BIOMECHANICAL ASSESSMENT AND ELECTROMAGNETIC INTERVENTION FOR DIABETIC ULCERS

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Biomechanical assessment and electromagnetic intervention for diabetic ulcers

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CERTIFICATE OF ORIGINALITY

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_____ (Signed)

__CHOI Ming Chun_____ (Name of student)

Abstract

Diabetic foot ulceration is a major risk factor for lower limb amputation. Novel assessments and treatments are therefore demanded for better management of diabetic foot ulcers in clinical settings. The present thesis hypothesizes that the novel biomechanical assessment can validly and reliably reflect the tensile properties and underlying collagen of diabetic ulcers whereas the pulsed electromagnetic field intervention can effectively exert changes in diabetic wounds in cellular, molecular and biomechanical aspects.

An optical coherence tomography-based air-jet indentation system was recently developed to objectively and quantitatively measure the indentation stiffness of the biological tissues in a non-invasive and non-contact manner. In the first study of the present thesis, a parallel comparison was made between the measurement of indentation stiffness by the indentation system and various tensile properties by conventional *ex vivo* tensile testing over 3 weeks in a biopsy punched wound model at the hindlimbs of streptozotocin-induced diabetic Sprague-Dawley rats. The relationship between the indentation stiffness and collagen visualized by picro-sirius red staining was also explored. The present study showed that the indentation stiffness was significantly negatively correlated to the tensile properties and the abundance and alignment of collagen fibres in different phases of diabetic wound healing.

Previous studies demonstrated that pulsed electromagnetic field (PEMF) could accelerate wound closure, increase myofibroblast population and

promote biomechanical strength in diabetic wounds. In the second study of the present thesis, we investigated whether the increased myofibroblast population induced by PEMF was associated with enhanced collagen deposition and alignment stained by picro-sirius red in square wounds inflicted at the back of diabetic rats. A significant increase in type I collagen fibres was observed in the PEMF group on day 7, but not day 10 or 14 compared to the control group. The abundance of type I collagen fibre was significantly positively correlated with the myofibroblast population on day 7. No significant between-group differences were found in collagen fibril alignment and collagen fibre orientation at any measured time points.

In the third study of the present thesis, we further examined whether PEMF delivered at different intensities might improve the tensile properties of diabetic wound healing using the biopsy punched wound model at the hindlimbs of diabetic rats. The present findings demonstrated that the PEMF delivered at 10mT could increase the energy absorption capacity of diabetic wounds in the early healing phase. PEMF, however, seemed to reduce the maximum stress and Young's modulus in the remodelling phase.

As chronic diabetic foot ulcers are frequently infected by *Pseudomonas (P.) aeruginosa* resistant to conventional antibiotic treatments, novel regimens are needed for controlling bacterial infections in chronic diabetic foot ulcers. In the fourth study of the present thesis, the effects of 5-mT PEMF (sham vs. 20-Hz vs. 72-Hz) in combination with 0 to 1 minimum inhibitory concentration (MIC) of gentamicin on the growth of *P. aeruginosa* was evaluated in terms of bacterial count at the baseline and after incubation. *P.*

aeruginosa incubated with different MICs of gentamicin was exposed to sham or active PEMF for 10 hours, and then further incubated without PEMF for 14 hours (i.e. 24-hour incubation totally). Twenty-Hz PEMF significantly inhibited the growth of *P. aeruginosa* under 1-MIC gentamicin. However, under sub-MIC levels of gentamicin, both 20-Hz and 72-Hz PEMF appeared to promote bacterial growth.

Publications arising from the thesis

Journal articles:

Choi, M. C., Cheung, K. K., Li, X., & Cheing, G. L. (2016). Pulsed electromagnetic field (PEMF) promotes collagen fibre deposition associated with increased myofibroblast population in the early healing phase of diabetic wound. *Archives of Dermatological Research, 308*(1), 21-29.

Choi, M. C., Cheung, K. K., Ng, G. Y., Zheng, Y. P., & Cheing, G. L. (2015). Measurement of diabetic wounds with optical coherence tomography-based air-jet indentation system and a material testing system. *Journal of Wound Care, 24*(11), 519-528.

Conference articles:

Choi, M. C., Cheung, K. K., Li, X., & Cheing, G. L. Y. (2014). Pulsed Electromagnetic Fields (PEMF) Promote Collagen Fibre Deposition Through Myofibroblast Proliferation in Early Diabetic Wound. *Archives of Physical Medicine and Rehabilitation, 95*(10), e99-e100.

Choi, M. C., Cheung, A. K. K., Li, X., & Cheing, G. L. Y. (2014). *Effects of Pulsed Electromagnetic Fields (PEMF) on Type I Collagen Fibre Deposition and Myofibroblast Population in Diabetic Wound Healing.* Paper presented at the 9th Pan-Pacific Conference on Rehabilitation cum 21st Annual Congress of Gerontology, Hong Kong, China.

Choi, M. C., Cheung, A. K. K., Ng, G. Y. F., Zheng, Y. P., & Cheing, G. L.

Y. (2013). *Biomechanical properties of diabetic wound measured with Optical Coherence Tomography (OCT)-based Air-jet Indentation System*. Paper presented at the Inaugural Symposium of HEALED Research Group "Health for Life", Hong Kong, China.

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LIST OF ABBREVIATIONS

AgNP	Silver nanoparticle
CFU	Colony-forming unit
cGMP	Cyclic guanosine monophosphate
CSPG	Chondroitin sulphate proteoglycan
DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
FGF	Fibroblast growth factor
IL	Interleukin
IQR	Inter-quartile range
MHB	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
NOS	Nitric oxide synthase
OCT	Optical coherence tomography
PEMF	Pulsed electromagnetic field
ROI	Region of interest
ROS	Reactive oxygen species
SD rats	Sprague-Dawley rats
SEM	Standard error of mean
STZ	Streptozotocin
TGF-β	Transforming growth factor beta

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Chapter 1

Introduction: literature review

1.1 Epidemiology and aetiology of diabetic foot ulcers

Diabetes mellitus (DM) is a popular non-communicable metabolic disease affecting millions of lives. According to the first global report on diabetes issued by World Health Organization recently (World Health Organization, 2016), 8.5% of adult population, which is equivalent to a total of 422 million adults worldwide are currently living with diabetes. In 2012, diabetes caused 1.5 million deaths (World Health Organization, 2016). Moreover, diabetes may lead to various complications that tremendously reduce patients' quality of life. These complications include retinopathy and blindness, nephropathy and kidney failure, chronic foot ulcers and lower limb amputation.

Among the diabetic population, the lifetime incidence of diabetic foot ulcers was estimated to be 25% (Singh et al., 2005). The prevalence of this population was 4-10% (Singh et al., 2005). Globally among different continents, the prevalence of ulceration in diabetic population was 3-13% (Zhang et al., 2016). Diabetic foot ulcers often lead to lower limb amputation due to poor ulcer management. Global Lower Extremity Amputation Study Group (2000) reported that 25-90% of all lower limb amputation cases were associated with diabetes. According to Ahmad et al. (2016), people with diabetes were exposed to 6 times more risk at amputation, compared to those without diabetes. A large scale review

conducted in the United Kingdom recently concluded that diabetic foot ulcer was significantly associated with an increased risk of death (Walsh et al., 2015). With the control of other major complications of diabetes, the hazard ratio for diabetic foot ulcer increasing risk of death was as high as 2.48 (Walsh et al., 2015). The 5-year survival rate was only 57.8% (Walsh et al., 2015).

The main problem of diabetic foot ulcers is that the ulcers do not heal and gradually become chronic. Increased inflammatory markers, peripheral polyneuropathy, vascular insufficiency, ischaemia, poor vision, limited joint mobility and ill-fitting footwear are all precipitating factors for diabetic foot ulcers (Dinh et al., 2012; Jeffcoate et al., 2003). Once accidental trauma occurs, the skin is easily wounded and different complications including peripheral polyneuropathy, ischaemia and infection impair healing and may lead to more serious consequences.

Peripheral polyneuropathy impairs sensation, innervation of small foot muscles and vasomotor functions (Gupta et al., 2014). The loss of protective sensation reduces patients' awareness of the pain and any traumas to their feet. Motor neuropathy alters normal foot movement patterns in gait and the pressure distribution on foot. Callus forms at points of increased pressure. The abnormal pressure distribution and callus were significantly associated with the occurrence of diabetic foot ulcers (Murray et al., 1996; Tang et al., 2015). Autonomic neuropathy leads to microvascular dysfunction because of arteriovenous shunting that reduces or even obstructs blood flow to the capillaries. This eventually contributes to ischaemia of the feet.

Among patients with diabetes, foot ischaemia can be caused by macrovascular problems such as atherosclerosis (Akbari et al., 1999; Huysman et al., 2009) or microvascular complications including vasomotor neuropathy as mentioned, thickened basement membrane, capillary wall fragility and thrombosis (Akbari et al., 1999; Giannini et al., 1995; Huysman et al., 2009). These dysfunctions decrease the efficiency of transportation of nutrients, oxygen and wastes in the feet. The feet with ischaemic problems are swollen, red and dry. Perspiration is lost. The skin is vulnerable to pressure and abrasion.

Infection is common in clinical diabetic foot ulcers. It was suggested that as many as 88.5% diabetic foot ulcer cases were found infected (Parsa et al., 2015). Diabetic foot ulcers are prone to infection because of immune dysfunction contributed by hyperglycaemia (Ahmed et al., 2016; Casqueiro et al., 2012; Geerlings et al., 1999). The bacteria that infect the ulcers secrete extracellular polymeric substances to form biofilm. The pathogenic biofilm in the ulcers is hypothesized to be one of the reasons a wound becomes chronic (Bjarnsholt et al., 2008). Indeed, different bacteria in biofilm secrete lytic enzymes and matrix metalloproteinases which digest not only the extracellular matrix of skin/wound such as fibroblast proteins and proteoglycans, but also immune cell mediators (McCarty et al., 2012). Apart from disturbing the structure of skin/wound and immune cell mediators, the pathogenic biofilm stimulates the host immune responses leading to chronic inflammation (Percival et al., 2015). Pathogenic biofilm resists not only the weakened host immunity in diabetic condition, but also

both natural and artificial antimicrobial compounds. In biofilm, the penetration rate as well as the uptake of antimicrobial compounds by the biofilm bacteria is reduced (Donlan et al., 2002). With the resistance to the antimicrobial compounds (Bjarnsholt et al., 2008; Chaudhry et al., 2016), biofilm infection in diabetic foot ulcers is difficult to treat. The persistent infection in the ulcers can spread and cause cellulitis and osteomyelitis, and more severely precipitate gangrene and then amputation (Jeffcoate et al., 2003).

Despite the effort by clinicians on providing the best medical care to people with diabetic foot ulcers, the outcome is still disappointing; amputation rate, prevalence and recurrence rate remain significant (Andrews et al., 2015; Apelqvist et al., 1993; Global Lower Extremity Amputation Study Group, 2000; Jeffcoate et al., 2003; Miller et al., 2014; Murray et al., 1996). More research should be conducted for the assessment and treatment of diabetic ulcers in order to improve the medical care for people with diabetes.

1.2 Pathologies of diabetic wounds

Wound healing can be divided into 3 distinct, sequential but overlapping phases: inflammatory, proliferative and remodelling phases. During the inflammatory phase, inflammatory cells migrate to the wound for phagocytosis to remove bacteria and foreign substances, and tissue debridement. Under diabetic condition, vasodilation is reduced; the hypoxic condition in the wound increases oxidative stress (Baltzis et al., 2014) as reactive oxygen species (ROS) are released from the mitochondria of

endothelial cells under hypoxia (Millar et al., 2007). Neutrophils and pro-inflammatory macrophages are abundant for a prolonged period, accompanied by increased levels of pro-inflammatory cytokines (Baltzis et al., 2014). Under all these conditions, chronic inflammation is characterized in diabetic wounds with the indication of increased ratio of M1 macrophages, which are pro-inflammation, to M2 macrophages, which control inflammation and enhance tissue healing (Nabzdyk et al., 2013).

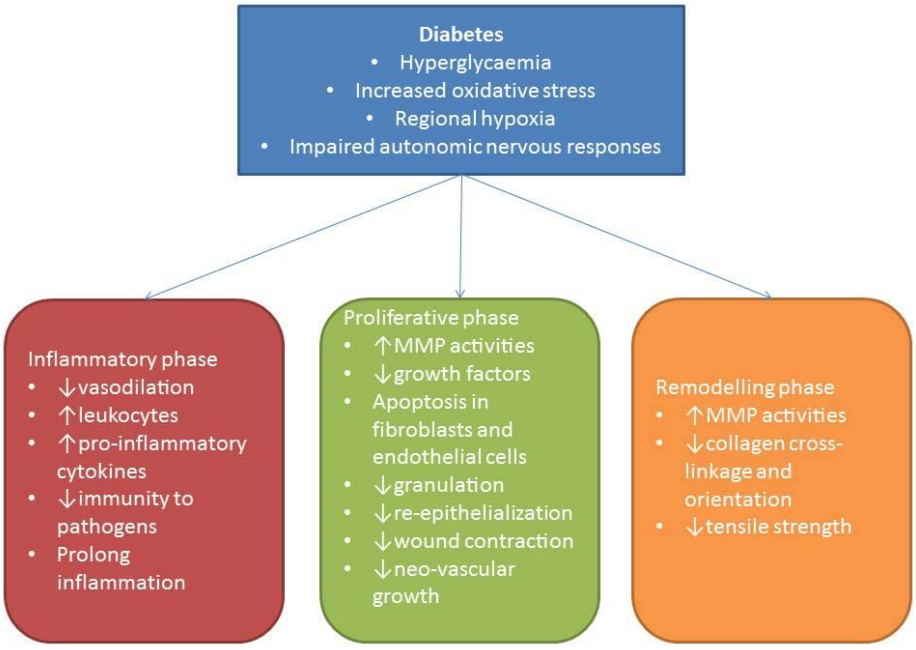
In the proliferative phase following the inflammatory phase, granulation tissue, extracellular matrix and new blood vessels are formed while the wound is re-epithelialized gradually. Diabetes, however, prolongs inflammation and delays formation of granulation tissues because of inadequate release of growth factors (Berlanga-Acosta et al., 2013). The persistence of macrophages together with the pro-inflammatory cytokines such as interleukin 1 beta (IL-1β) and tumour necrosis factor alpha (TNF-α) are related to the increased production of matrix metalloproteinases-2, -3, -8 and -9 (Ahmed et al., 2016; Berlanga-Acosta et al., 2013). Fibroblasts and endothelial cells are stressed by hyperglycaemia and increased oxidative stress, together with decreased levels of growth factors (Berlanga-Acosta et al., 2013). This results in the decreased recruitment and increased apoptosis of fibroblasts and endothelial cells (Baltzis et al., 2014; Berlanga-Acosta et al., 2013). Furthermore, transforming growth factor beta 1 (TGF-β1) and fibronectin critical to the differentiation of fibroblasts to myofibroblasts for wound contraction are deficient (Berlanga-Acosta et al., 2013) while TNF-α can suppress fibroblasts' expression of contractile protein, alpha smooth

muscle actin (Goldberg et al., 2007). As a result, diabetes impairs re-epithelialization, wound contraction, neo-vascular growth and extracellular matrix deposition (Baltzis et al., 2014; Berlanga-Acosta et al., 2013).

In the remodelling phase, collagen continues to be deposited and degraded that the deposition and degradation are balanced by the activities of fibroblasts and matrix metalloproteinases. During this process, the collagen fibres are better aligned and oriented. In diabetes, however, the increased matrix metalloproteinases continue to degrade collagen, disturbing the cross-link formation and orientation of collagen (Baltzis et al., 2014; Tsioufis et al., 2012). A study using diabetic and non-diabetic animal models echoed that the tensile strength of diabetic wounds was observed to be lower than that of the non-diabetic ones (Goudarzi et al., 2010).

In summary, diabetes impairs all inflammatory, proliferative and remodelling phases of wound healing (Figure 1.1) mainly due to hyperglycaemia, oxidative stress from increased ROS and advanced glycation end-products (Berlanga-Acosta et al., 2013; Peppa et al., 2011), regional hypoxia (Baltzis et al., 2014; Chao et al., 2009) and impaired autonomic nervous responses (Baltzis et al., 2014).

Figure 1.1: The impaired healing in diabetic wounds.



Disturbance of homeostasis raised from diabetes mellitus leads to different pathologies in the inflammatory, proliferative and remodelling phase of wound healing. MMP: matrix metalloproteinase.

As mentioned previously, diabetic wounds are vulnerable to pathogens due to the impaired innate immunity. Bacteria are one kind of pathogens that impair healing in diabetic wounds. Among different bacteria, the role of *Pseudomonas (P.) aeruginosa* in the chronicity of wounds has been extensively studied. Gjødsbøl et al. (2006) found that the chronic venous ulcers with resident *P. aeruginosa* were significantly larger in size than those without whereas other resident bacteria such as *Enterococcus faecalis* or *Escherichia coli* did not have significant effects on the size of chronic venous ulcers. Specifically from diabetic foot ulcers, *P. aeruginosa* has been found to be the most common species isolated (Parsa et al., 2015). Without timely elimination by polymorphonuclear leukocytes due to reduced innate immunity in diabetic condition, *P. aeruginosa* colonizes the wound, proliferates and secretes extracellular polymeric substances to form biofilm. At the same time, the bacterial species produces extracellular signal molecules, acylated homoserine lactones, for cell-to-cell signalling termed quorum sensing (Clinton et al., 2015; Williams et al., 2000). As the concentration of acylated homoserine lactones increase to a threshold, the bacteria start to produce different virulence factors which both enhance the survival and contribute to the pathogenicity of bacteria (Clinton et al., 2015; Pesci et al., 1997). Particularly, alginate, which is an important component of biofilm, enhances the survival of *P. aeruginosa* by strengthening the structure of biofilm (Nivens et al., 2001), preventing phagocytosis (Bjarnsholt et al., 2005; Bjarnsholt et al., 2008; Limoli et al., 2015), scavenging bactericidal free oxygen radicals secreted by polymorphonuclear

leukocytes (Bjarnsholt et al., 2005; Limoli et al., 2015; Simpson et al., 1989) and reducing the diffusion rate of antibiotic aminoglycosides (Allison et al., 1992; Bjarnsholt et al., 2005; Limoli et al., 2015); rhamnolipid can cause necrosis of polymorphonuclear leukocytes (Alhede et al., 2014; Bjarnsholt et al., 2005; Jensen et al., 2007); alkaline protease and elastase inhibit the actions of cytokines, and in turn impair the activation and proliferation of lymphocytes and macrophages (McCarty et al., 2012); elastase can also degrade the immunoglobulin G and disrupt the host's complement system (McCarty et al., 2012); lipopolysaccharide induces the release of pro-inflammatory cytokines (Bjarnsholt et al., 2008; McCarty et al., 2012); metalloproteinases shed the receptor of IL-6 from monocytes that may render bystander cells sensitive to pro-inflammatory IL-6 (Vollmer et al., 1996). The extracellular polymeric substances and proteases secreted by the bacteria can resist host immunity and antibiotics, provoke chronic inflammation, stimulate release of host matrix metalloproteinases, synergistically increase the activity of host matrix metalloproteinases and digest the extracellular matrix in the wound bed (Bjarnsholt et al., 2008; McCarty et al., 2012). Due to various pathologies contributed by *P. aeruginosa*, Bjarnsholt et al. (2008) hypothesized that *P. aeruginosa* at least partly caused the chronicity of wounds.

1.3 Biomechanical assessments for wounds

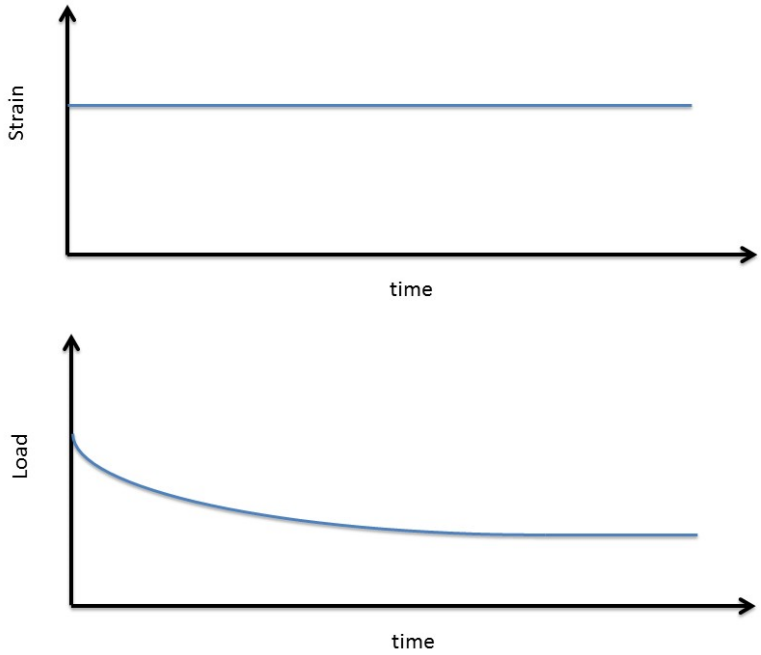
The biomechanical properties of wounds depend on the structural architecture and biological composition of wounds. As a defective form of

skin, the biomechanical assessments thus test the functional integrity and reflect the healing status of wounds. Tensile testing is a conventional assessment to measure the tensile biomechanical properties of excised wound specimens. The measurement can be categorized into low-strain or high-strain. Low-strain tensile biomechanical properties are measured at low strain and usually at lower load; the structure of the wound tissue is not disrupted during measurement. On the other hand, high-strain ones are obtained at high strain and usually at higher load that eventually lead to physical disruption of the wound tissue.

Low-strain measurement in tensile testing focuses mainly on the viscoelasticity of the wound tissue. The viscoelasticity of skin means that the skin possesses both viscous and elastic properties which are mainly contributed by collagen fibres and elastic fibres (Wilhelmi et al., 1998). The collagen fibres are arranged in a convoluted and woven manner in skin when not subjected to tensile stress (Wilhelmi et al., 1998). The elastic fibres are thin but much more elastic than collagen. Upon tensile load, the collagen fibres are straightened whereas the elastic fibres store the energy. The elastic fibres then restore the collagen fibres into the initial relaxed and wavy arrangement during unloading (Wilhelmi et al., 1998). This contributes to the elastic property of skin. However, upon slightly higher loading, the elastic fibres are microfragmented, and water is displaced from the network of collagen fibres; the collagen fibres are less likely to be restored to the relaxed convoluted state spontaneously (Wilhelmi et al., 1998). Microfragmentation of elastic fibres and water displacement of the

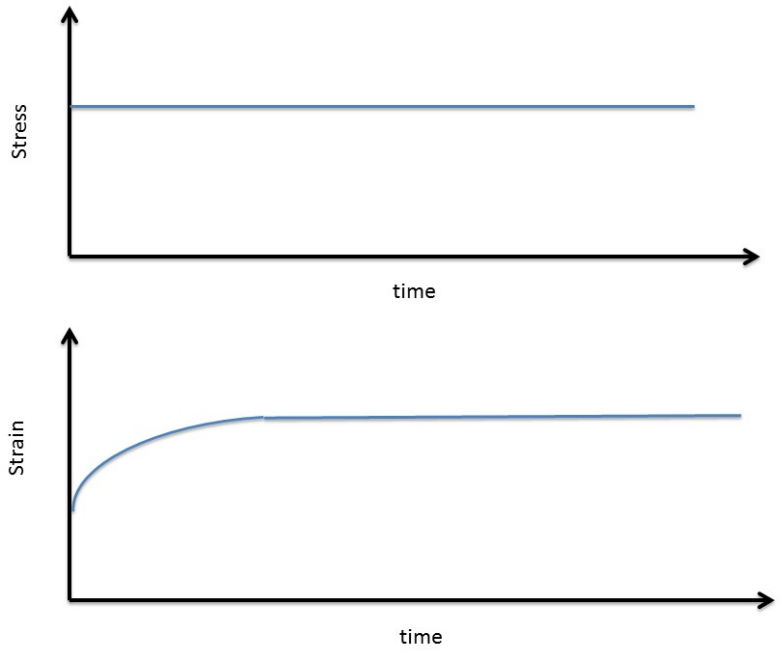
network of collagen fibres render the skin more viscous, i.e. the skin tends to dissipate energy to deform instead of storing energy to recoil. The energy from the tensile load is then dissipated in this process, which is called hysteresis (Wilhelmi et al., 1998). Under a constant strain, the effects of water displacement from collagen network and microfragmentation of elastic fibres accumulate over time; the load of the tissue decreases. This is referred as load-relaxation behaviour (Figure 1.2). Conversely, under constant stress, the deformation of the tissue increases; this is termed as creep behaviour (Figure 1.3). The load-relaxation and creep behaviours upon tensile loading of wounds are therefore related to the integrity of the network of elastic fibres and hydrated woven collagen fibres.

Figure 1.2: Load-relaxation behaviour.



Upon constant tensile strain, the load of a viscoelastic specimen decreases over time.

Figure 1.3: Creep behaviour.

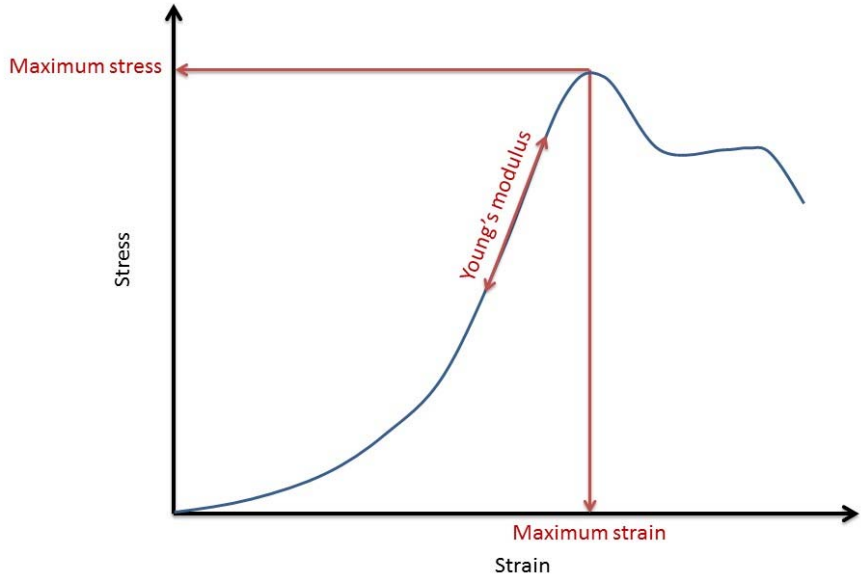


Upon constant tensile stress, the strain of a viscoelastic specimen increases over time.

For the high-strain measurement of tensile testing, collagen network comprising collagen abundance, alignment and orientation is the main contributor (Silver et al., 1992). Under high load and high strain, elastic fibres are mostly fragmented and their effect to the high-strain tensile biomechanical properties is minimal. Common high-strain biomechanical properties include maximum load (the maximum load of the wound tolerated before rupture), maximum stress (the maximum load normalized by the cross-sectional area of the wound), energy absorption capacity (the energy dissipated to deformation before rupture), Young's modulus (the slope of the linear phase in the stress-strain curve [Figure 1.4] representing the stiffness of wounds) and maximum strain (strain at the point of rupture) (Reddy et al., 2001; Williams et al., 1977). While maximum load is contributed by the mass of collagen, and the better collagen alignment and orientation (Sussman, 1973), maximum stress reflects only the collagen alignment and orientation as well as the density of cross-linkage among collagen filaments (An et al., 2004). Derived from the load-deformation curve (Figure 1.5) together with maximum load, energy absorption capacity depends on the mass of collagen and the elasticity of wounds (Williams et al., 1977). Young's modulus is the stiffness of wounds contributed by the alignment and orientation of collagen fibres but independent of the mass of tissue (Silver et al., 1992; Williams et al., 1977); greater Young's modulus means the wound is stiffer and greater stress is required to cause tissue deformation. Maximum strain is usually smaller with better collagen alignment and orientation, associated with greater maximum stress and

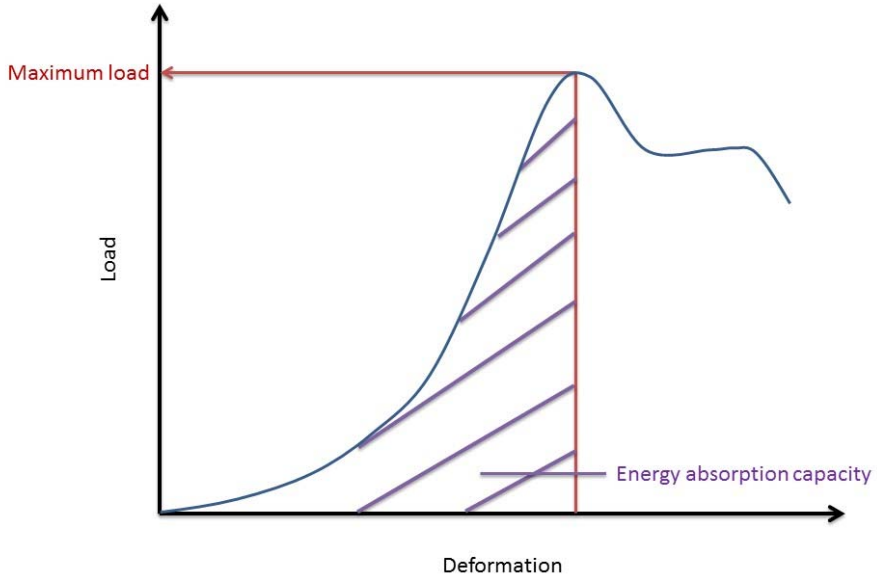
Young's modulus (Williams et al., 1977). Since greater tensile biomechanical strength is associated with more collagen abundance and better alignment and orientation of collagen fibres, the high-strain tensile biomechanical properties can therefore reflect the collagen abundance, alignment and orientation of healing wounds.

Figure 1.4: Tensile biomechanical properties derived from stress-strain curve.



During tensile testing, the stress (MPa) of the specimen increases with strain until rupture. The maximum stress (MPa) and maximum strain are derived at the point of rupture corresponding to the maximum load (N). The Young's modulus (MPa) is the slope of the linear elastic region of the stress-strain curve.

Figure 1.5: Tensile biomechanical properties derived from load-deformation curve.

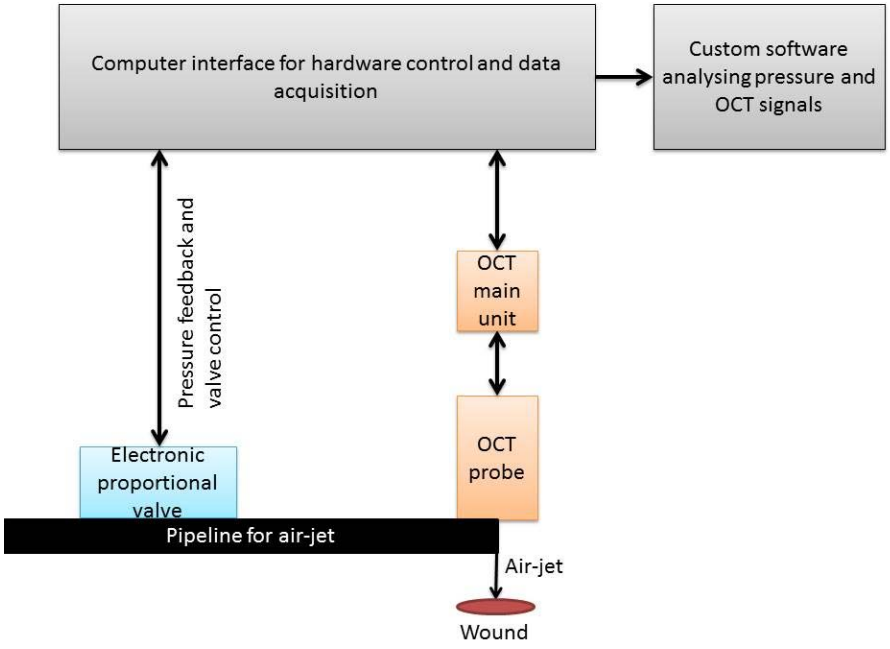


During tensile testing, the load (N) of the specimen increases with deformation (mm) until rupture. The maximum load (N) is derived at the point of rupture corresponding to the maximum stress (MPa). The energy absorption capacity (mJ) is the area under the load-deformation curve until rupture.

Although tensile testing is a conventional assessment for tensile biomechanical properties of wounds and can reflect the underlying structural architecture of collagen and elastic fibres, it requires the extraction of wound tissues from the body, thus limiting the usage to animal studies only. In other words, only non-invasive and low-strain biomechanical examinations are suitable for repeated measurements longitudinally over time without disturbing wound healing in clinical settings. For example, cutometry is a technology that applies suction force to deform the wounds to evaluate elasticity without destroying the wound tissues (Gabriel et al., 2015; Held et al., 2015). The elasticity may reflect collagen alignment and orientation, elastic fibres, viscosity of ground substances and even the subcutaneous tissues (Kim et al., 2006). However, cutometry is a contact-required method and the suction might disturb the delicate structure of the early healing wounds. Another example is ultrasonic elastography in which gentle, alternate compression and decompression are applied by the assessor onto the tissues, then a colour map indicating the resulting strain in the region of interest is displayed upon analysis by the system (Gnyawali et al., 2015). The compressive elasticity is mainly determined by the ground substances such as proteoglycans (Silver et al., 1992). Similar to cutometry, ultrasonic elastogaphy also requires the direct application of pressure to the wounds. In addition, it cannot provide quantitative data for between-sample comparisons due to uncontrolled compression applied manually by the assessor (Gnyawali et al., 2015), for which the inter-rater reliability may also be a concern.

Recently, our research collaborators developed an optical coherence tomography (OCT)-based air-jet indentation system to quantitatively measure soft tissues in a non-contact and non-invasive manner (Huang et al., 2009). It utilizes a stream of air to indent the wound bed and determine the indentation stiffness of the wound (Figure 1.6). As an indentation biomechanical measurement, it reflects the property of proteoglycans (Silver et al., 1992). Our earlier work has demonstrated that the OCT-based air-jet indentation system could validly and reliably measure the indentation stiffness of small muscles (Huang et al., 2009), plantar soft tissue (Chao et al., 2010) and diabetic foot ulcers (Chao et al., 2011) in human subjects. It can also record the increased stiffness in contracting small muscles (Huang et al., 2009), plantar soft tissue in aged people (Chao et al., 2010) and different parts of diabetic foot ulcer in patients (Chao et al., 2011). These studies have successfully explored the potential use of the OCT-based air-jet indentation system in *in vivo* biomechanical measurement of soft tissues. However, no studies to date have explored the use of indentation stiffness to reflect tensile properties and the underlying histology of diabetic wounds. Therefore, one of the aims of the present thesis was to establish the correlations between indentation stiffness, tensile properties and the underlying histology of collagen of diabetic wounds in order to provide evidence for the potential clinical use of the indentation stiffness measurement to assess diabetic foot ulcers and reflect the recovery status of biomechanical properties and collagen during wound healing.

Figure 1.6: Schematic diagram of the optical coherence tomography (OCT)-based air-jet indentation system.



Air-jet (a stream of air) of known force (N) after calibrated with the pressure is applied onto the wound. The indentation (mm) of the wound is measured by the OCT system. The indentation stiffness (N/mm) is then obtained after data analysis.

1.4 The effects of pulsed electromagnetic field (PEMF) on diabetic and non-diabetic wound healing

In clinical setting, PEMF is considered to be a new intervention for wound management (Costin et al., 2012; Gottrup et al., 2012; McGaughey et al., 2009). It has been shown that PEMF improved healing of clinical chronic ulcers by reducing wound size (Kwan et al., 2015; Sarma et al., 1997; Stiller et al., 1992; Todd et al., 1991), wound depth (Stiller et al., 1992), wound pain (Stiller et al., 1992) and swelling (Todd et al., 1991). Specifically for chronic diabetic foot ulcers, our research team has recently reported that PEMF exhibited a trend of accelerating wound closure, and a significant increase in cutaneous capillary diameter and flow rate (Kwan et al., 2015).

Increasing body of evidence in animal studies revealed that PEMF could improve wound closure (Athanasiou et al., 2007; Callaghan et al., 2008; Goudarzi et al., 2010; Matic et al., 2009; Ottani et al., 1988; Patino et al., 1996), inflammatory resolution (Athanasiou et al., 2007), wound contraction (Scardino et al., 1998), re-epithelialization (Athanasiou et al., 2007; Scardino et al., 1998), cell proliferation (Callaghan et al., 2008), neo-vascular growth (Athanasiou et al., 2007; Callaghan et al., 2008; Ottani et al., 1988), fibroblast population (Athanasiou et al., 2007), collagen deposition and maturation (Athanasiou et al., 2007; Ottani et al., 1988), and tensile strength of wounds (Goudarzi et al., 2010; Strauch et al., 2007). In diabetic wound models, both our research team and others showed that PEMF could improve wound closure (Callaghan et al., 2008; Cheing et al., 2014; Goudarzi et al., 2010), re-epithelialization (Cheing et al., 2014), cell

proliferation (Callaghan et al., 2008), neo-vascular growth (Callaghan et al., 2008), myofibroblast population (Cheing et al., 2014; Choi et al., 2016), collagen deposition (Choi et al., 2016) and tensile strength of wounds (Goudarzi et al., 2010). The studies described above used a wide range of PEMF parameters (e.g. from micro-tesla to tesla, from 1Hz to 50Hz) to produce similar effects; other studies used comparable parameters (e.g. from micro-tesla to milli-tesla, from 1Hz to 15Hz) but failed to find clinically meaningful results (Glassman et al., 1986; Gupta et al., 2009; Milgram et al., 2004). There is no consensus on the optimal set of PEMF parameters for promoting wound healing based on the literature.

Although the above studies have concluded that PEMF increased myofibroblast population, collagen deposition and tensile strength of diabetic wounds, it is important to examine the underlying mechanisms and explanations for the findings. Since myofibroblast is involved in collagen deposition during wound healing, it is possible that PEMF may enhance collagen deposition through promoting myofibroblast proliferation/differentiation. Therefore, the present thesis aims at providing evidence to support that the increased collagen deposition upon PEMF exposure may partially due to increased myofibroblast population in diabetic wound healing.

Existing studies investigating the effects of PEMF on tensile properties of diabetic wounds usually focus on tensile strength in the remodelling phase. The effects of PEMF on other tensile properties of diabetic wounds in earlier healing phases such as inflammatory and proliferative phase were

largely unknown. Therefore, another objective of the present thesis was to examine the influence of PEMF on different tensile properties, such as load-relaxation behaviour, maximum load, energy absorption capacity, maximum stress, Young's modulus and maximum strain, in different healing phases of diabetic wounds. As there is no consensus on the optimal PEMF parameters promoting wound healing, different intensities of PEMF were compared to probe for the optimal effects.

Apart from directly acting on wound healing, PEMF may also inhibit the growth of different bacteria that in turn altering the wound healing process especially in infected wounds. Bayir et al. (2015) showed that PEMF could inhibit the growth of planktonic *Staphylococcus aureus* and *Escherichia coli*. PEMF significantly reduced the biofilm cell biomass of *Helicobacter pylor*i (Di Campli et al., 2010). According to Matl et al. (2011), PEMF could increase the antibiotic efficacy of gentamicin to *Staphylococcus aureus*. PEMF augmented antibiotic efficacy of gentamicin even to the biofilm of *Staphylococcus epidermidis* (Pickering et al., 2003). These studies suggested a potential effect of PEMF on *P. aeruginosa*, which was hypothesized to at least partially contribute to the chronicity of wounds (Bjarnsholt et al., 2008). It is clear that the inoculation of *P. aeruginosa* to a diabetic wound model significantly impairs wound healing and causes chronic inflammation as a chronic wound model (Zhao et al., 2010; Zhao et al., 2012). However, no previous studies have fully addressed whether PEMF exerts influences on the growth/viability of *P. aeruginosa* in broth media, *in vitro* biofilm form, and biofilm on wounds. Therefore, the present

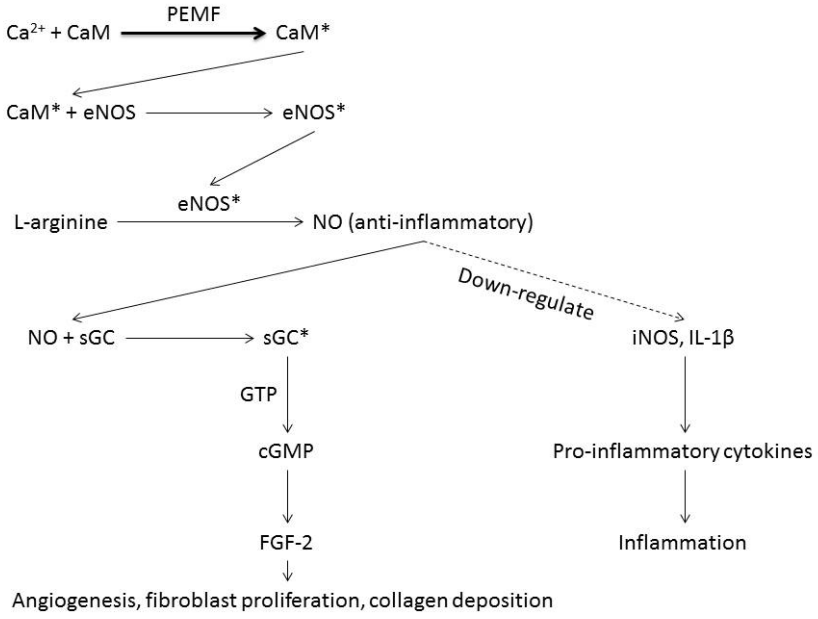
thesis was the first to investigate the effects of PEMF on P. aeruginosa in broth media, and would enlighten future studies on promoting chronic wound healing from an antimicrobial perspective.

1.5 The mechanisms of PEMF effect on wound healing

Although the mechanisms of the effect of PEMF on wound healing have not been adequately investigated, animal studies have provided some insights on the potential involvement of some molecules that are likely to participate in various key signalling cascades driving certain biological effects. Callaghan et al. (2008) showed that PEMF accelerated wound healing, promote angiogenesis and enhance tissue survival in diabetic wounds through a fibroblast growth factor 2 (FGF-2)-dependent pathway in *in vivo* mouse model. In an *in vitro* study, Vianale et al. (2008) demonstrated a decrease of pro-inflammatory chemokine production by PEMF in human keratinocyte cell line. Patruno et al. (2010) found that PEMF can enhance early activity of inducible and endothelial nitric oxide synthase (NOS) for cell proliferation in inflammation-stimulated human keratinocyte cell line; it may down-regulate inflammation and oxidative stress by reducing levels of cyclooxygenase-2, prostaglandin E2 and superoxide, accompanied by a decreased activity of catalase. Upon wound healing, nitric oxide generated by NOS plays important roles in inflammation, wound contraction, re-epithelialization, neo-vascular growth, cell migration, keratinocyte proliferation, collagen deposition (Isenberg et al., 2005). It is important to note that diabetic wounds are deficient in nitric oxide probably leading to

impaired extracellular matrix deposition, collagen deposition and breaking strength (Isenberg et al., 2005). By regulating level of nitric oxide, possibly via NOS, PEMF might therefore modulate both diabetic and non-diabetic wound healing. The activity of endothelial NOS is regulated by intracellular calcium fluxes or calmodulin (Isenberg et al., 2005). PEMF might enhance the binding of Ca^{2+} to calmodulin in a voltage-dependent manner to activate endothelial NOS, and in turn control the activity of inducible NOS and inflammation (Figure 1.7); this effect is dependent of the combination of intensity, frequency, pulse width and waveform of PEMF (Pilla et al., 2011). The nitric oxide from endothelial NOS can quickly trigger the generation of cyclic guanosine monophosphate (cGMP) (Isenberg et al., 2005) and lead to FGF-2 production that promotes angiogenesis and fibroblast proliferation (Pilla et al., 2011).

Figure 1.7: Simplified schematic representation of the proposed pulsed electromagnetic field (PEMF) transduction pathway in wound healing.



PEMF can enhance the activation of calmodulin (CaM) and then endothelial nitric oxide synthase (eNOS) which produces anti-inflammatory nitric oxide (NO). The NO from eNOS, on one hand down-regulates activities of inducible NOS (iNOS) and interleukin 1 beta (IL-1β), and on the other hand triggers the generation of cyclic guanosine monophosphate (cGMP), leading to release of fibroblast growth factor 2 (FGF-2). The effects would be anti-inflammation, angiogenesis, fibroblast proliferation and collagen deposition. sGC: soluble guanylyl cyclase; GTP: guanosine-5'-triphosphate; *activated form.

1.6 Four inter-related studies derived from the field of interest

As discussed in the previous parts, it is indicated that more assessment and treatment options should be studied to offer better evidences-based medical care and alleviate the global burden of diabetic foot ulcers. New biomechanical assessment and electromagnetic intervention were included in this thesis. The central hypothesis of the thesis for the assessment part was that the OCT-based air-jet indentation system could serve as a valid and reliable measurement of indentation stiffness of wound bed. This measurement can reflect the tensile properties as well as the underlying collagen abundance, alignment and orientation of diabetic wounds. For the intervention part, PEMF would effectively produce changes in the cellular, molecular and biomechanical properties of diabetic wounds. The central hypotheses led to 4 inter-related studies:

1. The use of OCT-based air-jet indentation system to assess the biomechanical property of diabetic wounds: correlations with conventional tensile testing and collagen abundance, alignment and orientation (referred as collagen histology in the thesis);
2. The effects of PEMF on myofibroblast and collagen histology, as well as their correlation, in diabetic wounds;
3. The effects of PEMF on different tensile biomechanical properties in various healing phases of diabetic wounds;
4. The effects of PEMF on *Pseudomonas aeruginosa*, which is a pathogenic bacterial strain commonly found in diabetic foot ulcers.

Chapter 2

Novel biomechanical assessment for diabetic wounds

2.1 Abstract

The biomechanical properties of diabetic foot ulcers are potential clinical measurements that reflect the integrity of the skin. Tensile testing is a conventional objective and quantitative biomechanical examination that can be applied for biological tissues such as wounds. However, since the testing procedures require excision and extraction of the tissues, it is not feasible to conduct tensile biomechanical measurements for diabetic foot ulcers on patients. Recently, a non-contact non-invasive optical coherence tomography (OCT)-based air-jet indentation system was developed (Chao et al., 2011; Huang et al., 2009) for measuring the indentation stiffness of biological tissues non-invasively. It allows clinical examination of biomechanical properties of diabetic wounds. In the first study of the present thesis, the indentation stiffness of diabetic wounds assessed by the OCT-based air-jet indentation system was correlated with the tensile biomechanical properties assessed by conventional tensile testing to determine whether the indentation stiffness reflects the actual tensile biomechanical properties of diabetic wounds. Histological examination was also conducted to examine the collagen recovery potentially reflected by the indentation stiffness.

One hundred and nineteen 10-week-old male Sprague-Dawley rats with streptozotocin-induced diabetes were used. Six-millimetre biopsy punched

wounds were induced on their hindlimbs. On post-wounding day 3, 5, 7, 10, 14 and 21, indentation stiffness (N/mm) of the wounds were assessed by using the OCT-based air-jet indentation system at wound centre and periphery. The tensile biomechanical properties including load-relaxation behaviour, total relaxation, load at 5% strain, maximum load, maximum stress, energy absorption capacity, Young's modulus and maximum strain of wound were assessed by tensile testing. In addition, wound samples were processed for collagen examination by the use of picro-sirius red polarized light microscopy. The collagen abundance, fibril alignment and fibre orientation were analysed by Fiji software.

For the whole study period, the indentation stiffness at both wound centre and periphery was significantly negatively correlated to the load at 5% strain (at wound periphery only), maximum load, maximum stress, energy absorption capacity, Young's modulus and maximum strain. Significant negative correlation was also found between the indentation stiffness at wound centre and collagen abundance, alignment and orientation. For the early phase of wound healing (day 3, 5 and 7), only the indentation stiffness measured at wound periphery was significantly negatively correlated to load at 5% strain, maximum load, energy absorption capacity and maximum strain. At the later phase of healing (day 10, 14 and 21), the indentation stiffness measured at wound centre and periphery was significantly negatively correlated with maximum load, maximum stress, energy absorption capacity (only at wound centre) and Young's modulus (only at wound periphery). The present study showed that the indentation stiffness

was correlated to the low- and high-strain tensile biomechanical properties, as well as the collagen histology in diabetic wounds.

2.2 Introduction

Diabetes mellitus (DM) is a systemic disease characterized by increased blood glucose level. Peripheral polyneuropathy, regional ischaemia in the limbs and diabetic foot ulceration are common diabetes-related complications. The predisposing factors for diabetic foot ulceration are multifactorial and the most well known risk factors include peripheral polyneuropathy, vascular insufficiency, plantar callus, elevated plantar pressures, history of previous foot ulceration and limited range of motion in the joints (Jeffcoate et al., 2003; Tang et al., 2015). Non-diabetic wounds usually heal well and the skin lesion can recover functionally in healthy individuals. Due to impaired sensation and circulation, diabetic wounds may be associated with repeated injuries, poor circulation and possibly infection, resulting in delayed healing over time and may subsequently lead to chronic foot ulcers (Bjarnsholt et al., 2008; Jeffcoate et al., 2003). Chronic diabetic foot ulcers do require medical attention.

Common clinical assessments for diabetic ulcers are wound size, wound colour, wound depth (tendon/capsule/bone involvements), presence of gangrene, infection and/or ischaemia (Gul et al., 2006; Oyibo et al., 2001). However, for monitoring the healing of the ulcers and predicting the prognosis, objective and quantitative measurements of wounds are lacking. Novel quantitative and reliable measurements may enhance the adoption of

the best strategies for treating the ulcers and preventing the recurrence. The biomechanical properties of wound bed which reflects the ultimate integrity of the wound can be a potential objective and quantitative measurement. Conventional tensile testing is commonly used in basic research to measure the tensile biomechanical properties; the low-strain tensile biomechanical properties, for which the testing may not cause permanent damage to the specimen, such as load-relaxation behaviour are contributed by the network of elastic fibres while the type, abundance, alignment and orientation of collagen are the major contributors to the high-strain ones, for which the testing involves breaking the specimen, such as maximum stress (Silver et al., 1992). Nevertheless, tensile testing cannot be conducted in clinical setting because the testing procedures require the excision of tissue sample from the living body. Recently, our research team has conducted a pilot study utilizing the OCT-based air-jet indentation system to assess the indentation stiffness of diabetic wounds (Choi et al., 2015) in a non-contact manner. Using the air-jet as an indenter, the wounds can be slightly indented without being contaminated or damaged from direct contact. Our earlier study demonstrated that the build-in air-jet device together with the OCT system can accurately and reliably measure the indentation force and deformation (Chao et al., 2011). Specifically for the current study, a pilot study using exactly the same testing procedures and animal wound model has been conducted to prove the reliability in the present experimental setting. The test-retest reliability was very high [ICC (3, 2) = 0.92]. Unlike in tensile testing involving the extraction and rupture of specimen, the

OCT-based air-jet indentation system deforms the wounds inwards temporarily to measure their biomechanical properties; the measurement is low-strain and non-contact in nature. The indentation loads are mainly absorbed by proteoglycans (Silver et al., 1992), which also regulate collagen deposition and maturation (Raghow, 1994; Reed et al., 2002), as well as the whole wound healing process (Schultz et al., 2009; Werner et al., 2003). Findings reported earlier by our team demonstrated that the indentation stiffness was negatively correlated with the tensile biomechanical properties in a diabetic wound model in rats (Choi et al., 2015). Nonetheless, that study did not differentiate the correlations in the early phase from the later healing phase and provide supporting quantitative histological findings. The objective of the present study was to explore the correlations between indentation stiffness and different tensile biomechanical properties of diabetic wounds over time (involving various healing phases). Since collagen is an important contributor to the biomechanical strength of skin/wound, the present study also examined the correlations between the indentation stiffness, and the collagen histology of diabetic wounds in a rat model.

2.3 Materials and methods

2.3.1 Animal handling and DM induction

The protocol of this study was approved by the Animal Subjects Ethics Sub-Committee of the administrating institution. One hundred and nineteen 10-week-old male Sprague-Dawley rats (300–400g) were used in the

experiment. All of the rats received humane care and the protocols were in compliance with the guidelines from the Animal Subjects Ethics Sub-Committee. The rats were first housed in groups of two to three at a temperature of 21^{o}C and 60% relative humidity under a 12-hour light-dark cycle. They were fed with a standard laboratory diet and sterile water *ad libitum*. After seven days of acclimatization, the rats were fasted for 12 hours before DM induction. For DM induction, streptozotocin was prepared in sterile citrate buffer adjusted to pH 4.4 at 10mg/ml. The resulting solution of streptozotocin was injected intra-peritoneally at a dosage of 50mg/kg body weight of the rats. Immediately before injection, the blood glucose level of the rats was measured to reject any hyper- or hypoglycaemic cases. After seven days, the blood glucose level of the rats was re-measured and monitored once a week throughout the experiment to ensure DM was successfully induced and sustained in the rats. Any rats with a blood glucose level lower than 16.7mmol/L were excluded from the study.

2.3.2 Wound induction

Before wound induction, the rats were anaesthetized by intra-peritoneal injection of mixtures of ketamine and xylazine at a dose of 100 and 10mg/kg body weight respectively. After shaving and cleansing, wounds were induced with a 6-mm biopsy punch on the lateral side of each hindlimb (about 3mm distal to the fibula head). The wounds were left opened and exposed without dressing. The rats were housed individually to prevent cannibalism. At the time points of harvest, photos of the wounds were taken.

The wound area was estimated by Fiji software (Schindelin et al., 2012). Percentage of wound area was defined as the area of the wound at a particular time point divided by the initial wound area (post-wounding day 0).

2.3.3 OCT-based air-jet indentation system and the testing protocols

Wounds were randomly selected for assessment using the OCT-based air-jet indentation system (Choi et al., 2015) on post-wounding day 3, 5, 7, 10, 14 and 21 in order to include data collected from different phases of wound healing. Immediately before the assessment, the rats were anaesthetized using the protocol described above. The detailed specifications of the OCT system have been described in our previous studies (Choi et al., 2015; Huang et al., 2009).

Briefly, the probe of the OCT device consisted of a 1mm air-jet bubbler together with a super-luminescent diode light source (DenseLight, DL-CS3055 A, Singapore) that operated at a central wavelength of 1310nm, a nominal -3dB spectral bandwidth of 50nm, and a nominal output power of 5mW. The OCT unit provided an axial resolution of 18mm and an imaging depth of approximately 2 to 3mm in highly scattered materials. An electronic proportional valve with pressure feedback (ITV 1030-311L-Q, SMC Corporation, Tokyo, Japan) at a measurement range of 0.5MPa was installed. The OCT software collected the signals and controlled the air valve with a step motor.

The system was used to measure the indentation stiffness of the wound bed in an *in vivo*, non-invasive, and non-contact manner. Our pilot study demonstrated that the indentation stiffness measurements made on scab-covered or scab-removed wounds were similar. In order to avoid triggering potential inflammation and swelling of the wound, which may interfere with the measurements, wound scabs were not intentionally removed before the assessment. There were 5 measurement sites including 1 at the centre of the wound, and 4 at the periphery (cephalic, caudal, medial, and lateral to the wound, 3mm from the centre of the wound with reference to the fibula). Two measurements were made at each site so a total of 10 measurements were taken at the 5 sites. Three cycles of loading and unloading at an indentation rate of about 0.13mm/s were applied and recorded, which lasted for approximately 30s in total. The maximum indentation force was about 0.012N. The stiffness coefficient (N/mm) in the force/deformation ratio was calculated to represent the indentation stiffness. Two trials of indentation measurements were conducted after preconditioning, with a 5-minute resting interval between the trials. The first loading and unloading cycle was the preconditioning cycle. Only the loading phases of the second and third cycles were utilized and averaged.

2.3.4 Tensile testing protocols

After the OCT-based air-jet indentation assessment was completed, the rats were sacrificed by cervical dislocation under anaesthesia or with an overdosed intra-peritoneal injection of ketamine and xylazine. The bodies

were kept frozen at -80^{o}C before tensile testing. Before the wound tissue was harvested for the examination, the rats were defrosted at room temperature of 25^{o}C for 6 hours.

A material testing system (MTS Synergie 200 machine, MTS 643.12F-24 extensometer: MTS Systems Corporation, Eden Prairie, MN, USA) and the TestWorks 4 Universal testing software (MTS Systems Corporation, Minnesota, USA) were used to measure the total relaxation, load at 5% strain, load-relaxation behaviour (total relaxation divided by load at 5% strain), maximum load, maximum tensile stress, energy absorption capacity, Young's modulus, and maximum strain of the wound tissue. The wound beds in a skin layer of 6.0 x 18.0mm^2 dimension were then dissected from the bodies along the fibula. The actual length, width, and thickness of the wound tissues were measured with a Vernier calliper. By the material testing system, the specimens were elongated to a 5% strain position at 0.167mm/s for 10 preconditioning oscillation cycles. The total relaxation, load at 5% strain and load-relaxation behaviour were recorded by elongating the specimens to 5% strain for 30s at the speed of 8.33mm/s. Then, the specimens were returned to -5% strain for 60s. The high-strain biomechanical properties were measured by elongating the specimen at the speed of 8.33mm/s until failure (Choi et al., 2015; Ng et al., 2004). Load and deformation were recorded at a sampling rate of 100Hz. During the whole testing procedures, the specimens were kept moist with normal saline under room temperature of 25^{o}C. The maximum load and energy absorption capacity were derived from the load-deformation curve while the maximum

stress, Young's modulus and maximum strain from the stress-strain curve (Reddy et al., 2001).

2.3.5 Histological analysis of collagen

The full-thickness wounds of randomly selected rats were harvested on post-wounding day 3, 5, 7, 10, 14 and 21 with 8-mm biopsy punches. The wound tissues were then processed and embedded with paraffin wax. Sections of 5μm thickness were prepared with a microtome. Deparaffinized sections were stained with picro-sirius red staining according to the standard procedures (Kiernan, 2002). Type I collagen fibres, which appeared red upon birefringence, at the wound centre was examined through circularly polarized light with a light microscope (Nikon Eclipse 80i, Nikon Corporation, Japan). Images were captured using a digital camera (Spot Flex 15.2 64 Mp Shifting Pixel, Diagnostic Instruments Inc., USA). Quantification of collagen was done by Fiji software. As the obvious picro-sirius red stain was all red without green portions, images were converted to 8-bit red channel for analysis. Amount of collagen was quantified in terms of area. Percentage of collagen abundance was the amount of collagen normalized by the area of dermis, where collagen is found. The intensity of the stain was measured to represent the alignment of collagen fibrils since better aligned collagen fibrils have greater birefringence and refract more red light to the objective of microscope (Montes et al., 1991). The Feret length of the top ten longest collagen fibres of the wound was determined to represent orientation of the collagen fibres.

Greater Feret length reflects greater orientation and anisotropy (Melis et al., 2002; Noorlander et al., 2002). By selecting six representative regions of interest (ROIs) in the dermis, the energy and coherency values from the Fiji plug-in, OrientationJ, were also obtained as parameters of orientation and anisotropy of collagen fibres (Rezakhaniha et al., 2012).

2.3.6 Statistical analysis

Pearson product-moment correlation coefficient was used to examine the correlation between the parameters of collagen examination and biomechanical properties measured by the OCT-based air-jet indentation system and tensile testing. Significance level was set at 0.05. The analysis was performed using IBM SPSS statistics (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.).

2.4 Results

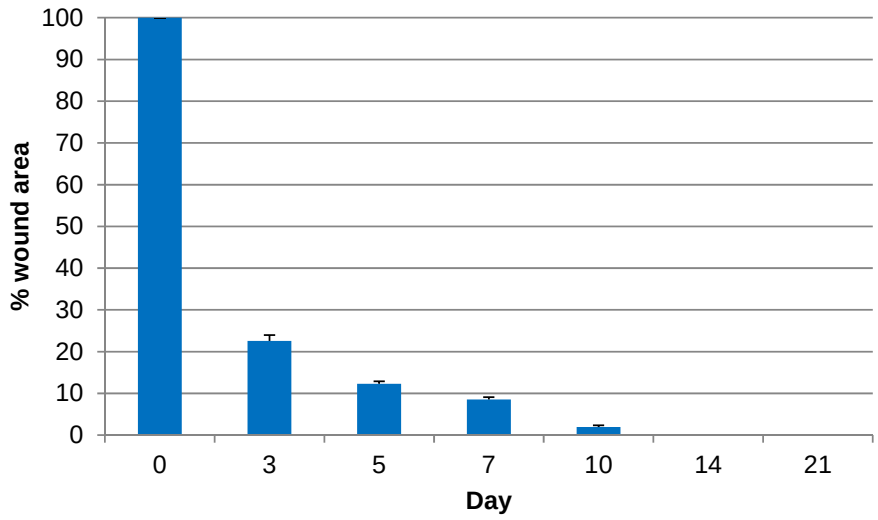
All wounds gradually healed over time without notable infection. No excessive exudates were observed and the wound margins were clear. Significant wound contraction occurred from day 0 to day 3. All wounds were grossly closed by day 14 (Figure 2.1) leaving visible but non-dystrophic scars on day 21.

Indentation stiffness of the wound centre and periphery was measured on day 3, 5, 7, 10, 14 and 21 (Figure 2.2). There was no significant change in the indentation stiffness at wound periphery over time and it was always smaller than that at the wound centre. The indentation stiffness of wound

centre in the early phase on day 3, 5 and 7 was greater than that in the later phase on day 10, 14 and 21. Based on this finding, correlation analysis between indentation stiffness and tensile biomechanical properties was separated into early and later phase respectively.

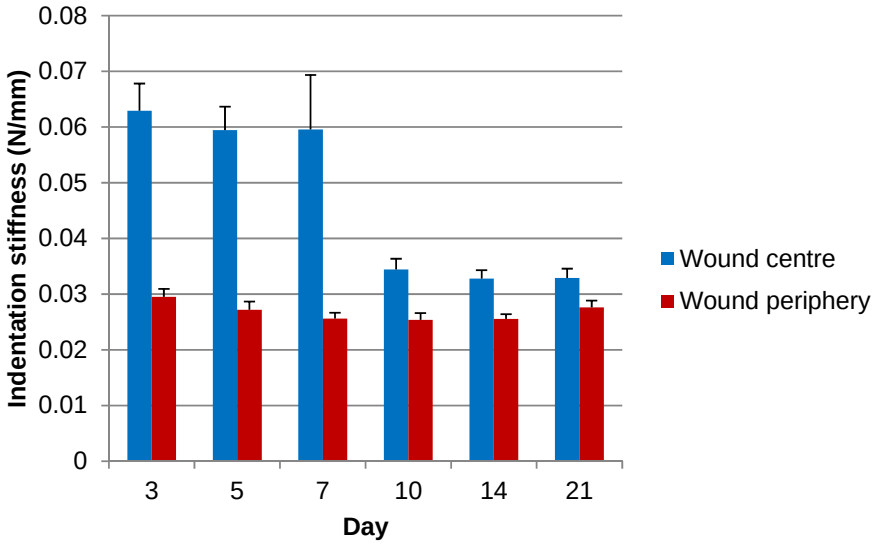
Tensile biomechanical properties and thickness over time are illustrated in Figure 2.3 and 2.4. Figure 2.5 shows representative images from picro-sirius red staining. The collagen deposition was obvious from day 10 and kept increasing until day 21.

Figure 2.1: The percentage of area of the diabetic wounds measured in 3 weeks.



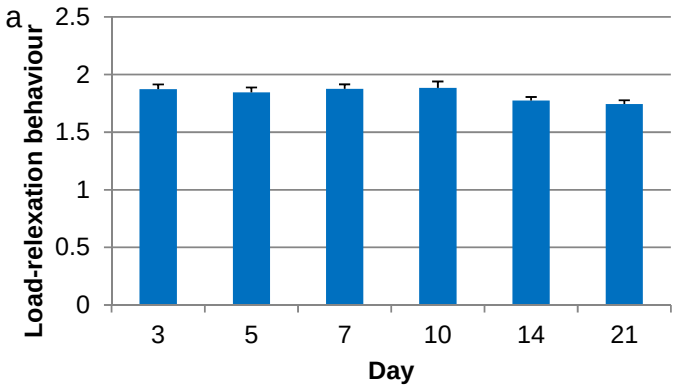
The percentage of wound area decreased over time. All wounds were closed by post-wounding day 14. The percentage of wound area was the wound area on the specific day divided by the initial wound area (day 0). Data are expressed as mean+SEM.

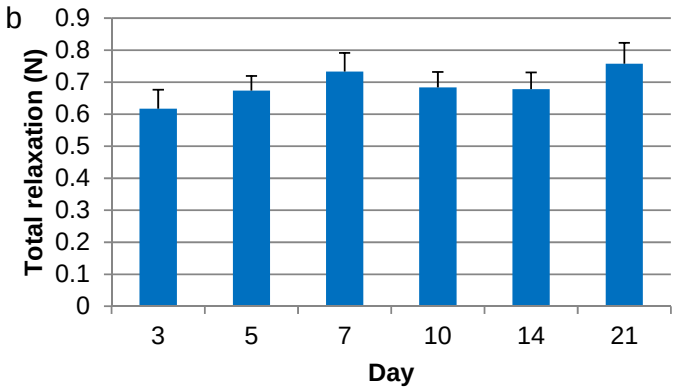
Figure 2.2: The indentation stiffness measured at the centre and periphery of the diabetic wounds in 3 weeks.

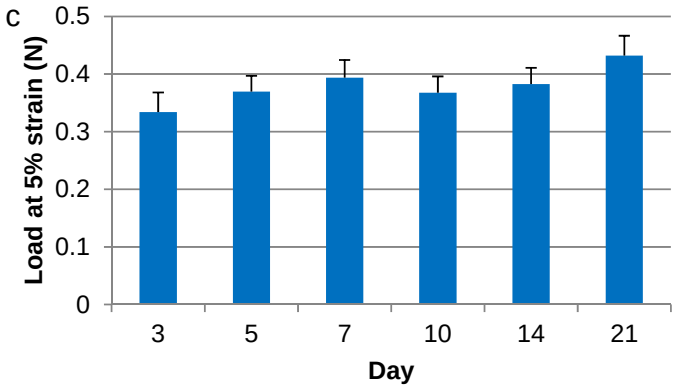


The indentation stiffness measured at wound centre on day 3, 5 and 7 was significantly greater than that on day 10, 14 and 21 ($p<0.05$). Data are expressed as mean+SEM.

Figure 2.3: The low-strain tensile properties (load-relaxation behaviour [a], total relaxation [b] and load at 5% strain [c]) of the diabetic wounds measured in 3 weeks.

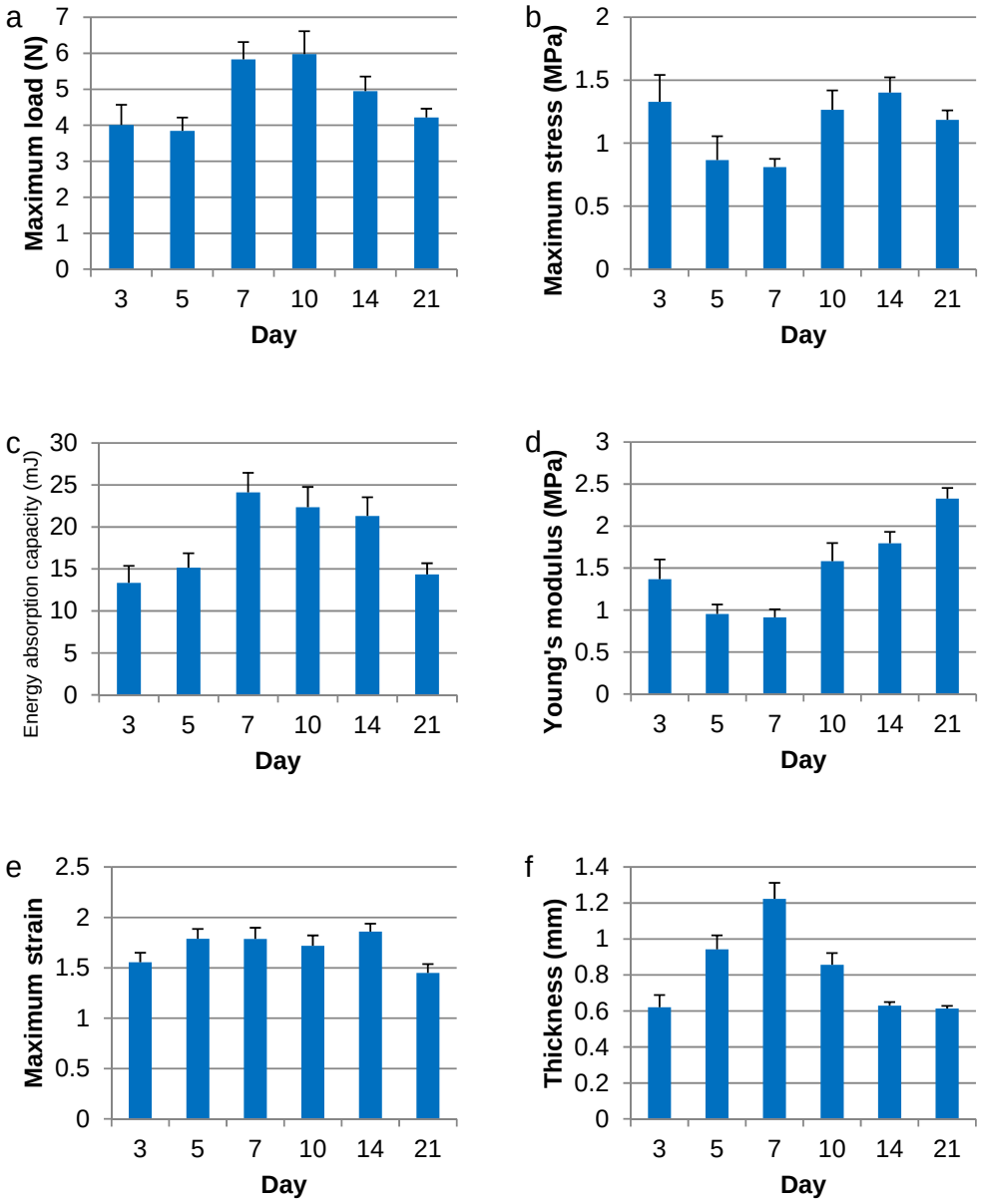






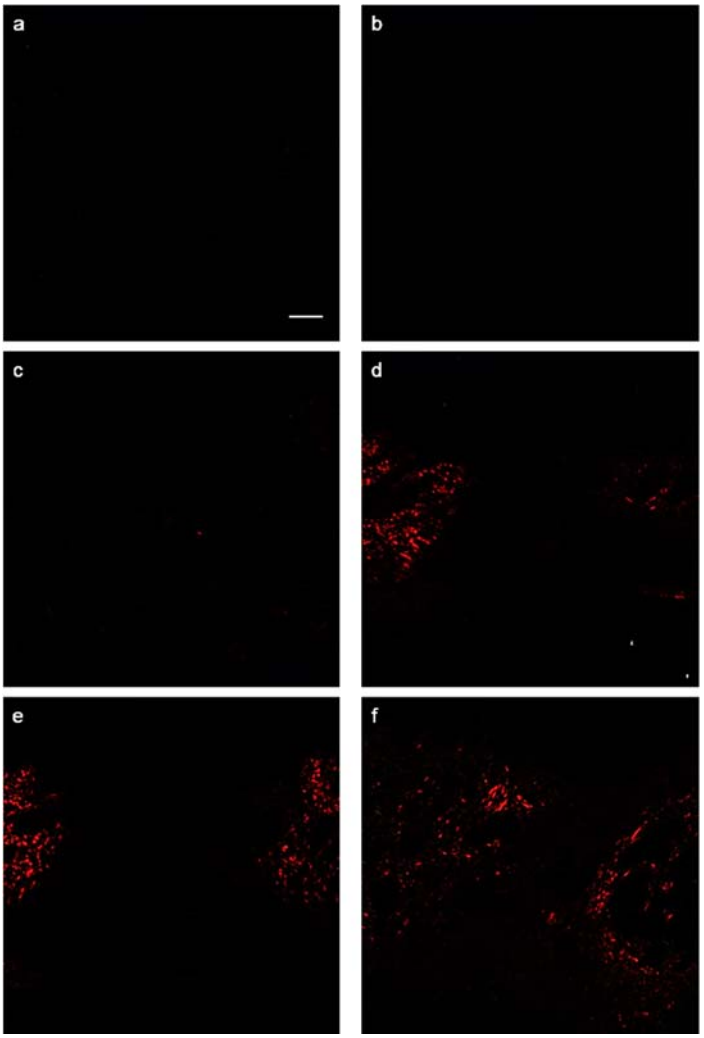
Data are expressed as mean+SEM.

Figure 2.4: The high-strain tensile biomechanical properties (maximum load [a], maximum stress [b], energy absorption capacity [c], Young's modulus [d] and maximum strain [e]) and thickness (f) of the diabetic wounds measured in 3 weeks.



Data are expressed as mean+SEM.

Figure 2.5: Representative picro-sirius red stained sections at the centre of the diabetic wounds examined under polarized light on post-wounding day 3 (a), day 5 (b), day 7 (c), day 10 (d), day 14 (e) and day 21 (f).



No collagen was observed to be deposited on day 3 and 5. Very limited collagen was observed on day 7. The abundance of collagen increased as the wounds healed on day 10, 14 and 21. Type I collagen fibres appeared red under polarized light. Scale bar: 150μm.

2.4.1 Correlations between indentation stiffness and tensile biomechanical properties

The correlations between indentation stiffness at wound centre and periphery, and tensile biomechanical properties of wound tissue were tabulated (Table 2.1). Indentation stiffness both at wound centre and periphery was significantly negatively correlated with all the high-strain tensile biomechanical properties from the pooled data of all time points. The indentation stiffness at wound periphery was also significantly negatively correlated with load at 5% strain which is a low-strain tensile biomechanical property ($r=-0.392$, $p=0.022$). Load at 5% strain (low-strain property) was significantly correlated to maximum strain (high-strain property) ($r=0.225$, $p=0.04$).

Besides, significant correlations were present between indentation stiffness at wound centre and periphery, between low-strain tensile biomechanical properties (load at 5% strain vs. load-relaxation behaviour [$r=-0.248$, $p=0.011$] and total relaxation [$r=0.97$, $p<0.001$]), and between high-strain tensile biomechanical properties (maximum load, maximum stress, energy absorption capacity, Young's modulus and maximum strain).

Table 2.1: The correlations between indentation stiffness and high-strain tensile biomechanical properties of diabetic wounds measured in 3 weeks.

		Indentation stiffness at wound centre (N/mm)	Indentation stiffness at wound periphery (N/mm)	Maximum load (N)	Maximum stress (MPa)	Energy absorption capacity (mJ)	Young's modulus (MPa)	Maximum strain
Indentation stiffness at wound centre (N/mm)	r		0.539	-0.325	-0.237	-0.338	-0.279	-0.306
	p		<0.001**	0.002**	0.025*	0.001**	0.008**	0.004**
Indentation stiffness at wound periphery (N/mm)	r	0.539		-0.409	-0.335	-0.348	-0.297	-0.29
	p	<0.001**		<0.001**	0.001**	0.001**	0.005**	0.006**

*p<0.05; **p<0.01

As the indentation stiffness at wound centre on the early wound healing phase (day 3, 5 and 7) was significantly different from that of the later phase (day 10, 14 and 21), the data obtained on the day 3, 5 and 7 (early phase) were separated from those from day 10, 14 and 21 (later phase) for subsequent correlation analysis.

In the early phase, the correlations between indentation stiffness at wound centre and tensile biomechanical properties did not reach significance, but the correlation between indentation stiffness at wound periphery and high-strain tensile biomechanical properties were mostly significant (Table 2.2).

In the later phase, the correlations between indentation stiffness at both wound centre and periphery, and high-strain tensile biomechanical properties were mostly significant (Table 2.3).

2.4.2 Correlations between indentation stiffness and collagen properties

Indentation stiffness at wound centre was significantly correlated with collagen abundance, percentage of collagen abundance, collagen fibril alignment, collagen fibre orientation, energy for anisotropy and coherency for anisotropy (Table 2.4). However, the correlations between indentation stiffness at wound periphery and the collagen properties did not reach statistical significance.

Table 2.2: The correlations between indentation stiffness and high-strain tensile biomechanical properties measured in the early healing phase (day 3, 5 and 7) of diabetic wounds.

		Indentation stiffness at wound centre (N/mm)	Indentation stiffness at wound periphery (N/mm)	Maximum load (N)	Maximum stress (MPa)	Energy absorption capacity (mJ)	Young's modulus (MPa)	Maximum strain
Indentation stiffness at wound centre (N/mm)	r		0.593	-0.068	-0.074	-0.094	-0.008	-0.274
	p		<0.001**	0.659	0.631	0.539	0.959	0.102
Indentation stiffness at wound periphery (N/mm)	r	0.593		-0.422	-0.278	-0.42	-0.212	-0.353
	p	<0.001**		0.004**	0.065	0.004**	0.161	0.017*

*p<0.05; **p<0.01

Table 2.3: The correlations between indentation stiffness and high-strain tensile biomechanical properties measured in the later healing phase (day 10, 14 and 21) of diabetic wounds.

		Indentation stiffness at wound centre (N/mm)	Indentation stiffness at wound periphery (N/mm)	Maximum load (N)	Maximum stress (MPa)	Energy absorption capacity (mJ)	Young's modulus (MPa)	Maximum strain
Indentation stiffness at wound centre (N/mm)	r		0.746	-0.41	-0.344	-0.391	-0.248	-0.241
	p		<0.001**	0.006**	0.022*	0.009**	0.108	0.115
Indentation stiffness at wound periphery (N/mm)	r	0.746		-0.386	-0.373	-0.294	-0.34	-0.122
	p	<0.001**		0.01*	0.013*	0.053	0.024*	0.429

*p<0.05; **p<0.01

Table 2.4: The correlations between indentation stiffness and collagen histology measured in the diabetic wounds.

		Collagen abundance (px^2)	% collagen abundance	Collagen fibril alignment index	Collagen fibre orientation index (px)	Energy for anisotropy	Coherency for anisotropy
Indentation stiffness at wound centre (N/mm)	r	-0.798	-0.804	-0.68	-0.723	-0.715	-0.689
	p	0.006**	0.005**	0.031*	0.018*	0.02*	0.028*
Indentation stiffness at wound periphery (N/mm)	r	-0.49	-0.454	-0.248	-0.429	-0.311	-0.455
	p	0.151	0.188	0.489	0.216	0.382	0.186

*p<0.05; **p<0.01

2.5 Discussion and conclusions

Our earlier study (Choi et al., 2015) reported the correlations between the indentation stiffness and the tensile biomechanical properties in the same wound model with a set of pooled data predominantly on post-wounding day 10. However, the sample size in that study was small. With a larger sample size, the present study confirmed the correlations between indentation stiffness and the high-strain (i.e. leading to breaking of specimen) tensile biomechanical properties in a set of pooled data with similar sample size at different time points. In the early wound healing phase, no significant correlation was found between indentation stiffness at wound centre and the tensile biomechanical properties. This might be due to the very limited collagen deposited at the wound centre (no visible red stain on day 3, 5 and 7 in Figure 2.5). In the later phase of healing, as more collagen is deposited at the wound centre and the collagen fibre alignment has improved, the wound centre could gradually contribute more to the tensile biomechanical properties. Therefore, the correlations become significant over time.

The present study also examined the collagen histology in order to explain the correlations between indentation and tensile biomechanical properties of diabetic wounds, and the results supported our earlier hypothesis that the correlations was related to the abundance and alignment of collagen in the dermis that contribute to the tensile biomechanical properties (Choi et al., 2015). Although the indentation stiffness is an indenting and low-strain property which should be contributed by proteoglycans, it may still reflect the collagen recovery in wounds because decorin, one of the proteoglycans

found in skin, interacts with various types of collagen (Brown et al., 1989; Tenni et al., 2002; Vogel et al., 1984) and may affect the tensile properties of skin (Carrino et al., 2000). Another proteoglycan, chondroitin sulphate proteoglycans (CSPGs) with great molecular size, dominates in the early phase of wound healing (before wound closure) while the amount of decorin with smaller molecular size increases after wound closure (Yeo et al., 1991). The decrease in molecular size of proteoglycans may be responsible for organization and maturation of collagen (Kuwaba et al., 2002). In the early phase, bigger CSPGs may contribute to greater indentation stiffness while collagen is being produced whereas in the later phase, smaller decorin may contribute to smaller indentation stiffness while collagen is under remodelling. Further study can be conducted to explore the relationships between indentation stiffness, proteoglycans and tensile biomechanical properties of diabetic wounds.

The present study showed that the indentation stiffness at wound centre was significantly negatively correlated with collagen abundance, alignment and orientation. The correlations between the indentation stiffness and collagen histology were even stronger than those between the indentation stiffness and tensile biomechanical properties. This suggested that indentation stiffness might represent the underlying collagen histology more than the tensile biomechanical properties. Nevertheless, the correlations between indentation stiffness and collagen histology of diabetic wounds appeared to be different in various phases of healing. Analysis of the correlations between indentation stiffness and collagen histology obtained in the early

and later phase was not presented separately in the result part. This was because the sample size in the early (day 3, 5 and 7) and later phases (day 10, 14 and 21) was not large enough. Nonetheless, our data suggested negative correlations between the indentation stiffness at wound centre and collagen histology ($|r|>0.5$) in both the early and later healing phases. In the later phase, the indentation stiffness at wound periphery was also negatively correlated to the collagen histology; the correlation was significant to collagen abundance ($p=0.03$). Due to the limited collagen abundance in the early phase (day 3, 5 and 7), the high indentation stiffness at wound centre might not only be explained by collagen itself. Apart from CSPGs with large molecular size, we postulated that it was related to oedema (Choi et al., 2015); the histological findings in the present study showed that oedema was present in the wounds on day 3, 5 and 7 but not on day 10, 14 and 21. This might partially explain the high indentation stiffness at wound centre in the early phase while low in the later phase. According to our unpublished pilot data, no oedema was observed in the wounds and the indentation stiffness was low at wound centre on day 1; there was no wound matrix or any granulation tissues at the wound centre on day 1. The data obtained on day 1 was therefore excluded in the present correlation study. Further study should be done to elucidate and explain the correlations between indentation stiffness, proteoglycans, collagen histology and oedema in early and later healing phase of diabetic wounds.

Apart from the correlations between the indentation stiffness, collagen and high-strain tensile biomechanical properties, the present study found

significant negative correlation between indentation stiffness at wound periphery and load at 5% strain (a low-strain tensile biomechanical property). As the load at 5% strain was also significantly negatively correlated to load-relaxation behaviour, these might imply that wounds with greater indentation stiffness at the periphery tends to have smaller load at 5% strain but greater load-relaxation behaviour. As the load-relaxation behaviour is an indicator for the viscoelastic property (Wilhelmi et al., 1998), the greater indentation stiffness at wound periphery might imply the wound tissue is more deformable with tensile loading. At the same time, greater indentation stiffness at wound periphery and smaller load at 5% strain correlated with smaller maximum strain. Therefore, the greater deformability in the early healing phase of the wounds might make them more vulnerable to mechanical stress. This is evidenced by the finding that the indentation stiffness at wound periphery was negatively correlated to the high-strain tensile biomechanical properties. Our findings on the correlations between low-strain and high-strain tensile biomechanical properties in wounds provided ground work for future clinical research on low-strain biomechanical assessments feasible for chronic wounds.

In conclusion, indentation stiffness, a low-strain indentation biomechanical measurement, was negatively correlated to high-strain tensile biomechanical properties in both early and later healing phase of diabetic wounds. The indentation stiffness was also negatively correlated to collagen abundance, alignment, orientation and anisotropy. Our findings provided evidence

supporting the potential clinical use of OCT-based air-jet indentation system for biomechanical assessment of wounds.

Note: Part of this chapter was published in a peer-reviewed journal (Choi et al., 2015) and presented at a conference (Choi et al., 2013).

Chapter 3

Pulsed electromagnetic field (PEMF) enhancing collagen deposition associated with an increase in myofibroblast population

3.1 Abstract

The management of diabetic ulcers is a challenge for healthcare professions. The recurrence rate and amputation rate related to diabetic foot ulcers are still high with the standard treatments (Andrews et al., 2015; Apelqvist et al., 1993; Global Lower Extremity Amputation Study Group, 2000; Jeffcoate et al., 2003; Miller et al., 2014). Novel treatments are needed to enhance healing and maintain the wound healed (Andrews et al., 2015).

Many studies have provided evidence that PEMF enhanced wound healing (Athanasiou et al., 2007; Detlavs et al., 1996; Ieran et al., 1990; Itoh et al., 1991; Ottani et al., 1988; Patino et al., 1996; Salzberg et al., 1995; Sarma et al., 1997; Stiller et al., 1992; Strauch et al., 2007). Particularly, Goudarzi et al. (2010) showed with animal model that PEMF can increase the biomechanical strength of the diabetic wounds. As the biomechanical strength is contributed mainly by collagen content in the wounds, PEMF may also improve the collagen deposition and alignment. Collagen deposition in wound healing is partly contributed by myofibroblasts (Desmoulière et al., 2003; Hinz et al., 2012; Tomasek et al., 2002; Vedrenne et al., 2012; Zhang et al., 1994), apart from fibroblasts. Our earlier study has

shown that PEMF accelerated diabetic wound closure and increased myofibroblast population (Cheing et al., 2014). Therefore, the second study of the present thesis evaluated the effects of PEMF on collagen fibre deposition, collagen fibril alignment and collagen fibre orientation. The potential relationships between collagen fibre deposition and myofibroblast population in diabetic wound healing were also examined.

Forty young male streptozotocin (STZ)-induced diabetic Sprague-Dawley (SD) rats were randomly assigned to either the PEMF group or control group. Square wounds of 2cm x 2cm were made at their back of the trunk. The PEMF group received daily exposure of PEMF to the wounds, while control group was handled in the same manner except that the PEMF device was not activated. Wound tissues harvested on post-wounding day 7, 10 and 14 were fixed, processed and sectioned. The abundance, fibril alignment and fibre orientation of type I collagen were quantified with picro-sirius polarization method and image analysis software. Myofibroblast population data were collected from the same batch of experiment and presented in our previous paper (Cheing et al., 2014). Correlation between myofibroblast population and collagen fibre deposition was examined. There was significantly greater abundance of type I collagen fibre in the PEMF group than in the control on day 7 ($p=0.013$), but not on day 10 or 14. No significant between-group differences were found in collagen fibril alignment and collagen fibre orientation at any measured time points. Positive correlation was found between collagen fibre deposition and myofibroblast population only on day 7 ($r=0.729$, $p=0.007$). In conclusion,

PEMF could significantly increase collagen fibre in the early phase of diabetic wound healing, which was associated with the enhancement of myofibroblast population.

3.2 Introduction

In mammals, skin is the largest organ that serves as the first line of defence against external abiotic (e.g. mechanical stress) and biotic (e.g. pathogens) stress, as well as thermal and osmotic regulation. Upon injury, the cutaneous system responses by undergoing a complex process of wound healing that consists of distinct but overlapping phases, namely inflammatory, proliferative and remodelling phases orchestrating in a highly organized fashion to allow morphological as well as functional recovery of skin. Wounds usually heal without prolonged delay and complications in healthy people. However, wound healing is delayed in people with diabetes mellitus (DM), one of the most common systemic diseases (Boulton et al., 2005). Impaired wound healing due to DM may often lead to unhealed open wound, gangrene and subsequently amputation due to severe bacterial infection. These obviously lead to disability and bring an impact on quality of life (Boulton et al., 2005; Faglia et al., 2001; Ghanassia et al., 2008; Jeffcoate et al., 2003; Lacle et al., 2012; Singh et al., 2005; Wild et al., 2004).

During the proliferative phase of wound healing, fibroblasts migrate into the wound matrix and a subpopulation of fibroblasts differentiates into myofibroblasts. Both fibroblasts and myofibroblasts are believed to be involved in deposition of extracellular matrix such as collagen fibres

(Tomasek et al., 2002). Collagen is among the most predominant extracellular matrix in skin that plays a key role in tissue architecture and homeostasis (Aumailley et al., 1998). The biomechanical strength of cutaneous wounds is mainly determined by the abundance of the type I collagen deposited during the proliferative phase as well as its re-organization during the subsequent remodelling phase (Montes et al., 1991). For skin as an anisotropic tissue in which its biomechanical properties are directionally dependent, alignment as well as the orientation of collagen fibre also contribute substantially to the biomechanical strength of skin (Noorlander et al., 2002; Ottani et al., 2001). Collagen deposition was significantly impaired in people with type 1 diabetes, with a 40% reduction in the level of hydroxyproline compared to the non-diabetic control (Black et al., 2003). Such reduction in collagen accumulation was not resulted from an increase in collagenase activity, and was independent of blood glucose control (Black et al., 2003). Furthermore, *in vitro* growth of wound fibroblasts from people with type 1 diabetes was significantly attenuated when compared with that from people without diabetes (Black et al., 2003). Their findings suggested that reduction in collagen accumulation in type 1 diabetes was associated with retarded fibroblast proliferation (Black et al., 2003).

Using an animal model, Schäffer et al. (1997) reported a 68% reduction in collagen deposition in an incisional wound model of STZ-induced diabetic rat when compared with the non-diabetic control. Since wound healing involves secretion of collagenous extracellular matrix to replace the tissue

or matrix loss from wounding (Diegelmann et al., 2004; Tomasek et al., 2002), the insufficient collagen deposition in DM will impair wound healing especially by reducing the biomechanical strength of the wound (Goudarzi et al., 2010) and consequently render the wound more vulnerable to mechanical stress (Diegelmann et al., 2004).

PEMF is a non-invasive treatment modality that has been shown to exert beneficial effects on diabetic-related complications (Jing et al., 2011; Lei et al., 2013; Musaev et al., 2003; Pan et al., 2013; Weintraub et al., 2009), possibly via improving microcirculation and angiogenesis (Pan et al., 2013; Tepper et al., 2004). In human, our earlier work showed that PEMF improve healing and microcirculation in diabetic foot ulcer (Kwan et al., 2015). While in STZ-induced diabetic rat, we have demonstrated that PEMF increases wound re-epithelialization during early phase of wound healing, which was accompanied by an increase in myofibroblast population (Cheing et al., 2014). It is well documented that the role of myofibroblasts during wound healing is primarily on wound contraction; the contribution of myofibroblasts to collagen deposition upon healing however has also received increasing attention (Desmoulière et al., 2003; Su et al., 2014; Vedrenne et al., 2012; Zhang et al., 1994).

The increased myofibroblast population induced by PEMF may potentially be related to increased collagen deposition. Goudarzi et al. (2010) showed that PEMF exposure can increase the biomechanical strength of wound tissue in diabetic rat, but whether it is due to enhanced collagen deposition has not yet been addressed. Though not done in a wound model, Ahmadian

et al. (2006) demonstrated that PEMF could increase the content of hydroxyproline, which is a component of mature collagen fibre, in rat skin. Nevertheless, direct association between PEMF treatment and collagen deposition in diabetic wounds has never been examined.

Therefore, the present study aimed to examine the effect of PEMF treatment on the deposition of type I collagen during diabetic wound healing and the potential relationship between myofibroblast population and collagen deposition. Besides, the unaddressed relationship between PEMF treatment and the functional organization of collagen fibres by examining the type I collagen fibril alignment and orientation were also explored.

3.3 Materials and methods

3.3.1 Animal handling and DM induction

Forty young male adult (300-400g, 9-11 weeks old) SD rats were used in the experiment. All of the rats received humane care and the protocols were in compliance with the guidelines from the Animal Subjects Ethics Sub-Committee of our university. The handling and DM induction procedures were described in Chapter 2.

3.3.2 Wound induction

One week after STZ injection, wounds were induced. Before wound induction, mixtures of ketamine and xylazine (Alfasan International, Woerden, Holland) were administered intra-peritoneally at the dosage of 100 and 10mg/kg body weight respectively. After shaving and cleaning, one

full thickness square wound of 2cm x 2cm was induced with a scalpel and a pair of scissors aseptically at the back of each rat. The wounds were then disinfected with betadine solution and temporarily covered with sterile gauze to minimize the chance of infection. The rats were then housed individually in cages to prevent cannibalism.

3.3.3 PEMF treatment

The wounded diabetic rats were randomly allocated into either PEMF treatment group (n=20) or control group (n=20). In the PEMF group, the rats were treated with PEMF for 60min in the morning daily starting from post-wounding day 1. During treatment, each rat was restrained individually in a transparent plastic cylindrical bottle with diameter of 10cm and height of 20cm. A commercially available PEMF unit (model XKC-600W; Magnetopulse International, Griffin, Australia) was used to deliver sinusoidal magnetic field with intensity of 5mT, frequency of 25Hz and pulse width of 40ms (Cheing et al., 2014). The parameters had been tested and confirmed with a hand-held magnetometer (Model 4048, F. W. Bell, Milwaukie, OR). For the control group, they were also restrained in the same way for 60min as the PEMF group did, except the PEMF device was inactivated.

3.3.4 Tissue preparation and picro-sirius red staining

Rats from PEMF-treated and sham control groups were randomly selected to be euthanized on day 7 (n=6 for each group, same for the followings),

day 10 (n=8) and day 14 (n=6). These time points were chosen according to different studies (Matic et al., 2009; Milgram et al., 2004; Patino et al., 1996).

After euthanizing the selected rats with carbon dioxide, full thickness of the wound tissues including the subcutaneous fat were excised. At least 5mm of the apparently intact skin away from the wound margin were excised together that the wound samples. The samples were immediately fixed with 4% paraformaldehyde in phosphate buffered saline (PBS, pH 7.4) overnight at 4°C, and then processed and embedded in paraffin wax. Sections of 5µm at the centre of the wounds were prepared for the histological staining. The sections on slides were first deparaffinized and hydrated, and stained with picro-sirius red stain according to standard procedures (Kiernan, 2002). The picro-sirius red staining was observed through circularly polarized light under a Leica DMRB microscope. Images were captured using a Leica DFC 490 digital camera (Leica Microsystems, Wetzlar, Germany).

3.3.5 Analysis of picro-sirius red staining

Collagen fibre deposition, collagen fibril alignment and collagen fibre orientation were measured quantitatively. The captured images were imported and analyzed using NIS Elements Advanced Research image analysis software (Nikon Instruments, Melville, NY).

Threshold of the images was selected with the intensity of the red stained portion to create a binary image. The dermis region where collagen fibres located was selected as the region of interest (ROI) (Montes et al., 1991).

The collagen fibre deposition was represented as the pixel area of bright red positive staining (redA) divided by the area of ROI (ROIA).

Supposedly, the alignment of collagen fibrils is positively associated with the intensity of birefringence (Montes et al., 1991), the intensity value of the image was analysed. The mean background intensity value (BGI) and the red stained mean intensity value (redI) were obtained. The alignment of collagen fibrils was represented as redI/BGI.

Apart from the alignment of collagen fibrils, the orientation of collagen fibres was also analysed quantitatively with reference to Melis et al. (2002) and Noorlander et al. (2002). The orientation of collagen fibres is determined by the continuity and the length of the collagen fibres. In the binary image, the length of each red stained item was computed and sorted with the software. Collagen fibre orientation was defined by the length of the longest axis of each red stained item. The top ten greatest lengths in pixels were averaged to obtain the orientation index. The greater the length, the more continuous are the collagen fibres and the better the orientation is.

3.3.6 Correlation between type I collagen deposition and myofibroblast population

The correlation between type I collagen deposition (redA/ROIA) and the semi-quantitative myofibroblast population score (4 for >75% of ROI; 3 for >50%; 2 for >25%; 1 for <25%) rated by two independent laboratory animal pathologists presented in our previous paper (Cheing et al., 2014) was tested.

3.3.7 Statistical analysis

Since Shapiro-Wilk normality test revealed that the data were not normal, non-parametric Mann-Whitney U tests were performed to examine the difference of the outcome measures for type I collagen fibre deposition, collagen fibril alignment, collagen fibre orientation and myofibroblast population between PEMF and control groups on different days. Spearman's rho test was conducted to examine the correlation between myofibroblast population and type I collagen fibre deposition. Significant level was set at 0.05. All the above statistical tests were done using IBM SPSS statistics (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.).

3.4 Results

Throughout the whole experimental period, there were no signs of infection in any of the wounds observed. The blood glucose levels of the rats maintained at a stably high level without significant group differences. Weight loss, frequent urination, increased consumption of food and water, and pink or red fur at the head and neck region were noted as observable characteristics of uncontrolled diabetes.

With picro-sirius red polarization microscopy, type I collagen fibres appeared red or orange with high intensity of birefringence. All these fibres were observed only in the dermal layer.

3.4.1 Type I collagen fibre deposition

As early as on day 7, type I collagen fibres could be clearly observed in PEMF group while in control, they were scarce (Figure 3.1). The type I collagen fibres deposition, represented by redA/ROIA increased steadily from day 7 to day 14 (Figure 3.2). This increase did not obviously plateau. The type I collagen fibre deposition in the wound tissues of PEMF group was also greater than that of control at each time point measured. However, significant difference between PEMF and control groups was only observed on day 7 ($p=0.013$). Although the collagen deposition was greater in the PEMF group than the control on day 10 and 14, the group difference did not reach significance (Figure 3.2).

3.4.2 Collagen fibril alignment

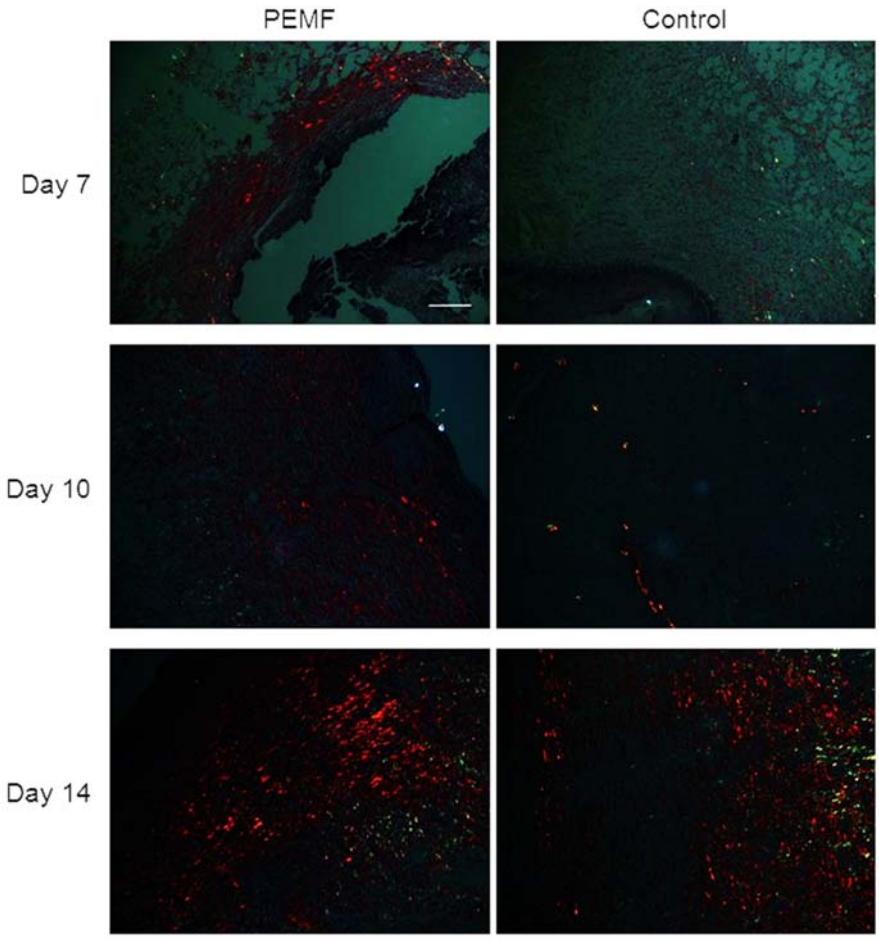
Collagen fibril alignment, represented by the intensity of birefringence, increased from day 7 to day 14 (Figure 3.1 and 3.3). The collagen fibril alignment was similar between groups. No significant differences between days or groups were found.

3.4.3 Collagen fibre orientation

In the plane of sections, no qualitative between-group difference was found in the orientation of collagen fibres in terms of parallelism to the epidermis (Figure 3.1). The collagen fibre orientation index increased steadily from day 7 to day 14 (Figure 3.4). Although the index in PEMF group was

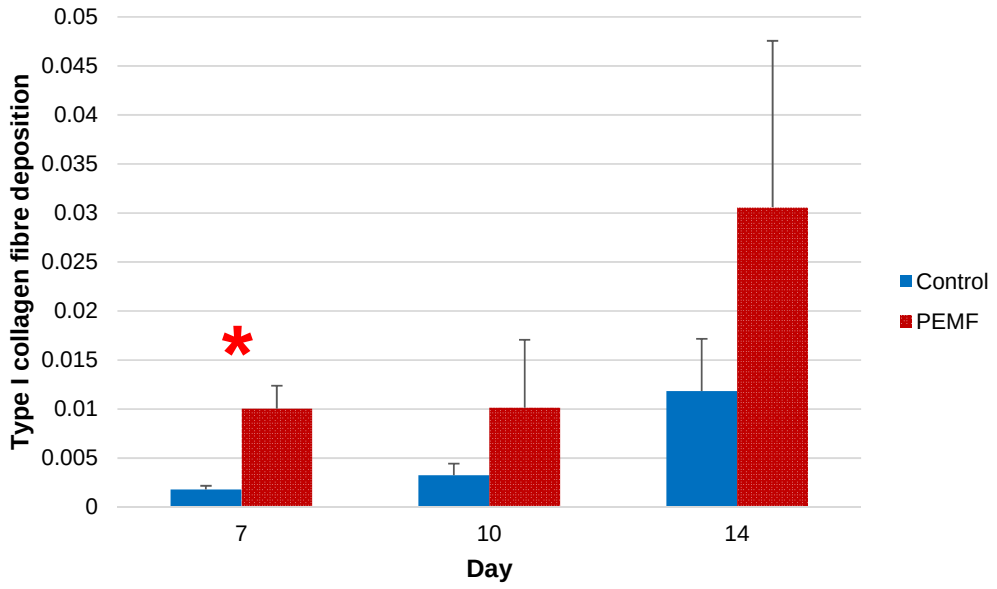
consistently greater than that in control, this difference did not reach statistical significance.

Figure 3.1: Representative picro-sirius red stained sections at the centre of the diabetic wounds from the PEMF and control groups examined under polarized light on post-wounding day 7, 10 and 14.



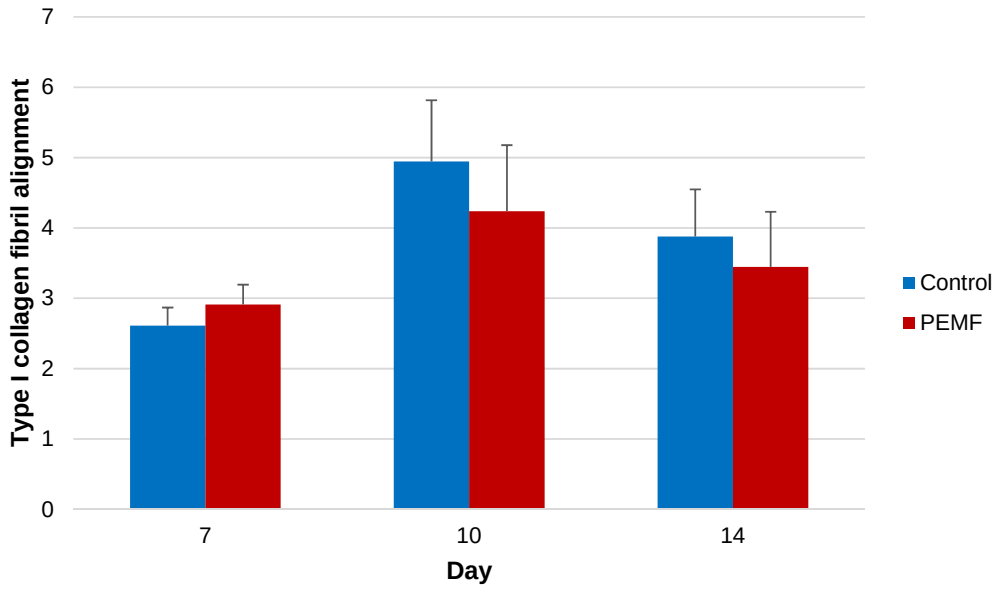
The PEMF group showed early type I collagen deposition whereas limited collagen was deposited in the control group on day 7. The PEMF group showed greater abundance of type I collagen fibres consistently than did the control group on day 10 and 14. Mature type I collagen fibres appeared as red while immature collagen fibres as green. Scale bar: 150μm.

Figure 3.2: The type I collagen fibre deposition of the diabetic wounds from the PEMF and control groups quantified on post-wounding day 7, 10 and 14.



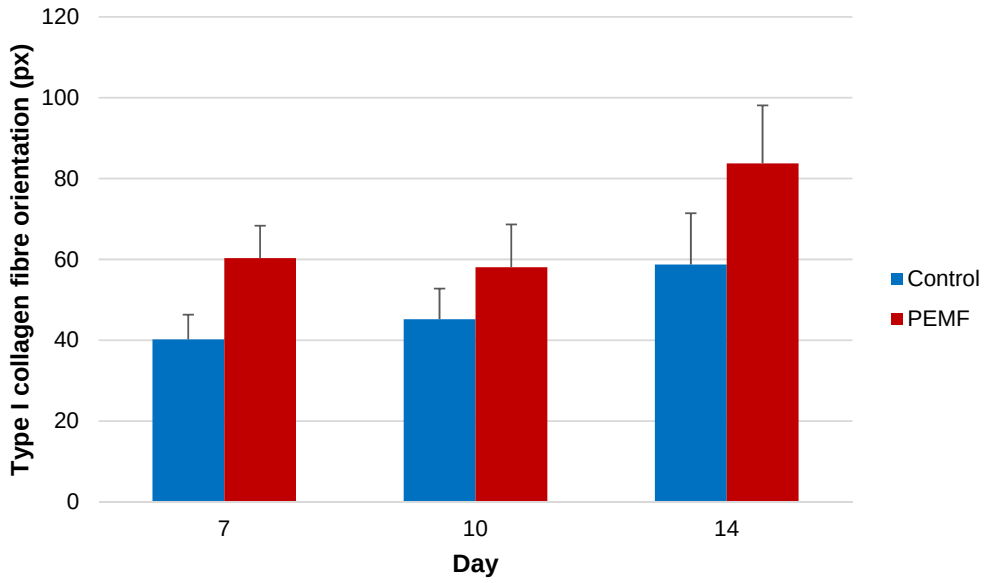
The area of red stained portion normalized by the area of dermis of the picro-sirius red stained sections at the wound centre was estimated as the type I collagen fibre deposition of the diabetic wounds. The PEMF group showed greater type I collagen fibre deposition consistently at the time points examined. Data are expressed as mean+SEM. *significant between-group difference ($p=0.013$).

Figure 3.3: The type I collagen fibril alignment of the diabetic wounds from the PEMF and control groups quantified on post-wounding day 7, 10 and 14.



The intensity of the red stained portion normalized by the background of the picro-sirius red stained sections at the wound centre was estimated as the type I collagen fibril alignment of the diabetic wounds. Data are expressed as mean+SEM.

Figure 3.4: The type I collagen fibre orientation of the diabetic wounds from the PEMF and control groups quantified on post-wounding day 7, 10 and 14.

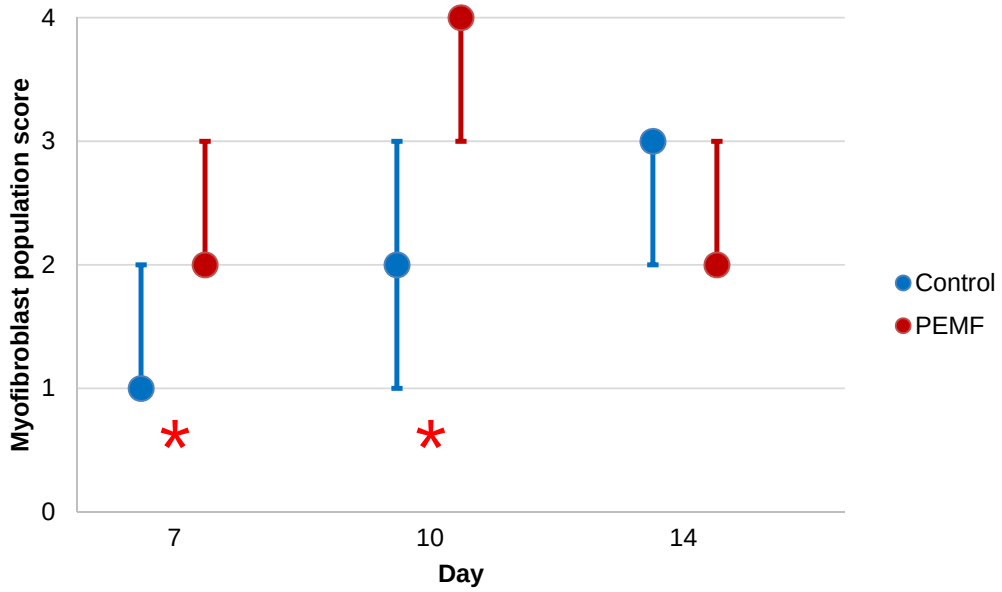


The top ten feret length of the red stained units of the picro-sirius red stained section of the wound centre was estimated as the type I collagen fibre orientation of the diabetic wounds. Data are expressed as mean+SEM.

3.4.4 Correlation between type I collagen fibre abundance and myofibroblast population

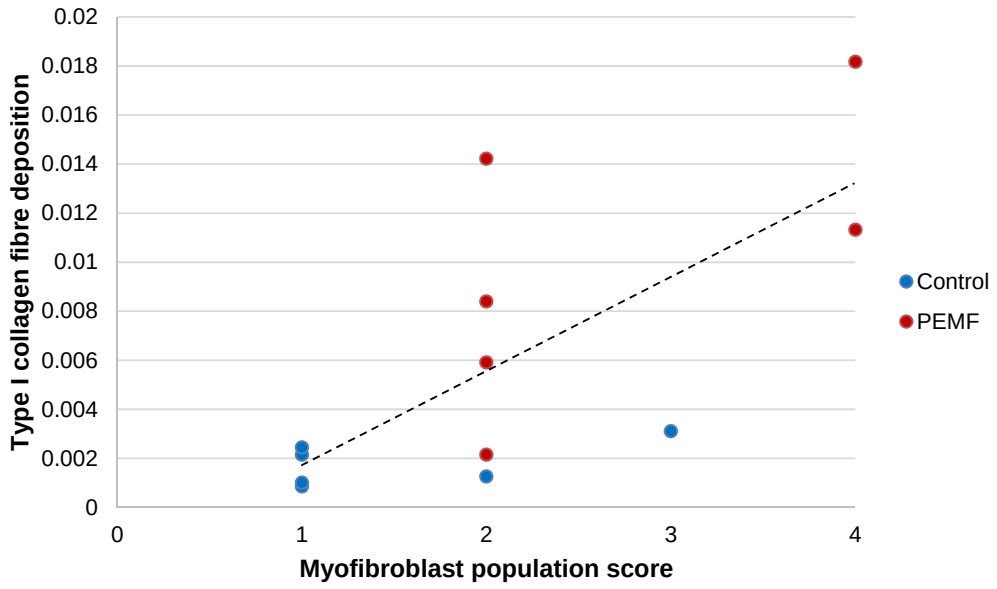
Our previous study reported a temporarily increase in myofibroblast population during early phase of diabetic wound healing upon PEMF treatment (Cheing et al., 2014). To investigate the potential relationship between the collagen deposition and the abundance of myofibroblasts, the redA/ROIA obtained was correlated with the semi-quantitative scores of myofibroblasts. As described previously (Cheing et al., 2014), PEMF treatment showed significantly more myofibroblast population on day 7 ($p=0.042$) and 10 ($p=0.024$) but become similar with control group from day 14 onwards (Figure 3.5). Strong positive correlation was significantly found between type I collagen fibre deposition and the population of myofibroblast on day 7 ($p=0.007$; Figure 3.6), but such significance in the correlation was no longer present on day 10 or day 14.

Figure 3.5: The myofibroblast population of the diabetic wounds from the PEMF and control groups quantified on post-wounding day 7, 10 and 14.



Myofibroblast population score was rated semi-quantitatively (0-4) by two independent raters according to the abundance of alpha smooth muscle actin-positive cells examined by immunohistology at the centre of the diabetic wounds. Data are expressed as median (dots) and IQR (error bars). *significant between-group differences (day 7: p=0.042; day 10: p=0.024).

Figure 3.6: The correlation between the type I collagen fibre deposition and myofibroblast population of the diabetic wounds from the PEMF and control groups examined on post-wounding day 7.



The positive correlation between the type I collagen fibre deposition and myofibroblast population score of the diabetic wounds on day 7 was statistically significant ($r=0.729$, $p=0.007$).

3.5 Discussion and conclusions

Diabetic wounds often show impairments in various cellular activities such as delayed wound closure and re-epithelialization, uncontrolled inflammatory response, and decreased fibroblast proliferation and collagen deposition, all of which together accounting for impaired healing in terms of poor functional recovery such as low tensile strength (Stadelmann et al., 1998). Myofibroblasts, a contractile form of fibroblasts, play a key role in wound contraction. Besides, they also involve in collagen deposition in a healing wound. PEMF has been shown to effectively accelerate wound healing, and our earlier study demonstrated that exposure of diabetic wounds with PEMF treatment increased myofibroblasts population (day 7 and day 10) in addition to enhanced wound closure (day 10 and day 14) and reduced epidermal gap (day 10) (Cheing et al., 2014). The present study adopted the wound tissues from the same batch of rats. Together with our previous studies, it has been found that PEMF promoted myofibroblast population, wound closure and collagen deposition in early phase of wound healing, but it did not produce significant changes in collagen orientation. To date, this is also the first study to examine the effect of PEMF on the structural arrangement of collagen in diabetic wounds quantitatively.

An earlier study reported that PEMF exposure increased the hydroxyproline content in the skin of non-diabetic rats (Ahmadian et al., 2006). Hydroxyproline is a common non-proteinogenic amino acid that is found almost exclusively in collagen (Bornstein et al., 1979). Measurement of hydroxyproline content is often used as an indicator for the collagen amount

or rate of collagen biosynthesis. It was found that PEMF treatment of 25Hz, either with an intensity of 2mT or 4mT for 8 days, showed a significant increase in hydroxyproline content in rat skin (Ahmadian et al., 2006). The treatment protocol applied as well as the timing, in which collagen content was found increasing significantly in that study, was very similar to our findings. Instead of examining the hydroxyproline content that reflects the total content of different collagen fibres, the present study directly examined the biomechanical strength-contributing type I collagen deposition by picro-sirius red polarization method. Type I collagen fibrils align in a more parallel way and appear thicker than type III collagen. Due to the anisotropy, the more parallel the fibrils align, the greater intensity of the birefringence is upon polarized light and so as the intensity of red stain (Montes et al., 1991). As highly birefringent collagen appears orange or red in picro-sirius red polarization method, this is a method to visualize specifically type I collagen and its fibril alignment based on the intensity of birefringence. The present study has demonstrated that PEMF increased the type I collagen deposition in the early phase (day 7) of diabetic wound healing. Our results also demonstrated that the type I collagen deposition in PEMF group tended to be consistently greater than that in the control on day 10 and 14, although the group difference did not reach significance. The non-significance may be due to the large standard deviation and small sample size, or it may be caused by a suboptimal treatment protocol that required further optimization. Nevertheless, the results from the present study suggested that PEMF could promote collagen synthesis and deposition in diabetic wound especially in

the early stage of wound healing (day 7).

Goudarzi et al. (2010) believed that the increased biomechanical strength in the healing diabetic wound upon PEMF treatment might be due to increased collagen fibre content and improved collagen fibril alignment (maturation). In the present study, however, PEMF did not significantly enhance type I collagen fibril alignment in diabetic wound healing. Since wound remodelling occurred late during healing, the time window for collagen fibril alignment observed in the present study may not be long enough as compared to Goudarzi et al. (2010), whose time point of measurement was 27 days post wounding. Of course it was also possible that the increased biomechanical strength obtained could be simply due to an increase in collagen deposition instead.

Conventionally, the spatial orientation of collagen fibres is determined by mathematical models with the input of the inclination angle and azimuth angle (Gasser et al., 2012). This method requires special equipment such as a polarizing light microscope with the universal rotatory stage. Expertise with good engineering or bioinformatics background is also expected. Describing only the general distribution of the collagen fibre orientation, the method ignores the orientation of the individual collagen fibres (Melis et al., 2002). A much easier way to quantitatively estimate the orientation of collagen fibres by measuring the average length of the top ten longest stained collagen fibre units only with the use of image analysis software was adopted (Noorlander et al., 2002). This method is valid based on the assumption that better oriented collagen fibres have greater continuity

(being longer). The possible limitation of this method is that it only considers one dimension which is the length without considering the actual orientation of the collagen fibres, whether perpendicular or parallel to the epidermis. However, among all the picro-sirius red stained sections, no collagen fibres oriented perpendicularly to the epidermis. Instead, they oriented more or less parallel to the epidermis.

Lee et al. (1997) found that PEMF could improve collagen fibre orientation in injured tendon with qualitative histological examination. Quantitatively, our finding showed that the collagen fibre orientation in PEMF group was consistently greater than that in the control, although the between-group difference did not reach the significance level. Such statistical non-significance would probably indicate that PEMF-mediated healing is predominantly by regeneration rather than by contraction and scarring in this wound healing model during our study period. This is supported by Milgram et al. (2004) that PEMF enhanced re-epithelialization but suppressed wound contraction in the early phase of wound healing; faster re-epithelialization may lead to more regeneration rather than scarring. However, the possibility that the rise in the collagen deposition measurement upon PEMF treatment in our model is mainly due to an increase in collagen density as a result of enhanced wound contraction could not be ruled out. It would be interesting to conduct further studies to confirm whether the collagen density measured is due to deposition but not wound contraction. Furthermore, the open-wound model in the present study may allow the wound to dry out which may encourage wound

contraction. This would require further investigation using splinted wound model with Tegaderm and Mastisol (Chung et al., 2010) to suppress wound contraction yet maintain the moisture of the wound to avoid drying out. Adopting the splinted wound model in both normal and diabetic rat may help define clearly the role of PEMF exposure on the biomechanical strength as well as on the wound contraction and collagen deposition.

It was long believed that the increase in collagen deposition is mainly due to an increase in fibroblast proliferation (Black et al., 2003). During cutaneous wound healing, however, it was suggested that myofibroblasts also play a role in synthesizing collagen (Hinz et al., 2012). The present study found that PEMF also increased type I collagen deposition, and demonstrated a significant positive correlation between myofibroblasts population and type I collagen content. During the later phase of wound healing, myofibroblasts gradually disappear while collagen is still being synthesized or under remodelling even after the wound has closed (Tomasek et al., 2002). Therefore, the correlation was not expected at the later time points. These data supported our hypothesis that PEMF promotes myofibroblast proliferation or differentiation that might lead to an increase in wound contraction and collagen deposition. However, the mechanisms of PEMF on promoting myofibroblast proliferation are unclear. Although PEMF-mediated release of FGF-2 was reported (Callaghan et al., 2008; Pan et al., 2013; Tepper et al., 2004), FGF-2 is known to be an inhibitor of myofibroblast differentiation (Ishiguro et al., 2009; Khouw et al., 1999). Therefore, FGF-2 is very unlikely to be involved in the increase of

myofibroblast population by PEMF. Instead, it is suspected that the effect of PEMF could be mediated at least in part via transforming growth factor beta (TGF-β), a potent regulator of myofibroblasts. It would be interesting to investigate if TGF-β signaling pathway plays a central role in the PEMF-stimulated myofibroblast proliferation and/or differentiation.

The present study showed that PEMF treatment promoted diabetic wound healing in terms of accelerated wound closure and increased collagen deposition, while the association of enhanced myofibroblasts population with collagen deposition suggested a potential mechanism of PEMF on wound healing that needed to be confirmed by future study. Under the present PEMF protocol, little influence was found on the collagen fibril alignment and the fibre orientation. Future studies should be performed to further optimize the treatment protocol of PEMF and determine the underlying mechanisms that promote myofibroblast population during diabetic wound healing.

Note: Part of this chapter was published in a peer-reviewed journal (Choi et al., 2016) and presented in conferences (Choi et al., 2014a; Choi et al., 2014b).

Chapter 4

Pulsed electromagnetic field (PEMF) as a potential treatment altering the biomechanical properties of diabetic wounds

4.1 Abstract

In Chapter 3, our findings showed that PEMF enhances collagen deposition associated with an increase in myofibroblast population in diabetic wounds. The previous studies by other research groups have only examined the tensile strength of the diabetic wounds at only one time point. In order to better reflect the structural recovery of diabetic wounds at different phases of healing, other tensile biomechanical properties such as load-relaxation behaviour, energy absorption capacity and Young's modulus at different time points should be included. Therefore, the third study of the present thesis investigated the effects of PEMF on the tensile biomechanical properties of diabetic wounds at different phases of healing. As various studies adopted different PEMF parameters generating inconsistent results, two different magnetic intensities of PEMF were included in the present study.

One hundred and eleven 10-week-old male streptozotocin (STZ)-induced diabetic Sprague-Dawley (SD) rats were randomly assigned to two PEMF groups and a sham control group. Six-millimetre biopsy punched full thickness wounds were made on the lateral side of their hindlimbs. The

PEMF groups received active PEMF delivered at 25Hz with intensity of either 2mT or 10mT daily, while the sham group was handled in a similar way but not exposed to PEMF. Wound tissues were harvested for tensile testing on post-wound days 3, 5, 7, 10, 14 and 21. Load-relaxation behaviour, maximum load, maximum stress, energy absorption capacity, Young's modulus and thickness of wound tissue were measured.

On post-wounding day 5, the PEMF group that received 10-mT intensity had significantly increased energy absorption capacity ($p<0.05$) and an apparent increase in the maximum load. However, the 10-mT PEMF group demonstrated a decrease in Young's modulus on day 14 ($p<0.05$). Both 2-mT and 10-mT PEMF groups showed a significant increase in the overall thickness of wound tissue ($p<0.001$) but a decrease in the overall maximum stress ($p<0.01$) of the wounds tissue.

The present findings demonstrated that the PEMF delivered at 10mT can improve energy absorption capacity of diabetic wounds in the early healing phase. However, PEMF (both 2-mT and 10-mT) seemed to decrease those material properties (maximum stress and Young's modulus) in the remodelling phase. PEMF could be a useful treatment for promoting the recovery of structural properties (maximum load and energy absorption capacity), but its exposure in the remodelling phase might cause potential delay in the recovery of material properties.

4.2 Introduction

Impaired wound healing is common in people with diabetes mellitus (DM) and diabetic foot ulceration is one of the well-known complications; the lifetime incidence among diabetic population was about 25% (Singh et al., 2005). Risk factors for diabetic foot ulcers include poor glycaemic control, ill-fitting footwear, peripheral polyneuropathy, ischaemia, poor vision and limited joint range of motion (Jeffcoate et al., 2003). Diabetic foot ulcers may progress into chronic ulcers which may lead to amputations severely decreasing the patients' quality of life (Boulton et al., 2005; Jeffcoate et al., 2003). Various authors agreed that current managements of diabetic foot ulcers were not effective enough to control the rate of amputation related to diabetic foot ulcers and novel treatments were encouraged to be adopted (Andrews et al., 2015; Global Lower Extremity Amputation Study Group, 2000; Jeffcoate et al., 2003; Miller et al., 2014).

PEMF has been used as an intervention for chronic ulcers in clinical studies yielding positive results that PEMF accelerated wound closure (Kwan et al., 2015; Sarma et al., 1997; Stiller et al., 1992; Todd et al., 1991), reduced wound pain (Stiller et al., 1992), enhanced healthy granulation (Stiller et al., 1992) and promoted circulation (Kwan et al., 2015). A systematic review also concluded that PEMF could significantly accelerate the healing of chronic ulcers (decubitus, venous and plantar) in patients (McGaughey et al., 2009).

On the other hand, animal studies also provide evidence for the use of PEMF in enhancing wound healing. Compared to non-diabetic animal

models, diabetic wounds exhibited impairments in wound closure (Callaghan et al., 2008; Goudarzi et al., 2010), neo-vascular growth (Callaghan et al., 2008) and tensile strength of wound (Goudarzi et al., 2010). PEMF has been shown to rescue the impairments, and accelerate wound closure (Callaghan et al., 2008; Goudarzi et al., 2010), promote vascular growth (Callaghan et al., 2008), improve blood circulation (Callaghan et al., 2008), increase myofibroblast population (Cheing et al., 2014), enhance collagen deposition (Choi et al., 2016) and increase tensile strength (Goudarzi et al., 2010) in the diabetic wound models.

Biomechanical properties of soft tissues are contributed by the underlying structural architecture and compositions of the matrix. For example, tensile biomechanical properties are mainly contributed by the amount of collagen, fibril alignment and fibre orientation. The assessment of biomechanical properties of injured soft tissues can thus reveal the functional outcome of healing. Nayci et al. (2001) found that PEMF improve the maximum stress of intestinal anastomosis in a rat model accompanied by an increase in hydroxyproline content, which is a composition of collagen. Recently in diabetic wounds, our research team has demonstrated that PEMF promotes collagen deposition (Choi et al., 2016). It is tempting to speculate that PEMF can also enhance various collagen-dependent biomechanical properties of diabetic wounds. Although Goudarzi et al. (2010) reported that 10-day exposure to PEMF increases the maximum stress of diabetic wounds, they did not report the changes in other tensile biomechanical properties such as maximum load, energy absorption capacity and Young's modulus

over time. Detailed assessment of the tensile biomechanical properties can provide more information on the structural recovery of the wounds and the effects of PEMF. Maximum load and energy absorption capacity are contributed by collagen abundance and orientation. However, maximum stress and Young's modulus does not depend on the mass, instead, they are mainly contributed by the quality, orientation and cross-link density of collagen fibres (Sussman, 1973; Williams et al., 1977). By measuring the biomechanical properties at different phases of wound healing, it can provide useful information on the effects of PEMF at specific wound healing time frame.

While investigating the effects of PEMF on wound healing, various research groups adopted different PEMF parameters. This may account for the contradictory findings reported in these studies. For example, two studies (Glassman et al., 1986; Ottani et al., 1988) adopted very different intensity, frequency, waveform and treatment time of PEMF; Ottani et al. (1988) showed that PEMF produced overall improvements on wound closure, neo-vascular growth and collagen deposition, which were contrary to the negative findings reported by Glassman et al. (1986). Pilla et al. (2011) proposed that the choice of PEMF parameters was crucial in determining the efficacy of PEMF. In order to evaluate whether intensity is a determinant of treatment protocol to enhance diabetic wound healing, the present study compared the effects of PEMF delivered at high and low intensities (in millitesla range commonly used clinically) on the tensile biomechanical properties of diabetic wounds at different phases of healing.

4.3 Materials and methods

4.3.1 Animal handling and DM induction

The present study protocol was approved by the Animal Subjects Ethics Sub-Committee of the administering institution. A total of 111 10-week-old male adult SD rats (300-400g) were used. All of the rats received humane care and the protocols were in compliance with the guidelines from the Animal Subjects Ethics Sub-Committee. The animal handling and DM induction procedures were described in Chapter 2.

4.3.2 Wound induction

Before wound induction, mixtures of ketamine and xylazine were administered intra-peritoneally at a dosage of 100 and 10mg/kg body weight respectively. After shaving and disinfecting with betadine and alcohol prep, the wounds were induced with sterile 6-mm biopsy punches on the lateral side of each hindlimb (about 3 mm distal to the fibula head). The wounds were left open and the rats were then housed individually to prevent cannibalism. A total of 215 wounds were used in this study. (Of the 111 rats, some wounds were randomly selected for other pilot studies that they were not included in the present study.) Photographs of wounds were taken on post-wounding day 0, 3, 5, 7, 10, 14 and 21 and wound area were analysed by the Fiji software. Percentage of wound area was calculated by dividing the wound area at the time point with the wound area on day 0, and then multiplied by 100%.

4.3.3 PEMF treatment

The rats were randomly allocated into sham and active PEMF groups. To examine the effects of high and low intensities, 2-mT and 10-mT PEMF treatment were included.

PEMF was generated by a commercially available device (BTL-4000, BTL Industries Ltd., UK). Starting from post-wounding day 1, the wound-bearing hindlimbs of the rats in the PEMF groups were exposed to 25-Hz PEMF with an intensity of either 2, or 10mT for 1 hour daily. During PEMF exposure, each rat was restrained in a plastic restrainer bag. The sham PEMF group was handled in a similar manner except the device was not activated.

4.3.4 Biomechanical testing

On post-wounding day 3, 5, 7, 10, 14 and 21, rats were randomly selected, euthanized and then kept frozen at -80^{o}C until the biomechanical testing. The rats were defrosted at room temperature for at least 6 hours before testing.

A constant speed material testing system (MTS Synergie 200 machine, MTS 643.12F-24 extensometer: MTS Systems Corporation, Eden Prairie, MN, USA) and the TestWorks 4 Universal testing software (MTS Systems Corporation, Minnesota, USA) were used to measure structural and material properties of the wound tissue.

The wound bed in a skin layer of 6.0mm x 18.0mm was dissected from each hindlimb of the rats along the fibula. The actual length, width and thickness

of the extracted tissue were measured with a Vernier calliper. The specimens were elongated by the material testing system to a 2.5% strain position at 10mm/min for 10 preconditioning oscillation cycles (Ng et al., 2004), followed by the elongation to 5% strain at a speed of 500mm/min and sustained for 30s. Load-relaxation was expressed as the change of load in the 30s normalized by the initial load. After the measurement of load-relaxation behaviour, the specimens were returned to the -5% strain position for 1min. The high-strain tensile biomechanical properties were measured by elongating the specimen at a speed of 500mm/min until failure (Ng et al., 2011). The speed of 500mm/min was adopted because this is commonly used in tendon/ligament testing, which can mimic the movement speed in daily life to increase the clinical relevance. Load and deformation were recorded at 100Hz. During the whole testing procedure, the specimens were kept moist. A load-deformation curve was plotted to examine the structural properties and the curve was transformed into a stress-strain curve by dividing the load with the cross-sectional area and the elongation with the original length to examine the material properties.

4.3.5 Statistical analysis

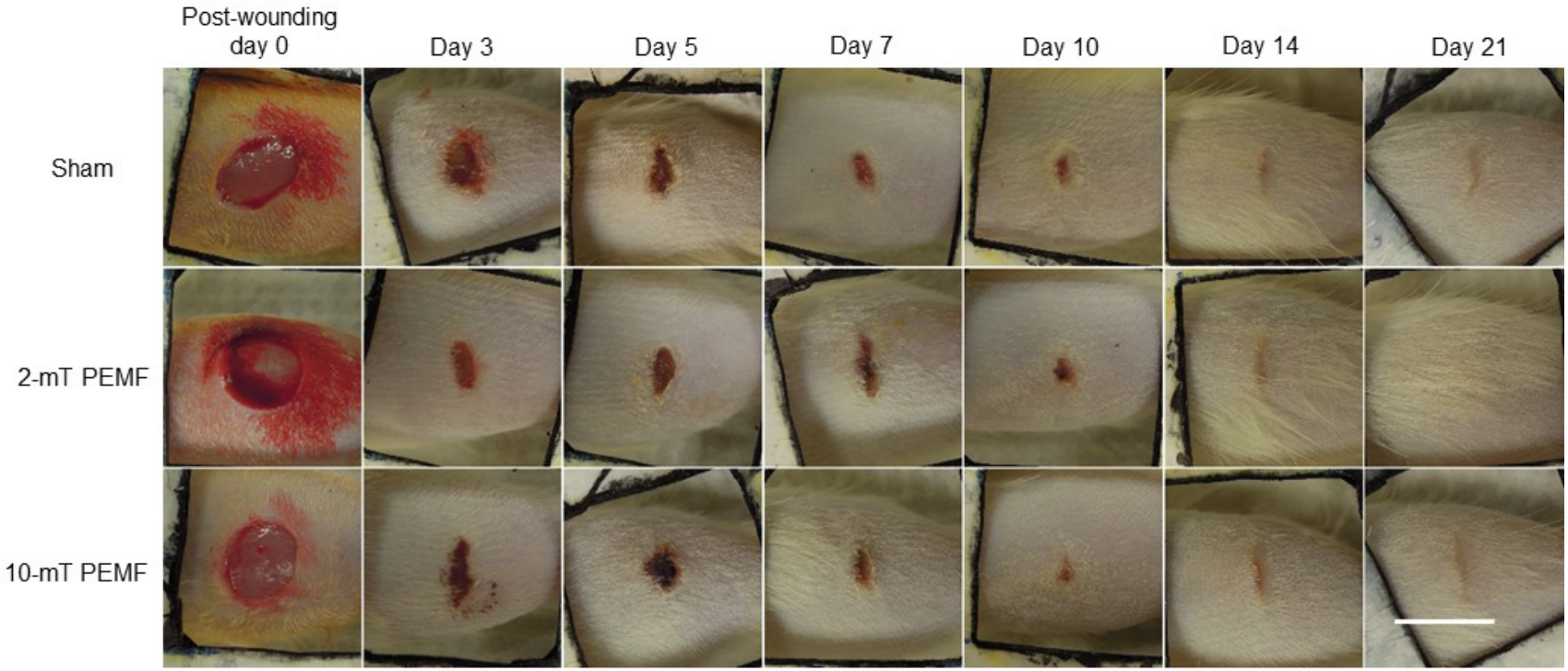
Two-way ANOVA with post-hoc LSD tests were used to examine the interactions between time points and PEMF intensities, and the overall group (sham, 2-mT and 10-mT PEMF) effects. One-way ANOVA with post-hoc LSD tests were used to test the effects of groups on the % wound area, biomechanical properties and thickness of the diabetic wounds at

different time points. Significance level was set as 0.05. The analysis was executed by IBM SPSS statistics (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.).

4.4 Results

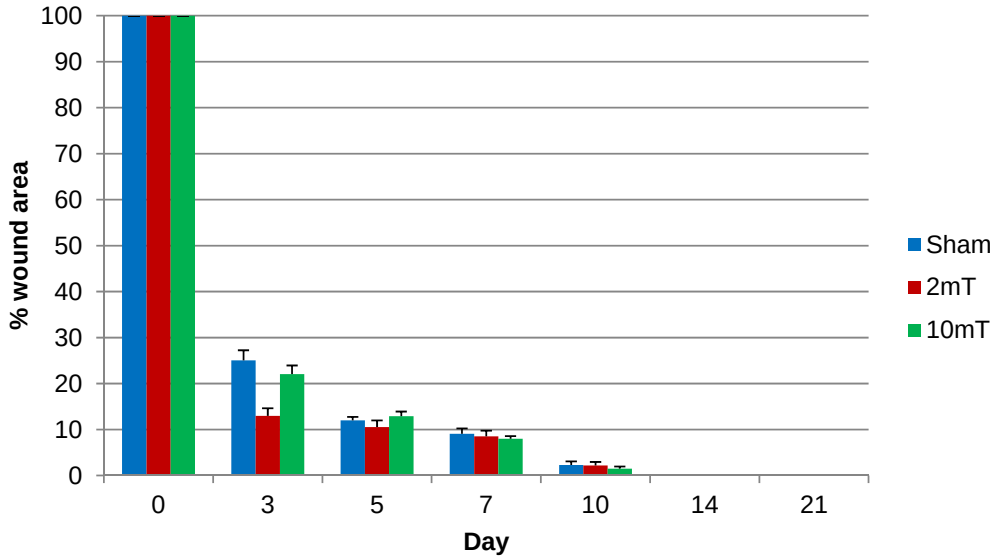
Throughout the experiment, hyperglycaemia was maintained and the body weight of rats decreased over time despite the increased consumption of water and food. These were typical signs of successful induction and maintenance of diabetic condition in the rats. No signs of wound infection were observed in any of the rats during the experiment. By day 14, all wounds had grossly closed up leaving no scab (Figure 4.1). There was significant group (PEMF intensity) x time interaction in percentage of wound area ($p=0.009$; Figure 4.2). Also, the wound size of the 2-mT PEMF group appeared to reduce on day 3. However, the between-group difference did not reach significance at any time point. No significant interactions or differences were found in the load-relaxation behaviour (Figure 4.3).

Figure 4.1: Representative photographs of the diabetic wounds from the 3 groups taken in 3 weeks.



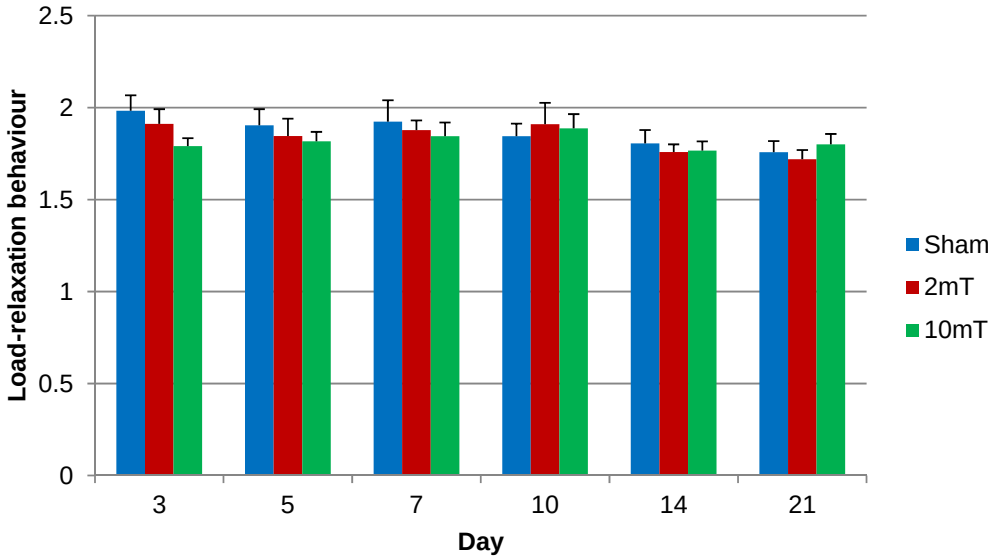
The diabetic wounds were treated with sham, 2-mT and 25-Hz, and 10-mT and 25-Hz PEMF in 3 weeks. All wounds were closed by day 14. The area of the wounds was slightly smaller in the 2-mT PEMF group. All photographs of the wounds were taken with reference to a 2cm x 2cm square scale. Scale bar: 1cm.

Figure 4.2: The percentage of wound area of the diabetic wounds from the 3 groups measured in 3 weeks.



The percentage of wound area was the area of wounds measured at a specific time point normalized by the initial wound area (day 0). Signification group (PEMF intensity) x time interaction was found ($p=0.009$). The area of the wounds from the 2-mT PEMF group tended to be smaller than that from the sham and 10-mT groups. Data are expressed as mean+SEM.

Figure 4.3: The load-relaxation behaviour of the diabetic wounds from the 3 groups measured in 3 weeks.

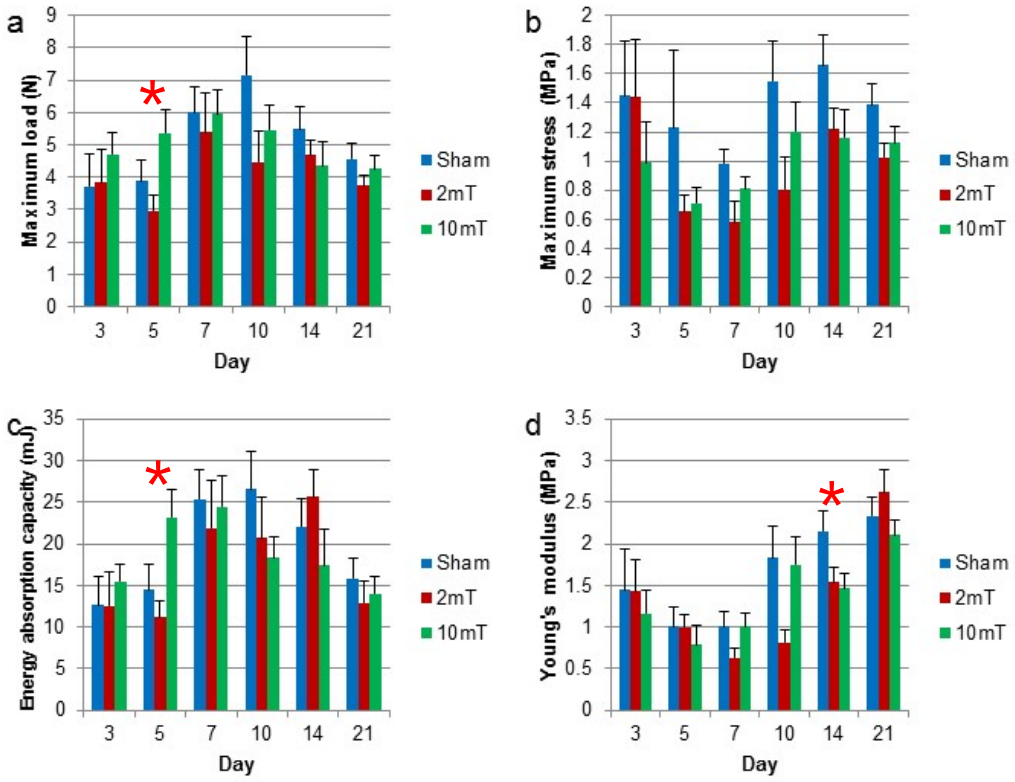


Data are expressed as mean+SEM.

As for high-strain biomechanical properties, there were significant overall between-group differences in maximum stress in which both PEMF groups were smaller than sham (sham vs. 2-mT: $p=0.004$; sham vs. 10-mT: $p=0.007$; Figure 4.4). On day 5, the energy absorption capacity of 10-mT PEMF group was significantly greater than that of the sham ($p=0.046$) and that of the 2-mT ($p=0.005$); the maximum load of the 10-mT was also significantly greater than that of the 2-mT ($p=0.01$). On day 14, the Young's modulus of the 10-mT group was significantly smaller than that of the sham group ($p=0.025$).

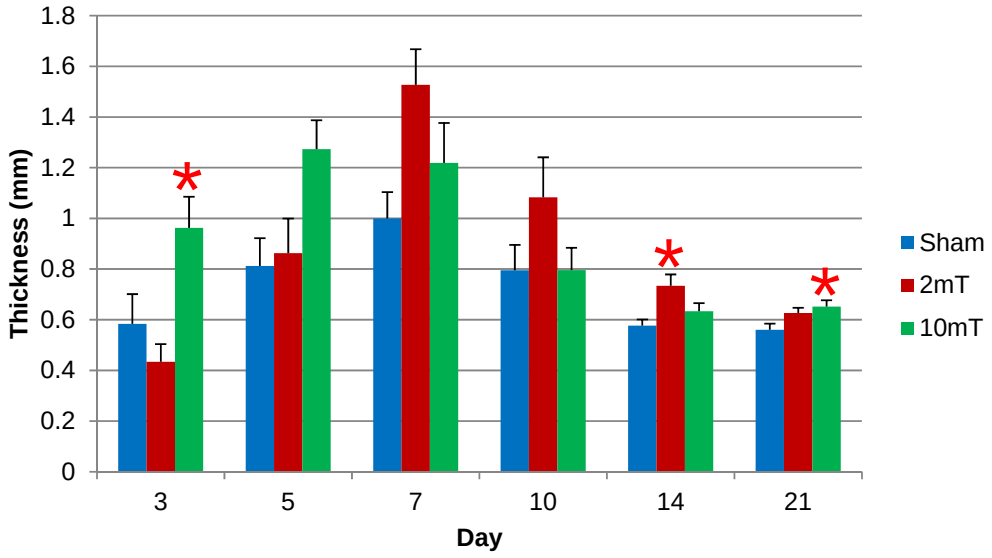
In terms of thickness of the wounds, there was significant group (PEMF intensity) x time interaction ($p<0.001$) and overall group effect ($p<0.001$). The wounds of both PEMF groups were thicker than those of the sham group (sham vs. 2-mT: $p=0.049$; sham vs. 10-mT: $p<0.001$; Figure 4.5). On day 3, the wounds of the 10-mT group was significantly thicker than those of the sham ($p=0.018$) and 2-mT group ($p=0.001$). The wound thickness of the 2-mT and 10-mT PEMF group was also significantly greater than that of the sham group on day 14 (sham vs. 2-mT: $p=0.002$) and 21 (sham vs. 10-mT: $p=0.008$) respectively.

Figure 4.4: The high-strain tensile biomechanical properties (maximum load [a], maximum stress [b], energy absorption capacity [c] and Young's modulus [d]) of the diabetic wounds from the 3 groups measured in 3 weeks.



The 10-mT PEMF group showed significantly greater energy absorption capacity than the sham and 2-mT group, and greater maximum load than the 2-mT group on day 5. However, the Young's modulus of the 10-mT group was significantly smaller than that of the sham group on day 14. Data are expressed as mean+SEM. *significant between-group difference ($p<0.05$).

Figure 4.5: The thickness of the diabetic wounds from the 3 groups measured in 3 weeks.



The 10-mT PEMF group showed significantly greater wound thickness than did the sham group on day 3 and 21, while the wound thickness of the 2-mT group was significantly greater than that of the sham on day 14. Data are expressed as mean+SEM. *significant between-group difference ($p<0.05$).

4.5 Discussion and conclusions

To our knowledge, it was the first study that examined the effects of PEMF on various tensile biomechanical properties of diabetic wounds over 3 weeks so as to cover the inflammatory, proliferative and part of the remodelling phases of wound healing. In terms of wound closure, the significant group (PEMF intensity) x time interaction in the present study suggested that PEMF might enhance wound closure which was consistent to those reported by other studies (Callaghan et al., 2008; Cheing et al., 2014; Goudarzi et al., 2010). In the present study, 2-mT PEMF appeared to enhance wound closure in the early phase of diabetic wound healing (Figure 4.1 and 4.2). Both Callaghan et al. (2008) and Goudarzi et al. (2010) found that PEMF could reduce the time for complete closure in diabetic wounds. While comparable PEMF parameters were applied, different diabetic wound models were adopted in the two previous studies compared to the present one. Callaghan et al. (2008) used a model with minimal wound contraction whereas Goudarzi et al. (2010) utilized large wounds. Both prolonged the wound closure for the observation of the wound-closure-promoting effect brought by PEMF. In the present study, the effect of PEMF on reducing time for wound closure might be masked by the effects of wound contraction and also the small wound size. Further research may consider various strategies such as the use of wound splinting to minimize wound contraction.

The present study evaluated the effects of PEMF on the load-relaxation behaviour but no between-group differences could be found. This might be because the load-relaxation behaviour is a low-strain tensile property which

is mainly contributed by the network of elastic fibres (Wilhelmi et al., 1998); the current protocol of PEMF treatment might not have significant beneficial effects on the recovery of the elastic fibres.

Instead of measuring the biomechanical strength of wounds at a single time point in the late phase of wound healing, different time points for biomechanical measurement were included in order to examine the biomechanical recovery in various healing phases. We found that 10-mT PEMF increased the maximum load and energy absorption capacity in the early phase only. However, after normalizing the maximum load with cross-sectional area as maximum stress, no significant difference was found between groups. This can be explained by the increased thickness in 10-mT group on day 3 and 5 compared to the sham group. Considering the composition of the skin, the thickness of the wound could be contributed by oedema and granulation tissue including extracellular matrix. With the improvement of the structural properties (maximum load and energy absorption capacity) which partly depend on the abundance of collagen, the PEMF-induced increase in wound thickness might probably be partly related to fibroblast/myofibroblast proliferation and collagen deposition. However, since the material properties (maximum stress and Young's modulus) of the diabetic wounds were not increased in PEMF groups, PEMF might not necessarily lead to better quality or alignment of the collagen fibres. In fact, our earlier studies (Cheing et al., 2014; Choi et al., 2016) showed that PEMF increased myofibroblast population and collagen deposition in the early phase of diabetic wounds, but not the collagen

quality and alignment. Although 2-mT PEMF did not appear to increase the thickness in the early phase, it might exert effects in the later phase by the time re-epithelialization was completed on day 10. This is supported by the trend of increased wound thickness on day 7, day 10 and significantly day 14, as compared with the sham group. These findings demonstrated that PEMF of different intensities may exert influences on diabetic wound healing at different time windows.

The maximum stress and Young's modulus depend on the alignment and orientation of collagen but not the mass of wound tissues (Williams et al., 1977). The overall smaller maximum stress and a significant decrease of Young's modulus in the PEMF groups than the sham group by day 14 reflected that PEMF might not promote collagen alignment and orientation especially in the late phase after re-epithelialization had completed. This finding was consistent to the result reported in our earlier study that PEMF did not promote alignment of the collagen during diabetic wound healing (Choi et al., 2016). Therefore, our findings did not support the notion that PEMF could increase the material properties of diabetic wounds after complete re-epithelialization, which was different from the report by Goudarzi et al. (2010), in which they found that PEMF significantly increased the maximum stress of diabetic wounds. In their study, the PEMF treatment was stopped on day 10, which was just before complete re-epithelialization. Hence, the treatment period corresponding to the wound healing phase appears to be a determining factor to the treatment outcome. Strauch et al. (2007) also reported PEMF-induced increment in tensile

strength, although in non-diabetic wounds. In their study, PEMF treatment was given even after complete re-epithelialization. The effective intensities used were 100μT and 5μT, which are very small as compared to other studies. Therefore, the intensity may also be another important determining factor for the treatment outcome. More studies should be done to develop an optimal set of PEMF parameters for treating diabetic wounds.

The present study showed that 10-mT PEMF can probably augment fibroblast/myofibroblast proliferation and collagen deposition in the early phase of healing; 2-mT PEMF may enhance the proliferation in the later phase. Nonetheless, PEMF of either intensity did not increase the maximum stress and Young's modulus after complete re-epithelialization. Conversely, the PEMF weakened the wounds in the remodelling phase. This might be because PEMF prolongs collagen deposition but suppresses the remodelling after complete re-epithelialization. Robotti et al. (1999) reported that PEMF decreased the breaking strength of incised tendon after 3 weeks in a chicken model; increased peri-tendinous adhesions were also noticeable in the PEMF group. Their findings suggested that fibrosis could be potentially promoted by PEMF treatment. It is therefore noteworthy to confirm whether fibrosis, which may be related to the PEMF-induced reduction in maximum stress in the present study, is present in the PEMF-treated diabetic wounds. The paradoxical effects of the PEMF resemble very much the roles of calpains, which are calcium-dependent proteases, in wound healing (Nassar et al., 2012). In a transgenic mice model, over-expression of calpastatin, which is an inhibitor to calpains, impaired re-epithelialization, suppressed

angiogenesis, decreased myofibroblast population and reduced collagen deposition in the early phase of wound healing. However, in the late phase, inhibition of calpains caused reduction in fibrosis. These findings reflected the important roles of calpain in promoting re-epithelialization, neo-vascular growth, myofibroblast differentiation, collagen deposition and fibrosis formation. Future studies to explore the relationships between the effects of PEMF and the endogenous calpains are warranted.

In conclusion, PEMF delivered at 10mT and 25Hz significantly increased energy absorption capacity and appeared to increase maximum load in the early proliferative phase, but it decreased Young's modulus in the remodelling phase of diabetic wounds. Throughout the time points examined, the current PEMF protocols (both the 2-mT and 10-mT) thickened the wounds but reduced the maximum stress of diabetic wounds.

Chapter 5

Pulsed electromagnetic field (PEMF) augmenting antibiotic effect of gentamicin to *Pseudomonas aeruginosa* (PAO1)

5.1 Abstract

Biofilm infection may be a major reason leading to chronic and non-healing wounds (Bjarnsholt et al., 2008). In diabetic foot ulcers, *Pseudomonas (P.) aeruginosa* is the most common bacteria found (Parsa et al., 2015). This species is also a common opportunistic bacteria in hospital (Bjarnsholt et al., 2008). Among various bacteria commonly found in infected chronic wounds, *P. aeruginosa* tends to result in larger wounds (Gjødsbøl et al., 2006). In order to explore the potential effects of PEMF on biofilm-infected diabetic foot ulcers, the fourth study of the present thesis was conducted to examine the effect of PEMF on the survival of *P. aeruginosa* (PAO1) with or without antibiotic gentamicin.

A 3 by 4 study design was adopted. *P. aeruginosa* in Mueller-Hinton broth (MHB) was randomly assigned to one of the 12 groups that had been exposed to either sham PEMF, 20-Hz or 72-Hz PEMF vs. 0, 0.25, 0.5 or 1 minimum inhibitory concentration (MIC) of gentamicin. The intensity of all active PEMF groups (PEMF frequency of either 20-Hz or 72-Hz) was set at 5mT. The exposure time was 10 hours. All 12 groups were incubated under 37^{o}C with aeration for 24 hours in total including the PEMF exposure time.

Bacterial colonies of each sample were counted at the baseline and after the 24-hour incubation.

With 10-hour 20-Hz PEMF radiation and gentamicin of 1 MIC, the growth of *P. aeruginosa* was significantly inhibited when compared to the sham PEMF group with 1-MIC gentamicin. Interestingly, in sub-MIC levels of gentamicin, both 20-Hz and 72-Hz PEMF seemed to promote the bacterial growth.

PEMF appeared to have differential influence on the growth of *P. aeruginosa* depending on the concentrations of gentamicin and parameters of PEMF. With sub-MIC levels of gentamicin, both 20- and 72-Hz PEMF increased the growth of *P. aeruginosa*. With the MIC level of gentamicin, 20-Hz PEMF decreased the viability of *P. aeruginosa*, but interestingly, 72-Hz PEMF enhanced the viability.

5.2 Introduction

Pseudomonas (P.) aeruginosa is a common opportunistic pathogen affecting immuno-compromised individuals. The pathogen causes lung infections especially in patients with cystic fibrosis (Talwalkar et al., 2016; Winstanley et al., 2016), urinary tract infections (Kumar et al., 2016) and chronic wound infections (Bjarnsholt et al., 2008; Parsa et al., 2015). The infections caused by the bacteria are usually chronic as they show resistance to the host immune responses (Bjarnsholt et al., 2008) and antibiotic treatments (Kaye et al., 2015). To enhance its survival, the pathogen secretes virulence factors including rhamnolipid to destroy polymorphonuclear leukocytes (Jensen et

al., 2007), and alginate to scavenge free oxygen radicals released by polymorphonuclear leukocytes (Bjarnsholt et al., 2005).

P. aeruginosa also secretes metalloproteinases and elastases which not only act against host immune chemicals, but also digest the extracellular matrix such as collagen, elastin and proteoglycans particularly in chronic wounds (McCarty et al., 2012; Supuran et al., 2001). To further complicate the situation, the metalloproteinases secreted by *P. aeruginosa* act synergistically with the host matrix metalloproteinases to decompose collagen prematurely before the remodelling phase of the healing process (McCarty et al., 2012). *P. aeruginosa* that cannot be eliminated by either the host immune system or antibiotics continuously stimulates the influx of polymorphonuclear leukocytes that may lead to tissue damage as a result of increased production of cytotoxic enzymes, free oxygen radicals and inflammatory mediators (Percival et al., 2015). These biochemical signals can cause persistent damage to the wound and, together with the bacteria and its proteases, delay the entire wound healing process with a prolonged inflammatory phase.

Common treatments for chronic ulcers include dressing, debridement and antibiotic therapy (Andrews et al., 2015; Cooper et al., 2015). Focusing on the mitigation of risk factors, specific treatments may apply to a specific type of chronic ulcers. For examples, chronic venous ulcers are usually treated with compression dressings and venous surgery to eliminate venous reflux and obstruction (Cooper et al., 2015), while chronic diabetic foot ulcers may require offloading and surgery to manage ischemia (Andrews et

al., 2015; Jeffcoate et al., 2003). Despite the multidisciplinary care, the outcome of chronic diabetic foot ulcers is still unsatisfactory (Andrews et al., 2015). Due to the therapeutic challenge, more effective interventions for chronic diabetic foot ulcers are constantly in demand (Andrews et al., 2015; Game et al., 2012).

Emerging evidence supports PEMF to be an effective therapeutic approach for promoting wound healing. Previous studies demonstrated that PEMF reduced the wound size (Kwan et al., 2015; Salzberg et al., 1995; Sarma et al., 1997; Stiller et al., 1992; Todd et al., 1991), healing time (Itoh et al., 1991), recurrence rate (Ieran et al., 1990), swelling (Todd et al., 1991) and wound pain (Stiller et al., 1992), but increased microcirculation (Kwan et al., 2015). Animal studies have shown that PEMF enhanced healing of acute wounds without infections (Athanasiou et al., 2007; Matic et al., 2009; Ottani et al., 1988; Patino et al., 1996; Strauch et al., 2007). Although the mechanisms remain unclear, PEMF could be a potential treatment option for chronic ulcers.

As most chronic ulcers are colonized by bacteria, it was believed that bacterial infection played an important role in the chronicity of the ulcers (Bjarnsholt et al., 2008; Gjødsbøl et al., 2006; Parsa et al., 2015). It is noteworthy to investigate whether PEMF exerts effects on the bacteria infecting chronic wounds. So far, no studies have examined the effects of PEMF on evidently infected chronic wounds, but *in vitro* studies have shown that PEMF inhibited the growth of *Staphylococci* in the presence (Matl et al., 2011; Pickering et al., 2003) or absence of antibiotics (Ahmed

et al., 2013). The effects of PEMF on bacterial growth depend on the PEMF parameters. When PEMF delivers at low frequencies (0.1 or 0.3Hz) and high intensity (15mT), PEMF increases the viability of Gram-positive bacteria (Moore, 1979). Since Gram-negative bacteria are more resistant to antibiotics than Gram-positive ones, investigations on the responses of Gram-negative bacteria to PEMF are noteworthy. For a Gram-negative strain, Piyadasa et al. (2016) demonstrated that short exposure (3 hours) to PEMF at very high frequency (~100kHz) promoted the growth of *Escherichia (E.) coli*. In contrast, longer exposure (7 hours) to PEMF inhibited the growth of *E. coli*. In their study, when the *E. coli* was cultured with a sub-lethal dose of silver nano-particles (AgNPs) which metabolically compromise bacteria, PEMF conversely inhibited the viability of the *E. coli* independent of the length of exposure. The mechanism behind has not been elucidated yet. It was postulated that PEMF alters electrostatic balance of the membrane components (Inhan-Garip et al., 2011) and modifies the penetration rate for antimicrobial agents into the bacterial cell (Segatore et al., 2012). Specifically for *P. aeruginosa*, Moore (1979) showed that PEMF delivered at very low frequency (0.3Hz) could increase the viability, but when higher intensities (30 and 60mT) were applied, PEMF may reduce the viability of *P. aeruginosa*. Segatore et al. (2012) found that PEMF (2mT and 50Hz) could immediately increase the susceptibility of *E. coli* and *P. aeruginosa* to aminoglycoside antibiotics at 4-, 6- and 8-hour incubation. However, the effect disappeared after 24-hour incubation which may be due to adaptation of bacteria to PEMF exposure.

In clinical setting, antibiotics are commonly prescribed to manage wound infection in patients (Andrews et al., 2015; Cooper et al., 2015), it is clinically important if PEMF can enhance the action of antibiotics on the growth or survival of Gram-negative *P. aeruginosa*. An *in vitro* study is important to determine the effect of PEMF on the bacterial species alone under controlled environment without the interference from the host immune system. Therefore, the present study aimed at investigating the effects of PEMF on the growth of *P. aeruginosa* in broth culture in the presence or absence of gentamicin after 24-hour incubation. To avoid the adaptation of bacteria, shorter exposure time (<24 hours) was used.

5.3 Materials and methods

5.3.1 Preparation of suspension of *P. aeruginosa* (PAO1)

Frozen stock of *P. aeruginosa* PAO1 was streaked on a Luria-Bertani (LB) agar plate and incubated for 24 hours under 37^{o}C. Morphologically similar colonies were picked and each of which was transferred into 3ml MHB for 24-hour incubation under 37^{o}C with aeration. The concentration of this bacterial suspension was about 10^9 colony-forming units (CFU)/ml. The suspensions were then diluted by 1000 folds in MHB and the resulting *P. aeruginosa* suspensions were about 10^6 CFU/ml.

5.3.2 Determination of MIC of gentamicin against *P. aeruginosa*

As the lowest concentration of an antibiotic leading to inhibition of a specific bacterial strain at a visible level, the MIC of gentamicin for PAO1

was determined through serial 2-fold dilution. One-millilitre gentamicin of different concentrations was added into 1ml *P. aeruginosa* suspension standardized at 0.5 McFarland standard. After 24-hour incubation under 37^{o}C with aeration, the lowest concentration of gentamicin leading to a visibly clear suspension was determined as the MIC. In the present study, the MIC was tested to be 0.3125mg/L, and the resulting bacterial concentration under the MIC level of gentamicin was about 10^5 CFU/ml.

5.3.3 Mixing suspension of *P. aeruginosa* with different concentrations of gentamicin

Gentamicin of 0, 0.5, 1 and 2 MIC was prepared in sterilized distilled water. One millilitre of each concentration was then mixed with 1ml of the diluted *P. aeruginosa* suspension in 5ml test tubes. The resulting concentrations of gentamicin were 0, 0.25, 0.5 and 1 MIC respectively.

5.3.4 PEMF treatment protocol and grouping

A commercially available PEMF device (BTL-5000, BTL Industries Ltd., UK) was used. According to previous studies (Matl et al., 2011; Pickering et al., 2003), frequencies of 20 and 72Hz were chosen as PEMF parameters, both at 5-mT intensity. Sham group was included. The two PEMF groups were treated with PEMF for 10 hours. The sham group was not exposed to PEMF. All groups were incubated for 24 hours at 37^{o}C with aeration in total.

5.3.5 Enumeration of viable bacterial cells

At baseline and 24 hours, 1µl of the *P. aeruginosa*-gentamicin mixture was withdrawn from each sample and serially 10-fold diluted for viable bacterial count. Each of the diluted samples was spread onto LB agar plate and incubated for 24 hours at 37^{o}C. Bacterial colonies were then counted.

5.3.6 Statistical analysis

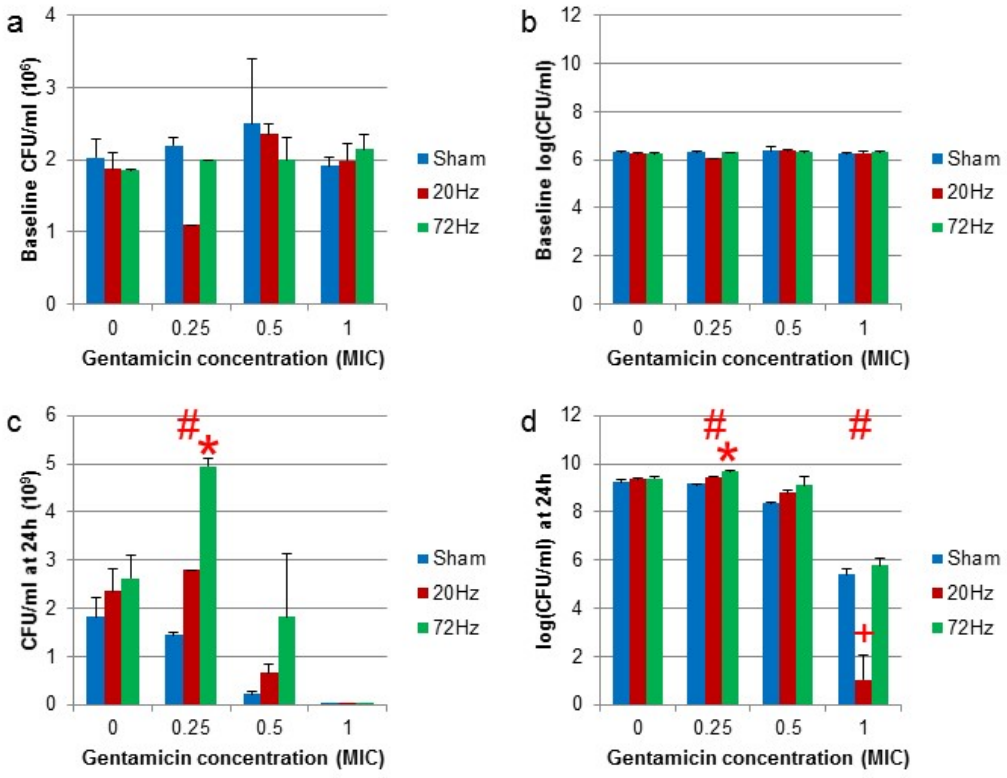
Two-way repeated measure ANOVA was performed to analyse the within-group differences in bacterial counts and the interaction between time and treatment groups for different MICs. One-way ANOVA was used to probe the between-group differences of CFU/ml and log(CFU/ml) for different MICs. Two-way ANOVA was performed to test the between-group differences and the PEMF x MIC interaction at 24 hours. Significance level was set as 0.05. The analysis was performed using IBM SPSS statistics (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.).

5.4 Results

Two independent experiments with duplication for each group were conducted and the findings are summarized in Figure 5.1. The baseline bacterial count, CFU/ml and log(CFU/ml), of all groups were not significantly different from each other at different MICs. After 24-hour incubation, both the CFU/ml and log(CFU/ml) at 0 and 0.25 MICs increased significantly from the baseline ($p<0.05$). At 1 MIC, both the CFU/ml and

log(CFU/ml) of sham ($p=0.007$ and $p=0.047$, respectively) and 20-Hz PEMF group ($p=0.004$ and $p=0.016$ respectively), but not 72-Hz PEMF group, significantly decreased from the baseline. At sub-MIC levels, the 72-Hz PEMF group had higher bacterial counts than the 20-Hz PEMF and sham groups after 24 hours. Statistical significance was observed between 72-Hz PEMF and sham groups at 0.25 MIC (CFU/ml: $p=0.004$; log[CFU/ml]: $p=0.003$). Interestingly, at 1 MIC, log(CFU/ml) of 20-Hz PEMF group was significantly lower than that of sham and 72-Hz PEMF groups ($p=0.003$ and $p=0.002$ respectively). Significant PEMF group x time (baseline and 24 hours) interactions were found for both CFU/ml and log(CFU/ml) at 0.25 MIC ($p=0.004$ and $p=0.001$ respectively), and log(CFU/ml) at 1 MIC ($p=0.001$). The PEMF x MIC interactions for both CFU/ml and log(CFU/ml) were also significant in 24 hours ($p=0.02$ and $p<0.001$ respectively).

Figure 5.1: The population of *P. aeruginosa* with irradiation of sham, 20-Hz or 72-Hz PEMF, and different concentrations of gentamicin at the baseline (a: CFU/ml; b: log[CFU/ml]) and after 24-hour incubation (c: CFU/ml; d: log[CFU/ml]).



Data are expressed as mean+SEM. *significantly different from sham PEMF group (p=0.004 for CFU/ml and p=0.003 for log[CFU/ml]); +significantly different from sham (p=0.003) and 72-Hz group (p=0.002); #significant PEMF x time interaction (at 0.25 MIC, p=0.004 and p=0.001 for CFU/ml and log[CFU/ml] respectively; at 1 MIC, p=0.001 for log[CFU/ml]).

5.5 Discussion and conclusions

This is the first study that showed the effects of combining PEMF and gentamicin on *P. aeruginosa*, which is a Gram-negative bacterial species associated with clinical conditions including chronic wounds, cystic fibrosis and urinary tract infections. Interestingly, the effects of PEMF and gentamicin on *P. aeruginosa* depend on PEMF parameters and gentamicin concentrations. Under sub-MIC levels of gentamicin, PEMF enhanced the growth of *P. aeruginosa* with a dose-dependent response in which higher PEMF frequency (72Hz) enhanced greater bacterial growth than did the lower frequency (20Hz). Under MIC level of gentamicin, the bacterial growth or survival were significantly suppressed; the effects of PEMF were however different. The PEMF delivered at higher frequency (72Hz) maintained the survival of *P. aeruginosa*, whereas the PEMF at lower frequency (20Hz) significantly compromised it. Together with the significant time x group interaction at MIC level, the 20-Hz PEMF was shown to act synergistically with gentamicin to suppress the survival of *P. aeruginosa*.

Our findings demonstrated that PEMF influences the growth of Gram-negative bacteria in an antibiotic concentration-dependent manner. Piyadasa et al. (2016) recently reported the effects of PEMF on another Gram-negative bacterium, *E. coli*. Although the PEMF parameters were not fully reported in their study, they showed that PEMF alone, without any antibacterial agents, stimulated the growth of *E. coli*. Nonetheless, PEMF in combination of AgNPs significantly reduced the viability of *E. coli*. AgNPs

exert the bactericidal effect by physically contacting the bacteria (Agnihotri et al., 2013), increasing the oxidative and metabolic stresses (Soni et al., 2014) to the cell wall (Mirzajani et al., 2011), cell membrane (Agnihotri et al., 2013; Soni et al., 2014) and deoxyribonucleic acid (DNA) (Soni et al., 2014). AgNPs may eventually rupture bacterial cell wall, denature proteins, block respiration and cause bacterial death (Kumar et al., 2008). Gentamicin, on the other hand, exerts its bactericidal effect by a more specific pathway. As an aminoglycoside, it irreversibly binds to the ribosome and interferes with protein translation (Lando et al., 1973). The affected ribosome misreads the messenger ribonucleic acid and produces non-functional or toxic peptides. The mechanisms of the actions of AgNPs and gentamicin are completely different. Nevertheless, PEMF can act synergistically with these two antimicrobial agents. Future studies can further examine the mechanism of the synergistic antibacterial effect of PEMF and aminoglycosides or a broad spectrum of antimicrobial agents.

It was found that 20-Hz PEMF augmented the antibiotic effect of gentamicin. Instead of attenuating or suppressing the growth of bacteria, it appeared to kill most of the bacteria (3 of out 4 bacterial suspension yielded 0 CFU/ml). One possibility is that the 20-Hz PEMF might alter the electrostatic balance of the membrane components (Inhan-Garip et al., 2011), making the membrane more negatively charged to increase the rate of cationic gentamicin penetration (Oncul et al., 2016; Segatore et al., 2012). It might also regulate other bacterial metabolism and induce additional stress with 1-MIC gentamicin (Del Re et al., 2009). Our findings indicated that

PEMF delivered at different frequencies (i.e. 72-Hz PEMF) produced the opposite effect on bacterial growth. The observed nonlinearity ('windows') of biological effects may be explained by Binhi et al. (2002) that PEMF at a specific frequency interferes with various ions to a different extent, which modulates the rate of ion-protein binding, subsequently leading to different biological effects. At lower concentrations of gentamicin, however, PEMF might increase the membrane permeability and metabolism (Oncul et al., 2016) that promote nutrient entrance and growth (Nascimento et al., 2003). Gusev et al. (2005) also hypothesized that PEMF may excite protons in the medium that can theoretically be utilized as an energy source by bacteria for growth.

Ahmed et al. (2013) studied the influence of PEMF to *Staphylococcus (S.) aureus*, a Gram-positive strain, in the absence of any antimicrobial agents. They examined the effects of PEMF delivered at different frequencies and intensities, demonstrating that PEMF of all tested parameters inhibited the growth. These findings contradict those reported by Piyadasa et al. (2016) and the present study. However, with gentamicin, Matl et al. (2011) demonstrated that PEMF delivered at 20Hz and 5mT augments the antibiotic effect against *S. aureus*. This is comparable with the present findings at the MIC level of gentamicin. Indeed, other studies evaluating the effects of PEMF on growth and viability of bacteria generated contradicting results (Ahmed et al., 2013; Belyaev, 2011; Cellini et al., 2008; Del Re et al., 2004; Inhan-Garip et al., 2011; Matl et al., 2011; Moore, 1979; Nascimento et al., 2003; Oncul et al., 2016; Piyadasa et al., 2016; Segatore et al., 2012).

The contradicting results should be partly contributed by the different PEMF parameters adopted. The specific PEMF parameters that enhance or suppress growth or viability of bacteria remain uncertain.

Inhan-Garip et al. (2011) demonstrated that PEMF exerted different structural changes on Gram-positive and negative bacteria; PEMF disturbed the integrity of cell wall and cell membrane of Gram-negative bacteria while leading to the heterogeneity of cytoplasm, condensation of DNA and possible abnormal septation in Gram-positive strains. However, PEMF may increase the susceptibility to antimicrobial agents in both Gram-positive and negative bacteria (Matl et al., 2011; Pickering et al., 2003; Piyadasa et al., 2016; Segatore et al., 2012). More studies are needed to reveal the mechanisms of PEMF on the viability of Gram-positive and negative bacteria. The potential structural changes of *P. aeruginosa* bacterial cell induced by PEMF can be examined by electron microscopy in the future.

Although the mechanism of PEMF, in the presence of absence of antibiotics, on bacterial viability is unclear, it might be related to the regulation of ion-protein binding by PEMF leading to further signalling cascades (Binhi et al., 2002). One example is calcium signalling involving calcium-binding proteins. In eukaryotic cells, it has been shown to promote the binding of calcium ion (Ca^{2+}) to calmodulin and activate the calmodulin-dependent nitric oxide pathway (Pilla, 2013). The pathway generates nitric oxide, cyclic adenosine monophosphate and cyclic guanosine monophosphate to promote tissue healing (Pilla et al., 2011). Even though the structure of bacteria as prokaryotes is different from that of eukaryotes, prokaryotes

have similar calcium-binding proteins (Dominguez et al., 2015). The calcium signalling pathways are involved in a wide range of metabolic activities and affect cell cycle, gene expression, proteolysis, virulence, stress resistance etc. (Dominguez et al., 2015). For example, Sarkisova et al. (2014) showed that the calcium-binding EF-hand protein (PA4107) is responsible for virulence, iron acquisition and biosynthesis of pyocyanin, proteases and stress response proteins of *P. aeruginosa* under high Ca^{2+} concentration (5 or 10mM).

Since most clinically problematic diabetic wound infections originate from biofilm, future work should involve the biofilm form of *P. aeruginosa*. Previous studies have shown that PEMF exerted adverse effects on biofilm development and maintenance in other bacterial species with or without antibiotics (Di Campli et al., 2010; Pickering et al., 2003). Specifically, PEMF may alter the expression of surface protein that in turn changes the surface charge of bacteria (Di Campli et al., 2010; Smith, 2005). This would affect the cell adhesion to a surface and biofilm development. PEMF may also induce stress to bacteria and alter their morphology and gene expression that could potentially affect their biofilm development and maintenance (Del Re et al., 2009; Di Campli et al., 2010; Inhan-Garip et al., 2011; Williams et al., 2006).

Future studies to explore the potential effect of PEMF on the potency of gentamicin are warranted. The potential dose-dependent effect of PEMF delivered at different frequencies should be examined with a larger sample size. Wider range of gentamicin concentrations should also be included in

future studies in order to confirm the antibiotic-augmentation effect of PEMF of specific concentrations.

In conclusion, PEMF enhances the viability of *P. aeruginosa*, which is a Gram-negative strain with clinical importance, under sub-MIC levels of gentamicin. At the MIC level of gentamicin, 20-Hz PEMF augments the antibiotic effect and significantly suppresses the viability of *P. aeruginosa*. In contrast, PEMF delivered at 72-Hz slightly enhances the survival of *P. aeruginosa*.

Chapter 6

Conclusions and Suggestions for Future Research

Because the current assessment and treatment regimens in clinical settings appear to be inadequate in preventing lower limb amputation due to uncontrolled diabetic ulceration, more research should be done in identifying effective assessments and interventions in the future. Our findings supported the use of the recently developed optical coherence tomography-based air-jet indentation system to serve as an objective, quantitative and non-invasive clinical biomechanical assessment tool that allows repeated measurements for diabetic wounds. The indentation stiffness of the wounds measured by this system correlated significantly with the tensile biomechanical properties as well as the underlying collagen abundance, alignment and orientation over time. As the indentation stiffness may also be closely related to the content of proteoglycans that regulate collagen deposition and maturation, more studies should be done to reveal the relationships between indentation stiffness, proteoglycans and other extracellular matrix of diabetic wounds in different phases of healing. In future clinical trials, indentation stiffness measurement can be used to explore not only the predictive validity on diabetic ulcers, but also the pre- or post-ulceration skin of people with diabetes.

In order to identify a clinical treatment for augmenting diabetic wound healing, the present thesis examined the effects of pulsed electromagnetic field (PEMF) on diabetic wounds. The present findings demonstrated that PEMF was effective in promoting collagen deposition (more than 5-fold

increase compared with the no-PEMF control), which was associated with an increase in myofibroblast population, and enhancing energy absorption capacity (about 1.6 times greater than the sham PEMF control) of diabetic wounds in the early phase of wound healing. However, the PEMF protocols adopted by the present thesis could not promote collagen alignment and orientation as well as maximum stress and Young's modulus especially in the remodelling phase of diabetic wounds. Different potential mechanisms including calcium-dependent, fibroblast growth factor 2-dependent, calpain-depdendent and nitric oxide-dependent pathways of PEMF on promoting myofibrolast population, collagen deposition and material properties in diabetic wounds should be explored. The potential effects of PEMF on proteoglycans which regulate collagen deposition and maturation can also be examined. PEMF protocols with different intensities, frequencies, waveforms and treatment duration should be compared to identify optimal protocol for treating diabetic wounds. The treatment effects on diabetic wound healing should eventually be tested in clinical trials.

The present thesis demonstrated that apart from promoting healing in diabetic wounds, PEMF might also act as a potential adjunctive treatment to boost the efficacy of antibiotics for treating diabetic wound infection. *Pseudomonas (P.) aeruginosa* was used as the model bacteria due to its significant role in the infection and development of chronic wounds. PEMF delivered at 5mT and 20Hz could significantly reduce the viability of *P. aeruginosa* 4-log under gentamicin at minimum inhibitory concentration. In contrast, the 5-mT and 72-Hz PEMF appeared to increase the growth of the

P. aeruginosa 3-fold. More detailed responses of wound-infecting bacteria as well as the underlying mechanisms to PEMF should be clarified. These include the potential structural changes and calcium-dependent metabolic changes induced by different PEMF protocols with different frequencies, intensities and waveform. The *in vitro* studies should be followed by *in vivo* ones and clinical trials to evaluate the outcomes of PEMF treating bacterial infected diabetic wounds.

Appendix - Preview of published journal articles

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ORIGINAL PAPER

Pulsed electromagnetic field (PEMF) promotes collagen fibre deposition associated with increased myofibroblast population in the early healing phase of diabetic wound

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Abstract The present study evaluated the effects of PEMF on collagen fibre deposition, collagen fibril alignment and collagen fibre orientation. The potential relationships between collagen fibre deposition and myofibroblast population in diabetic wound healing were also examined. Forty young male streptozotocin-induced diabetic Sprague–Dawley rats were randomly assigned to PEMF group or control group. 2 cm × 2 cm square wounds were made at their back. The PEMF group received daily exposure of PEMF to the wounds, while control group was handled in the same manner except that the PEMF device was not activated. Wound tissues harvested on post-wounding day 7, 10 and 14 were fixed, processed and sectioned. The abundance, fibril alignment and fibre orientation of type I collagen were quantified with picro-sirius polarization method and image analysis software (Nikon NIS Element AR). Myofibroblast population data were adopted from our previous study. Correlation between myofibroblast population and collagen fibre deposition was examined. There was significantly greater abundance of type I collagen fibre in the PEMF group than in the control on day 7 ($P = .013$), but not on day 10 or 14. No significant between-group differences were found in collagen fibril alignment and collagen fibre orientation at any measured time points. Positive correlation was found between collagen fibre deposition and myofibroblast population only on day 7 ($r = .729$, $P = .007$). In conclusion, PEMF can significantly increase collagen fibre in the early phase of diabetic wound healing, which is associated with the enhancement of myofibroblast population.

Keywords Type I collagen · Diabetic wound · Pulsed electromagnetic fields · Myofibroblast · Rat model

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Introduction

In mammals, skin is the largest organ and the first line of defence against external abiotic (e.g. mechanical stress) and biotic (e.g. pathogens) stresses, as well as thermal and osmotic regulation. Upon injury, the cutaneous system responses by undergoing a complex process of wound healing that consists of distinct but overlapping phases, namely inflammatory, proliferative and remodelling phases orchestrating in a highly organized fashion to allow morphological as well as functional recovery of skin. Wounds usually heal without prolonged delay and complications in healthy people. However, wound healing is delayed in people with diabetes mellitus (DM), one of the most common systemic diseases [5]. Impaired wound healing due to DM may often lead to unhealed open wound, gangrene and subsequently amputation due to severe bacterial infection. These obviously lead to disability and bring an impact on quality of life [5, 11, 13, 17, 22, 35, 42].

During the proliferative phase of wound healing, fibroblasts migrate into the wound matrix and a subpopulation of fibroblasts differentiates into myofibroblasts, which is believed to be involved in deposition of extracellular matrix such as collagen fibres [39]. Collagen is among the most predominant extracellular matrices in skin that plays a key role in tissue architecture and homeostasis [2]. The biomechanical strength of cutaneous wounds

Measurement of diabetic wounds with optical coherence tomography-based air-jet indentation system and a material testing system

• **Objective:** Material testing system is a conventional but destructive method for measuring the biomechanical properties of wound tissues in basic research. The recently developed optical coherence tomography-based air-jet indentation system is a non-destructive method for measuring these properties of soft tissues in a non-contact manner. The aim of the study was to examine the correlation between the biomechanical properties of wound tissues measured by the two systems.

• **Method:** Young male Sprague-Dawley rats with streptozotocin-induced diabetic were wounded by a 6 mm biopsy punch on their hind limbs. The biomechanical properties of wound tissues were assessed with the two systems on post-wounding days 3, 7, 10, 14, and 21. Wound sections were stained with picro-sirius red for analysis on the collagen fibres. Data obtained on the different days were charted to obtain the change in biomechanical properties across the time points, and then pooled to examine the correlation between measurements made by the two devices. Qualitative analysis to determine any correlation between indentation stiffness measured by the air-jet indentation system and the orientation of collagen fibres.

• **Results:** The indentation stiffness is significantly negatively correlated to the maximum load, maximum tensile stress, and Young's modulus by the material testing system (all $p<0.05$). The orientation of collagen changes with the indentation stiffness over time.

• **Conclusion:** Our findings support the use of optical coherence tomography-based air-jet indentation system to evaluate the biomechanical properties of wounds in a non-contact manner. It is a potential clinical device to examine the biomechanical properties of chronic wounds *in vivo* in a repeatable manner.

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biomechanical properties; diabetic wound; rat model; OCT-based air-jet indentation system

The biomechanical strength of the skin is the ultimate measure that reflects the skin's actual capacity to tolerate physical deformities during bodily movements or external stress. Upon wounding, the biomechanical strength is impaired but it will gradually recover during the healing process. Thus, the maximum tensile stress, maximum load, energy absorption capacity, tensile stiffness (Young's modulus), and maximum strain of the wound tissue are important indicators of the biomechanical strength of the wound during healing.[1] Clinically, chronic ulcers can be assessed on redness, hardness, itchiness and pain.[2] However, these measures are just semi-quantitative and subjective. Objective measures include wound area, Doppler flowmetry and skin thickness measured by high-frequency ultrasound.[2] These measurement methods are conducted in a direct contact manner and cannot directly measure the actual strength of the wounds. Therefore, there is a lack of objective, quantitative and non-contact clinical measurement method for assessing chronic wounds.

The biomechanical properties of wound tissue are conventionally measured in animal studies using the material testing system (MTS). Although MTS testing can show the tensile strength of wound tissues, it is *ex vivo*, destructive and hence a non-repeatable method. Its use is limited to cross-sectional measurements but not prospective and repeated measurements that are commonly performed in clinical settings. Also, MTS testing involves the excision of skin tissue from one intact piece of an integument network into a small strip of fragment. Such excision has been shown to potentially disturb the network and the biomechanical properties of the strip, the properties of which then turn out to be different from those of the intact network.[3] Therefore, MTS does not seem to be a feasible test for measuring wound strength in the clinical setting. Moreover, the loading applied in the MTS

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Appendix - Conference posters

Pulsed Electromagnetic Fields (PEMF) Promote Collagen Deposition through Myofibroblast Proliferation in Early Diabetic Wound

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Introduction

It is well known that healing for diabetic wound is delayed and the biomechanical strength of wound tends to be weaker than that of the non-diabetic ones. This may because collagen deposition is impaired in diabetic wounds and type I collagen fibres contribute greatly to the biomechanical strength of the healing wound.

Previous studies have shown that myofibroblast is essential in wound healing. The deficits of myofibroblast proliferation may account for the delayed healing in diabetic wounds. Recent studies suggested that myofibroblast plays more crucial role in collagen deposition than fibroblast in the wound healing process. PEMF has been shown to accelerate diabetic wound healing and improves its biomechanical strength.

The objective of the present study was to determine whether PEMF exerts its effects through enhancing type I collagen fibre deposition and myofibroblast proliferation. We hypothesized that PEMF could increase type I collagen fibre deposition through increasing myofibroblast proliferation.

Methods

Forty young male streptozotocin-induced diabetic Sprague-Dawley rats were used, and a 2cm x 2cm wound was induced at the back. They were randomly assigned to either the PEMF (n=20) or control group (n=20). For the PEMF group, the rats were restrained and their wounds were exposed to 60min of PEMF daily for 2 weeks at 5mT, 25Hz and with a 40ms pulse width daily. The control group was restrained and handled in the similar way except the PEMF device was not activated.

The wound tissues were harvested on post-wounding day 7, 10 and 14. They were fixed and processed into paraffinized blocks and then sectioned. With picro-sirius red, type I collagen was stained red when examined under polarized light. Its abundance was quantified using imaging software (NIS elements AR, Nikon Instruments). The abundance of type I collagen was expressed as red stained area (redA) / region of interest (ROI).

Myofibroblast population was examined by fluorescent immuno-histochemistry against α-smooth muscle actin (α-SMA), and the α-SMA immunoreactivity was scored using semi-quantitative ratings (4 for >75% of ROI; 3 for >50%; 2 for >25%; 1 for <25%) by two independent experts in laboratory animal pathology.

Mann-Whitney U tests were performed to examine the difference of the outcome measures for type I collagen fibre deposition and myofibroblast population between PEMF and control groups on different days. Spearman's rho test was conducted to examine the correlation between myofibroblast population and type I collagen fibre deposition. Significant level was set at 0.05. All the above statistical tests were done using IBM SPSS statistics 20.

Results

Both PEMF and control groups showed increasing type I collagen from day 7 to 14. However, the type I collagen content in PEMF group was consistently greater than that in the control on day 7, 10 and 14 (Figure 1). Significantly greater content of type I collagen was found in the PEMF group than in control only on day 7 (P=.013) but not on day 10 and 14.

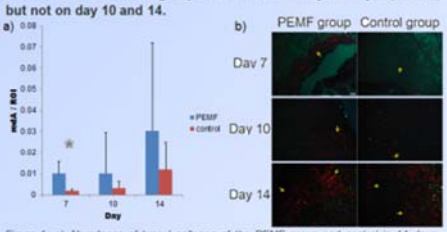

Figure 1: a) Abundance of type I collagen of the PEMF group and control in 14 days; *P<0.05; redA: red stained area, ROI: region of interest. b) Representative picro-sirius red stained sections from the PEMF and control groups on day 7, 10 and 14; arrows indicating stained type I collagen fibres; scale bar: 100 μm.

On day 7 and 10, myofibroblasts population in the PEMF group was significantly greater than in control (day 7: P=.042; day 10: P=.024) (Figure 2). On day 14, myofibroblasts population was similar in both groups.

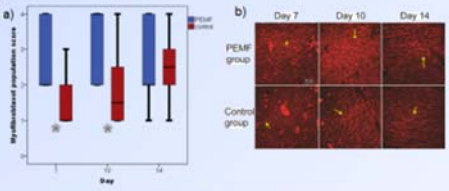

Figure 2: a) Myofibroblast population of the PEMF group and control in 14 days; *P< 05.
b) Representative sections of α-SMA-immunoreactive myofibroblasts from PEMF and control groups on day 7, 10 and 14; note that the hollow circular shaped stains were blood vessels but not myofibroblasts; arrows indicating the stained myofibroblasts; scale bar: 50 μm.

A strong and positive correlation was found between collagen fibre deposition and myofibroblast population on day 7 (r=.729, P=.007) (Figure 3).

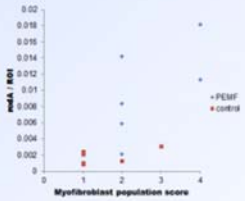

Figure 3: Correlation between the abundance of type I collagen and myofibroblast population on day 7; redA: red stained area, ROI: region of interest.

Discussion

Previous study demonstrated that PEMF can increase hydroxyproline content in skin of non-diabetic rats. Our study is the first one to illustrate that PEMF can increase the actual type I collagen content in diabetic wound. This may account for the increase in biomechanical strength of PEMF-treated diabetic wound shown by other studies as type I collagen fibres are the major contributor to the biomechanical strength of skin.

We have also demonstrated earlier that PEMF improves wound closure and re-epithelialization.[1] The present study suggested that PEMF may enhance type I collagen fibre deposition through augmenting myofibroblast proliferation or differentiation. This implies that the increase in biomechanical strength of PEMF-treated diabetic wound could be at least partly contributed by the increased myofibroblast proliferation that subsequently promotes type I collagen fibre deposition.

Further studies on signaling pathway leading to an increase in myofibroblast population that accelerates diabetic wound healing is warranted. Also, the identification of optimal parameters of PEMF for promoting healing of diabetic wound at the later stage of wound healing can be done.

Clinical Relevance

Our findings support the use of PEMF to promote healing of diabetic ulcers. Clinical study should be conducted to confirm whether similar findings can be shown in patients.

Conclusion

PEMF increases collagen fibre deposition and myofibroblast population in the early phase of diabetic wound healing. The increase in collagen fibre deposition in early diabetic wound could be due to the enhancement of myofibroblast proliferation.

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BIOMECHANICAL PROPERTIES OF DIABETIC WOUND MEASURED WITH OPTICAL COHERNECE TOMOGRAPHY (OCT)-BASED AIR-JET INDENTATION SYSTEM

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Background & Aims

OCT-based air-jet indentation system is a recently developed device that can measure the biomechanical property of soft tissues by applying air-jet to compress and deform the tissue. The present study made use of this device to measure the stiffness of wound bed in a non-contact, non-invasive, *in vivo* and repeatable manner, as compared to the data obtained by Material Testing System (MTS), which is a conventional but destructive method measuring mechanical properties of an excised soft tissue specimen in an *in vitro* manner. The study aims at exploring the correlation between biomechanical properties of wound tissue measured by the OCT-based air-jet indentation system and MTS.

Materials & Methods

33 young male streptozotocin-induced diabetic Sprague-Dawley rats were wounded with 6 mm biopsy punch on their hind limbs. The biomechanical properties of wound tissues were assessed with the OCT-based air-jet indentation system and MTS on post-wounding day 3, 7, 10, 14 and 21. Data obtained on different days were pooled to examine the correlation of measurement made by the two devices.

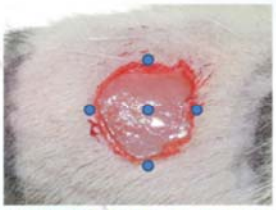

Figure 1: The sites of wound were marked for OCT-based air-jet indentation system measurement (centre, cephalic, caudal, lateral, medial)

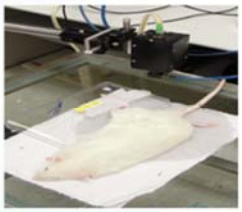

Figure 2: Set-up of OCT-based air-jet indentation system

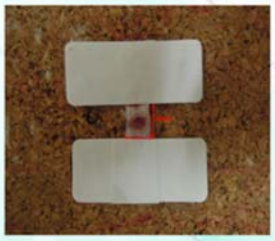

Figure 3: Specimen prepared for MTS assessment

Figure 4: Specimen being tested on MTS

Results

During the 3-week period of assessment, the maximum force, maximum strength, energy absorption capacity and Young's modulus obtained by MTS all exhibited a general increasing trend. The indentation stiffness significantly increased just after wounding, peaked on day 3 and became similar to that before wounding.

By using non-parametric Spearman's rho tests, the indentation stiffness of wound bed measured by the OCT-based air-jet indentation system is significantly negatively correlated to maximum load, maximum strength, energy absorption capacity and Young's modulus obtained by using MTS. (Table 1)

			Tested with OCT-based air-jet indentation system	
			Stiffness at wound centre	Stiffness at wound periphery
Tested with MTS	Maximum load	r	-0.429	-0.414
		p-value	0.025*	0.025*
	Maximum strength	r	-0.599	-0.489
		p-value	0.001**	0.007**
	Energy absorption capacity	r	-0.383	-0.357
		p-value	0.048*	0.058
	Young's modulus	r	-0.477	-0.389
		p-value	0.012*	0.037*

Table 1: The correlations between mechanical properties by OCT-based air-jet indentation system and MTS. *: $p<0.05$; **: $p<0.01$

Conclusion & Discussion

The biomechanical properties measured by the 2 systems are significantly negatively correlated to each other. OCT-based air-jet indentation system may reflect the tensile biomechanical properties of wounds in a non-contact, non-invasive, in vivo and repeatable manner. Smaller indentation stiffness, which is associated with greater tensile strength, may be related to better collagen alignment.

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