Staphylococcus aureus. Indeed, there are two brands of nAg dressing available. Lee et al. (2010) compared the efficacies of nAg-containing dressing materials for treating a full-thickness mice model infected by methicillin-resistant Staphylococcus aureus. They found that the dressing with a higher concentration of nAg was superior in rate and time for wound healing.

There have also been studies showing that nAg had a significant anti-inflammatory action on animal models (Wright et al., 2002; Wong et al., 2009; Nadworny et al., 2010b; Bisson et al., 2013) and human cells, (Shin et al., 2007; Aparna Mani et al., 2015). The detailed action will be elaborated in the next section. Since nAg dressing has a more potent antibacterial effect than Ag dressing, nAg dressing is enjoying increasing popularity in clinical use.

2.10.2.2. *Types of honey dressings*

There are many types of honey (e.g. MH, Yemen honey, clover honey, royal jelly honey, Pakistan honey) used for dressing management in different countries. Among all types of honey, only MH has been categorized as medical grade and formally approved by the U.S. Food and Drug Administration as well as Conformité Européene Marking. The use of MH in dressing management has increased in popularity, and there is relatively more evidence for its use when compared with other types of honey.

2.11. Action of nanocrystalline silver and Manuka honey

2.11.1. Antibacterial effect

As mentioned as above, DFU is prone to bacterial infection because of the impaired local circulation and the decreased activity of white blood cells. Impaired local circulation is related to a decrease in endothelium-derived vasodilators, leading to vasoconstriction (Clayton & Elasy, 2009) and PAD. In addition, the activities of white blood cells are impaired due to the decreased

accumulation of leukocytes (Galkowska et al., 2005) as well as the decrease in the polarization and activation of macrophages (Kanter et al., 2013). The antibacterial action of nanocrystalline silver and manuka honey are illustrated as follows.

2.11.1.1. *Nanocrystalline silver*

Nanocrystalline silver (nAg) can increase the surface area that is in contact with the wound surface (Kon & Rai, 2013). The bactericidal effect of nAg depends on its size. It is preferable for nAg to be around 1-10 nm in size, as this is the size at which direct interaction can occur with the surface of the cells and within the bacteria (Morones et al., 2005). Thus, nAg can increase the bioactivity and solubility of silver to allow chemical reactions to take place (Fong & Wood, 2006). Apart from size, the shape of nAg can also affect their bactericidal effect (Pal et al., 2007). As regards antibacterial action, Lok et al. (2006) revealed that nAg appears to be significantly more efficient than silver ions. nAg and Ag^+ share the following common bactericidal effects. Silver ions can interact with thiol group-containing enzymes, such as NADH-dehydrogenase II in the respiratory system (Matsumura et al., 2003). This will lead to the formation of hydroxyl radicals and to an attack on the cell itself, and subsequently to deoxyribonucleic acid (DNA) damage (Gordon et al., 2010). Silver ions also induce apoptotic pathways and damage DNA inside the bacteria, leading to their death (Kon & Rai, 2013; Pandian et al., 2010; Pal et al., 2007).

In vitro evidence shows that nAg^+ has a unique antibacterial action on cells that Ag does not have. There is an electrostatic attraction between nAg^+ and the

negative charge cell membranes of bacteria. nAg binds to the modified phospholipid bilayer and induces a massive leakage of protons (Dibrov et al., 2002). When nAg^+ anchors to the bacterial cell wall and causes structural change by forming irregular-shaped "pits" on the bacterial outer membrane, the permeability of the membrane changes and becomes porous (Sondi & Salopek-Sondi, 2004). In an electro spin resonance spectroscopy study, Kim et al. (2007) further discovered that nAg generates free radicals, which can damage the bacteria cell membrane. The cell will then progressively release lipopolysaccharides and membrane protein, which will ultimately cause the cell to die. Mirzajani et al. (2011) further investigated the effect of nAg on Gram-positive Staphylococcus aureus (S. aureus). They discovered that nAg exerted on the peptidoglycan of a bacterial wall and decomposed the peptide part of the cell wall. By attaching to the bonds of glycan strands composed of N-acetylglucosamine and N-acetylmuramic acid, nAg destroyed the bonds and released muramic acid. As a consequence, "pits" were generated on the bacterial cell membrane. However, the exact mechanism that occurred on the Gram-negative membrane remain unknown. McQuillian et al. (2012) continued working on Gram-negative bacteria, and investigated the effect of nAg on Escherichia coli (E. coli). Using inductively coupled plasma mass spectrometry, they discovered that the primary mechanism of nAg was to interact with and dissolve the outer and inner membranes of a cell. Ag^+ was then released into the cell and affected a transcriptional response.

In addition, nAg can dephosphorylate the peptide substrate on tyrosine residues. Mijakovic et al. (2005) demonstrated that the phosphorylation of protein

substrate in bacteria can influence bacterial sign transduction. Shrivastava et al. (2007) found that the inhibition of this mechanism by nAg can influence the signal transduction and stop the growth of bacteria. In summary, the overall effect of nAg is both to inhibit the reproduction of bacteria and also to kill them directly.

2.11.1.2. *Manuka honey*

Manuka honey (MH), which comes from Leptospermum scoparium in New Zealand, exhibits antibacterial activity (Mandal & Mandal, 2011). Our body of knowledge on its antibacterial mechanism remains incomplete. The antibacterial action of MH is mainly based on its physical properties and on the active ingredient that it contains. First, honey has an osmotic effect, drawing moisture from the environment and dehydrating bacteria (Mandal & Mandal, 2011). This effect is reduced after dilution by wound exudate (Ahmed et al., 2003). Second, the pH value of MH is between 3.2 and 4.5. This acidic nature can inhibit the growth of most micro-organisms, such as E. coli, Pseudomonas aeruginosa (P. aeruginosa), and Streptococcus pyogens (Stephen-Haynes, 2004; Molan, 2001; Molan, 1996). Third, methylogloxal (MGO) is one of the phytochemical factors with antibacterial activity that have been identified within MH. In vitro studies have revealed that the MGO in MH contains the majority of the non-peroxide activity, and that the amount of MGO is closely related to the level of antibacterial activity (Adams et al., 2008; Mavric et al., 2008). Ordinary honey has a limited amount of MGO, ranging in concentration from 1.6-135 mg/kg, compared to 38-725 mg/kg in manuka honey (Adams et al., 2008). A minimum inhibitory concentration (MIC) for E. coli and S. aureus was observed at 1.1mM of MGO

(Mavric et al., 2008). Atrott and Henle (2009) also found a linear correlation between MGO levels in 61 samples of manuka honey and antibacterial activities. There were also some other unidentified biochemical substances, which later laboratory studies revealed also contribute to antimicrobial activity. Kwakman et al. (2011) identified some cationic and non-cationic compounds that contributed to bactericidal activities against different types of bacteria. Kato et al. (2012) found a glycoside of methyl syringate called "Leptosin," which had a positive correlation with antibacterial activity in MH.

Unfortunately, the exact mechanism contributing to the bactericidal activity of MH remains largely unknown (Adams et al., 2008). The antibacterial action of MH was recently explored with the hope of elucidating the related mechanism, but its precise mode of antibacterial action is only just beginning to be understood. In vitro studies have demonstrated that cell division is interrupted and cell separation cannot occur following the formation of septa on staphylococcus aureus (S. aureus) (Henriques et al., 2010) and MRSA (Jenkins et al., 2011) when these bacteria are exposed to MH. Roberts et al. (2012) observed extensive cellular lysis of P. aeruginosa at an MIC concentration of 12% w/v in MH. The honey-treated cells were unable to form microcolonies, and two target genes were identified as being involved in the process. In addition, Packer et al. (2012) found that MH causes two different proteins to be down-regulated and one to be up-regulated on S. aureus. These two proteins had roles to play in ribosomal function, protein synthesis, the metabolic process, and transcription. Merckoll et al. (2009) discovered that honey has bactericidal effects on both planktonic and biofilm-embedded bacteria, since bactericidal substances in

honey can penetrate into the biofilm. Maddocks et al. (2012) further found that MH decreases the formation of biofilm by inhibiting the Streptococcus pyogenes from binding to fibronectin. This binding is important for the colonization of bacteria and the development of biofilm (Bonifait et al., 2008). Iron is necessary to sustain the growth of bacteria. P. aeruginosa produced two extensively characterized siderophores to capture iron (Simon et al., 2009). Kronda et al. (2013) further discovered that MH decreased the production of siderphores at both ¼ and ½ MIC, showing that MH impeded the growth of the cells. So far, the above in vitro evidence suggests that there is no single mechanism to antimicrobial action, but that a combination of factors results in diverse modes of antibacterial inhibition and killing.

Compared with antiseptics that decrease the bacterial count within minutes, the antibacterial activity of MH is much slower (Simon et al., 2009). The latest laboratory studies explain this phenomenon. Kwakman et al. (2011) showed that MH does not have a rapid antibacterial effect against different kinds of bacteria in the first 2 hours, but that its potency increases after 24 hours. The reason for the slow onset action relates to the fact that MH lacks major factors involved in rapid antibacterial activity, such as bee defensin-1 and hydrogen peroxide. Adams et al. (2009) found that the main antibacterial ingredient MGO forms through the conversion of dihydroxyacetone. The concentration of MGO is low in freshly produced honey, but increases after storage at 37°C.

MH has an antibacterial effect on different microorganisms in vitro. The rate of inhibition depends on the species of bacteria and the concentration of honey

(Ahmed et al., 2003; Efem and Iwara, 1992). Cooper et al. (1999) found that MH would still prevent the growth of S. aureus if diluted by a further 7- to 14-fold in vitro. Cooper et al. (2002a) continued this work, discovering that 17 strains of pseudomonas isolated from infected burn wounds could be killed by MH, even when diluted more than 10-fold. Hammond and Donkor (2013) found that the corresponding MIC and minimal bactericidal concentration of Clostridium difficile on MH were both 6.25% (v/v). Kwakman et al. (2011) further discovered that the bactericidal activity of MH could kill Bacillus subtilis, P. aeruginosa, and E. coli. Maddocks et al. (2012) identified the bactericidal effect of MH on streptococcus pyogenes in both planktonic cultures and biofilm. MH has also been found to kill antibiotic-resistant bacteria. Cooper et al. (2002b) found that seven strains of vancomycin-resistant enterococci were inhibited by MH at 4.61 ± 0.51% (v/v). French et al. (2005) demonstrated that MH inhibited 18 strains of antibiotic-resistant coagulase-negative staphylococci at dilutions of down to 29.9 ± 1.9% (v/v). Interestingly, MH has a synergistic antibacterial effect with antibiotics. Five antibiotics and MH combinations were found that improve antibacterial effectiveness in vitro (Jenkins and Cooper, 2012). However, MH cannot kill all micro-organisms. Lusby et al. (2005) revealed that MH is unable to inhibit Serratia marcescens and Candida albicans.

However, the effect of MGO in diabetic ulcers is debatable. One of the molecular alternations of DFU is the excessive oxidative stress. MGO changed the structure and function of immunological enzymes to form AGEs, reducing the efficiency of the peripheral blood immune-cell response, which can impair the wound-healing process (Price and Knight, 2009; Majton, 2011). In an in vitro study, Sassi-Gaha

et al. (2010) found that highly reactive dicarbonyls attacked the lysine, arginine (Arg) and cysteine residues of long-lived proteins (e.g. collagens) to form irreversible AGEs, causing changes in collagen pathophysiology. Therefore, there is clearly a paucity of high-quality human studies relating to the use of topical honey to treat diabetic ulcers. A summary of the above antibacterial effects of nAg and MH is shown in Table 2.4.

Table 2.4. Mechanism of antibacterial effect of nAg and MH

Author	Nature of study	Mechanism of antibacterial effect of nAg
Kon & Rai, 2013	Review	Increases the surface area contacting the wound surface
Morones et al., 2005	In vitro	Has a direct interaction with the cell surface and within the bacteria with diameter around 1-10 nm
Pal et al., 2007	In vitro	The bactericidal action of nAg with a truncated triangular shape exceeds that of nAg with a spherical or rod shape
Matsumura et al., 2003	In vitro	Interacts with thiol group of respiratory enzymes
Gordon et al., 2010	Animal study	Inactivates the key enzyme by blinding the thiol group, forming free radicals, and subsequently damaging DNA
Pandian et al., 2010	In vitro	Interacts with and condenses phosphorous-containing DNA and cytoplasm (apoptosis), inhibits cell replication
Dibrov et al., 2002	In vitro	Binds to the modified phospholipid bilayer and induces a massive leakage of protons
Sondi & Salopek-Sondi, 2004	In vitro	Attaches the negatively charged cell membrane by forming “pits”, making the membrane porous and resulting in leakage of intracellular content
Kim et al., 2007	In vitro	Bacteria release cellular content after the permeability of the cell membrane increases, leading to cell death
Mirzajani et al., 2011	In vitro	Destroyed the bonds of glycan strands composed of N-acetylglucosamine and N-acetylmuramic acid in the cell membrane of Gram +ve bacteria and causing “pits” to form
McQuillan et al., 2012	In vitro	Interacts with the outer and inner membrane of Gram –ve bacteria, and then membrane dissolves; Ag^{+} releases into the cell and affect a transcriptional response
Mijakovic et al., 2005 Shrivastava et al., 2007	In vitro	Phosphorylation of the protein substrate in bacteria can influence bacterial sign transduction and cell cycle progression

Table 2.4. Mechanism of antibacterial effect of nAg and MH (cont'd)

Author	Nature of study	Mechanism of antibacterial effect of MH
		Physical property
Mandal & Mandal, 2011	Review	Osmotic effect can draw water from bacteria and dehydrate them
Molan, 2001	Review	Acidity (pH 3.2-5.5) can inhibit the growth of most micro-organisms
		Active ingredient
Adams et al., 2008	In vitro	High concentration of MGO ranged from 38 to 828 mg/kg as compared with non-MH
Mavic et al., 2008	In vitro	MGO ranged from 38-761 mg/kg and could inhibit E. coli and S. aureus at 1.1 mM
Atrott & Henle, 2009	In vitro	MGO ranged from 189 to 835 mg/kg and was directly responsible for antibacterial property
Kwakman et al., 2011	In vitro	Glycoside of methyl syringate called "Leptosin" correlated positively with antibacterial activity
Kato et al., 2012	In vitro	Other than MGO, cationic and non-cationic compounds contributed to antibacterial activity
		Mechanism of action
Henriques et al., 2010 Jenkins et al., 2011	In vitro	Honey-treated cells fail to advance the progress of cell division and separation • S. aureus • MRSA
Roberts et al., 2012	In vitro	Extensive cell lysis on P. aeruginosa at MIC 12% (w/v) after 60 minutes of exposure to MH
Paker et al., 2012	In vitro	Ribosomal function interfered with S. aures including protein synthesis, the metabolic process, & transcription
Maddocks et al., 2012	In vitro	Inhibition of the binding of Streptococcus pyogenes to fibronectin and the development of biofilm
Kronda et al., 2013	In vitro	Limits P. aeruginosa in capturing iron, and impedes its growth
		Bactericidal activity on different micro-organisms
Cooper et al., 1999	In vitro	Streptococcus pyogens
Cooper et al., 2002	In vitro	Pseudomonas species
Hammond & Donkor, 2013	In vitro	Clostridium difficile
Kwakman et al., 2011	In vitro	MRSA, Bacillus subtilis, E. coli, P. aeruginosa
Maddocks et al., 2012	In vitro	Streptococcus pyogenes
Cooper et al., 2002	In vitro	Vancomycin-resistant enterococci
French et al., 2005	In vitro	Antibiotic-resistant strains of coagulase-negative staphylococci

2.11.2. Anti-inflammatory effect

Since DFU is prone to inflammation because of the molecular alternation in high MMP, cytokine and oxidative stress, the up-regulation of these enzymes will destroy the ECM and result in poor ulcer healing. High concentrations of MMP-1, -8 and -9 were found in non-healing DFU (Lobmann et al., 2002; Muller et al., 2008). The elevated pro-inflammatory cytokines of TNF-α, IL-1 and IL-6 lead to impaired collagen synthesis and angiogenesis, resulting in chronic inflammation (Lobmann et al., 2005; McLennan et al., 2006; Trengove et al., 2000; Chan et al., 2012).

Poor control of diabetes leads to excessive free radical production, resulting in biological damage and poor ulcer healing (Van den Berg et al., 2008; Soneja et al., 2005). The anti-inflammatory actions of MH and nAg can target the molecular alternation of DFU. Their anti-inflammatory mechanisms are illustrated below.

2.11.2.1. *Nanocrystalline silver*

Compared with the antibacterial action of silver, the exact mechanism of its anti-inflammatory action is still unclear. To the best of my knowledge, the studies that have been published on the subject have focused on the observable effect of the anti-inflammatory action of silver, instead of on the detailed molecular mechanism. The animal and in vivo evidence showing the anti-inflammatory effect of nAg is as follows. Wright et al. (2002) discovered that a full-thickness contaminated wound in a porcine model treated with nAg healed more quickly and was accompanied with a decrease in MMP activities as well as with the

stimulated apoptosis of PMNLs. They suggested that enhanced cellular programmed cell death (apoptosis) would decrease the number of cells to release cellular content with numerous cytotoxic compounds such as proteases, oxygen radicals, and various acids, in turn decreasing local inflammation. Several animal studies revealed that nAg was as effective as topical steroids and immunosuppressive drugs in decreasing allergic contact dermatitis by suppressing TNF-α and IL-12 and inducing the apoptosis of inflammatory cells (Bhol et al., 2004; Bhol & Schechter, 2005; Wong et al., 2009).

Nadworny et al. (2010a) continued the work in different animal models and echoed the previous findings. They further discovered that nAg had the ability to promote healing because some kinds of growth factors were elevated after topical nAg intervention. Using a porcine model, Nadworny et al. (2010b) found that treatment with solutions containing nAg resulted in a decrease in pro-inflammatory cytokines TNF-α and IL-8 expression, with an increase in IL-4, EGF, keratinocyte growth factor (KGF), and KGF-2 expression. Again using a porcine model, Nadworny and colleagues (2010b) also discovered that nAg was mainly deposited in the epidermis, but that cell apoptosis was significant in the dermis and minimal in the epidermis. They therefore proposed that the anti-inflammatory effects of nAg were induced by interactions with cells in the top layers of the skin, which then released biological signals resulting in widespread anti-inflammatory activity. nAg induced the down-regulation of TNF-α and IL-8 expression, as well as the up-regulation of IL-4, IL-10, EGF, KGF, and KGF-2. Bisson et al. (2013) furthered the work on the latest nAg topical dressing, demonstrating that nAg dressings had a significant inflammatory effect on a non-

infected inflammatory mice model, equivalent to that obtained using a topical steroid cream.

In addition, there is some in vivo evidence showing that nAg has an anti-inflammatory effect. Shin et al. (2007) performed the work on human cells. The results showed that TNF-α and interferon-γ were significantly inhibited at low concentrations of nAg of over 3 ppm. Aparna Mani et al. (2015) examined the effect of synthetic nAg on healthy human cells. They also showed that all three cytokines (TNF-α, IL-1β, and IL-6) were inhibited at concentrations ranging from 10-20 μg/ml. Obviously, from the published animal and human studies, the anti-inflammatory effect on nAg is clear, although the exact pathway is still unknown. Interestingly, all histological findings demonstrated apoptosis of the inflammatory cells induced by nAg, so that avoiding the inflammatory cells produces chemoattractants to induce further inflammation upon bursting. In addition, all of the molecular findings indicated that the TNF-α expression was down-regulated. In diabetic wound healing, impairing the proliferation of fibroblasts has been linked to an increase in the level of TNF-α (Kaiser & Polk, 1997). When the level of TNF-α level is inhibited, there is a significant increase in fibroblast proliferation (Siqueira et al., 2010).

2.11.2.2. *Manuka honey*

Honey has been shown to reduce both acute and chronic inflammation, although the mechanism for this anti-inflammatory action is not entirely understood (Pieper, 2009). The antioxidants found in honey are considered to be important

determinants of anti-inflammatory activity (Molan, 2006). The antioxidant properties of honey are beneficial in counteracting advanced glycation and lipoxidation end products, which can induce oxidative stress and inflammation in diabetics (Molan and Betts, 2008). Natural honey contains flavonoids, phenolic acids, and other enzymes. All of the active components work together to provide a synergistic anti-inflammatory and antioxidant effect (Vallianou et al., 2014). Chan et al. (2013) revealed that pinobanksin, pinocembrin, luteolin, and chrysin were the major flavonoids found in MH, accounting for 61% of the total flavonoids. Flavonoids are known for their anti-inflammatory activity. Cho et al. (2004) found that chrysin was able to suppress the activity of pro-inflammatory enzymes, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOs). Raso et al. (2001) discovered that several flavonoids could inhibit the expression of COX-2 and iNOs in a concentration-dependent manner.

Honeys from different botanical origins have different components. The following in vitro studies give a clear picture of the anti-inflammatory action of MH. Henriques and colleagues (2006) discovered that MH had the strongest antioxidant capacity among the varieties of honey that were tested, and that it was able to quench the added free hydroxyl radicals within 5 minutes of being added. This antioxidant capacity contributed to the ability of MH to resolve chronic inflammations, including ulcers. In addition, Tonks et al. (2003) revealed that MH stimulated both the pro-inflammatory cytokines TNF-α and IL-1β and the anti-inflammatory cytokines IL-6 from monocytes. Tonks and his colleagues (2007) further found that a 5.8-kDa component isolated from MH stimulated these cytokines via a toll-like receptor (TLR) 4. After heat treatment, sugar syrup

and MH lose this function. This explains why supermarket honey cannot be used as medicinal honey for treating wounds. Van den Berg et al. (2008) also discovered that the phenolic constituents of MH were able to inhibit the production of ROS and scavenge superoxide anion. Recently, an in vitro study from the University of Waikato found that MH increased both the expression of the pro-inflammatory cytokine TNF-α and the anti-inflammatory cytokines Il-10 and IL-1, and the growth factors PDGF and TGF-β. ROS production by phagocytosis was also down-regulated in the presence of MH (Bean, 2012). These findings are in alignment with previous in vitro studies, and explain why MH may allow inflammation to proceed at a modulated level while simultaneously allowing healing to occur.

Leong et al. (2011) determined that MH decreased the production of superoxides by neutrophil in vitro and decreased odema and leukocyte infiltration in a mice model, but were unable to pinpoint the specific content contributing to the inflammatory action. Tomblin et al. (2014) further discovered that this anti-inflammatory activity of MH directly correlated to the phenolic content through a TLR1/ TLR2 signalling pathway. The higher phenolic content produced an elevated anti-inflammatory effect. This result echoed the findings of Leong et al. (2011) on the specific content inside MH that is responsible for the anti-inflammatory function. The anti-inflammatory actions of nAg and MH are summarized in Table 2.5.

Table 2.5. Mechanism of anti-inflammatory effect of nAg and MH

Author	Nature of study	Mechanism of anti-inflammatory effect of nAg
Wright et al., 2002	Animal study	Reduces the activity of MMPs and stimulates the apoptosis of PMNs, leading to a decrease in the release of cytotoxic compounds such as proteases and oxygen radicals
Bhol et al., 2004	Animal study	Effectively decreases allergic contact dermatitis on a guinea pig model, similar to topical steroids
Bhol & Schechater, 2005	Animal study	Suppresses the activities of TNF-α and IL-12 and induces the apoptosis of inflammatory cells
Wong et al., 2009	Animal study	Down-regulates the production of TNF-α without having a significant toxic effect on a peritoneal adhesion model
Nadworny et al. 2010a	Animal study	Decreases TNF-α and IL-8, and increases IL-4, EGF, KGF, & KGF-2
Nadworny et al., 2010b	Animal study	Down-regulates TNF-α and IL-8, and up-regulates IL-4, IL-10, EGF, KGF, and KGF-2
Bisson et al., 2013	Animal study	Demonstrates a significant inflammatory effect, equivalent to that which results from using topical steroid cream
Shin et al., 2007	In vivo	TNF-α and interferon-γ are significantly inhibited at low concentrations of nAg
Aparna Mani et al., 2015	In vivo	TNF-α, IL-1β, and IL-6 are inhibited at concentrations ranging from 10-20 μg/ml

Table 2.5. Mechanism of anti-inflammatory effect of nAg and MH (cont'd)

Author	Nature of study	Mechanism of anti-inflammatory effect of MH
		Active ingredient
Chan et al., 2013	In vitro	Pinobanksin, pinocembrin, luteolin and chrysin are the major flavonoids found in MH; low levels of quercetin and galangin were also detected
Raso et al., 2001	In vitro	Quercetin and galangin inhibit the expression of COX-2 and iNOs in a concentration-dependent manner
Cho et al., 2004	In vitro	Chrysin suppresses the activity of pro-inflammatory enzymes
Tonks et al., 2007	In vitro	5.8-kDa component isolates of from MH stimulated pro-inflammatory cytokines TNF-α, IL-1β and the anti-inflammatory cytokines IL-6 via toll-like receptor (TLR) 4
Tomblin et al., 2014	In vitro	Phenolic content is directly correlated to anti-inflammatory activity of MH through a TLR1/ TLR2 signalling pathway
		Mechanism of action
Henriques et al., 2006	In vitro	The formation of free radicals such as hydroxyl radicals is inhibited, and this contributes to resolving chronic inflammation
Tonks et al., 2003	In vitro	Pro-inflammatory cytokines TNF-α and IL-1β as well as anti-inflammatory cytokine IL-6 from monocytes are stimulated
van den Berg et al., 2008	In vitro	ROS production and scavenge superoxide anions are inhibited
Bean, 2012	In vitro	The pro-inflammatory cytokine TNF-α and the anti-inflammatory cytokines Il-10 and IL-1, as well as the growth factors PDGF and TGF-β, are up-regulated ROS production by phagocytosis is down-regulated
Leong et al., 2011	In vitro Animal study	The production of superoxides by neutrophil decreased Leukocyte infiltration and odema in the mice model decreased

2.11.3. Bacterial resistance to silver and MH

No studies have been conducted on bacterial resistance in MH. However, some reports have documented bacterial resistance to Ag/ nAg on possible wound pathogens, including Providencia stuartii (Wenzel et al., 1976), Enterobacter cloacae (Gayle et al., 1978), Pseudomonas species (Haefeli et al., 1984), Acinetobacter baumannii (Deshpande & Chopade, 1994) and E. coli (Gupta et al., 2001). Laboratory studies found that the efflux of silver ions (Li et al., 1997) or the acquisition of genetic material in the form of plasmids was the mechanism of a silver-resistant E coli mutant (Gupta et al., 1999). Some researchers have claimed that the clinical incidence and probability of multiple mutations remain low even though silver resistance has been documented (Chopra, 2007; Cooper & Gray, 2012). Unlike antibiotics, silver attacks more than one of the target specific sites (Percival et al., 2005), and there is no direct evidence that silver resistance mechanisms confer cross-resistance to antibiotics (Chopra, 2007). Percival et al. (2008) continued the work on Ag-containing wound dressings in vitro. They concluded that despite evidence of genetic resistance to silver, all strains were killed following a maximum of 48 hours of exposure to the dressings. Therefore, both Ag and nAg were safe to use clinically.

2.11.4. Cytotoxicity of silver and honey

No studies have been conducted on the cytotoxicity of MH. By contrast, the cytotoxicity of silver is an issue that has been debated. Van den Du Toit and Page (2009) compared a honey (30% medical grade honey gel) group, an acticoat

(nAg dressing) group and a control (untreated) group, finding that the nAg group had poor keratinocyte and fibroblast survival, proliferation and migration. Plas et al. (2008) found that silver dressings induced rapid cell death within two hours; they recommended the use of silver dressings only on critically contaminated wounds. Zou et al. (2012) compared different pairs of Ag-based and non-Ag-based dressings with basic materials in vitro. The results showed that human fibroblasts extracted from diabetic patients decreased in viability by 54-70% and collagen synthesis by 48-68% when they came into contact with the Ag-based dressings compared with non-Ag-based dressings. They did not suggest discarding Ag dressings but stated that such dressings should be used with caution when treating non-infected diabetic wounds. In vitro and animal studies have shown that silver dressings have significant cytotoxic effects on keratinocytes and fibroblasts (Burd et al., 2007; Poon & Burd, 2004). Therefore, the international consensus on the use of silver is that it should be discontinued if wound infection is no longer present (International Consensus, 2012).

Surprisingly, findings from recent clinical studies do not support the view that using modern silver dressings can lead to cytotoxicity. Lansdown et al. (2002b) claimed that toxicity from dressings containing silver is rare because in modern dressings the silver is in a controlled-release preparation and some of the silver ions bind to the protein of the wound exudate. Lansdown et al. (2005) discovered that the ions from a silver dressing penetrated only several millimeters into the wound bed. Karlsmark et al. (2003) noted that the serum silver level for patients treated with silver dressings was no higher than the reference value, although five patients experienced a temporary increase in their

silver level. Gago et al. (2008) provided further evidence that a high level of Ag rapidly reduces infection and results in the faster healing of infected chronic wounds.

Tshukudu et al. (2010) performed an in vitro study on cell viability. A different honey-based (medical grade multi-floral honey from Bulgaria) dressing and different silver-based (Ag and nAg) dressings were tested. They found that all the dressing extracts showed variable effects on cell viability and that the exposed cells showed a similar morphology. Acticoat (nAg with alginate) was the most toxic to cells, with less than 30% viability. Interestingly, the group treated with Atrauman silver (Ag tulle contains triglycerides) demonstrated an increase in the number of viable cells as compared with the control group, but we could not establish any solid conclusion on whether the triglycerides contributed to the viability of the cells. In general, nAg still had a marked cytotoxic effect on cells in comparison with the tested honey. The available information indicates that the findings from both in vivo and in vitro studies are inconsistent, and that knowledge on the cytotoxicity of Ag and nAg dressings is incomplete. There is no evidence to show that Ag or nAg dressings are unsafe for use in human patients.

To conclude, the inconsistent findings that were reported on the antibacterial and cytotoxic effects of Ag/nAg compared with honey dressings might have depended on the types of silver or honey dressings that were tested. Therefore, the findings from one type of dressing might not be generalizable to other types of dressing using similar ingredients.

2.12. Research evidence of Ag or nAg and honey

2.12.1. Effect of Ag or nAg dressings on wounds other than DFU

The use of Ag or nAg dressings on burn wounds is a common clinical practice nowadays, and several studies have investigated their effect. Different databases were searched for Ag or nAg on burn wounds in clinical studies over the past 10 years. One meta-analysis and five RCTs were found. Rashaan et al. (2014) conducted a meta-analysis and found that non-silver dressing was preferred over silver dressing in the treatment of partial thickness skin burns in children. Among the five RCTs, four of them compared Ag versus nAg dressings, Ag dressing versus silver sulfadiazine, and nAg dressing versus silver sulfadiazine on second degree burns (Verbelen et al., 2014; Silverstein et al., 2011; Opasanon et al., 2010; Muangman et al., 2006). Only one RCT compared Ag dressing with non-Ag dressing (Caruso et al., 2006). The result showed that wound healing between the Ag dressing and non-Ag dressing groups was similar. To conclude, the clinical effectiveness of Ag/nAg dressing was not yet confirmed, although it was a common clinical intervention for burn wounds.

2.12.2. Effect on Ag or nAg-impregnated dressings on DFU

According to the in vitro and animal studies in the previous section (tables 2.4 and 2.5), nAg had antibacterial and anti-inflammatory effects that could target the biochemical alternation of DFU. Studies also found that there was a correlation between reductions in bacterial burden/ wound inflammation and

wound healing in both animal (Guthrie et al., 2014; Tian et al., 2007) and clinical studies (Miller et al., 2010a).

To date, no unifying theories have been established through the above basic science research and brought into the clinical context. Nevertheless, researchers have investigated Ag or nAg topical dressings from laboratory studies and tried to link their findings with the observable clinical outcomes. Different databases have been searched for reviews on the effect of Ag/nAg dressings on DFU. For published studies related to Ag dressing compared with non-Ag dressing, two case series studies (Rayman et al., 2005; Mir, 2013), two randomized controlled trials (RCT) (Gottrup et al., 2013; Jude et al., 2007), and one meta-analysis (Bergin & Wright, 2011) have been identified in the past 10 years. Bergin and Wright (2011) reviewed 16 studies, including case studies and RCTs. They concluded that it was difficult to draw solid conclusions with regard to the effectiveness of using Ag dressing on DFU, since there was no high quality RCTs that met the inclusion criteria. After the meta-analysis, a case study found that Ag dressings were effective (Mir, 2013). However, the case study design is a low level of evidence and subject to bias. Gottrup et al. (2013) showed that silver collagen dressing was more effective than collagen dressing in DFU healing, but the combined effect of silver and collagen could not be eliminated in this study. Therefore, there was no solid clinical evidence reported regarding on the use of Ag/nAg on DFU. There was a research gap identified regarding the use of Ag/nAg dressing on DFU.

In the past 10 years, only one case series study (Cahn & Kleinman, 2014) investigated the effect of nAg on DFU. Cahn's study used a non-surgical approach to treat six patients with diabetic foot abscesses. The abscesses were treated with topical oxygen and drained using nAg foam rope (Polymen® Wic® Silver Rope). The effects of debridement and oxygen therapy on diabetic foot abscesses could not be excluded. The patients varied in terms of their history of diabetes (5 to 20 years) and ankle brachial index score (0.57 to 1.63). The duration of their treatment ranged from two to eight months. In addition, three patients suffered from PAD. During the period that they were being treated for diabetic foot abscesses, they also underwent percutaneous revascularization. Although all of the patients with diabetic foot abscess and osteomyelitis had completely healed within two to nine months by treatments using debridement, a topical oxygen extremity chamber, and Polymen® Wic® Silver Rope, we could not determine the sole effect of nAg foam in this study. Therefore, there is no clinical evidence of nAg on DFU healing.

2.12.3. Effect on MH dressings on wounds other than DFU

In the past 10 years, there were numbers of clinical trials studying MH on various types of wounds and the results were arguable. For those RCT with vigorous design, the result showed that MH had no superior effect. However, the relative small scale RCT and prospective study found that MH was effective. McIntosh & Thomson (2006) found that MH was inferior to paraffin tulle gras on surgical wounds after partial toenail avulsion in 100 patients. The duration of complete wound healing in paraffin tulle gras group was significant shorter than

that of MH group. Although there was 13% of loss to follow up in this study, there was a clear sample size calculation, randomization and concealment. Similarly, Bardy et al. (2011) discovered that it had no significant difference between MH and golden syrup in their effect on radiation-induced oral mucositis in 127 patients. Despite the fact that per-protocol analysis was used in this study, the low withdrawal rate (less than 2%), clear blinding and well-defined priori sample size calculation minimized the risk of bias of the result. In addition, Robson et al. (2009) also revealed that there was no significant difference on time to healing between MH and standard local practice (using different types of dressings on different types of wounds) in an open-label randomized controlled trail. In Robson et al.'s study, the risk of bias was low because of the robust study design including clear sample size calculation, intervention protocol, randomization and concealment.

On the other hand, the effect of MH on venous ulcer was not clear. Jull et al. (2008) conducted a large community-based RCT with over 300 patients and found MH did not significantly improve venous ulcer healing when compared with usual care which composed of different types of dressing according to the local practice. The design of Jull et al.'s study was vigorous with clear defined protocol, sample size calculation, randomization and concealment. However, Gethin & Cowman (2009) had a contradictory result and found the mean wound reduction rate in MH group was significantly higher than hydrogel group. In Gethin's study, there was no clear report on blinding and concealment. Risk of bias was found in this study.

In a retrospective case review, Thomas et al. (2011) found that MH was an effective treatment for chronic pilonidal sinus wound. The nature of the study and the small sample size (n=17) may lead the result in subject to risk of bias. In another prospective cohort, Gethin et al. (2008) discovered that MH was effective in reducing the wound size in various types of ulcer. However, the nature of study and short duration of follow-up (2 weeks) may subject to the bias of the result. In brief, the above studies included different kinds of ulcers with different pathologies. The common point was that these ulcers had either inflammation or the combination of inflammation and infection. This was similar to the DFU. Until now, it is not free from doubt to confirm or reject the effect of MH in different types of wounds although the above vigorous designed RCTs all showed that the MH did not demonstrate superior effect when compared to other treatment options. There was still room for certain case reviews or prospective studies to demonstrate the effect of MH on wound other than DFU.

2.12.4. Effect of honey-related dressings on DFU

In DFU, the local factors that hinder wound healing are a high bioburden and a high inflammatory response. MH has been confirmed to have such properties as shown above (tables 2.4 and 2.5). Studies have shown MH to be effective in promoting wound healing in an in vitro rat aortic ring (Rossiter et al., 2010) and a horse model (Carnwath et al., 2014; Bischofberger et al., 2013). However, there is limited high-quality evidence to show its effect in clinical study. I reviewed single case studies, case series, and RCTs on the effect of honey or related products on DFU that had been published in the past 10 years, finding six single

case studies (Eddy & Gideonsen, 2005; Lotfy et al., 2006; Candeías & Cardoso, 2011; Mohamed et al., 2012; Mohamed et al., 2014a; Mohamed et al., 2014b), five case series studies (Abdelatif et al., 2008; Makhdoom et al., 2009; Moghazy et al., 2010; Siavash et al., 2011; Surahio et al., 2014), one controlled trial (Shukrimi et al., 2008) and three RCTs (Siavash et al., 2015; Mohamed, 2013; Kamaratos et al., 2014). Various kinds of honey products were applied as interventions in the published studies. For studies related to MH and DFU, only one published case series study (Gethin & Cowman, 2005) and two RCTs (Al Saeed, 2013; Kamaratos et al., 2014) were found on the use of MH in leg ulcerations (Table 2.6). Gethin and Cowman (2005) reported eight cases series studies on the use of MH in leg ulcerations. Only one involved DFU, while the others involved leg ulcerations with different etiologies. Therefore, this study does not represent DFU findings.

Al Saeed (2013) performed an RCT on 59 patients using an MH-impregnated dressing against tulle on DFU. The results showed that the honey dressing was superior in terms of the proportion of healing, rate of amputation, and time to eradicate the infection. However, the methodology of the study was flawed. There was no report on how the randomization, concealment, and double blinding were performed. The criteria for inclusion and exclusion in the study were not stated clearly. The antibiotics that were used and any adverse events were not reported. According to the Cochrane Collaboration's "risk of bias" criteria (Higgins and Green, 2011), there was a high risk of bias in selection, performance, and detection.

Kamaratos et al. (2014) performed another RCT on 63 patients using MH tulle against saline-soaked gauze. The result was that the intervention group healed significantly more quickly than the control group. Since the blinding of the outcome assessors was not clearly reported, a high risk of detection bias was found. A high risk of selection bias relating to the randomization sequence was predictable, as the participants were assigned to groups I and II in an alternate manner. The inclusion and exclusion criteria were not clearly reported. Like Al Saeed's study (2013), this study also failed to report the use of antibiotics and adverse events, which were the confounding factors of DFU healing throughout the study. Overall, we were not confident enough to come to a solid conclusion regarding the superior effect of MH dressing over conventional dressing because a high risk of bias in the design was identified in both of these studies. A high-quality study with a vigorously designed RCT on DFU is needed to enrich the body of knowledge in this area.

Table 2.6. The clinical evidence on MH topical dressings on DFU

Author	Nature of study	No. of subjects	Intervention	Funding	Major findings	Comments
Gethin & Cowman, 2005	Case series	8	Manuka honey	Not stated	A mean initial wound size of 5.62 cm for all wounds decreased to 2.25 cm at the end of the four-week treatment period.	There was a high risk of detection bias because the outcome assessors were not blinded. There was a high risk of selection bias because no inclusion and exclusion criteria were mentioned. Only one out of eight ulcers was DFU.
Al Saeed, 2013	RCT	59	Manuka honey-impregnated dressing vs. tulle	Self-funded	The percentage of ulcers healed in the honey group (61.3%) was significantly higher than in the control group (11.5%). There were significantly fewer toe amputations in the honey group (9.7%) compared with the control group (34.6%).	There was a high risk of selection and performance bias because randomization, concealment, and double blinding were not reported. Inclusion and exclusion criteria were not clearly stated. Episodes of antibiotic use and any adverse events were not reported and compared between groups.
Kamaratos et al., 2014	RCT	63	Manuka honey tulle vs. saline-soaked gauze	Self-funded	The two groups did not differ significantly in the percentage of ulcers healed at the16-week follow-up session. The mean healing time in the honey group of 31 ± 4 days was significant shorter than the 43 ± 3 days for the control group.	There was a high risk of detection bias because the blinding of the outcome assessors was not clearly reported. There was a high risk of selection bias because no true randomization was performed. Inclusion and exclusion criteria were not clearly reported. Episodes of antibiotic use and any adverse events were not reported and compared between groups.

2.12.5. Antibacterial effect of Ag/nAg and MH dressings on DFU

In this section, we review the studies conducted in the past 10 years comparing the antibacterial effects (Table 2.7) of silver and honey. Nasir et al. (2012) conducted an in vitro study to compare the antibacterial effect of hydrofiber silver (aquacel Ag) and hydrofiber soaked with MH. They found that that hydrofiber Ag had a greater zone of inhibition (ZOI) than MH for Gram-negative bacteria, but no statistical test was performed for the comparison. Guthrie et al. (2014) obtained a similar result. They carried out a study on a mice traumatic model contaminated with S. aureus, and revealed that the nAg group had statistically significantly lower bacterial counts than the MH group. However, Bradshaw (2011) conducted an in vitro study aiming to compare iodine, MH and different commercial brands of Ag wound dressings, and obtained a contradictory result that was not significantly different in ZOI among the three groups. Nevertheless, a significant difference in ZOI was observed between different commercial brands of Ag dressings. There could be several reasons for these inconsistent findings. First, the nature of the dressing materials, as a carrier medium to hold the active ingredient of Ag or honey, might have affected the antibacterial activity. Second, the origins of the honey used in these studies differed. Third, the concentrations of Ag in different commercial brands varied.

No clinical study has been conducted to investigate the antibacterial effectiveness between Ag/nAg and MH on DFU. In addition, there has been no related clinical study to investigate the anti-inflammatory effect of these two categories of dressings on DFU.

Table 2.7. The comparison between antibacterial effects of silver and honey

Authors	Funding	Nature of studies	Major findings
		Antibacterial effect	
Nasir et al., 2010	University-funded	In vitro	Aquacel Ag (hydrofiber Ag) had a greater zone of inhibition than MH-soaked Aquacel in Gram-negative bacteria
Guthrie et al., 2014	Self-funded	Animal study	Acticoat (nAg dressing) can reduce the bacteria burden more effectively than MH in a heavily contaminated mice model
Bradshaw, 2011	University-funded	In vitro	There is no significant difference in antibacterial activity between honey and silver dressings, but there is a significant difference in the strength of activity among different brands of silver dressings
Lund-Nielsen et al., 2011a	Self-funded	In vivo	There is no significant difference in the qualitative bacteriology of malignant wounds between honey-coated and silver-coated dressings

2.13. Clinical evidence of the comparison between MH and nAg dressings on DFU healing

No clinical studies have been conducted to compare conventional, honey and silver dressings in different types of ulcers including DFU. In addition, clinical studies comparing the clinical effectiveness of honey and silver have been very limited. The literature from the past 10 years comparing silver and honey were reviewed. Three RCTs (Malik et al., 2010; Baghel et al., 2009; Lund-Nielsen et al., 2011b) and one retrospective study (Gupta et al., 2011) were published on their effects on burns and malignant wounds. The studies investigated the effect of different types of honey in comparison with silver sulphadiazine on burn wounds; these studies are therefore excluded from analysis in this review (Malik et al., 2010; Baghel et al., 2009; Gupta et al., 2011). All three studies found that

different types of honey were more effective than silver sulfadiazine in burn wounds. Further details of the results are given in the discussion chapter. Only one RCT was published on the use of nAg against MH dressings on malignant wounds (Lund-Nielsen et al., 2011b). It compared the application of MH and nAg on malignant wounds in 75 patients and found no differences between MH and nAg in wound healing, cleanliness, mal-odor control, and wound pain. The design of this study clearly reported the inclusion and exclusion criteria, randomization procedure, and allocation concealment. Baseline demographic data between the groups were compared prior to the analysis. However, this study still contained some methodological flaws. There was no blinding of outcome assessors, and a per-protocol analysis was used instead of an intention-to-treat analysis. In addition, this study did not report the confounding factors on the use of antibiotics and adverse events that affected wound healing. The study was subject to a high risk of detection and attrition bias. Importantly, no study has been published that compares nAg and MH in DFU.

2.14. Conclusion on evidence: research gap identification

Based on the above literature review, we identified a gap in the knowledge on the effectiveness of nAg/Ag dressing on DFU. Importantly, nAg is a relatively new product of dressing that has a more potent and unique antibacterial effect from the basic science point of view when compared with Ag. nAg also has well-documented unique anti-inflammatory effects as equated with Ag. As regards honey, MH dressing is the most popular and has a relatively stronger evidence

base compared with other types of honey dressings (Kamarato et al. 2014; Al Saeed 2013).

Three additional research gaps were recognized. Firstly, there was no clinical evidence for nAg dressing on DFU. Also, MH dressing has more clinical evidence as compared with nAg dressing on DFU healing although the bias was identified in some previous studies testing the effect of MH. Secondly, there were no studies comparing nAg and MH on DFU in terms of the healing effect and using anti-inflammatory biomarkers as an outcome in measuring wound healing. Lastly, a pilot study to test the feasibility and acceptance of nAg is also not available for us to consider the possibility of carrying out a study testing the effect of nAg on DFU healing.

2.15. Summary

This chapter reviewed all aspects related to DFU and its healing. Firstly, the different phases of the acute normal healing process were discussed. Secondly, the macroscopic pathway of the developing DFU was illustrated. Thirdly, the molecular alternation of DFU, its classification and specific assessment were reviewed, and the factors affecting DFU healing and the standard treatment for DFU were identified. Fourthly, the commonly used topical dressing materials for DFU, nAg and MH, were comprehensively reviewed. The evidence for nAg and MH in previous studies was reviewed and criticized. Lastly, the research gap was identified to justify the significance of the present study.

The research student published a manuscript in the international journal "Evidence-based Complementary and Alternative Medicine" (Tsang et al. 2015). The full text of this manuscript is attached in Appendix 1. It covers the discussion of the sections 2.4 "alternation of healing in DFU", 2.11 "action of nAg and MH dressing", 2.12.2 "effect of Ag or nAg-impregnated dressings on DFU", 2.12.4 "effect of honey related dressing on DFU", 2.12.5 "antibacterial effect of Ag/ nAg and MH dressing on DFU" and 2.13 "clinical evidence of the comparison between MH and nAg" in this chapter.

Chapter 3 – Conceptual framework

3.1. Introduction

There are two main parts in this chapter. The first part is to explain how the conceptual framework to be developed to guide the study, testing the preliminary effect of nAg. Besides, the corresponding aim and objectives for the preliminary effect were highlighted. In the second part, the aim and objectives of the feasibility of the study protocol and acceptability of the interventions in this pilot study were discussed. Lastly the significance of the pilot study is revealed.

3.2. Development of the conceptual framework

The conceptual framework was developed based on the literature review, which was thoroughly discussed in Chapter 2. This framework was used to guide on testing the preliminary effect of nAg.

3.2.1. DFU healing

Wound healing is considered as the granulation tissue to mature into a scar and complete wound closure with re-epithelialization. When a wound is in the healing process, the wound size is decreasing. In addition, there is reduced inflammation continuously as evidence by the down-regulation of MMPs and pro-inflammatory cytokines in wound fluid (Lobmann et al. 2002; Chan et al. 2012). At the same time, the infection status is also minimized as decreasing

bacteriology and clinical signs of infection (Fonder et al., 2008; Plummer & Albert, 2008).

Ulcer healing, inflammation and infection are closely associated. "Ulcer healing" is the direct clinical parameter for measuring DFU healing, and it can be indicated by complete wound healing and decrease in ulcer size. "Inflammation" and "Infection" are two obstacles to hinder DFU healing. If the inflammation and infection are well controlled, DFU healing likely occurs. Since there is prolonged inflammation in non-healing DFU (Muller et al., 2008; Chan et al., 2012; Van den Berg et al., 2008), the inflammatory status of DFU is an indicator of DFU healing. Inflammatory status is associated with the concentration of biomarkers in wound fluid including "total protein" and the inflammatory markers "MMP-9", "TNF-α" and "IL-1α" in wound fluid (Muller et al., 2008; Lobmann et al., 2005). Infection status is associated with the number and types of bacteria as well as the clinical signs of infection including local swelling, erythema, local tenderness, local warmth and purulent discharge as well as the systematic inflammatory response (Lipsky et al., 2012a).

3.2.2 Identification of parameters for DFU healing

Based on the above discussions and the literature review of Chapter two, eight parameters of DFU healing were identified as follows (Table 3.1).

Table 3.1. The parameters of DFU healing

DFU healing	
Ulcer healing	• Proportion of complete healing • Percentage of change in ulcer size
Inflammation	Wound fluid concentration • Total protein • MMP-9 • TNF-α • IL-1α
Infection	• Bacteriology (types of micro-organisms) • Severity of wound infection

3.2.3. Interventions

According to the published studies on DFU management, as discussed in the literature review, patient education on self-care management in DM and DFU, debridement, off-loading of the local ulcer and related ulcer care are effective in DFU healing. The application of topical dressing is one of the important approaches used in local ulcer care. nAg and MH dressings are now used more often in clinical practice for DFU healing. They are well documented to have the anti-inflammatory and antibacterial effect (Aparna Mani et al., 2015; Tomblin et al., 2014; Pandian et al., 2010; Kato et al., 2012). On the contrary, conventional dressing "paraffin tulle" does not have these effects. However, the effect of nAg on DFU healing is unknown and no comparison between the effects of nAg and MH on DFU healing is established, which are the gaps identified in Chapter two.

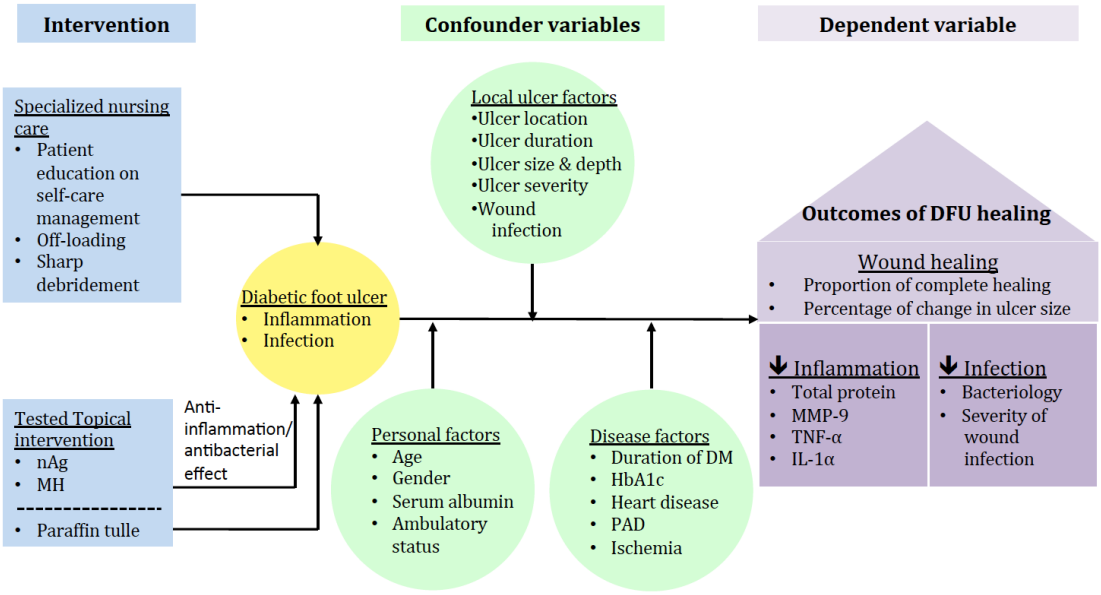
3.2.4. Risk factors affect DFU healing

As mentioned in Chapter 2, the factors affecting DFU healing can be categorized into personal, disease and local ulcer factors. Among the personal factors, advanced age, male gender, lower serum albumin level and lower ambulatory status are obstacles to DFU healing (Prompers et al., 2008; Pickwell et al., 2013; Zhang et al., 2013; Marston, 2006; Leung et al., 2001). As regards the disease factors, diabetes control including longer duration of diabetes and higher HbA1c level, as well as co-morbidities including heart disease, PAD, ischemia and renal failure decreased the probability of DFU healing (Marston, 2006; Gershater et al., 2009; Prompers et al., 2008; Pickwell et al., 2013; Ince et al., 2007; Leung et al., 2001). Finally, in terms of local ulcer factors, the distal region of the ulcer, the larger the ulcer size and depth, longer duration of the ulcer and ulcer infection increased the difficulty of ulcer healing (Prompers et al., 2008; Pickwell et al., 2013; Gershater et al., 2009; Marston, 2006; Margolis et al., 2002; Leung et al., 2001). These factors are summarized in Table 2.2 of chapter 2. All these factors influencing the healing of DFU through different pathways have been thoroughly discussed in the literature review chapter.

3.3. Introduction of the conceptual framework

Based on the above discussions, which were retrieved from part of the literature review discussed in Chapter two, the conceptual framework to guide the pilot study testing the preliminary effect of nAg was developed (Figure 3.1).

Figure 3.1. Conceptual framework



nAg and MH topical dressings, which have the antibacterial and anti-inflammatory effect, are generally use in DFU since inflammation and infection on DFU are not uncommon. With the combination of interventions containing topical dressings and specialized nursing care (patient education for self-care management, off-loading of ulcers and sharp debridement), they decrease the inflammatory and infectious status of DFU and eventually promote the healing in DFU. The DFU healing is indicated by the parameters of wound healing (proportion of complete ulcer healing and percentage of change in ulcer size), decreased inflammation (increased concentration of total protein and decreased MMP-9, TNF-α, IL-1α in the wound fluid) and decreased infection (bacteriology and severity of wound infection). However, several factors, which include personal, disease and local ulcer factors, affect the DFU healing outcomes. It is proposed in the framework that the specialized nursing care and each type of

dressing material contribute to DFU healing and decrease inflammation and inflection status of DFU, but the personal, disease and local ulcer factors as confounders affect DFU healing outcomes

3.4. Study aim and objectives on the preliminary effect of nAg on DFU healing

3.4.1. Aim on the preliminary effect

According to the above conceptual framework, the aim and objectives regarding the preliminary effect of nAg were identified. The aim was to test the preliminary effect of nAg against MH and conventional dressings on healing DM foot ulcer. The objectives addressing the aim were following.

3.4.2. Objectives on the preliminary effect

According to the literature review, the level of evidence for MH dressing was relatively higher, thus it was the comparison group in this study. The nAg dressing group was the intervention group. The conventional dressing (paraffin tulle) was the control group. In order to address the study aim, there were three categories of objectives including ulcer healing, inflammation and infection categories. They were listed as below. Since the ultimate goal of the effectiveness of topical dressing was "ulcer healing", it was the primary outcome. "Inflammation" and "infection" were the secondary outcomes.

3.4.2.1. *Ulcer healing*

- To examine the differences of the three groups using nAg, MH and conventional dressings on DFU in **the proportion of complete healing** after the completion of the interventions
- To examine the differences of the three groups using nAg, MH and conventional dressings on DFU in **ulcer size reduction** after the completion of the interventions

3.4.2.2. *Inflammation*

- To test the differences in concentration of **total protein** in wound fluid among the three groups using nAg, MH and conventional dressings on DFU after the completion of the interventions
- To examine the differences in concentration of **matrix metalloproteinase (MMP)-9** in wound fluid among the three groups using nAg, MH and conventional dressings on DFU after the completion of the interventions
- To examine the differences in concentration of **tumor necrosis factor-alpha (TNF-α)** in wound fluid among the three groups using nAg, MH and conventional dressings on DFU after the completion of the interventions
- To examine the differences in the concentration of **interleukin-1 alpha (IL-1α)** in wound fluid among the three groups using nAg, MH and conventional dressings on DFU after the completion of the interventions

3.4.2.3. *Infection*

- To examine the differences in **bacteriology** among the three groups using nAg, MH and conventional dressings on DFU after the completion of the interventions
- To examine the difference in **severity of wound infection** among the three groups using nAg, MH and conventional dressings on DFU after the completion of the interventions

3.4.3. Hypotheses on the preliminary effect of nAg

Three groups of alternative hypotheses were noted below.

3.4.3.1. *Ulcer healing*

- The nAg dressing group has a significant higher proportion of complete ulcer healing of DFU than the MH and conventional dressing groups after the completion of the interventions.
- The nAg dressing group has a significant greater size reduction of DFU than the MH and conventional dressing groups after the completion of the interventions.

3.4.3.2. *Inflammation*

- The nAg dressing group has a significant increase in the level of total protein than the MH and conventional dressing groups after the completion of the interventions.
- The nAg dressing group has a significant decrease in the level of MMP-9

than the MH and conventional dressing groups after the completion of the interventions.

- The nAg dressing group has a significant decrease in the level of TNF-α than the MH and conventional dressing groups after the completion of the interventions.
- The nAg dressing group has a significant decrease in the level of IL-1α than the MH and conventional dressing groups after the completion of the interventions.

3.4.3.3. *Infection*

- The nAg dressing group has a significant decrease in bacteriology than the MH and conventional dressing groups after the completion of the interventions.
- The nAg dressing group has a significant decrease in the severity of wound infection than the MH and conventional dressing groups after the completion of the interventions.

3.5. Feasibility and acceptability of the study

A pilot study is used to provide experience in the running of the trial and to explore any problems to that they can be corrected before the main study begins (Whitehead et al. 2014). In general, a pilot study should address all elements of the planned trial and ensure that the study is feasible and will be conducted in a manner that reduces threats to study validity (Tickle-Degnen, 2013). By carrying out the study protocol to test the preliminary effect, the ultimate goal of this pilot

was to know how feasible of the study protocol and the intervention acceptance of participants.

3.5.1. Study aim and objectives on feasibility and acceptability

3.5.1.1. *Aims on feasibility and acceptability*

The **primary** aims of this pilot study focused on the processes of running a main study. The aims were:

- to investigate the feasibility of the trial protocol
- to test the acceptability of the interventions

3.5.1.2. *Objectives on feasibility and acceptability*

Feasibility

- To assess the feasibility of the process to recruit participants (referral, screening/ enrollment, selection criteria, recruitment, multi-center approach, randomization and blinding)
- To explore the feasibility of interventions (clinical visits, education, off-loading, debridement and topical dressings)
- To explore the feasibility of outcome measures

Acceptability

- To identify the reasons for study attrition and evaluate the participants' acceptability of the interventions

- To evaluate the participants' adherence to the interventions

3.6. Significance of study

The present pilot study is the first trial to test the feasibility, acceptability, preliminary effect of nAg on DFU, against MH and conventional dressings. Through this pilot study, we would identify the strength and weakness of the study design and the study protocol for improvement in the main study. Its findings would potentially contribute to the following.

3.6.1. Further study preparation

Through the feasibility test of recruitment, we can identify whether there was any methodology flaw and hence improvement in the ways for participants' recruitment in the future main study. By exploring the feasibility of the pilot study design, we could assess the whole study process so that the study protocol, logistic and resources could be reviewed. It may be considered to make necessary changes in the main study. In addition, by testing the feasibility of outcome measures, we could examine the appropriateness of the outcome selection as well as the instrument used in the measurement of those study outcomes. Moreover, it also explored the acceptability of the interventions by participants, referrals by clinicians and intervention providers, including student investigators, nurses in the GOPD as well as community nurses. The findings let us know how to modify the interventions to minimize the barriers of the

participants to participate in this study and increase intervention adherence of the participants and minimize the attrition rate.

Apart from the future main study, the preliminary effect of nAg dressing against MH dressing and traditional dressing could contribute to other similar wound management studies. With the combination of clinical and laboratory findings as the study outcomes, the scope of nursing research on wound management is extended. The triangulation of both clinical and laboratory data used in this pilot study can become a reference for conducting similar wound management studies in the future. Since the previous scope of the nursing research on wound care focused mainly on the clinical outcome on the topical dressing and the social aspect of patients, the preliminary result of both clinical and laboratory data could end up in other similar wound care researches. It may potentially act as a new direction of nursing research on wound care. In addition, the potential positive preliminary effect on DFU healing could likely increase motivation, commitment and confidence of researchers to conduct further similar studies.

3.6.2. Potential development of evidence-based clinical practice on the use of topical dressings

This is the first pilot trial for the preliminary effect of nAg dressing on DFU healing. Although it is a pilot study with small sample size, it would potentially provide evidence-based clinical practice to professional healthcare providers including nurses for their reference in the selection and application of topical dressing materials. Through the clinical application of this preliminary finding of

nAg from this pilot study by nurses, it would potentially constitute the new practical knowledge to underpin the clinical nursing practice on wound management in DFU.

3.6.3. Contribution to nursing profession

Nursing research has a tremendous influence on current and future professional nursing practice (Tingen et al. 2009). Selection and application of topical dressings are one of the important aspects in wound care management for nursing profession. The potential positive preliminary evidence would let nurses take it as a reference for improvement of nursing knowledge, practice, education and research on wound management especially in DFU.

3.7. Summary

Based on the literature review, the conceptual framework was developed to guide this study testing the preliminary effect of nAg on DFU healing. All the objectives on the three aspects “feasibility”, “acceptability” and “preliminary effect” were identified. Those hypotheses related to testing the “preliminary effect” on the clinical performance as well as biochemical markers related to DFU healing were revealed. Finally, the significance of this pilot study including future study preparation, evidence-based clinical practice and contribution to nursing were discussed.

Chapter 4 – Methodology

4.1. Introduction

This chapter is consisted of two parts. In the first part, it is related to the methodology of the preliminary effect. The trial design, participants, interventions, outcomes, sample size, randomization with sequence generation, allocation concealment mechanism and implementation, blinding as well as statistical methods are all developed according to the CONSORT guideline (Schulz et al., 2010). The second part is the main aim of this pilot study which is the illustration of the methodology regarding the feasibility and acceptability tests. In order to let readers know the details of the study design and the protocol for better understanding of the feasibility and acceptability test, the methodology of the preliminary test on the nAg effect was discussed first. Then, the feasibility and acceptability are illustrated afterwards. Lastly, the safety evaluation and ethical considerations are also discussed.

PART I - Methodology: the test on the preliminary effect of nAg

4.2. Study design

To address the research gaps identified, nAg, and MH dressings, which have antibacterial and anti-inflammatory effect, as well as paraffin tulle are the topical

dressings together with the specialized nursing care (patient education for self-care management, off-loading of ulcers and sharp debridement) as the study interventions to three different groups of patients with DFU having inflammation and/or infection.

An open-labeled, three parallel groups, balanced randomization (1:1:1), multi-center design was adopted in this randomized control trail. This study was registered on ClinicalTrial.gov with registration number NCT025779.

4.2.1. Level of evidence and choice of design

This pilot study adopted RCT because it is the strongest level of evidence below systematic review in research design for studies examining treatment interventions. RCT is the most scientifically rigorous method of hypothesis testing and is regarded as the gold standard trial for evaluating the effectiveness of interventions (Akobeng, 2005). It can enhance researcher understanding of the connection between individual characteristics and their respective treatment responses (Velengtas et al. 2012). The strength of the RCT rests on its excellent internal validity, which is based largely on the power of randomization to ensure that the only difference between treatment arms that is the exposure of the treatment of interest (Booth and Tannock, 2014).

In an RCT, investigator needs to demonstrate that the intervention/treatment works, which frequently requires that the study be performed in a well-defined population. Besides, an RCT is designed to answer a single question or small number of questions about treatment efficacy that are usually narrow in scope (Ho et al. 2008). In this pilot study, we have a well-defined population (patients with DFU) and focused scope of study aim (the effectiveness of topical nAg dressing). Therefore, the RCT design was the most appropriate design used in this study.

4.2.2. Selection of comparison and control groups

An RCT is a type of study in which participants are randomly assigned to one of two or more clinical intervention. A three-group design was used in the present RCT. The advantage of using three groups design is to determine the efficacy of the intervention treatment against the relatively well-established treatment and the standard conventional care. The primary purpose of the control groups is to control for threats to internal validity. The control group is to receive the usual treatment and their outcome is compared with that of the intervention group (Levin, 2007). On the other hand, the goal of an active-control trial is to determine whether the new intervention is more effective than the current one. The active comparator receives a currently accepted intervention in an active-control arm (Resnik, 2008). For this kind of study, the active comparator can determine whether an experimental intervention is superior or inferior to some other form of treatment (Freedland et al. 2011).

As the research gaps and the strength of evidence identified in chapter two, the selection of intervention group, comparison and control groups were nAg, MH and paraffin tulle respectively. It was because the research gaps were identified as no evidence of nAg dressing group on DFU, no research evidence on comparing nAg and MH dressing on DFU. Besides, MH group had relatively stronger evidence to support its effect. In addition, dressing with conventional dressing (paraffin tulle) was the classic and conventional care for wound care including DFU.

4.2.3. Multi-center approach

Importantly, RCT has a well known limitation is time-consuming and difficult to recruit sufficiency patients in clinical study (Ho et al. 2008). Therefore, a multi-center approach was adopted in order to recruit enough subjects in a short period.

4.3. Participants

Convenience sampling was adopted and a convenience sample recruited in the present study.

4.3.1. Inclusion criteria for selection of participants

a. History of type 2 diabetes mellitus and

b. Living in the community and

c. Age 40 or over with foot ulcer and

d. Ulcer size equal to or larger than 1 cm in diameter and

e. Ulcer located at or below malleolar region of foot and

f. Superficial ulcer, ulcer penetrating to tendon or capsule and

g. Ulcer without infection, mild and moderate infection and

h. No foreseeable surgery within the 12-week study period

Type 2 diabetes accounts for 90-95% of diabetes (American Diabetes Association, 2004). The major type of DFU is within this population, therefore it was selected as the target population. Age being a risk factor for healing DFU, most patients with DFU are over 40 (Prompers et al., 2008). The size of the ulcer is another risk factor affecting diabetic healing. Ulcer size is negatively correlated with the time of ulcer healing (Pickwell et al., 2013). In addition, when an ulcer is too small, the discrepancy of ulcer measurement is higher using the line tracing method (see section 4.6) and it is also too difficult to collect wound fluid.

As the location and condition of the ulcer are factors associated with DFU healing, patients with the above criteria were recruited in order to decrease the discrepancy among groups. In order to ensure the participants could remain involved throughout the 12-week study period, they were only recruited when there was no foreseeable surgery within the 12-week period.

4.3.2. Exclusion criteria for selection of participants

a. HbA1c level ≥ 10% or

b. Severe ischemia with ABI ≤ 0.4 or

c. Renal failure or

d. Severe ulcer infection or

e. Ulcer deep into bone and joint or

f. Osteomyelitis or

g. Known case of venous ulcer or varicose vein or

h. Known case of benign or malignant tumor or

i. Known to have any auto-immune disease or

j. Known allergy to MH/ nAg or

k. Participation in other experimental treatment studies

The control of diabetes is one of the key factors in DFU healing. Poor diabetic control will affect ulcer healing (Marston, 2006). According to a report by the Health Resources and Services Administration of the United States Department of Health and Human Services (2012), poor glycemic control was defined as HbA1c more than 9%. In addition, for patients with HbA1c more than 10%, the average blood glucose was higher than 13 mmol/l and categorized as indicating very poor glycemic control (Diabetic Multidisciplinary Team, 2009). In order to minimize the factors hindering DFU healing, participants with HbA1c levels higher than 10% were excluded from the present study.

Margolis et al. (2008) found that PAD was highly associated with lower limb amputation in patients with DFU (HR 7.71; 95% CI 5.29-1.26). A low ABI value, which indicates poor distal circulation and a high risk of PAD, resulted in a poor wound healing prognosis. Therefore, patients with ABI ≤ 0.4 were excluded from the study. Renal failure also affects ulcer healing since the small vessels are severely impaired (Gershater et al., 2009). Patients with a history of renal failure,

including those receiving peritoneal dialysis and hemodialysis, were also excluded.

When DFU is accompanied by severe infection, patients usually require admission for intravenous antibiotics (Lipsky et al., 2012b). According to the published literature, the exposed bone in the wound bed is susceptible to osteomyelitis (Dinh et al., 2008). Intravenous antibiotics and debridement are the appropriate management for the condition (Lipsky et al., 2012b), rather than antibacterial topical dressing. Therefore, DFUs with severe infection, exposed bone and osteomyelitis were beyond the scope and contra-indicated for the topical application of dressing materials. They were excluded from the present study.

In addition, co-morbidities including venous ulcer, tumor and autoimmune disease would affect the healing of DFU, and patients with a history of allergy to honey or silver were not suitable subjects for the topical dressings. Further, if the patients were participating in other studies, this might be contraindicated to the tested interventions or logistical arrangements in this study. Therefore, any of these factors resulted in exclusion from this study.

4.4. Settings

Patients who met the inclusion and exclusion criteria were recruited from the Orthopedics and Traumatology (O&T) wards and the specialty outpatient department (SOPD) of O&T in Hospital A, O&T wards of Hospital B as well as a

general outpatient department (GOPD). All of these departments are under the management of the Hospital Authority in Hong Kong. Hospitals A and B are the two regional hospitals in Kowloon and the New Territories respectively. Their O&T departments receive patients with acute orthopedic problems, including diabetic ulcers, from their own regions. The O&T SOPD in Hospital A followed up patients after discharge and also received new referrals from primary health care settings. In addition, the GOPD provided community care services to its local region, including a family medicine clinic, an integrated nurse and allied health clinic, a podiatry clinic and a wound dressing clinic.

4.5. Participant recruitment

4.5.1. Referral mechanism

In order to ensure the sufficient number of participants, the potential participants were approached in a number of ways. Among the hospitalized patients of Hospital A, potential participants were either recruited directly by the student investigator or referred by the O&T surgeon. Some potential participants in Hospital A were recruited by the student investigator once they had been discharged from Hospital A, if they agreed to participate in the study. Other potential participants in the O&T SOPD of Hospital A were referred by O&T surgeons to the student investigator during the follow-up in the O&T SOPD. Among the hospitalized patients in Hospital B, the O&T nurse specialist referred potential participants to the student investigator directly once they had been discharged from hospital B. The student investigators also went to the GOPD

weekly and recruited potential participants directly while they were attending the wound dressing clinic.

4.5.2. Recruitment procedure

Before recruitment of the potential subjects, they were screened by the student investigator in the pre-study visit to the O&T nurse clinic visit of hospital A, to confirm their eligibility to participate in this study.

- During the pre-study visit, the student investigator explained all the study details to potential participants, as well as the reason for the screening. The content of the explanation was according to the explanation notes either in Chinese (appendix 2) or English (appendix 3).
- After their agreement to accept the screening, participants were screened for the inclusion and exclusion criteria using the clinical screening form (appendix 4) as well as the screening tests.
- The screening tests included:
    - Measure body temperature, blood pressure and pulse
    - Take blood for HbA1c and serum albumin if there were no latent 3-month blood results
        - Assess the ulcer severity and size measurement by SEWSS (appendix 5)
        - ABI measurement (appendix 6)
- Participants with HbA1c ≥ 10% or ABI ≤ 0.4 were triaged to routine care.
- The informed written consent in Chinese (appendix 7) or English (appendix 8) of participants who met all the inclusion criteria and none of the exclusion criteria were obtained before the nurse clinic appointments were

arranged and before the commencement of interventions.

4.5.3. Criteria for withdrawal of participants

- The participants were withdrawn from the trial if they met any one of the following criteria:
  - Non-compliance with the prescribed dressing method for 2 consecutive visits. For example, participants changed the prescribed dressing method on their own.
  - More than one absence from the scheduled follow-up
  - Severe adverse events, including undergoing an operation during the trial period
  - Allergy to the treatment products after using them
  - Verbal decision to terminate the treatment

4.5.4. Sample size estimation

The main objective of pilot study is to test the practicability of the study. A pilot study is used to evaluate the feasibility and acceptability of the study design, study protocol and interventions. As it is the pilot study, the estimation of sample size for the main study is not applicable here.

Julious (2005) recommended that the sample size of pilot study was 12 per group. Hertzog (2008) suggested that the sample size of pilot study should range from 10 to 40. However, from the clinical experience of the student investigator, it is quite hard to find eligible participants. Besides, the main study aim is the

feasibility and acceptability test in this pilot study. Therefore, 10 per group were targeted in this pilot study and the total number of 30 participants was planned.

4.6. Interventions

The interventions are specialized nursing care and application of topical dressings, which are common care management to Hong Kong patients with DFU in this study. In specialized nursing care, it includes patient education on self-care management in diabetes control and diabetic foot, off-loading of the local ulcer area, combating infection and debridement. After the specialized nursing care, the student investigator applied the nAg, MH or paraffin tulle dressings to the DFUs of the participants in the experimental, comparison and control groups respectively.

4.6.1. Interventions: participants' clinic visits

All participants were scheduled for eight visits to the O&T nurse clinic of Hospital A. They attended the nurse clinic for follow-up by the student investigator weekly for the first four weeks and then biweekly until week 12 in the follow-up period. In addition, he checked the participants' vital signs to confirm that their health status in each clinic visit. Research assistant A monitored their adverse events (AEs). AEs included any event that affected wound healing, such as wound infection, taking oral antibiotics, any operation or any event that required hospitalization. The student investigator also performed the following interventions in the clinic visits.

4.6.1.1. *Patient education on diabetic control and foot care*

Patient education on the diabetic control and foot care is the crucial element on DFU healing (Ren et al. 2014). In the first clinic visit, the student investigator would perform patient education on self-care management in diabetic control and diabetic foot care for all participants by using the standard education booklets produced by the Department of Health. The importance of diabetic drug and diet compliance was reinforced for all participants. They were also educated on shoes, as well as foot and nail care. Their understanding was checked after the individual teaching session and any queries were clarified at the first clinic visit. In the subsequent visit, the student investigator monitored their compliance with the drug and diet protocol and responded to their queries regarding DM, DFU and self-management issues before the study interventions.

4.6.1.2. *Off-loading*

Off-loading is important for DFU healing since direct pressure on ulcer wound compromise ulcer healing (Brem et al., 2004). According to the literature review in chapter two, customer-made insole (CMI) was used in this study. A colleague with expertise in orthotics was consulted on making standard CMI so that the student investigator could provide standard off-loading insoles to participants who needed them, after their enrollment in the study. This arrangement was to ensure that all the participants were receiving sufficient pressure relief over the local ulcer during the study period. In order to improve patient compliance with using CMI in the study, their compliance was monitored and reinforced at every clinic visit. If there was any improper function of off-loading, the orthotics

experts were consulted again for amendment of the footwear. Stick and heel walking method were also educated to participants for those with forefoot plantar ulcer. The student investigator also performed callus debridement by scalpel at every clinic visit whenever necessary, especially on the peri-ulcer region, so as to decrease the pressure created by callus.

4.6.1.3. *Combating infection and sharp debridement*

In order to decrease the severity and chance of bacterial infection, the student investigator also showed all participants on how to clean the affected foot before having it dressed by nurses in the community. Demonstration by the student investigator and return demonstration by participants was performed at the first clinic visit. In order to detect any wound infection in the participants, research assistant A assessed the signs and symptoms of local and systemic infection at every clinical visit.

If the participants had a moderately infected DFU according to the clinical signs of infection, they were referred to their medical colleagues for oral antibiotics. Therefore, research assistant A reviewed the severity of infection in order to identify the infection status and oral antibiotic prescription of all participants at each clinical visit. If the infection status was severe, they were referred for admission to hospital for intravenous antibiotic injection, and their participation in the study was terminated. The standard operation procedure for handling wound infection is given in appendix 9.

Sharp debridement is the gold standard for the management of non-viable tissue in DFU (International Best Practice Guidelines, 2013). So, sharp debridement is one of our important components of specified nursing care. The student investigator cleansed the ulcer and debrided the biofilm and non-viable tissue by scalpel if necessary after the verbal consent of participants at every clinic visit, in order to decrease the bacterial loading on the surface of the ulcer. The student investigator who is a nurse consultant is competent to perform debridement on all participants in this study. The student investigator also stimulated all the exposed tendons and avascular tissue by needle or blade until it bled. This intervention was used as cellular recruitment to the local area for angiogenesis and granulation formation. If there was a need to perform sharp debridement for the non-viable tissue or stimulate the vascularity of the avascular tissue, the student investigator performed this procedure every time the patient attended the clinic. When the participants experienced pain on the first time of sharp debridement procedure, the student investigator would advise them to take analgesic one-hour before the procedure. The pain level of participants would be reassessed again on the subsequent sharp debridement procedure.

4.6.1.4. *Topical dressing application*

The student investigator prescribed the specific dressing materials to the participants according to their group allocation. There are two types of nAg dressing available in the market, namely type A and B. nAg dressing of type A had higher concentration than type B. The evidence showed that type A was more effective in killing P. aeruginosa (Margeret et al., 2006) and also higher

efficiency in ulcer healing (Lee et al., 2010). Therefore, the nAg dressing of type A was used in this study.

At every clinic visit, the student investigator used soap and tap water to clean the whole affected foot of each participant first in the nurse clinic of hospital A. Then, he performed the dressing change by cleansing the ulcer with sterile water under aseptic technique. Finally, the nAg, MH and paraffin tulle dressings were applied to the ulcers according to the group allocation by the student investigator in each visit. Apart from these clinic visits, each participant was required to go to the GOPD for GOPD nurses or community nurses to clean the ulcer and apply the specific dressing material daily. A prescription in which a specific type of dressing material noted was given to each participant after each clinic visit for the participant to give it to the GOPD nurse or community nurse for action. The details of the study protocol on the first visit and subsequent visits were shown in appendix 10.

4.6.1.5 *Monitoring participants' compliance*

During every clinic visit, the student investigator monitored participants' compliance with the diet control, foot care, off-loading and dressing regimen. At every clinic visit, the student investigator asked all participants and their carers for details of their daily DFU care. If non-compliance on diet control, foot care, off-loading (including the use of CMI and technique) and the prescribed specific dressing materials was discovered, the investigator explored the obstacles and helped them to solve the problem. At the same time, they were reminded that they would be withdrawn from the study if non-compliance was observed at two

consecutive visits. Community nurses from the CNS or nurses from the general outpatient department (GOPD) monitored their compliance when the participants received their wound dressing.

4.7. Study outcomes

There were totally eight outcomes for testing the preliminary effect in this study. They were further divided into three categories, namely ulcer healing, inflammation and infection. "Ulcer healing" included "proportion of complete healing" and "percentage of change in ulcer size". "Inflammation" comprised the concentration of biomarkers, including "total protein", "MMP-9", "TNF-α" and "IL-1α" in wound fluid. "Infection" included "bacteriology" and "clinical signs of wound infection". Among all the outcomes, the "proportion of complete ulcer healing" was the primary outcome. The others were all secondary outcomes. The outcome record form (appendix 11) was designed by the student investigator to record the results of the following outcomes.

4.7.1. Proportion of complete ulcer healing

Complete ulcer healing, defined as complete wound closure by scarring tissue without any observable raw areas or areas covered by crust. Research assistant A, an experienced registered nurse, assessed whether the ulcer was completely healed at each clinic visit. These were held in the 1st, 2nd, 3rd, 4th, 6th, 8th, 10th, and 12th weeks of this study, corresponding to the time points of data collection at T_0, T_1, T_2, T_3, T_4, T_5, T_6, and T_7 respectively. When the ulcer was healed after assessment by research assistant A, she took a clinical photo of it. The photo

would then be passed to research assistant B, who was an advanced practice nurse and experienced in wound management, to verify complete wound healing status. If no consensus could be reached between research assistants A and B regarding complete wound healing, an appointment was arranged with the participant in order to enable research assistants A and B to assess the ulcer together, to confirm whether it was completely healed.

4.7.2. Ulcer size reduction

A digital wound measurement device produced by a wound product company was used to measure the wound dimensions in terms of length, width and area. This digital tablet can convert a line tracing into a true area measurement. The detailed operation procedure for this digital wound measurement device is given in appendix 12. While the ulcer was being cleansed and debrided by the student investigator, research assistant A waited outside the nurse clinic. After the cleansing and debridement procedure, research assistant A would be called into the clinic to measure the wound size. She used the same device to measure the size of the ulcer at every clinic visit (T_0, T_1, T_2, T_3, T_4, T_5, T_6, and T_7), and recorded the length, width and area of the ulcer in the outcome record form.

4.7.3. Concentration of biochemical markers

The biochemical markers included total protein, MMP-9, TNF-α and IL-1α. After the ulcers were rinsed with normal saline, the student investigator collected the wound fluid from the ulcers of all participants in the clinical visits to assess the concentration of each marker. A standard operation procedure for wound fluid collection was established (Appendix 13).

After the collection of wound fluid, the student investigator measured the biochemical markers. All the samples were placed in an ice bag and transferred to the biochemical laboratory in order to measure the concentration of all biomarkers. The protein content for all sample wound fluid was quantified and standardized using the bovine serum albumin standard. A standard operating procedure for the measurement of total protein concentration inside the wound fluid was developed, as shown in appendix 14. Inflammatory cytokines including TNF-α and IL-1α, as well as matrix metalloproteinase MMP-9, were measured using a commercial Enzyme-linked immunosorbent assay (ELISA) human kit according to the manufacturer's protocols (Abcam, USA).

ELISA was used as the standard measurement method for examining the biomarkers in the wound fluid (Leng et al., 2008). For MMP-9, the wound fluid was diluted 4000-fold before measurement. The minimum detectable dose was less than 10 pg/ml. For TNF-α and IL-1α, the wound fluid was diluted 400-fold before measurement. The minimum detectable doses of TNF-α and IL-1α were less than 8 pg/ml and 0.5 pg/ml respectively. The concentration of each biomarker was recorded by the student investigator in the outcome record form.

4.7.4. Bacteriology

As mentioned in section 2.9.4.2 of chapter 2, the most appropriate method for counting bacteriology is wound swab taking by Levine technique. The detailed standard operating procedure for taking wound swabs is shown in appendix 15. Using the Levine technique, the student investigator took wound swabs from the ulcers of all participants at every clinic visit (T_0, T_1, T_2, T_3, T_4, T_5, T_6, and T_7) after

cleansing the ulcers with normal saline. Then, the swabs were sent to the hospital laboratory for measurement of the resulting culture. The types and quantity of bacteria from the wound swab were recorded on the outcome record form.

4.7.5. Severity of wound infection

According to section 2.9.4.1 of chapter 2, the quantification of DFU infection was based on the "ISDA and IWGDF classification of diabetic foot infection" (Appendix 9). The classification was based on both local and systemic clinical signs of wound infection. The severity of the wound infection was categorized into uninfected, mild, moderate and severe. Research assistant A assessed the severity of infection in the DFU according to the classification at every clinic visit (T_0, T_1, T_2, T_3, T_4, T_5, T_6, and T_7).

4.8. Other study variables

Apart from outcome variables as mentioned in the previous section, there were baseline variables and variables for adverse event recorded in the instruments. Besides, the time for these data collection was discussed.

4.8.1. Baseline variables: demographics and risk factors

There were three groups of risk factors including personal, disease and ulcer risk factors. The details of the risk factors were shown in conceptual framework Figure 3.1 of Chapter three. All these risk factors were recorded in the risk record form (appendix 16), which was designed by the investigator. Its content

validity was 1.0 after reviewing by an orthopedic nurse specialist, a nurse consultant and a medical consultant. Research assistant A assessed all participants for these factors at the first clinical visit T_0.

Severity of ulcer, which was one of the local ulcer factors, was assessed by a scoring system "Saint Elain wound score system" (SEWSS). This scoring system (appendix 5) based on the anatomical location, factors contributing to wound healing (ischemia, infection, edema and neuropathy) and tissue involvement (depth, size and wound healing phase) calculates the score to classify into grades one, two and three to indicate the severity of DFU. The corresponding score of grade one, two and three were ≤ 10, 11-20 and 21-29 respectively. Higher scores implied a lower probability of wound healing. A score ≤ 10 meant that the DFU was likely to heal. A score of 11-20 suggested that the probability of wound healing depended on the wound management method. Scores higher than 21 implied that the DFU had poor healing potential (Martínez-De Jesús, 2010). Research assistant A charted the wound score during the first clinic visit at T_0 for the baseline comparison among three groups.

4.8.2. Variables for adverse event

Adverse effects and taking oral antibiotics, which could affect DFU healing, were identified using the event record form (Appendix 17). It was designed by the student investigator. Therefore, at each clinical visit, research assistant A, who was the experienced nurse, asked the participants whether they had experienced adverse events, or if they were taking oral antibiotics. The research assistant A then recorded the data in this form.

4.9. **Randomization**

4.9.1. Sequence generation

Block randomization was used in the present pilot study. The randomization plan was generated by an online internet randomization software (http://www.randomization.com). The present study involved three treatment groups. A block size of ten (i.e. 1 to 10) was used, and four blocks were generated with group numbers 1, 2 or 3. The advantage of using this block size was that the unpredictability and small inequalities among groups were preserved. The student investigator demonstrated to research assistant C how to use this online software and asked her to perform return demonstration to ensure she got it correctly. Firstly, the research assistant C numbered the 40 envelopes in sequence from 1 to 40. Then, she generated a sequence (40 numbers) of randomized group numbers (i.e. 1, 2 or 3) and printed these numbers onto small pieces of paper. She then put the randomized group numbers (1, 2 or 3) into the corresponding sequence of envelopes (1 to 10), numbered in chronological order. For example, she put group number 3 into the first opaque envelope and labeled it as number 1. Each envelope was sealed after being numbered from 1 to 10. Lastly, the numbered sealed envelopes in chronological order were passed to the student investigator for enrollment of participants.

4.9.2. Allocation concealment

When eligible participants were enrolled into the study, the student investigator opened the sealed envelope in front of each participant according to the sequence of enrollment (number of sequence, e.g. 1, 2, 3, 4, 5). He assigned

participants to interventions based on the randomized group number inside the opaque and sealed envelope. Throughout the process, the student investigator was unaware of the randomization plan and the allocation sequence.

4.10. Blinding

Research assistant A measured the size of the ulcer and other study variables including the wound score, the risk factors in the case record form, and the severity of ulcer infection, as well as adverse events and oral antibiotics in the event record form. Research assistant B confirmed the healing status of the ulcer. They were both blinded to group allocation and unaware of the three different types of topical dressing materials applied to the participants.

In order to make sure the research assistant A was blinded, research assistant A waited outside the clinic during each dressing change. The student investigator would cleanse the wound, perform thorough debridement and use the scalpel to remove any staining by topical dressings on the wound edge before research assistant A entered into the clinic. The research assistant A then measured the wound size and took a clinical photo in each clinic visit. When research assistant A found that the wound was completely healed during the assessment, she passed the electronic clinical photo to the research assistant B. Then, the research assistant B verified the healing status of the ulcer, so that she could be blinded as to the type of dressing material used. Both of the research assistant A and B were very experience and specialized in wound care.

Since the physical appearance and smell of the three types of dressing materials were different, participants could not be blinded in the research process. In addition, the student investigator performed all the interventions to all three groups so that it was not possible to blind. The community nurses and GOPD nurses were also unable to be blinded because they removed the old dressing and applied the new one, so that they knew which type of dressing was being used. Besides, the collection of wound fluid and the data collection of the concentration of biomarkers in the biochemical laboratory were highly specialized procedures. It was necessary for the student investigators, rather than the research assistants, to perform the procedures and data collection of biomarkers. Therefore, it was not possible to blind the student investigators.

4.11. Data analysis

4.11.1. Descriptive statistics and baseline data comparison

Descriptive data analysis was used to present the participants' demographics (age and gender), as well as the personal, disease and local ulcer risk factors affecting DFU healing. Frequency count and percentage were used on nominal and ordinal data. Mean and standard deviation (SD) were used on interval and scale data. The demographics and risk factors were compared among groups in order to detect any baseline variances. The nominal and ordinal data were compared by Fisher's exact test. The scale data were compared by Kruskal-Wallis test. The level of significance was set at 0.05. Adjustments were made if unequal demographics or risk factors were found among groups.

4.11.2. Statistical tests on study outcomes

All the analysis was carried out according to the **intention-to-treat** principle. All participants were counted after randomization. The detailed statistical tests according to outcomes were as follows:

- The outcome of "proportion of complete ulcer healing": **Fisher exact test** was used to compare the proportion of complete ulcer healing at 12 week.
- The outcome of "percentage of change in ulcer size": **Kruskal-Wallis test** was used to compare the change among the three groups.
- The laboratory outcomes of "total protein", "MMP9", "TNF-α", "IL-1α" concentrations: **Kruskal-Wallis test** was used to compare the difference at week 1 and week 4 among groups and **Wilcoxon Signed Rank test** was applied to compare the difference between week 1 and wee 4 within group.
- The laboratory outcome on "bacteriology": **Kruskal-Wallis test** was used to assess for the change between week 1 and week 4 among the three groups.
- The outcome of "severity of wound infection": **Fisher's exact test** was used to compare each week among the three groups and **Kruskal-Wallis test** was used to compare among the three groups at a whole.

4.11.3. Comparison between per-protocol analysis and the intention-to-treat principle

A sensitivity analysis was performed to assess the impact on protocol violation of conclusions drawn from the intention-to-treat principle. Comparison of proportion of complete healing among the three groups was made by **Fisher exact test** under the principle of **per-protocol analysis** and the **intention-to-treat principle**. Participants who were lost to follow-up or discontinued the study for different reasons were all excluded in the per-protocol analysis. All participants were included in the intention-to-treat analysis after randomization. The data, which collected on their last visit to the clinic, was assumed that their wound condition remained unchanged until the end of the trial period. Any difference in the result implied that participants who were lost to follow up and discontinued the study had different study outcomes from those who stayed until the end of the study.

4.11.4. Data quality assurance

In order to ensure the data quality, research assistant A was assigned to verify that the source document and other trial records were accurate, complete, up-to-date and maintained. The student investigator trained research assistant A in the data quality assurance process. The student investigator also checked the data entry sheet with raw data every month. The responsibilities of research assistant A were as follows:

- Check the accuracy and completeness of the case screening form and

the consent form.

- Verify that the laboratory data in the outcome record form was consistent with the corresponding laboratory result.
- Check that all enrolled participants' withdrawals and dropouts from this trial were reported and explained on the outcome record form.

4.11.5. Reporting and handling of missing data

There were three categories of confounder variables, namely the personal, disease and local ulcer factors. Variables in the personal and disease factors were related to participants' demographics and co-morbidities. Data in local ulcer factors consisted of ulcer parameters and had five variables. For outcome variables related to "ulcer healing" and "infection", there were eight points of observation from T_0 to T_7 in this study. The outcome variables related to "inflammation" were collected from T_0 to T_3 with four points of observation. For the adverse events, there were seven points of observation, from T_1 to T_7.

There were 23 variables in total. The total number of subjects was 31. Therefore, the total number of data items was 1519. The total missing data represented 4.02% of the whole. The variables related to confounder variables were collected at T_0. There were no missing data on confounders. For the outcome variables, the missing data were due to 3 participants withdrawing from the study for various reasons. In addition, for those outcome variables related to biomarkers, the reason for the missing data was because the ulcer was too small and it was not possible to collect wound fluid. The missing data in this category was 5.65%.

Similarly, the reason for missing data in the adverse events was related to participant withdrawal (5.52%). A summary of the missing data is shown in table 4.1.

Table 4.1. Summary of the percentage of missing data

Category of variables	No. of variables	No. of data	No. of missing data	Percentage of missing data
Personal factors	4	124	0	0
Disease factors	5	155	0	0
Local ulcer factors	5	155	0	0
Outcomes	8	868	49	5.65
Adverse events	1	217	12	5.52
Total	**23**	**1519**	**61**	**4.02**

In this study, there were eight points of observation, from T_0 to T_7. The method used for handling missing data on outcome variables related to ulcer healing was the last observation carried forward.

4.12. Training of research assistants

Research assistant A was responsible for collection the data on ulcer size and other variables. In order to ensure her measurement accuracy, she was trained in using the digital wound measurement device and used forms to collect the data provided by the student investigator. Firstly, the student investigator

demonstrated on how to use the wound measurement device. Then, the research assistant A was requested to perform returning demonstration until she got the skill on measurement. She and the student investigator then measured the size of 30 wounds in order to check the accuracy of her measurement. These wounds were not the DFU in the study. Pearson's correlation test revealed a high correlation (R^2=0.992), indicating that research assistant A's measurements were similar to those of the student investigator who was specialized in wound care, so that research assistant A's measurement could be considered accurate.

Using the above 30 wounds, the student investigator trained research assistant A in assessing the severity of wound infection and the SEWSS wound score. The training method was also demonstration and return demonstration. The result indicated a high correlation between the student investigator's assessment of the severity of the wound infection (R^2=0.980) and the SEWSS wound score (R^2=0.990). She was also trained through demonstration and return demonstration in recording the baseline data on the case record form and the adverse events on the event record form.

In addition, research assistants A and B were appointed to assess the wound status of complete wound healing. The student investigator demonstrated the assessment skills and requested for returned demonstration. The student investigator assessed 30 wounds (20 healing and 10 non-healing) independently, which were not the DFU in the study, and took photos of the wounds. Research assistant A examined the patients and research assistant B examined the electronic photos and identified those that were completely healed. The results

of Pearson's correlation test revealed a high agreement between the student investigator and research assistant A (R^2=0.999), student investigator and research assistant B (R^2=0.999), research assistant A and B (R^2=1.000) indicating that the assessments of research assistants A and B on complete wound healing were as accurate as those of the student investigator.

PART II - Methodology: the feasibility and acceptability test

4.13. Feasibility

4.13.1. Feasibility of recruitment process

In order to test the feasibility of the recruitment process, the number of referral made by the clinicians in different departments and hospitals, the number of screening, enrollment and recruitment of participants were counted by the student investigator. The feedbacks from the refused patients to participate in the study were collected in order to identify the reasons for their refusal. Besides, the appropriateness of selection criteria, the adequacy of the multi-center approach and the randomization procedure were reviewed through the self-reflection by the student investigator and interview of research assistant. Further, the blinding was analyzed through the self-reflection of student investigator and the interview of the research assistants.

4.13.2. Feasibility of interventions

The feasibility of interventions included the appropriateness of duration and frequency of clinic visit for the interventions, education on diabetes management and foot care, referral and checked for off-loading device, sharp debridement, selection of topical dressings by student investigators as well as application of topical dressings by intervention providers. Moreover, the request and compliant from participants were collected. Besides, the student investigator performed the self-reflection and interviewed the participants during the clinic visit for assessing and evaluating the feasibility on the duration of each intervention as well as identifying any safety issues and difficulty encountered during the interventions.

After the completion of the pilot study, a structured questionnaire, which was designed by the student investigator, was used to collect the feedback (appendix 18) on the feasibility of the interventions from the point of view of the intervention providers (exclude student investigator) who were the community nurses and nurses in the GOPD helping to change the dressings to the participants. This structured questionnaire consisted of four items including the clarity of the dressing prescription, the handling of the topical dressing, workload and time management. They were asked to rate their agreement level on each item in a five-point Likert scale from one (lowest rating) to five (highest rating). The higher score indicated the higher level of agreement. In addition, there was one open-ended item asking additional comments of the intervention providers on the interventions. Two experienced advanced practice nurses in the

GOPD and one experienced advanced practice nurse in the community nursing service performed the content validity. The content validity index was 0.90.

4.13.3. Feasibility of outcome measures

The feedback and comments on the training, support, data collection, and use of the instruments were collected using a structured questionnaire (Appendix 19) from the two research assistants A and B. The structured questionnaire, which was designed by the student investigator, consisted of four items including the adequacy of training received, support, perceived competency on the data collection using the instruments and workload. They were asked to self-rating their agreement level on each item in a five-point Likert scale from one (lowest rating) to five (highest rating). The higher score indicated the higher level of agreement. In addition, there is one open-ended item to collect additional comments on outcome measures from research assistants. One ward manager and two experienced advanced practice nurses in the O&T department of Hospital A performed the content validity. The content validity index was 1.00.

There were eight outcomes related to the preliminary effect. The feasibility of the outcome measures was evaluated including wound healing status, wound size measurement, wound fluid collection and measuring method, bacteriology as well as severity of infection. Their feasibility were assessed and reported through the reflection and re-visit by the student investigator in the research process.

4.14. Acceptability

4.14.1. Acceptance of the interventions and study attrition

The retention rate and the reasons for study attrition was recorded and analyzed. The acceptability on the four interventions including patient education, debridement, off-loading and experience on using topical dressings from the participants was collected by the student investigator through the interview at the end of each clinic visit.

4.14.2. Adherence of participants to interventions

The intervention on the adherence of diabetic control in terms of HbA1c and serum albumin level were collected and compared on the first and last visit in order to check the diet compliance of participants. Moreover, their adherence on foot care, off-loading devices and the dressing regimen were also revealed by the comments of participants through the same interview as above in the O&T nurse clinic at the end of each clinic visit.

4.15. Ethical consideration

In the design of this clinical trial, ethical issues were considered in several areas. Firstly, ethical approval was obtained from the ethical review sub-committee of the HKPU and Hospital A before the commencement of the study. Secondly, an explanation was given to participants by the student investigator, using a

standard information sheet, so that each of them was able to make their own decision with full understanding. This also conformed to the ICH Good Clinical Practice (GCP) guideline of the Helsinki Declaration. Thirdly, written informed consent was obtained from participants by the student investigator before the commencement of the study. In addition, the possible risks in this study were handled in a number of ways. All the recruited participants were checked for allergy history to make sure that they were not allergic to the tested material. If there is any serous adverse event occurs, the ethical review sub-committee in both HKPU and hospital A were informed and reported within 15 calendar days. This reporting criteria and timeframe was followed the ICH GCP guideline E1 (Karlberg & Tsang, 1998).

Besides, the participants were told that they had the right to withdraw from the study at any time without any penalty. After withdrawal, it did not affect their health care services received. Further, no participant names or identity numbers was written down on all the study records. A number would be given to each particpants. Each participant would assign a code. The entire documents related to this study were coded according to the corresponding participants. No participant name was written on study related documents. Therefore, anonymity and confidentiality were followed strictly. Furthermore, all electronic document including the data sheet and digital images of the ulcers were stored in locked folders with password of the student investigator's computer. Lastly, all study related documents were locked and archived for at least one year after the study, according to ICH GCP guidelines.

4.16. Summary

This chapter covered the details of the methodology in this pilot study including the practicability of the study and the intervention effectiveness. The design was adhered to The International Conference on Harmonization of Good Clinical Practice Guideline and the content was followed to the reporting format of the consort statement. The overall trial design, participant selection with justifications, sample size estimation, interventions, outcomes, method of randomization with allocation concealment mechanism, blinding and statistical methods were discussed. The intervention protocol including the training of research assistants was highlighted. The methodology on the feasibility and acceptability were discussed. Lastly, the ethical considerations of this study were illustrated.

Chapter 5 – Results

5.1. Introduction

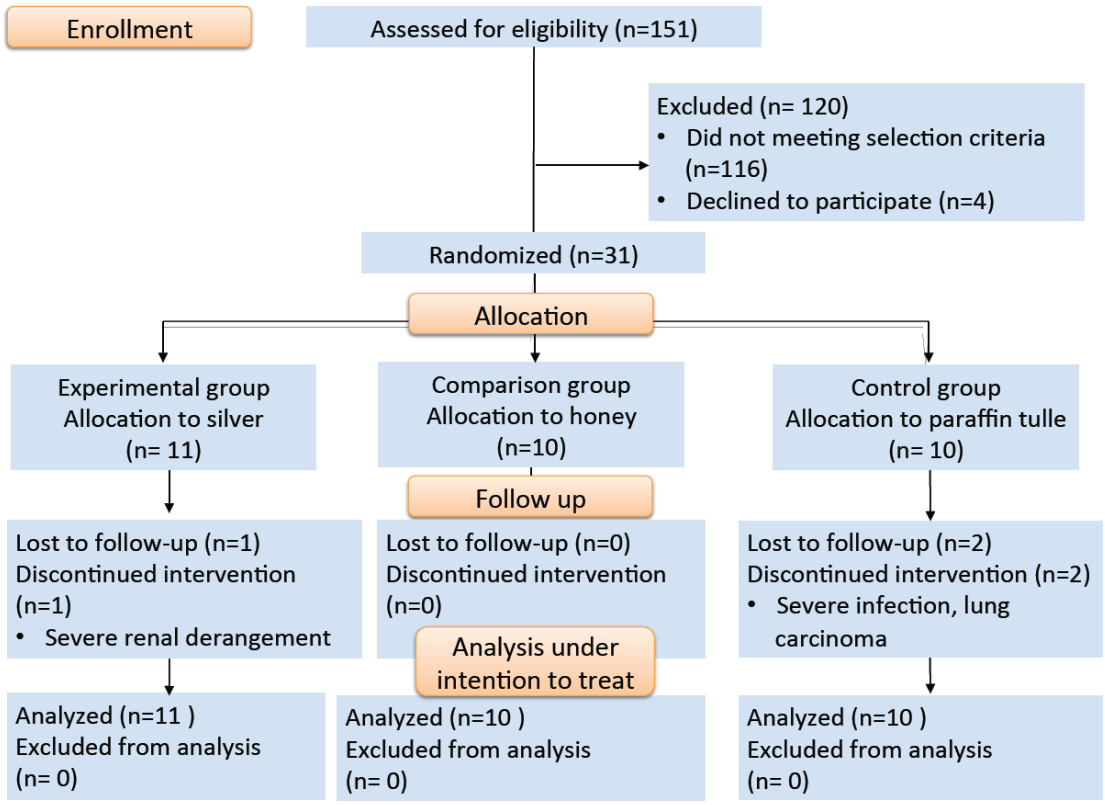
This chapter is divided into two parts. In order to let readers better understand the results of the feasibility and acceptability test, the result of the preliminary effect was discussed first, followed by the feasibility and acceptability results. The first part was the preliminary effect of nAg dressing. The three categories of the results in effectiveness including "ulcer healing", "inflammation" and "infection" were presented. The second part was the **main study results** of this pilot study. They were the feasibility of the study design and protocol and acceptability of interventions. Lastly, the effect size was calculated based on the result of the preliminary effect.

PART I – Result: Preliminary effect of nAg

5.2. Number of participants

As shown in the CONSORT flow diagram (Figure 5.1), a total of 151 patients were screened. Of these, 116 did not meet the selection criteria and four declined to join the study. We originally planned to recruit 30 participants and there was one extra referral of participant received on the last day of recruitment. Finally, we recruited 31 eligible participants to join this study. They were recruited from Hospital A, Hospital B and one GOPD.

Figure 5.1. CONSORT flow diagram



5.3. Demographics and identified risk factors

5.3.1. Participants' profile

There were 18 males (58.1%) and 13 females (41.9%), with 24 (77.4%), 5 (16.5%) and 2 (6.5%) participants recruited from Hospital A, Hospital B and the GOPD respectively. Their average age was 64.97 years (SD=11.34). The duration of diabetes was 14.45 years (SD=9.7). The average HbA1c level was 8.06 mmol/ L (SD=1.59). The average size of the ulcers was 8.36 cm^2 (SD=12.54). The depth of the ulcer was one of the important factors hindering the progress of ulcer

healing. According to the University of Texas Classification on ulcer depth, only two ulcers (6.45%) were in “Group 0” which involved dermis. Others ulcers were all deep to skin, tendon or capsule. 15 ulcers (48.39%) were in “Group 1” which involved full thickness skin loss. 14 ulcers (45.16%) were in “Group 2” that were deep to tendon or capsule. As mentioned in Chapter four, the SEWSS was used to measure the severity of DFU. All of the ulcers scored from 9 to 18, with a mean score of 13.45 (SD=2.17). All the wound scores were below 20 indicating that those wounds had the healing potential (Martínez-De Jesús, 2010). The participant profile and the participants’ personal, disease factors and local ulcer risk factors were shown in Table 5.1.

Table 5.1. Participant demographics and personal, disease and ulcer factors

		n=31	Percentage	Mean	SD
Origin of participants	Hospital A	24	77.42		
	Hospital B	5	16.13		
	GOPD	2	6.45		
Personal factors					
Age (years)				64.97	11.34
Gender	Male	18	58.06		
	Female	13	41.94		
Serum albumin level (mmol/ L)				34.52	5.90
Ambulatory status	Ambulatory	31	100		
	Chair bound	0	0		
	Bed bound	0	0		
Disease factors					
Duration of diabetes (years)				14.45	9.70
HbA1c level (mmol/ L)				8.06	1.59
Heart disease	Yes	15	48.39		
	No	16	51.61		
PAD	Yes	2	6.45		
	No	29	93.55		
Ischemia [Ankle brachial index (ABI)]				1.07	0.21
Local ulcer factors					
Ulcer location on foot	Toe amputation	11	35.49		
	Dorsum	4	12.90		
	Plantar	4	12.90		
	Plantar to dorsum	1	3.23		
	Medial malleolus	2	6.45		
	Anterio-medial ankle	3	9.68		
	Lateral malleolus	4	12.90		
	Heel	2	6.45		
Ulcer duration (weeks)				11.16	12.61
Ulcer size (cm^2)				8.36	12.54
Ulcer depth	0	2	6.45		
	1	15	48.39		
	2	14	45.16		
	3	0	0		
Ulcer severity (score range 0-30)				13.45	2.17
Severity of wound infection	None	16	51.6		
	Mild	12	38.7		
	Moderate	3	9.7		

5.3.2. Comparison of participants' baseline information on demographics and risk factors among three groups

The present study consisted of three groups: the nAg (experimental), MH (comparison) and conventional (control) groups. All the personal, disease and local ulcer factors identified in the previous chapter, which affected DFU healing, were compared among groups (Table 5.2). The eligible participants were recruited from three different venues, namely Hospital A, Hospital B and a GOPD. Their distribution was not significantly different among the groups. There was also no significant difference among the groups in personal, disease and local ulcer factors. This implies that their baseline information was similar and comparable across all three groups.

Table 5.2. The comparison of baseline information on demographics and risk factors among groups

		nAg (n=11)	MH (n=10)	Conventional (n=10)	p-value
Origin of participants	Hospital A	9	7	8	0.134
	Hospital B	2	3	0	
	GOPD	0	0	2	
Gender	M	7	4	7	0.433
	F	4	6	3	
Age (years) [mean (SD)]		63.36 (11.31)	65.60 (11.42)	66.1 (12.31)	0.948
Serum albumin level (mmol/ L) [mean (SD)]		32.55 (6.99)	33.80 (4.89)	37.40 (4.84)	0.147
Ambulatory status	Ambulatory	11	10	10	>0.9999
Duration of diabetes (years) [mean (SD)]		14.82 (10.44)	13.30 (9.63)	15.20 (9.88)	0.921
HbA1c level (mmol/ L) [mean (SD)]		8.27 (1.32)	8.30 (2.26)	7.59 (0.99)	0.574
Heart disease	Yes	4	5	6	0.604
	No	7	5	4	
PAD	Yes	1	0	1	>0.9999
	No	10	10	9	
Ischemia (ABI) [mean (SD)]		1.06 (0.20)	1.03 (0.24)	1.13 (0.21)	0.944

* $p \leq 0.05$

Table 5.2. The comparison of baseline information on demographics and risk factors among groups (cont'd)

		nAg (n=11)	MH (n=10)	Conventional (n=10)	p-value
Ulcer location on foot	Toe amputation	7	2	2	0.391
	Dorsum	1	2	1	
	Plantar	0	1	3	
	Plantar to dorsum	1	0	0	
	Medial malleolus	0	1	1	
	Anterio-medial ankle	1	2	0	
	Lateral malleolus	1	1	2	
	Heel	0	1	1	
Ulcer duration (weeks) [mean (SD)]		11.45 (6.67)	12.80 (10.54)	14.70 (8.12)	0.401
Ulcer size (cm^2) [mean (SD)]		8.68 (6.84)	10.98 (8.03)	8.28 (7.27)	0.495
Ulcer depth	0	0	1	1	0.867
	1	5	5	5	
	2	6	4	4	
	3	0	0	0	
Ulcer severity SWESS (score 0-30) [mean (SD)]		13.27 (2.15)	14.40 (1.90)	12.27 (2.31)	0.195
Severity of wound infection	None	7	3	6	0.498
	Mild	3	6	3	
	Moderate	1	1	1	

* $p \leq 0.05$

5.4. The confounders affecting ulcer healing throughout 12 weeks

5.4.1. Episodes of using oral antibiotics at different time points over 12 weeks

The use of oral antibiotics was a confounder in affecting changes in bacteriology on the DFU and the clinical signs of wound infection. As a result, it would affect ulcer healing. The number of episodes of participants taking oral antibiotics was counted among groups. At every clinic visit, one participant taking oral antibiotics was counted as one episode. There were totally 30 episodes of participants taking antibiotics in the study period: 12 in the nAg group, 11 in the MH group and 7 in the conventional group (Table 5.3). Among these episodes, around half involved participants continuing their antibiotics upon discharge from hospital. Only five episodes were of participants commencing oral antibiotics during the study period. Two were in the nAg group, one in the MH group and two in the conventional group. In the nAg group, one participant was on prophylactic antibiotics because of the need to perform another medical procedure in the general clinic, and another was taking oral antibiotics to reduce calf swelling. In the MH group, one participant was on oral antibiotics because she found generalized blisters after self-administration of traditional Chinese medicine. One participant in the conventional group had a chest infection and was prescribed antibiotics by primary physician. The other had a wound infection found at the final visit in week 12, and required antibiotics.

In addition, every single time point was compared among groups using Fisher's exact test (Table 5.3). There were no significant differences among the three groups, revealing that there were no significant differences in the distribution of antibiotic use among the three groups throughout the 12-week study period. This implies that the confounder "use of oral antibiotics" did not affect the comparison of ulcer healing among groups.

Table 5.3. The use of antibiotics among the three groups over 12 weeks

Week	Use of antibiotics	nAg (n=11)	MH (n=10)	Conventional (n=10)	p-value
1	No	6	4	6	0.740
	Yes	5	6	4	
	Healed	0	0	0	
2	No	9	7	9	0.291
	Yes	2	3	1	
	Healed	0	0	0	
3	No	9	9	8	>0.9999
	Yes	2	1	1	
	Healed	0	0	1	
4	No	9	9	9	0.755
	Yes	2	1	0	
	Healed	0	0	1	
6	No	8	9	9	>0.9999
	Yes	1	0	0	
	Healed	2	1	1	
8	No	7	7	6	>0.9999
	Yes	0	0	0	
	Healed	4	3	4	
10	No	4	5	6	0.604
	Yes	0	0	0	
	Healed	7	5	4	
12	No	2	5	5	0.174
	Yes	0	0	1	
	Healed	9	5	4	
Total number of episodes of taking antibiotics		12	11	7	

* $p \leq 0.05$

5.4.2. Episodes of adverse events at different time points over 12 weeks

Adverse events happened within the study period that might have affected ulcer healing. Six episodes of adverse events occurred during the study period. Four were in the conventional group (Table 5.4), and there was one each in the MH and nAg groups. The four adverse events in the conventional group were lung carcinoma, chest infection, blisters found near the ulcer due to friction on walking, and wound infection. One subject in the MH group found generalized blisters on the body caused by an allergic reaction after taking traditional Chinese medication. An oral steroid drug was prescribed to the participant by a doctor in the family clinic. Another adverse event, in the nAg group, was an episode of calf swelling. It was not caused by the DFU, since the wound healing progress was satisfactory at the time. However, the calf swelling affected the DFU healing because of the compromised distal microcirculation after swelling.

When comparing the distribution of adverse events at different time points of assessment using Fisher's exact test, none of them were significant, indicating that the confounder "episode of adverse event" was no different among the groups and did not affect the comparison of ulcer healing among groups. A summary of the results is shown in Table 5.4.

Table 5.4. The adverse events among groups over 12 weeks

Week	Infectious status	nAg (n=11)	MH (n=10)	Conventional (n=10)	p-value
1	No	11	10	10	>0.9999
	Yes	0	0	0	
	Healed	0	0	0	
2	No	11	10	10	>0.9999
	Yes	0	0	0	
	Healed	0	0	0	
3	No	10	10	7	0.210
	Yes	1	0	2	
	Healed	0	0	1	
4	No	11	10	8	0.193
	Yes	0	0	1	
	Healed	0	0	1	
6	No	9	8	9	>0.9999
	Yes	0	1	0	
	Healed	2	1	1	
8	No	7	7	6	>0.9999
	Yes	0	0	0	
	Healed	4	3	4	
10	No	4	5	6	0.604
	Yes	0	0	0	
	Healed	7	5	4	
12	No	2	5	5	0.174
	Yes	0	0	1	
	Healed	9	5	4	
Total number of adverse events		1	1	4	

* $p \leq 0.05$

5.5. Results on "ulcer healing"

"Ulcer healing" was the first category of results related to effectiveness in this study. The results are presented according to the sequence of the hypothesis as stated in section 3.7 of chapter 3.

5.5.1. Proportion of complete healing at week 12

5.5.1.1. *Intention-to-treat principle*

Under the intention-to-treat principle, all the participants were included in the analysis after the randomization procedure. On using the Fisher's exact test, the proportions of complete healing at the end of week 12 were 81.8%, 50% and 40% in the nAg, MH and conventional groups respectively (Table 5.5). However, there was no significant difference among the three groups on using the Fisher's exact test with p-value 0.135.

Table 5.5. The proportion of complete ulcer healing among the three groups at week 12 under intention-to-treat principle (n=31)

	Treatment group						p-value
	nAg (n=11)		MH (n=10)		Conventional (n=10)		
	no.	%	no.	%	no.	%	
Healing	9	81.8	5	50	4	40	0.135
Non-healing	2	18.2	5	50	6	60	

* $p \leq 0.05$

5.5.1.2. *Per-protocol analysis*

There was one participant in the nAg group and two participants in the conventional groups that were on discontinuation of interventions before week 12 (Figure 5.1). They were excluded in the per-protocol analysis. The

proportions of complete healing at the end of week 12 were 90%, 50% and 50% in the nAg, MH and conventional groups respectively (Table 5.6). Similar to the result in the intention to treat principle, the result was not significant with p-value 0.106 on using the Fisher's exact test.

Table 5.6. The proportion of complete ulcer healing among the three groups at week 12 under per-protocol analysis (n=28)

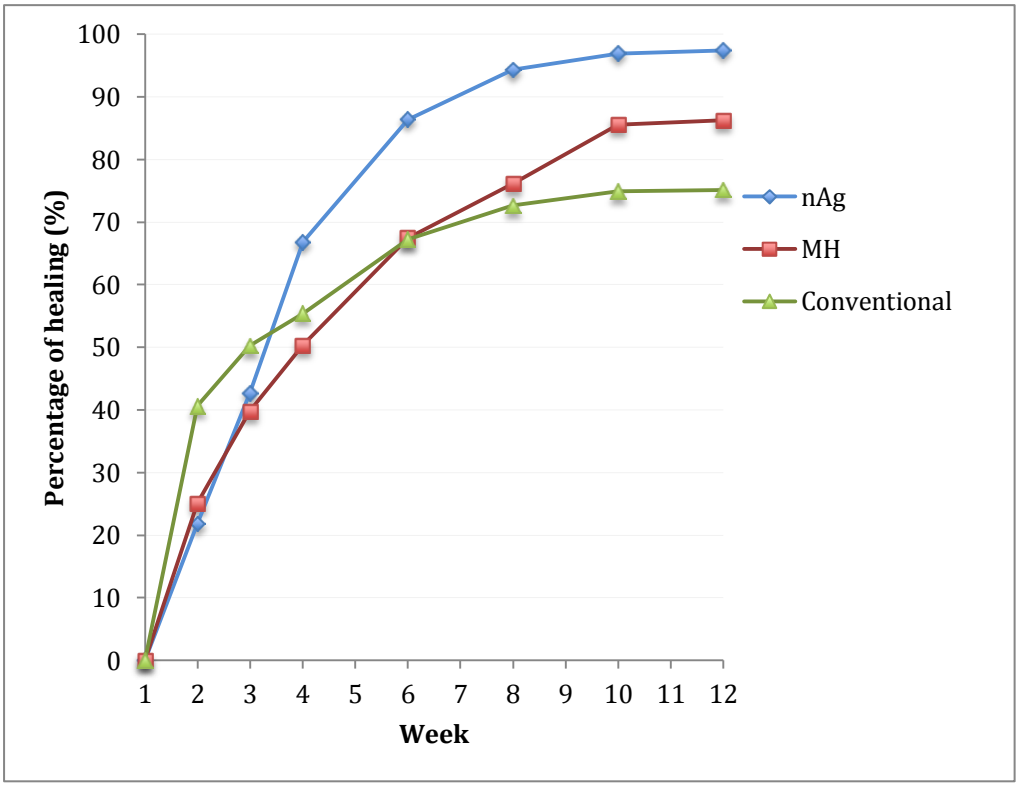
	Treatment group						p-value
	nAg (n=10)		MH (n=10)		Conventional (n=8)		
	no.	%	no.	%	no.	%	
Healing	9	90.0	5	50	4	50	0.106
Non-healing	1	10.0	5	50	4	50	

* $p \leq 0.05$

5.5.2. Ulcer size reduction over 12 weeks

The ulcer size reduction in terms of percentage of area reduction was examined over the 12-week study period among the three treatment groups. The average ulcer size reduction in each group is shown in Figure 5.2.

Figure 5.2. Ulcer size reduction in each group



For the change in ulcer size at 12-week among the three groups, nAg group had the greatest ulcer size reduction (97.45%, SD=5.98). The MH group and the convention group were 86.25% (SD=15.98) and 77.51% (SD=26.40) respectively. However, the result did not show significant difference among the groups (p=0.071) on using the Kruskal-Wallis test. The summary of the result was shown in Table 5.7. Although the result was not significant, nAg group still had the greatest decrease percentage in ulcer size reduction when compared with MH and conventional groups.

Table 5.7. The change in ulcer area at week 12 among three groups

	Treatment group			p-value
	nAg	MH	Conventional	
% of ulcer size reduction at 12-week [SD]	97.45 [5.98]	86.25 [15.98]	77.51 [26.40]	0.071

* $p \leq 0.05$

5.5.3. Summary of the "ulcer healing" findings

A summary of the results on ulcer healing is shown in Table 5.8. nAg had the highest proportion of complete ulcer healing and greatest ulcer size reduction over the 12-week study period although the statistics were not significant.

Table 5.8. Summary of the ulcer healing findings

Results	Significance
Proportion of complete ulcer healing over 12 weeks	NS
Ulcer size reduction over 12 weeks	NS

NS: Non-significance

5.6. **Results on "inflammation"**

"Inflammation" was the second category of results. It was related to the concentration profile of biochemical markers found in the wound fluid.

5.6.1. Concentration profile of total protein over the first 4 weeks

The levels of total protein inside wound fluid varied among groups. Their mean and standard deviation were shown in Table 5.9. It showed that the average level of total protein inside wound fluid increased in week 4 when compared with week 1 in MH group. On the other hand, the corresponding average levels decreased in both nAg and MH group. As a whole, there was no significant difference in the average levels among groups in week 1 (p=0.804) and week 4 (p=0.810) on using the Kruskal-Wallis test. On comparing the levels of total protein within group in week 1 and week 4 by Wilcoxon signed rank test, there was no significant difference with p-values 0.345, 0.893 and 1.000 in nAg, MH and conventional groups respectively (Table 5.9).

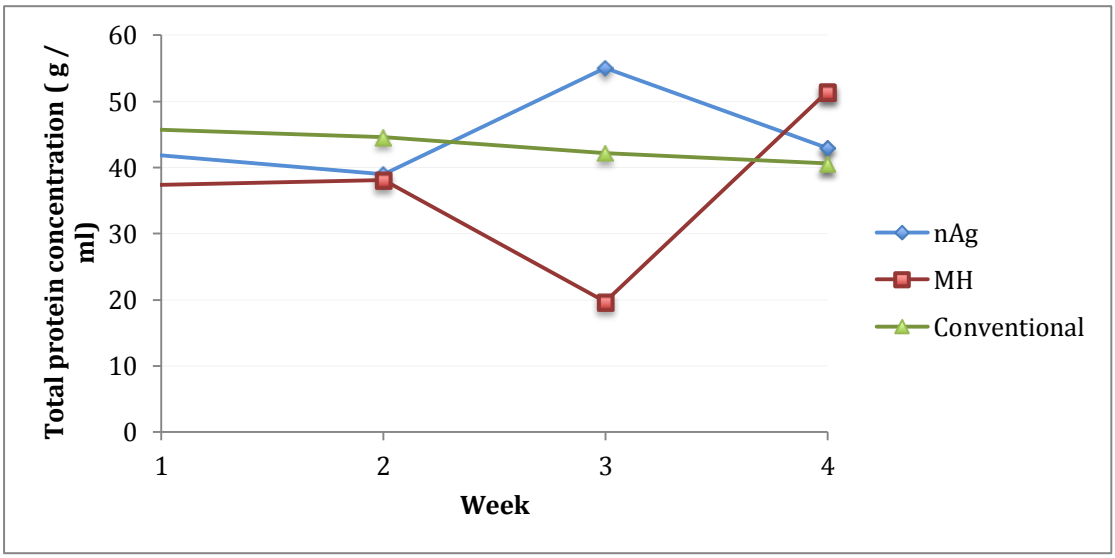
Table 5.9. The average levels of total protein at week 1 and week 4 among groups

	nAg (g/ ml) mean [SD]	MH (g/ ml) mean [SD]	Conventional (g/ ml) mean [SD]	p-value
Week 1	44.66 [33.06]	36.70 [35.42]	46.79 [28.72]	0.804
Week 4	42.93 [27.28]	51.43 [34.69]	40.62 [19.99]	0.810
p-value	0.345	0.893	1.000	

* $p \leq 0.05$

In addition, changes in trend were analyzed among the groups. There was an up and down trend in the nAg and MH groups. Total protein decreased steadily in the conventional group. The average concentration profile among the three groups is shown in Figure 5.3.

Figure 5.3. The average concentration profile of total protein among the three groups



5.6.2. Concentration profile of MMP-9 over the first 4 weeks

Similar to the total protein level, the variance in the MMP-9 level among groups was considerable. The dispersion of the concentration of MMP-9 is illustrated in Table 5.10. Using the Kruskal-Wallis test, there was no difference among the groups at both week 1 (p=0.063) and week 4 (p=0.089). On comparing the levels of MMP-9 on individual group between week 1 and week 4 by Wilcoxon signed rank test, there was no significant difference in nAg (p=0.465), MH (p=0.500) and conventional groups (p=1.000). The details of the results are shown in Table 5.10.

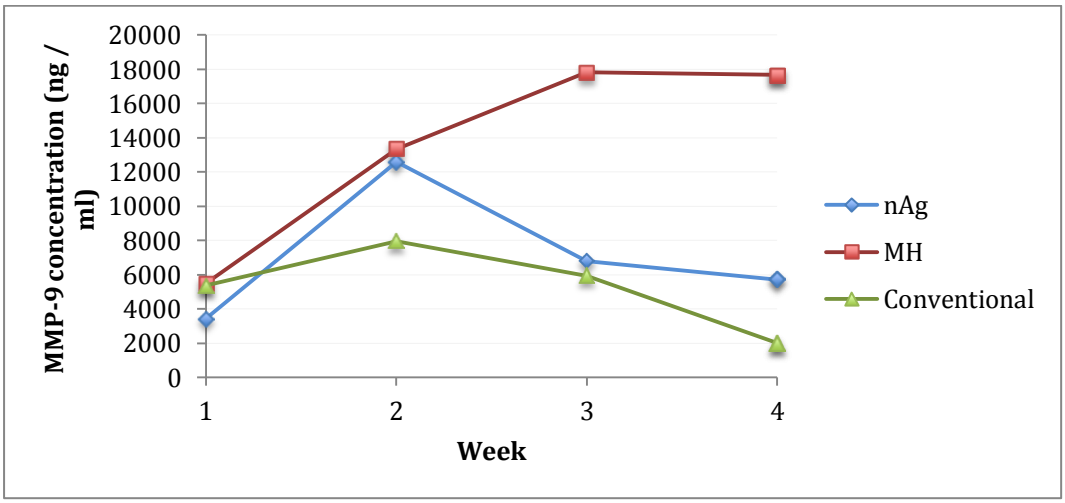
Table 5.10. The average levels of MMP-9 at week 1 and week 4 among groups

	nAg (ng/ ml) mean [SD]	MH (ng/ ml) mean [SD]	Conventional (ng/ ml) mean [SD]	p-value
Week 1	3412.38 [5322.27]	5655.76 [7163.14]	5365.84 [13725.55]	0.063
Week 4	5720.59 [10480.71]	17672.54 [11276.75]	1987.39 [2892.48]	0.089
p-value	0.465	0.500	1.000	

* $p \leq 0.05$

When observing the change of the MMP-9 average concentration over the first four weeks, the change in concentration among the groups was considerable, as shown in Figure 5.4. An upward trend was noted in the MH group, while the nAg and conventional groups reported a non-linear trend.

Figure 5.4. The average concentration profile of MMP-9 among the three groups



5.6.3. Concentration profile of TNF-α over the first 4 weeks

Similar to MMP-9, changes in the TNF-α concentration in wound fluid were extensive within each group over the 4-week period. The Kruskal-Wallis test showed no significant difference in the levels of TNF-α at both week 1 (p=0.209) and week 4 (p=0.562) among groups. On comparing the level of TNF-α at week 0 and week 4 among groups by Wilcoxon signed rank test, there were no significant differences in each group with p-values in 0.068, 0.800 and 0.414 in nAg, MH and conventional groups respectively (Table 5.11).

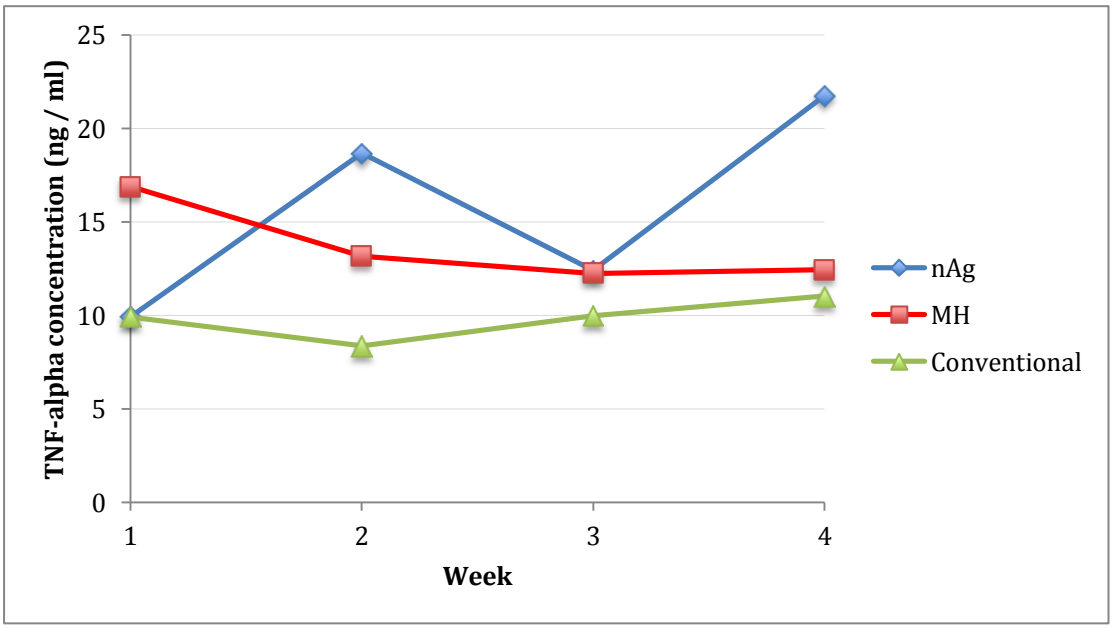
Table 5.11. The average levels of TNF-α at week 1 and week 4 among groups

	nAg (ng/ ml) mean [SD]	MH (ng/ ml) mean [SD]	Conventional (ng/ ml) mean [SD]	p-value
Week 1	9.93 [6.21]	24.89 [26.76]	9.92 [8.83]	0.209
Week 4	21.77 [18.26]	12.44 [9.13]	11.03 [8.51]	0.562
p-value	0.068	0.800	0.414	

* $p \leq 0.05$

On assessing the trend in each group, the TNF-α in the MH group decreased, whereas that in the nAg and conventional groups increased. By contrast, the TNF-α in the conventional group remained more or less constant (Figure 5.5).

Figure 5.5. The average concentration profile of TNF-α among the three groups



5.6.4. Concentration profile of IL-1α over the first 4 weeks

For IL-1α, the variations in level within the groups was high. The levels of change in IL-1α were not in the same direction among the three groups, similar to MMP-9 and TNF-α. Based on the Kruskal-Wallis test, there were no significant differences among groups in the levels of IL-1α at both week 1 and week 4. On comparison the level of IL-1α among groups by Wilcoxon signed rank test, there were no significant differences in all the three groups between week 1 and week 4. The respective p-values in nAg, MH and conventional groups were 0.715, 0.686 and 0.109 (Table 5.12).

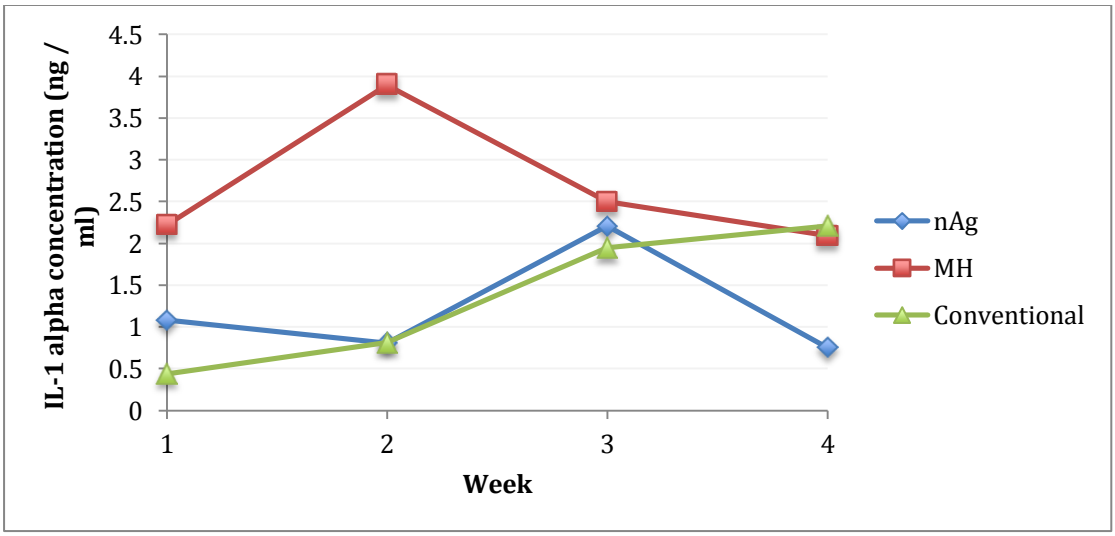
Table 5.12. The average levels of IL-1α at week 1 and week 4 among groups

	nAg (ng/ ml) mean [SD]	MH (ng/ ml) mean [SD]	Conventional (ng/ ml) mean [SD]	p-value
Week 1	1.31 [1.17]	1.93 [1.32]	0.62 [0.71]	0.184
Week 4	0.57 [0.96]	2.08 [2.28]	2.21 [2.70]	0.380
p-value	0.715	0.686	0.109	

* $p \leq 0.05$

Trends in the changes in IL-1α concentration differed across the three groups, similar to the tendency found in MMP-9 and TNF-α. There was a steadily increasing trend in the conventional group. However, no obvious linear trends were found in the nAg and MH groups (Figure 5.6).

Figure 5.6. The average concentration profile of IL-1α among the three groups



5.6.5. Summary of the "inflammation" findings

According to the above findings, the nAg did not show a significant difference in the levels of MMP-9, TNF-α and IL-1α than the MH and conventional dressing groups. In addition, there was no difference in the concentration of all the tested biomarkers between week 1 and week 4 in each group. The following table 5.13 is a summary of the findings.

Table 5.13. Summary of the "inflammation" findings over the first 4 weeks

		Total protein	MMP-9	TNF-α	IL-1α
Within group comparison "week 1 and week 4"	nAg group	NS	NS	NS	NS
	MH group	NS	NS	NS	NS
	Conventional group	NS	NS	NS	NS
Among 3 groups comparison	Week 1	NS	NS	NS	NS
	Week 4	NS	NS	NS	NS

NS: Non-significance

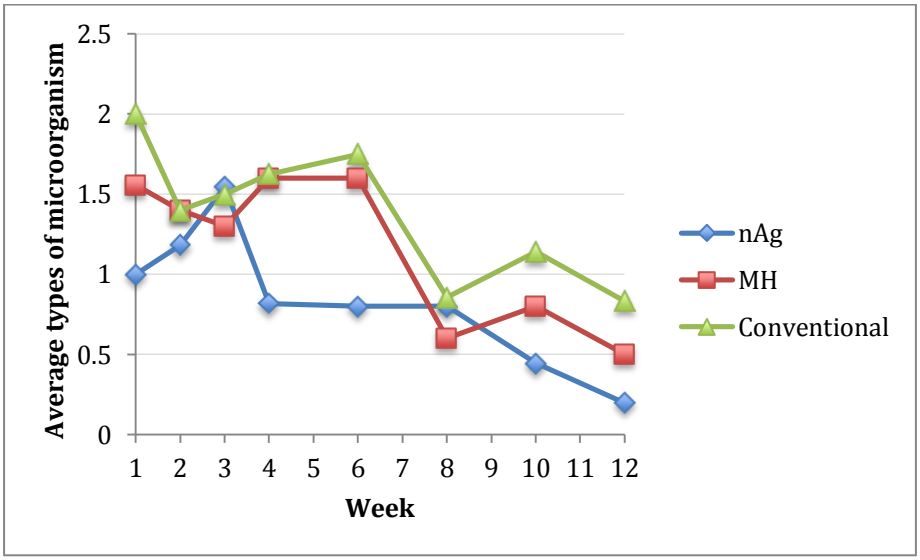
5.7. Results on "infection"

"Infection" is the third category of results, and consists of "the bacteriology of the ulcer" and "the severity of wound infection".

5.7.1. Bacteriology over 12 weeks

The types of microorganism found among the groups all showed a decreasing trend over the 12-week study period, demonstrating that the types of microorganism decreased throughout the study period. The decreasing trend of all three groups is shown in Figure 5.7.

Figure 5.7. The average types of microorganisms among the three groups



The average types of microorganism at week 1 among the groups are shown in Table 5.14. However, on using the Kruskal-Wallis test, there was no significant difference in the types of microorganism at both 1st week 1 and 12th week among the three groups. When the types of microorganism were compared between 1st week 1 and 12th week by Wilcoxon signed rank test, there was a significant decrease in the types of microorganism in nAg (p=0.024) and conventional (p=0.031) groups. Nevertheless, it was not significant in MH group (p=0.084) (Table 5.14).

Table 5.14. The average types of microorganism among the three groups at week 1 and week 12

Week	nAg		MH		Conventional		p-value
	Types of micro-organism	SD	Types of micro-organism	SD	Types of micro-organism	SD	
1	1.00	1.00	1.56	1.59	2.00	1.25	0.182
12	0.18	0.60	0.50	1.27	1.00	1.16	0.107
p-value	0.024*		0.084		0.031*		

* $p \leq 0.05$

In short, by integrating the above findings in this section, both nAg and conventional groups showed a significant difference in bacteriology during the study period. Nevertheless, the nAg group did not show a significant decrease in bacteriology as compared with the MH and conventional groups.

5.7.2. Severity of wound infection over 12 weeks

In order to assess the severity of infection objectively, the ISDA and IWGDF classification of diabetic foot infection (DFI) (Lipsky et al., 2012a) was adopted in this study. In the nAg and MH groups, no severe wound infection occurred during the study period. There was one episode of severe wound infection in the conventional group. The subject required admission for intravenous antibiotics. There were eight observation points throughout the 12 weeks of the study period, at weeks 1, 2, 3, 4, 6, 8, 10 and 12.

At each time point, the degree of severity of infection was compared among groups using Fisher's exact test. The results are demonstrated in Table 5.15, which showed that there were no significant differences at the different observation points among the three groups throughout the 12-week study period.

5.7.3. Summary of the "infection" findings

The results in the category of "infection" were similar. There were no differences among the three groups in bacteriology on the severity of wound infection at different observation points in the study period.

Table 5.15. The severity of wound infection at different observation points among the three groups

Week	Infectious status	Treatment group			p-value
		nAg (n=11)	MH (n=10)	Conventional (n=10)	
1	None	7	3	6	0.498
	Mild	3	6	3	
	Moderate	1	1	1	
	Severe	0	0	0	
	Healed	0	0	0	
2	None	10	6	9	0.068
	Mild	1	4	0	
	Moderate	0	0	1	
	Severe	0	0	0	
	Healed	0	0	0	
3	None	10	9	8	>0.9999
	Mild	1	1	0	
	Moderate	0	0	0	
	Severe	0	0	0	
	Healed	0	0	1	
4	None	10	8	9	0.074
	Mild	1	2	0	
	Moderate	0	0	0	
	Severe	0	0	0	
	Healed	1	0	1	
6	None	8	8	9	0.315
	Mild	0	1	0	
	Moderate	1	0	0	
	Severe	0	0	0	
	Healed	2	1	1	
8	None	6	7	6	0.836
	Mild	0	0	0	
	Moderate	1	0	0	
	Severe	0	0	0	
	Healed	4	3	4	
10	None	3	5	6	0.090
	Mild	1	0	0	
	Moderate	0	0	0	
	Severe	0	0	0	
	Healed	7	5	4	
12	None	2	5	4	0.301
	Mild	0	0	0	
	Moderate	0	0	1	
	Severe	0	0	1	
	Healed	9	5	4	

* $p \leq 0.05$

PART II – Results: Feasibility and Acceptability

5.8. Feasibility of recruitment process

5.8.1. Referral

In total, 31 participants were enrolled in this pilot study. 67.8% (n=21) was recruitment from the student investigator. Only 16.1% (n=5) was referral by O&T surgeons from Hospital A and 16.1% (n=5) was referral by nurses from Hospital B. The percentage of referral by other surgeons and nurses was low. When the student investigator approached the doctors and nurses for patients' referral, none of them refused the request. However, one of the O&T doctors claimed that his clinical duties were heavy and the time schedule was tight, and he may forget to make the referral to the student investigator.

5.8.2. Screening and enrollment

A total of 151 patients were screened. Of these, 116 did not meet the selection criteria and the proportion of fit for enrollment was 23.2%. The reasons for exclusion were due to severe ulcer infection, ulcer deep into bone and joint, osteomyelitis as well as renal failure. 35 patients were eligible to join the study. Among those excluded patients (116 patients), severe ulcer infection as well as ulcer deep into bone and joint accounted for 70.7% (82 patients) of the patients.

5.8.3. Participant's selection criteria

The inclusion criteria were selected because the DFUs with these characteristics had a high healing potential and could be treated by topical dressings. On the other hand, the exclusion criteria were the conditions that were not suitable for the use of topical dressing materials. For those DFUs with the characteristics of the exclusion criteria, they had the low healing potential and out of the scope of using topical dressing for healing. Based on the clinical experience of the student investigator, there was a chance of deterioration of DFU when the selected topical dressing materials were used. In Hong Kong, only patients with severe DFU would be admitted to the hospital. It was the reason why there was a low percentage of enrollments in the present pilot.

5.8.4. Recruitment

As mentioned before in the first part, we recruited 31 participants from 35 eligible patients. Four of them declined to join. The recruitment rate was calculated as number of participants enrolled divided by the number of eligible patients and it was 88.6%. The reasons for the refusal of the participants are as follows. Two patients stated that they were unable to be followed up weekly; one patient expressed that her family members did not want her to enroll in the RCT and another patient stated that he would be out of town soon during the scheduled follow-up period. The details of the CONSORT flow diagram were shown in Figure 5.1.

5.8.5. Multi-center approach

In the early planning stage of this study, hospital A was the only study center. However, the recruitment was only 12 participants in the first year of the commencement of study. The student investigator then contacted the other hospitals and got one hospital to participate in this pilot study. He also went to one GOPD for recruitment. As a result, a multi-center approach was adopted in the present study. Although a multi-center approach is considered to be helpful in increasing the number of participants to be recruited, the recruitment rate was still low.

5.8.6. Randomization

In the present pilot study, block randomization procedure was used through the online software as stated in Chapter 4. The procedure was smooth, which was performed by research assistant C. The generated sequence of sealed envelopes was in chronological order and passed to the student investigator for enrollment of participants. According to the feedback from research assistant C, she had no problem on the randomization procedure. In addition, there was no participant who refused the allocation. The demographic data and risk factors were evenly distributed among groups (Table 5.2).

5.8.7. Blinding

In this pilot study, the student investigator, community nurses and nurses in GOPD were not blinded because they were the intervention providers to put the dressing onto the wound. The student investigator also collected the wound fluid and performed the laboratory tests on wound fluid. Research assistant A was blinded during group allocation as she assessed the wound healing status, wound size, ulcer severity, severity of wound infection and adverse events of the participants. The research assistant B was also blinded during group allocation when she assessed the wound healing status.

From the self-reflection of the student investigator, it was not possible to blind all the other intervention providers because they had to perform the dressing change and the appearances of the topical dressings were different. In addition, the student investigator was also not suitable to be blinded because he had to apply the topical dressings according to the group allocation. Also, he was the data collector who collected wound fluid and perform the wound fluid analysis in the laboratory. It might potentially pose bias on getting the laboratory result and plotting the graph.

According to the comment of research assistant A, the blinding was effective in this pilot study. She was unable to know the group allocation and also failed to identify which type of dressing was used on the participants when performing the above assessments. In addition, research assistant B expressed that she was

completely blinded over group allocation during assessment of the wound healing status through clinical photo.

5.9. Feasibility of interventions

5.9.1. Frequency and duration of clinic visit

5.9.1.1. *Frequency of clinic visit*

The frequency of visit was once per week in the first four weeks and then alternative week from weeks five to twelve. There were eight clinic visits in total in 12 weeks. There was no participant who expressed that the total duration of visit was too long or too short. Moreover, there was no participant who requested to decrease the number of clinic visit. However, there were two participants who requested to increase the number of visit to weekly because they thought that the increased frequency of specialized nursing care would facilitate the wound healing process.

In addition, some participants expressed that complete wound healing occurred within one week in-between the clinic visit. The verbatim report below shows that the wound healing occurred in-between clinic visit.

"In the previous visit, the ulcer was almost healed. According to the comment by the community nurse, the ulcer was completely healed five days after the previous visit." (Participant 23)

Therefore, the present frequency of the visit and the number of time points for data collection in this pilot study may not reflect the accurate time of healing of the DFU. In addition, another issue related to the frequency of clinic visit was the wound fluid collection. In the present pilot study, most of the ulcers did not allow the collection of wound fluid after week four. Since the wound size became small, it was hard to collect the wound fluid. The increase of frequency of clinic visit would increase the time point of wound fluid collection. In short, the frequency of clinic visit should be re-considered for improvement.

From the self-reflection of the student investigator, the dropout rate was low in the present pilot study. If the frequency of the visit increases in future main study and other similar studies, the possible increase of the dropout rate may likely occur. Therefore, a balance between these two issues should be made and some strategies should be considered to minimize the dropout rate in the main study.

5.9.1.2. *Duration of clinic visit*

The time for each clinic visit to perform all interventions in the first clinic visit (T_0) and the subsequent visits (T_1-T_7) was about 1.5–2 hours and 1–1.5 hours respectively. In the first clinic visit, the education on self-care management and the clarification on the concept of participants were carried out to the participants. Thus, the duration of each clinic visit was longer than that of a regular clinic visit which was about 30 minutes to one hour. During the clinic

visits, all participants accepted the duration of the visit and no participant requested to leave earlier.

5.9.2. Patient education on self-care management

In this study, the student investigator performed all the interventions to the participants and they were carried out in the O&T nurse clinic of Hospital A. Generally, some participants expressed that the contents were appropriate and it was easy to understand. The education session was useful to help them on the clarification of the misconception. Two verbatim reports below show that they found the education was workable for their self-management of DFU.

"Previously, I only concerned the dressing material used on the wound. Now, I understood the frequent showering of the foot and the ulcer before the dressing is also important for the diabetic foot ulcer." (Participant 5)

"The shoes that I wear today were particularly for the foot dorsum ulcer. I perceive that it is good as suggested by the salesman. Now, I know that I need to purchase a diabetic foot because of the foot deformity." (Participant 9)

Since all the necessary information on self-care management was performed in the first clinic visit, most of the participants claimed that they could understand and remember the contents. However, some aged participants could not remember all the self-care issues. If the aged participants came along with their relatives, their relatives helped to repeat the education contents, which they did

not fully understand to them. This was easier for them to get the concept. In short, the contents of the education were appropriate. However, a single education session on self-care management during the first clinic visit in this pilot study may be overloaded for some aged participants.

5.9.3. Off-loading

In this study, the method of off-loading included using the CMI, walking aids, heeling walking and callosity debridement. The CMI was supplied by the colleague with expertise in orthotics. The CMI was produced and usually delivered to the participants smoothly within one week. The participants had no complication on using the CMI. Therefore, the arrangement and logistics for the CMI were feasible in this pilot study.

Furthermore, the participants who needed a walking stick for heeling walking bought the stick themselves. The student investigator demonstrated to the participants on using walking stick and heeling walking. They could return the demonstration correctly after the education. The student investigator did not receive any compliant on this arrangement. Therefore, the arrangement was feasible to them.

In addition, the callosity debridement over the peri-wound region was another off-loading method. On the present clinic visit schedule, the callosity could be debrided timely so that it could not create extra pressure over the DFU. Importantly, callosity debridement was a procedure that needed a special

training. It was found that not all of the nurses were able to perform the procedure.

"Most of the nurses in the GOPD seldom help me remove the callosity. Previously, I asked the nurses in the GOPD to help me debride the callosity but I was refused by her." (Participant 12)

Therefore, it reflected that the callosity debridement was feasible in this pilot study provided that the intervention providers had the skill to perform. The frequency of callosity debridement in this study was appropriate to reduce the local pressure effectively.

5.9.4. Sharp debridement

The equipment needed for the sharp debridement procedure included scalpel, scissors and forceps. The procedure should be performed in a room or compartment with privacy. Based on the above requirements, this procedure could be performed in an ordinary clinic setting. Throughout this pilot study, there was no adverse event or uncontrolled bleeding occurred during the sharp debridement procedure. Most of the participants did not have any compliant on the procedure. Only some of the participants complained that there was a certain degree of pain during the sharp debridement but they could tolerate. The pain score was ranged from zero to eight out of ten. There were 9.7% participants (n=3) had the pain score that equivalent to or more than seven out of ten. For those participants experienced pain during the procedure, they were advised to

take the oral analgesic one hour before the clinic visit. Therefore, it was feasible to perform the sharp debridement in this pilot study. In addition, similar to the callosity debridement, the sharp debridement procedure was only performed by the student investigator, instead of by other nurses in this study. One of the participants expressed that the community nurses did not perform the debridement upon request. Therefore, the pain control and the skill of the intervention providers on sharp debridement were crucial in the main study and other similar studies.

5.9.5. Topical dressing

5.9.5.1. *Selection of dressing materials*

Throughout the pilot study, there was no participant who requested to change the treatment arm after randomization. There was also no adverse event occurred related to the topical dressing materials.

There was no dropout participant related to the use of dressing materials. The experience from the participants showed that it was feasible to use those topical dressings although some of them had mild pain (tingling sensation). Nine participants (29.0%) expressed that there was mild pain on the use of nAg dressing and honey dressing. The pain lasted for few hours especially on the first application. The range of pain scores on the 2nd clinic visit (T_1) for nAg and MH dressings for the nine participants were one to three (mean=1.77; SD=2.38) and one to four (mean=2.00; SD=2.26) out of 10 respectively. The verbatim reports

below showed the feasibility of the topical dressings from the participants' point of view.

"I feel mild tingling sensation after the application of the nAg dressing after a few hours. It subsides in the afternoon." (Participant 18)

"I had mild irrigating sensation during the first week of the application of the MH dressing. The average pain score was 2 to 3 out of 10. It was acceptable because the irritation only lasted for 3 to 4 hours." (Participant 19)

5.9.5.2. *Topical dressing application*

The nurses in GOPD or community nurses performed the dressing change in-between clinic visit. From the comments of the participants, the nurses in GOPD and community nurses performed the standard dressing change with the prescribed dressing materials. The participants told the student investigator that the nurses in GOPD dressed their ulcers according to the prescription.

"I've brought the prescription note that you gave me to the GOPD. The nurses used the dressing method on me for the dressing change according to your prescription." (Participant 1)

However, some participants expressed that the nurses could not help them to remove the biofilm during the dressing change.

"After you've taught me in the teaching session, I got to know that there is a biofilm over the ulcer surface of the foot dorsum. I tried to remove it during showering before dressing in GOPD. However, I could not remove the biofilm. Then, I asked the nurse in GOPD to remove it but she was unable to remove it." (Participant 16)

From the point of views of student investigator and participants, the GOPD nurse and community nurses could follow the prescription to perform the standard dressing procedure. But they were unable to perform the additional skills (e.g. biofilm removal and sharp debridement) on dressing management.

In addition, the feasibility of dressing material application was also explored from the point of views of other intervention providers including community nurses and nurses in GOPD. The feedback from 10 nurses in different centers of GOPD and community nurses who had the experience to take care of the participants with DFUs in this study was collected (Appendix 17). Their role was to perform the dressing change as the standard dressing procedure according to the dressing instruction. Overall, the mean scores of all items were above 4 out of 5 except handling the MH dressing (score=3.9). The following is the summary of the result (Table 5.16).

Table 5.16. The summary of feedback from intervention providers (N=10)

Item no.		Mean (SD)
1	The prescription of dressing method is clear enough for you to follow.	4.80 (0.422)
2	The dressing is easy to handle. • nAg dressing • MH dressing • Paraffin tulle	 4.50 (0.527) 3.90 (0.738) 4.60 (0.516)
3	You can handle the workload to perform dressing change on participants in this study.	4.40 (0.516)
4	You have enough time to perform the dressing change.	4.50 (0.527)

The MH dressing got the lowest rating on the degree of difficulty in handling the dressing. Some nurses reported that MH dressing was sticky by using forceps and gloves.

"MH dressing keeps sticking on my forceps and even my surgical gloves."

In brief, there was no problem on the dressing materials from the point of views of the participants although some of them expressed that mild tingling sensation on using nAg and MH. On the topical dressing application, the other intervention providers could follow the dressing method, which was prescribed by the student investigator. However, some of the nurses showed deficiency in skills in the application of MH dressing.

5.10. **Feasibility of outcome measures**

5.10.1. Training and support to research assistants on outcome measures

The two research assistants performed the data collection on outcome measures in this pilot study. Research assistant A helped in ABI measurement, wound score charting, wound size measurement, wound healing status assessment, grading the severity of infection and adverse event assessment. She felt that the training was enough with score four out of five. When there was a problem, it was easy for her to seek help and clarify the query (score=4). But she was not so confident of performing data collection (score=3). Among different categories of data collection, she lacked confidence in wound size measurement and grading the severity of infection. Both of them had the score three. Research assistant A was confident in the other categories of data collection including ABI measurement, wound score charting, wound healing status and adverse event assessment. All of them scored five. On the other hand, she expressed that the time schedule was tight occasionally for the data collection (score=3).

Research assistant B was responsible for verifying the wound healing status. She was an experienced advanced practice nurse. She was confident of verifying the healing status (score=5). She felt that training was enough and easy to get help and clarify when there were queries (score=5). Research assistant B felt that there was enough time for the data collection. Below is the summary of the feedback of research assistant A and B (Table 5.17).

Table 5.17. The summary of the feedback of research assistants (N=2)

Items	RA-A	RA-B
Training is enough for you to complete the data collection.	4	5
When there is a problem during the data collection, it is easy for you to seek help.	4	5
When there is a query after the data collection, you can clarify the query timely.	4	5
You are confident of performing the data collection after the training.	3	5
Research assistant A • ABI measurement • Chart wound score • Wound size measurement • Assess wound healing status • Assess severity of infection • Assess adverse event	5 4 3 5 3 5	
Research assistant B • Verify wound healing status		5
You have enough time to collect the data	3	5

Research assistant A scored three on the items of data collection (wound size measurement and assessing the severity of wound infection) and time management. In the open-ended item, research assistant A pointed out the difficulties in measuring size of small wounds and assessing severity of infection.

"When the wound size becomes small, I need to perform the tracing of the ulcer margin very carefully. Otherwise, the error may increase. Sometimes, I want to

further clarify with others on the severity of infection according to the clinical signs especially on the sign of erythematous around the ulcer and the local edema." (Research assistant A)

This is the reason for research assistant A scored three on those items on data collection. She had also the written comment on time management.

"The time schedule is tight sometimes especially when I need to take care of other clinical duties." (Research assistant A)

In short, it reflected that research assistant A perceived that there was a problem on the digital wound measuring device on measuring small wounds and the training on assessing the severity of infection was not sufficient.

5.10.2. Assessment of wound healing status

The two research assistants assessed the wound healing status in this pilot study. One of them assessed the healing status in the clinic and another assessed through electronic photo. From the interview of both research assistants, they expressed that there was no problem on the assessment because they were experienced nurses on wound management. Their assessment on wound healing in the pilot study was the same. As a matter of fact, if there was a difference in the perception on healing status, the participants would be called back to the clinic for reassessment. This arrangement may create bias when the reassessment was to be performed several days later, although it did not happen in this pilot study.

From the self-reflection of the student investigator, the reason of the employment of research assistant B to verify the wound healing status was due to the doubt of the ability of research assistant A since she was not as experienced as research assistant B. Therefore, the ability and the experience of research assistant A was one of the concern in the future main study.

5.10.3. Wound size measurement

The digital wound measurement device was used to measure the wound size. As mentioned in the previous section, research assistant A was not confident in the wound size measurement and her self-evaluation score was only three out of five although wound measurement was performed three times and the average wound size was obtained. The difficulties she mentioned above were the possible reason for her decreased confidence.

From the observation of the result on wound size measurement, which was performed by research assistant A, the student investigator found that most of the small wound size had 10% or above differences following the three-time measurement. Therefore, this digital wound measure device may not be suitable for small wounds.

5.10.4. Wound fluid collection and measurement

Micropipette and transparent dressing were used to collect the wound fluid (Appendix 14). The student investigator performed the collection and

measurement. It was easy for him to manage the method after the appropriate and sufficient training he received. The original plan was to collect the wound fluid till week 12. However, it was found that the amount of wound fluid collected after week 4 was not enough for the analysis by ELISA in most of the ulcers. The amount of wound fluid was much decreased when the ulcers became smaller. In the first 4 weeks, the amount of wound fluid collected was ranged from 50 to 500 μL. After week 4, there was only 5 to 10 μL wound fluid collected in every clinical visit. Therefore, in testing the concentration of biochemical markers, the wound fluid after week 4 was too little to be analyzed and reported. The ELISA method was not the most appropriate analytical method for those wounds with small amount of wound fluid.

5.10.5. Bacteriology

The analysis of bacteriology should include the types of microorganisms and their corresponding quantity. Since the sample size was small in this pilot study, we only analyzed the types of microorganisms. The quantity of each type of microorganisms among groups and longitudinally within the 12 weeks could not be compared because it was not possible to perform numerous sub-group analyses in the small sample size. Therefore, the present result did not show a complete picture in bacteriology. The quantity of each type of microorganisms was also an important parameter for comparison.

5.10.6. Severity of wound infection

The severity of wound infection was categorized into none, mild, moderate and severe according to the local and systemic signs of infection. According to the structure evaluation of research assistant A, she rated her confidence on grading the severity of wound infection was three out of five. The reason for her decreased confidence was stated in the above section. From the self-reflection of student investigator, it showed that the training of research assistant on this aspect in the pilot study was not enough. There was a concern on the accuracy on grading the severity of wound infection by the research assistant if she did not have enough clinical experience.

5.11. **Acceptability of interventions**

5.11.1. Attrition rate

Among all the 31 participants enrolled in this study, there were only three participants withdrawn from this study. The rate of withdrawal was 9.7%. All the withdrawals were related to own disease factors. None of them was related to the acceptability of interventions. In the experimental (nAg) group, one participant discontinued the intervention because she had severe renal derangement and wished to withdraw from the study. In the comparison (MH) group, there was neither loss to follow up nor premature termination. In the conventional group, two participants discontinued the intervention: one suffered a severe chest infection and required hospitalization, and the other was

diagnosed with lung carcinoma during the study period and needed to withdraw from the study because this disease would affect the DFU healing.

5.11.2. Patient education in self-care management

In general, the participants accepted the concept of self-care management. However, there was a query in some participants about the education related to "showering before dressing". It is because this concept is contradictory to the traditional Chinese practice on wound management. Some participants asked several times about the suitability of tap water on wounds. One of the participants questioned about this issue as follows.

"*When showering the ulcer before dressing, is it easy to have wound infection? Is tap water clean enough for the procedure*" (*Participant 10)*

From the self-reflection of the student investigator, the patient education on this aspect was one of the concerns in the future main study and it is necessary to plan strategies to tackle this issue.

5.11.3. Off-loading

All of the participants accepted the use of CMI. However, some young participants did not accept the use of stick. The reasons for the non-compliance would be further elaborated in the compliance section. In addition, all the participants accepted and appreciated the intervention on callosity debridement

for off-loading. No participant refused the procedure. In the first clinic visit, the student investigator explained to the participants the importance of off-loading. This may be the reason why all accepted the callosity debridement procedure. Some of the participants even reminded the student investigator of performing the callosity debridement during clinic visits.

5.11.4. Sharp debridement

No participant refused the intervention on sharp debridement during clinic visit. Before performing the sharp debridement procedure in each clinical visit, the student investigator also obtained oral consent from the participants to confirm their final decision on the previous written consent for participating this study. It had a certain degree of pain for the intervention. The result of the pain score of sharp debridement as presented as above. In general, the pain level was acceptable to all of the participants. The verbatim reports below are some examples for the acceptability.

"It only had minimal pain during the sharp debridement since my sensation level of my foot was low." (Participant 22)

"I felt moderate to severe pain on average during the procedure. But there was a sharp pain during the puncture onto the wound bed. The pain level was about 7 to 8 out of ten. You say that it can facilitate the healing process so it is acceptable to me." (Participant 25)

From the reflection of student investigator, the pain control needed to be managed in the future main study although no participants refused the sharp debridement procedure.

5.11.5. Topical dressing

No participant asked for changing the allocated topical dressing nor were they withdrawn from the study related to the acceptability of topical dressing. The participants in the conventional group were also satisfied with the topical dressing. It was because they saw the improvement with the topical dressing applied. The verbatim report below demonstrates the acceptance.

"Thank you for your care and the use of topical dressing. The improvement is obvious. The wound is almost healed over these several weeks of dressing."

Noteworthy is that the MH dressing has a distinct sweet smell, which comes from manuka honey. No participant felt that it was an unpleasant smell. Some of the participants liked the smell indeed. One of the participants had the malleolar ulcer and mentioned:

"I like the smell of honey from the MH dressing. My grandchild even shows the curiosity about using honey as dressing material on the ulcer." (Participant 20)

In short, from the student investigator's and intervention provider's perspective, it was found that all of the participants accepted the three types of topical dressings.

5.12. **Adherence to intervention**

5.12.1. Education on diabetic control and foot care

5.12.1.1. *Diabetic control*

The average level of HbA1c for all the participants at week 1 was 8.02 mmol/L (SD=1.64) and at the end study was 7.68 mmol/L (SD=1.54) with p=0.219 by using the t-test. Although it was not significant, the HbA1c level was decreased. On the other hand, the nutritional level was increased. The average level of serum albumin was 34.71 mmol/L (SD=5.99) and 38.04 mmol/L (SD=6.53) at week 1 and week 12 respectively (p≤0.0005). The details are shown in Table 5.18.

Table 5.18. HbA1c and serum albumin level at week 1 and week 12 (n=31)

	Week 1	Week 12	p-value
HbA1c level (mmol/ L) [mean (SD)]	8.02 (1.65)	7.68 (1.54)	0.219
Serum albumin(mmol/L) [mean (SD)]	34.71 (5.99)	38.04 (6.53)	≤0.0005**

* p ≤ 0.05
**p ≤ 0.00005

The result indicated that the participants improved the control of diabetes with the selection of appropriate food. It was thanks to the effect on education and monitoring.

5.12.1.2. *Foot care*

As mentioned before, all the participants were educated on performing showering, including the ulcer before the dressing by community nurses in the daily care. Five participants (16.1%) still did not perform the procedure after the 3rd clinic visit (T_2). The reason for the non-compliance was the fear of infection after the advice of other doctors and nurses.

"Some doctors and nurses told me previously that the ulcer should not be showered with tape water. I am afraid of wound infection so I am reluctant to do so."
(Participant 7)

The student investigator checked their compliance by the foot hygiene status in each clinic visit. After further reinforcement and clarification, all of them performed the practice on showering on the 4th clinic visit. In short, the compliance on showering before dressing was a concern on patient education on self-management since it was contradictory to our traditional Chinese concept and it was also affected by the perception of some doctors and nurses. From the reflection of the student investigator, the compliance of foot care in this aspect was another concern in the future main study.

5.12.2. Off-loading

All participants (100%) used the prescribed CMI during the study period. Some participants expressed that the comfort level increased after using the CMI. The verbatim report below demonstrates the good compliance on using the CMI.

"The wound pain over the foot plantar decreased after using the CMI." (*Participant 8*)

However, there were two participants (6.5%) with forefoot plantar ulcer who were not compliance with the use of stick and heeling walking method strictly. The verbatim report below illustrates the reason of their non-compliance.

"I did not accept the use of stick in my relatively young age and I did not feel any pain when the foot stepped on the ground." (Participant 12)

The age of the above participant was about forty. Therefore, the reasons for the non-compliance on using stick were the self-image in relatively young participants as well as painlessness when stepping.

5.12.3. Topical dressing

We envisaged the non-compliance of the dressing method for two consecutive visits; they would be withdrawn from the study. There was no participant

withdrawal case due to the non-compliance of the dressing method. They were all 100% compliance with the dressing method.

5.13. **Effect size calculation for future study**

The calculation of the effect size from this study was used to plan the sample size in the future main study. To measure the effect size of the study, we had to use the primary study outcome, which was the proportion of complete ulcer healing. However, the healing incidence did not have means and standard deviation that were needed to calculate the effect size. Therefore, we adopted the outcome of wound size reduction to analyze the effect size of this study. According to the present sample size, the effect size, standard deviation and the mean of ulcer size reduction, the effect size of the study was calculated based on the online effect size calculator (Ellis, 2009).

The mean ulcer size reduction of nAg, MH and conventional dressing were 97.45% (SD=5.98), 86.25% (SD=15.98) and 77.51% (SD=26.40) respectively. The effect sizes of Cohen for the three comparison groups including the nAg & MH dressings, nAg & conventional dressings and MH & conventional dressing were 0.93, 1.04 and 0.49 respectively.

Since our study objective is to assess the effect of nAg, the effect between nAg and conventional dressings is our major concern. The effect size between nAg and conventional dressings is chosen (1.04). The sample size calculation is based on t-test. By the online sample size calculator G*Power by using 5% significance

level with a statistical power of 80%, the total sample size is 45 subjects and 15 subjects for each group. Based on our result on attrition rate 9.7%, the estimated total sample size is 48 subjects and 16 subjects per group.

5.14. **Summary**

In the first part of this chapter, the result on preliminary effect of the nAg dressing was presented. The participants' profiles in different groupings were not significant among the three groups. In addition, the confounders of "episodes of the use of oral antibiotics" and "episodes of adverse events" were not significantly different among groups.

In the first category, the findings were related to "ulcer healing". Although they were not significant on the proportion of complete healing and the percentage of the change in ulcer size, the nAg dressing had an observable highest value as compared with the MH and conventional dressings. In the second category regarding findings on "inflammation", it had no significant results on both within group and overall group comparison. In the last category of findings on "infection", there were no significant differences among groups in terms of the changes in bacteriology and signs of wound infection. However, both nAg group and conventional group had significant decrease in the types of bacteria throughout the study. A summary of all the results on preliminary effect of nAg is shown in table 5.19.

Table 5.19. Summary on the preliminary effect of nAg

Categories	Outcomes	Within group comparison			Overall groups comparison
		nAg	MH	Conventional	
Ulcer healing	Proportion of complete healing				NS
	Percentage of change in ulcer size				NS
Inflammation	Total protein	NS	NS	NS	NS
	MMP-9	NS	NS	NS	NS
	TNF-α	NS	NS	NS	NS
	IL-1α	NS	NS	NS	NS
Infection	Bacteriology	S	NS	S	NS
	Severity of wound infection				NS

S: Significance
NS: Non-significance

In the second part, the results on the feasibility of the study protocol and acceptability of interventions of this pilot study were presented. The summary of the results on the feasibility and acceptability, which needs improvement in the future main study, are as follows.

- Feasibility of the study protocol
  - Low referral rate
  - Low enrollment rate
  - Insufficient and inappropriate study centers
  - Non-blinding of group allocation on wound fluid analysis
  - Insufficient frequency and long duration of clinic visit

  - Single education session with too many contents delivered
  - Inadequate skill training on MH dressing application to other intervention providers
  - Inadequate training of research assistant A in data collection on grading the severity of wound infection
  - The different time of complete wound healing assessment by research assistant A and B
  - Inappropriate wound size measurement device
  - Inappropriate wound fluid measuring method
  - Inadequate analysis on bacteriology (types and quantity of microorganism)
- Acceptability and adherence of interventions
  - Insufficient reinforcement and attention on patient education in self-care management including DFU cleansing and stick walking
  - Optimize pain control on sharp debridement

Lastly, the power and sample size for the future main study were revealed.

Chapter 6 – Discussion

6.1. Introduction

In the first part of this chapter, the results on the feasibility of the study design and study protocol and acceptability of interventions were discussed. In the second part, the preliminary effect of nAg dressing was discussed. The three categories of findings including "ulcer healing", "inflammation" and "infection" were compared with previous similar studies and critically discussed.

PART I – Discussion: Feasibility and Acceptability

6.2. Feasibility of recruitment process

Several issues were identified regarding the recruitment process in this pilot study. They were the low referral rate, low enrollment rate because of the strict selection criteria, the inappropriate and insufficient study centers as well as recruitment rate. The above issues were discussed in this section.

6.2.1. Referral

Although regular reminders were given to the doctors and nurses concerned who could make the referral, the rate of referral was low (22.6%). The student investigator recruited the majority of the participants directly. There were several reasons for the low referral. Since the doctors and nurses in Hong Kong were very busy with their duties, it was difficult for them to prioritize the referral in their daily jobs. This situation was supported by Foster et al. (2015),

who stated clinician's perception on few eligible patients and the difficulty in prioritizing the referral due to time constraints. Furthermore, Thoma et al. (2010) reviewed that the reasons for the low referral rate was likely caused by the clinician's preference for certain therapies. In this study, some doctors thought that surgery (e.g. partial thickness skin graft) could help patients to heal their ulcers in a short period of time. Some nurses suggested using other interventions (e.g. topical pressure wound therapy) instead of using topical dressing materials. These barriers were unlikely to be managed so replying on clinician's referral for recruitment of participants was unlikely to be feasible.

6.2.2. Enrollment, selection criteria and study center

In our pilot study, the study settings were two hospitals and one GOPD. As mentioned, many DFUs of the patients in the hospitals were rather severe to be excluded in the pilot study and also the potential participants in the GOPD had already started the special dressing by specialty nurses or podiatrists so it was not possible to change their treatment plan and recruit them to the pilot study. These are reasons for the low recruitment rate in this pilot study. There were several similar RCTs (Cazzell et al. 2015; Nasiri et al. 2015; Li et al. 2015) on DFU but the percentage of recruitment in these studies was higher than that in our present pilot study because the recruitment was conducted in the clinic settings. Cazzell et al. (2015) conducted a study on DFU, which compared small intestine submucosa tri-layer matrix (intervention arm) against standard care (control arm). The percentage of enrollment was 78.5% because it included less severe DFU and the study setting was in 11 outpatient DFU centers. Nasiri et al. (2015)

conducted another RCT on testing the topical olive oil on healing DFU. The percentage of enrollment was 51.5%. It included the topical intervention with less severe DFU, and the study setting was also set in a diabetic clinic. Li et al. (2015) carried out a RCT on testing the effect of autologous platelet-rich gel on DFU in a regional hospital in China. The percentage of enrollment was 32.1%. This gel should be used in non-infected DFU so the study must include non-infected DFUs. As the DFU of patients in hospital settings tend to be either moderately or severely infected, the percentage of enrollment was relatively lower in this study than that in the previous studies discussed above.

In short, the evidence has been provided from the above previous studies that clinics tend to be a more favorable setting for recruiting patients with less severe DFUs. In addition, the number of study centers also affected the number of recruitment and enrollment of participants. This is evidenced by Cazzell et al.'s (2015) study, which had a large study sample obtained from 11 centers in United States within 15 months.

6.2.3. Recruitment

In our study, the recruitment rate was high (88.6%) and comparable with previous studies. The recruitment rate of Hu et al. (2016), Cazzell et al. (2015), Nasiri (2015) and Li et al. (2015) were 98.1%, 97.6%, 77.3% and 100% respectively. The reasons of the non-participants in this pilot study were unable to follow up regularly in the twelve-week study period and unwilling to join. The high recruitment rate in this study was likely that the rapport was built between

the patients and the student investigator through daily care before the enrollment of study. Moreover, there was a clear explanation to the potential participants for the purpose and advantage of the study and the logistics of care during the study by the student investigator. The student investigator found that the explanation to the patients on the advantage of enrollment in the study was a more useful strategy to attract their participation in the study, resulting in an increased the recruitment rate. The advantage included the shorter period of waiting time for follow-up and the potential improvement in their DFU healing. However, in the future main study, the recruitment may not be carried out in the hospital. The rapport between intervention providers and potential participants may not be as strong as that in this pilot study. Therefore, strategies to attract their participations should be considered.

6.2.4. Summary of the feasibility on recruitment process

The shortcomings of the recruitment process in this pilot study were identified as follows. They are inadequate referrals by doctors and nurses, hospitals as study settings being less favorable than clinics in the community, and insufficient number of study settings adopted. They all should be addressed to increase the recruitment rate of participants for the future main study.

6.3. Feasibility of randomization and blinding

Arain et al. (2010) pointed out that one of the purposes of the pilot study was to ensure the randomization procedure running smoothly. In the pilot study,

research assistant C performed the randomization well and the randomization procedure was smooth. It was related to the sufficient training to the research assistant as to how to use the online software. Demonstration and return demonstration were performed. The student investigator performed the clarification on the procedure with research assistant C in order to make sure of her understanding. It indicated that the training to the research assistant was important.

The optimal strategy of blinding was to minimize the likelihood of differential treatment of outcomes as many individuals as possible in a trial (Karanicolas et al. 2010). In this pilot study, the student investigator, community nurses and participants were not blinded because they were the intervention providers. Obviously, the non-pharmacological interventions are often harder to blind. The lack of blinding does not automatically indicate a methodologically unsound trial as the objective outcome measure may prevent the bias in other ways (Kent, 2012). In this pilot study, effective blinding of two data collectors (research assistants A and B) on group allocation was adopted although the RA-A was not confident of measuring the wound size on small wounds and assessing the severity of wound infection. However, the collection and laboratory and the wound fluid analysis of each participant was performed by the student investigator. As he was also the intervention provider, potential bias may be created on the execution of the laboratory results. The blinding of laboratory testing needs to be re-considered in the future main study in order to strengthen the blinding and minimize the potential bias.

6.4. **Feasibility of interventions**

6.4.1. Frequency and duration of clinic visit

In this pilot study, the frequency of clinic visit was weekly in the first 4 weeks and then bi-weekly till week 12. The duration of each clinic visit was about one to two hours. The problems we identified are inaccurate time of wound healing within the 12 weeks of the study period and long duration of each clinic visit.

For the study design on the frequency of clinic visit, Li et al. (2015) performed a RCT on chronic refractory DFU and followed the participants in 12 weeks with a frequency wound assessment every three days. The increased frequency of wound assessment showed a higher accuracy of time of wound healing. Lavery et al. (2014) carried out a RCT on chronic DFU that followed up the participants daily till week 12. Similar to Li et al.'s study, the daily follow-up of participants resulted in an increased accuracy of time of complete wound healing (42 days in the intervention group versus 69.5 days in the control group). On comparing with our research design, the studies in Li et al. (2015) and Lavery et al. (2014) were more accurate to assess the real time of complete wound healing. For the more accurate assessment of real time for complete wound healing in the main study, the frequency of clinic visits and the length of trial period should be re-considered. However, Rabinowitz et al. (2009) analyzed in a meta-analysis that the dropout rates were significantly influenced by the length of the trial period. Therefore, the dropout issue should be taken into account and minimized if there

would be an increase of the frequency of clinic visit and length of trial period in the future main study.

In addition, the duration of each clinic visit was long, especially the first clinic visit in which long education for self-care management was provided. The reason for the long duration is the several tasks performed including intervention and data collection in each clinic visit. Undoubtedly, long duration of each clinic visit is likely to increase the participants' drop out rate in studies although this did not happen in the pilot study. To minimize the dropout rate in the main study, the duration of clinic visit and the education section should be shortened.

6.4.2. Patient education on self-care management

As reported in the result chapter, the contents of the patient education were appropriate to the participants for their self-management of DFU and diabetes. However, some aged participants could not remember some steps if they were not accompanied by their relatives who could take important notes for them and remind them. However, it is not feasible to request all aged participants to be accompanied by their relatives to attend the education section in the main study. In addition, the effectiveness of long education section especially to aged participants will be decreased because their attention period may be short. Therefore, the length and contents of the education section and also the education strategies especially to aged participants should be well planned for the main study in view of the effectiveness. Gucciardi et al. (2007) conducted a

survey on the design of education interventions to patients. The majority of patients wished to spend 20–30 minutes each time for the education session.

6.4.3. Off-loading and sharp debridement

Regarding the off-loading and sharp debridement, it was smooth and no special issue was found. Callosity and sharp debridement were the crucial part in these interventions. They are also a risky procedure and should be performed by a well-trained, qualified and competent health care professional (Bryant & Nix, 2016).

6.4.4. Topical dressing

On topical dressings, the participants accepted the allocated topical dressing in each group. No participants requested to change their group after they had been assigned. A high acceptance of group assignment was received. There were several reasons for the participants' acceptance of the group allocation and allocated topical dressing. Supported by Jenkins & Fallowfield's (2000) study, the trust relationship and rapport between the participants and the student investigator in most cases before the enrollment is the reason for the acceptance of the participants in the pilot study. Besides, there was no complication occurred and the pain level of participants was low in the application of the topical dressings, so the topical dressings were feasible and acceptable to the participants. From the comments of the participants, the intervention providers

(community nurses and nurses in the GOPD) followed the dressing prescription well in performing the standard dressing change.

According to the view of other intervention providers, it was found that the dressing prescription was clear. The amount of workload and the time management for the dressing change were sufficient. It was because the dressing procedure they performed was similar to their standard daily practice. The dressing procedure did not take too much time on each participant. However, some of the nurses found that the MH dressing was sticky covering their forceps and contributed to the viscous and sticky property (Molan, 2002; Alam et al. 2014).

6.4.5. Summary of the feasibility on interventions

To sum up this section, we identified the inadequate frequency of clinic visit and the inadequate length of trial period that resulted in inaccuracy of wound healing time and missing complete wound healing. Besides, there was a long duration of clinic visit that might have impact on the attrition rate. In addition, the competence of the intervention providers on the callosity debridement, sharp debridement and topical dressings application were also a concern. These all should be addressed well for the future main study.

6.5. Feasibility of outcome measures

6.5.1. Training and support to research assistants on outcome measures

Although both of research assistants A and B perceived that the training and support were enough, research assistant A, who was the experienced registered nurse, was not confident about data collection on the wound size measurement and grading the severity of wound infection. It was because using the wound tracing method would create errors if the wound size was small. In addition, grading the severity of wound infection was mostly based on the clinical signs of infection. Occasionally, the clinical signs of wound infection in this pilot study were not concrete so it was difficult for the research assistant to have a definite conclusion.

In addition, research assistant A felt that the workload was heavy to her. It may be due to the fact that she needed to perform all of the assessments in the pilot study. Importantly, her work was not only confined to the study and she also needed to take care of other clinical duties. On the other hand, research assistant B, who was the experienced advanced practice nurse, only needed to verify the wound healing status and the workload was not heavy. She had no problem with the verification of wound healing status and the workload was not heavy to her. Therefore, the experience requirement, training, time management and the workload management of research assistants are important issues, which we have to well address for the quality of research in the main study and other studies in the future.

6.5.2. Clinical Outcome measures

In the pilot study, when there was a discrepancy found in the wound healing status between research assistants A and B, the participant would be arranged an extra clinic visit for the assessment. Although the appointment of the clinic visit would be arranged as soon as possible, it would create a time lag on the date of complete ulcer healing which would definitely affect the result on proportion of complete ulcer healing.

From the investigation of the student investigator, it was found that there was around 10% discrepancy on wound size measurement on small wound in repeated measurement by using the present digital wound measurement device, because line tracing of the wound edge was needed during the measurement process. Wendelken et al. (2011) identified the drawbacks on wound size measurement by line tracing. Firstly, tracings are often complicated by condensation that causes “fogging” of the film surface, which obscures the wound margins. Secondly, lighting conditions often cause reflections and shadows that can prevent the clinician from assessing the wound. Thirdly, it is occasionally hard to recognize migrating epithelium and to determine wounded from unwounded skin. Because of the above, the line tracing method may affect the internal consistency of the study and affect the reliability of this important outcome assessment.

6.5.3. Laboratory outcome measures

In the present study, it was found that the amount of wound fluid collected was not enough after the 4th week for the wound fluid analysis due to the decrease in wound size. It was because, unlike other kinds of wounds (e.g. venous ulcer, traumatic wound), the amount of wound fluid in DFU was relative low (Schmohl et al. 2012). ELISA method was used in the present pilot study for the enzyme measurement including total protein and three enzymes (MMP-9, TNF-α and IL-1α). By using the ELISA method, the requirement of the amount of wound fluid was relatively high.

Indeed, the wound fluid analysis is used to provide deeper insights for the causes of delay wound healing (Löffler et al. 2011). Until now, there was no solid evidence on the appropriate duration on the wound fluid collection. Muller et al. (2008) examined the change of MMPs in DFU. The result demonstrated that the decrease in MMPs concentration was found in the 8th week to the 12th week. Therefore, there was a chance that the change in the concentration occurred after the 4th week. However, we only analyzed the wound fluid till week 4 in this pilot study. Thus, the short duration of wound fluid analysis and the inadequate data of biochemical markers would restrict the assessment of the trend and interpretation of changes of their concentrations during the DFU healing process. This may be reason for the non-alignment between the changes in concentration of biochemical markers and wound size. Because of the lack of solid evidence on the appropriate period of wound fluid analysis, it can only hypothesize that more data on the concentration of the biochemical markers collected can facilitate the

analysis on the healing process and assess a clearer relationship between the changes on the concentration of biochemical markers and the healing outcomes. In order to have analyses with a small amount of wound fluid, other analytical method instead of ELISA should be considered.

On the bacteriology analysis in this study, we only analyzed the types of microorganism. Because of the small sample size, the quantity of the corresponding types of bacteria was unable to be compared among groups. The bacteriology assessment should include the types and quantity of microorganisms (Gardner et al. 2007) for fully investigated would infection so the future main study should involve these two types of analysis.

6.5.4. Summary of the feasibility on outcome measures

In this section, we discussed several issues on outcome measures. They included clinical experience, training and workload management of research assistants, the wound healing status verified by the two research assistants, the wound size measurement device and would fluid analysis method, and complete picture on bacteriology assessment.

6.6. Acceptability and adherence of interventions

6.6.1. Attrition rate

In our pilot study, the attrition rate was not high (9.7%) and all the attrition was related to the patient own disease factors and was not related to the study intervention. Our attrition rate was lower than that of similar DFU studies ranging from 10% to 21% (Zelen et al. 2016; Cazzell et al. 2015; Nasiri et al. 2015). Zelen et al. (2016) carried out a RCT on allogenic human dermis in DFU. The attrition rate was 10%. All of the attrition was related to DFU infection occurred during intervention. Cazzell et al. (2015) performed a study on topical porcine small intestine submucosa in DFU and the attrition rate was 20.7%. The reasons for the attrition included patients' decision, non-compliance, protocol violation and adverse events. Nasiri et al. (2015) conducted a RCT on the application of olive oil in DFU. The attrition rate was 11.7% because the participants were lost along the follow-up period.

Page & Persch (2013) also pointed out that the perceived benefit of the subjects and regular follow-up appointment at similar time of the same day in every week were the strategy to decrease the attrition rate. In this pilot study, the participants knew their DFUs decreased in size during the study period. Moreover, the time for clinic visit was almost at the similar time of the same day in every week. Therefore, it was easy for them to remember the time of the next clinic visit. With this study benefit and study design, different from Cazzell et al.'s (2015) study, we did not have non-compliance and protocol violation.

Other reasons for the low attrition rate in this study was the ease to comply with the topical dressing and minimal side effect of the interventions (Gabriel & Mercado, 2011). In this study, the main intervention was the topical dressing. Because of the perceived benefit from the topical dressings by the participants, they had the good compliance with the topical intervention. In addition, the side effect was only mild tingling sensation after several hours of application of MH and nAg dressings. The participants could well tolerate. Because of the above reasons, the attrition rate in this pilot study was low.

6.6.2. Patient education on self-care management

In this study, the average HbA1c level of participants was decreased, whereas the corresponding average serum albumin level was significant increased when compared with the 1st week and the 12th week. It implied that the participants had well control on the diet and the intake of nutritional food. This implication let us know that they adhered to diabetes self-care management. Al Hohair's study (2013) investigating the effectiveness of the impact of health education to diabetes control reported similar result as our study. It was reported that the HbA1c level of the participants was significant decreased from 8.2% to 7.5% ($p<0.001$), which indicated that the health education on diabetes was effective. There are some factors for improving patients' adherence to diabetes self-care management including building a good rapport with the patients, continuous monitoring and reinforcement of the health behavior (Delamater, 2006). The above factors also applied to our present study. The reasons for good adherence to the diabetes control were related to the clear explanation of the importance of

diabetic control through education on self-management in the first clinic visit. Moreover, reinforcement of the participants' health behavior during each clinic visit and building up a good rapport with them were performed throughout the study period.

Regarding the self-care management on foot care, it was found that 16.1% (3 participants) did not perform showering before dressing after the 3rd clinic visit. In a comprehensive review, Jin et al. (2008) analyzed that patients' belief in the therapy was effective and the perceived benefit was strongly related to their adherence. Moreover, patients' belief and attitude, cultural factors as well as the interpersonal dynamics between carers and patients can affect the patients' adherence (Martin et al. 2005). In the area of foot ulcer care, many patients or even health care workers in our culture thought that chronic ulcers could not receive showering with clean tap water or boiled water directly. Although there is no published literature to address the myth about wound care in traditional Chinese culture, it is common for us to answer patient's query on his worrying about showering the wound with tape water before dressing. Therefore, the adherence to this self-care was relatively low in Chinese culture when compared with other diabetic foot ulcer care concept. Therefore, their misconception in this area should be addressed to improve their self-management of DFU.

6.6.3. Off-loading

The result of this study found that all the participants were willing to use the customer-molded insole. However, two participants (6.5%) did not adhere to the

stick and heel walking method. Both of them were middle-aged, whereas the old aged participants had good compliance. Indeed, age was one of the factors to affect adherence. Jin et al. (2008) analyzed that aged people usually had good adherence to the medical treatment and middle-aged patients had relatively poor adherence and intended to make compliant on the given interventions. Vickery et al. (2008) pointed out that this presented a negative relationship on mobility and self-esteem. Keneckt et al. (2001) revealed that self-esteem was the crucial factor to affect a patient's adherence to medical intervention.

Resnik et al. (2009) further identified the use of mobility aids was associated with aging, physical decline and stigmatizing of weakness. Therefore, for the middle-aged participants in this study, the use of stick for walking may signify an image of getting old and weak. In addition, they may not have any experience of using the stick before. Therefore, the adherence to using walking stick was lower in this age group.

Although all of the participants said that they agreed to the off-loading intervention, some of their adherence was different. As a researcher, we not only interviewed the participants, but also needed to closely monitor their behaviors. We also needed to identify their barriers in order to help them to overcome the obstacles.

6.6.4. Sharp debridement

No participant refused the procedure because they perceived that the sharp debridement procedure was good for their wound healing after the clear explanation on the purpose of the procedure by the student investigator. In addition, they observed that there was a gradual ulcer healing during the 12-week study period. Most participants, however, had mild to moderate pain. A small portion of participants had high pain intensity. They tolerated the pain instead of refusing the procedure. None of the participants refused to attend the clinic or were withdrawn from the study because of the pain issue. The participants accepted and adhered to the procedure because of the perceived benefit of the intervention (Jin et al. 2008). At the end of the first visit in this pilot study, the pain was assessed and we advised them to take analgesics. The majority of participants took panadol 500mg one hour before the sharp debridement procedure. Although the participants took the analgesics, they still suffered from pain during the debridement. As the participants had to receive the sharp debridement procedure, it was not appropriate to let them experience uncontrolled pain because it might increase the dropout rate.

Campbell & Edwards (2012) further highlighted that the ethnic differences in pain perception and pain management method existed. However, there was still no solid evidence on the pain tolerance of Chinese population as compared with other ethics. Rosenthanl et al. (2001) applied the topical anesthetic lignocaine/ prilocaine cream onto the wound bed 30 minutes before the sharp debridement

procedure. As compared with the control group, the topical analgesic could significantly reduce the pain level ($p<0.0001$).

6.6.5. Summary of the acceptability and adherence of interventions

In this section, the attrition rate, non-adherence to showering over the affected foot with tap water before dressing, using stick by the middle-aged participants and wound pain during debridement procedure were discussed. They are required to be managed well in the main study.

PART II – Discussion: Preliminary Effect of nAg

The discussion of the preliminary effect of nAg in this part was based on the three outcomes as presented in Chapter five, which included ulcer healing, inflammation and infection. The overall results showed that there was no significance difference in these three outcomes among nAg, MH and conventional groups. The non-significant results on these outcomes were related to two common reasons. Firstly, the sample size was small and unable to detect the small differences among groups. Secondly, the specialized nursing care including patient education on self-care management, off-loading and sharp debridement decrease the level of differences in the study outcomes.

6.7. Ulcer healing

To our knowledge, there are no previous studies comparing nAg and MH on DFU. Only few studies compared nAg and conventional dressings and also MH and conventional dressings for DFU. The discussions of the results of this study in wound healing were extended to the previous studies with other types of Ag and other types of honey for different types of wounds with inflammation and infection.

6.7.1. Proportion of complete ulcer healing

The present pilot study found that the proportion of complete ulcer healing for participants using the nAg dressing was higher than those using the MH dressing and conventional dressings while those who used MH dressing had higher proportion than those using conventional dressing (nAg > MH > conventional) in DFU healing but they did not reach statistical significance.

The possible major reasons for this non-significant result were due to the two reasons as described above, including the small sample size and the specialized nursing care to all participants. The specialized nursing care was most likely to help the wound healing. In turn, it would reduce the level of differences in the infection and inflammation status of the DFUs among the three groups. It might have result in the non-significant finding in complete wound healing.

The previous experimental studies showed that nursing education could improved patients' knowledge of diabetes, diet control, symptom control, foot care and self-efficacy of foot care (Seyyedrasooli et al. 2015; MakkiAwouda et al. 2014). In the present study, patient education was given during the first visit. The results illustrated participants' high adherence to diet control, foot care (except showing before dressing), off-loading (except middle-aged participants) and dressing regimen. Their understanding and adherence were also monitored during every clinic visit.

In addition, the invasive procedure of sharp debridement was performed to all participants in each clinic visit. From the basic science of wound healing, sharp debridement of DFU on the dead tissue or the avascular tissue until bleeding could stimulate the non-migratory edge epithelium, recruit the cells like neutrophil and macrophage. In turn, it could facilitate immune cell response (Wolcott et al. 2009). They could release chemoattractants like various growth factors and cytokines to the site of injury (Golinko et al. 2008). Indeed, sharp debridement could disrupt and remove mature biofilms (White, 2011). Therefore, it could reduce bacterial loading and inflammation by removing biofilm, and thus the sharp debridement procedure could facilitate ulcer healing.

6.7.1.1. *Discussion on the nAg and conventional dressings*

There was no previous study testing the nAg effect on DFU against conventional dressings. However, there was one previous study investigating the effectiveness of nAg dressing against conventional dressing (plain gauze) on military wounds

with debridement (Fries et al 2014) and it was reported that there was no statistically difference in the days of healing between groups but the days of wound healing in the nAg group were slightly longer than those of the control group indicating that the conventional dressing (plan gauze) is more effective than nAg in wound healing. The inconsistent findings of Fries et al's study and our pilot study are likely caused by the different types of wounds. In our pilot study, there were mild to moderate infection on DFUs and one to three types of microorganisms cultured in DFUs on the first week. Majority of the ulcers were chronic wounds. However, in Fries et al.'s study, about half of the wounds yielded negative culture of microorganism on the intervention and control group respectively. They were acute wounds. The low bacterial loading in Fries et al.'s study might decrease the efficiency of antibacterial effect in nAg dressing. In short, the level of infection and inflammation of wounds between these two studies were different. This may be the difference of the results in the two studies.

6.7.1.2. *Discussion on other types of silver versus local standard dressings*

There was a RCT on DFU conducted by Gottrup et al (2013) to compare the effects of collagen silver and the standard treatment on DFU healing. In the control group, the local standard dressings used in Gottrup et al.'s study were based on the standard treatment protocol in the study center. The study result revealed that significantly higher percentage of healing in the collagen silver group. However, collagen silver contains collagen and silver, which contributed

to DFU healing simultaneously. As the single effect of silver in Gottrup et al's study was unknown, it is difficult to compare their study to our pilot study.

6.7.1.3. *Discussion on MH versus conventional dressing*

The present pilot study revealed that the MH dressing had a higher proportion of healing at the 12th week although it was not significant. There were two RCTs on DFU testing the effect of MH against the conventional dressings (Kamaratos et al 2012, Al Saeed 2013). Kamaratos et al.'s study (2012) found that higher proportion of DFUs in MH dressing group were healed when compared with conventional dressing group although it was also not significant. Indeed, the proportion of healing of all participants in Kamaratos et al.'s study was higher than that reported in the present study. This could be explained by the fact that Kamaratos et al.'s study excluded the participants with ABI < 0.9 so the ulcers had a better blood perfusion, which may result in higher proportion of complete wound healing.

Al Saeed (2013) reported that MH dressing group had a significantly higher percentage of DFU healing than conventional dressing group at six weeks and six months interval respectively. The proportion of healing in Al Saeed's study was much higher than that reported in the present study. It might be due to the reason that surgical interventions including toe amputation were performed to all participants during the study period. This might have facilitated the ulcer healing in Al Saeed's study. The present pilot study, Kamaratos et al.'s and Al Saeed's studies all found that MH had a higher proportion of healing of DFUs

than conventional dressings although the difference was not statistically different in our pilot study.

6.7.2. Ulcer size reduction

Ulcer size reduction is a sign of ulcer healing. It was a study outcome to indicate wound healing in wound care research (Gethin & Cowman, 2008; Miller et al., 2010; Malik et al., 2010). The percentage of the size reduction was the highest in the nAg group (nAg > MH > conventional) but the differences were not statistically significant. The reasons of the insignificance were the same as the proportion of complete ulcer healing. There was no previous study comparing the differences of effects among nAg, MH and conventional dressings on DFU. The following discussions extended to the previous studies on other types of wounds.

6.7.2.1. *Discussion on nAg versus MH dressing to other types of wounds*

There was no previous study tested the effect of nAg dressing against MH dressing on DFU but there was a randomized control study to compare the effects of nAg and MH on wound size reduction of malignant wounds (Lund-Nielsen et al. 2011b). The result revealed that the nAg group had lower percentage of reduction in wound size, than the MH group after the four weeks intervention but the difference was not statistically significant. This result is inconsistent with our pilot study result indicating that higher percentage of wound size reduction in nAg group. The inconsistent findings were probably

related to the difference in wound types. Although malignant wounds also have inflammation and infection for which MH and nAg dressings are considered as appropriate topical dressings for wound healing, the pathology of DFU and malignant wounds are totally different. On the one hand, DFU contains normal cells and the healing goes through the normal four phases of wound healing process. On the other hand, because of the rapid growing of tumor cells, malignant wounds may not have wound healing due to the uncontrolled active dividing cells. This may be the reason for the different findings obtained in the two studies.

6.7.2.2. *Discussion on MH dressing versus usual care to other types of wounds*

The present study found that the MH group was higher in the percentage of ulcer size reduction than conventional group, even though it was not significant. The result was similar to Jull et al.'s open-label RCT (2008b). Jull et al.'s investigated the effect of the MH dressing against usual care on venous ulcer. The result showed that the percentage in ulcer size reduction in the MH dressing group was higher than the usual care but both groups were not statistically significant. The usual care included different types of dressing materials that were available in nursing service such as alginate, hydrofiber and foam. The district nurse determined the usual care according to their practice. The usual care in Jull et al.'s study was various and the wounds were venous ulcer, which were different from the present study. However, both studies' results were similar, reporting that the MH dressing was better in wound size reduction.

To sum up in this section, the present pilot study reported that the nAg dressing had a potentially higher effectiveness than MH and conventional dressings in DFU healing in terms of complete wound healing and wound size reduction although both results did not reach significance. The major reasons were the small sample size and specialized nursing care.

6.8. Inflammation

There are several biochemical markers in wound fluid, including total protein and cytokines TNF-α and IL-1α as well as MMP-9, which are pro-inflammatory biochemical markers. The concentration of these biochemical markers at the 1st week was not significantly different among the three dressing groups indicating that the baseline of these biochemical markers in these three groups was similar. The results on these biochemical markers were discussed below.

6.8.1. Concentration of total protein

In the present study, there were up and downtrends of the total protein concentration in the nAg and MH dressing groups and there was a decreasing trend in the conventional dressing group. In general, the trend of total protein concentration in the three groups was not along with wound healing in the 4-week interval. In addition, there was no significance difference in the concentration between groups at the 4th week and within group between the 1st and the 4th week. In the recent 15 years, there were no studies investigating the change of the total protein level of wound fluid in relation to the wound healing.

Until 2000, James et al.'s study (2000) reported that total protein level of good healing wounds was significantly higher than poor healing wounds. In this pilot study, the observed ulcer healing in chronological order was nAg, MH and conventional group. However, only MH had an increased average total protein concentration on week 4 when compared with week 1. Therefore, the result of this present study in this area was inconsistent with that of James et al.'s study (2000).

6.8.2. Concentration of MMP-9

As shown in the result, the trend of MMP-9 concentration in the nAg, MH and conventional dressing groups were up and down. Same as the total protein concentration, the trend of MMP-9 concentration in the three groups was not along with DFU healing in the 4-week interval. There were no significant differences between groups at the 1st week and the 4th week. There were no differences within group neither when comparing the 1st week and the 4th week in the three groups. On observing the average MMP-9 concentration profile at week 1 and week 4, the MH group had an increase in concentration, whereas both nAg and conventional groups showed a decrease in concentration.

This observable result implied that the nAg and MH dressing groups had poor healing as compared with conventional dressing group because the activities of MMP-9 declined when wound started to heal (Rayment et al. 2008b; Moore et al., 2007). However, as reported above, the nAg dressing group had better observable ulcer healing. Therefore, it was found in this study that the change of

concentration level of MMP-9 was not aligned with the reduction rate of DFU size, which was different from the results of the previous studies.

According to previous studies, it was found that the change in concentration of MMP-9 was negatively correlated to the wound healing status (Trengove et al., 1999; Rayment et al., 2008b, Cullen et al., 2002). The concentration of MMP-9 declined in wound healing in progress. There were several possible reasons for the contradictory findings between the present study and the previous studies. The possible reasons would be discussed in the next section of 6.3.4.

6.8.3. Concentration of cytokines TNF-α and IL-1α

It was found in the present study that the trend of cytokines concentration in the three groups was up and down in the 4-week interval. There was no obvious increasing or decreasing trend. This tendency was the same as the trend of total protein and MMP-9 concentration. In both cytokines TNF-α and IL-1α concentrations, there was no significance in the between groups in the 1st week and the 4th week as well as within groups in the 1st week and the 4th week.

According to the basic science in the literature review, the inflammatory cells produced the pro-inflammatory cytokines including TNF-α and IL-1α. They were activated in the acute inflammation and the concentration decreased when the wounds started to heal (Mohd Yussof et al. 2012; Digelmann and Evans, 2004). However, the nAg group had a higher reduction rate of DFU size and also had a higher TNF-α concentration level than the conventional group. Same as the

MMP-9, the TNF-α concentration was not aligned with the findings in wound healing. In IL-1α, the concentration was lower in the nAg dressing group than in the conventional dressing group, which was aligned with the reduction rate of DFU size.

The non-aligned results of TNF-α concentration and DFU healing in this study were inconsistent with the laboratory studies. Trengove et al. (2000) studied the wound fluid sample from chronic venous ulcer and found the up-regulation of IL-1 and TNF-α in chronic non-healing ulcers. Similar to the previous reasons, the chronicity of DFU in the present study differed from Trengove et al.'s study. In addition, Chan et al. (2012) found in an in vitro study that the neutralization of TNF in diabetic wounds improved the angiogenesis. On the contrary, in the present study, the increased levels of these pro-inflammatory cytokines were observed in the healing phase.

In addition, the association of TNF-α and wound healing was also found in clinical study. Fox et al. (2015) pilot clinical study with five patients with venous leg ulcers found that they had a higher level of TNF-α before intervention and the level of TNF-α was decreased at week four and the wound size was decreased.

6.8.4. Possible explanations for the inconsistent results on biochemical markers

In summary, the result on "inflammation" was associated with the biochemical markers including MMP-9, TNF-α and IL-1α. This present study found that the concentration trend of these biochemical markers did not align with the basic science. The concentration level of these biochemical markers also did not align with the reduction in DFU size. As mentioned before, the possible major reasons for the inconsistent results in this study were the small sample size and specialized nursing care given. The possible reasons were discussed below.

6.8.4.1. *Small sample size and short duration of wound fluid collection*

Small sample size in this present pilot study may be one of the reasons for the non-aligned results between the concentrations of biomarkers and wound healing status. The limited amount of wound fluid collection was another possible explanation. The wound fluid of DFUs was limited when compared with other types of ulcers, e.g. traumatic ulcer. It was because the size of DFU was usually small. The wound fluid of most of the ulcers in the present study could not be collected after the first four weeks. Four week's time might not be long enough for evaluating the trend of concentration and also the wound healing incidence was not high enough in four week's time for assessing the alignment of biomarker concentration and wound healing status. Therefore, the trend of the concentration of the biomarkers did not align with both basic science and clinical result of wound healing. As discussed in the previous chapter, the small sample

size was related to the inappropriate and insufficient study centers in this pilot study.

6.8.4.2. *Specialized nursing intervention: serial sharp debridement*

Serial sharp debridement was performed on every clinical visit during the study period. There were different degrees of bleeding after the debridement procedure. Apart from debridement, the avascular tissue was stimulated to bleed because most of the ulcers involved in this study had tendon and fascia exposed. As a result, acute minor trauma was performed periodically during the study period. The related interventions produced new acute minor trauma and wounding in order to attract neutrophil, cell adhesion molecule and fibroblast to migrate to the local area. The cells were being stimulated, in turn, produced MMPs and cytokines to the local area (Shah et al. 2012). These interventions may probably act as an important confounder to increase the level of MMPs and cytokines. This is the possible reason for not having aligned association between the concentration of biomarkers and the wound healing status.

Utz et al. (2010) investigated the prediction of MMPs in a traumatic wound failure. They collected the wound fluid at the time of debridement in the operation theatre. All the patients were followed for 30 days. Those traumatic wounds with failure closure by skin graft or dehiscence were considered as poor healing. They compared the wound fluid between the two groups. The result showed that MMP-9 was not significant and MMP-3 was significantly lowered in the poor healing group.

Although Utz et al's study (2010) investigated the changes of MMPs in acute traumatic wounds with serial debridement. The wound fluid was not collected through direct aspiration. Rather, the wound fluid was recovered from the foam dressing of vacuum assisted closure device. As discussed in the literature review, the recovery of wound fluid from the absorbent dressing may affect the enzymes concentration inside wound fluid. Besides, the vacuum assisted closure could influence the level of MMPs and cytokines in wound fluid (Glass et al. 2014). These may be the reason for the Utz's result, which was different from the previous studies. Indeed, MMP-9 was an important biomarker for wound healing (Lobmann et al., 2002; Mutter et al., 2008). Therefore, the effect of serial debridement to the changes of MMP level was still unknown.

6.8.4.3. *Infected chronic wound*

Most of the previous studies in this area mainly focused on the chronic non-healing and non-infected wound. Some of them compared the acute wound fluid against the chronic wound fluid (Trengove et al. 1999). One of the studies focused on the non-infected ulcer (Cullen et al. 2002) or chronic non-infected ulcer only (Trengove et al. 2000; Rayment et al. 2008b).

However, in the present pilot study, chronicity of ulcers varied. All the ulcers had bacterial colonization or different degree of local wound infection. It is noteworthy that all of the DFU in this study were in the healing stage during the study period. For infected or heavy colonized wounds, the bacterial endotoxin and biofilm did affect the level of MMPs and cytokines inside the wound fluid.

The continued presence of bacteria within the wound leaded to a further prolonged inflammatory response and further increases in pro-inflammatory mediators, which could up-regulate the host expression (McCarty and Percival, 2013). Furthermore, bacteria could also produce their own proteases that would enhance the overall protease activity (Lantz, 1997). The above might have contributed to non-aligned correlation laboratory and clinical results of this study, which was not supported by the previous studies.

To sum up this section, the changes in the concentration of the biochemical markers not aligning with the clinical observation of wound healing was related to the small sample size, short duration of fluid collection and serial sharp debridement. In addition, our present pilot study's result was not supported by previous studies. It was related to the different nature of ulcers studied and different wound fluid collection method.

6.9. Infection

In the infection category, there were bacteriology and severity of wound infection. The results of these two study outcomes were discussed below.

6.9.1. Changes in bacteriology

From the within-group analysis, the respective mean of the types of microorganism was significantly different in both nAg and conventional groups between the 1st week and the 12th week. However, the mean of the types of

microorganism were not significantly different in the MH group between the 1st week and the 12th week. From the between-group analysis, the mean of the types of microorganism were not significantly different at 1st week and 12th week. In short, there was no significant difference in between groups on the changes of the mean of the types of microorganism between the 1st week and the 12th week although the significant decrease in the types of bacteria within group was found in nAg and conventional groups.

This result was similar to those reported in the vitro and clinical studies. In the in vitro study, Bradshaw (2011) found that there was no significant difference between nAg and MH dressing on common wound pathogen including E. coli, S. aureus and P. aeruginosa. Kamaratos et al.'s clinical study (2012) revealed that there was no significant difference in presence of sterile DFUs between their MH and conventional dressing groups with 63 patients. However, higher percentage of ulcers became sterile in the MH dressing group than that in the conventional dressing group during the 1st week, 2nd week and 4th week. Trial et al.'s study with 42 patients (2012) compared the number of microorganisms in the ulcers with mixed etiologies between silver dressing and silver-free dressing. The results showed that there was no significant difference in the number of microorganisms between groups regardless of the taking of antibiotics during the 15 days of the study period although there was a trend of improving in the silver group. The relative risks of non-Ag group to nAg group were all more than one but they were not statistically significant.

The possible reasons of the insignificant result on the mean of the types of microorganism between groups in our present pilot study were due to several reasons. Firstly, the potency of the antibacterial effect between the nAg and MH dressing groups, nAg and conventional dressing groups and MH and conventional dressing was small, so the small sample size could not detect the difference statistically. Secondly, the specialized nursing care to all groups including showering before dressing as well as repeated debridement of the biofilm and non-viable tissue all contributed to the decrease in concentration in microorganism on the wound bed. It may further decrease the difference of microorganism between groups. In turn, it was difficult to detect the small difference in microorganism among groups. Therefore, no significant difference was detected among groups in the types of microorganism in this study.

In addition, the analysis of the change in bacteriology should include the types and quantity of microorganism (Gardner et al. 2007). In the present pilot study, owning to the small sample size, only the types of microorganism were analyzed. The corresponding quantity of microorganism was not compared among groups. This also needs to be addressed in the future study.

6.9.2. Severity of wound infection

In the present pilot study, there was no significant difference in severity of wound infection among the three groups at each week from the 1st week to the 12th week although the nAg and MH dressings had the antibacterial effects. The result from our study was not supported by previous studies.

An animal study investigating the relative antibacterial potency between MH and nAg revealed that nAg was more effective than MH in killing S. aureus in a rabbit model (Guthrie et al. 2014). When looking at the comparison study between nAg or MH against the conventional dressings in clinical studies, the results were controversial. Gottrup et al. (2013) compared the silver collagen against the control group with the local standard dressings on DFU and found that the silver collagen group had no signs of infection, but around one third of the participants in the control group had infection. Al Saeed (2013) compared the effects of MH dressing versus paraffin tulle on DFU. It showed that MH dressing combined with surgery had an obvious shorter time to eradicate infection than the control group with conventional dressings combined with surgery.

The present study result on the severity of wound infection was different from these previous studies. The possible reasons for this difference are as follows. Firstly, specialized nursing care on patient education on self-care management was given to each participant. They were required to perform showering by running water before dressing. The specialized nursing care on sharp debridement could also remove the biofilm and the non-viable tissue. These procedures could further decrease the bacterial concentration on the wound bed in the three groups. This was likely to cause the level of differences among groups in severity of infection to decrease and the small sample size was unable to test this decreased differences. However, the participants in Gottrup et al.'s (2013) and Al Saeed et al.'s (2013) studies did not have these interventions. Secondly, the episode of antibiotics was not reported and compared in both Gottrup et al.'s (2013) and Al Saeed et al.'s (2013) studies. Although their results

indicated that nAg and MH effectively eradicated infection in their studies, the important confounder on the episode of antibiotics was not compared between groups. Their study results may be subject to bias.

To sum up this part, the possible reason for the insignificant results on the changes in the mean types of microorganism and the severity of wound infection among groups were related to the small sample size as well as specialized nursing care on showering before dressing and debridement. In addition, our present pilot study's result was not supported by previous studies. It was related to the fact that those previous studies did not compare the confounder of episode of antibiotics to compare between groups.

6.10. **Summary**

In the first part, the results of the feasibility on recruitment process, interventions and outcome measures, and the results of the acceptability of the interventions were discussed with literature support. In the second part, the results on clinical and laboratory data were triangulated, explained, discussed and compared with the basic science, in vitro and clinical studies. The possible reasons for the inconsistent results were also discussed.

Chapter 7 – Conclusion

7.1. Introduction

In this chapter, the results of this pilot study were summarized and its strengths were discussed. The implications for this pilot study and the recommendations for conducting the main study which would potentially develop an evidence-based clinical practice and contribute to nursing profession. Lastly, the limitations and suggestions for further studies were also discussed.

7.2. Summary of the results

The prevalence of DFU was increasing (Singh et al. 2005). The impact in terms of the quality of life for patient (Chu et al. 2014) and cost of caring were high (American Diabetes Association, 2008). According to the in vitro evidence, nAg dressing appear to be useful in DFU healing (Chan et al., 2013) since nAg can target to the biochemical deficiencies of DFU. However, the clinical evidence to show the effectiveness was limited. The primary aim of this pilot study was to explore the feasibility of the study design and acceptability of the interventions for promoting DFU healing before the main study to be conducted. The secondary aim was to test the preliminary effect of nAg dressing on DFU healing. The primary study outcome was the proportion of complete ulcer healing and the secondary outcomes were percentage of change in ulcer size, would fluid concentration of total protein, MMP-9, TNF-α and IL-1α; bacteriology and the severity of wound infection.

7.2.1. Summarized results of the feasibility and acceptability test

In the feasibility of the recruitment process (referral, screening and enrollment, participant's selection criteria, recruitment, multi-center approach, randomization and blinding), several areas were identified for improvement in the main study. They were low referral rate, low enrollment rate, inappropriate and inadequate numbers of study centers and non-blinding of group allocation on collection and analysis of wound fluid.

In the feasibility of interventions, the long duration of each clinical visit, insufficient frequency of clinic visit, insufficient length of trial period, lengthy education session, inadequate pain control on sharp debridement as well as inadequate skills of intervention providers on callosity, sharp debridement and the application of specific types of the topical dressing were identified. In the feasibility of outcome measures, several issues were identified. They were insufficient confidence and skills of research assistant regarding assessing the severity of wound infection and measurement of wound size, workload management of research assistants, requirement of verification in wound healing status, inappropriate device for wound size measurement and wound fluid analysis method as well as the analysis on bacteriology (types and number of microorganisms). All these issues have to be managed for improving the feasibility of the main study.

Regarding to the acceptability of interventions by participants, the following issues required to improve in the main study were identified including the

attrition rate, unsatisfactory compliance of the participants with showering before wound dressing and use of stick in middle-aged participants.

7.2.2. Preliminary effect of nAg on DFU healing

The secondary aim of this pilot study was to investigate the preliminary effect of nAg against the MH and conventional dressings in healing DFU. The results were grouped into three categories in terms of healing, inflammation and infection. The nAg dressing group showed no significant difference on proportion of complete healing and reduction of ulcer size as compared with the MH dressing and conventional dressing groups over the 12 weeks study period. However, the nAg group had the highest proportion of DFU healing followed by the MH group and conventional group at the end of week 12.

It was found that the trend of concentrations of all biomarkers fluctuated, instead of showing one direction within the 4-week interval, different from the basic science on these biomarkers. There was no significant difference in the concentration of all biochemical markers (total protein, MMP-9, TNF-α and IL-α) within groups between 1st week and week 4 and between groups at the 4th week. The relationship between the concentration level of all biomarkers and the wound healing status was in contrast to the basic science on would healing and also to the previous studies. The nAg dressing group did not demonstrate a significant decrease in bacteriology and severity of wound infection when compared with the MH and conventional dressing groups. However, the significant within-group differences were found in the level of decreasing

bacteria in the nAg and conventional dressing groups between 1st week and 12th week but it was not in the MH dressing group.

7.3. Strength of the study

This was the first pilot RCT to compare the preliminary effect of the nAg dressing, MH dressing and conventional dressings on DFU healing and also at the same time to explore the feasibility of this study design and the acceptability of the interventions. Apart from its vigorous design and tight control with three treatment groups, this preliminary test had both clinical outcomes and laboratory outcomes, which were categorized as ulcer healing (clinical outcomes), inflammation and infection (laboratory outcomes) to indicate the preliminary effect of nAg. The clinical and laboratory data were triangulated to address the study objectives and hypotheses. Under this design, the study provided multi-dimensional directions to evaluate the effectiveness of nAg when it was compared with MH and conventional dressings on DFU healing.

This pilot study also adopted the multi-center approach for recruitment of the participants. This approach in the main study would strengthen the external validity of the study results and generalizability of the study. Moreover, the specialized care was performed by one intervention provider, the student investigator so that the variances were minimized. The two trained research assistants, who were responsible for data collection, were blind to the group allocation so the detection bias was minimized.

7.4. Recommendations for future main study

The future main study should be improved the feasibility of the study design and acceptability of the interventions to strengthen the interval and external validity of the study. According to the results and the discussion, several areas required for improvement were identified. They were recruitment process, blinding, interventions (clinic visit, education on self-care management, debridement and off-loading), length of the trial period, intervention providers, research assistants, outcome measurement method and device, and minimization of attrition rate.

7.4.1. Recruitment process

The following strategies are recommended for improving the recruitment resulting in large and diverse sample to be obtained for the main study.

7.4.1.1. *Study settings*

In Hong Kong, the patients with DFU admitted into hospitals usually have severe infected ulcer. The DFUs with bone exposed and deep wound infection were not uncommon for hospitalized patients. However, these types of ulcers are not suitable for testing the effectiveness of topical dressing in wound care research. Therefore, hospitals are not the favorable settings for recruitment of participants in the main study. Instead, centers in the community and long-term institutional settings are favorable settings to recruit participants with less severe DFUs.

7.4.1.2. *Multi-center approach*

In order to facilitate the recruitment and minimize the type II error, a multi-center design for a larger and more diverse sample is recommended. The participants can be recruited from both community and long-term institutionalized care settings including GOPD, orthopedic clinics, wound and diabetic clinics, homes for the aged as well as day care centers. After recruitment of participants, the interventions will be performed in the specialized clinics (e.g. diabetic center, orthopedic nurse clinic, wound clinic).

7.4.1.3. *Referral*

In this study, the referral rate by clinicians and nurses was low although regular reminder was given. If hospitals will not be the study settings, referrals from doctors and nurses will not be adopted. Instead, it is recommended to invite in-charge or key person of each center as a study coordinator for each study setting to identify and refer potential participants to principle investigator according to the selection criteria for screening of trained research assistants with nursing background.

7.4.2 Blinding

In order to minimize the bias on the wound fluid collection and analysis, the blinding of group allocation of trained research assistants with nursing background responsible for would fluid collection and also trained research

assistants with bio-medical science background for would fluid analysis in laboratory.

7.4.3. Interventions

7.4.3.1. *Clinic visit and length of the study trial*

In this pilot study, the exact time of healing was not accurate in this pilot study due to the relatively low follow up frequency. In addition, when the DFUs healed after 12 weeks, we did not know the time for complete wound healing. In the future main study, it is recommended to increase the clinic visit frequency to daily. During each visit, a trained research assistant with nursing background as intervention providers is employed. The research assistant will perform dressing change daily and sharp debridement if necessary after wound assessment. In addition, she will reinforce and monitor the participants on the compliance of the self-care practice in off-loading and foot care. Besides, the length of trial period is recommended to extend to 16 weeks. It was because previous study showed that the healing rate of DFU was more than 90% in 16 weeks of study period (Kamaratos et al. 2012).

7.4.3.2. *Patient education on self-care management*

In order to facilitate the understanding, acceptance and compliance on the care practice educated in the session of self-care management, it is suggested to separate the education session from the first visit. It is because the duration of

first clinic visit is too long and the risk of drop out may increase. Besides, the content of the education session in the pilot was too much to be remembered by and draw sufficient attention of participants especially aged participants. In order to solve the problem, a single one to one education session is recommended before the commencement of the first clinic visit. The content of the education session will be re-focused and concise so as to shorten the duration.

As most of participants in the pilot study were older people, appropriate education strategies should be adopted. In order to facilitate participants' learning, each of them would be given a training pamphlet on the self-care tips on diabetes control and foot care, which would be written in words in large font and supplemented with some photos for explanation. Besides, demonstration of required skills will be performed by intervention providers and return demonstration of participants will be performed to assess their understanding. The areas that we identified for poor acceptance and non-compliance in the pilot study (showering before wound dressing as well as stick and heeling walking) will be emphasized and also explore their views on these two issues in the education session for intervention providers' further observation and actions to overcome their cultural barriers and change their misconception if necessary.

7.4.3.3. *Sharp debridement*

Since some of the participants experienced moderate to severe pain during the sharp debridement procedure, appropriate pain management should be well

adopted to minimize participants'' suffering and their dropout due to pain. Participants would be asked to take analgesic one-hour before the procedure if it is subscribed by doctors. If participants do not have the analgesic to be prescribed, intervention providers would ask doctors in clinics to prescribe for them if available. If there is no available doctor in the clinic, the participants will be advised to go to the GOPD to request for analgesic. When the pain scores of participants are still high than five out of ten during the procedure, topical analgesic (e.g. xylocaine jelly) would be added to wound for five minutes for pain control.

7.4.4. Outcome measures

7.4.4.1. *Wound fluid collection and measurement*

In this study, there was limited wound fluid after week four. It is thus suggested to collect the wound fluid two times per week by a trained research assistant with nursing background. Moreover, another method called "flow cytometry" is suggested for analyzing the biochemical markers in wound fluid. Singh et al. (2016) and Tecilazich et al. (2013) used flow cytometry for the analysis of the enzymes in the wound fluid on DFU study. The main advantage of using flow cytometry on performing the biochemical markers analysis was small amount of fluid required and it can test more than one type of biochemical markers simultaneously. In general, the flow cytometry provide sensitivity equal to or better than the conventional ELISA counterpart, require less sample processing

time, and have the ability to measure multiple analysis simultaneously in samples as small as 20 μL or less of sample (Nolan & Mandy, 2006).

7.4.4.2. *Bacteriology*

In the pilot study, we did not analyze the quantity of each type of microorganism because of the small sample. Apart from types of microorganism, the corresponding quantity of each types of microorganism will be also analyzed in the future main study in order to have a complete picture of bacteriology in wounds.

7.4.5. Intervention providers

In the future main study, participants would be recruited from different centers but clinic visits would be only in either diabetic, orthopedic nurse or wound clinics. One fulltime intervention provider would be in each of these clinics to perform study interventions in participants' clinic visits. Intervention providers should be experienced registered nurses having experience in wound management on DFU, callosity and sharp debridement.

The training of intervention providers to ensure their competence in performing the study interventions is the crucial part for the success implementation of study interventions. The student investigator would train up intervention providers on the required skills of the study interventions. As application of MH dressing, callosity, debridement and removal of biofilm were identified in the

pilot study for improvement; the student investigator would pay more attention and efforts to the training on these several areas. Return demonstration of intervention providers would be required to ensure their competency in the required skills

7.4.6. Training and support to research assistants

The research assistants would be responsible for screening the potential participants and collection of all data including wound fluid so they would be blinded of group allocation. One of the comments from the research assistant in the pilot study was the heavy workload. In the future study, the research assistants would be full-time experienced wound nurse who only work for the main study so over-workload issue can be solved. According to the feedback from the research assistant in the pilot study, she was not confident in collection of some data. Therefore, training would be provided to all research assistants in the future main study. The training provided by the student investigator would include the use of all assessment tools and equipment before the commencement of the main study. The area, which the research assistant was not confident to perform, on the identification of severity of wound infection will be emphasized and closely observed in the training. Return demonstration of all research assistants would be required to ensure their competence in the skills required. The inter-rater agreement between the student investigator and each research assistant should be 90% or above on a total of 30 patients. Any problem encountered by research assistants can directly contact the student investigator for his support and problem solving.

In the pilot study, a research assistant who was an experienced advance practice nurse verified the assessment of wound healing by another research assistant who was a registered nurse with less experience in wound care. However, in the main study, verification of wound healing assessment would not be necessary. It is because research assistants responsible for wound healing assessment would be experienced wound nurses. After the intensive training by the student investigator and the inter-rater agreement test to ensure their accuracy of assessment before commencement of the main study, verification of the wound healing assessment would not be necessary.

The line tracing method was not accurate enough to measure the size of small wounds in the pilot study. In the main study, the latest web camera software with iPad would be adopted to measure wound size in order to reduce the bias on the measurement. The training to research assistants on using this software and inter-rater agreement test would be carried out to ensure research assistants' accuracy in this measurement.

7.5. Implications of the study

This study has implications for preparation of the main study, evidence-based clinical practice and nursing profession contribution. The study results shed new light on these three aspects.

7.5.1. Preparation for the future main study

This pilot study identified the strengths and weaknesses of the feasibility of the study design and the acceptability of the interventions. The recommendations would be made to address the weaknesses regarding feasibility and acceptability issues for improvement in the future main study. It also serves as a reference for other similar pilot studies for preparing main studies.

Unlike this pilot study, the previous wound management studies in nursing were mostly focused on the clinical outcome and/or the social science aspects. Triangulation of both clinical and laboratory results to provide the evidence on wound healing in this pilot study has extended the scope of wound management research in nursing. Further, based on the results from the preliminary effect of nAg dressing on DFU, the present trial informs the sample size for the future trial. Using the average percentage of ulcer size reduction and standard deviation from each intervention group, the effect size and sample size of the future study are calculated. It can serves as a reference on similar wound care research.

7.5.2. Potential development of evidence-based clinical practice in nursing

Usually, front-line health care providers base on their clinical judgment to decide type of topical dressing in wound care management. This pilot study has provided preliminary evidence to frontline healthcare providers including nurses on the choice of topical dressing in healing DFU. The future main study which would address the weaknesses regarding the study design and feasibility

of the interventions would potentially develop an evidence-based practice on specialized care and selection of appropriate topical dressing in DFU management for nurses in clinical settings.

7.5.3. Potential development of nursing profession

This study also has implications for education to frontline nurses. They have to be educated on selection criteria for patients to be applied the nAg dressing and also be trained on applying the nAg dressing materials appropriately. In addition, the specialized nursing care in this study including sharp debridement, off-loading and focus of education for self-management of patients has to be educated and trained to improve knowledge and skills of nurses in DFU management. The development and improvement of nurses' knowledge, practice and education in DFU management through the main study would contribute to nursing profession.

7.6. Limitations of the study

Firstly, there are some issues regarding the internal validity of this pilot study. Because of the low referral rate and the insufficient study centers causing small sample size, this pilot trial was not enough power to minimize the risk of Type I and Type II errors in testing the preliminary effect of nAg. Besides, because of the intervention providers put the topical dressing onto the wound, it resulted in no blinding used for this study. Both the participants and intervention providers were aware of the interventions. This limitation may affect the internal validity

of the design and the interpretation of the findings. Moreover, the non-blinding of the wound fluid analysis performed by the student investigator may induce bias on analyzing the result and plotting the result of the biochemical markers concentration from the raw data. As a doctoral degree student project, the research funding was insufficient to employ a research assistant to perform the laboratory testing. It may affect the integrity of the internal validity of the study.

In addition, there was insufficient frequency of clinic visit and induced insufficient observation points to assess the outcomes on ulcer healing in this study. The assessment on complete healing and ulcer size reduction may not be accurate enough to detect the differences among groups. Further, the single education with too much content delivered, some of the participants may not get fully understanding on the self-care issues. The compliance of interventions may be affected and it would affect the outcomes of this pilot study.

Furthermore, regarding to the outcome assessment, there were several limitation discovered. Inadequate confidence of research assistant on the grading of the severity of infection may induce error on the study outcome. It can affect the internal validity of the study outcome assessment. The inappropriateness of wound digital wound size measurement device for small wounds induced another bias of the wound size measurement. The unsuitable wound fluid measuring method (ELISA) caused insufficient observational points for the change of the concentration of biochemical markers so that the long-term change in the concentrations of those makers and the correlation with the clinical outcome could not observe. Further, the evaluation of bacteriology

should include the types of microorganisms and the quantity of each type of microorganisms (e.g. none, scanty, moderate and severe). In the present study, only the types of microorganisms were compared among groups. The corresponding quantity was not taken into account. It was because further sub-groups analysis could not be carried out given the small sample size.

There are two types of nAg dressing products (Type A and Type B) available in the market. They have different concentrations of nAg. Margaret et al. (2006) revealed that Type B was less satisfactory for P. aeruginosa than Type A. A recent comparison study found that the rate of wound size decreased was higher in Type A when compared with Type B (Lee et al. 2010). In the present study, Type A of nAg was used for the experimental group. Therefore, the study results may not be applied to the patients who applied Type B of nAg dressing. Similarly, the results of the present study may not be generalized to other types of Ag and honey dressings.

Moreover, in between the clinic visits, different nurses in GOPD cleaned and changed the dressing of the participants and different community nurses changed the dressing during their home visit to the participants. The prescription of one type of dressing according to the group allocation was given to each participant and s/he presented the prescription to the nurses in the GOPD or community nurses who cleaned and changed the dressing. The nurses possessed different experiences and skill levels. Some of the nurses may perform more than the prescription (e.g. biofilm removal from the wound bed). Such extra skill could facilitate the ulcer healing. This was considered as a confounder

of wound healing in this study. However, this kind of handling is indeed the nursing practice in Hong Kong and hence poses difficulties in avoiding this confounder.

The recruitment of participants in this study was under restricted inclusion and exclusion criteria. These selected participants were those mostly beneficial to the topical intervention. In addition, it was the sampling bias. Convenience sampling method was used so that the participants in this study might not represent the overall population of patients with DFU. The generalization of the results would be limited if these would be adopted in the main study.

7.7. Conclusion

This was the first pilot RCT to test the feasibility of the study design, acceptability of the interventions and the preliminary effect of nAg on DFU. The study results were summarized. The proportion of complete wound healing and reduction rate of ulcer size in the nAg group is the highest among the three groups although the differences were not statistically significant. The concentration level of the biomarkers in wound fluid was not aligned with the healing status of ulcers but this is the first trial to have both clinical and laboratory outcomes to indicate wound healing, which has extended wound care research in nursing. Several weaknesses of the feasibility and acceptability were summarized. The strengths of the study including the rigorous design, multi-center approach for recruitment of the participants and blinding of group

allocation for two research assistants responsible for collection of clinical data were discussed.

The recommendations were provided to improve several areas, which affect the internal and external validity of the main study At last, this study has implications for preparation of the main study and potential development of evidence-based practice on specialized care and use of appropriate topical dressing in DFU management and potential development of nursing profession. The limitations of this pilot study were also discussed.

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Review Article

The Anti-Inflammatory and Antibacterial Action of Nanocrystalline Silver and Manuka Honey on the Molecular Alternation of Diabetic Foot Ulcer: A Comprehensive Literature Review

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Honey and silver have been used since ancient times for treating wounds. Their widespread clinical application has attracted attention in light of the increasing prevalence of antibiotic-resistant bacteria. While there have been a number of studies exploring the anti-inflammatory and antibacterial effects of manuka honey and nanocrystalline silver, their advantages and limitations with regard to the treatment of chronic wounds remain a subject of debate. The aim of this paper is to examine the evidence on the use of nanocrystalline silver and manuka honey for treating diabetic foot ulcers through a critical and comprehensive review of in vitro studies, animal studies, and in vivo studies. The findings from the in vitro and animal studies suggest that both agents have effective antibacterial actions. Their anti-inflammatory action and related impact on wound healing are unclear. Besides, there is no evidence to suggest that any topical agent is more effective for use in treating diabetic foot ulcer. Overall, high-quality, clinical human studies supported by findings from the molecular science on the use of manuka honey or nanocrystalline silver are lacking. There is a need for rigorously designed human clinical studies on the subject to fill this knowledge gap and guide clinical practice.

1. Introduction

The use of silver as a prophylactic and treatment for infections and other diseases dates back to about 100 BC, when it was used for this purpose by the ancient Greeks and Romans [1]. In the late nineteenth century, there was a resurgence of interest in using silver compounds to treat venereal diseases and eye infections [2]. The topical application of honey was also a common practice for centuries [3] by the Egyptians, Greeks, Romans, and Chinese [4]. The early Egyptians of around 1650 BC were the first to use honey as a component in the topical treatment of wounds, as evidenced from the text of the Smith papyrus [5]. A document from 1392 details wound care practices, including the use of honey, in the Middle Ages [6]. However, the use of honey came to be considered outmoded around the 1940s, following the advent of antibiotics. With the recent increase in multiresistant bacteria due to the overuse of antibiotics in the past few decades, the potential of honey and silver in the management of various chronic wounds such as diabetic foot ulcers, venous ulcers, and pressure ulcers has spurred new interest in the wound care community [7].

Appendix 1

2. The Challenge of Managing Diabetic Foot Ulcers

Diabetic foot ulcer (DFU) is the focus of the present review because of the high prevalence of the disease and the burden it places on the health system. It is estimated that diabetes affects 8.3% of the global population or 382 million people [8]. This number continues to grow, making DFU a major public health problem [9]. Foot ulcer is a common complication, affecting 4%–10% persons with diabetes mellitus [10] and preceding over 85% of amputations in this population of patients [11]. The risk of complication increases with time. The cumulative incidence of DFU increases from 27.3% during the first year of diagnosis to 76.4% five years after the initial diagnosis. The rate of amputation increases from 12.5% to 47.1% [12].

The cost of caring for people with diabetes is exorbitantly high, amounting to $174 billion in the United States in 2007, of which foot ulceration accounted for 24% to 31% [13]. Stockl et al. [14] revealed that the average cost per DFU episode was $13,179, and greater in the case of deep ulcers with coexisting infection and circulation problems (as evaluated using the Wagner classification system). Apart from the financial impact of DFU, patients with DFU experience many limitations in their physical, social, and vocational activities (especially those who were required to undergo an amputation), leading to poor health-related quality of life (HRQOL) [15, 16].

There are multiple factors behind the development of DFU, with neuropathy, peripheral vascular disease (PVD), and foot deformity being the most prominent risk factors [17, 18]. The lack of protective sensation predisposes a person with diabetes to suffering from repetitive trauma and ulcers that do not heal due to poor circulation [19]. Wound healing can be further complicated by bacterial infections [20, 21] and prolonged inflammation [22–25]. The reasons for such prolonged and excessive inflammation are still unclear [22, 26], but they likely involve bacterial colonization, biofilms, and recurrent tissue trauma [27]. Apart from the obvious clinical predisposing risk factors, recent studies have revealed that complex cellular and molecular aberrations such as poor extracellular matrix (ECM) formation, high levels of matrix metalloproteinases (MMPs), and other proinflammatory cytokines, as well as oxidative stress, are responsible for delayed healing [28].

2.1. Cellular Abnormalities. The exact mechanisms behind poor wound healing remain elusive [29]. Loots et al. [30] showed a diminished proliferative capacity and an abnormal morphology of fibroblasts in wounds related to diabetes. Galkowska et al. [31] found in an in vitro study that the healing process of diabetic foot ulcers may be hampered by mechanisms that reduce the accumulation of leukocytes. Waltenberger et al. [32] performed a chemotaxis assay using isolated monocytes from diabetic patients and found that monocytes are less responsible for the vascular endothelial growth factor (VEGF) when compared with normal person. Using immunohistochemistry techniques, Usui et al. [33] discovered that keratinocyte migration and differentiation were impaired along the margin of chronic ulcers in patients with diabetes mellitus. Albiero et al. [34] discovered that wound healing delayed as a result of diabetes was associated with the defective recruitment, survival, and proliferation of BM-derived endothelial progenitor cells in mice. Macrophages isolated from diabetic mice also exhibit greater infiltration by inflammatory M1 macrophages and may contribute to impaired diabetic wound healing [35].

2.2. Poor ECM Formation and High Levels of MMPs. The ECM formation is defective in DFU. ECM creates a scaffold for cellular attachment, which is crucial for wound healing. Blakytny and Jude [29] stated that the disruption in the formation of new ECM as well as the diminished stimulation of cell proliferation results in the lack of a proper scaffold for cellular attachment.

MMPs are zinc-dependent endopeptidases, and their inhibitors are called tissue inhibitors of matrix metalloproteinases (TIMPs). They are excreted by a variety of connected tissue, fibroblasts, keratinocytes, proinflammatory cells such as neutrophil, and macrophage. Those MMPs are regulated by hormones, growth factors, and cytokines in response to signals [36, 37]. The functions of MMPs include influencing cell migration, promoting cellular proliferation apoptosis, modulating growth factors and their receptors, and degrading the structural components of ECM during the remodeling of tissue [37, 38].

In diabetic patients, hyperglycaemia activates the pathways of the mitogen-activated protein kinase to stimulate the production of cytokine and promote inflammation [39]. The high level of MMPs is also the pathological alternation in DFU in biochemical terms. The overexpression of MMPs and elastase breaks down the components of ECM and inhibits growth factors [40]. Lobmann et al. [41] compared the MMP levels of 20 patients with diabetic foot ulcers with those of 12 patients with traumatic ulcers. The results showed that the concentrations of MMP-1 and MMP-9 increased 65-fold and 14-fold, respectively, in the diabetic ulcer biopsies. Muller et al. [42] conducted another cohort study on sixteen patients with neuropathic diabetic ulcers. The results echoed those of Lobmann's study. The levels of MMP-8 and MMP-9 decreased in the good healer group and remained stable in the poor healer group during the 12-week follow-up period.

2.3. High Proinflammatory Cytokines. To heal ulcers, proinflammatory cytokines can chemotactically draw inflammatory cells into the injured area [43]. Lobmann et al. [27] stated that the upregulation of TNF-α and IL-1 stimulates the synthesis of MMP-1 and inhibits the synthesis of collagen. Trengove et al. [44] found in an in vitro study the upregulation of interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) in chronic nonhealing ulcers. On the other hand, decreased levels of all proinflammatory cytokines can improve wound healing. Chan et al. [45] found in an in vitro study that the neutralization of TNF in diabetic wounds improves the angiogenesis.

2.4. High Oxidative Stress. People with diabetes usually have hyperglycemia. There is increasing evidence to suggest

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a causal link between hyperglycemia and oxidative stress leading to cellular damage [40]. Excessively high levels of free radicals cause damage to cellular proteins, membrane lipids, and cell nucleic acids and eventually lead to cell death [46]. In people with diabetes, free radicals (superoxide anion and hydroxyl radial) are formed in disproportionately high levels by glucose oxidation, the nonenzymatic glycation of proteins, and the subsequent oxidative degradation of glycated proteins. The glycated proteins develop further reactions to form advanced glycation end products (AGEs) [47, 48]. The accumulation of AGEs causes the upregulation of proinflammatory cytokines and MMPs that will degrade ECM through the production of reactive oxygen species (ROS) [29]. The production of peroxynitrite anion and peroxynitrous acid [49] can also lead to biological damage [50]. The molecular alternations of DFU are summarized in Table 1.

Because of the molecular alternations of DFU shown in Table 1, DFU is difficult to treat clinically. Many clinicians attempt to apply different topical dressing materials to treat DFU. However, few high-quality studies have been conducted to guide clinical practice on the application of topical dressing materials. In the recent Cochrane database on systematic reviews, no papers related to honey and silver met the inclusion criteria in this review because of the poor quality of the papers [51, 52]. Even though many randomized controlled trials have recently been conducted, they suffer from methodological flaws related to the design of the research, including a small sample size, deficient randomization or blinding, and poor statistical analysis. Moreover, it is difficult to pool together and analyze trials on dressing materials because of the diversity of the studies [53]. At the same time, honey and silver are widely used by clinicians to treat DFU because of their antibacterial effect. In the present review, we examine the evidence relating to the anti-inflammatory and antibacterial actions of nanocrystalline silver (nAg) and manuka honey (MH) to determine their effects on the molecular alternation of DFU.

3. Action of Nanocrystalline Silver

3.1. Antibacterial Effect of nAg. nAg can increase the surface area that is in contact with the wound surface [54]. The bactericidal effect of nAg depends on its size. It is preferable for nAg to be around 1–10 nm in size, as this is the size at which direct interaction can occur with the surface of the cells and within the bacteria [55]. Thus, nAg can increase the bioactivity and solubility of silver to allow chemical reactions to take place [112]. Apart from size, the shape of nAg can also affect their bactericidal effect. Pal et al. [56] found that the bactericidal action of a truncated triangular shape exceeds that of a spherical as well as a rod shape. As regards antibacterial action, Lok et al. [113] revealed the exposure of *Escherichia coli* (*E. coli*) to nAg for a short time. They discovered that the mechanisms of nAg and silver ions were similar but that nAg appears to be significantly more efficient than silver ions. nAg and Ag^+ share the following common bactericidal effects. Silver ions can interact with thiol group-containing enzymes, such as NADH-dehydrogenase II in the respiratory system [57]. This will lead to the formation of hydroxyl radicals and to an attack on the cell itself and subsequently to DNA damage [58]. Besides, silver ions induced apoptotic pathways inside the bacteria, leading to their death [54, 56]. Pandian et al. [59] conducted an in vitro study to echo the apoptotic pathways. They found that bacteria that showed evidence of apoptosis and deoxyribonucleic acid (DNA) were fragmented after exposure to nAg.

In vitro evidence shows that nAg^+ has a unique antibacterial action on cells. There is an electrostatic attraction between nAg^+ and the negative charge cell membranes of bacteria. nAg binds to the modified phospholipid bilayer and induces a massive leakage of protons [60]. When nAg^+ anchors to the bacterial cell wall and causes structural change by forming irregular-shaped "pits" on the bacterial outer membrane, the permeability of the membrane changes and becomes porous [61]. In an electron spin resonance spectroscopy study, Kim et al. [62] further discovered that that nAg generates free radicals, which can damage the bacterial cell membrane. The cell will then progressively release lipopolysaccharides and membrane protein, which will ultimately cause the cell to die. Mirzajani et al. [63] further investigated the effect of nAg on Gram-positive *Staphylococcus aureus*. They discovered that nAg exerted on the bacterial peptidoglycan cell wall and specifically decomposed its peptide part. By attaching to the bonds of glycan strands composed of N-acetylglucosamine and N-acetylmuramic acid, nAg destroyed the bonds and released muramic acid. As a consequence, "pits" were generated on the bacterial cell membrane. However, the exact mechanism that occurred on the Gram-negative membrane remained unknown. McQuillian et al. [64] continued working on Gram-negative bacteria and investigated the effect of nAg on *Escherichia coli*. Using inductively coupled plasma mass spectrometry, they discovered that the primary mechanism of nAg was to interact with and dissolve the outer and inner membranes of a cell; then Ag^+ released into the cell and affected a transcriptional response.

In addition, nAg can dephosphorylate the peptide substrate on tyrosine residues. Mijakovic et al. [65] demonstrated that the phosphorylation of protein substrate in bacteria can influence bacterial sign transduction. Shrivastava et al. [66] found that the inhibition of this mechanism by nAg can influence the signal transduction and stop the growth of bacteria. In summary, the overall effect of nAg is both to inhibit the reproduction of bacteria and to kill them directly.

3.2. Bacterial Resistance to Silver. However, some reports have documented bacterial resistance to silver on possible wound pathogens. The pathogens included *Providencia stuartii* [114], *Enterobacter cloacae* [115], *Pseudomonas* species [116], *Acinetobacter baumannii* [117], and *E. coli* [118]. Laboratory studies found that the efflux of silver ions or the acquisition of genetic material in the form of plasmids was the mechanism of a silver-resistant *E. coli* mutant [119].

Some researchers have claimed that the clinical incidence and probability of multiple mutations remain low even though silver resistance has been documented [120, 121]. Unlike antibiotics, silver attacks more than one of the target specific sites [122] and there is no direct evidence

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Table 1: The molecular alternations of DFU.

Number	Author	Nature of study	Cellular abnormalities
[30]	Loots et al., 1999	In vitro	Fibroblasts decrease proliferative capacity and abnormal morphology
[31]	Galkowska et al., 2005	In vivo	Leukocytes decrease accumulation
[32]	Waltenberger et al., 2000	In vitro	Monocytes in diabetic patient are less reactive to VEGF
[33]	Usui et al., 2008	In vivo	Impaired migration and differentiation of keratinocytes
[34]	Albiero et al., 2011	Animal study	Reduced in the recruitment, survival, and proliferation of endothelial progenitors at the site of the injury
[35]	Kanter et al., 2012	Animal study	Decreased in the polarization and activation of macrophages
Number	Author	Nature of study	Poor ECM formation
[29]	Blakytny and Jude, 2006	Review	AGEs cause the upregulation of MMPs and cytokines that degrades ECM through the production of ROS
[40]	Sibbald and Woo, 2008	Review	The overexpression of MMPs and elastase breaks down the components of ECM and inhibits growth factors
Number	Author	Nature of study	High levels of MMPs
[41]	Lobmann et al., 2002	In vivo	MMP-1 and MMP-9 increased 65-fold and 14-fold, respectively, in diabetic ulcer biopsies
[42]	Muller et al., 2008	In vivo	MMP-8 and MMP-9 remained stable in the poor healer group but decreased in the good healer group
Number	Author	Nature of study	High proinflammatory cytokines
[27]	Lobmann et al., 2005	Review	The upregulation of TNF-α and IL-1 stimulated the synthesis of MMP-1 and inhibited the synthesis of collagen
[39]	McLennan et al., 2008	Review	Hyperglycaemia activates the pathways of mitogen-activated protein kinase to stimulate cytokine production and promote inflammation
[44]	Trengove et al., 2000	In vivo	IL-1, IL-6, and TNF-α are upregulated in chronic nonhealing ulcers
[45]	Chan et al., 2012	In vitro	Neutralization of TNF improves the angiogenesis
Number	Author	Nature of study	High oxidative stress
[47]	van den Berg et al., 2008	In vitro	Free radicals (superoxide anion and hydroxyl radicals) are formed by the oxidative degradation of glycated proteins, which subsequently form AGEs
[49]	Soneja et al., 2005	Review	The production of peroxynitrite anion and peroxynitrous acid can lead to biological damage

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that silver resistance mechanisms confer cross-resistance to antibiotics [120]. Percival et al. [123] continued the work on Ag-containing wound dressings in vitro. They concluded that despite evidence of genetic resistance to silver, all strains were killed following a maximum of 48 hours of exposure to the dressings.

3.3. Anti-Inflammatory Effect of nAg. Compared with the antibacterial action of silver, the exact mechanism of its anti-inflammatory action is still unclear. To our knowledge, the studies that have been published on the subject have focused on the observable effect of the anti-inflammatory action of silver, instead of on the detailed molecular mechanism. The animal and in vivo evidence showing the anti-inflammatory effect of nAg are as follows. Wright et al. [67] discovered that a full-thickness contaminated wound in a porcine model treated with nAg healed more quickly and was accompanied with a decrease in MMP activities as well as with the stimulated apoptosis of polymorphonuclear leukocytes (PMNs). They suggested that enhanced cellular programmed cell death (apoptosis) would decrease the number of cells to release cellular content with numerous cytotoxic compounds such as proteases, oxygen radicals, and various acids, in turn decreasing local inflammation. Bhol et al. [68] had similar findings. Through clinical observations, they found that topical nAg cream was effective at decreasing allergic contact dermatitis on a guinea pig model and that the effect was similar to that achieved using topical steroids and immunosuppressive drugs. Bhol and Schechter [69] further extended their work using a rat model. From a histological and immunohistochemical examination, they discovered that nAg had the ability to inhibit contact dermatitis by suppressing TNF-α and IL-12, as well as inducing the apoptosis of inflammatory cells. Similar to previous findings, Wong et al. [70] showed that nAg was effective at decreasing inflammation through the downregulation of the production of TNF-α, without a significant toxic effect on a peritoneal adhesion model of mice.

Nadworny et al. continued the work that had been conducted on the effect of nAg by using different animal models. The results echoed the previous findings. They further discovered that nAg had the ability to promote healing because some kinds of growth factors were elevated after the topical nAg intervention. Using a porcine model, Nadworny et al. [71] found that treatment with solutions containing nAg resulted in a decrease in proinflammatory cytokines TNF-α and IL-8 expression, with an increase in IL-4, epidermal growth factor (EGF), keratinocyte growth factor (KGF), and KGF-2 expression. Again using a porcine model, Nadworny and colleagues [72] also discovered that nAg was mainly deposited in the epidermis but that cell apoptosis was large in the dermis and minimal in the epidermis. They therefore proposed that the anti-inflammatory effects of nAg were induced by interactions with cells in the top layers of the skin, which then released biological signals resulting in widespread anti-inflammatory activity. nAg induced the downregulation of TNF-α and IL-8 expression, as well as the upregulation of IL-4, IL-10, EGF, KGF, and KGF-2. Bisson et al. [73] furthered the work on the latest nAg topical dressing for a histopathological analysis involving mice. The result demonstrated that nAg dressings have a significant inflammatory effect on a noninfected inflammatory skin model, equivalent to that obtained using topical steroid cream.

In addition, there is some in vivo evidence showing that nAg has an anti-inflammatory effect. Shin et al. [74] performed the work in human cells. The result showed that TNF-α and interferon-γ were significantly inhibited at low concentrations of nAg of over 3 ppm. Mani et al. [75] examined the effect of synthetic nAg on healthy human cells. They also showed that all three cytokines (TNF-α, IL-1β, and IL-6) were inhibited at concentrations ranging from 10 to 20 μg/mL. Obviously, from the published animal and human studies, the anti-inflammatory effect of nAg is clear although the exact pathway is still unknown. Interestingly, all histological findings demonstrated apoptosis of the inflammatory cells induced by nAg, so that avoiding the inflammatory cells produces chemoattractants to induce further inflammation upon bursting. In addition, all of the molecular findings indicated that the TNF-α expression was downregulated. In diabetic wound healing, impairing the proliferation of fibroblasts has been linked to an increase in the level of TNF-α [124]. When the level of TNF-α level was inhibited, there was increase in the number of fibroblasts proliferation significantly [125]. Therefore, we can hypothesize that the anti-inflammatory effect of nAg may be beneficial to the healing of DFU. The actions of nAg are summarized in Table 2.

3.4. Clinical Evidence on the Use of nAg-Impregnated Dressings on DFU. To date, no unifying theories have been established through the above basic science research and brought into the clinical context. Nevertheless, researchers have investigated Ag or nAg topical dressings and tried to link their findings with the observable clinical outcome. Unfortunately, there have been few clinical studies on the effect of nAg on DFU. We searched different databases for studies on the effect of Ag or nAg dressings on DFU that had been published in the past 10 years and identified one case report [126], two case series studies [127, 128], two randomized controlled trials (RCT) [129, 130], and one meta-analysis [52]. Since the effect of nAg is more potent than Ag and the molecular mechanism is not exactly the same, the non-nAg studies were excluded from the analysis. Only one case series study investigated the effect of nAg. Cahn and Kleinman [127] used a nonsurgical approach to treat six patients with diabetic foot abscesses. The abscesses were treated with topical oxygen and drained using nAg foam rope (Polymen Wic Silver Rope). The effect on debridement and oxygen therapy on the diabetic foot abscesses could not be excluded. In this case series study, the patients varied in terms of their history of diabetes (5 to 20 years) and ankle brachial index score (0.57 to 1.63). The duration of their treatment ranged from two to eight months. In addition, three patients suffered from peripheral vascular disease. During the period that they were being treated for diabetic foot abscesses, they also underwent percutaneous revascularization. Although all of the patients with diabetic foot abscess and osteomyelitis had completely healed within

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Table 2 Modes of action of nAg

Number	Author	Nature of study	Mechanism of ant bacterial action
[54]	Kon and Rai, 2013	Review	Increases the surface area contacting the wound surface
[55]	Morones et al., 2005	In vitro	Have a direct interaction on the cell surface and within the bacteria with a diameter of around 1–10 nm
[56]	Pal et al., 2007	In vitro	The bactericidal action of nAg with a truncated triangular shape exceeds that of nAg with a spherical or rod shape
[57]	Matsumura et al., 2003	In vitro	Interacts with the thiol group of respiratory enzymes
[58]	Gordon et al., 2010	Animal study	Inactivates the key enzyme by blinding the thiol group, forming free radicals, and subsequently damaging DNA
[59]	Pandian et al., 2010	In vitro	Interacts with and condenses phosphorus-containing DNA and cytoplasm (apoptosis) and inhibits cell replication
[60]	Dibrov et al., 2002	In vitro	Binds to the modified phospholipid bilayer and induces a massive leakage of protons
[61]	Sondi and Salopek-Sondi, 2004	In vitro	Attaches the negatively charged cell membrane by forming "pits," making the membrane porous and resulting in leakage of intracellular content
[62]	Kim et al., 2007	In vitro	Bacteria release cellular content after the permeability of the cell membrane increases, leading to cell death
[63]	Mirzajani et al., 2011	In vitro	Destroyed the bonds of glycan strands composed of N-acetylglucosamine and N-acetylmuramic acid in the cell membrane of Gram +ve bacteria and causing "pits" to form
[64]	McQuillan et al., 2012	In vitro	Interacts with the outer and inner membrane of Gram −ve bacteria, and then membrane dissolves; Ag^{+} releases into the cell and affects a transcriptional response
[65]	Mijakovic et al., 2006	In vitro	Phosphorylation of the protein substrate in bacteria can influence bacterial sign transduction and cell cycle progression
[66]	Shrivastava et al., 2007		
Number	Author	Nature of study	Mechanism of anti-inflammatory action
[67]	Wright et al., 2002	Animal study	Reduces the activity of MMPs and stimulates the apoptosis of PMNs, leading to a decrease in the release of cytotoxic compounds such as proteases and oxygen radicals
[68]	Bhol et al., 2004	Animal study	Effectively decreases allergic contact dermatitis on a guinea pig model, similar to topical steroids
[69]	Bhol and Schechter, 2005	Animal study	Suppresses the activities of TNF-α and IL-12 and induces the apoptosis of inflammatory cells
[70]	Wong et al., 2009	Animal study	Downregulates the production of TNF-α without having a significant toxic effect on a peritoneal adhesion model
[71]	Nadworny et al., 2010	Animal study	Decreases TNF-α and IL-8 and increases IL-4, EGF, KGF, and KGF-2
[72]	Nadworny et al., 2010	Animal study	Down regulates TNF-α and IL-8 and upregulates IL-4, IL-10, EGF, KGF, and KGF-2
[73]	Bisson et al., 2013	Animal study	Demonstrates a significant inflammatory effect, equivalent to that which results from using topical steroid cream
[74]	Shin et al., 2007	In vivo	TNF-α and interferon-γ are significantly inhibited at low concentrations of nAg
[75]	Mani et al., 2015	In vivo	TNF-α, IL-1β, and IL-6 are inhibited at concentrations ranging from 10 to 20 μg/mL

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two to nine months by treatments using debridement, a topical oxygen extremity chamber, and Polymen Wic Silver Rope, we could not determine the sole effect of nAg foam in this study.

3.5. Cytotoxicity Effect of Silver on Modern Wound Dressings. The cytotoxicity of silver is an issue that has been debated. Lansdown [131] claimed that toxicity from dressings containing silver is rare because in modern dressings the silver is in a controlled-release preparation and some of the silver ions bind to the protein of the wound exudate. On the other hand, in vitro and animal studies have shown that silver dressings have significant cytotoxic effects on keratinocytes and fibroblasts [132, 133]. Van den Plas et al. [134] found that silver dressings induced rapid cell death within two hours; they recommended the use of silver dressings only on critically contaminated wounds. Zou et al. [135] compared different pairs of Ag-based and non-Ag-based dressings with basic materials in vitro. The result showed that human fibroblasts, which were extracted from diabetic patients, decreased in viability by 54–70% and collagen synthesis by 48–68% when they came into contact with the Ag-based dressings compared with the non-Ag-based dressings. They did not suggest discarding Ag dressings but stated that such dressings should be used with caution when treating noninfected diabetic wounds. Therefore, the international consensus on the use of silver is that silver should be discontinued if wound infection is no longer present [136].

However, the findings from recent human studies do not support the view that using modern silver dressings could lead to cytotoxicity. Karlsmark et al. [137] noted that the serum silver level for patients treated with a silver dressings was no higher than the reference value, although five patients experienced a temporary increase in their silver level. Gago et al. [138] provided further evidence that a high level of Ag rapidly reduces infection and results in the faster healing of infected chronic wounds. Lansdown et al. [139] discovered that the ions from a silver dressing penetrate only several millimeters into the wound bed. The available information indicates that the findings from the in vitro studies are inconsistent and that knowledge on the cytotoxicity of Ag and nAg dressings is incomplete. Based on our existing knowledge, it can be said that silver has both anti-inflammatory and antibacterial properties. Indeed, toxicity from the long-term use of silver in clinical practice cannot be completely ruled out, especially when silver is used as an anti-inflammatory moderator instead of an antibacterial agent. Further research is needed in this area to ensure the clinical safety of using silver dressing therapy over the long term [140]. There is both a knowledge gap and a clinical query with regard to the use of silver as an anti-inflammatory moderator for treating wounds with no infection but with inflammation.

4. Action of Manuka Honey

4.1. Antibacterial Action of Manuka Honey. MH, which comes from *Leptospermum scoparium* in New Zealand, exhibits antibacterial activity [76]. Our body of knowledge on its antibacterial mechanism remains incomplete. The antibacterial action of MH is mainly based on its physical properties and on the active ingredient that it contains. First, honey has an osmotic effect, drawing moisture from the environment and dehydrating bacteria [76]. This effect is reduced after dilution by wound exudate [141]. Second, the pH value of MH is between 3.2 and 4.5. This acidic nature can inhibit the growth of most microorganisms, such as *E. coli*, *Pseudomonas aeruginosa* (*P. aeruginosa*), *Salmonella* species, and *Streptococcus pyogenes* [77, 142, 143]. Third, methylglyoxal (MGO) is one of the phytochemical factors with antibacterial activity that have been identified within MH. In vitro studies have revealed that the MGO in MH contains the majority of the nonperoxide activity and that the amount of MGO is closely related to the level of antibacterial activity [78, 79]. Ordinary honey has a limited amount of MGO, ranging in concentration from 1.6 to 135 mg/kg, compared to 38–725 mg/kg in manuka honey [78]. A minimum inhibitory concentration (MIC) for *E. coli* and *Staphylococcus aureus* (*S. aureus*) was observed at 1.1 mM of MGO [79]. Atrott and Henle [80] also found a linear correlation between MGO levels in 61 samples of manuka honey and antibacterial activities. There were also some other unidentified biochemical substances in the MH, which later laboratory studies revealed to also contribute to antimicrobial activity. Kwakman et al. [81] identified some cationic and noncationic compounds that contributed to bactericidal activities against different types of bacteria after the neutralization of MGO. Kato et al. [82] found a glycoside of methyl syringate called "Leptosin," which had a positive correlation with antibacterial activity in MH.

Unfortunately, the exact mechanism contributing to the bactericidal activity of MH remains largely unknown [78]. The antibacterial action of MH was recently explored with the hope of elucidating the related mechanism, but its precise mode of antibacterial action is only just beginning to be understood. In vitro studies have demonstrated that cell division is interrupted and cell separation cannot occur following the formation of septa on *S. aureus* [83] and MRSA [84] when these bacteria are exposed to MH. Roberts et al. [85] observed extensive cellular lysis of *P. aeruginosa* at an MIC concentration of 12% w/v in MH. The honey-treated cells were unable to form microcolonies and two target genes were identified as being involved in the process. In addition, Packer et al. [86] found that MH causes two different proteins to be downregulated and one to be upregulated on *S. aureus*. These two proteins had roles to play in ribosomal function, protein synthesis, the metabolic process, and transcription, indicating that MH could interfere with the ribosome or its translational capacity. Merckoll et al. [144] discovered that honey has bactericidal effects on both planktonic and biofilm-embedded bacteria, since bactericidal substances in honey can penetrate into the biofilm. Maddocks et al. [87] further found that MH decreases the formation of biofilm by inhibiting the *Streptococcus pyogenes* from binding to fibronectin. It has this effect because this binding is important for the colonization of bacteria and the development of biofilm [145]. Iron is necessary to sustain the growth of bacteria. *P. aeruginosa* produced two extensively characterized siderophores to capture iron [146]. Kronda et al. [88] further discovered

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that MH decreased the production of siderophores at both 1/4 and 1/2 MIC, showing that MH impeded the growth of the cells. So far, the above in vitro evidence suggests that there is no single mechanism to antimicrobial action but that a combination of factors results in diverse modes of antibacterial inhibition and killing.

Compared with antiseptics that decrease the bacterial count within minutes, the antibacterial activity of MH is much slower [146]. The latest laboratory studies explain this phenomenon. Kwakman et al. [81] showed that MH does not have a rapid antibacterial effect against different kinds of bacteria in the first 2 hours but that its potency increases after 24 hours. The reason for the slow onset action relates to the fact that MH lacks major factors involved in rapid antibacterial activity, such as bee defensin-1 and hydrogen peroxide. The main active ingredient in the antibacterial effect of MH is MGO. Adams et al. [147] found that MGO forms through the conversion of dihydroxyacetone. This conversion is a nonenzymatic reaction that takes place in the presence of proteins or amino acids. The concentration of MGO is low in freshly produced honey but increases after storage at 37°C.

MH has an anti-antibacterial effect on different microorganisms in vitro. The rate of inhibition depends on the species of bacteria and the concentration of honey [141, 148]. Cooper et al. [89] found that MH would still prevent the growth of *S. aureus* if diluted by a further 7- to 14-fold in vitro. Cooper et al. [90] continued the work and discovered that 17 strains of *pseudomonas* isolated from infected burn wounds could be killed by MH, even when diluted more than 10-fold. Hammond and Donkor [91] investigated the bactericidal effect of *Clostridium difficile* on MH. They found that the corresponding MIC and minimal bactericidal concentration (MBC) were both 6.25% (v/v). Kwakman et al. [81] further discovered that the bactericidal activity of MH could kill *Bacillus subtilis*, *P. aeruginosa*, and *E. coli*. Maddocks et al. [87] identified the bactericidal effect of MH on *Streptococcus pyogenes* in both planktonic cultures and biofilm. Apart from that, MH can also kill antibiotic-resistant bacteria. Cooper et al. [92] found that seven strains of vancomycin-resistance *Enterococci* were inhibited by MH at 4.61 ± 0.51% (v/v). French et al. [93] demonstrated that MH inhibited 18 strains of antibiotic-resistant coagulase-negative *Staphylococci* at dilutions of down to 29.9 ± 1.9% (v/v). Interestingly, MH has a synergistic antibacterial effect with antibiotics. Five antibiotics and MH combinations were found that improve antibacterial effectiveness in vitro [149]. However, MH cannot kill all microorganisms. Lusby et al. [150] revealed that MH is unable to inhibit *Serratia marcescens* and *Candida albicans*.

However, the effect of MGO in diabetic ulcers is debatable. Price and Knight [151] pointed out that MGO changed the structure and function of immunological enzymes to form AGEs and reduced the efficiency of the peripheral blood immune-cell response. Majtan [152] further pointed out that manuka honey contains high levels of MGO and speculated that patients with diabetes may be placed at risk by the use of manuka honey because of its direct negative effect on cells or its indirect effect on the formation of AGEs, which could impair the wound-healing process. In an in vitro study, Sassi-Gaha et al. [153] found that highly reactive dicarbonyls attacked the lysine, arginine (Arg), and cysteine residues of long-lived proteins (e.g., collagens) to form irreversible AGEs, causing changes in collagen pathophysiology. Therefore, there is clearly a paucity of high-quality human studies relating to the use of topical honey to treat diabetic ulcers.

4.2. Anti-Inflammatory Action of Manuka Honey. Honey has been shown to reduce both acute and chronic inflammation, although the mechanism for this anti-inflammatory action is not entirely understood [154]. The antioxidants found in honey are considered to be important determinants of anti-inflammatory activity [155]. The antioxidant properties of honey are beneficial in counteracting advanced glycation and lipoxidation end products, which can induce oxidative stress and inflammation in diabetics [156]. Natural honey contains flavonoids, phenolic acids, and other enzymes. All of the active components work together to provide a synergistic anti-inflammatory and antioxidant effect [157]. Chan et al. [94] revealed that pinobanksin, pinocembrin, luteolin, and chrysin were the major flavonoids found in MH, accounting for 61% of the total flavonoids. Low levels of quercetin and galangin were also found. Flavonoids are known for their anti-inflammatory activity. Cho et al. [96] found that chrysin was able to suppress the activity of proinflammatory enzymes, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOs). Raso et al. [95] discovered that several flavonoids, including quercetin and galangin, also inhibited the expression of COX-2 and iNOs in a concentration-dependent manner.

Honeys from different botanical origins have different components. The following in vitro studies give a clear picture of the anti-inflammatory action of MH. Henriques and colleagues [99] discovered that MH had the strongest antioxidant capacity among the varieties of honey that were tested and that it was able to quench the added free hydroxyl radicals within 5 minutes of being added. This antioxidant capacity contributed to the ability of MH to resolve chronic inflammations, including ulcers. In addition, Tonks et al. [100] revealed that MH stimulated both the proinflammatory cytokines TNF-α and IL-1β and the anti-inflammatory cytokine IL-6 from monocytes. Tonks and his colleagues [97] further found that a 5.8 kDa component isolated from MH stimulated these cytokines via toll-like receptor (TLR) 4. After heat treatment sugar syrup or MH loses this function. This explains why supermarket honey cannot be used as medicinal honey for treating wounds. van den Berg et al. [47] also discovered that the phenolic constituents of MH were able to inhibit the production of ROS and scavenge superoxide anion. Recently, researchers from the University of Waikato investigated the anti-inflammatory activity of MH in vitro. Bean [101] found that MH increased both the expression of the proinflammatory cytokine TNF-α and the anti-inflammatory cytokines Il-10 and IL-1 and the growth factors PDGF and TGF-β. ROS production by phagocytosis was also downregulated in the presence of MH. These findings are in alignment with previous in vitro studies and

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explain why MH may allow inflammation to proceed at a modulated level while simultaneously allowing healing to occur.

Leong et al. [102] determined that MH decreased the production of superoxides by neutrophil in vitro and decreased odema and leukocyte infiltration in a mice model but were unable to pinpoint the specific content contributing to the inflammatory action. Tomblin et al. [98] further discovered that this anti-inflammatory activity of MH directly correlated to the phenolic content through a TLR1/TLR2 signalling pathway. The higher phenolic content produced an elevated anti-inflammatory effect. This result echoed the findings of Leong et al. [102] on the specific content inside MH that is responsible for the anti-inflammatory function. The antibacterial and anti-inflammatory actions of MH are summarized in Table 3.

4.3. The Clinical Evidence on the Use of MH on DFU. In DFU, the local factors that hinder wound healing are a high bioburden and a high inflammatory response. MH has been confirmed to have such properties in vitro. Unfortunately, there is limited high-quality evidence to show its effect in vivo. We reviewed single case studies, case series, and randomized controlled trials on the effect of honey or related products that had been published in the past 10 years. Six single case studies [158–163], five case series studies [164–168], one controlled trial [169], and three RCTs [104, 105, 170] were found on DFU. Various kinds of honey products were applied as interventions in the published studies. Different kinds of honey have various active ingredients and concentrations, so they have been excluded from the present analysis. Only one published case series study [103] was found on the use of MH in leg ulcerations with DFU and two RCTs on DFU [104, 105] (Table 4). Gethin and Cowman [103] reported eight cases on the use of MH in leg ulcerations. Only one case involved DFU, while the others involved leg ulcerations with different etiologies. They revealed that the mean initial wound size was 5.62 cm^2, decreasing to 2.25 cm^2 at the end of the four-week study. No inclusion and exclusion criteria were mentioned for the study and subject to a high risk of detection bias although the outcome assessor was blinded.

Al Saeed [104] performed an RCT using MH impregnated dressing against tulle on DFU. The result showed that the honey dressing was superior in terms of the proportion of healing, rate of amputation, and time to eradicate the infection. Unfortunately, the methodology of the study was flawed. No report was made on how the randomization, concealment, and double blinding were performed. The criteria for inclusion and exclusion in the study were not stated clearly. The antibiotics that were used and any adverse events were not reported. According to the Cochrane Collaboration's "risk of bias" criteria [171], there was an unclear risk of bias in selection, performance, and detection. Kamaratos et al. [105] performed another RCT using MH tulle against saline soaked gauze. The result was that the intervention group healed significantly more quickly than the control group. An unclear risk of detection bias was found in that the blinding of the outcome assessors was not clearly reported. A high risk of selection bias relating to the randomization sequence was predictable, as the participants were assigned to groups I and II in an alternate manner. The inclusion and exclusion criteria were not clearly reported. Although it was found in both RCTs that MH was more effective than tulle dressing, we were not confident enough to come to a solid conclusion because of the high risk of bias in the design of the research. Therefore, a high-quality study with a vigorously designed RCT for DFU is needed to enrich the body of knowledge in this area.

5. Comparison of Nanocrystalline Silver and Manuka Honey Dressing

According to the review in the preceding sections, both nAg and MH have clear antibacterial and anti-inflammatory effects in vitro. In this section, we review the studies conducted in the past 10 years comparing the antibacterial effect, cytotoxicity (Table 5), and clinical effectiveness of silver and honey.

5.1. Antibacterial Effect. Nasir et al. [106] conducted an in vitro study to compare the antibacterial effect of hydrofiber Ag (aquacel Ag) and hydrofiber soaked with manuka honey. They found that that hydrofiber Ag had a greater zone of inhibition (ZOI) than MH for Gram-negative bacteria, but no statistical test was performed for the comparison. Guthrie et al. [107] obtained a similar result. They carried out an animal study on a mice traumatic model contaminated with *S. aureus* and revealed that the nAg group had statistically significantly lower bacterial counts than the MH group. However, Bradshaw [108] conducted an in vitro study and obtained a contradictory result. Bradshaw compared different iodine, honey, and silver wound dressings and found no significant overall difference in ZOI among the three groups. Nevertheless, a significant difference in ZOI was observed between different types of Ag dressings against each bacterium. There could be several reasons for the inconsistent findings. First, the nature of the dressing materials, as a carrier medium to hold the active ingredient of Ag or honey, might have affected the antibacterial activity. Second, the origins of the honey used in these studies differed. Third, the chemical nature of silver and its concentrations in different commercial brands varied.

In addition, Lund-Nielsen et al. [109] conducted a single blinded RCT to study the qualitative bacteriology in malignant wounds using honey-coated and silver-coated dressings. No significant differences were found between the groups. However, this result may not be generalizable to other types of wounds since malignant wounds are characterized by continuous tissue deterioration with a large volume of necrotic tissue and slough. This may lead to a high bioburden.

5.2. Cytotoxicity. Surprisingly, the in vitro findings comparing the cytotoxicity of silver and honey are inconsistent, since silver has well-known cytotoxic effect, as shown in previous in vitro studies. Du Toit and Page [110] conducted an in vitro comparison of both types of dressing with regard to the cell morphological effects of keratinocytes and fibroblasts.

Appendix 1

Table 3: Modes of action of manuka honey.

Number	Authors	Nature of studies	Major findings on antibacterial action
			Physical property
[76]	M. D. Mandal and S. Mandal, 2011	Review	Osmotic effect can draw water from bacteria and dehydrate them
[77]	Molan, 2001	Review	Acidity (pH 3.2–5.5) can inhibit the growth of most microorganisms
			Active ingredient
[78]	Adams et al., 2008	In vitro	High concentrations of MGO ranged from 38 to 828 mg/kg as compared with non-MH
[79]	Mavric et al., 2008	In vitro	MGO ranging from 38 to 761 mg/kg can inhibit *E. coli* and *S. aureus* at 11 mM
[80]	Atrott and Henle, 2009	In vitro	MGO ranged from 189 to 835 mg/kg and was directly responsible for the antibacterial property
[81]	Kwakman et al., 2011	In vitro	Glycoside of methyl syringate called "Leptosin" correlated positively with antibacterial activity
[82]	Kato et al., 2012	In vitro	Other than MGO, cationic and noncationic compounds contributed to antibacterial activity
			Mechanism of action
[83]	Henriques et al., 2010	In vitro	Honey-treated cells fail to proceed cell division and separation
[84]	Jenkins et al., 2011		(i) *S. aureus*(ii) MRSA
[85]	Roberts et al., 2012	In vitro	Extensive cell lysis on *P. aeruginosa* at MIC 12% (w/v) after 60 minutes of exposure to MH
[86]	Packer et al., 2012	In vitro	Ribosomal function on *S. aureus* interfered including protein synthesis, the metabolic process, and transcription
[87]	Maddocks et al., 2012	In vitro	Inhibition of the binding of *Streptococcus pyogenes* to fibronectin and the development of biofilm
[88]	Kronda et al., 2013	In vitro	Limit *P. aeruginosa* to capture iron and impede its growth
			Bactericidal activity on different microorganisms
[89]	Cooper et al., 1999	In vitro	*Streptococcus pyogenes*
[90]	Cooper et al., 2002	In vitro	*Pseudomonas* species
[91]	Hammond and Donkor, 2013	In vitro	*Clostridium difficile*
[81]	Kwakman et al., 2011	In vitro	MRSA, *Bacillus subtilis*, *E. coli*, *P. aeruginosa*
[87]	Maddocks et al., 2012	In vitro	*Streptococcus pyogenes*
[92]	Cooper et al., 2002	In vitro	Vancomycin-resistant *Enterococci*
[93]	French et al., 2005	In vitro	Antibiotic-resistant strains of coagulase-negative *Staphylococci*
Number	Authors	Nature of studies	Major findings on anti-inflammatory action
			Active ingredient
[94]	Chan et al., 2013	In vitro	Pinobanksin, pinocembrin, luteolin, and chrysin are the major flavonoids found in MH; low levels of quercetin and galangin were also detected
[95]	Rao et al., 2001	In vitro	Quercetin and galangin inhibit the expression of COX-2 and iNOs in a concentration-dependent manner
[96]	Choi et al., 2004	In vitro	Chrysin suppresses the activity of proinflammatory enzymes
[97]	Tonks et al., 2007	In vitro	5.8-kDa component isolated from MH stimulates the proinflammatory cytokines TNF-α and IL-1β and the anti-inflammatory cytokines IL-6 via toll-like receptor (TLR) 4
[98]	Tomblin et al., 2014	In vitro	Phenolic content is directly correlated to the anti-inflammatory activity of MH through a TLR1/TLR2 signalling pathway

Appendix 1

Table 3: Continued.

Number	Authors	Nature of studies	Major findings on anti-inflammatory action
			Mechanism of action
[99]	Henriques et al., 2006	In vitro	The formation of free radicals such as hydroxyl radicals are inhibited and contribute to resolving chronic inflammation
[100]	Tonks et al., 2003	In vitro	Proinflammatory cytokines TNF-α and IL-1β as well as anti-inflammatory cytokine IL-6 from monocytes are stimulated
[47]	van den Berg et al., 2008	In vitro	ROS production and scavenge superoxide anions are inhibited
[101]	Bean, 2012	In vitro	The proinflammatory cytokine TNF-α and the anti-inflammatory cytokines IL-10 and IL-1 and the growth factors PDGF and TGF-β are upregulated
			ROS production by phagocytosis is downregulated
[102]	Leong et al., 2012	In vitro	The production of superoxides by neutrophil decreased
		Animal study	Leukocyte infiltration and odema in the mice model decreased

Appendix 1

Table 4: The clinical evidence on manuka honey topical dressings on DFU.

Number	Author	Nature of study	Number of subjects	Intervention	Funding	Major findings	Comments
[103]	Gethin and Cowman, 2005	Case series	8	Manuka honey	Not stated	A mean initial wound size of 5.62 cm for all wounds decreased to 2.25 cm at the end of the four-week treatment period.	There was a high risk of detection bias because the outcome assessors were not blinded. There was unclear risk of selection bias because no inclusion and exclusion criteria were mentioned. Only one out of eight ulcers was DFU.
[104]	Al Saeed, 2013	RCT	59	Manuka honey impregnated dressing versus tulle	Self-funded	The percentage of ulcers healed in the honey group (61.3%) was significantly higher than in the control group (11.5%). There were significant fewer toe amputations in the honey group (9.7%) compared with the control group (34.6%).	There was an unclear risk of selection and performance bias because randomization, concealment, and double blinding were not reported. Inclusion and exclusion criteria were not clearly stated. The use of any antibiotics and any adverse events were not reported.
[105]	Kamanos et al., 2014	RCT	63	Manuka honey tulle versus saline soaked gauze	Self-funded	The two groups did not differ significantly in the percentage of ulcers healed at the 16-week follow-up session. The mean healing time in the honey group of 31 ± 4 days was significantly shorter than the 43 ± 3 days for the control group.	There was an unclear risk of detection bias because the blinding of the outcome assessors was not clearly reported. There was a high risk of selection bias because no true randomization was performed. Inclusion and exclusion criteria were not clearly reported. Adverse effects were not reported.

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Table 5: Comparison of the antibacterial effect and cytotoxicity of honey and silver.

Number	Authors	Funding	Nature of studies	Major findings
				Antibacterial effect
[106]	Nasir et al., 2010	University-funded	In vitro	Aquacel Ag (hydrofiber Ag) had a greater zone of inhibition than MH-soaked aquacel in Gram-negative bacteria
[107]	Guthrie et al., 2014	Self-funded	Animal study	Acticoat (nAg dressing) can reduce the bacterial burden more effectively than MH in a heavily contaminated mice model
[108]	Bradshaw, 2011	University-funded	In vitro	There is no significant difference in antibacterial activity between honey and silver dressings, but a significant difference in the strength of activity among different brands of silver dressings
[109]	Lund-Nielsen et al., 2011	Self-funded	In vivo	There is no significant difference in the qualitative bacteriology of malignant wounds between honey-coated and silver-coated dressings
				Cytotoxicity
[110]	Du Toit and Page, 2009	Self-funded	In vitro	Marked cytotoxicity with high nonviability staining and cell-scoring was observed in the nAg group (Actioat) compared with the honey group (L-Mesitran: medical grade natural honey from Netherlands) and the control group
[111]	Tshukudu et al., 2010	Company-sponsored	In vitro	There was no significant difference between the best-performing silver and honey-based dressing extracts with regard to cell viability

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Compared with the honey (30% medical grade honey gel) group and the control (untreated group), the Acticoat (nAg dressing) group had poor keratinocyte and fibroblast cell proliferation. Cell survival, migration, and shape were negatively affected. Tshukudu et al. [111] performed another similar in vitro study on cell viability. A different honey-based (medical grade multifloral honey from Bulgaria) dressing and different silver-based (Ag and nAg) dressings were tested. They found that all the dressing extracts showed variable effects on cell viability and that the exposed cells showed a similar morphology. Acticoat (nAg with alginate) was the most toxic to cells, with less than 30% viability. Interestingly, the group treated with Atrauman silver (Ag tulle contains triglycerides) demonstrated an increase in the number of viable cells as compared with the control group, but we could not establish any solid conclusion on whether the triglycerides contributed to the viability of the cells. In general, nAg still had a marked cytotoxic effect on cells in comparison with the tested honey.

To conclude, the inconsistent findings that were reported on the antibacterial and cytotoxic effects of silver compared with honey dressings might have depended on the types of dressings that were tested. Therefore, the findings from one dressing might not be generalizable to other dressings using similar ingredients.

5.3. The Clinical Evidence from Comparisons between MH and nAg Dressings. In vivo comparisons between honey and silver have been very limited. We reviewed the literature from the past 10 years comparing silver and honey. To our knowledge, three RCTs [172–174] and one retrospective study [175] were published on their effect on burns and malignant wounds. Only one comparative study was published on the use of nAg and MH dressings on malignant wounds. The other studies investigated the effect of different types of honey in comparison with silver sulphadiazine on burn wounds; these studies are therefore excluded from analysis in this review. No comparative study was published on nAg and MH in DFU. Lund-Nielsen et al. [174] compared the application of MH and nAg on malignant wounds and found no differences between MH and nAg in wound healing, cleanliness, mal-odor control, and wound pain. The design of this study was good, with clearly reported inclusion and exclusion criteria, randomization procedure, and allocation concealment. Baseline demographic data between the groups were compared prior to the analysis. Unfortunately, this study still contained some methodological flaws. There was no estimation of the power of the required sample size, no blinding of outcome assessors, and a per-protocol analysis was used instead of an intention to treat analysis. The result was a high risk of detection and attrition bias.

6. Conclusion

To conclude, from the findings of the in vitro and animal studies, both MH and nAg have clear antibacterial and anti-inflammatory effects. They seem to have the capacity to potentially influence the pathogenetic role of a number of mechanisms that contribute to impaired healing and chronicity of DFU. In addition, the in vitro and animal studies produced inconsistent findings on the relative potency of the antibacterial and cytotoxic effects of silver and MH. This may be due to the different types of dressings that were used, with different concentrations of active ingredients. Importantly, there is limited evidence on the clinical effectiveness of MH, nAg, and nAg versus MH in DFU. Only some limited case studies series and loosely designed RCTs in a related area were found. This literature review points to a clear future direction for research in a related area. Based on the best available in vitro evidence, we can justify our practice of using silver or honey in terms of the molecular science. However, there is no strong evidence to show that there is an absolute clinical benefit to using nAg or MH ingredients as topical dressings to treat DFU. For now, we cannot conclude whether nAg or MH is more suitable for treating DFU. Therefore, there is a need for a vigorously designed clinical study with human participants to investigate the solo effect of MH and nAg and to compare their clinical effectiveness.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Ka-Kit Tsang wrote the paper. Enid Wai-Yung Kwong, Kevin Y. Woo, Tony Shing-Shun To, Joanne Wai-Yee Chung, and Thomas Kwok-Shing Wong reviewed the paper.

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治療資訊說明

比較藥用銀海藻敷料丶麥盧卡蜂蜜敷料和常規敷料對治療糖尿腳傷口成效之隨機對照試驗

我們誠邀你參與上述之研究。這個研究是由香港理工大學護理系博士生曾家傑先生進行，由鄺惠容博士監督；並已得到香港理工大學之人類實驗對象操守小組委員會（HSESC）批准進行（HSESC 編號：HSEARS20120415001）。這一次研究是由香港理工大學的研究經費資助。

今次研究目的是比較藥用銀海藻敷料丶麥盧卡蜂蜜敷料和常規敷料對治療糖尿腳傷口的成效。我誠邀你參與這一項研究，因為你現在有糖尿脚傷口，而你的參與將有助改善糖尿腳傷口治療的知識應用。

如果你同意參與這一項研究，你將會有同等機會接受銀海藻敷料丶麥盧卡蜂蜜敷料或常規敷料治療。如果你被隨機分配接受蜂蜜敷料療程，你的傷口就會用麥盧卡蜂蜜海藻敷料。如果你被分配到納米銀敷料組別，你的傷口就會用納米銀海藻敷料。相反，如果你被分配到接受常規敷料療程，你的傷口就會用常規消毒藥水和紗布。

以下是關於三個組別的敷料資料。麥盧卡蜂蜜和納米銀有相同的功用，但是它們有不同的作用機制。它們也是可以用在局部或全層皮膚失去的傷口；功能包括消炎、殺死傷口上的細菌和幫助傷口癒合。另外，常規消毒藥水亦可以有效殺死傷口上的細菌；紗布可保護傷口。

如果你同意參與這項研究；首先，我會跟你量血壓、量度傷口大小、抽血檢查糖尿控制和營養。之後，我會致電通知你是否適合參加。如果合適，我會再致電通知你前來。在第二次回來，我會跟你簽研究同意書和轉介義肢矯型部做鞋墊。弄好之後，我會再安排你回來。之後，在一、二、三、四、六、八、十及第十二個星期再回來覆診。當正式開始研究的時候，我會把傷口拍照片、種菌和抽取傷口滲液去化驗。之後，在每個星期，我們會再次跟你抽取傷口滲液去化驗，亦會取滲液種菌。在每一次覆診，之前的檢查也會按時候跟你做。如果我驗到有什麼特別，我會跟你解釋及因應情況去處理你的問題。還有，我會檢查你活動能力，會轉介你去社康姑娘或街症洗傷口。我會寫張洗傷口紙給姑娘，在你不回來覆診的其它時間，他們就會依照我寫的處方給你洗傷口。

你回來覆診共九次；每一次回來，醫院是會收取六十元覆診費；但敷料和消毒藥水是免費的。我會給與你用到下一次覆診。如果你的傷口有腐肉，我會給你做清創程序。而這一個程序也是平常覆診的常規治療。如果你用蜂蜜和納米銀敷料的時候，有些病人會有輕微刺痛的感覺。如果你用了之後發覺真的有刺痛，我建議你通知我，我會跟你檢查和跟進情況。

在每一次覆診，我會給你解釋你的治療成效和進展。你的參與純屬自願，你有權隨時退出而不受任何懲罰。如果你決定不參與這一項研究，你會分流到一般

的常規治療；而日後的治療不會因為不參加研究而有任何影響。但是如果我發現你連續二次不照指示的方法洗傷口、不按時回來覆診超過一次、在研究其間需要做手術，我們會終止你的測試。有少量病人，對敷料有嚴重敏感或傷口有嚴重發炎；雖然風險很少，但是我們亦會終止你的測試。之後，我將會轉介你到專科診所接受治療。

你的個人資料會<u>完全保密</u>，只會用在研究用途。在未得到你的同意前，你的個人資料(包括姓名和聯絡地址)將絕對不會向研究人員以外的任何人公開。你所有的研究檔案，包括電子相片，只會用研究編號來作記錄。在另一方面， 通過簽訂書面同意書，您即授權臨床研究倫理委員會和監管機構直接核查您的研究數據。如果您對作為研究參與者所享有的權利有任何疑問，可以致電 2958 同九龍中/東聯網臨床研究倫理委員會聯絡。你亦可以聯絡研究員（伊利沙伯醫院骨科顧問護師曾家傑），電話是 3506

如果你想獲取更多的資料，可以致電鄺惠容博士，電話是 2766 。你亦可以聯絡曾家傑先生，電話是 3506 。曾先生的辦工室地址是加士居道 30 號伊利沙伯醫院 C 座 3 樓；電郵地址是 btkkz02@

如果你有任何關於這項研究的投訴，可以書面聯絡香港理工大學研究事務處人類實驗對象操守小組委員會秘書呂小姐，來信請註明該研究所屬之部門及負責研究員。

鄺惠容博士
首席研究員

INFORMATION SHEET

A randomized controlled trial on the effectiveness of nanocrystalline silver, honey alginate and conventional dressing in healing DM foot ulcer

You are invited to participate on a study conducted by Mr. Tsang Ka Kit, who is a post-graduate student of the Department of Nursing in The Hong Kong Polytechnic University under the supervision of Dr. Enid Kwong. The project has been approved by the Human Subjects Ethics Sub-committee (HSESC) of The Hong Kong Polytechnic University (HSESC Reference Number: HSEARS20120415001). This study is funded by the research grant of Hong Kong Polytechnic University.

The aim of this study is to test the effectiveness and the healing ability of the nanocrystalline silver alginate, Manuka honey alginate and conventional dressing materials to DM foot ulcer. We invite you to participate in this study because your participation has an important contribution to enrich the knowledge for the treatment of DM foot ulcer.

If you decide to participate, you will be randomized in either honey dressing group, silver dressing group or conventional dressing group in equal chance. If you are randomized to the honey-dressing group, Manuka honey with alginate "Medihoney gel sheet" will be topically applied to your foot ulcer. If you are randomized to silver-dressing group, nanocrystalline silver with alginate "Acticoat absorbent" will be employed. On the other hand, if you are assigned to the conventional group, antiseptics and paraffin gauze will be used.

Here is some information about the three groups of dressing materials. Both nanocrystalline silver and Manuka honey have similar function but in different mechanisms. They are also indicated for ulcers from partial thickness to full thickness loss. They have anti-inflammatory effect, kill bacteria and have the function to promote ulcer healing. Moreover, antiseptics in the conventional group can also kill bacteria. The outer gauze covering can act as protection to the ulcer.

If you agree to participate in the study, we will measure your blood pressure as well as ulcer size involving your foot ulcer, blood taking for HbA1c level and serum albumin level at the beginning. Then, you will be informed the suitability of getting into the study through telephone. If you are eligible for the intake criteria, you will be invited to go to this clinic again. Obtaining written informed consent and refer colleague from Prosthetic & Orthotic department for off-loading device will be scheduled on your next visit. When the in-sole is available, I will arrange follow up at 1-, 2-, 3-, 4-, 6-, 8-, 10- and 12-week. Clinical photo, wound swab will be taken and wound fluid will be collected at the beginning of the study. Then, wound fluid and wound swab will be collected at every clinic visit. In each visit, the above examination will be performed according to schedule. If we identify any abnormalities during the examination, you will be notified and I will manage your problem accordingly.

Moreover, community nurse (CNS) or general outpatient clinic (GOPC) will be referred in between clinic visit according to your mobility level. The responsible community nurse from the CNS or nurses in the GOPD will perform the dressing change daily or on alternate days according to the instruction in-between the clinic visit time. All the involved nurses in

the community will receive the standard instruction sheets regarding the dressing methods for the respective dressing materials.

In the total 9 clinic visits, you need to pay the clinic attendance fee of $60 as usual. For those dressing materials, they are free of charge and I will provide to you in sufficient amount till next clinic visit. If there is non-viable tissue on your ulcer, I will perform conservative sharp debridement to you. This is the usual practice on the ulcer management in the clinic. For the whole study process, the care is the same as our usual care.

When using Manuka or nanocrystalline silver dressing materials, some of the users may experience minor tingling sensation. This discomfort feeling is normal. If you have this discomfort, you are suggested to tell me and I will assess your condition.

During every clinic visit, any new information that may be relevant to you including your ulcer condition and the effectiveness of the current treatment will be explained and discussed with you. In the study period, you have every right to withdrawn from the study before or during the measurement without penalty of any kind. After withdrawal, you will be triage to routine care and your withdrawal does not affect our care given to you. On the other hand, if I discover that you are non-compliance to the dressing method for 2 consecutive visits, absence from the schedule follow-up for more than 1 time or undergoing operation during the trial period, you will be informed to withdraw from the study. Besides, if you are allergy to the treatment products or severe infection although the chance is very minimal, I will also terminate from the study. Then, I will refer you to the medical colleagues for treatment.

All of your personal information will be kept **completely confidential** and will be used for research purpose only. This means that your record (including your name and contact address) can only be accessed by the research team. All your records including clinical photos will use research code for labeling. At the same time your study data will be granted to the research ethical committee and the regulatory authorities for data verification.

If you have any questions related to your rights as a research subject, please contact Research Ethics Committee (Kowloon Central/ Kowloon East) at 3506 . If you would like to get more information about this study, please contact Dr. Enid Kwong on tel. no. 2766 or Mr. Tsang Ka Kit on tel. no. 3506 ; mailing address: Block C, 3/F, Queen Elizabeth Hospital, 30 Gascoigne Road, Kowloon and email address: btkkz02@

If you have any complaints about the conduct of this research study, please do not hesitate to contact Ms Kath Lui, Secretary of the Human Subjects Ethics Sub-Committee of The Hong Kong Polytechnic University in writing (c/o Research Office of the University) stating clearly the responsible person and department of this study.

Dr. Enid Kwong
Principal Investigator/ Chief Investigator

Case code _____

Clinical Screening Form

Inclusion criteria	**Yes**	**No**
History of type 2 diabetes mellitus		
Living in the community		
Age 40 or above with foot ulcer		
Ulcer with size equals or larger than 1 cm in diameter		
Ulcer located at or below malleolar region of foot		
Superficial wound, wound penetrates to tendon or capsule		
Ulcer without infection or mild to moderate infection		
Subject with no foreseeable surgery within 12-week study period		
Exclusion Criteria	**Yes**	**No**
HbA1c level equals or high than 10 percent		
Severe ischemia with ankle-brachial index (ABI) ≤ 0.4		
Ulcer severe infection		
Ulcer deep into bone and joint		
Osteomyelitis		
Known case of venous ulcer or varicose vein		
Known case of benign or malignant tumor		
Known to have any auto-immune disease		
Known allergy to MH/ nAg		
Participation in other experimental treatment studies		

ABI	Chronic ulcer on *Lt / Rt side	
	Lt	Rt
Brachial		
Dorsalis pedis		
Posterior tibialis		
ABI calculation		

N.B.: - Yes / No ✓as appropriate
- * delete as appropriate
- For any selected subject, all the inclusion criteria should be "Yes" and exclusion criteria should be "No"

Saint Elain Wound Score System (SEWSS) for Diabetic Foot Ulcer

			Score
Anatomical	Location	Phalanges/ digits	1
		Metatarsal	2
		Tarsal	3
	Topographic aspects	Dorsal or plantar	1
		Lateral or medial	2
		Two or more	3
	Affected zones	One	1
		Two	2
		Entire Foot (multiple wounds)	3
Aggravating Factors	Ischemia	No (ABI = 0.9 – 1.1)	0
		Mild (ABI = 0.7 – 0.89)	1
		Moderate (ABI = 0.5 – 0.69)	2
		Severe (ABI < 0.5)	3
	Infection	No	0
		Mild (Erythema < 2cm, induration, tenderness, warmth, and purulent discharge)	1
		Moderate (Erythema > 2cm, muscle. Tendon, or bone or joint infection)	2
		Severe (Systemic inflammatory response)	3
	Edema	No	0
		Periwound	1
		Bilateral secondary to systemic disease	2
	Neuropathy	No	0
		Protective sensation or vibration diminished	1
		Loss of protective sensation or vibration	2
		Diabetic neuro-osteo arthropathy (Charcot)	3
Affected Tissues	Depth	Superficial (skin only)	1
		Deep ulcer (below dermis)	2
		All layers (bone and joint)	3
	Area	Small < 10 cm^2	1
		Medium 10 – 40 cm^2	2
		Large > 40 cm^2	3
	Wound healing phase	Epithelialization	1
		Granulating	2
		Inflammatory	3
		Total score	

Ankle-brachial index measurement

1. A sphygmomanometer, ultrasound gel, portable ultrasound doppler are needed.

2. Ask the patient to lie in a supine position for approximately 10 minutes.

3. Measure the brachial blood pressure on both arms. The higher of the 2 systolic pressures is used to measure the brachial systolic pressure.

4. Place a pressure cuff around the leg above the ankle. Palpate the dorsalis pedis and posterior tibial artery pulses. Then apply ultrasound gel on the skin over the arteries. Place the doppler probe over one of these arteries to identify a signal.

5. Inflate the blood pressure cuff and carefully deflate until a sound is heard. This is the ankle systolic pressure and becomes the numerator in the equation.

6. Calculate the ABI by dividing the higher brachial into the higher ankle pressure.

$$\text{ABPI} = \frac{\text{Highest pressure at the ankle for the leg}}{\text{Highest brachial pressure obtained for both arms}}$$

$$= \frac{\text{Ankle Systolic Pressure}}{\text{Brachial Systolic Pressure}}$$

參與研究同意書

比較藥用銀海藻敷料丶麥盧卡蜂蜜敷料和常規敷料對治療糖尿腳傷口成效之隨機對照試驗

本人_____同意參與由研究生曾家傑先生開展並由鄺惠容博士監督的上述研究。

本人知悉此研究所得的資料可能被用作日後的研究及發表，但本人的私隱權利將得以保留，即本人的個人資料不會被公開。

研究人員已向本人清楚解釋列在所附資料卡上的研究程序，本人明瞭當中涉及的利益及風險；本人自願參與研究項目。

本人知悉本人有權就程序的任何部分提出疑問，並有權隨時退出。而這將不會影響本人所接受的服務或權益。

本人明白所有個人資料會完全保密，並只會用於研究用途。

本人明瞭和同意有關此項研究所需要做的檢查，包括：

- 量體温和血壓
- 抽血
- 傷口種菌、傷口滲液化驗
- 量度傷口大少
- 傷口影相
- 轉介義肢矯形部同事做鞋墊
- 洗傷口
- 傷口清創

參與者姓名 _____
參與者簽署 _____
研究人員姓名 _____
研究人員簽署 _____
日期 _____

副本致 : 參與者 ／ 研究員 ／ 研究檔案

CONSENT TO PARTICIPATE IN RESEARCH

A randomized controlled trial on the effectiveness of nanocrystalline silver, honey and conventional dressing in healing DM foot ulcer

I _____ hereby consent to participate in the captioned research conducted by Mr. Tsang Ka Kit (research student) and supervised by Dr. Enid Kwong.
I understand that information obtained from this research may be used in future research and published. However, my right to privacy will be retained, i.e. my personal details will not be revealed.
The procedure as set out in the attached information sheet has been fully explained. I understand the benefit and risks involved and have had the opportunity to ask questions. My participation in the project is voluntary.
I acknowledge that I have the right to question any part of the procedure and can withdraw at any time, without giving any reason, without my medical care of legal rights being affected.

I understand that sections of any of my medical notes may be looked at by responsible individuals from the current research project or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.

I confirm that I have understood and agreed the treatment protocol in order to complete the research process including

- Checking body temperature and blood pressure
- Blood taking
- Wound swab culture and wound fluid collection
- Ulcer size measurement
- Ulcer photo taking
- In-sole making and amendment
- Wound dressing changing
- Conservative sharp debridement on wound

Name of participant _____

Signature of participant _____

Name of researcher _____

Signature of researcher _____

Date _____

Copies to: Subject / Researcher / File

Appendix 9

Handling of wound infection

- Infection classification of the International Consensus on the Diabetic Foot (Lipsky et al., 2004a; Lipsky et al, 2004b)

Classification	Definition
Uninfected	Wound lacking purulence or any manifestations of inflammation
Mild	■ Infection involving the skin and subcutaneous tissue only, with no involvement of deeper tissues. ■ No systematic sings and symptoms. ■ No other causes of an inflammatory response. ■ Presence of ≥2 manifestations of inflammation • Local tenderness or pain • Local warmth • Localized swelling or induration • Erythematous ≤ 0.5-2 cm around the ulcer • Purulent discharge
Moderate	■ Infection involving structures deeper than skin and subcutaneous tissues (eg. abscess, osteomyelitis, septic arthritis, or necrotizing fascitis) ■ Erythematous (cellulitis) extending > 2 cm around an ulcer in addition to one of the following • Edema • Tenderness • Heat • Purulent discharge ■ No signs of a systematic inflammatory response and metabolic stable
Severe	■ Systemic toxicity or metabolic instability, manifested by two or more of the following • Fever • Chills • Tachycardia • Hypotension • Confusion • Vomiting • Leukocytosis • Acidosis • Severe hyperglycemia • Azotemia

- If the infection status was mild to moderate, participant continues the study.
- If there is moderate signs of infection, he/ she was referred to medical colleague of O&T department QEH for oral antibiotic according to the wound swab result.
- If the infection status was severe, he/ she would be terminated the study.
- He/ she then referred to admission for intravenous antibiotics injection

Study protocol

After the screening of eligible patients as mentioned in the previous section, they would be invited into this study. The protocol for the first visit was as follows.

- The student investigator performed the patient education on diabetic control and foot care as well as referred the participants to make the CMI as indicated.
- In addition, vital signs were measured.
- After removing the old dressing, the wound was cleansed with normal saline.
- Wound fluid was collected by covering the wound with transparent dressing for 30mins to 2 hours until sufficient wound fluid was collected by micropipette.
- Then, the wound was cleansed with soap and water and rinsed with sterile water.
- If there was non-viable tissue, sharp debridement was performed by the student investigator. The scab and crust of the peri-ulcer skin were also removed by scalpel.
- The research assistant A was then called into the consultation room to measure the wound size and take the photo. The assistant was also collected all other related data including risk factors assessment and wound scoring.
- The student investigator then covered the wound by using the topical dressing according to the group allocation result.

There are seven subsequent visits for each subject. In each visit, the following procedures were performed.

- The student investigators measured the vital signs of participants, check for the compliance of participants and the condition of the CMI.
- The procedure was repeated as in the first visit.
- In addition, the research assistant A would assess for any adverse event.
- In the last visit, the student investigator would take the blood to check for the HbA1c and serum albumin in order to collect the objective data for diet control.

The following table is a summary of the screening and data collection in each visit.

Assessment	Pre-study	Week	Clinic visit							
			1st	2nd	3rd	4th	6th	8th	10th	12th
Screening	X									
Visit to O&T nurse clinic			X	X	X	X	X	X	X	X
Time point for data collection			T_0	T_1	T_2	T_3	T_4	T_5	T_6	T_7
Demographic data			X							
Ulcer swab culture			X	X	X	X	X	X	X	X
Collect wound fluid			X	X	X	X	X	X	X	X
Wound size measurement	X		X	X	X	X	X	X	X	X
AE / SAE monitoring				X	X	X	X	X	X	X
Assessment of taking oral antibiotics			X	X	X	X	X	X	X	X

Outcome Record Form

Date of assessment _____

Case code _____

Treatment group honey/ silver/ conventional*

No. of week _____

Wound size _____cm²				
Wound healed or not Yes / No*				
Wound swab culture result Date : _____	Types of bacteria _____ _____ _____ _____	Number of bacteria _____ _____ _____ _____		
Clinical sign of infection	none / mild / moderate / severe*			
Concentration of biomarkers in wound fluid	Total protein _____	MMP9 _____	IL-1α _____	TNF-α _____
HbA1c level	Date : _____ _____ mmol/ L			
Serum albumin level	Date : _____ _____ mmol/ L			

N.B. : *delete when appropriate

Operation procedure on digital wound measurement device

- Turn on the digital wound measurement device.
- Remove the white backing layer from grid paper.
- Place the grid area over the wound. Position the top of the tracing grid in the direction of the patient's head.
- Trace around the edge of the wound margin onto the grid using a permanent marker pen. Care must be taken to remain within the grid area.
- Remove the contaminated wound contact layer from the tracing grid quickly and carefully. Dispose of as clinical waste.
- Place the tracing grid on the surface of the digital unit, fitting the holes in the tracing grid over the pegs on the unit surface.
- Place the stylus at a point on the wound tracing and press the switch on the stylus and wait unit the tracing symbol appears in the display.
- Whilst holding the coversheet/ tracing grid security on the surface of the unit, trace the wound outline on the grid without lifting the stylus. Ensure the tracing is started and finished at the same point.
- When processing is completed, the device will BEEP and display the area measurement in cm^2 in the digital display.
- The wound size measurement is performed three times and the average wound size is obtained.

Reference:

Instruction manual of Smith & Nephew Visitrak Digital. Available online at http://www.medicalplus-pt.com/conteudo/uploaded/videos/pdfs/VisitrakDigital_manual.pdf Retrieved on 4

Would fluid collection for laboratory analysis

Wound fluid was collected in the first four week of clinic visit.

- Ulcer is washed with sterile water before the fluid collection.
- Apply an occlusive dressing – Tegaderm (3M, USA) over the ulcer.
- Exudate accumulated under the dressing after 30 minutes to 2 hour.
- The fluid is extracted by micropipette and collected in 0.4ml Protein Lobind tubes (Eppendorf, Hamburg, Germany).
- The wound fluid samples are centrifuged at 6000 revolutions per minutes (rpm) for 30 minutes, aliquoted and stored at -20 °C until further analysis.
- The protein content for all samples is quantitated and standardised using the BCA Protein assay kit (Pierce Biotechnologies, Rockford, IL, USA).
- The protein content for all samples is quantitated and standardised using the bovine serum albumin (BSA) standard.
- Pro-inflammatory cytokines TNF-α and IL-1α as well as MMP-9 inside the wound fluid are measured by using commercial enzyme-linked immunosorbent assay (ELISA) human kit according to the manufacture's protocols (Abcam, USA).

Total protein measurement inside wound fluid

- Pipette 50μL of reserve osmosis (RO) water into 5 appendorf (polypropylene tubes)
- Pipette 50μL (1000 μg/ml) of protein into the first polypropylene tube
- Repeat the procedure by pipetting 50μL of mixed solution from one tube to others so that serial dilution of 500μg/ml, 250μg/ml, 125μg/ml, 62.5μg/ml and 0μg/ml are prepared
- The protein standards and samples are assayed in duplicate. 10μL of each standard and sample solution are pipetted into separate microtiter plate wells
- Prepare dye reagent (Bio-Rad protein Assay) in 1:5 dilution of stock (600μL stock + 2400μL H_2O = 3ml)
- 200μL diluted dye reagent is added to each well and mixed thoroughly using a microplate mixer
- The plate was incubated at room temperature for at least 5 minutes
- The absorbance is measured at 595nm using a Benchmark Plus™ microplate spectrophotometer
- A relative measurement of protein concentration of sample is obtained from standard curve

Collect wound swab for culture

Levine's Technique

- Cleanse the wound with normal saline
- Select an area near the center of the wound free of necrotic tissue and debris
- Using the wound swab with culture medium
- Swab is then rotated over a 1 cm^2 area for 5 seconds with sufficient pressure to extract fluid form within the wound tissue
- Send swab to laboratory immediately

Appendix 16

Risk Factor Record Form

Case code _____ Treatment group silver / honey / conventional*

Date of assessment _____

Personal risk factor	Gender : M / F	Age : _____ years old
	Ambulatory status: Ambulatory (walk independent/ walk with aids) /Non-ambulatory (chair-bound/ bed bound)*	
	Serum albumin level Date : _____ _____ mmol/ L	
	Smoking history : Yes/ No* If yes, smoking for ____years ____ pack/ day	
Disease risk factor	History of DM : _____ years DM drugs : nil / oral / subcutaneous	
	HbA1c level Date : _____ _____ mmol/ L	
	PAD : Yes / No	
	Ischemia : Yes / No	
	Heart disease :	
	Others :	
Ulcer risk factor	Ulcer location:	
	Ulcer duration: _____weeks	
	Ulcer size _____cm	
	Ulcer depth : UT classification 0 / 1/ 2 / 3*	
	Wound infection : none / mild/ moderate/ severe	

N.B. : *delete when appropriate

Event Record Form

Case code _____ Treatment group silver / honey / conventional*

Date of assessment _____

Taking antibiotics	Yes / No*
	Name of oral antibiotics _____
Adverse event	Required admission : Yes / No*
	_____ _____ _____

N.B. : *delete when appropriate

Adverse event is any event that affected the ulcer healing
For examples, chest infection, UTI.

Feedback from intervention providers

Intervention provider: Community nurse / Nurse in GOPD*

	1 (lowest rating)	2	3	4	5 (highest rating)
The prescription of dressing method is clear enough for you to follow.	1	2	3	4	5
The dressing is easy to handle. ▪ nAg dressing ▪ MH dressing ▪ Paraffin tulle	 1 1 1	 2 2 2	 3 3 3	 4 4 4	 5 5 5
You can handle the workload to perform dressing change on participants in this study.	1	2	3	4	5
You have enough time to perform the dressing change.	1	2	3	4	5

* delete as appropriate

Additional comments:

_____

_____

Feedback from data collectors

Data collector: Research assistant A/ B*

	1 (lowest rating)	2	3	4	5 (highest rating)
Training is enough for you to complete the data collection.	1	2	3	4	5
When there is a problem during the data collection, it is easy for you to seek for help.	1	2	3	4	5
When there is a query after the data collection, you can clarify the query timely.	1	2	3	4	5
You are confident in performing the data collection after the training.	1	2	3	4	5
Research assistant A • ABI measurement • Chart wound score • Wound size measurement • Assess wound healing status • Assess severity of infection • Assess adverse event	1 1 1 1 1 1	2 2 2 2 2 2	3 3 3 3 3 3	4 4 4 4 4 4	5 5 5 5 5 5
Research assistant B • Verify wound healing status	1	2	3	4	5
You have enough time to collect the data	1	2	3	4	5

* delete as appropriate

Additional comments:

_____

_____

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